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Pharmacology and Immunological Mechanisms of an Herbal Medicine, ASHMI™ on Allergic Asthma

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Abstract

Allergic asthma is a chronic and progressive inflammatory disease for which there is no satisfactory treatment. Studies reported tolerability and efficacy of an anti-asthma herbal medicine intervention (ASHMI) for asthma patients, developed from traditional Chinese medicine. To investigate the pharmacological actions of ASHMI on early- and late-phase airway responses (EAR and LAR), Ovalbumin (OVA)-sensitized mice received 6 weeks of ASHMI treatment beginning 24 h following the first intra tracheal OVA challenge. EAR were determined 30 min following the fourth challenge and LAR 48 h following the last challenge. ASHMI effects on cytokine secretion, murine tracheal ring contraction and human bronchial smooth muscle cell prostaglandin (PG) production were also determined.

ASHMI abolished EAR, which was associated with significantly reduced histamine, leukotriene C4, and OVA-specific IgE levels, as well as LAR, which was associated with significantly reduced bronchoalveolar lavage fluid (BALF) eosinophils, decreased airway remodeling, and lower Th2 cytokine levels in BALF and splenocyte cultures. Furthermore, ASHMI inhibited contraction of murine tracheal rings and increased production of the potent smooth muscle relaxer PGI₂. ASHMI abrogation of allergic airway responses is associated with broad effects on asthma pathological mechanisms.

Keywords

allergic asthma; traditional Chinese herbal medicine; airway hyperreactivity; Th2 cytokines and airway smooth muscle contractility

Introduction

Over the past several decades, the number of individuals with atopic diseases, such as asthma, has increased dramatically in industrialized countries. Current conventional medications for these chronic disorders are not fully satisfactory and prolonged use often causes serious side effects. Effective and safe alternative medicines are needed to improve asthma treatment. In recent years, there has been increasing interest in complementary and alternative medicine (CAM) (Bielory et al., 2004; Li, 2009). Traditional Chinese medicines (TCMs) have been used to treat asthma for centuries in China and other Asian countries and are still in the medical mainstream in these countries. TCM, as with CAM therapy, is beginning to play a role in the US healthcare system. In recent years, a small but increasing number of well-controlled clinical trials have provided evidence that some TCMs may have potential for developing botanical drugs (Li, 2009). Our previous clinical study of adult, moderate–severe, persistent asthma patients showed that the Anti-asthma Herbal Medicine Intervention (ASHMI) increased lung function (FEV1), reduced symptom scores, and decreased β -2 agonist use, without adverse effects, such as adrenal or immune suppression commonly seen with prednisone (Wen et al., 2005). Patients in the ASHMI-treated group showed no abnormal findings in hematology, serum chemistry, or electrocardiograms (Wen et al., 2005). ASHMI is the first herbal medicine to receive US Food and Drug Administration (FDA) investigational new drug approval for clinical trials for treating asthma. A phase I study confirmed that ASHMI is safe and well tolerated clinically (Kelly-Pieper et al., 2009). A phase II study is underway. ASHMI may prove to be an effective alternative therapy for asthma. However, its pharmacological and immunological actions have not been fully elucidated. Allergic asthma is a complex disease and its end point is airway hyperresponsiveness (AHR) (bronchoconstriction). Asthma is expressed clinically as an early phase airway reaction (EAR), occurring within 30 min of relevant antigen (Ag) exposure, followed by a late phase airway reaction (LAR) 9–12 h later. EAR is triggered by the release of histamine and leukotrienes (LT) from IgE-activated mast cells, whereas LAR is mediated by infiltration of inflammatory cells, primarily eosinophils (Barrios et al., 2006). Th2 cytokines play a central role in both EAR and LAR (Barrios et al., 2006). GATA-3 is the key transcription factor underlying Th2 cell differentiation and memory (Lohning et al., 2002). It is also suggested that abnormal airway smooth muscle (ASM) tone, increased ASM contraction and reduced ASM relaxation are the determinants of AHR (Borger et al., 2006). In this study, we tested the effects of ASHMI on EAR and LAR in a mouse model of chronic asthma and investigated relevant pharmacological and immunological mechanisms underlying these effects in vivo and in vitro. We demonstrated, for the first time, that an herbal formula exerts a wide range of pharmacological actions on multiple mechanisms underlying allergic asthma.

Methods

ASHMI preparation and quality control

ASHMI is composed of 3 herbs, inspected for identity and quality by Duan WL, a pharmacologist specializing in traditional Chinese medicine (Department of Material Medica, Weifang Asthma Hospital, Weifang, China). Based on organoleptic and

microscopic examination, the raw herbal materials used in ASHMI were identified as the fruiting body of *Ganoderma lucidum* (Ling-Zhi), the roots of *Sophora flavescens* Ait (Ku-Shen), and the roots and rhizome of *Glycyrrhiza uralensis* Fischer (Gan-Gao), respectively (Table 1). Voucher specimens of the raw herbs are archived in the botanical chemistry laboratory, Center for Chinese Herbal Medicine for Allergy and Asthma, Mount Sinai School of Medicine, New York. ASHMI was provided by the Sino-Lion Pharmaceutical Company (Weifang, China) as previously described (Kelly-Pieper *et al.*, 2009). In brief, herbs were boiled together twice in water. The decoctions were combined, concentrated under reduced pressure, and dried. The yield of ASHMI extract was 11.5%. Product quality and consistency were monitored by high performance liquid chromatography (HPLC) fingerprinting (Fig. 1), as previously described (Kelly-Pieper *et al.*, 2009). In brief, 50 mg of ASHMI extracts were dissolved in 10 ml of H₂O. The solution was transferred to a separatory funnel, and extracted with 5 ml of *n*-butanol three times. The combined extracts were dried and dissolved in 2 mL 50% aqueous methanol; 10 μ L of solution was then analyzed using a Waters Alliance 2695 HPLC system with photodiode array detector (2996; Waters Corporation, Milford, MA, USA). Data were acquired and processed with Waters' Empower software. The separation was achieved on Zorbax SB-C18 column (150 $\times$ 4.6 mm; 5 μ m particle size) from Agilent Technologies (Santa Clara, CA, USA). The mobile phases include 0.15% H₃PO₄ (A) and acetonitrile (B). The separation gradient started at 2% of B to 48% for 75 min and further increased to 70% in 4 min. The flow rate was adjusted to 1.0 mL/min and the column temperature was set to be 27 °C. All the chemicals and solvent used were HPLC grade from Fisher Scientific (Pittsburgh, PA, USA). Identity of compounds was established by LC-MS analysis (Fig. 1) using commercially available standards (ChromaDex, Inc., Irvine, CA, USA). LC-MS spectra were obtained using a LCT premier TOF mass spectrometer (Waters Corporation, Milford, MA, USA) coupled to Waters Alliance 2695 HPLC system. 10 μ L sample was analyzed under the same gradient condition described above. The mass spectra were collected from *m/z* 50 to 1000 in W optics mode and positive electrospray mode (ESI+). The desolvation gas flow was set to 500 L/h at a temperature of 350 °C. The cone gas was set to 40 L/h and the source temperature was set to 110 °C. The capillary voltage and the cone voltage were set to 3200 v and 25 v respectively. In order to generate accurate and reproducible results, all analyses were acquired using lock spray with Leucine, Enkephalin ([M+H]⁺ = 556.2771) as the reference. Heavy metal, pesticide, and microbial test results met the standards (Table 1). Endotoxin levels in ASHMI were tested using the Pyrogen Plus assay kit (<0.03 EU/ml, limit of sensitivity) (Lonza, Hopkinton, MA, USA). No endotoxin was detected (Srivastava *et al.*, 2009).

Antigen sensitization/challenge, ASHMI treatment—BALB/c mice (6 weeks old) were purchased from Jackson Laboratory (Bar Harbor, ME, USA). Standard guidelines for the care and use of animals were followed (Institute of Laboratory Animal Resources Commission of Life Sciences, 1996). To generate a chronic asthma model, mice were sensitized weekly intraperitoneally (i.p.) with 100 μ g OVA (TypeV; Sigma-Aldrich, St Louis, MO, USA) and 2 mg of alum in 0.4 ml of phosphate buffered saline (PBS) for 2 weeks, and were challenged intratracheally (i.t.) with 100 μ g OVA in 50 μ L PBS weekly for 3 weeks, then 2 consecutive daily challenges (4th and 5th challenges) 4 weeks later. To determine whether ASHMI prevents the progression of chronic asthma, 4.5 mg of ASHMI

in 0.5 ml of water was administered intragastrically (i.g.) twice daily for 6 weeks beginning one day after the initial i.t. challenge. The dose was determined by a conversion table of equivalent human to animal dose ratios based on body surface area (Xiu, 1986). Additional OVA-sensitized/challenged mice received 0.5 ml water, i.g. twice daily for 6 weeks as sham treatment controls. Naïve mice served as normal controls. EAR was determined 30 min following the 4th challenge (Fig. 2A) and LAR was determined 2 days following the 5th challenge (Fig. 3A).

Measurement of peak expiratory flow in EAR—Peak (maximal) expiratory flow (PEF) (ml/min) was used as a parameter to determine the severity of bronchial constriction. PEF values were monitored using a plethysmograph chamber (Kent Scientific Corporation Torrington, CT, USA) as previously described (Li *et al.*, 2004) with some modifications. Thirty min following the 4th OVA i.t. challenge, the mice were placed in the plethysmograph chamber system consisting of a head (anterior) chamber separated from the body (posterior) chamber by a latex collar. Bias airflow was passed through the anterior chamber and allowed to exit through a direct airflow sensor. Signals were collected and parameters derived using a Kent RSP001 respiratory data acquisition system (Kent Scientific Corporation, Torrington, CT, USA).

Measurements of plasma and BALF histamine, leukotriene (LT) C₄, and plasma Ag-specific IgE levels

Blood was obtained via retro-orbital puncture immediately following PEF measurements, and plasma was obtained. In an additional experiment, 4–5 mice from each group were sacrificed following immediate PEF measurement and bronchoalveolar lavage fluids (BALF) were obtained. Plasma and BALF histamine and LT C₄ levels were determined by specific ELISA as described by the manufacturers (ImmunoTECH Inc., Marseille, France; Cayman Chemical, Ann Arbor, MI, USA, respectively) (Chavez *et al.*, 2006). Plasma Ag-specific IgE levels were determined by ELISA as previously described (Li *et al.*, 1998).

Measurement of late phase AHR

AHR to acetylcholine (ACh) provocation was measured 2 days following the 5th challenge by measuring the Airway Pressure Time Index (APTI) (Kent Scientific Corporation, Torrington, CT, USA), a classical invasive measure of AHR, as previously described (Busse *et al.*, 2007; Levitt and, Mitzner, 1988).

BALF cell differential counts and lung histology

Immediately after APTI measurement, lungs were lavaged, and BALF and cytospin slides were prepared. Cell differential counts were obtained by counting at least 500 cells per slide by light microscopy after staining with HEMA 3 (Fischer Scientific, Pittsburgh, PA, USA). Lung samples were fixed in neutral buffered formaldehyde, and embedded in paraffin. Five-micron sections were stained with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS) for analysis of inflammatory cells and goblet cells.

Quantitative collagen assay

Newly formed collagen content of homogenized lung samples was measured using the Sircol Collagen Assay (Biocolor Ltd, Carrickfergus, UK) a quantitative colorimetric method as described previously (Jain *et al.*, 2002). In brief, frozen non-lavaged right lungs were homogenized in 1 mL of ice-cold PBS with a protease inhibitor (Roche Diagnostics, Indianapolis, USA). The homogenates were gently shaken for 12 h at 4°C and centrifuged at 15,000×g for 60 min to remove debris. The supernatant fluid (250 µL) was used for the Sircol™ assay, according to manufacturer's instructions. Optical density readings were made at 540 nm. Results were expressed as µg collagen per mg total protein that was determined by the Bradford Protein Assay (BioRad, Hercules, CA, USA).

Quantification of cytokines in BALF

Mice were sensitized and challenged, and treated with or without ASHMI as in Fig. 3A. Twenty-four h following the 5th challenge, mice were sacrificed, BALF collected, and cytokines measured by ELISA according to the manufacturer's instructions (PharMingen, San Diego, CA, USA).

Measurement of tracheal ring contractile responses

Mouse tracheal rings were prepared as previously described (McGraw *et al.*, 2003). Tracheas from OVA-sensitized/challenged mice 24 h following the 3rd challenge (OVA), and naïve mice were excised, and cut into 3 mm rings. Tracheal rings were mounted on a Myograph System Model 610 M (AD Instruments, Denver, CO, USA) and allowed to rest in physiological salt solution (PSS [In g/L- NaCl: 6.95, KCl: 0.35, MgSO₄ .7 H₂O: 0.28, KH₂PO₄: 0.1561, CaCl₂ .2 H₂O: 0.36, NaHCO₃: 2.1, EDTA: 0.01, Glucose: 1.09]) with continuous oxygenation at 37 °C for 30 min with no applied tension. Rings were then slid over a pair of mounting pins held in the organ chamber. After establishing stable tension, the contractile potential of each ring was first assessed by measuring the contractile response to 60 mM KCl. After washing, tracheal rings were treated with or without ASHMI (100 µg/ml) for 30 min. This dose was based on our preliminary dose-dependent study ranging from 12.5–200 µg/ml, which showed that 100 µg/ml generated maximal inhibition of ring contraction to ACh in a non-toxic manner (data not shown). Contractile responses were evaluated with increasing doses of ACh (1×10^{-9} to 1×10^{-3} M). Results are expressed as percent of contraction to 60 mM KCl as previously described (Justice *et al.*, 2001). To determine whether ASHMI-mediated relaxation of ASM, involved activation of the β_2 AR, the β_2 AR antagonist ICI118551 (50 µM, Sigma, St Louis, MO, USA) was added to ASHMI treated rings 30 min prior to ACh stimulation. This dose approaches the higher doses of ICI 118,551 used in previous studies (Dupuis *et al.*, 2005; Roberts *et al.*, 1999). Also, in data not shown, we tested ICI 118,551 at doses of 1.8, 5.5, 16.6, 50 and 150 µM. Contractile responses were evaluated with increasing doses of ACh (1×10^{-9} to 1×10^{-4} M).

Human tracheal smooth muscle cell culture and prostanoids measurement

Human tracheal smooth muscle cells (HTSMCs, ScienCell Research Lab, San Diego, CA, USA) were cultured in Smooth Muscle Cell Medium (ScienCell Research Lab, San Diego, Ca, USA) containing 10% growth factor and 1% penicillin streptomycin. When the cultures

were over 90% confluent, cells were treated with 0.25% (w/v) trypsin/0.53 mM EDTA for 5 min at 37 °C. The digestion was neutralized with culture medium and cells were counted. For analysis, cells were seeded in 48-well cell culture plate at 1×10^4 cells/0.5 ml/well and incubated overnight. Medium was aspirated and cells were washed once with sterile PBS. New medium alone or medium containing 7 different concentrations of ASHMI (0, 31.25, 62.5, 125, 250, 500, 1000 µg/ml) were added to the wells, and supernatants were harvested 30 min or 6 h later. Levels of PGI₂, PGE₂, PGD₂, PGF₂α and TxA₂ were determined by ELISA (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer's instructions.

Statistical methods

Data were analyzed using Sigma-Stat 2.03 software (Systat Software, Inc., Chicago, IL, USA). For data that satisfied normality testing, differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by pairwise testing using Bonferroni's adjustment. For data that appeared very skewed (non-normal), differences between groups were analyzed by ANOVA on Ranks followed by all pairwise comparisons. *P* values ≤ 0.05 were considered significant.

Power

We computed power and sample size (Power = 0.8, $\alpha = 0.05$) based on our preliminary results of ASHMI effects on PEF and APTI, 4–5 mice per experimental group were needed to demonstrate statistically significant results.

RESULTS

ASHMI treatment abolished EAR and reduced histamine, LTC₄, and Ag-specific IgE levels

To determine the effect of ASHMI on EAR, PEF was used as a parameter, and measurements were performed 30 min following the 4th OVA i.t. challenge (Fig. 2A). Lower PEF values indicate bronchoconstriction. PEF of sham-treated mice was less than 50% of naïve mice ($p < 0.05$, Fig. 2B), indicating severe bronchoconstriction. In contrast, PEF values of the ASHMI-treated group remained normal and slightly higher than naïve mice, demonstrating that ASHMI completely blocked antigen-induced EAR. Compared to the sham-treated mice, ASHMI-treated mice also had significantly lower plasma and BALF histamine (Fig 2C and 2E, $p < 0.05$), and LTC₄ levels (Figs 2C–2F, $p < 0.001$). ASHMI also significantly suppressed Ag-specific IgE synthesis ($p < 0.001$, Fig. 2G) whereas IgG1 and IgG2a levels were unaffected (data not shown).

ASHMI treatment prevented LAR, and reduced airway inflammation and remodeling

To determine the effect of ASHMI on LAR, APTI was used as a parameter and measured 2 days following the 5th OVA i.t. challenge (Fig. 3A). Increased APTI values indicate AHR to ACh provocation. APTI levels of sham-treated mice were significantly higher than naïve mice (Fig. 3B, $p < 0.001$), indicating induction of AHR. However, APTI levels of ASHMI-treated mice were essentially the same as naïve mice. The percent of eosinophils in BALF from the ASHMI-treated groups were significantly lower than in sham-treated mice ($p < 0.001$, Fig. 3C). Collagen content of lungs of ASHMI treated mice was significantly less than that of sham treated mice ($p < 0.05$, Fig. 3D), and was similar to that of naïve mouse

lungs. Histological analysis showed that airways in sham-treated mice contained many goblet cells, whereas few goblet cells were present in airways of the ASHMI-treated group (Fig. 3E). Concordant with the BALF data, infiltration of inflammatory cells was also reduced in ASHMI-treated mice (data not shown).

ASHMI altered T cell cytokine profiles in allergic mice

BALF from sham-treated mice contained substantial levels of Th2 cytokines including IL-4, IL-5 and IL-13, but no Th1 cytokine (IFN- γ), or T regulatory cytokines (IL-10 or TGF- β) (Fig. 4), indicating a Th2 dominant response. In contrast, IL-4, IL-5 or IL-13 was undetectable in BALF from ASHMI treated mice whereas IFN- γ , IL-10 and TGF- β were markedly elevated, indicating specific Th2 suppression.

ASHMI directly reduces airway smooth muscle contractility

ASHMI not only eliminated EAR, but PEF levels in the ASHMI-treated group appeared to be even higher than in the naïve group (Fig. 2B). Also, ASHMI eliminated AHR to ACh provocation even though eosinophil inflammation was not completely eliminated (Figs 3B and 3C). These data prompted us to hypothesize that ASHMI's potent effect on bronchoconstriction also involved a direct effect on airway smooth muscle contractility. To assess this possibility, we used murine tracheal rings isolated from asthmatic and naïve mice and compared their contractile response to ACh stimulation in the presence and absence of ASHMI. Contractile responses of tracheal rings from asthmatic mice markedly increased in response to incremental doses of ACh as compared to that of naïve mice, demonstrating hypercontractility of ASM from mice with asthma (Fig. 5A). ASHMI virtually abolished ACh-induced contraction of tracheal rings from both asthmatic and naïve mice ($p < 0.01$ – 0.001 vs. untreated rings, Fig. 5A). This markedly decreased response was reversible because responses to KCl and ACh 1 h after washout were essentially the same as pretreatment responses (data not shown). β_2 -adrenergic receptor (β_2 AR) activation is a major pathway in airway relaxation and β_2 AR agonists are widely used as bronchodilators by asthma patients (Billington and Penn, 2003). We therefore tested the effects of the β_2 AR antagonist ICI 118,551 on ASHMI inhibition of ASM hypercontractility. Data shown in Fig. 5B shows that even at 50 μ M, a concentration well above previous reported doses (Bilski *et al.*, 1983), ICI 118,551 did not restore ACh-induced hypercontractility to ASHMI-treated rings of asthmatic mice (Fig. 5B), indicating that ASHMI effects on ASM are unlikely to be mediated by β_2 AR stimulation. In other data not shown ICI 118,551 tested at doses of 1.8, 5.5, 16.6, 50 and 150 μ M did not alter ASHMI-induced suppression of tracheal contractility to ACh.

ASHMI increased human bronchial smooth muscle cell PGI₂ production

Prostanoid subtypes determine ASM tone and maintain a balance in the airways. PGI₂ and PGE₂ mediate ASM relaxation whereas PGD₂, TXA₂ and PGF₂ α mediate ASM contraction (Clarke *et al.*, 2008). We investigated whether prostanoids played a role in ASHMI-mediated ASM relaxation. We found that ASHMI significantly increased PGI₂ production by HTSMCs beginning at 500 μ g/ml after 30 min or 6 h cultures ($p < 0.05$, Figs 6A and 6B) without cytotoxicity (data not shown). There was no significant increase in

PGD2 production at any doses or time points (Figs 6C and 6D) where levels remained below 10 pg/ml in all conditions tested. There was no significant increase in PGE2, TXA2 or PGF2 α production at any time points and doses (data not shown).

Discussion

Growing concern over the increased rate of asthma in industrialized countries, together with increasing reports of adverse side effects of current asthma drugs has intensified attempts to find new drug therapies. The importance of developing new generations of botanical medicines, which target multiple pathological mechanisms for complicated diseases, has been recently reemphasized (Wagner and Ulrich-Merzenich, 2009). TCM has long been used to treat asthma; however, there is little scientific evidence that Chinese herbal medicines affect multiple mechanisms relevant to asthma. Utilizing a murine model of allergic asthma, we demonstrated for the first time that ASHMI exhibits broad pharmacological actions relevant to asthma. ASHMI eliminated EAR following antigen challenge. This effect was associated with a marked reduction in blood and lung histamine and LTC4 levels, and significant reduction in Ag-specific IgE levels. ASHMI also eliminated AHR to ACh provocation 2 days following the last antigen challenge, and reduced pulmonary inflammation and remodeling. In addition, consistent with the safety data in humans (Wen *et al.*, 2005; Kelly-Pieper *et al.*, 2009), no abnormality of liver or kidney functions was detected following treatment in this model (data not shown). Given the safety profile and efficacy demonstrated in this model, ASHMI may prove to be a novel and effective phytomedicine for asthma. Th2 cytokines play a critical role in orchestrating and perpetuating inflammation in asthmatic airways. These cytokines promote B cells switching to IgE production, mast cell maturation, eosinophilic inflammation, smooth muscle contraction and increased mucus production (Epstein, 2006). We showed that ASHMI abolished Th2 cytokines IL-4, IL-5 and IL-13 in BALF. ASHMI suppression of Th2 responses might be a major mechanism underlying ASHMI effects on EAR and LAR. IFN- γ is an important cross regulator of Th2 cytokines, and a recent study found that low IFN- γ production in the first year of life was a predictor of childhood wheezing (Stern *et al.*, 2007). TGF- β and IL-10, T regulatory cytokines, have been suggested to be the mechanism underlying allergen immunotherapy (Jutel *et al.*, 2003). In this study, we showed that ASHMI increased lung IFN- γ , TGF- β and IL-10 levels in BALF of ASHMI-treated mice, suggesting a beneficial immunomodulatory effect. Increased IFN- γ , TGF- β and IL-10 levels may contribute to ASHMI suppression of Th2 responses in lungs of ASHMI-treated mice in this model. Increased ASM contraction and/or reduced smooth muscle relaxation in chronic asthma contributes to excessive airway narrowing, characteristic of asthmatic attacks (Billington and Penn, 2003). In this study, we showed that ASHMI virtually abolished ACh-induced contractility of tracheal rings from asthmatic and naïve mice. Furthermore, given that the contractility of ASHMI treated murine tracheal rings from asthmatic and normal mice was lower than untreated tracheal rings of naïve mice, and that PEF values were modestly higher in ASHMI treated compared to naïve mice, ASHMI may induce ASM relaxation. The potent acute bronchodilators β 2AR agonists are the most widely used asthma drugs. However, chronic use of these agents can result in loss of protective effect and/or increased airway hyperreactivity (Hall 2004). In this study, we showed that a β 2-AR

antagonist did not alter ASHMI suppression of ACh-induced contractility of tracheal rings, indicating that ASHMI effects do not involve the β 2AR. PGI2 is a potent relaxer of ASM. We showed that ASHMI-induced PGI2 production by HTSMCs, but had minimal or no effect on other prostanoid subtypes, suggesting that PGI2 could play a role in ASHMI-mediated airway relaxation. In conclusion, we demonstrated that the herbal formula ASHMI exhibits a wide range of pharmacological actions on pathophysiological processes responsible for EAR and LAR in asthma. These effects were associated with beneficial immunomodulatory effects on Th1/Th2 and T regulatory cytokines, modulation of airway smooth muscle contractility and increased smooth muscle cell secretion of the potent smooth muscle relaxant prostaglandin PGI2. All these actions would be of benefit to human asthma patients, and may explain, at least in part, the clinical efficacy of ASHMI demonstrated in asthma patients. Given our observations in the current study, ASHMI may prove to be an effective new phytomedicine for asthma. Further studies to identify active compounds and underlying mechanisms responsible for ASHMI's pharmacological and immunological actions are underway.

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Figure 1. 3D-HPLC-DAD fingerprint of the ASHMI extract

Alkaloids, flavonoids, and triterpenoids in ASHMI extract are shown by labels. Peak analysis and assignment were performed using standard samples and/or LC-MS method. Matrine, sophocarpine, oxymatrine, kushenol O, trifolirhizin correspond to the compounds from *Radix Sophorae Flavescentis*. Ganoderic acid B, D, A, H, and F and ganolucidic acid D correspond to *Ganoderma lucidum*; Liquiritin, liquiritigenin, 7, 4'-dihydroxyflavone, Isoanonin, 3',7'-Dihydroxy-4'-methoxy-isoflavone, and glycyrrhizic acid correspond to compounds from *Radix glycyrrhiza*.

Figure 2. Early ASHMI treatment abolished EAR and reduced histamine, LT C4 and Ag-specific IgE levels

(A) Mice were sensitized, challenged, and treated with ASHMI as described in the protocol.

(B) PEF values were measured 30 min following the 4th challenge. Plasma samples were obtained immediately following PEF measurement. Histamine (C) and LTC₄ (D) levels were measured by ELISA.

In separate experiments mice were sacrificed immediately following PEF measurement and BALF were obtained from mice in each group and pooled. BALF histamine levels (D) and BALF LT C4 levels (E) were determined. (F) Plasma OVA-specific IgE levels were determined by ELISA. Results of B, C, D and G are expressed as means ± SEM for each group of mice (n = 10–15/group from 2–3 sets of experiments. *, p < 0.05 vs. sham; **, p < 0.01 vs. sham; *** p < 0.001 vs. sham; # p < 0.05 vs. naive; ###, p < 0.001 vs. naive. Results of E and F are means ± SEM of triplicates from pooled BALF of 4 mice. Sham, OVA sensitized/challenged mice received water as sham treatment. ASHMI, OVA

sensitized/challenged mice received ASHMI treatment. Naïve, not sensitized/challenged, nor treated.

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Figure 3. ASHMI treatment eliminated LAR and reduced airway inflammation and remodeling (A) Mice were sensitized, challenged, and treated with ASHMI as described in the protocol.

(B) APTI following ACh provocation was measured 2 days after the 5th OVA challenge.

(C) Percent eosinophils in BALF immediately following APTI was determined. (D) Total lung collagen content was measured in homogenized lung supernatants. Lung histology

analysis (E) shows numerous goblet cells (PAS positive cells, purple color) in airway from a sham-treated mouse, markedly reduced mucus goblet cells in airways of an ASHMI-treated mouse and the absence of goblet cells in airways of a naïve mouse. Results of B and C are expressed as means $\pm$ SEM for each group of mice ($n = 10-15$ /per group from 2 sets of experiments. ***, $p < 0.001$ vs. sham; ###, $p < 0.001$ vs. naïve. Results of (D) are Mean $\pm$ SEM of 5-6 mice from each group. *, $p < 0.05$; vs. sham; #, $p < 0.05$ vs. naïve.

Figure 4. Immunomodulatory effect of ASHMI on T cell cytokine profiles

In a separate experiment, mice were sensitized, challenged, and treated with ASHMI as in Fig 3A. Mice were sacrificed 24 h following the 5th challenge and BALF was collected from each group of mice ($n = 4$) and pooled. Cytokine levels were determined by ELISA. Results are expressed as means $\pm$ SEM of triplicates.

Figure 5. ASHMI modulation of airway smooth muscle contractility

(A) Effect of ASHMI on tracheal ring contractility. Tracheal rings from OVA sensitized/challenged (OVA) or naive mice were pretreated with or without ASHMI for 30 min. Contractile responses to increasing doses of ACh were measured. Results are expressed as mean $\pm$ SEM of rings. ##, $p < 0.01$ and ### $p < 0.001$ vs. OVA. ***, $p < 0.001$ vs. OVA + ASHMI. (B) Effect of β_2 -adrenergic receptor antagonist on ASHMI mediated effect on tracheal rings. Tracheal rings from OVA mice were pretreated with ASHMI in the presence or absence of β_2 -adrenergic receptor antagonist ICI 11855 (50 μ M) for 30 min. Contractile responses to increasing doses of ACh were measured. Results are expressed as mean $\pm$ SEM of 4–5 rings.

***, $p < 0.001$ vs. OVA + ASHMI, ###, $p < 0.001$ vs. OVA + ASHMI + ICI118,551. OVA, tracheal rings from OVA sensitized and challenge mice; Naive, tracheal rings from naive mice; OVA+ASHMI, tracheal rings from OVA sensitized and challenge mice pretreated with ASHMI; Naive + ASHMI, tracheal rings from naive mice pretreated with ASHMI.

Figure 6. ASHMI induced the release of PGI₂ production by human tracheal smooth muscle cells (HTSMCs)

HTSMC were cultured for 30 min and 6 h with or without ASHMI at different concentrations as indicated. Supernatants were then harvested and PGI₂ levels (**A**) and (**B**) and PGD₂ levels (**C**) and (**D**) were determined by measuring levels of PGI₂ metabolite 2,3-dinor-6-keto PGF1α and PGD₂ metabolite 11β-PGF2α. Data are mean ± SEM of triplicate ELISA. *p < 0.05 vs. untreated. The Y axis of PGI₂ (**A**) and (**B**) and PGD₂ (**C**) and (**D**) are not on the same scale.

Table 1

Chinese name	Ling-Zhi	Ku-Shen	Gan-Cao
% in ASHMI formula	62.5%	28.1%	9.4%
Synonyms	Reishi	Light yellow sophora root	Licorice root
Pharmaceutical name	Ganoderma	Radix Sophorae Flavescentis	Radix Glycyrrhiza
Plant Species Information	<i>Ganoderma lucidum</i> Karst.	<i>Sophora flavescens</i> Ait.	<i>Glycyrrhiza uralensis</i> Fischer.
Part used	Fruiting body	Root	Root and rhizome
Family	Polyporaceae	Leguminosae	Leguminosae
Geographical location	Shandon, China	Hubei, China	Neimeng, China
Harvest time	August and September	June and July	Autumn
Processing	Clean and dry in the shade	Remove the remains of rootstock and rootlet, wash clean, soak briefly cut into thick slices and dry	Eliminate foreign matter, wash clean, soften thoroughly, cut into thick slices, cool in the air
Storage	Preserve in a dry place, protected from molds and moths	Preserve in a dry place	Preserve in a well-ventilated dry place, protected from moths.
Traditional Uses	General weakness, cough, asthma, insomnia, indigestion	dysentery, jaundice, pruritis edema, dysuria, eczema	sore throat, cough
Modern Uses	Nightmares, neurasthenia, coronary heart disease, arrhythmia, asthma	chronic hepatitis B, leukocytopenia	bronchitis, gastroduodenal ulcers
Heavy metals			
Lead ¹ (mg/kg)	<0.85	<0.87	<0.23
Mercury ² (mg/kg)	<0.1	<0.1	<0.1
Pesticides			
Cypermethrin ³ (mg/kg)	0.047	0.02	0.01
Dichlorvos ⁴ (mg/kg)	0.031	0.007	0.01

¹ Recommended residue limits <5.0 mg/kg *;

² Recommended residue limits <0.2 mg/kg *;

³ Recommended limits 0.5 mg/kg (**);

⁴ Recommended residue limits <0.1 mg/kg ***: Green Trade Standards of Importing & Exporting Medicinal Plants & Preparations, Published by Ministry of Foreign Trade and Economic Cooperation, P.R. China, 2001 <http://www.cas.ac.cn/html/Dir/2001/04/23/5754.htm>. **: Maximum Residue Limit in the European Union, (Reg.(EC) 396/2005. (<http://www.nda.agric.za/docs/plantquality/MRL%20Export%20lists/table%20grapes.pdf>; <http://www.upasitearesearch.org/news/26.pdf>)