

A NEW COMPOUND, 1H,8H-PYRANO[3,4-C]PYRAN-1,8-DIONE, SUPPRESSES AIRWAY EPITHELIAL CELL INFLAMMATORY RESPONSES IN A MURINE MODEL OF ASTHMA

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Clinical and experimental studies have established eosinophilia as a sign of allergic disorders. Activation of eosinophils in the airways is believed to cause epithelial tissue injury, contraction of airway smooth muscle and increased bronchial responsiveness. As part of the search for new anti-asthmatic agents produced by medicinal plants, the effects of 270 standardized medicinal plant extracts on cytokine-activated A549 human lung epithelial cells were evaluated. After several rounds of activity-guided screening, the new natural compound, 1H,8H-Pyrano[3,4-c]pyran-1,8-dione (PPY), was isolated from *Vitex rotundifolia* L. To elucidate the mechanism by which the anti-asthmatic responses of PPY occurred in vitro, lung epithelial cells (A549 cell) were stimulated with TNF- α , IL-4 and IL-1 β to induce the expression of chemokines and adhesion molecules involved in eosinophil chemotaxis. PPY treatments reduced the expression of eotaxin, IL-8, IL-16 and VCAM-1 mRNA significantly. Additionally, PPY reduced eotaxin secretion in a dose-dependent manner and significantly inhibited eosinophil migration toward A549 medium. In addition, PPY treatment suppressed the phosphorylation of p65 and ERK1/2, suggesting that it can inhibit the MAPK/NF- κ B pathway. To clarify the anti-inflammatory and anti-asthmatic effects of PPY in vivo, we examined the influence of PPY on the development of pulmonary eosinophilic inflammation in a murine model of asthma. To accomplish this, mice were sensitized and challenged with ovalbumin (OVA) and then examined for the following typical asthmatic reactions: an increase in the number of eosinophils in BALF; the presence of Th2 cytokines such as IL-4 and IL-5 in the BALF; the presence of allergen-specific IgE in the serum; and a marked influx of inflammatory cells into the lung. Taken together, our results revealed that PPY exerts profound inhibitory effects on the accumulation of eosinophils into the airways while reducing the levels of IL-4, IL-5, and IL-13 in the BALF. Therefore, these results suggest that PPY may be useful as a new therapeutic drug for the treatment of allergic asthma.

Exposure to environmental hazards causes outbreaks of allergic diseases, such as allergic asthma,

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atopic dermatitis and allergic rhinitis. However, such exposure is inevitable, as evidenced by more than 50 million people in the United States being affected by allergic diseases every year. These diseases cost the US health care system approximately \$18 billion annually (1). Therefore, institutional and industrial studies are being conducted to elucidate the mechanism by which allergies occur and develop therapies to cure, or prevent their symptoms (2).

The initial symptoms of asthma include airway inflammation in which eosinophils play a crucial role (3). Eosinophils are always present in excess in the airways of asthma patients, however, their accumulation decreases as the symptoms of asthma decrease. Eosinophils exert their effects by selectively migrating from the peripheral blood and adhering to vascular endothelial cells (4). They are then transported to the airways by chemokines, where they infiltrate and cause inflammation. Eotaxin is a major eosinophil-specific chemokine that is released in the respiratory epithelium following allergic stimulation. Eotaxin not only causes eosinophil migration, but is also generated within eosinophils, thereby increasing the impact of eosinophil migration (5). The expression of eotaxin is regulated by mitogen-activated protein (MAP) kinases (MAPK), which also participate in airway remodeling by inducing many other chemokines (6-7).

Natural products capable of inhibiting eosinophil function and/or infiltration are desirable treatments for asthma and other allergic diseases (8). We previously reported that Moutan Cortex Radicis suppresses the secretion of eotaxin by inhibiting NF- κ B activation (9-10). Many previous studies have suggested that activated NF- κ B plays a crucial role in the increased expression of many inflammatory genes involved in airway inflammation in asthma (11-14) and the regulation of eosinophils (15). As part of the search for new anti-asthmatic agents produced by medicinal plants, the effects of 270 standardized medicinal plant extracts on cytokine-activated A549 human lung epithelial cells were evaluated using our standard eotaxin ELISA assay. The extract of *Vitex rotundifolia* Linn fil., which is widely used as a traditional herbal medicine in various Asian countries for the treatment of migraine pain and allergic diseases (16), was selected for

activity-guided fractionation due to its excellent attenuation of eotaxin secretion in A549 cells. After several rounds of activity-guided fractionations, 1H,8H-Pyrano[3,4-c]pyran-1,8-dione (PPY) was isolated as a pale yellow powder. Characterization of this compound revealed that it had not previously been isolated from natural sources. The effects of PPY on lung epithelial cell inflammatory responses were then evaluated in vitro and shown to exert an anti-inflammation effect. We then confirmed that PPY exerted an anti-asthmatic effect by inhibiting the development of pulmonary eosinophilic inflammation in a murine model of ovalbumin-sensitized allergic asthma.

MATERIALS AND METHODS

UV and IR spectra were recorded on a Hitachi U-3000 spectrophotometer and a Bio-Rad FTS 135 FT-IR spectrometer, respectively. 1D and 2D NMR experiments were performed on a Bruker Avance 400 MHz FT-NMR instrument with tetramethylsilane (TMS) as internal standard. EIMS was obtained on a JEOL JMS 700 Mstation HRMS spectrometer. Column chromatography was carried out with Si gel 60 (70-230 mesh, Merck, Darmstadt, Germany). TLC analysis was performed on precoated 0.25 mm thickness Kieselgel 60 F₂₅₄ (Merck, Germany) plates with visualization under UV light (254 and 365 nm) and 10% (v/v) sulfuric acid spray followed by heating (120°C, 5 min).

Plant Material

The fruits of *Vitex rotundifolia* Linne fil (Verbenaceae) were purchased from Sun Ten Pharmaceutical (Taipei, Taiwan). A voucher specimen (No. E10) has been deposited at the Herbarium of College of Oriental Medicine, Kyung Hee University, Korea.

Extraction and Isolation

The fruits of *V. rotundifolia* (3 kg) were extracted with MeOH (5 × 10L) for 24 h by percolation. The solvent was evaporated *in vacuo* to afford an MeOH extract (100 g), which was then suspended in water (1 L), and partitioned with *n*-hexane (5 × 1L), EtOAc (5 × 1L), and *n*-BuOH (3 × 1L), sequentially. The EtOAc extract (17 g) was separated by silica gel column chromatography (ϕ 4.5 cm; silica gel 70-230 mesh, 500 g) using gradient mixtures of MeOH in CHCl₃ (0→50 %, and washed with MeOH 100%, 5L each) as mobile phases, affording 1H,8H-Pyrano[3,4-c]pyran-1,8-dione (235 mg, 0.0078% w/w).

Cell culture

Human type II-like epithelial lung cells (A549 cells) were obtained from the Korean Cell Line Bank (Cancer Research Institute, Seoul, Korea). These cells were then cultured in tissue culture plates (Corning, New York, USA) in RPMI medium (Invitrogen, Rockville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, MA, USA) and penicillin-streptomycin (Invitrogen) at a density of 1×10^6 cells/ml. The plates were then incubated at 37°C under 100% humidity and 5% CO_2 . The cells were sub-cultured every 3–4 days to maintain the density at 1×10^6 cells/ml.

Animals

Eight-week-old male BALB/C mice weighing 22–25 g were purchased from Charles River Korea (Sungnam, Korea). The mice were housed conventionally in plastic cages in a room that was maintained at 22–24 °C with a 12-h light-dark cycle, during which time they were given free access to water. All animal experiments were conducted in accordance with the NIH guidelines and approved by the Institutional Animal Care and Use Committee at Kyung Hee University.

Measurement of Th1/Th2 cytokine production in CD4^+ T cells

Splenocytes were prepared by disrupting the spleen with a syringe in complete medium (RPMI 1640 with 10% FBS) and the cells washed in RPMI medium, followed by lysis of erythrocytes using ammonium chloride reagent (BD Bioscience, San Diego, CA, USA). The cells were then counted and viability was determined by trypan blue exclusion. Subsequently, CD4^+ T cells were isolated from CD4^+ (L3T4) microbeads (Miltenyi Biotec, Bergisch, Germany) as directed by the manufacturer.

CD4^+ T cells were resuspended in a complete medium (RPMI 1640 with 10% FBS), then activated with plate-bound 10 $\mu\text{g/ml}$ anti-CD3 (BD Bioscience, San Diego, CA, USA) and 2 $\mu\text{g/ml}$ anti-CD28 (BD Bioscience, CA, USA) for 24 h. Naïve T cells were incubated with 5 ng/ml rIL-12 (BD Bioscience), 10 $\mu\text{g/ml}$ anti-IL-4 (BD Bioscience) and 50 U/ml rIL-2 for Th1 differentiation. For Th2 cell differentiation, 10 ng/ml rIL-4 (BD Bioscience) and 10 $\mu\text{g/ml}$ anti-IL-12 (BD Bioscience) were added to the culture medium in the presence of 50 U/ml of rIL-2. The cells were incubated in the absence or presence of PPY (0.1, 1, 10 $\mu\text{g/ml}$). CD4^+ T cells were activated with plate-bound 10 $\mu\text{g/ml}$ anti-CD3 mAb and 2 $\mu\text{g/ml}$ anti-CD28 Ab. The cell-free culture supernatants were then collected and assayed for eotaxin using enzyme-linked immunosorbent assay kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

OVA sensitization and challenge

Mice were sensitized by intraperitoneal injection of

100 μg of OVA (Grade V; Sigma Aldrich, St. Louis, MO, USA) absorbed in 20 mg of aluminum hydroxide (Sigma-Aldrich) in 0.2 mL of sterile phosphate-buffered saline (PBS) on days 0 and 7. Next, the mice were administered dexamethasone (0.5 mg/kg in PBS) (Sigma-Aldrich) or PPY (5 $\mu\text{g/kg}$ in PBS) intraperitoneally 30 min prior to being challenged with aerosolized OVA on forty-two consecutive days (chronic model). Negative controls were injected and exposed to PBS ($n=6$ per group).

Eotaxin inhibition assay

Cells were plated at a density of 1×10^6 cells/ml in 6-well flat bottomed plates (Corning, New York, USA). Upon reaching confluency, the medium was changed with serum free RPMI, after which the samples were incubated for an additional 24 h. The cells were then stimulated with a combination of IL-1 β (10 ng/ml) (Biosource, Camarillo, CA, USA), TNF- α (100 ng/ml) (Biosource) and IL-4 (100 ng/ml) (Biosource) and then incubated for another 24 h. Next, various concentrations of PPY (0.01, 0.1, 1, 10, 100 $\mu\text{g/ml}$) were added to the medium, after which the cells were incubated for an additional 48 h (17). The cell-free culture supernatants were then collected and assayed for eotaxin using enzyme-linked immunosorbent assay kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions (18).

Chemotaxis assay

The human eosinophilic leukemia cell line, EoL-1, was obtained from the RIKEN cell bank (Wako Pure Chemical Industries, Tokyo, Japan). To ensure precise results, EoL-1 cells (19) were starved in serum-free media with 0.2% bovine serum albumin for one day prior to the assay. A549 cells were then stimulated with TNF- α (100 ng/ml), IL-4 (100 ng/ml) and IL-1 β (10 ng/ml) for 24 h, after which they were treated with various doses of PPY for 48 h. The supernatants were then used for a chemotaxis assay using a QCM_{TM} chemotaxis 96-well (5 μM) Cell Migration Kit (Millipore, Billerica, USA) according to the manufacturer's instructions. Briefly, 150 μl of the supernatants were added to the lower well of the kit. Next, the upper well was placed on the lower well and seeded with EoL-1 cells at a density of 1×10^4 cells/ml in 100 μl of RPMI media. Following incubation for 4 h, the media in the upper well was removed, after which a new lower well filled with 100 μl of cell detachment buffer was attached to the upper well for 30 min. Next, 75 μl of supernatant from the first lower well, 75 μl of cell detachment buffer (included in kit; Millipore, Billerica, USA) from second lower well, and 50 μl of dye solution (dye:lysis buffer = 1:75) were then added to a flat-bottomed well plate (total volume = 200 μl). Next, 150 μl of this mixture was transferred onto a well plate

appropriate for fluorescence reading. The absorbance at 484/527 nm was then measured using a Fluoroskan Ascent fluorometer (Labsystems, Helsinki, Finland). Each combination was assayed in triplicate.

Real time PCR analysis

Total cellular RNA was extracted using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocols, after which the concentration of total RNA was quantified by determining the optical density at 260 nm (OD 260). The reaction mixtures were then subjected to 40 cycles of denaturation at 95°C for 15 sec and annealing at 60°C for 1 min. Real-time PCR was performed on a GeneAmp 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I as the dsDNA-specific binding dye and continuous fluorescence monitoring (Table I).

MAPK and NF- κ B activation

Activation of NF- κ B and the MAPK phosphorylation state was analyzed using a Bio-Plex phosphoprotein assay and a total target protein assay (Bio-Rad, Hercules, CA, USA), respectively, according to the manufacturer's instructions. Briefly, A549 cell lysates were collected at 30 min, 1 h and 3 h using a Bio-Plex Cell Lysis Kit (20). To evaluate NF- κ B activation, nuclear extracts were prepared and the protein concentrations were adjusted to 600 μ g/ml. Fifty microliters of coupled beads that recognized phosphorylated and total ERK1/2, p38, JNK and NF- κ B were then added to the 96-well filter plate and then washed twice. The same volume of cell lysates was then added to the plate, and the samples were then incubated with the beads for 15-18 h. Next, 25 μ l of detection antibodies were washed and then added to the plates, which were subsequently incubated for 30 min. Fifty microliters of streptavidin-PE (included in kit, Bio-Rad) was then added, after which the samples were washed and incubated in the dark for 10 min. The samples were then rinsed, after which 125 μ l of resuspension buffer was added and the phosphorylation levels were then analyzed using a Luminex 100TM analyzer and the Bio-Plex Manager software (Bio-Rad).

Cell counts in bronchoalveolar lavage fluid (BALF)

The mice were sacrificed, after which bronchoalveolar lavage was conducted (21) by instilling 1 mL saline into the lungs through a tracheal cannula and then gently aspirating the fluid. This process was repeated three times, after which the total cell counts were determined by counting the cells in samples of bronchoalveolar lavage fluid (BALF) using a haemocytometer. In addition, the fluid samples were cytopun onto glass slides using a cytopsin centrifuge (Cellspin, Hanil, South Korea)

(400 $\times$ g for 4 min) and then stained with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI, USA). The BALF supernatant was stored at -70°C for subsequent evaluation of cytokines.

Measurement of BALF cytokines

The concentrated BALF supernatants were analyzed for the presence of IL-4, IL-5, IL-13 and IFN- γ using enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, Camarillo, CA, USA) as previously described (9). The levels of IgE in serum were measured by sandwich ELISA using Mouse Ovalbumin specific IgE Assay kit (AbD Serotec, Kidlington, UK) according to the manufacturer's instructions.

Lung histology

At 24 h after the last challenge, the mice were sacrificed and the lungs and trachea were removed (22). The tissues were then fixed with 4% (v/v) paraformaldehyde (Sigma Aldrich, St. Louis, MO, USA), after which the specimens were dehydrated and embedded in paraffin. For histological examination, 4 μ m sections of fixed embedded tissues were cut using a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and then sequentially stained with hematoxylin 2 and eosin-Y. Hyperplasia of goblet cells within the bronchial epithelium was assessed by counting cells in periodic acid Schiff (PAS)-stained section. For immunohistochemistry of smooth muscle actin (SMA), 4 μ m sections of lower trachea and lung tissue were treated with 0.3% H₂O₂-methanol for 20 min to block endogenous peroxidase; these were subsequently incubated at 4°C overnight with anti-SMA (Smooth Muscle Actin) mouse monoclonal antibody (1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing in PBS, the slide was treated with biotinylated secondary antibody for 20 min, streptavidin-horse radish peroxidase (HRP) for 20 min. Signals for immunoreactivity 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).

Statistical analysis

Statistical analysis of the data was conducted using the Prism 4.02 software (GraphicPad Software Inc. San Diego, CA, USA) and SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA). All data shown represent the mean S.E.M., and statistical analysis was performed using one-way ANOVA for multiple comparisons. The Mann-Whitney U test was applied to compare differences between two samples. In all cases, *P* values < 0.05 were

considered significant.

RESULTS

Characterization of 1H,8H-Pyrano[3,4-c]pyran-1, 8-dione

The extracted compound gave a molecular ion peak at m/z 165.0114 $[M+H]^+$ in the HREIMS, which corresponds to the elemental formula $C_8H_4O_4$. The UV spectrum of PPY exhibited absorption maxima at 256 nm, indicating the presence of aromatic conjugating system(s). The ^1H spectrum of this compound exhibited signals characteristic of *ortho*-coupled protons at δ_H 7.88 (2H, d, $J = 8.7$ Hz, H-3, H-6) and 6.82 (2H, d, $J = 8.7$ Hz, H-4, H-5), which were correlated with the ^{13}C NMR signals at δ_C 131.6 (C-3, C-6) and 114.6 (C-4, C-5) in the HSQC NMR spectrum. In addition, ester group signals at δ_C 168.7 (C-1, C-8) and quaternary carbon signals at δ_C 162.0 (C-4a) and 121.3 (C-8a) were also observed in the ^{13}C NMR spectrum. Furthermore, the HMBC correlations of H-3 and H-6/C-1, C-8, C-4, C-5, and C-4a indicated the presence of α -pyrones, and the HMBC correlations of H-4 and H-5/C-3, C-6, C-4a, and C-8a indicated that these α -pyrones were linked to C-4a and C-8a. Based on these findings, the structure of this compound was determined to be 1H,8H-Pyrano[3,4-c]pyran-1, 8-dione (Fig. 1). Although artificial synthesis of this compound has previously been reported (23), this chemical has not previously been isolated from natural sources.

Inhibition of eotaxin secretion by PPY

Human lung epithelial A549 cells were stimulated with TNF- α , IL-4 or IL-1 β to induce the expression of chemokines and adhesion molecules involved in eosinophil chemotaxis. We then used ELISA to investigate the suppression of eotaxin secretion by treatment with various concentrations of PPY. The results of the ELISA revealed that eotaxin secretion (Fig. 2) was decreased by 45 % in response to treatment with PPY at a concentration of 1 μ g/ml, and by over 70% in response to treatment with PPY at a concentration of 10 μ g/ml.

PPY inhibits eosinophil migration

Eosinophil migration was determined by measuring the optical density (OD) at 484/527 nm.

Table I. Sequences of primer used for real-time PCR.

Gene	Oligonucleotide Sequences
Hu GAPDH	FW 5'-TTC ACC ACC ATG GAG AAG GC-3' RV 5'-GGC ATG GAC TGT GGT CAT GA-3'
Hu Eotaxin	FW 5'-CAC ACC TTC AGC CTC CAA CAT G-3' RV 5'-CCA CTG GTG ATT CTC CTG TAG C-3'
Hu RANTES	FW 5'-CTG CCT CCC CAT ATT CCT CGG-3' RV 5'-GAG TTG ATG TAC TCC CGA ACC C-3'
Hu ICAM-1	FW 5'-TGA GGA GAG ATC ACC ATG GAGC-3' RV 5'-GCC AGG GAA CAG ACC ACG GT-3'
Hu VCAM-1	FW 5'-TTC ACG AGG CCA CCA CTC ATC-3' RV 5'-ATG ACT TCC AAG CTG GCC GTG-3'
Hu IL-8	FW 5'-TGT GTT GGC GCA GTG TGG TC-3' RV 5'-TGT GTT GGC GCA GTG TGG TC-3'
Hu IL-16	FW 5'-CTC GCT CAA CCT TTC AGA GCT-3' RV 5'-GAC ATG GAT GCC GTC TAA TTG C-3'
Mu GAPDH	FW 5'-ACA CAT TGG GGG TAG GAA CA-3' RV 5'-AAC TTT GGC ATT GTG GAA GG-3'
Mu eotaxin	FW 5'-CAG ATG CAC CCT GAA AGC CAT-3' RV 5'-TGC TTT GTG GCA TCC TGG AC-3'
Mu RANTES	FW 5'-TGC CCT CAC CAT CAT CCT CAC-3' RV 5'-GGC GGT TCC TTC GAG TGA CA-3'
Mu VCAM-1	FW 5'-ATG CCT GTG AAG ATG GTC GC-3' RV 5'-TGG TCA GAA CGG ACT TGG AC-3'
Mu ICAM-1	FW 5'-ATG GCT TCA ACC CGT GCC AA-3' RV 5'-AGC TTC CAG TTG GGT CCA CT-3'

The supernatant of pre-stimulated A549 cells that were not treated with PPY was used to normalize the OD value of other groups. A trans-well migration assay (Fig. 3) revealed that eosinophil (EoL, human eosinophil cell) migration toward the chamber containing the A549 cell media from cells that were treated with 10 μ g/ml PPY was reduced by up to 48%. These results indicate that PPY treatment of A549 cells leads to the suppression of eosinophil migration.

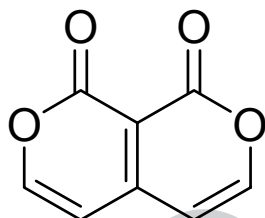
Chemokine and adhesion molecule expressions in A549 cells

We also measured the mRNA expression levels of eotaxin and eosinophil-related chemokines and adhesion molecules using real-time PCR. Treatment with PPY suppressed the Eotaxin and VCAM-1 mRNA expression by 46% and 37%, respectively (Fig. 4). In addition, IL-8 mRNA levels were decreased by approximately 30% in response to

Table II. Bronchoalveolar differential cell counts.

	Control	OVA	Dexamethasone	PPY
Total cells ($\times 10^4$)	5.33 $\pm$ 4.50	17.42 $\pm$ 7.27 [#]	11.00 $\pm$ 5.17	8.66 $\pm$ 2.30
Eosinophils ($\times 10^4$)	1.50 $\pm$ 0.84	5.43 $\pm$ 3.99 [#]	1.17 $\pm$ 0.98 [#]	1.00 $\pm$ 0.00*
Neutrophils ($\times 10^4$)	21.33 $\pm$ 20.43	13.71 $\pm$ 11.06	16.50 $\pm$ 7.64	1.33 $\pm$ 0.58*
Lymphocytes ($\times 10^4$)	17.33 $\pm$ 18.06	40.86 $\pm$ 21.28	7.00 $\pm$ 8.12 [#]	19.67 $\pm$ 13.87
Monocytes ($\times 10^4$)	459.83 $\pm$ 32.46	440.00 $\pm$ 25.86	475.33 $\pm$ 8.45	478.00 $\pm$ 13.45

Mice were treated with saline, OVA administered via inhalation plus saline (OVA), OVA administered via inhalation plus 0.5 mg/kg Dexamethasone (OVA+DEX) or OVA administered via inhalation plus 5 μ g/kg PPY (OVA+PPY). BAL cells were then separated using a Cytospin, after which they were stained with H&E. Differential cell counting was then performed using standard morphological criteria. Data are expressed as the mean $\pm$ S.E.M ($n=6$). SPSS version 10.0 (SPSS, Inc., Chicago, IL, USA) was used to perform the statistical analyses. The Mann-Whitney U test was applied to compare differences between two samples (control and OVA, OVA only and OVA + Dexamethasone, OVA only and OVA + PPY). * $P<0.05$, [#] $P<0.01$



1H,8H-Pyrano[3,4-c]pyran-1,8-dione

Fig. 1. Structure of 1H,8H-Pyrano[3,4-c]pyran-1,8-dione.

treatment with PPY at a concentration of 10 μ g/ml. Furthermore, IL-16 reduction was also reduced by 24% in response to treatment with PPY at a concentration of 10 μ g/ml. However, the levels of ICAM-1 and RANTES did not change.

PPY inhibits ERK1/2 and NF- κ B activation

To better understand the signal pathways involved in the activity of PPY, we used a Luminex 100TM analyzer and Bio-Plex Manager software to investigate the possible involvement of PPY in the MAPK (Fig. 5A) cascade. Specifically, we evaluated the effects of PPY on ERK1/2, p38 and the c-Jun N-terminal protein kinase (JNK), as well as activation of NF- κ B (Fig. 5B). The activation of NF- κ B and ERK1/2 was substantially suppressed in the presence of 10 μ g/ml PPY; however, no significant

changes were observed in the activation of JNK and p38. These results suggest that the inhibition of TNF- α , IL-4 and IL-1 β induced eotaxin secretion in response to PPY treatment is mediated through reduced activation of the ERK1/2 and NF- κ B signaling pathways in A549 cells.

Effects of PPY on Th1/Th2 cytokine production CD4⁺ T cells

To investigate the direct effect of PPY on Th1/Th2 specific cytokine production in CD4⁺ T cells, the amount of IL4, IL5, IFN- γ and TNF- α were quantitated in the supernatant from Th1/Th2 polarized CD4⁺ T cells. The level of IL-4 (Fig. 6) and IL-5 is significantly decreased in CD4⁺ T cells in Th2 skewed condition upon PPY treatment, while no significant changes were observed in the production of IFN- γ and TNF- α in Th1 condition. These results imply that PPY has a direct effect on Th2 lineage development of CD4⁺ T cell decreasing Th2 specific cytokine production.

Effect of PPY on BALF cytokines and OVA-specific serum IgE

The Th2 cytokines, including IL-4 and IL-5, are important with regard to the initiation and propagation of allergic inflammatory responses. Although Th2 cytokines promote airway inflammation in asthma, Th1 cytokines protect against allergic disease and

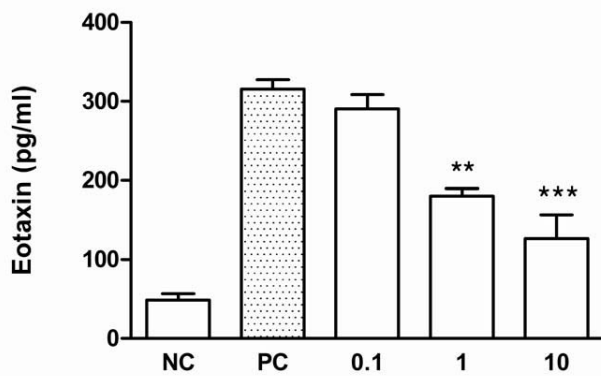


Fig. 2. Eotaxin inhibition assay of 1H,8H-Pyrano[3,4-c]pyran-1,8-dione in stimulated A549 cells. The amount of eotaxin in the supernatant was measured by ELISA. NC (negative control); unstimulated A549 cell supernatant, PC (positive control); cytokine-stimulated A549 cell supernatant. Data are presented as the mean $\pm$ S.E.M. *** $p < 0.001$ compared to control. ** $p < 0.01$ compared to control.

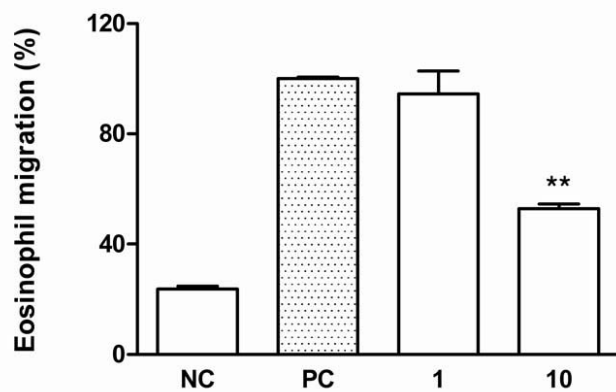


Fig. 3. PPY treatments lead to the suppression of eosinophil migration. A549 cells were stimulated by TNF- α , IL-4, and IL-1 β and then treated with PPY. The supernatants were then used for a chemotaxis assay as described in the materials and methods section. Data are presented as the mean $\pm$ S.E.M. C: control (TNF- α , IL-4, and IL-1 β stimulation. No PPY treatment) ** $p < 0.01$ compared to the control.

asthma. The levels of IL-4, IL-5, IL-13 and IFN- γ were determined by ELISA to determine whether treatment with PPY affects the secretion of cytokines in the BALF. As shown in Fig. 7A, treatment with PPY led to a 34% reduction in the amount of IL-5; however, the levels of IL-4, IFN- γ and IL-13 did not differ significantly in the treatment and the control

groups. Although the extent of the changes did not reach the statistically significant level, the amount of IL-4 and IL-13 resulted in a decreasing pattern. In addition, the OVA-specific IgE levels in serum were significantly inhibited by 43% compared to the control group (Fig. 7B)

Inhibitory effects of PPY on the influx of inflammatory cells in BALF

To evaluate the effect of PPY on the recruitment of cells into the airway, we investigated total lung cells, lymphocytes, neutrophils, macrophages and eosinophils in BALF. The total number of cells and eosinophils in the BALF of the OVA-challenged group were 3.4-fold and 3.6-fold (Table II) higher than those in the control group, respectively. And the number of eosinophils observed in the BALF of the PPY-treated group was inhibited by 82% compared to the OVA-challenged group.

Detection of mRNA from lung tissue by real time PCR

To investigate the effects of PPY on mRNA expression on lung tissue, total cellular RNA was extracted from lung cells that were treated with or without PPY in the presence or absence of OVA sensitization and inhalation. The eotaxin, VCAM-1, ICAM-1 and RANTES mRNA was then quantified by real time PCR analysis. The PPY exerted a strong suppressive effect on eotaxin and VCAM-1 mRNA expression (Fig. 8), however, the levels of ICAM-1 and RANTES did not differ significantly in the treatment and the control groups.

The effect of PPY on airway remodeling

Histological sections of lung tissue from mice that were exposed to OVA exhibited airway inflammation and were found to have infiltrating eosinophils in the peribronchial regions of the lung (Fig. 9A). Conversely, airway inflammation was decreased in histological sections of lung tissue obtained from mice that were treated with dexametasone and PPY. Periodic acid Schiff (PAS)-positive mucos-counting goblet cells around the bronchial airways were detected in the OVA-treated groups. On the other hand, PPY treatment substantially decreased PAS-positive goblet cells around the bronchial airway (Fig. 9B). In addition,

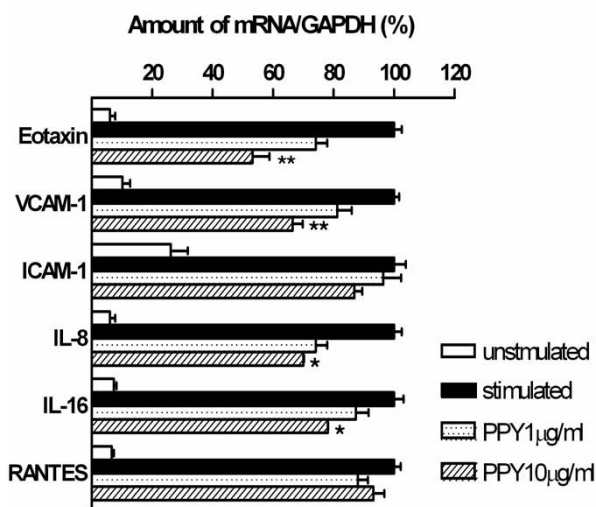


Fig. 4. Effects of PPY on mRNA levels as determined by real-time RT-PCR. Real-time PCR assays were conducted to quantify the mRNA levels of eotaxin, RANTES, IL-8, IL-16, ICAM-1 and VCAM-1. The expression rates were normalized using GAPDH mRNA expression as a control. NC: negative control (unstimulated A549 cells), PC: positive control (TNF- α , IL-4 and IL-1 β stimulated A549 cells). Data are presented as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. positive control as determined by one-way ANOVA.

OVA-challenged mice showed expression of SMA in the peribronchial muscle layer of the lung, and PPY treatment abrogated the expression of these proteins (Fig. 9C). These findings demonstrate that PPY has a potential to be effective on the airway remodeling.

DISCUSSION

TNF- α and IL-1 β are key regulators of immune and inflammatory genes (24-25). Conversely, IL-4 is a pivotal cytokine that is produced by Th2 cells and associated with allergic diseases that support Th2 development (26-27). In this study, we demonstrated that eotaxin, RANTES, ICAM-1, VCAM-1, IL-8 and IL-16 mRNA expression is induced in A549 cells that have been stimulated with the pro-inflammatory cytokines, TNF- α , IL-4 and IL-1 β . However, only eotaxin and VCAM-1 mRNA expression decreased significantly in response to treatment with PPY both in vitro and in vivo (28). The expression of eotaxin is regulated by mitogen-activated protein kinases (MAPK). In addition to eotaxin regulation, the

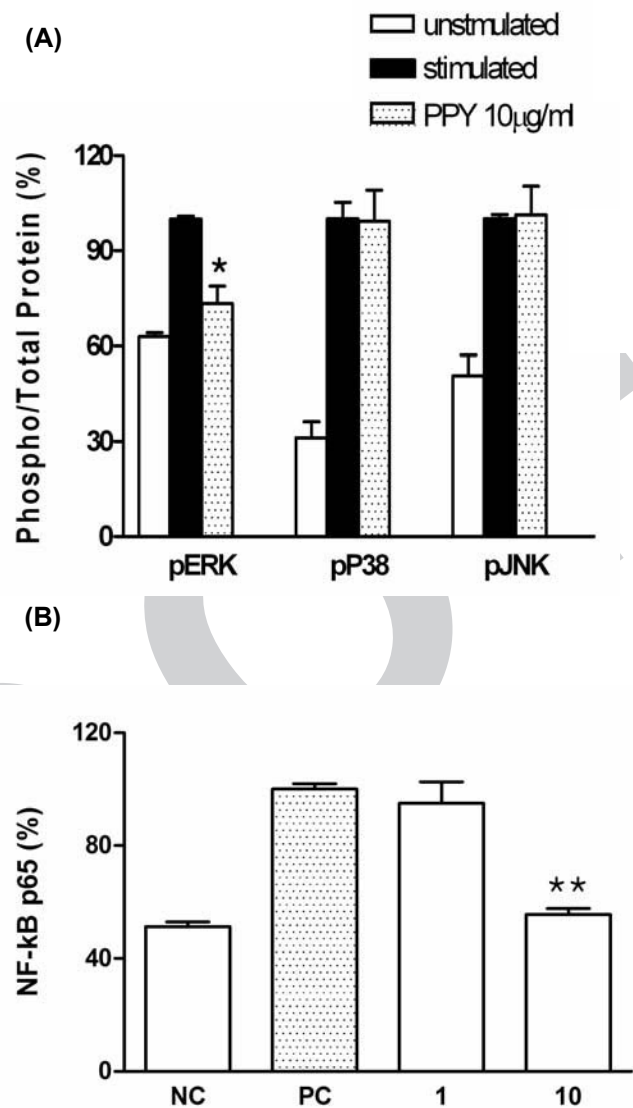


Fig. 5. Multiplex analysis of MAPK (A) and NF- κ B (B) activation. Serum-starved A549 cells were treated with TNF- α , IL-4, and IL-1 β and then treated with PPY (10 μ g/ml) for 3 h. The cell lysates were then harvested using a Bio-Plex Cell Lysis Kit. The fluorescence intensity from antibody coupled beads that recognized phosphorylated and total ERK1/2, p38, JNK and NF- κ B were analyzed using a Luminex 100TM analyzer. NC (negative control); supernatant from un-stimulated A549 cells, PC (positive control); supernatant from cytokine-stimulated A549 cells. Data are presented as the mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$ vs. positive control as determined by one-way ANOVA.

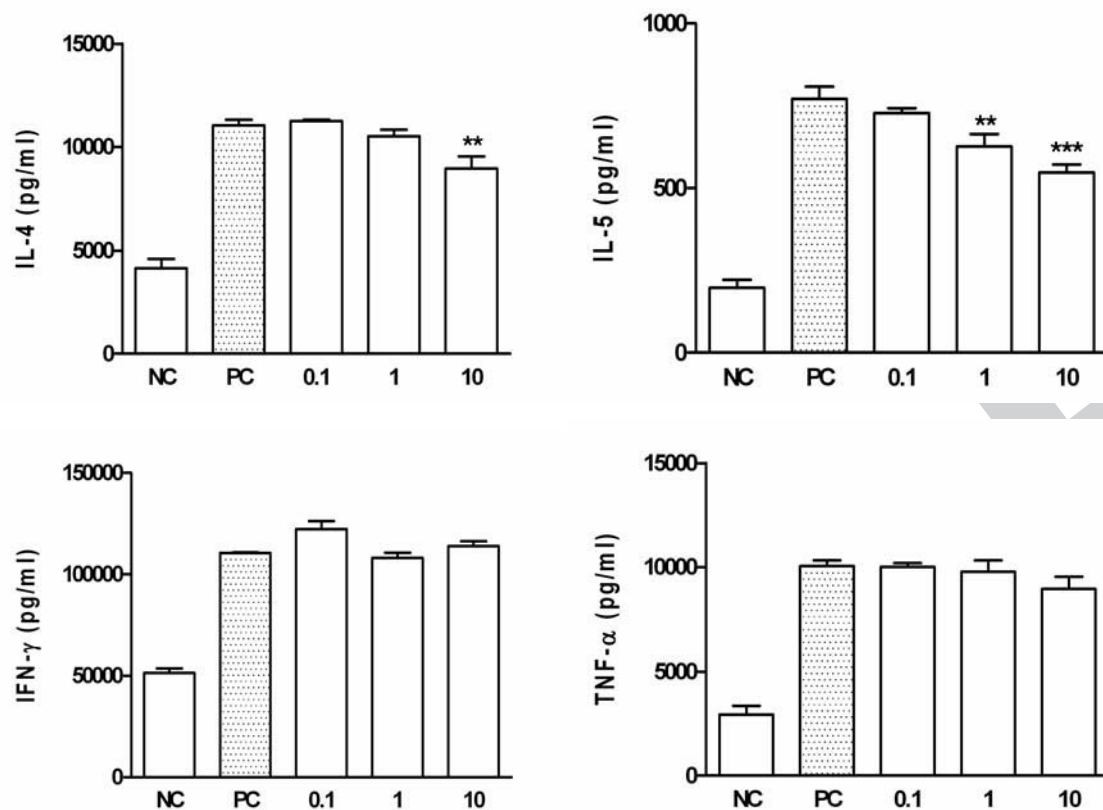


Fig. 6. Effects of PPY on cytokine production from Th1/Th2 cells. Naïve CD4⁺ T cells were incubated with rIL-12 and anti-IL-4 Ab for Th1 differentiation. For Th2 cell differentiation, rIL-4 and anti-IL-12 Ab were added to the culture medium in the presence of rIL-2. T cells were then incubated in the absence or presence of PPY (0.1, 1, 10 μ g/ml). CD4⁺ T cells were activated with plate-bound 10 μ g/ml anti-CD3 mAb and 2 μ g/ml anti-CD28 Ab. The amounts of cytokine in the medium were quantitated using ELISA. Data are presented as the mean $\pm$ S.E.M. * P <0.05, ** P <0.01 vs. control as determined by one-way ANOVA.

MAPK cascade participates in airway remodeling by inducing many other chemokines. The MAPK superfamily has been divided into three groups, the extracellular signal-regulated kinases, p42/p44 (ERK) and p38/MAPK (HOG), and the c-Jun N-terminal kinase/stress activated protein kinase (JNK/SAPK). Eotaxin is induced by cytokine stimulation via NF- κ B and p38/MAPK (14), whereas ICAM-1 is induced via ERK and JNK (29) and VCAM-1 is induced via NF- κ B and p38/MAPK. It is important to note that the factors that PPY regulates, eotaxin and VCAM-1, are both regulated by the NF- κ B signaling pathway. However, there is a series of reports demonstrating that the expression of ICAM-1 and RANTES were also regulated by NF- κ B (36-38). Therefore, the detailed mechanisms of PPY on

controlling only a few inflammatory related genes downstream of NF- κ B remain to be elucidated.

The eotaxin level is associated with the degree of subepithelial fibrosis and eosinophilic airway inflammation. Although eotaxin recruits eosinophils in asthma and other allergic diseases, it also induces eosinophil migration into the bronchoalveolar space and the peritoneal cavity, which is a hallmark of allergic disease. Therefore, we performed a chemotaxis assay to evaluate the inhibition of eosinophil migration by PPY. As expected, eosinophil migration was significantly inhibited by treatment with PPY. We also demonstrated that the pharmacological effect of PPY is mediated by blocking activation of the NF- κ B and ERK1/2 signaling pathways. Taken together, these results

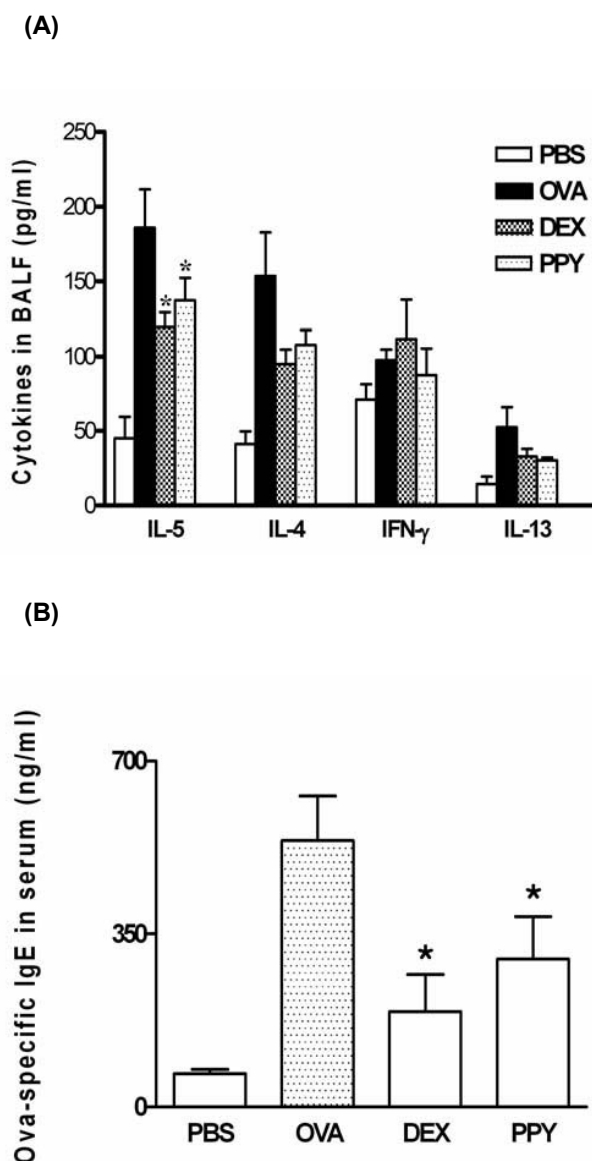


Fig. 7. Effects of PPY on cytokines and the immunoglobulin E level in BALF from an OVA-induced murine model of asthma. BALB/C mice were administered various treatments via injection and then administered OVA via inhalation for 14 days to induce asthma. At the end of the experiment, the BALF was collected and the cytokine production was analyzed by ELISA. In addition, blood was collected by cardiac puncture and the levels of OVA-specific IgE in the samples were then evaluated by ELISA. (A) Cytokines in BALF (B) OVA-specific IgE in serum. PBS: Normal BALB/C mice, OVA: Ovalbumin inhalation (control), DEX: OVA + Dexamethasone (0.5mg/kg), PPY: OVA + PPY (5 μ g/kg). Data are presented as the mean $\pm$ S.E.M (n=6). * P <0.05 vs. positive control as determined by one-way ANOVA.

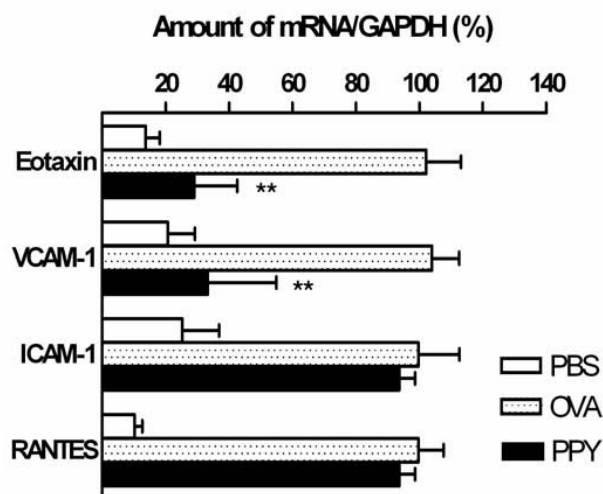


Fig. 8. Effects of PPY on eotaxin, RANTES, VCAM-1 and ICAM-1 mRNA expression in the lung tissue collected from an OVA-induced murine model of asthma. BALB/C mice were treated by injection and then administered OVA via inhalation for 14 days to induce asthma as described in the materials and methods section. At the end of the experiment, the lungs were removed from the mice and analyzed by real-time PCR. PBS: Normal BALB/C mice (Control), OVA: Ovalbumin inhalation, PPY: OVA + PPY (5 μ g/kg). Data are presented as the mean $\pm$ S.E.M (n=6). ** P <0.01 vs. positive control as determined by one-way ANOVA.

strongly suggest that PPY has the potential for use in the treatment of asthma and allergic disease through inhibition of eotaxin secretion and eosinophil migration into the airway epithelium (30-31).

Bronchial asthma is characterized by reversible airway obstruction in response to allergens, chronic eosinophilic airway inflammation, and non-specific airway hyper-responsiveness (AHR) (32). In this study, we used an OVA-induced murine model of allergic airway disease and found an increase in the number of eosinophils in BALF, a marked influx of inflammatory cells into the lung around blood vessels and airways, airway luminal narrowing, the development of AHR, the detection of IFN- γ and Th2 cytokines, including IL-4 and IL-5 in the BALF, and the presence of allergen-specific IgE in the serum of control mice. In many cases, IL-4 is capable of inducing the rolling and adhesion of circulating eosinophils to endothelial cells. These

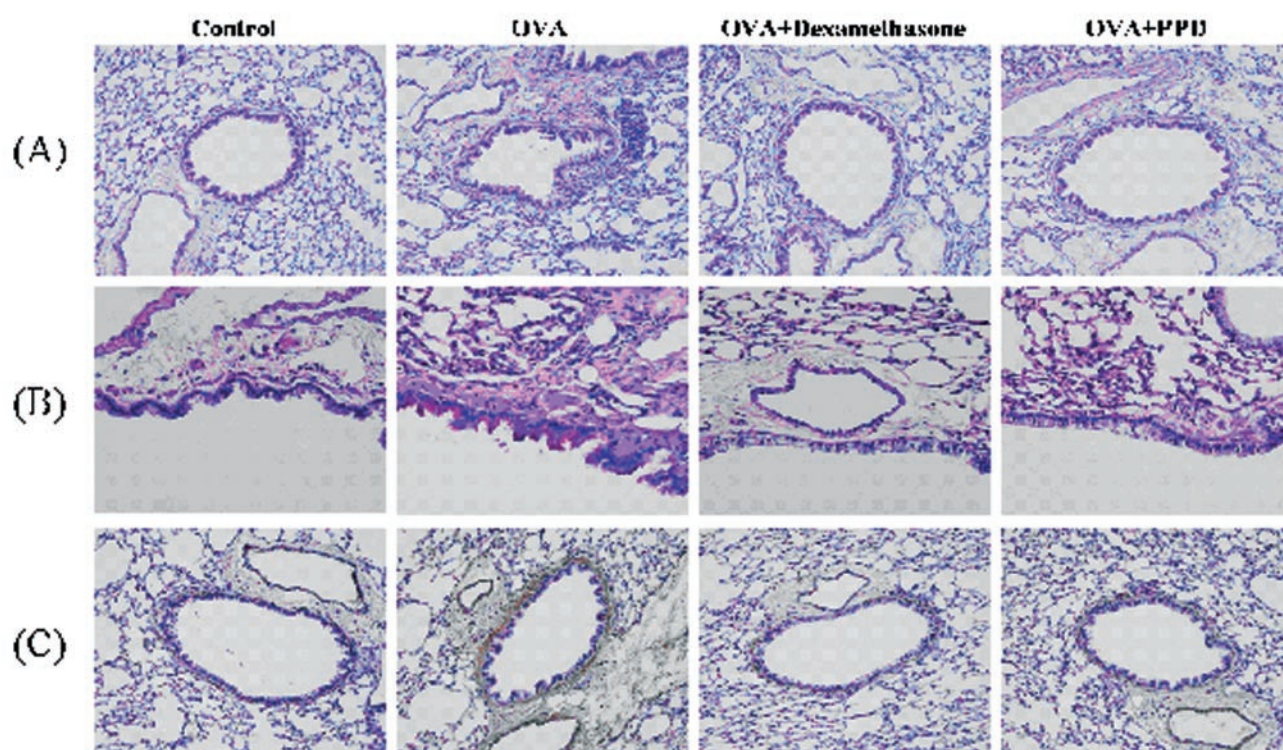


Fig. 9. Effects of PPY on the airway remodeling of lung tissue [H&E (A), PAS (B) and SMA immunohistochemistry (C)] in OVA-induced mice. Sections were obtained from the lungs of mice that were treated with PBS (Control), OVA, OVA plus 0.5mg/kg Dexamethasone and OVA plus 5 μ g/kg PPY ($\times 200$).

eosinophils can then be attracted to target tissues by both IL-5 and chemokines (33-34). In addition, IL-4 and IL-13 are required for AHR induction, which occurs in response to the induction of allergen-specific IgG1 and IgE production. Mice lacking IL-5 (IL-5 knockout mice) (35) fail to develop airway eosinophilia in response to allergen exposure unless the animals are reconstituted with IL-5, which demonstrates that IL-5 is crucial for the induction of airway eosinophilia. Furthermore, treatment of wild-type mice with a neutralizing anti-IL-5 Ab (35) strongly reduces the eosinophilic inflammatory response induced by allergens. Taken together, these findings indicate that the inhibitory mechanisms of PPY on IL-4 and IL-5 production may involve suppression of the eosinophil influx into the airways. In addition, the administration of PPY resulted in down-regulation of the Th2 cytokines, IL-4 and IL-5, in Th2 skewed conditions; however, there was no significant increase in levels of the Th1 cytokine,

IFN- γ , TNF- α . Although further studies will be required to elucidate the effect of PPY on the Th1-Th2 balance, these results suggest that treatment with PPY could suppress the Th2 response, at least partially, without augmenting the Th1 response. In addition, these results indicate that the impairment of Th2 cytokine production in response to treatment with PPY may be related to a decreased level of eosinophils in BALF and a decreased number of leukocytes in the lung tissue.

In conclusion, the administration of PPY prior to the final airway OVA challenge resulted in a significant inhibition of characteristic asthmatic reactions. However, the blood cell sub-population did not change significantly in response to treatment with PPY (data not shown). Furthermore, PPY did not have any cytotoxicity in all tested concentrations on A549 cells (data not shown) and there were no signs of liver or kidney dysfunction in the PPY-treated mice (data not shown). Therefore, these

results indicate that PPY has the potential for use as a new therapeutic drug for the treatment of allergic asthma.

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REFERENCES

1. Elsner J, Escher SE, Forssmann U. Chemokine receptor antagonists: a novel therapeutic approach in allergic diseases. *Allergy* 2004; 59:1243-58.
2. Factor P. Gene therapy for asthma. *Mol Ther* 2003; 7: 148-52.
3. Kay AB. Asthma and inflammation. *J Allergy Clin Immunol* 1991; 87:893-910.
4. Ponath PD, Qin S, Ringler DJ, et al. Cloning of the human eosinophil chemoattractant, eotaxin. Expression, receptor binding, and functional properties suggest a mechanism for the selective recruitment of eosinophils. *J Clin Invest* 1996; 97: 604-12.
5. Jose PJ, Griffiths-Johnson DA, Collins PD, Walsh DT, Moqbel R, Totty NF, Truong O, Hsuan JJ, Williams TJ. Eotaxin: a potent eosinophil chemoattractant cytokine detected in a guinea pig model of allergic airways inflammation. *J Exp Med* 1994; 179:881-7.
6. Djukanovic R, Roche WR, Wilson JW, Beasley CR, Twentyman OP, Howarth RH, Holgate ST. Mucosal inflammation in asthma. *Am Rev Respir Dis* 1990; 142:434-57.
7. Djukanovic R, Wilson JW, Britten KM, Wilson SJ, Walls AF, Roche WR, Howarth PH, Holgate ST. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am Rev Respir Dis* 1992; 145:669-74.
8. Sa-Nunes A, Rogerio AP, Medeiros AI, Fabris VE, Andreu GP, Rivera DG, Delgado R, Faccioli LH. Modulation of eosinophil generation and migration by *Mangifera indica* L. extract (Vimang). *Int Immunopharmacol* 2006; 6:1515-23.
9. Kim J, Lee H, Lee Y, et al. Inhibition effects of Moutan Cortex Radicis on secretion of eotaxin in A549 human epithelial cells and eosinophil migration. *J Ethnopharmacol* 2007; 114:186-93.
10. Chung HS, Kang M, Cho C, et al. Inhibition of nitric oxide and tumor necrosis factor-alpha by moutan cortex in activated mouse peritoneal macrophages. *Biol Pharm Bull* 2007; 30:912-6.
11. Matsukura S, Stellato C, Plitt JR, Bickel C, Miura K, Georas SN, Casolaro V, Schleimer RP. Activation of eotaxin gene transcription by NF-kappa B and STAT6 in human airway epithelial cells. *J Immunol* 1999; 163:6876-83.
12. Jedrzkiewicz S, Nakamura H, Silverman ES, Luster AD, Mansharamani N, In KH, Tamura G, Lilly CM. IL-1beta induces eotaxin gene transcription in A549 airway epithelial cells through NF-kappaB. *Am J Physiol Lung Cell Mol Physiol* 2000; 279:L1058-65.
13. Wong CK, Zhang JP, Ip WK, Lam CW. Activation of p38 mitogen-activated protein kinase and nuclear factor-kappaB in tumour necrosis factor-induced eotaxin release of human eosinophils. *Clin Exp Immunol* 2002; 128:483-9.
14. Wong CK, Wang CB, Ip WK, Tian YP, Lam CW. Role of p38 MAPK and NF-kB for chemokine release in coculture of human eosinophils and bronchial epithelial cells. *Clin Exp Immunol* 2005; 139:90-100.
15. Ward C, Chilvers ER, Lawson MF, Pryde JG, Fujihara S, Farrow SN, Haslett C, Rossi AG. NF-kappaB activation is a critical regulator of human granulocyte apoptosis in vitro. *J Biol Chem* 1999;274:4309-18.
16. Shin TY, Kim SH, Lim JP, et al. Effect of *Vitex rotundifolia* on immediate-type allergic reaction. *J Ethnopharmacol* 2000; 72:443-50.
17. Cheng G, Ueda T, Eda F, Arima M, Yoshida N, Fukuda T. A549 cells can express interleukin-16 and stimulate eosinophil chemotaxis. *Am J Respir Cell Mol Biol* 2001; 25:212-8.
18. Sanmugalingham D, De Vries E, Gauntlett R, Symon FA, Bradding P, Wardlaw AJ. Interleukin-5 enhances eosinophil adhesion to bronchial epithelial cells. *Clin Exp Allergy* 2000; 30:255-63.
19. Terakawa M, Tomimori Y, Goto M, Hayashi Y, Oikawa S, Fukuda Y. Eosinophil migration induced by mast cell chymase is mediated by extracellular

- signal-regulated kinase pathway. *Biochem Biophys Res Commun* 2005; 332:969-75.
20. Mu H, Ohashi R, Yan S, Chai H, Yang H, Lin P, Yao Q, Chen C. Adipokine resistin promotes in vitro angiogenesis of human endothelial cells. *Cardiovasc Res* 2006; 70:146-57.
 21. Lai WQ, Goh HH, Bao Z, Wong WS, Melendez AJ, Leung BP. The role of sphingosine kinase in a murine model of allergic asthma. *J Immunol* 2008; 180: 4323-9.
 22. Lee KS, Lee HK, Hayflick JS, Lee YC, Puri KD. Inhibition of phosphoinositide 3-kinase delta attenuates allergic airway inflammation and hyperresponsiveness in murine asthma model. *Faseb J* 2006; 20:455-65.
 23. Creak GA, Liew SE, Tan SF. Pyranopyrandiones: a molecular orbital study. *Journal of the Singapore National Academy of Science* 1973; 3:223-226.
 24. Vecchiarelli A, Siracusa A, Cenci E, Puliti M, Abbritti G. Effect of corticosteroid treatment on interleukin-1 and tumour necrosis factor secretion by monocytes from subjects with asthma. *Clin Exp Allergy* 1992; 22:365-70.
 25. Lilly CM, Nakamura H, Kesselman H, et al. Expression of eotaxin by human lung epithelial cells: induction by cytokines and inhibition by glucocorticoids. *J Clin Invest* 1997; 99:1767-73.
 26. Seder RA, Paul WE, Ben-Sasson SZ, LeGros GS, Kagey-Sobotka A, Finkelman FD, Pierce JH, Plaut M. Production of interleukin-4 and other cytokines following stimulation of mast cell lines and in vivo mast cells/basophils. *Int Arch Allergy Appl Immunol* 1991; 94:137-40.
 27. Broide D, Sriramarao P. Eosinophil trafficking to sites of allergic inflammation. *Immunol Rev* 2001; 179:163-72.
 28. Bentley AM, Durham SR, Robinson DS, Menz G, Storz C, Cromwell O, Kay AB, Wardlaw AJ. Expression of endothelial and leukocyte adhesion molecules interacellular adhesion molecule-1, E-selectin, and vascular cell adhesion molecule-1 in the bronchial mucosa in steady-state and allergen-induced asthma. *J Allergy Clin Immunol* 1993; 857-68.
 29. Krunkosky TM, Jarrett CL. Selective regulation of MAP kinases and chemokine expression after ligation of ICAM-1 on human airway epithelial cells. *Respir Res* 2006; 7:12.
 30. Halden Y, Rek A, Atzenhofer W, Szilak L, Wabnig A, Kungl AJ. Interleukin-8 binds to syndecan-2 on human endothelial cells. *Biochem J* 2004; 377:533-8.
 31. Pease JE, Williams TJ. Eotaxin and asthma. *Curr Opin Pharmacol* 2001; 1:248-53.
 32. Gonzalo JA, Lloyd CM, Kremer L, Finger E, Martinez AC, Siegelman MH, Cybulsky M, Gutierrez-Ramos JC. Eosinophil recruitment to the lung in a murine model of allergic inflammation. The role of T cells, chemokines, and adhesion receptors. *J Clin Invest* 1996; 98:2332-45.
 33. Bochner BS, Schleimer RP. The role of adhesion molecules in human eosinophil and basophil recruitment. *J Allergy Clin Immunol* 1994; 94:427-38.
 34. Zhu Z, Homer RJ, Wang Z, Chen Q, Geba GP, Wang J, Zhang Y, Elias JA. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin Invest* 1999; 103:779-88.
 35. Tanaka H, Kawada N, Yamada T, Kawada K, Takatsu K, Nagai H. Allergen-induced airway inflammation and bronchial responsiveness in interleukin-5 receptor alpha chain-deficient mice. *Clin Exp Allergy* 2000; 30:874-81.
 36. Kudo T, Lu H, Wu JY, Graham DY, Casola A, Yamaoka Y. Regulation of RANTES promoter activation in gastric epithelial cells infected with *Helicobacter pylori*. *Infect Immun* 2005; 73:7602-12.
 37. Holden NS, Catley MC, Cambridge LM, Barnes PJ, Newton R. ICAM-1 expression is highly NF-kappaB-dependent in A549 cells. No role for ERK and p38 MAPK. *Eur J Biochem* 2004; 271:785-91.
 38. Newton R, Holden NS, Catley MC, Oyelusi W, Leigh R, Proud D, Barnes PJ. Repression of inflammatory gene expression in human pulmonary epithelial cells by small-molecule IkappaB kinase inhibitors. *J Pharmacol Exp Ther* 2007; 321:734-42.