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SKI3301, a purified herbal extract from *Sophora tonkinensis*, inhibited airway inflammation and bronchospasm in allergic asthma animal models in vivo

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Abstract

Ethnopharmacological relevance

Sophora tonkinensis (Leguminosae, ST) is a traditional herbal plant in Korea and China.

Its roots and rhizomes have been used to dissipate heat, to clear toxic material and to

treat acute pharyngolaryngeal infections and sore throats.

Aim of study

In this study, we tried to investigate the anti-inflammatory and anti-asthmatic effects of a purified extract (SKI3301) from *Sophora tonkinensis* using *in vitro* enzyme assay models and ovalbumin (OVA)-induced asthma animal models.

Materials and methods

The effect of SKI3301 on pro-inflammatory enzymes such as 5-lipoxygenase, phosphodiesterase 3 & 4, and thromboxane synthase was assayed *in vitro*. BALB/c mice were sensitized with OVA/Alum ip injection and nebulized with OVA to induce airway inflammation. Bronchoalveolar lavage (BAL) fluid was collected and analyzed for leukocytes infiltration and IL-5 production along with lung histopathology. Guinea pigs passively sensitized with anti-OVA antiserum were used to investigate the effect of SKI3301 on bronchospasm *in vitro* and *in vivo*.

Results

SKI3301 potently inhibited the activities of 5-lipoxygenase, phosphodiesterase 3 & 4, and thromboxane synthase. Orally administered SKI3301 attenuated the total leukocytes and eosinophil infiltration and IL-5 level in BAL fluids. Histopathological changes associated with lung inflammation were also reduced by SKI3301. SKI3301 inhibited OVA-induced contraction of isolated trachea from sensitized guinea pigs. SKI3301 also protected OVA-induced bronchoconstriction in the sensitized guinea pigs. Maackiain, one of 3 major components of SKI3301, was effective in inhibiting 5-lipoxygenase and OVA-induced airway inflammation.

Conclusion

In this study, SKI3301 potently inhibited pro-inflammatory enzymes and attenuated

OVA-induced bronchospasm in animal model of allergic asthma. These results suggest that SKI3301 may have therapeutic potential for allergic asthma.

Keywords: SKI3301, *Sophora tonkinensis*, Airway inflammation, Asthma, Maackiain

Introduction

Asthma is a chronic inflammatory airway disease with a variable degree of airflow obstruction. It is characterized by bronchial inflammation with prominent eosinophil infiltration, airway remodeling, bronchial hyper-responsiveness, cough, chest tightness and wheeze (Lemanske and Busse, 2003; Martinez and Vercelli, 2013). Recent report revealed that asthma affects 300 million adults and children worldwide and the occurrence is steadily increasing with the global industrialization (Braman, 2006). For the treatment of asthma, inhalational corticosteroids (ICS) with or without long-acting β_2 -agonists (LABA), are frequently used (FitzGerald and Shahidi, 2010; Rottier and Duiverman, 2009). Inhaled corticosteroids are the most effective controller medications currently available but a serious concern on side effects like osteoporosis and immunosuppression, and resultant poor compliance is constantly looming over its chronic use (Bjermer, 2001; Jonasson et al., 1999; Newton et al., 2010). Inhaled β_2 -agonists are currently the medications of choice for the relief of bronchoconstriction and exercise-induced bronchoconstriction but patients can frequently develop tolerance after repeated use. Furthermore, Asthma is an inflammatory disorder in which the small airways of the lung play an important role. However, inhaled therapies have the disadvantage of limited penetration into the peripheral airways and an inability to treat the systemic component of asthma (Bjermer, 2001). In this context, many efforts have been made to

develop a novel oral anti-asthma medication which can overcome the drawbacks of the conventional anti-asthma drugs (Barnes, 2006; Mullane, 2011).

Asthma is a highly complex disease in which multiple factors such as inflammatory cytokines, lipid mediators, eosinophil infiltration and aberrant CD4⁺ T lymphocyte (Th1/Th2) balance are involved and, therefore, modulation of a single factor can hardly produce a clinically significant outcome (Apter, 2014; Wenzel, 2013). Corticosteroids still remain the sole effective therapy for asthma since they can suppress multiple inflammatory mechanisms but on the other hand, they accompany many side effects (Newton et al., 2010; Rottier and Duiverman, 2009). Specific therapies targeted on single cytokines or inflammatory mediators are associated with fewer side effects but they might be less effective than corticosteroids. Asthma is a very complex disease so it is unlikely that targeting a single receptor or mediator will be greatly effective (Barnes, 2010). Except biologics, the one and only novel class of anti-asthma therapy introduced in the last 30 years are leukotriene antagonists (e.g. montelukast). The most effective approaches may be combination therapies that suppress multiple cytokines and a range of redundant and disconnected pathways that separately contribute to asthma pathogenesis (Hansbro et al., 2011). This suggests that novel asthma therapies need to have a broad spectrum of anti-inflammatory activity and at the same time they should be free from safety issues. In this background, herbal medicine which can harbor multiple components with a broad spectrum of anti-inflammatory activities has attracted a large attention.

Sophora tonkinensis (Leguminosae, ST) is a traditional herbal medicine, namely Shan-Dou-Gen in China and Korea. Its roots and rhizomes have been used to dissipate heat, to clear toxic material and to treat acute pharyngolaryngeal infections and sore

throats. Previous reports regarding chemical constituents and biological evaluations disclosed that flavonoids in the *S. tonkinensis* might modulate inflammatory cytokines (Lee et al., 2015; Yoo et al., 2014b; Chae et al., 2016a and 2016b). Based on the traditional usage and recent findings, *S. tonkinensis* extract may be effective in airway diseases such as asthma but no experimental evidence currently exists to support this notion to our knowledge.

In this study, we tried to investigate the anti-inflammatory and anti-bronchospasmic effects of a purified extract (SKI3301) and its major compounds from *S. tonkinensis* using in vitro enzyme assay models and ovalbumin-induced asthma animal models.

1. Materials and methods

1.1. Reagents

SKI3301 was prepared as the method described at the literature (Yoo et al., 2014b). 10 kg of *Sophora tonkinensis* Gagnep. root and rhizome of 0.5 ~ 2.0 cm in length is twice extracted with 70 L of about 50 % (v/v) ethanol-water mixture in a extraction unit at a temperature of 80 °C. The extract is filtered and is then concentrated by evaporation *in vacuo* until its volume becomes 10 L. The concentrate is twice partitioned by 10 L of water-saturated *n*-butanol. The upper phase is collected and filtered. The upper phase is then concentrated by evaporation *in vacuo*. Trifolirhizin and maackiain was purchased from ChromaDex. Sophoranone were obtained from Elcom Science. The plant material used in the present study was identified by Professor Young Bae Seo, College of Pharmacy, Seoul National University. A voucher specimen (SKC01) has been deposited at the herbarium of the College of Pharmacy, Seoul National University. SKI3301 extract (Lot No. SOTB07-10-07, which contained 4.76% of TF, 1.84% of Maack, 3.17% of SPN, and 0.25% of ABF) was provided (Fig 1) by Life Science R&D Center, SK

Chemicals (Sungnam, Republic Korea).

1.2. In vitro inflammatory enzyme assay

1.2.1. 5-lipoxygenase (5-LO) inhibition assay

Peripheral blood mononuclear leukocytes (PBML) were isolated from peripheral blood by density gradient centrifugation by following the method described by Peltier et al (Peltier et al., 2009). PBML were preincubated with compounds or vehicle for 15 min at 37 °C. The reaction was initiated by adding calcium ionophore A 23187 (Sigma C7522, USA) in DMSO and terminated after 15 min by adding 20 µl of 1N HCl to stop the reaction. Neutralize the reaction with 20 µl of 1N NaOH. Centrifuge assay plate for 10 min at 3,000 rpm. The supernatant was analyzed for LTB₄ assay by enzyme immunoassay kit (Assay Design Inc. 900068)

1.2.2. Phosphodiesterase 4 (PDE4) inhibition assay

Human U937 monocytic cell was cultured at 37°C in a humidified atmosphere with 5% CO₂ in RPMI 1640 medium supplemented with 10% fetal calf serum. The cells harvested by centrifugation for 10 min at 1,500 rpm at 4 °C. The medium was removed and the cell pellet rinsed with PBS (150 mM NaCl, 2.5 mM KCl, 1.5 mM KH₂PO₄, 8 mM Na₂HPO₄, pH 7.4). The solution was centrifuged at 10,000 rpm for 20 min 4 °C. The pellet was resuspended with 20 mM Tris-HCl, 1 mM MgCl₂, 0.1 mM EDTA, 3mM DTT, pH 7.1. The solution went through gel-filtration column (Hi PrepTM 16/60 Sephacryl S-300 HR, code No: 17-1167-01) to get more purified enzyme. The enzyme was stored at -70 °C and ready for use. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, test compounds or vesicles and an appropriate amount of the

enzyme, and was preincubated at 25 °C for 15 min. The reaction was initiated by and 1 μ M cold cAMP and 0.01 μ M [^{3} H] cAMP and incubate for 20 min at 25 °C. The reaction was terminated by boiling for 5 min and then chilled on ice for 5 min. 100 μ g of snake venom (Sigma V7000) were added and incubated for 10 min at 37 °C. And then 200 μ l of AG1-X2 resin (Bio-Rad, 140-1251) in water were added and allowed resin to settle down for 5 min. The [^{3} H]adenosine in clear supernatant fluid was counted in a liquid scintillation counter.

1.2.3. Thromboxane synthase (TxS) inhibition assay

Human concentrated platelet was placed into centrifuge tube and washed with equal volume of HBSS and then centrifuged at 2,500 rpm for 15 min. The pellet was resuspended with 20mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1mM DTT, 1mM Benzamidine, 1 mM MgCl₂. and centrifuged at 2,500 rpm for 15 min. The pellet was resuspended with 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1mM DTT, 1 mM Benzamidine, 1 mM MgCl₂. The solution was centrifuged at 10,000 rpm for 30 min. The supernatant was taken and centrifuged at 375,000 rpm for 60 min at 4 °C. The pellet was resuspended with 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 1mM DTT, 1mM Benzamidine, 1 mM MgCl₂ and stored at -70 °C and ready for use. Test compounds or vehicle and adequate amount of enzyme in 10mM Tris-HCl, pH 7.4 were combined and preincubated for 15 min at 25 °C. 10 mM PGH₂ was added to initiate the reaction and incubated for 3 min at 25 °C. 20 μ l of 1N HCl was added to stop the reaction. Neutralize the reaction with 20 μ l of 1N NaOH. Centrifuge assay plate for 10 min at 3,000 rpm. The supernatant was analyzed for Thromboxane assay by enzyme immunoassay assay kit (Assay Design Inc.

900002)

1.2.4. Phosphodiesterase 3 (PDE3) inhibition assay

Concentrated platelet from human was placed into centrifuge tube and was centrifuged at 4,200 rpm for 20 min at 4 °C and then the supernatant was discarded. The pellet was resuspended with 50mM Tris-HCl, pH 7.4. The solution was centrifuged at 4,200 rpm for 20 min at 4 °C. The pellet was resuspended with 20 mM Tris-HCl, 1 mM MgCl₂, 0.1 mM EDTA, 3 mM DTT, pH 7.1. The solution was centrifuged at 8,000 rpm for 10 min at 4 °C. And then the supernatant went through ion-exchange column (Hi Prep™ 16/10, code No: 17-5090-01) to get more purified enzyme, the enzyme was stored at -70 °C and ready for use. The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, test compounds or vesicles and an appropriate amount of the enzyme, and was preincubated at 25 °C for 15 min. The reaction was initiated by adding 1 μM cold cAMP and 0.01 μM [³H] cAMP and incubate for 20 min at 25 °C. The reaction was terminated by boiling for 5 min and then chilled on ice for 5 min. 100 μg of snake venom (Sigma V7000) were added and incubated for 10 min at 37 °C. And then 200 μl of AG1-X2 resin (Bio-Rad, 140-1251) in water were added and allowed resin to settle down for 5 min. The [³H]adenosine in clear supernatant fluid was counted in a liquid scintillation counter.

1.3. Animals

Specific pathogen-free female BALB/c mice, aged 6 – 8 weeks, and guinea pigs aged 7 ~ 8 weeks were purchased from the SLC (Japan). The animals were maintained at 22 ± 2 °C temperature, 50 ± 5 % relative humidity with a 12 h cycle of light and dark.

They had free access to water and food *ad libitum*. All the protocols for the animal studies (SKIACUC-P-011, SK-IACUC-140108-1) were approved by the internal animal ethics committee of SK Chemicals.

1.4. Induction of airway inflammation and drug administration

The mice were sensitized with an intraperitoneal injection of 20 µg OVA (Albumin from chicken egg white, Grade V. Sigma), which was emulsified in 2 mg Alum (Inject Alum, Pierce), in a total volume of 100 µl on days 1, 7, and 14 days. At day 21, mice were grouped as following: 1) OVA-sensitized plus challenge with PBS; 2) sensitization plus challenge with 1% OVA and treated with 1% CMC; 3) sensitization plus challenge with 1% OVA and treated bid with compounds for 3 days from day 22. For oral administration, SKI3301 was suspended in 1% CMC. Trifolirhizin and maackiain were suspended in 0.5% MC. Sophoranone was suspended in 30% PEG and 70% corn oil because of its low solubility. The sensitized mice were challenged through the airways with 1% OVA in PBS for 50 min which was delivered by a jet nebulizer (Hugo Sachs Electronick, Hugstetten Germany) driven by compressed air at 20 psi. OVA inhalation was done for 50 min at days 22 and 24 after the initial sensitization. Twenty-four hours after the last challenge (day 25), the mice were sacrificed for bronchoalveolar lavage and histopathological examination.

1.5. Collection of bronchoalveolar lavage (BAL) fluid

The lungs of the mice were cannulated and ice-cold PBS (total 1.5 ml) was instilled into the lungs. Bronchoalveolar lavage fluid (BAL) fluid was obtained by aspiration three times via tracheal cannulation. Each BAL fluid was centrifuged and the

supernatant collected and stored at -70°C. Total inflammatory cell number in BAL fluid was measured using cell counter (MS9-5H, Melet Schloesing Lab. Osny France). For differential cell count, 100 µl of BAL fluid was loaded onto a slide and centrifuged (200g, 4°C, 10 min) to fix the cells onto the slide using a cytospin (Shandon Cytospin 4 Cyto centrifuge. Thermo Electron Corp. MA. USA). Cell pellets was suspended in 0.5 ml PBS, and 100 µl of each solution was spun onto a slide. After slides were dried, cells were fixed and stained using Shandon Kwik-Diff stains (Thermo Electron Corp. MA, USA)

1.6. Measurement of interleukin 5 (IL-5) in BAL fluid

IL-5 in BAL fluid was measured using mouse specific ELSIA kit (M5000, R&D Systems Inc. MN USA) according to the manufacturer's instructions. Briefly, a monoclonal antibody specific for mouse IL-5 has been pre-coated onto a microplate. Samples are reacted into the wells and incubated for 2 hours at room temperature. After washing, horseradish peroxidase enzyme labeled antibody IL-5 reacted at 2 hours at room temperature. The microtiter strips are then washed to remove unbound antibody-enzyme reagent. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The yellow color generated read on a microplate reader at 450nm.

1.7. Preparation of lung tissues and histopathology

After BAL fluid was obtained, the lung tissues were fixed in 10% neutral buffered formalin and they were embedded in paraffin wax and then cut into 4 µm thickness sections. Sections were stained with hematoxylin and eosin (Sigma, USA) for general

morphological assessment or periodic acid Schiff (PAS) stain (Sigma, USA) for the detection of mucus-secreting cells (goblet cells) hyperplasia. The evaluation of histopathological findings was performed under light microscopy by two pathological investigators in a blinded fashion. The histopathological findings of each section were classified into four characteristic categories of observation (perivascular or periairway infiltration of inflammatory cells, alveolitis and goblet cell hyperplasia). The parameters were graded from score 0 to 3, with 0 indicating not remarkable lesion (-), 0.5 indicating minimal lesion ($\pm$), 1 indicating mild lesion (+), 2 indicating moderate lesion (++), and 3 indicating remarkable lesion (+++).

1.8. Measurement of OVA-induced contraction of trachea isolated from sensitized guinea pigs

The guinea pigs (400 ~ 500 g) were passively sensitized with an intravenous injection of anti-OVA anti-serum (titer 1000 ~ 3000) in a total volume of 1.5 ml/kg at 48 hr before use. The trachea were isolated and cut into ring approximately 2 mm wide. The trachea rings were opened by cutting the bone area and tied each ends. The trachea rings were suspended in 10 ml organ baths containing Krebs-Henseleit solution bubbled with 95% O₂ and 5% CO₂ and maintained at 37°C. The pH of the equilibrated buffer was 7.4

Trachea contractions were measured with force transducer (Biegestab K30 Type 351, Hugo Sachs Elektronik, March/Friburg, Germany) and transducer coupler (Type A, Coulborn Instruments, PA, USA). After the initial tension was adjusted 1 g, the tissue was treated with carbachol (100 μ g/ml). After measuring contraction, trachea was washed with new Krebs-Henseleit solution and stabilized for 30 min. This procedure

was repeated and we used only the trachea showing consistent response. After washing and stabilizing, the trachea was treated with test compounds for 5 min and then OVA was added to induce contraction to the bath. The tissue was incubated with indomethacin 2 μ M for 30 min to exclude effect of prostanoids (Redkar-Brown and Aharony, 1989). The OVA-induced contraction was measured for 30 min and then carbachol (100 μ g/ml) was added again to know the contraction reproducibility. The contraction was expressed as a percentage of the maximum carbachol response.

1.9. Measurement of OVA-induced bronchoconstriction of sensitized guinea pigs

The sensitized guinea pigs were pretreated with pyrilamine maleate (10 mg/kg) and propranolol (0.1 mg/kg) 30 min before aerosolized 1% OVA challenge for 2 min. Compounds were orally administered 1 hour before 1% OVA challenge. The bronchoconstriction was measured using Double Chamber Plethysmograph Box (HSE, Germany) installed with plethysmometer. Airway resistance was measured for 15 minutes as an indicator of bronchospasm.

1.10. Statistical analysis

All values are expressed as mean $\pm$ SEM. The statistical significance of the difference was assessed by one way ANOVA followed by Dunnet test or Turkey test. *P* values lower than 0.05 were considered significant. SigmaStat (Sigma Stat, Jandel Co., CA, USA) was used for statistical analysis.

2. Results

2.1. Effects of SKI3301 on pro-inflammatory enzymes

To explore the anti-asthmatic activities of SKI3301, we investigated the effect of SKI3301 on representative pro-inflammatory enzymes involved in asthma (Fig. 1), 5-lipoxygenase, phosphodiesterase 3/4 and thromboxane synthase. As a result, SKI3301 potently and concentration-dependently inhibited 5-lipoxygenase, phosphodiesterase 3/4 and thromboxane synthase with the IC₅₀ values of 1.61 µg/ml, 11.8 µg/ml, 0.634 µg/ml and 5.56 µg/ml, respectively.

2.2. Effect of SKI3301 on the infiltration of inflammatory cells and IL-5 in BAL fluid *in vivo*

To examine the effects of SKI3301 on asthmatic airway inflammation *in vivo*, we induced airway inflammation in BALB/c mice by ovalbumin (OVA) inhalation and evaluated the number of total leukocytes and eosinophils in the BAL fluid (Fig. 2A). OVA inhalation caused a marked influx of leukocytes into BAL fluid. Compared to the saline group, the OVA group had a 5.4-fold increase in total cells recovered from BAL fluid and the majority (66%) of the infiltrated inflammatory cells was eosinophils while in the saline group, eosinophil was less than 1% of total leukocytes. In the meantime, SKI3301 treatment significantly attenuated the increased numbers of total leukocytes and eosinophils in BAL fluid after OVA inhalation (Fig. 2A). Montelukast also showed a significantly suppressive effect on leukocytes infiltration into BAL fluid.

OVA inhalation induced significant IL-5 elevation in BAL fluid (Fig. 2B). SKI3301 treatment significantly inhibited the IL-5 increase in BAL fluid with dose-relating manner, further supporting the suppressive effect of SKI3301 on eosinophil

infiltration. MK also significantly inhibited the IL-5 level in BAL fluid.

2.3. Effect of SKI3301 on histopathology of lung tissues

Hematoxylin and eosin staining and periodic acid-Schiff (PAS) staining were performed on the lung tissues to analyze the effects of SKI3301 on histological feature of asthma. Histological analyses revealed typical pathologic features of asthma in the OVA-challenged mice (Fig. 3). The lung tissue of OVA inhalation group showed dense peribronchial and perivascular inflammation due to leukocyte infiltration (Fig. 3A) and mucus hyper-production by goblet cells within the bronchi when compared with the NC group (Fig. 3B). SKI3301 reduced the degree of inflammatory cell infiltration and hyperplasia of goblet cell. These results indicate that SKI3301 reduces the degree of pathological inflammation in lung tissues in asthma.

2.4. Effect of SKI3301 on OVA-induced contraction of trachea isolated from sensitized guinea pigs

The administration of specific antigen to isolated trachea from sensitized guinea pigs results in a rapid developing and prolonged contraction (Jones et al., 1988). When added to isolated trachea from sensitized guinea pigs, OVA induced 84.9% contraction compared to maximum contraction induced by carbachol (Fig. 4). SKI3301 inhibited the OVA-induced contraction of isolated trachea in a concentration-dependent manner. Rolipram (RP), a phosphodiesterase inhibitor, significantly inhibited OAV-induced contraction of the isolated trachea, but, MT failed to show significant inhibition effect.

2.5. Effect of SKI3301 on OVA-induced bronchoconstriction of passively sensitized conscious guinea pigs

The guinea pigs passively sensitized were pretreated with pyrilamine maleate to be protected from immediate death upon OVA challenge and with propranolol to potentiate bronchoconstriction (Anderson et al., 1983; Heuer, 1991). The aerosolized 1% OVA challenge to passively sensitized guinea pigs resulted in a rapid developing of bronchoconstriction (Fig. 5). Orally administered SKI3301 also reduced significantly the OVA-induced bronchoconstriction of the sensitized guinea pigs at the doses of 200 and 400 mg/kg. Meanwhile, SKI3301 100 mg/kg and montelukast had no significant effects on OVA-induced bronchoconstriction of passively sensitized conscious guinea pigs.

2.6. Effect of SKI3301 components on inflammatory enzymes and airway inflammation

SKI3301 contains 3 major compounds, trifolirhizin, maackiain, and sophoranone. At *in vitro* enzyme assay, maackiain and sophoranone inhibited 5-lipoxygenase (5-LO) and their IC₅₀ were 7.9 μ M and 1.6 μ M. Trifolirhizin had no inhibition effects on 5-LO, PDE3, and PDE4. At *in vivo* study, only maackiain treatment groups significantly attenuated the increased numbers both of total leukocytes and eosinophils in BALF after OVA inhalation (Fig. 6). Trifolirhizin showed 22.6% inhibition effect on eosinophil increase in BALF, but it's not statistically significant ($p = 0.081$). Meanwhile, sophoranone 30 mg/kg showed no significant inhibition effects on total leukocytes and eosinophils increase in BALF compared to vehicle (30% PEG).

Discussion and conclusion

Allergic asthma is an inflammatory disease characterized by eosinophilic airway inflammation, mucus hyper-secretion, and bronchial hyper-responsiveness. Oral treatment of SKI3301 alleviated the OVA-induced asthmatic changes such as leukocytes infiltration in BAL fluid and histopathological changes of the lung tissue in sensitized BALB/c mice challenged with OVA. Potent and broad spectrum inhibition of pro-inflammatory enzymes, 5-LO, PDE3/4, and thromboxane synthase by SKI3301 may underlie its anti-asthmatic, anti-bronchospasmic and anti-inflammatory effects *in vivo*. It is well known that inflammatory enzymes such as 5-LO, PDE3/4, and thromboxane synthase play important roles in the development of asthma. 5-LO catalyzes the conversion of arachidonic acid (AA) into leukotriene A₄ (LTA₄) and 5-hydroperoxyeicosatetraenoic acid. LTA₄ can then be converted either into LTB₄ by LTA₄ hydrolase or into LTC₄ by LTC₄ synthase (Werz and Steinhilber, 2006). LTB₄ is a potent chemo-attractant for neutrophils, eosinophils and monocytes, leading to an adherence of phagocytes to the vessel walls, a neutrophil degranulation and a release of superoxide anions (Nagy et al., 1982; Okada et al., 1996). LTC₄ and its metabolite, LTD₄, on the other hand, work by acting as potent bronchoconstrictor that increase a vascular permeability as well as stimulate a mucus secretion from airways (Wenzel, 2003). 5-LO inhibitor, zileuton, as well as CysLT1 receptor antagonists, montelukast, pranlukast and zafirlukast, are now available for the treatment of asthma (Werz and Steinhilber, 2006).

PDE₄ is considered as a promising anti-inflammatory target for several reasons: 1) leukocyte functions are suppressed by cAMP, 2) PDE₄ is the predominant isoform present in inflammatory and immune cells, 3) and inhibitors of PDE₄ negatively

regulate the functions of almost all proinflammatory and immune cells and exert widespread anti-inflammatory activities in animal models of asthma (Fan Chung, 2006). The PDE₄ inhibitors, roflumilast and cilomilast have been reported to improve pulmonary function and symptoms in both asthma patients and animal models.

Cilostazol, a PDE₃ inhibitor, reduced bronchial responsiveness in healthy volunteers and asthmatics (Fusimura et al., 1995; Fusimura et al., 1997). Siguazodan, a PDE₃ inhibitor, was more effective than rolipram as a muscle relaxant in both large and small airways, and siguazodan, but not rolipram, potentiated the relaxant effect of isoprenaline on LTD₄-contracted smooth muscle (Torphy et al., 1993). The recognition that PDE₄ inhibitors are anti-inflammatory and PDE₃ inhibitors are bronchodilators has lead to research on combination effects and the development of dual inhibitor (Abbott-Banner & Page, 2014; Matera et al., 2014). Although PDE₃ inhibitors appear to have little effect on T-cell but PDE₃ inhibitors may potentiate the effect of PDE₄ inhibitors on T-cell activation (Giembycz et al., 1996).

Thromboxane A₂ (TXA₂), a metabolite of arachidonic acid, is a potent bronchoconstrictor and has been proposed to play an important role in bronchial asthma by inducing and developing bronchial hyperresponsiveness and bronchoconstriction (Jones and Saroea, 1992). It was already shown that TXA₂ modifiers, receptor antagonists and synthase inhibitors were able to suppress the antigen-induced immediate bronchoconstriction and to attenuate the bronchial hyperresponsiveness in asthmatic patients (Fujimura et al., 1986; Beasley et al., 1989; Fujimura et al., 1990)

SKI3301 also significantly inhibited IL-5 production in BAL fluid. Among Th2-specific cytokines, IL-5 is known to be a key cytokine in the growth, the differentiation and the release of bone marrow eosinophil into the peripheral circulation, and it also promotes

the recruitment, activation, and survival of eosinophil in the site of inflammation (Foster et al., 2002; Takatsu and Nakajima, 2008). The activated eosinophil acts as an effector cell in allergic diseases by releasing cytotoxic granule proteins such as leukotrienes. The inhibitory activities of SKI3301 both on eosinophil infiltration and IL-5 might be interrelated.

Allergic asthma is a pulmonary disease that causes difficulty in breathing due to airway obstruction. SKI3301 significantly reduced the OVA-induced bronchoconstriction of the sensitized guinea pigs both in vivo and in vitro. Meanwhile montelukast did not significantly inhibited OVA-induced contraction. It may be a result from specific and selective activity of montelukast against LTD₄ receptor. Jones et al. reported that montelukast antagonized contractions of guinea pig trachea induced by LTD₄ but it failed to antagonize contraction of guinea pig trachea induced by LTC₄, serotonin, acetylcholine, histamine, prostaglandin D₂, U-44069 (Jones et al., 1995). This may be other mechanisms like PDE3/4 may be involved in OVA-induced bronchoconstriction (Underwood et al., 1994) and only broadly effective agents like SKI3301 may be effective against allergen-induced bronchoconstriction.

Among the 3 major components of SKI3301 (trifolirhizin, maackiain and sophoranone), trifolirhizin and maackiain might contribute the effects of SKI3301 on airway inflammation. Trifolirhizin was reported that it could not only inhibit airway smooth muscle contraction to acetylcholine but also relax pre-contracted airway smooth muscle (Yang et al., 2013). Trifolirhizin is a maackiain glycoside and was metabolized to maackiain when administered orally to rat (Yoo et al., 2014a). Maackiain is known to inhibit mixed lymphocyte reaction (MLR) in mouse T cells (Kim et al., 2013) and in our studies it inhibited 5-LO activity and reduced OVA-induced airway inflammation.

Sophoranone also inhibited 5-LO activity and was known to have significant ROS inhibitory activity (Atta-ur-Rahman et al., 2012). However, sophoranone failed to inhibit airway inflammation *in vivo* at our study and it may be related with its low absorption when administered orally (Yoo et al., 2014a).

In summary, these results demonstrated that SKI3301 inhibited inflammatory enzymes and significantly decreased the OVA-induced airway inflammation and bronchospasm that is characteristic feature of asthma. Taken together, these results indicate that SKI3301 is a potential herbal medicine for asthma treatment.

Conflict of interest

The authors have declared no conflict of interest statement in this article

Author contributions

HY, KHR and YWC designed this work. HY, MK and SP conducted the *in vivo* and *in vitro* experiments. HY and YWC prepared the chemical analysis of SKI3301. HY, MK, SP, KHR, HSC, JK and YWC analyzed the experimental data. HY, HSC, JK and YWC wrote the first draft of manuscript. All authors approved the final manuscript.

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Fig 1. Inhibitory effect of SKI3301 on 5-lipoxygenase, phosphodiesterase 3/4, and thromboxane synthase. A) 5-LO activity in human PBML cells. LTB₄ production was measured by enzyme immunoassay. B) PDE4 enzyme activity in human U937 cells. The [³H]adenosine production was measured from [³H]cAMP + cAMP. C) TxS activity in human platelets. Thromboxane B₂ production from PGH₂ was measured using enzyme immunoassay kit. D) PDE3 enzyme activity in purified enzyme from human platelet. The [³H]adenosine production was measured from [³H]cAMP + cAMP.

Fig 2 SKI3301 inhibited the infiltration of inflammatory cells and IL-5 level in BAL fluid. Female BALB/c mouse was challenged with OVA inhalation (at day 22, 24) to induce allergic inflammation after sensitization with ovalbumin and alum (at day 0, 7, 14). BAL fluid was obtained from each group 1 day after last challenge, and the number of total lymphocytes and eosinophils (A) and IL-5 level were determined (B). Animals were orally administered with vehicle or compounds (at day 22 ~ 24). Results are shown as mean ± SEM. NC: normal control; MT: montelukast. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ vs vehicle.

Fig 3 SKI3301 reduced the intensity of eosinophil and lymphocyte infiltration into peribronchial and peribronchiolar space, alveolitis, and goblet cell hyperplasia. Lung sections from OVA-challenged mice were fixed, sectioned, and stained with hematoxylin and eosin to assess airway inflammation or periodic acid Schiff (PAS) reagent to assess goblet cell hyperplasia. The intensity was graded as described in section 2. NC: normal control; MT: montelukast. H&E staining x 40; PAS staining x 200.

Fig 4 Effects on OVA-induced contraction of isolated trachea from sensitized guinea pigs. Naive guinea pigs were sensitized by intravenous injection of anti-OVA-antiserum (1.5 ml/kg) and the guinea pig were used 48 h later. Isolated trachea was pretreated with 10 nM indomethacin before 10 µg/ml OVA challenge. The contraction was expressed as a percentage of the maximum carbachol response. Each data represents the mean ± SEM (n=5~6). ** $p < 0.01$ vs vehicle.

Fig 5 OVA-induced bronchoconstriction of passively sensitized conscious guinea pigs. Naive guinea pigs were sensitized by intravenous injection of anti-OVA-antiserum (1.5 ml/kg) and the guinea pig were used 48 h later. The sensitized guinea pigs were pretreated with pyrilamine maleate (10 mg/kg) and propranolol (0.1 mg/kg) 30 min before aerosolized 1% OVA challenge for 2 min. Compounds were orally administered 1 hour before 1% OVA challenge. The bronchoconstriction was measured using Double

Chamber Plethysmograph Box (HSE, Germany) installed with plethysmometer. Airway resistance was measured for 15 minutes as an indicator of bronchospasm. AUC15: The area under the airway resistance curve. Each data represents the mean $\pm$ SEM (n=12). *, $p < 0.05$ vs vehicle.

Fig 6 Maackiain inhibited the infiltration of inflammatory cells in BAL fluid and reduced the intensity of eosinophil and lymphocyte infiltration into peribronchial and peribronchiolar space. Female BALB/c mouse was challenged with OVA inhalation (at day 22, 24) to induce allergic inflammation after sensitization with ovalbumin and alum (at day 0, 7, 14). BAL fluid was obtained from each group 1 day after last challenge, and the number of total lymphocytes and eosinophils were determined. Lung sections from OVA-challenged mice were fixed, sectioned, and stained with hematoxylin and eosin to assess airway inflammation. The intensity was graded as described in section 2. H&E staining x 40

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Fig. 1

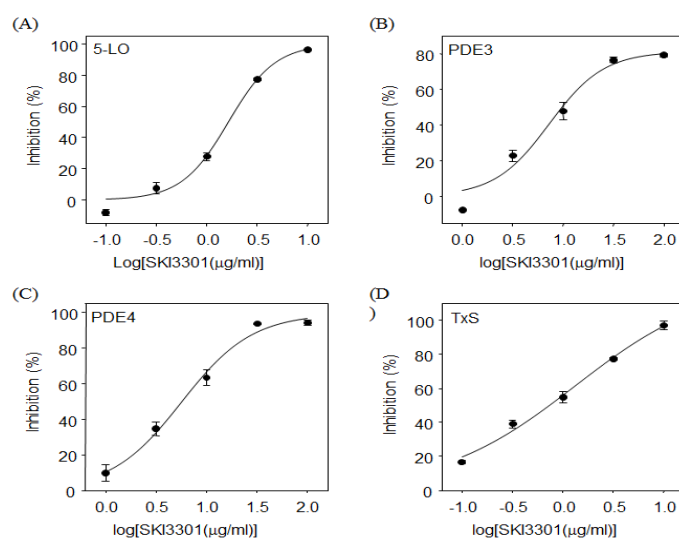


Fig. 2

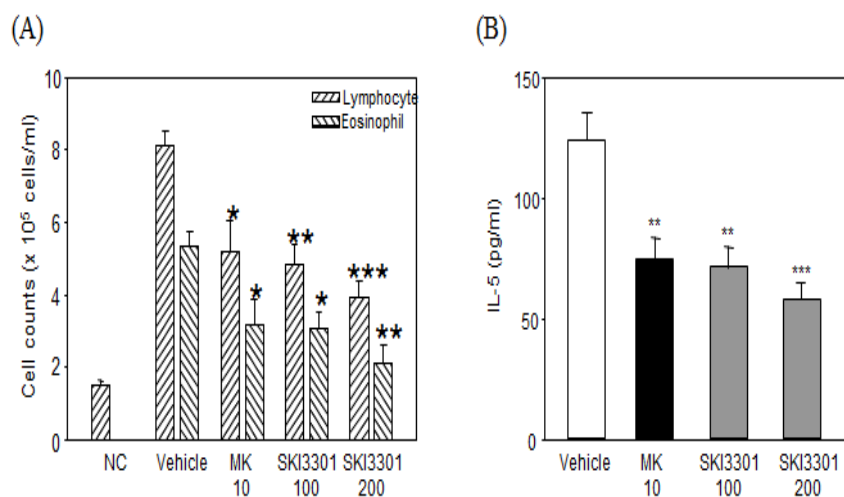


Fig. 3

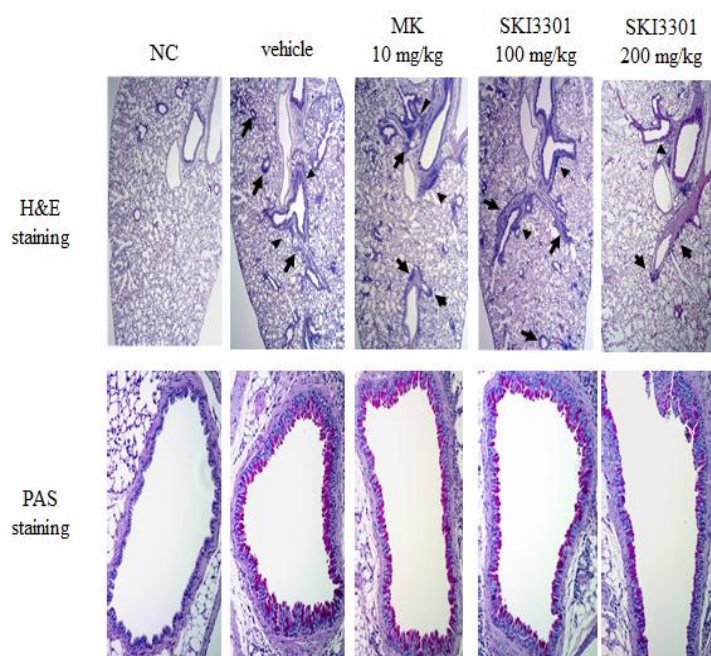


Fig. 4

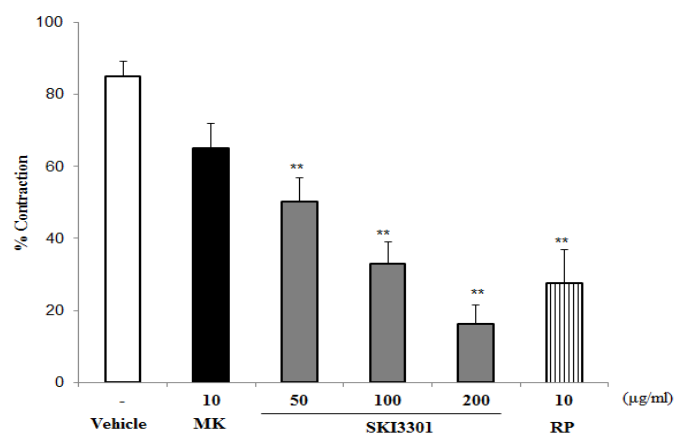


Fig. 5

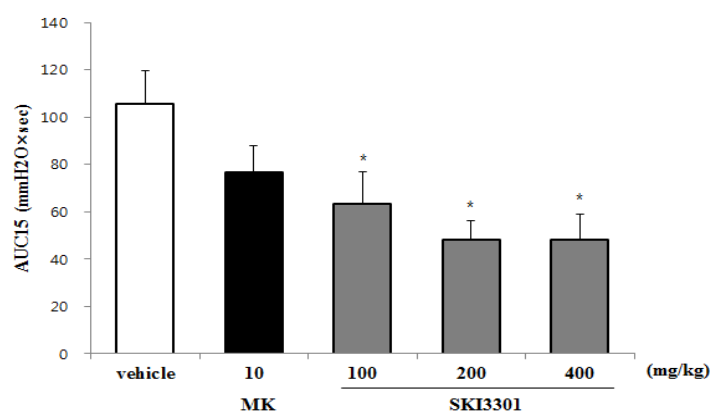
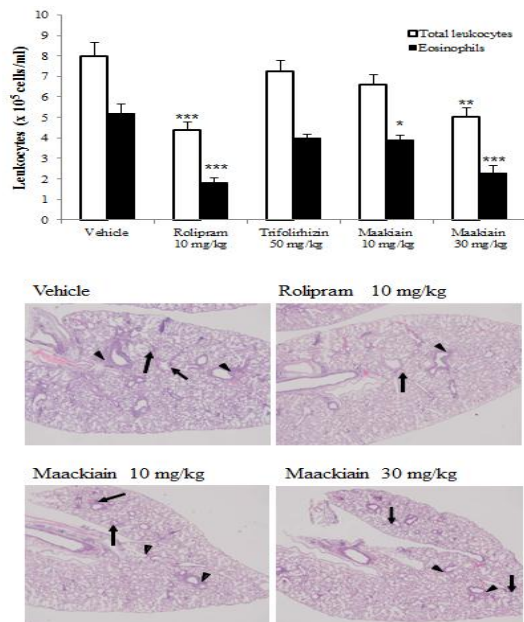
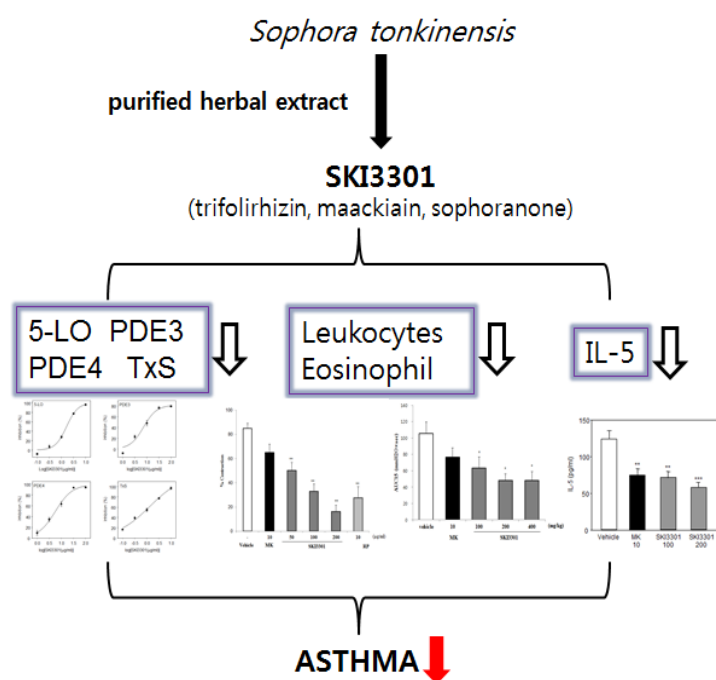


Fig. 6



Graphical Abstract



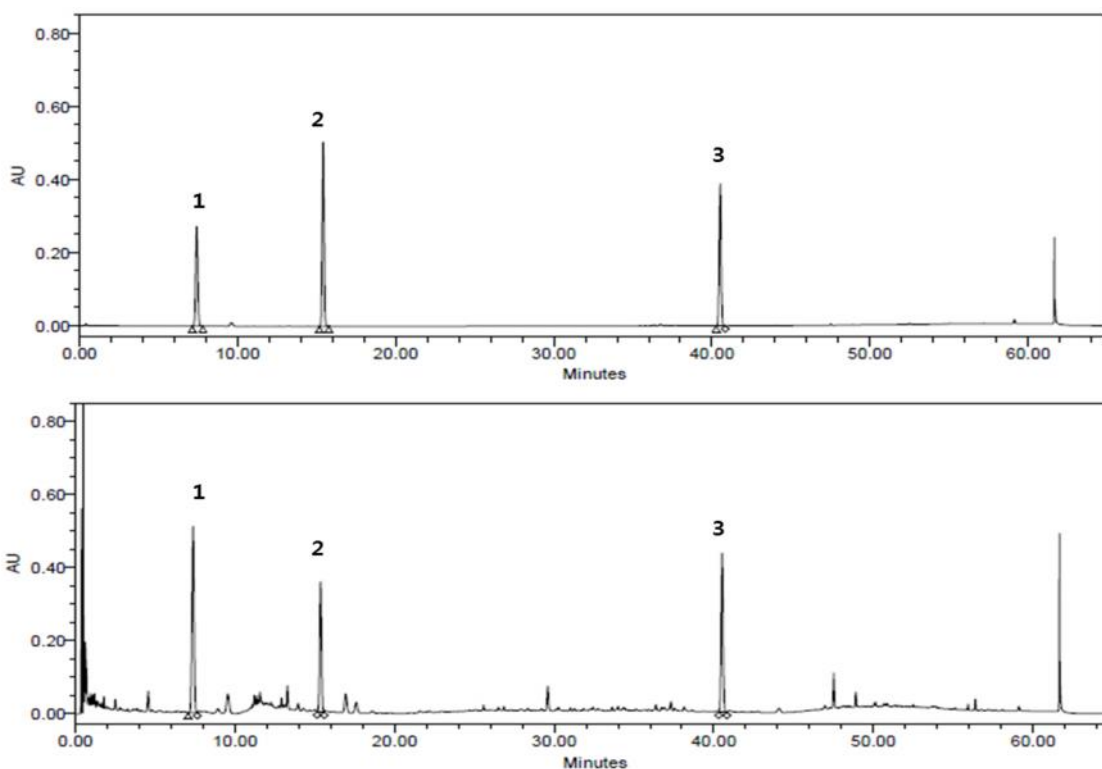


Figure S1. Chromatogram of SOTB07-10-07 and three reference standards. (A) HPLC-UV chromatogram of three reference compounds. (B) HPLC-UV chromatograms of SOTB07-10-07 chromatogram;

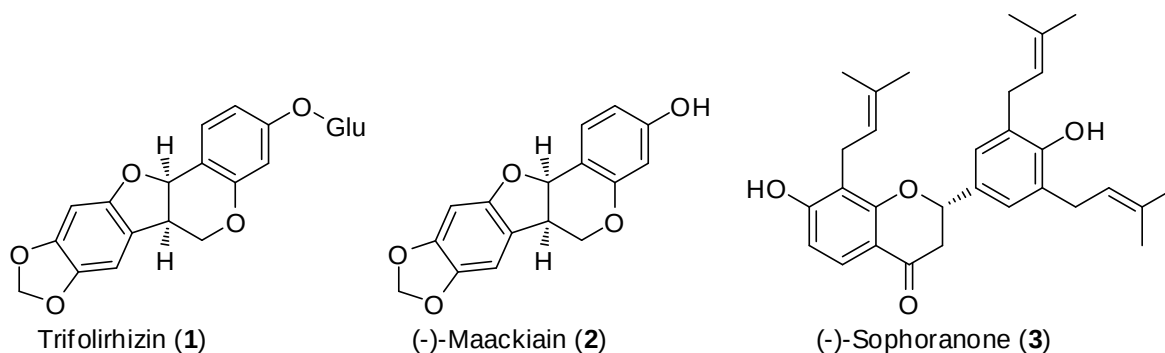


Figure S2. The structures of major compounds in SOTB07

Table S1. : The quantitative of analytic markers in SOTB07

Serials	Analytic marker	Contents
1	Trifolirhizin	4.76
2	(-)-Maackiain	1.84
3	(-)-Sophoranone	3.17

