



The effects of the standardized herbal formula PM014 on pulmonary inflammation and airway responsiveness in a murine model of cockroach allergen-induced asthma



Kyung-Hwa Jung^{a,1}, Hei-Lim Choi^{b,1}, Soojin Park^a, Geunhyeog Lee^c, Miran Kim^c, Joon-Ki Min^b, Byung-Il Min^b, Hyunsu Bae^{a,d,*}

^a Department of Physiology, College of Korean Medicine, Kyung Hee University, #1 Hoekidong, Dongdaemoon-ku, Seoul 130-701, Republic of Korea

^b Department of East-West Medicine, Kyung Hee University, #1 Hoekidong, Dongdaemoon-ku, Seoul 130-701, Republic of Korea

^c Central Research Institute, Hanlim Pharm. Co. Ltd., 1007 Yoobang Dong, Yongin, Kyunggi Do, Republic of Korea

^d Institute of Korean Medicine, Kyung Hee University, #1 Hoekidong, Dongdaemoon-ku, Seoul 130-701, Republic of Korea

ARTICLE INFO

Article history:

Received 27 September 2013

Received in revised form

24 March 2014

Accepted 17 April 2014

Available online 28 May 2014

Keywords:

Asthma

Cockroach

Airway hyperresponsiveness

PM014

ABSTRACT

Ethnopharmacological relevance: PM014 is a modified form of the Chung-Sang-Bo-Ha-Tang (CSBHT) herbal formula that has been used to treat chronic pulmonary diseases in Korea for centuries. Previously, we developed a formulation of PM014 based on a series of in vitro and in vivo screening efforts that comprises seven herbal extracts. The PM014 formula includes the root of *Rehmannia glutinosa*, the cortex of *Paeonia suffruticosa*, the fruit of *Schizandra chinensis*, the root of *Asparagus cochinchinensis*, seeds of *Prunus armeniaca*, the root of *Scutellaria baicalensis* and the root of *Stemona sessilifolia*. Asthma is a chronic inflammatory disease of the lungs that is characterized by wheezing, bronchial contraction, and chest tightness. In addition, the airway becomes hypersensitive and narrows through an inflammatory reaction mediated by Th2 cells. The present study was conducted to evaluate the ability of PM014 to prevent allergic airway inflammation and to attenuate airway responses in a cockroach allergen-induced mouse model.

Materials and methods: Mice sensitized to and challenged with cockroach allergen were treated with oral administration of PM014. Airway resistance was determined by whole body plethysmography. In addition, Th2 cytokines and immune cell profiles of bronchoalveolar lavage (BAL) fluid and inflammatory mediators in serum were analyzed by ELISA. A series of histological examinations were also conducted to demonstrate the effects of PM014 on airway remodeling, goblet cell hyperplasia and inflammatory responses in the lung.

Results: PM014 significantly inhibited the number of total cells, eosinophils, neutrophils, macrophages and lymphocytes in the BAL fluid of mice that were challenged with cockroach allergen. In addition, PM014 reduced the levels of Th2 cytokines (IL-4, IL-5 and IL-13) in the BAL fluid and inflammatory mediators such as IgE in the serum, as measured by enzyme-linked immunosorbent assay (ELISA). Histopathological analysis also showed that PM014 substantially inhibited eosinophil infiltration into the airway, goblet cell hyperplasia and smooth muscle hypertrophy.

Conclusions: In this study, our results indicate that PM014 has significant effects on allergic airway inflammation upon exposure to cockroach allergen in a mouse model. According to these outcomes, PM014 may have therapeutic potential as a treatment for allergic asthma.

© 2014 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Asthma is a chronic lung disease of the airway that affects approximately 300 million adults and children worldwide (Braman, 2006). The clinical symptoms of asthma are characterized by wheezing, coughing, shortness of breath, and chest tightness. These typical symptoms and functional abnormalities are due to lung inflammation and airway remodeling (Noble et al., 2012). Asthma is a form of immune dysfunction

* Corresponding author at: Department of Physiology, College of Korean Medicine, Kyung Hee University, #1 Hoeki-dong Dongdaemoon-ku, Seoul, 130-701, Republic of Korea. Tel.: +822 961 9316; fax: +822 962 9316.

E-mail addresses: jkhk242@naver.com (K.-H. Jung), estella277@hanmail.net (H.-L. Choi), ih_sj@naver.com (S. Park), duddms0910@naver.com, coolman@hanlim.com (G. Lee), kmr@hanlim.com (M. Kim), drminjk@naver.com (J.-K. Min), mbi@khu.ac.kr (B.-I. Min), hbae@khu.ac.kr (H. Bae).

¹ These authors contributed equally to this work.

(Tsujiyama et al., 2013). Asthma is a multi-cellular process involving helper T cells, eosinophils, mast cells and neutrophils (Wardlaw et al., 2000, 2002; Brightling et al., 2003; Humbles et al., 2004; Holgate, 2009). Additional, a key feature of allergic asthma is the recognition of allergens and subsequent sensitization that leads to a T helper type 2 (Th2) cytokine response (Desai and Brightling, 2009). Dendritic cells in the airway epithelium and submucosa engulf and process allergens and present them to T cells in association with several important co-stimulatory molecules (Matsuse et al., 2012). Subsequent T cell polarization toward a Th1 or Th2 phenotype is partially under the influence of dendritic cell-derived interleukin (IL)-12. Increased IL-12 levels drive the inflammatory response toward a Th1 bias, whereas the Th2 phenotype predominates in allergic asthma. Th2 cells play a central role in controlling the allergic response through the production of cytokines such as interleukins IL-4, IL-5, and IL-13 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Larche et al., 2003). These cytokines regulate the main processes involved in immunoglobulin (IgE) production and lead to the differentiation and activation of eosinophils (Bax et al., 2012). Eosinophils are involved in the structural changes that occur in the airways of asthma patients, including goblet cell hyperplasia, enlarged submucosal mucus glands, and increased airway smooth muscle (ASM) mass. The pathogenesis described above leads to bronchial hyperresponsiveness and causes airflow obstruction (Kawakami and Kita, 2005; Lee et al., 2009).

Herbal medicine has a long history of use in humans in Asian countries and is well received by patients. Chung-Sang-Bo-Ha-Tang (CSBHT) has especially been used for centuries to treat lung diseases such as asthma in Korea (Roh et al., 2005). However, CSBHT contains 18 species of medicinal herbs, and it is difficult to standardize the herbal formula. Therefore, CSBHT was modified into the PM014 formula, which contains 7 species of medicinal herbs and was proven effective in a murine chronic obstructive pulmonary disease (COPD) model in a previous study (Lee et al., 2012).

In this study, we investigated the effects of PM014 on allergic airway inflammation via a cockroach allergen-induced mouse model. Cockroach allergens are important sensitizing agents that may contain significant allergenic activity and are a leading cause of allergic asthmatic allergic exacerbation in many parts of the world (Rosenstreich et al., 1997). Several studies have indicated that childhood exposure to cockroach allergens led to the development of specific allergic sensitization, risk for persistent asthma and bronchial hyper-responsiveness with a significant loss of lung function (Platts-Mills et al., 2000). In this regard, a strong relationship between indoor allergic sensitization and the exacerbation of asthma symptoms has been demonstrated for cockroach allergens (Litonjua et al., 2001; Arizmendi et al., 2011). The results of our study showed the effects of PM014 in terms of immunological changes such as decreased Th2 cytokines, serum immunoglobulin levels and histopathological changes of lung tissues in a mouse model of cockroach allergen-induced airway inflammation response.

2. Materials and methods

2.1. Reagents

PM014 containing 7 species of medicinal herbs was purchased from Kyung Hee Herb Pharm (Seoul, South Korea). Each herb in PM014 was cut and mixed in a total amount of 2100 g according to the ratios indicated in Table 1.

The mixture was extracted with distilled water (2100 mL) under reflux for 3 h at 90–100 °C and then filtered through a

Table 1

Formula of PM014.

Formula of PM014	Amount (g)	Chemical marker
Root of <i>Rehmannia glutinosa</i>	600	5-HMF
Cortex of <i>Paeonia suffruticosa</i>	300	Paeoniflorin
Fruit of <i>Schizandra chinensis</i>	300	Schizandrin
Root of <i>Asparagus cochinchinensis</i>	300	Asparagine
Seed of <i>Prunus armeniaca</i>	225	Amygdalin
Root of <i>Scutellaria baicalensis</i>	225	Baicalin
Root of <i>Stemona sessilifolia</i>	150	Stemonine
Total	2100	

25 µm sieve. The residue was successively re-extracted with distilled water (1680 mL) using the same method. Whole supernatant was concentrated at 60 °C under a vacuum using an evaporating system and vacuum dried to 70 brix (1254 g). Viscous extracts were mixed with 260 g of dry cornstarch and subsequently vacuum dried at 60 °C. Finally, 1103 g (yield 40%) of dried extract powder was obtained. For quality assurance, four standard materials were selected to represent the herbal medicines in PM014 and were analyzed by HPLC (Fig. 1). Three different batches of PM014 were quantified in triplicate (Table 2). After considering the variation of the standard materials in different batches of PM014, the minimum quantities of standard material in PM014 (1 g) were determined to be Paeoniflorin > 0.43 mg, Schizandrin > 0.12 mg, Baicalin > 7.26 mg, and Amygdalin > 2.48 mg.

HPLC analysis was performed to quantify standard materials in PM014 as shown in supplemental Fig. 1. Each compound was analyzed for three independent batches with triplicate data. Furthermore, the standardized herbal formula of PM014 has been approved for the Investigating New Drug program by the Ministry of Food and Drug Safety, Republic of Korea (ID:20130030575).

2.2. Animals

Balb/c mice (6–7 weeks of age weighing 20–25 g) were purchased from Charles River Korea (Seoungnam, South Korea). All mice were kept under pathogen-free conditions in an air conditioned environment with a 12 h light/dark cycle. In addition, all mice had free access to food and water during these experiments. This experimental study was approved by the Institutional Animal Care and Use Board of Kyung Hee University (KHUASP (SE)-12-005).

2.3. Induction of airway inflammation and animal treatment

The experimental schedule was performed according to the previous study (Choi et al., 2013). Briefly, the mice were sensitized by intraperitoneal (i.p.) injections with 10 mg of cockroach allergen (CKA) (Hollister-Stier, Spokane, WA, U.S.A.) in incomplete Freund's adjuvant (Sigma-Aldrich, St. Louis, MO, U.S.A.) on days 0 and 14. Subsequently, mice received an intratracheal (i.t.) challenge with cockroach allergen (1% CKA in phosphate-buffered saline [PBS]) on days 28–30 and 5% CKA in PBS on day 31.

CKA-sensitized mice were treated with PM014 (50, 100 and 200 mg/kg body wt) by oral administration 2 h before CKA challenge. The negative control (CON) and CKA-challenged (CKA) groups were treated only with PBS by oral administration, and the positive control group was treated with Montelukast sodium (10 mg/kg body wt) suspended in PBS by oral administration 2 h before CKA challenge. Mice were analyzed using non-invasive lung function measurements (All Medicus, Seoul, Korea) to assess AHR. On day 35, mice were sacrificed and their lung tissues were collected for other analyses. These experiments were performed twice to clarify that PM014 has

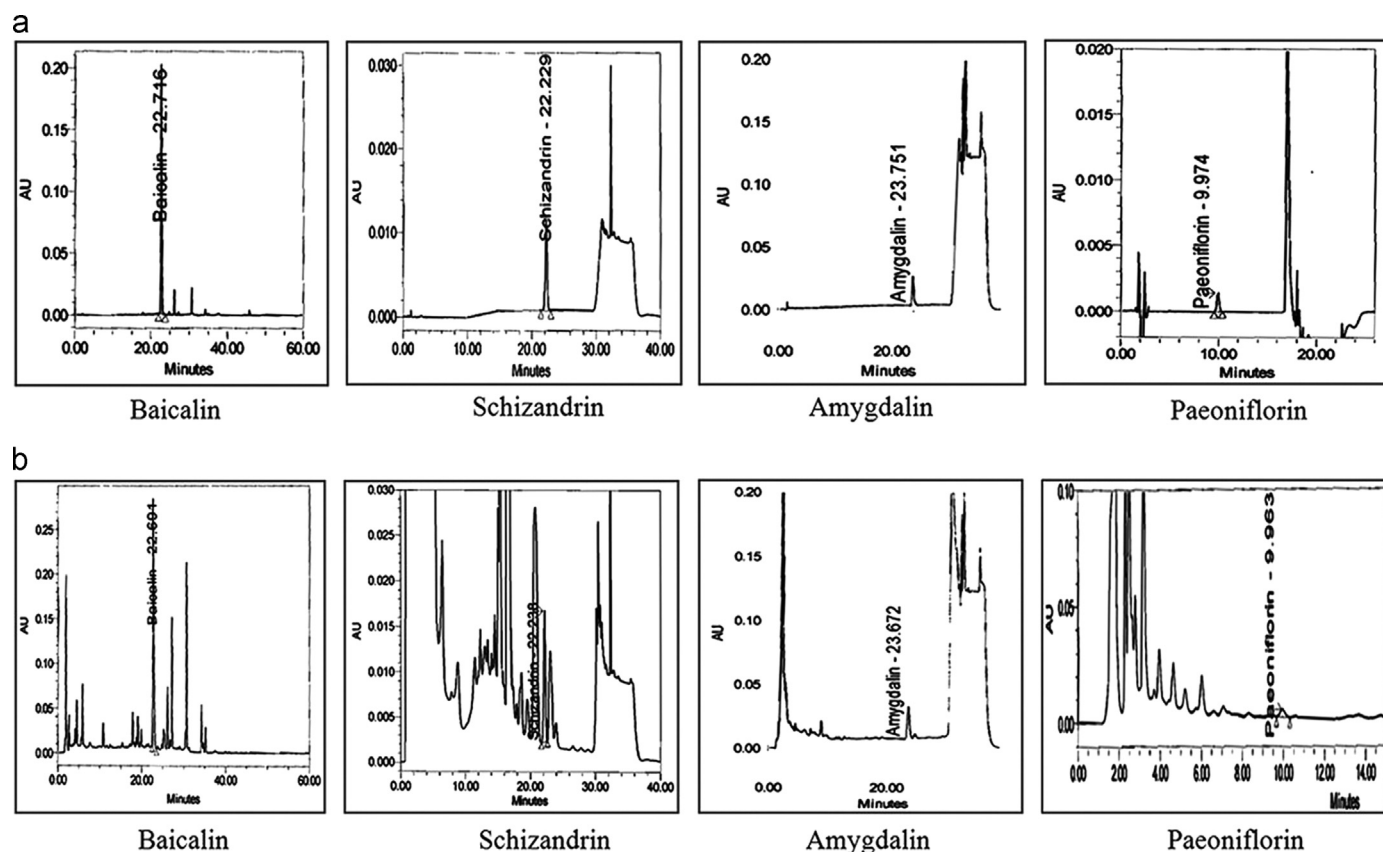


Fig. 1. HPLC chromatograms of standards and PM014. The UV detectors recorded an Amygdalin peak at 214 nm, a Schizandrin peak at 250 nm, and Paeoniflorin and Baicalin peaks at 254 nm. The Capcell PakC18 Column (250 × 4.6 mm, 5 μm) for Amygdalin and the Waters Atlantis T3 Column (150 × 4.6 mm, 5 μm) for Schizandrin, Paeoniflorin and Baicalin were selected for the HPLC study. The mobile phases were comprised of 10% and 100% Me–OH for Amygdalin, 20% and 100% Me–OH for Schizandrin, 14% ACN with 1% acetic acid and 100% ACN for Paeoniflorin and 0.1% acetic acid and 100% ACN for Baicalin. The gradient programs were analyzed per time at a flow rate of 1.0 ml/min for Amygdalin, Paeoniflorin and Baicalin and 1.5 ml/min for Schizandrin using a commercial splitter. The injection volumes were 10 μl for Amygdalin, Paeoniflorin and Baicalin and 20 μl for Schizandrin. (A) Standards and (B) PM014.

Table 2
Quantitative analysis of standard materials.

Compound	Batch number	Quantity (mg/g)	Quantity (mg/g)
Baicalin	20401	11.748 ± 0.318	11.638 ± 0.110
	20402	11.418 ± 0.233	
	20403	11.748 ± 0.296	
Paeoniflorin	20401	0.924 ± 0.022	0.902 ± 0.022
	20402	0.858 ± 0.019	
	20403	0.924 ± 0.029	
Schizandrin	20401	0.165 ± 0.001	0.167 ± 0.001
	20402	0.167 ± 0.000	
	20403	0.170 ± 0.000	
Amygdalin	20401	3.828 ± 0.029	3.916 ± 0.058
	20402	3.894 ± 0.044	
	20403	4.026 ± 0.01	

significant effects on the attenuation of cockroach allergen-induced airway inflammatory responses (Fig. 2).

2.4. Measurement of airway hyperresponsiveness (AHR)

Non-invasive measurements of airway responsiveness were used in this study (All Medicus, Seoul, South Korea). Airway responsiveness was assessed in unrestrained conscious mice 24 h after the last challenge with CKA as described in other studies (Hamelmann et al., 1997; Kim et al., 2013). Mice were placed in a barometric plethysmographic chamber (All Medicus, Seoul, Korea) and baseline readings were taken for 3 min. The enhanced pause

was calculated according to the following formula: $[(\text{expiratory time/relaxation time} - 1) \times (\text{peak expiratory flow/peak inspiratory flow})]$. Penh was calculated according to the manufacturers' protocol and is a dimensionless value that represents a function of the proportion of maximal expiratory to maximal inspiratory box pressure signals. Penh was used as a measure of airway resistance to methacholine. The results were expressed as the percentage increase in Penh after challenge with each concentration of methacholine, where the baseline Penh value (after saline challenge) is expressed as 100%. Penh values averaged for 3 min after each nebulization were evaluated. The results were expressed as the relative increase in Penh following challenge with each concentration of methacholine (0, 25, 50 and 100 mg/ml).

2.5. Analysis of bronchoalveolar lavage (BAL) fluid

Mice were sacrificed on day 35. BAL fluid was collected by slow infusion and extraction of 1 mL of ice cold PBS. This procedure was repeated three times, and the lavages were pooled. Recovered BAL fluid (70–80%) was centrifuged at 1300 rpm for 10 min. The cell pellets were resuspended in 1 ml of PBS and adhered to glass slides using cytocentrifugation (Sandon, Waltham, MA, U.S.A.). Total viable cell counts were determined with a hemacytometer using trypan blue exclusion. Additionally, differential counts of eosinophils, neutrophils, lymphocytes, and macrophages were determined from Diff-Quick stained (Life Technologies, Auckland, New Zealand) cytospin smears of BAL fluid samples ($5 \times 10^5/200 \mu\text{l}$ cells) obtained from individual mice. Furthermore, when we performed cell counts in BAL fluid, one researcher randomly

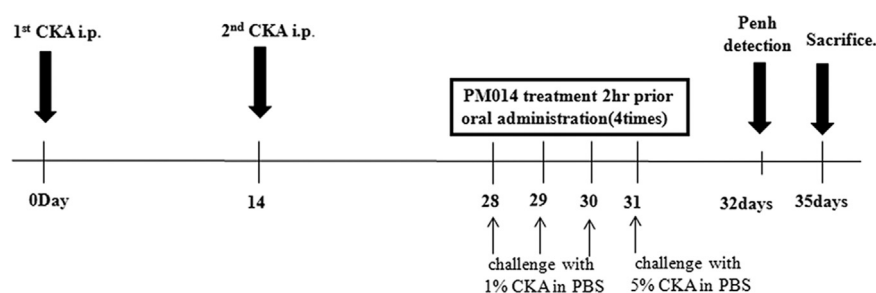


Fig. 2. Experimental time schedule. Sensitization: CKA via i.p. injection (day 0, 14). Challenge: CKA via i.t. (days 28–31). PM014 treatment: Oral administration 2 h prior to CKA challenge. AHR was measured 24 h after the last CKA challenge (day 32). Mice were sacrificed on day 35, and BAL fluid, blood and lung tissues were collected.

selected BAL cell slides and another researcher performed the BAL cell counts in a blinded manner. The results are expressed as total cell number $\times 10^4$. BAL fluid was then centrifuged and the supernatants were kept at -80°C in an ultraslow freezer until further use.

2.6. Measurement of cytokines in BAL fluid using enzyme-linked immunosorbent assay (ELISA)

The concentrations of the Th2 cytokines such as IL-4, IL-5 and IL-13 were measured using a quantitative sandwich enzyme-linked immunoassay kit (BD Pharmingen, San Diego, CA, U.S.A. for IL-4 and IL-5; R&D, Minneapolis, MN, U.S.A. for IL-13). A 96-well microtiter plate (Costar, Corning, NY, U.S.A.) was incubated overnight at 4°C with anti-mouse IL-4, IL-5 and IL-13 monoclonal antibodies in coating buffer, washed with PBS containing 0.05% Tween-20 (Sigma, St. Louis, MO, U.S.A.) and blocked with 5% FBS in PBS for 1 h at room temperature. Subsequently, 100 μl of BAL fluid was loaded, and the plate was incubated for 2 h at room temperature followed by incubation with secondary peroxidase labeled biotinylated anti-mouse IL-4, IL-5 and IL-13 monoclonal antibodies in assay diluents for 1 h. Finally, the plates were treated with TMB substrate solution (BD Pharmingen, San Diego, CA, U.S.A.) for 30 min. The reaction was then stopped by adding 50 μl of stop solution. The optical density was measured at 450 nm using a microplate reader (SOFT max PRO, version 3.1 software, Sunnyvale, CA, U.S.A.). All results were normalized to the total BAL fluid protein content in each sample. The protein concentrations were determined using a BCA kit (Pierce Biotechnology Inc., IL, U.S.A.).

2.7. Determination of IgE titers using ELISA

For serum IgE quantification, 96-well microtiter plates (Costar, Corning, NY, U.S.A.) were coated with anti-mouse IgE monoclonal antibody. Serum was diluted with 5% FBS in PBS (assay diluents) at 1:250, and IgE measurements were determined by standardized sandwich ELISA (BD Pharmingen, San Diego, CA, U.S.A.) according to the manufacturer's protocol. Optical density was measured at 450 nm using a microplate reader (SOFT max PRO, version 3.1 software, Sunnyvale, CA, U.S.A.).

2.8. Preparation of lung tissues and histology

Lung tissues were removed from the mice, and the right lower lobes of the lungs were removed for histological analysis. Four percent paraformaldehyde fixing solution was infused into the lungs. The specimens were dehydrated and embedded in paraffin. For histological examination, 4- μm sections of embedded tissues were cut using a rotary microtome, placed on glass slides, deparaffinized, and stained sequentially with hematoxylin and eosin (H&E). Images of lung tissue sections stained with H&E and hyperplasia of goblet cells within the bronchial epithelium were

assessed and periodic acid Schiff-stained (PAS) sections were acquired with an Olympus BX51 microscope (Olympus, Tokyo, Japan) equipped with a DP71 digital camera (Olympus, Tokyo, Japan) under $200\times$ and $400\times$ magnification. For immunohistochemistry (IHC) detection of Myosin regulatory light polypeptide 9 (MYL9), 4- μm sections of lower trachea and lung tissues were treated with 0.3% H_2O -methanol for 20 min to block endogenous peroxidase activity. The sections were subsequently incubated at 4°C overnight with an anti-MRCL3/MRLC2/MYL9 (FL-172) rabbit polyclonal antibody (1: 50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). After the slides were incubated with avidin-biotin peroxidase complexes (ABC kit, Vector Laboratories, Burlingame, CA, U.S.A.), the color was developed with 3, 3'-diaminobenzidine tetrachloride (DAB; Zymed Laboratories, South San Francisco, CA, U.S.A.). After immunohistochemical staining, the slides were counterstained with Harris's hematoxylin for 1 min, mounted with Canada balsam (Show Chemical Co., Ltd., Tokyo, Japan) and examined under an Olympus BX51 light microscope (Olympus, Tokyo, Japan) equipped with a DP71 digital camera (Olympus, Tokyo, Japan). Four bronchioles were randomly selected from sections of the slides, and cross-sections of lung parenchyma were captured, digitized and evaluated using Image Pro-Plus 5.1 software (Media Cybernetics, Inc., Silver Spring, MD, U.S.A.) (Lederlin et al., 2012; Gorman et al., 2013).

2.9. Statistical analyses

Data are presented as the mean $\pm$ SEM. Analyses of data were performed using the GraphPad Prism software (GraphPad Software, La Jolla, CA, U.S.A.). The differences between each group were determined via one-way ANOVA followed by Newman-Keuls Multiple Comparison tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. The effects of PM014 on airway hyperresponsiveness (AHR)

To determine the inhibitory effects of PM014 on AHR, whole body barometric plethysmographic analysis was performed (Fig. 3).

The Penh value of the CKA group was significantly higher than that of the CON group at 100 mg/ml of methacholine. However, there were no significant responses with 0, 25, and 50 mg/ml of methacholine. The PM014 (50, 100 and 200 mg/kg mg/kg) group showed significantly reduced Penh values when compared to the CKA group treated with 100 mg/ml of methacholine. Additionally, the MK group showed Penh values similar to that of the CON group. These results indicate that PM014 can relieve airway hyperresponsiveness during allergic airway inflammation.

3.2. The effects of PM014 on total and inflammatory cell levels in BAL fluid

The airway inflammation model using cockroach allergen was established with *Balb/c* mice to examine the suppressive effects of PM014. The number of total cells, neutrophils, lymphocytes and macrophages increased significantly in the CKA group when compared to the CON group, implying that the challenge of airway inflammation was successful. This result indicated that cockroach antigen challenge caused a marked influx of leukocytes into the BAL fluid. However, treatment groups with PM014 (50, 100 and 200 mg/kg) or MK group showed a remarkable decrease in the total number of leukocytes, eosinophils, neutrophils, lymphocytes

and macrophages in BAL fluid when compared to the CKA group (Fig. 4).

3.3. The effects of PM014 on cytokine production

ELISA was used to evaluate differences in the expression of inflammatory molecules such as the Th2 cytokines IL-4, IL-5 and IL-13, between the treatment groups. Th2 cytokines (IL-4, IL-5 and IL-13) were augmented in the CKA group when compared to the CON group. The levels of IL-4, IL-5 and IL-13 were decreased in the MK group. Importantly, doses of PM014 (50, 100 and 200 mg/kg) significantly suppressed the secretion of IL-4, IL-5 and IL-13 in BAL fluid in a dose-dependent manner when compared to the CKA group. Our data demonstrate that there were strong and dose-dependent effects of 50–200 mg/kg of PM014 on the secretion of IL-4, IL-5 and IL-13. Most of all, treatment with doses of 200 mg/kg PM014 showed a potent suppressive effect on the secretion of Th2 cytokines that was much stronger than MK treatment (Fig. 5).

3.4. The effects of PM014 on serum IgE levels

An important component of the allergic airway inflammation model is the production of inflammatory mediators such as IgE. Therefore, serum IgE levels were measured in CKA-challenged mice and the PBS, MK and PM014 (50, 100 and 200 mg/kg) treated groups. The IgE in serum significantly increased in the CKA group when compared to the CON group, indicating that the induction of allergic airway inflammation was successful. The concentration of serum IgE levels were significantly reduced in the PM014 (50, 100 and 200 mg/kg) treated groups and the MK group when compared to the CKA group (Fig. 6).

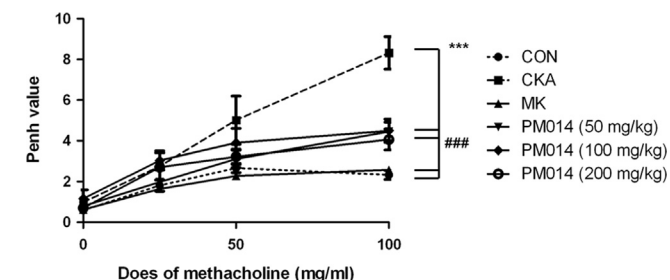


Fig. 3. The effects of PM014 on airway hyperresponsiveness in a CKA-challenged asthma model. Twenty-four hours after the final intratracheal CKA or PBS administration, whole body barometric plethysmographic analysis was performed to evaluate the inhibitory effects of PM014 on airway hyper-responsiveness. The mice were stimulated with increasing doses of aerosolized methacholine (0, 25, 50 and 100 mg/ml). PBS-challenged mice treated with PBS (CON), CKA-challenged mice treated with PBS (CKA), CKA-challenged mice treated with Montelukast (10 mg/kg) (MK), CKA-challenged mice treated with PM014 (50, 100 and 200 mg/kg) (PM014). Concentrations are indicated. Data are shown as the mean $\pm$ S.E.M. Statistical analyses were conducted by one-way ANOVA followed by a Newman–Keuls Multiple Comparison test ($***p < 0.001$ vs. CON, $###p < 0.001$ vs. CKA; $n = 5-6$).

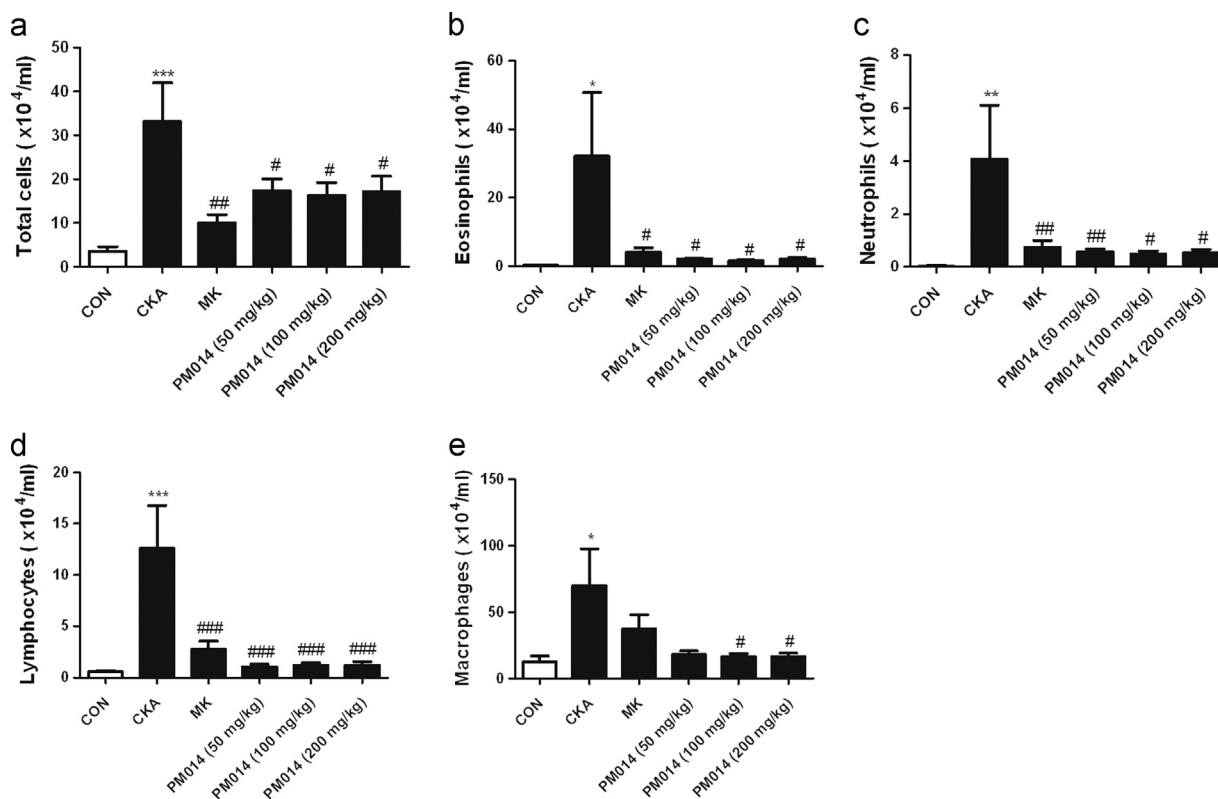


Fig. 4. The effects of PM014 on total cell counts and cell differentiation in BAL fluid. BAL fluid was obtained from the lungs of mice. (a) Total cell number counts, (b) eosinophil counts, (c) neutrophil counts, (d) lymphocyte counts and (e) macrophage counts. Data are shown as the mean $\pm$ S.E.M. Statistical analyses were conducted via one-way ANOVA followed by a Newman–Keuls Multiple Comparison test ($***p < 0.001$, $**p < 0.01$, $*p < 0.05$ vs. CON, $###p < 0.001$, $##p < 0.01$, $#p < 0.05$ vs. CKA; $n = 5-6$).

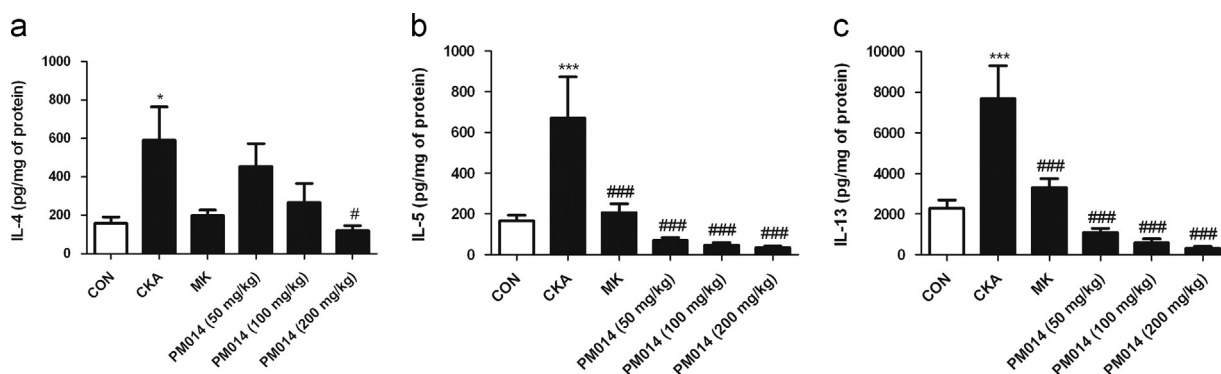


Fig. 5. The effects of PM014 on IL-4, IL-5 and IL-13 levels in BAL fluid. BAL fluid was collected by infusion and extraction of ice-cold PBS. The concentrations of the cytokines IL-4, IL-5 and IL-13 were measured with a quantitative sandwich enzyme-linked immunoassay kit. (a) levels of IL-4, (b) levels of IL-5, (c) levels of IL-13. Data are shown as the mean $\pm$ S.E.M. Statistical analyses were conducted via one-way ANOVA followed by a Newman–Keuls Multiple Comparison test (** $p < 0.001$, * $p < 0.05$ vs. CON, ### $p < 0.001$, # $p < 0.05$ vs. CKA; $n = 5-6$).

3.5. The effects of PM014 on CKA-induced lung histomorphologic changes

To observe whether PM014 exerted effects on CKA-challenged lung tissue damage and inflammatory cell infiltration into airways, lung tissue sections were stained with H&E. Histological sections of lung tissue from mice that were challenged with CKA exhibited airway inflammation and were found to have heavy infiltration of inflammatory cells in the peribronchial regions of the lung. Changes observed in lung tissue sections indicated that the PM014 (50, 100 and 200 mg/kg) treated groups displayed less inflammatory cells in the peribronchial regions when compared to the CKA group (Fig. 7).

To observe goblet hyperplasia, we stained lung sections with PAS stain (Fig. 8).

The overproduction of mucus and goblet cell hyperplasia were observed in the bronchial airways of the CKA group and the MK and PM014 (50, 100 and 200 mg/kg) treatment groups but not in those obtained from the CON group. To evaluate airway remodeling, smooth muscle hyperplasia was detected by IHC using an MYL9 antibody. The CKA-challenged mice showed expression of MYL9 in the bronchial muscle layer of the lung, and the PM014 treatment decreased the area of this smooth muscle layer (Fig. 9A and B).

These findings demonstrate that PM014 has the potential to counteract allergic asthma-associated airway remodeling.

4. Discussion

Asthma is a chronic respiratory disease characterized by bronchial inflammation and airway hyperresponsiveness (Nials and Uddin, 2008). It is a leading cause of morbidity and mortality in critically ill patients. Montelukast is a leukotriene receptor antagonist that is used for the maintenance of asthma. Leukotriene, a lipid mediator, is derived from eosinophils, mast cells, monocytes, and basophils, and causes bronchial smooth muscle contraction and exacerbates lung inflammation in asthma (Muz et al., 2006). However, Montelukast has side effects such as anxiety and other adverse neuropsychiatric effects (Kelly and Nelson, 2003). Accordingly, there is a need for the development of alternatives which are safe and effective for the treatment of allergic airway inflammation diseases such as asthma.

Asthma and allergies are the most common diseases associated with cockroach infestation, especially in inner-cities where it affects up to 80% of asthmatic children sensitized and exposed to allergens produced by *Blattella germanica*. In addition, cockroach

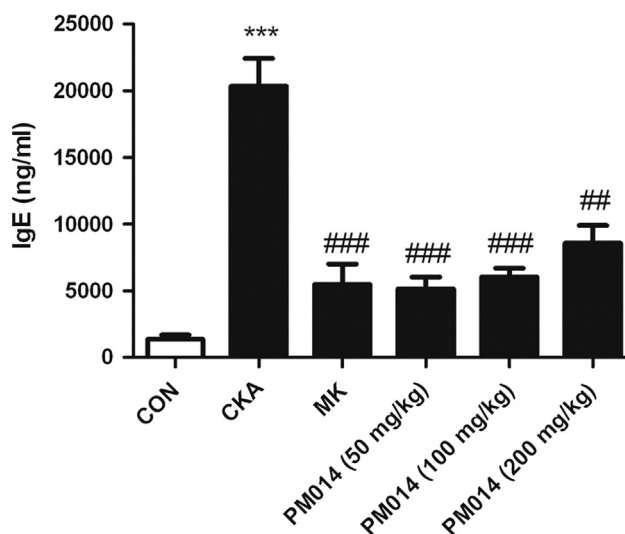


Fig. 6. Serum levels of IgE. Serum was collected from the retro-orbital plexus. The amount of IgE was evaluated by ELISA. PBS-challenged mice treated with PBS (CON), CKA-challenged mice treated with PBS (CKA), CKA challenged mice treated with Montelukast (10 mg/kg) (MK), CKA-challenged mice treated with PM014 (50, 100 and 200 mg/kg) (PM014). Concentrations are indicated. Data are shown as the mean $\pm$ S.E.M. Statistical analyses were conducted via one-way ANOVA followed by a Newman–Keuls Multiple Comparison test (** $p < 0.001$ vs. CON, ### $p < 0.001$, # $p < 0.05$ vs. CKA; $n = 5-6$).

allergens appear to have a greater effect on asthma morbidity than dust mites or pet allergens in these children (Arruda et al., 2001; Gruchalla et al., 2005).

In asthma, the CD4⁺ T cells (a type of lymphocyte) are the crucial mediators of Th2 type immune responses in the bronchial airway (Barnes, 2001). Allergen specific Th2 cells mediate an allergic response through the secretion of cytokines such as IL-4, IL-5 and IL-13 (Klein Wolterink et al., 2012). IL-4 mediates important proinflammatory functions in asthma, including the induction of the IgE isotype switch, promotion of eosinophil transmigration across the endothelium, stimulation of goblet cell metaplasia, mucus production and Th2 lymphocyte differentiation leading to the release of IL-5 and IL-13 (Dabbagh et al., 1999; Borish et al., 2001; Lee et al., 2009). IL-5 is involved in B cell differentiation and mast cell recruitment, and particularly influences eosinophil activation, maturation, and recruitment (Lee et al., 2009, 2012; Wegmann, 2011). IL-13 induced pathologic changes including infiltration of eosinophils, epithelial damage, hyperplasia of goblet cells, transformation of airway fibroblasts to myofibroblasts and smooth muscle hyperactivity are often observed (Ingram and Kraft, 2012). IL-13 is also involved in airway

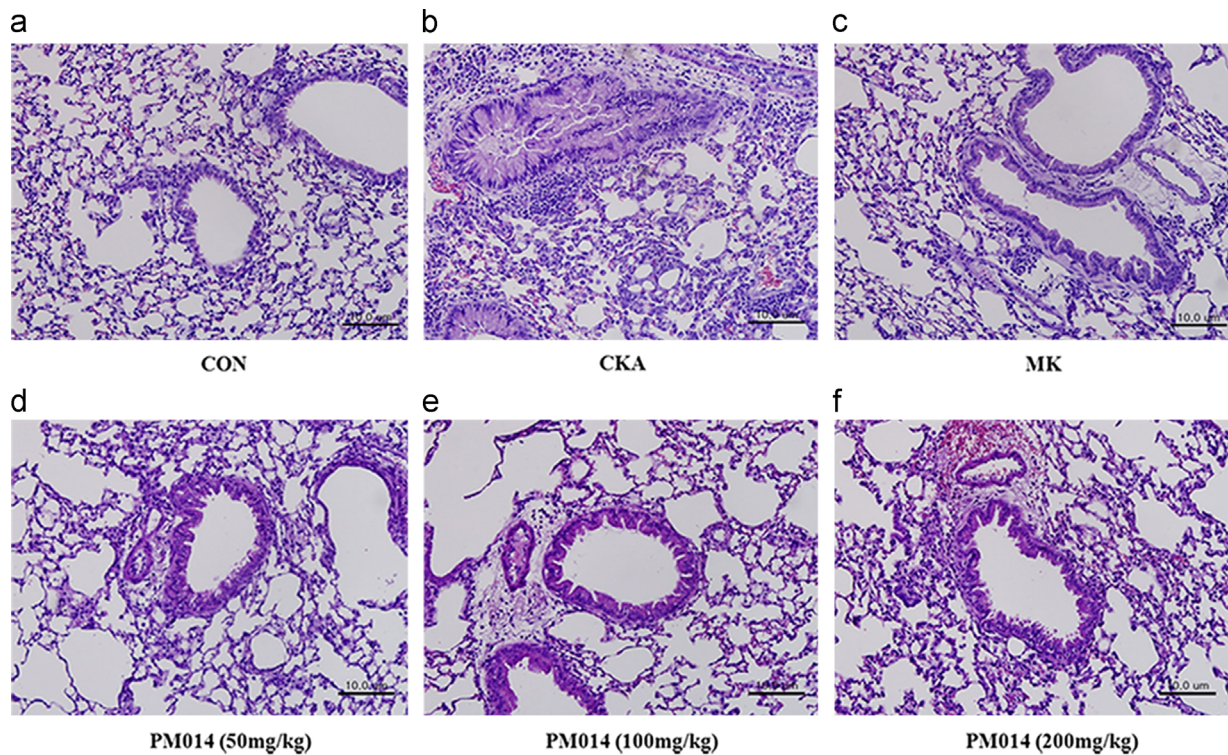


Fig. 7. The effects of PM014 on histopathological changes in lung tissues from CKA-challenged allergic asthma mice. Lung tissues were stained with H&E to observe inflammatory cell infiltration (magnification $200\times$) as described in the Materials and Methods section. (a) mice treated with PBS only (CON), (b) CKA-challenged mice treated with PBS (CKA), (c) CKA-challenged mice treated with Montelukast (10 mg/kg) (MK), (d) CKA-challenged mice treated with PM014 (50 mg/kg) (PM014 (50 mg/kg)), (e) CKA-challenged mice treated with PM014 (100 mg/kg) (PM014 (100 mg/kg)), (f) CKA-challenged mice treated with PM014 (200 mg/kg) (PM014 (200 mg/kg)). Concentrations are indicated.

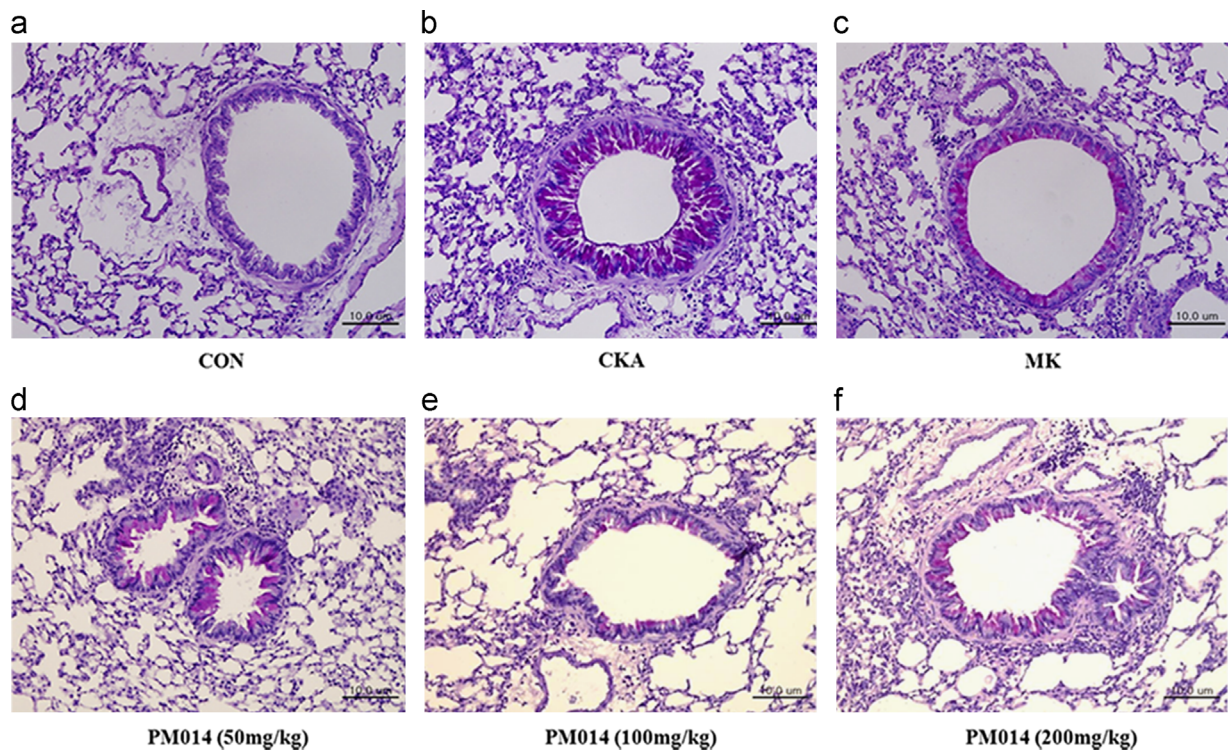


Fig. 8. The effects of PM014 on airway goblet cell hyperplasia and mucus production. Lung tissues were stained with PAS (magnification $200\times$) as described in Section 2. (a) Mice treated with PBS only (CON), (b) CKA-challenged mice treated with PBS (CKA), (c) CKA-challenged mice treated with Montelukast (10 mg/kg) (MK), (d) CKA-challenged mice treated with PM014 (50 mg/kg) (PM014 (50 mg/kg)), (e) CKA-challenged mice treated with PM014 (100 mg/kg) (PM014 (100 mg/kg)), (f) CKA-challenged mice treated with PM014 (200 mg/kg) (PM014 (200 mg/kg)).

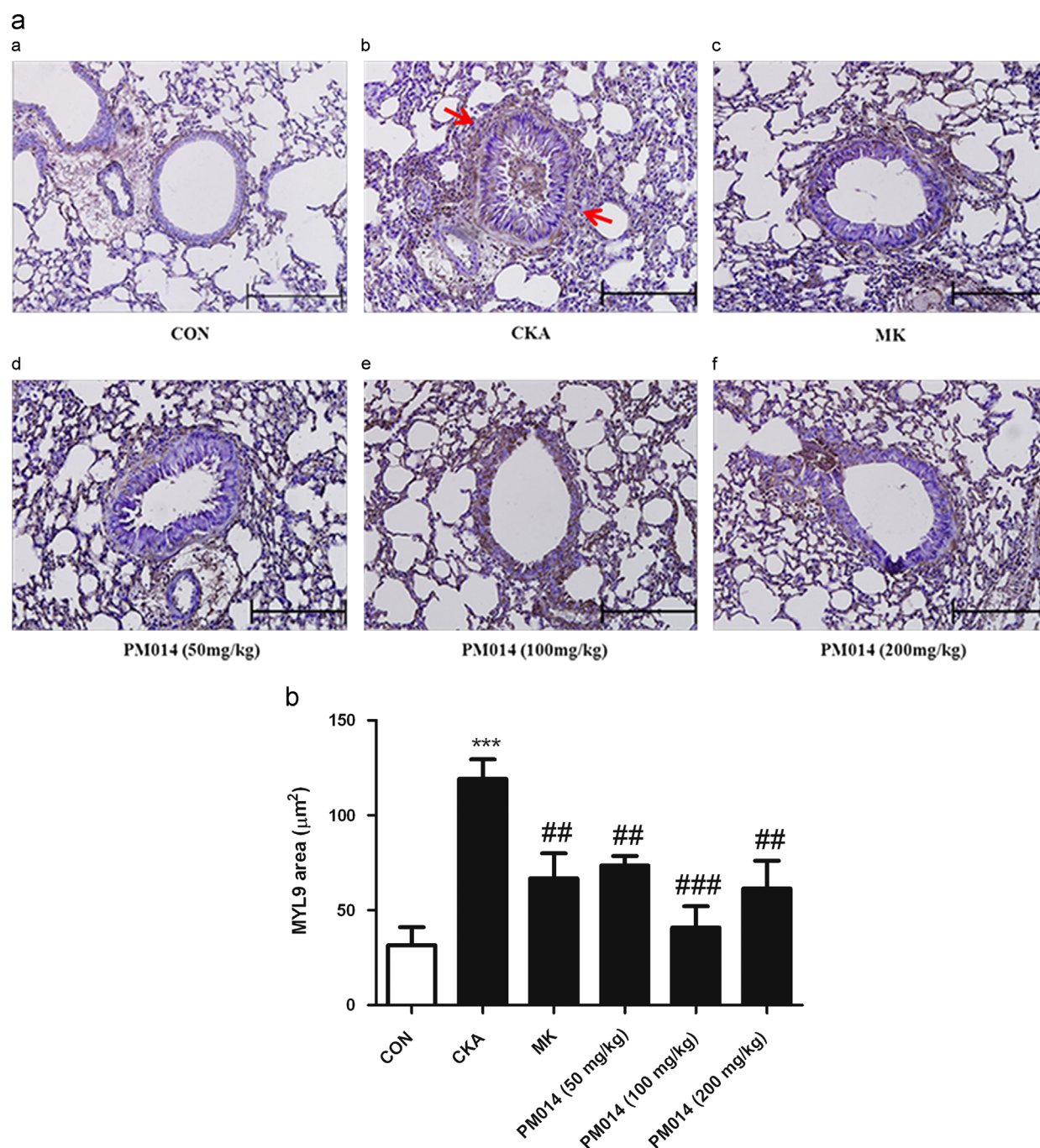


Fig. 9. The effects of PM014 on airway remodeling in lung tissue and MYL9 immunohistochemistry in CKA-challenged mice. Lung tissue sections were stained with anti-MYL9 antibodies and detected by immunohistochemistry as described in Section 2. (A) Representative immunohistochemistry of the smooth muscle MYL9 expression in a subject with allergic inflammation and enlarged view of a smooth muscle bundle. The red arrows indicate MYL9 positive smooth muscle layer (brown color indicates positivity, magnification $200\times$). (a) PBS-challenged mice treated with PBS; CON, (b) CKA-challenged mice treated with PBS; CKA, (c) CKA-challenged mice treated with Montelukast (10 mg/kg); MK. (d–f) CKA-challenged mice treated with PM014; PM014 (50, 100 and 200 mg/kg) as indicated. (B) Graphs represent the area of MYL9 positive smooth muscle in bronchial was calculated based on the immunohistochemical images and were measured using the Image Pro-Plus 5.1 software (Media Cybernetics, Inc., Silver Spring, MD, U.S.A.) at magnifications of $200\times$. Data are shown as the mean $\pm$ S.E.M. Statistical analyses were conducted via one-way ANOVA followed by a Newman–Keuls Multiple Comparison test (*** $p < 0.001$ vs. CON, ### $p < 0.001$, ## $p < 0.01$ vs. CKA; $n = 5–6$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hyperresponsiveness (AHR) and promotes IgE isotype switching in B cells and mucus secretion in the airway mucosa (Wills-Karp et al., 1998). Eosinophils, which are important mediators of epithelial damage in asthma, release a plethora of mediators and cytotoxic products, including major basic protein (MBP), eosinophil peroxidase, eosinophil cationic protein, and up to 28 cytokines, growth factors, and chemokines. Eosinophils also release bronchoconstriction mediators such as leukotriene C4 (LTC4), which are elevated during the later

stages of asthmatic reactions (Wardlaw et al., 2000; Humbles et al., 2004; Bradding, 2008; Wegmann, 2011).

Asthmatic airway remodeling is characterized by various structural changes including abnormal epithelium formation, subepithelial membrane thickening, alteration of the extracellular matrix (ECM) deposition, neo angiogenesis, mucus gland hypertrophy, and increased numbers of peribronchial muscle layers. The functional consequences of airway remodeling contribute to an

increased susceptibility to asthma exacerbation (Payne et al., 2003; Black et al., 2012). In addition, increased levels of MYL9, which is the central regulator of cellular contraction, have been found in the peribronchial muscle layers obtained from asthmatics (Xu et al., 2011; Prakash, 2013).

In our study, the effects of PM014 on allergic airway inflammation were investigated using a cockroach allergen mouse model, which is one of the widely used models that simulate asthma (Campbell et al., 1998). PM014 is a modified formulation of Chung-Sang-Bo-Ha-Tang (CSBHT) and contains 7 major species also found in CSBHT. In general, traditional Korean medicine has been used with a modified formulation according to the prescription theory mainly described in “Donguibogam (UNESCO’s memory of the world program). The PM014 formula includes root of *Rehmannia glutinosa*, cortex of *Paeonia suffruticosa*, fruit of *Schizandra chinensis*, root of *Asparagus cochinchinensis*, seed of *Prunus armeniaca*, root of *Scutellaria baicalensis* and root of *Stemona sessilifolia*. Individual herbs found in PM014 have been shown to have various biological effects. Some PM014 compounds such as schisandrin, monoterpene glycosides and *Scutellaria baicalensis* are reported to have anti-inflammatory effects (Guo et al., 2008; Yoon et al., 2009; Zhu and Fang, 2013). In addition, the biological activities of compounds isolated from *Stemona sessilifolia* exhibit antitussive effects (Yang et al., 2009). In another study, root of *Rehmannia glutinosa* exhibited anti-allergic effects (Kim et al., 1998). The therapeutic potency of PM014 should be attributed to its combined and synergistic effects on multiple targets as a result of its diverse components.

In previous studies, the herbal formula PM014 attenuated lung inflammation in a murine model of chronic obstructive pulmonary disease (COPD) (Lee et al., 2012). COPD and asthma are both characterized by pulmonary and systemic inflammation. Therefore, we investigated the anti-inflammatory effects of PM014 in a mouse model of cockroach allergic inflammation with respect to airway function, allergen-induced inflammatory infiltrates in the airways, Th2 cytokine production, serum immunoglobulin levels and histopathological changes in lung tissues. Our results show that PM014 treatment markedly improved the Penh value of the cockroach allergen-induced mice (CKA group). Penh has been used to evaluate changes in lung function and as a method to evaluate airway responsiveness in many other studies (Lundblad et al., 2002; Chung et al., 2013). However, Penh is indirect method to measure non-specific airway hyperresponsiveness (AHR). Recently, most of researchers, however, used other method prior to Penh, because it is evaluated as not complete way to assess lung mechanical function in mice (Lundblad et al., 2002; Bates et al., 2004). We also investigated the changes in leukocyte levels including eosinophils, neutrophils, macrophages and lymphocytes. Eosinophils are particularly important factors in allergic airway inflammation related to respiratory diseases such as asthma. In the present study, PM014 (50, 100 and 200 mg/kg) treatment significantly reduced the total cell number of leukocytes and the influx of macrophages, eosinophils, neutrophils, and lymphocytes in BAL fluid when compared to the CKA group. These results suggest that a dose of 50 mg/kg of PM014 is likely the maximum concentration needed to induce airway inflammation inhibitory effects for these criteria. In addition, the levels of Th2 cytokines (IL-4, IL-5 and IL-13) were markedly reduced. The 200 mg/kg of PM014 treatment showed a particularly significant inhibition of Th2 cytokine (IL-4, IL-5 and IL-13) secretion by the inflammatory response. Serum IgE concentrations also significantly decreased following PM014 treatment. In the meantime, we investigated whether PM014 had effects on the histological changes of the lung tissue. All concentrations of PM014 exhibited lower amounts of inflammatory cells such as infiltrating eosinophils in the peribronchial regions and goblet cell hyperplasia around the bronchial airway. Furthermore,

expression of MYL9 in the peribronchial muscle layer of the lung was abrogated. When taken together, these results and those of the previous study suggest that PM014 is another alternative for the clinical treatment of lung inflammation.

5. Conclusions

In summary, our investigation demonstrated that the standardized herbal formula PM014 inhibited the development of allergic inflammation in the cockroach allergen-induced airway response in mice. The effects of PM014 suggest that it has potential for use in the treatment of allergic asthma-associated airway remodeling. However, further studies to elucidate the mechanisms by which the effects of PM014 occur should be conducted to aid in the discovery of new therapeutic agents for the prevention of asthma.

Acknowledgments

This study was supported by Grants from the Traditional Korean Medicine R&D Project, Ministry for Health & Welfare & Family Affairs (B100053), and the National Research Foundation of Korea (NRF) funded by the Korean Ministry of Education, Science and Technology (No. 2011-006220).

References

- Arizmendi, N.G., Abel, M., Puttagunta, L., Asaduzzaman, M., Davidson, C., Karimi, K., Forsythe, P., Vliagoftis, H., 2011. Mucosal exposure to cockroach extract induces allergic sensitization and allergic airway inflammation. *Allergy Asthma and Clinical Immunology* 7, 22.
- Arruda, L.K., Vailles, L.D., Ferriani, V.P., Santos, A.B., Pomes, A., Chapman, M.D., 2001. Cockroach allergens and asthma. *Journal of Allergy and Clinical Immunology* 107, 419–428.
- Barnes, P.J., 2001. Th2 cytokines and asthma: an introduction. *Respiratory Research* 2, 64–65.
- Bates, J., Irvin, C., Brusasco, V., Drazen, J., Fredberg, J., Loring, S., Eidelman, D., Ludwig, M., Macklem, P., Martin, J., Milic-Emili, J., Hantos, Z., Hyatt, R., Lai-Fook, S., Leff, A., Solway, J., Lutchen, K., Suki, B., Mitzner, W., Pare, P., Pride, N., Sly, P., 2004. The use and misuse of Penh in animal models of lung disease. *Am. J. Respir. Cell Mol. Biol.* 31, 373–374.
- Bax, H.J., Keeble, A.H., Gould, H.J., 2012. Cytokine IgE action in mast cell activation. *Frontiers in Immunology* 3, 229.
- Black, J.L., Panettieri Jr., R.A., Banerjee, A., Berger, P., 2012. Airway smooth muscle in asthma: just a target for bronchodilation? *Clinics in Chest Medicine* 33, 543–558.
- Borish, L.C., Nelson, H.S., Corren, J., Bensch, G., Busse, W.W., Whitmore, J.B., Agosti, J. M., 2001. Efficacy of soluble IL-4 receptor for the treatment of adults with asthma. *Journal of Allergy and Clinical Immunology* 107, 963–970.
- Bradding, P., 2008. Asthma: eosinophil disease, mast cell disease, or both? *Allergy Asthma Clinical Immunology* 4, 84–90.
- Braman, S.S., 2006. The global burden of asthma. *Chest* 130, 4S–12S.
- Brightling, C.E., Bradding, P., Pavord, I.D., Wardlaw, A.J., 2003. New insights into the role of the mast cell in asthma. *Clinical and Experimental Allergy* 33, 550–556.
- Campbell, E.M., Kunkel, S.L., Strieter, R.M., Lukacs, N.W., 1998. Temporal role of chemokines in a murine model of cockroach allergen-induced airway hyper-reactivity and eosinophilia. *Journal of Immunology* 161, 7047–7053.
- Choi, M.S., Park, S., Choi, T., Lee, G., Haam, K.K., Hong, M.C., Min, B.I., Bae, H., 2013. Bee venom ameliorates ovalbumin induced allergic asthma via modulating CD4+CD25+ regulatory T cells in mice. *Cytokine* 61, 256–265.
- Chung, H.S., Kim, Y., Oh, S.J., Kim, H., Choi, S.I., Zhang, Y., Jeong, J.H., Bae, H., 2013. A synthetic compound, 4-acetyl-3-methyl-6-(3,4,5-trimethoxyphenyl)pyrano [3,4-c]pyran-1,8-dione, ameliorates ovalbumin-induced asthma. *Bioorganic and Medicinal Chemistry* 21, 6359–6365.
- Dabbagh, K., Takeyama, K., Lee, H.M., Ueki, I.F., Lausier, J.A., Nadel, J.A., 1999. IL-4 induces mucin gene expression and goblet cell metaplasia in vitro and in vivo. *Journal of Immunology* 162, 6233–6237.
- Desai, D., Brightling, C., 2009. Cytokine and anti-cytokine therapy in asthma: ready for the clinic? *Clinical and Experimental Immunology* 158, 10–19.
- Gorman, S., Weeden, C.E., Tan, D.H., Scott, N.M., Hart, J., Foong, R.E., Mok, D., Stephens, N., Zosky, G., Hart, P.H., 2013. Reversible control by vitamin D of granulocytes and bacteria in the lungs of mice: an ovalbumin-induced model of allergic airway disease. *PLoS One* 8, e67823.
- Gruchalla, R.S., Pongracic, J., Plaut, M., Evans 3rd, R., Visness, C.M., Walter, M., Crain, E.F., Kattan, M., Morgan, W.J., Steinbach, S., Stout, J., Malindzak, G., Smartt, E., Mitchell, H., 2005. Inner City Asthma Study: relationships among sensitivity,

- allergen exposure, and asthma morbidity. *Journal of Allergy and Clinical Immunology* 115, 478–485.
- Guo, L.Y., Hung, T.M., Bae, K.H., Shin, E.M., Zhou, H.Y., Hong, Y.N., Kang, S.S., Kim, H.P., Kim, Y.S., 2008. Anti-inflammatory effects of schisandrin isolated from the fruit of *Schisandra chinensis* Baill. *European Journal of Pharmacology* 591, 293–299.
- Hamelmann, E., Schwarze, J., Takeda, K., Oshiba, A., Larsen, G.L., Irvin, C.G., Gelfand, E.W., 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *American Journal of Respiratory and Critical Care Medicine* 156, 766–775.
- Holgate, S.T., 2009. Novel targets of therapy in asthma. *Current Opinion in Pulmonary Medicine* 15, 63–71.
- Humbles, A.A., Lloyd, C.M., McMillan, S.J., Friend, D.S., Xanthou, G., McKenna, E.E., Ghiran, S., Gerard, N.P., Yu, C., Orkin, S.H., Gerard, C., 2004. A critical role for eosinophils in allergic airways remodeling. *Science* 305, 1776–1779.
- Ingram, J.L., Kraft, M., 2012. IL-13 in asthma and allergic disease: asthma phenotypes and targeted therapies. *Journal of Allergy and Clinical Immunology* 130, 829–842 (quiz 843–844).
- Kawakami, T., Kitauro, J., 2005. Mast cell survival and activation by IgE in the absence of antigen: a consideration of the biologic mechanisms and relevance. *Journal of Immunology* 175, 4167–4173.
- Kelly, H.W., Nelson, H.S., 2003. Potential adverse effects of the inhaled corticosteroids. *Journal of Allergy and Clinical Immunology* 112, 469–478 (quiz 479).
- Kim, H., Lee, E., Lee, S., Shin, T., Kim, Y., Kim, J., 1998. Effect of *Rehmannia glutinosa* on immediate type allergic reaction. *International Journal of Immunopharmacology* 20, 231–240.
- Kim, S.R., Kim, D.I., Kang, M.R., Lee, K.S., Park, S.Y., Jeong, J.S., Lee, Y.C., 2013. Endoplasmic reticulum stress influences bronchial asthma pathogenesis by modulating nuclear factor kappaB activation. *Journal of Allergy and Clinical Immunology* 132, 1397–1408 (e1311).
- Klein Wolterink, R.G., Kleinjan, A., van Nimwegen, M., Bergen, I., de Bruijn, M., Levani, Y., Hendriks, R.W., 2012. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. *European Journal of Immunology* 42, 1106–1116.
- Larche, M., Robinson, D.S., Kay, A.B., 2003. The role of T lymphocytes in the pathogenesis of asthma. *Journal of Allergy and Clinical Immunology* 111, 450–463 (quiz 464).
- Lederlin, M., Ozier, A., Dournes, G., Ousova, O., Girodet, P.O., Begueret, H., Marthan, R., Montaudon, M., Laurent, F., Berger, P., 2012. In vivo micro-CT assessment of airway remodeling in a flexible OVA-sensitized murine model of asthma. *PLoS One* 7, e48493.
- Lee, H., Han, A.R., Kim, Y., Choi, S.H., Ko, E., Lee, N.Y., Jeong, J.H., Kim, S.H., Bae, H., 2009. 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. *International Journal of Immunopathology and Pharmacology* 22, 591–603.
- Lee, H., Kim, Y., Kim, H.J., Park, S., Jang, Y.P., Jung, S., Jung, H., Bae, H., 2012. Herbal formula, PM014, attenuates lung inflammation in a murine model of chronic obstructive pulmonary disease. *Evidence Based Complementary and Alternative Medicine* 2012, 769830.
- Litonjua, A.A., Carey, V.J., Burge, H.A., Weiss, S.T., Gold, D.R., 2001. Exposure to cockroach allergen in the home is associated with incident doctor-diagnosed asthma and recurrent wheezing. *Journal of Allergy and Clinical Immunology* 107, 41–47.
- Lundblad, L.K., Irvin, C.G., Adler, A., Bates, J.H., 2002. A reevaluation of the validity of unrestrained plethysmography in mice. *Journal of Applied Physiology* 93, 1198–1207 (1985).
- Matsuse, H., Hirose, H., Fukahori, S., Tsuchida, T., Tomari, S., Kawano, T., Fukushima, C., Kohno, S., 2012. Regulation of dendritic cell functions against harmful respiratory pathogens by a cysteinyl leukotrienes receptor antagonist. *Allergy and Rhinology (Providence)* 3, e30–34.
- Muz, M.H., Deveci, F., Bulut, Y., Ilhan, N., Yekeler, H., Turgut, T., 2006. The effects of low dose leukotriene receptor antagonist therapy on airway remodeling and cysteinyl leukotriene expression in a mouse asthma model. *Experimental and Molecular Medicine* 38, 109–118.
- Nials, A.T., Uddin, S., 2008. Mouse models of allergic asthma: acute and chronic allergen challenge. *Disease Models and Mechanisms* 1, 213–220.
- Noble, P.B., Ansell, T.K., James, A.L., McFawn, P.K., Mitchell, H.W., 2012. Airway smooth muscle dynamics and hyperresponsiveness: in and outside the clinic. *Journal of Allergy (Cairo)* 2012, 157047.
- Payne, D.N., Rogers, A.V., Adelroth, E., Bandi, V., Guntupalli, K.K., Bush, A., Jeffery, P.K., 2003. Early thickening of the reticular basement membrane in children with difficult asthma. *American Journal of Respiratory and Critical Care Medicine* 167, 78–82.
- Platts-Mills, T.A., Rakes, G., Heymann, P.W., 2000. The relevance of allergen exposure to the development of asthma in childhood. *Journal of Allergy and Clinical Immunology* 105, S503–508.
- Prakash, Y.S., 2013. Airway smooth muscle in airway reactivity and remodeling: what have we learned? *American Journal of Physiology – Lung Cellular and Molecular* 305, L912–933.
- Roh, G.S., Seo, S.W., Yeo, S., Lee, J.M., Choi, J.W., Kim, E., Shin, Y., Cho, C., Bae, H., Jung, S.K., Kwack, K., 2005. Efficacy of a traditional Korean medicine, Chung-Sang-Bo-Ha-Tang, in a murine model of chronic asthma. *International Immunopharmacology* 5, 427–436.
- Rosenreich, D.L., Eggleston, P., Kattan, M., Baker, D., Slavin, R.G., Gergen, P., Mitchell, H., McNiff-Mortimer, K., Lynn, H., Ownby, D., Malveaux, F., 1997. The role of cockroach allergy and exposure to cockroach allergen in causing morbidity among inner-city children with asthma. *New England Journal of Medicine* 336, 1356–1363.
- Tsujiyama, I., Mubassara, S., Aoshima, H., Hossain, S.J., 2013. Anti-histamine release and anti-inflammatory activities of aqueous extracts of citrus fruits peels. *Orient Pharmacy and Experimental Medicine* 13 (13), 175–180.
- Wardlaw, A.J., Brightling, C., Green, R., Woltmann, G., Pavord, I., 2000. Eosinophils in asthma and other allergic diseases. *British Medical Bulletin* 56, 985–1003.
- Wardlaw, A.J., Brightling, C.E., Green, R., Woltmann, G., Bradding, P., Pavord, I.D., 2002. New insights into the relationship between airway inflammation and asthma. *Clinical Science (London)* 103, 201–211.
- Wegmann, M., 2011. Targeting eosinophil biology in asthma therapy. *American Journal of Respiratory Cell and Molecular Biology* 45, 667–674.
- Wills-Karp, M., Luyimbazi, J., Xu, X., Schofield, B., Neben, T.Y., Karp, C.L., Donaldson, D.D., 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282, 2258–2261.
- Xu, W., Hong, W., Shao, Y., Ning, Y., Cai, Z., Li, Q., 2011. Nogo-B regulates migration and contraction of airway smooth muscle cells by decreasing ARPC 2/3 and increasing MYL-9 expression. *Respiratory Research* 12, 14.
- Yang, X.Z., Zhu, J.Y., Tang, C.P., Ke, C.Q., Lin, G., Cheng, T.Y., Rudd, J.A., Ye, Y., 2009. Alkaloids from roots of *Stemona sessilifolia* and their antitussive activities. *Planta Medica* 75, 174–177.
- Yoon, S.B., Lee, Y.J., Park, S.K., Kim, H.C., Bae, H., Kim, H.M., Ko, S.G., Choi, H.Y., Oh, M.S., Park, W., 2009. Anti-inflammatory effects of *Scutellaria baicalensis* water extract on LPS-activated RAW 264.7 macrophages. *Journal of Ethnopharmacology* 125, 286–290.
- Zhu, X., Fang, Z.H., 2013. New monoterpene glycosides from the root cortex of *Paeonia suffruticosa* and their potential anti-inflammatory activity. *Natural Product Research* 28, 301–305.