Evaluation of the therapeutic potential and underlying mechanisms of synephrine, a component of Kampo medicine, against allergic rhinitis

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Tomoko Hommura¹,²*, Katsuaki Dan³, Sho Kanzaki², Kenji Watanabe⁴,⁵ and Kaoru Ogawa²

Abstract: The mechanisms of action of Kampo medicines as treatments for allergic rhinitis are unknown. In this study, we aimed to identify novel potential therapeutic agents for allergic rhinitis and to elucidate their underlying mechanisms. Different components of Kampo medicines (crude drugs) were screened for their ability to inhibit the secretion of thymic stromal lymphopoietin (TSLP), a cytokine secreted during allergen exposure. Synephrine (SYN) exhibited the strongest inhibitory effect. In an early-phase allergic reaction, histidine decarboxylase (HDC) and its receptor are activated, leading to the secretion of TSLP. Mucins are thought to be produced as a late-phase reaction. SYN inhibited TSLP at the mRNA and protein level. Increased expression of the HDC protein was confirmed in tissues of patients with allergic rhinitis. In addition, SYN inhibited TSLP at the mRNA and protein level.

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PUBLIC INTEREST STATEMENT
We screened 55 Kampo drugs in an attempt to discover novel therapeutic agents for allergic rhinitis. The three major symptoms of allergic rhinitis are sneezing, rhinorrhea, and nasal congestion. The response mechanism is classified into an early-phase reaction, mainly involving IgE, mast cells, and basophils, and a late-phase reaction, mainly involving Th2 responses. The early-phase reaction is indicated to cause sneezing and rhinorrhea, and the late-phase reaction is indicated to cause eosinophil-associated nasal congestion, mucous secretion, and tissue remodeling. Histamine is important in the early-phase reaction, and leukotrienes are important in both early- and late-phase reactions. Our results demonstrate that the natural drug synephrine exerts its effects on the reaction pathways of both early- and late-phase reactions. This single drug showed efficacies similar to those of histamine H1 receptor antagonists and leukotriene antagonists and may become a novel therapeutic agent for amelioration of all three major symptoms of allergy.
levels and inhibited mucin 5AC mRNA expression. Its inhibitory effects on both early- and late-phase allergic reactions indicate that SYN can serve as a novel therapeutic agent with potential leukotriene antagonist-like activity.

Subjects: Allergology & Clinical Immunology; Immunology; Complementary & Alternative Medicine

Keywords: allergic rhinitis; Kampo; synephrine; histamine H1 receptor; histidine decarboxylase; leukotriene antagonist

1. Introduction

Allergic rhinitis is treated with various agents, including histamine (HA) synthesis inhibitors, HA release inhibitors, HA receptor (HR) antagonists, steroids, and anti-leukotriene agents. Many Kampo medicines have been used in the treatment of allergic rhinitis, which is supported by empirical evidence. However, their mechanisms of action are largely unknown.

Allergic rhinitis develops when HA is produced as a result of the allergen-induced priming of the nasal mucosa. The three major symptoms of allergic rhinitis are sneezing, rhinorrhea, and nasal congestion. HA is involved in sneezing and rhinorrhea, while leukotrienes and other chemical mediators are involved in nasal congestion, besides HA.

HA, which is responsible for the onset of allergic rhinitis, is a biologically active substance discovered by Dale and Laidlaw (1910). It is biosynthesized from the amino acid histidine by histidine decarboxylase (HDC). HA is stored in mast cells, which are present in large numbers in the skin and mucosae, as well as in basophils and the gastrointestinal tract. In one mechanism of HA release, antigens bind to immunoglobulin E (IgE) receptors on the surface of mast cells or basophils, resulting in their degranulation. In another mechanism, HDC is activated by inflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, granulocyte–macrophage colony-stimulating factor, or IL-3, resulting in the release of HA. HA, along with many other chemical mediators, is known to induce an inflammatory response via HRs. Four HRs (H1R, H2R, H3R, and H4R) have been identified, and all have been reported to be expressed in the nasal mucosa (Nakaya, Takeuchi, & Kondo, 2004). In particular, H1R and H4R are considered to be intricately involved in allergies. Furthermore, H4R is known to have a higher affinity for HA than H1R has, while H1R and H4R are known to have little homology with each other.

Other factors associated with respiratory allergic diseases include leukotrienes, thymic stromal lymphopoietin (TSLP), and the mucin (MUC) gene family. Leukotrienes, which are known to be mediators that trigger respiratory allergic diseases (Shirasaki, 2008), are important in the synthesis of mucus (Shimizu, Hirano, Majima, & Sakakura, 2000). In human airway epithelial cells, leukotrienes induce the secretion of mucin 5AC (Shirasaki, Kanaizumi, Seki, & Himi, 2015). TSLP, which is produced by epithelial cells, is known to promote T helper 2 (Th2) cell responses. In human airway epithelial cells, TSLP mRNA expression levels are significantly increased by stimulation with Toll-like receptor (TLR) 2, TLR3, TLR8, and TLR9 ligands, as well as with IL-4, IL-13, IL-1β, and TNF-α (Kato, Favoreto, Avila, & Schleimer, 2007). In addition, the expression of TLR2, TLR3, and TLR4 is enhanced in the nasal mucosa of patients with allergic rhinitis (Fransson et al., 2005). Various Th2 cytokines, such as IL-4 and IL-13, have been demonstrated to be involved in allergic rhinitis (Benson, Adner, & Cardell, 2001). Enhanced expression of IL-1β and TNF-α has also been reported in the affected nasal mucosa in allergic rhinitis (Bachert, Hauser, Prem, Rudack, & Ganzer, 1995; Lee et al., 2016; Tyurin et al., 2017).

The MUC gene family encodes for mucin proteins, which are the main components of the nasal discharge. To date, the genes MUC1 to MUC21 have been discovered. The MUC genes primarily
expressed in the nasal mucosa are MUC1, MUC2, MUC4, MUC5AC, MUC5B, MUC7, and MUC8 (Ali & Pearson, 2007). The MUC genes primarily expressed in goblet cells, submucosal gland mucous cells, and serous cells are MUC5AC, MUC5B, and MUC8, respectively. In addition, MUC2, MUC5AC, MUC7, and MUC8 are considered secretory proteins. Allergic rhinitis is characterized by increased expression of MUC5AC, which is primarily secreted from epithelial cells, and MUC5B, which is secreted from epithelial and submucosal cells (Ali, 2009). Similar patterns of expression have been reported in asthma (Ordonez et al., 2001).

The response mechanism of allergic rhinitis is divided into an early-phase reaction, which primarily involves IgE, mast cells, and basophils, and a late-phase reaction, which primarily involves Th2 responses. The early-phase reaction triggers symptoms such as sneezing and rhinorhea, while the late-phase reaction triggers symptoms associated with eosinophils, such as nasal congestion, mucus production, and tissue remodeling. HA is critical in the early-phase reaction, while leukotrienes are important in the early- and late-phase reactions (Shimizu, Shimizu, Hattori, & Majima, 2003). TSLP has recently been reported to contribute to both early- and late-phase reactions in a murine model of allergic rhinitis (Akasaki et al., 2016). The MUC gene family members are involved in the mucus production, indicating that they are primarily associated with the late-phase reaction.

Previous studies have not comprehensively analyzed the pathway from early-phase to late-phase allergic reactions or the pathological mechanisms of allergic responses at the gene level. Therefore, we aimed to investigate the mechanisms in each step of allergic responses in a presumed signal transduction pathway. First, we screened 55 components of Kampo medicines (Table 1) for their inhibitory effects on TSLP, which is involved in both early-phase and late-phase reactions, and selected synephrine (SYN) as a novel therapeutic candidate for allergies. Moreover, to identify the site of SYN action, we chose fexofenadine (FEX) as the H1R antagonist, L-carbocisteine (LCC) as the expectorant (mucolytic agent), pranlukast (Pran) as the anti-leukotriene agent, and Sho-seiryu-to (Japanese name) [Chinese name: Xiao-qing-long-tang (XQLT)] as a Kampo medicine. LCC inhibits the increase in the generation of MUC5AC-derived mucin, which is the chief component of the airway-secreted fluid (Ishibashi, Kobayashi, Idesawa, Taniguchi, & Matsuzawa, 2004). Sho-seiryu-to was found to be effective in a double-blind, placebo-controlled trial for allergic rhinitis (Baba, 1995). We evaluated SYN against these comparators in terms of its effects on HDC, H1R, H4R, TSLP, MUC5AC, and MUC5B mRNA expression.

2. Materials and methods

2.1. Reagents
The Kampo medicine Sho-seiryu-to was purchased from Tsumura & Co. (Tokyo, Japan), while the components of Kampo medicines (crude drugs) were purchased from Sigma–Aldrich (St. Louis, MO, USA). The Kampo medicine and crude drugs were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 1 mg/mL and stored at −80 °C until use. Before use, the drugs were diluted 1,000-fold, to a final concentration of 1 μg/mL. The final concentration of DMSO was 0.1%. The TSLP standard was purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Molecular biology reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Cells
Human nasal epithelial cells (HNEpCs) were purchased from PromoCell (Heidelberg, Germany). They were cultured in airway epithelial cell growth medium (PromoCell), without additional supplements, in an atmosphere of 5% CO2 at 37 °C, according to the provided protocol. The cells were seeded in a 96-well plate and cultured for 1–2 days until 80–90% confluence was reached and were then used for experiments.
Table 1. List of components of Kampo medicines

<table>
<thead>
<tr>
<th></th>
<th>Component</th>
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<th>Component</th>
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<tr>
<td>1</td>
<td>Albiflorin</td>
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<td>[6]-Gingerol</td>
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<td>2</td>
<td>Alisol A</td>
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<td>Ginsenoside Rb1</td>
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<td>3</td>
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<tr>
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<td>Glabridin</td>
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<td>Glycyrrhetic Acid</td>
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<td>Homogentisic Acid</td>
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<td>Isoliquiritigenin</td>
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<td>(E)-Cinnamaldehyde</td>
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<td>Limonin</td>
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<td>Synephrine</td>
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<td>Liquiritigenin</td>
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<td>Tangeretin</td>
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<td>Palmatine Chloride</td>
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</table>
2.3. Extraction and purification of RNA from human nasal mucosal tissue

Tissue RNA extraction and purification were performed with the TRIzol® reagent (Gibco BRL, Life Technologies, Täby, Sweden). Nasal mucosal tissue, preserved at −80 °C, was sliced into thin sections and homogenized in TRIzol®. To remove proteins, the samples were incubated for 5 min at room temperature. Then, chloroform was added, and the samples were vortexed for 15 s, followed by incubation for 2–3 min at room temperature and centrifugation at 12,000 × g for 15 min at 4 °C. The RNA-containing upper phase was transferred to a new tube, followed by the addition of 0.5 mL of isopropyl alcohol and incubation at room temperature for 10 min. Then, the tube was centrifuged at 12,000 × g for 15 min at 4 °C, and the RNA pellet formed was washed with 75% ethanol, air-dried, and dissolved in 20 μL of RNase-free water. After measuring the RNA concentration, the rest of the solution was stored at −80 °C for reverse transcription.

2.4. Reverse transcription reaction

A reverse transcription reaction of purified RNA was performed with the PrimeScript™ RT master mix (Takara Bio, Inc., Shiga, Japan) according to the manufacturer’s protocol. Briefly, a master mix containing 2 μL of 5× PrimeScript™ buffer, 0.5 μL of PrimeScript™ RT enzyme mix I, 0.5 μL of oligo (dT) primer, and 0.5 μL of random hexamers was added to 500 ng of an RNA template. RNase-free water was added to obtain a total reaction volume of 10 μL. The reverse transcription reaction was carried out for 15 min at 37 °C in a TaKaRa thermal cycler (Takara Bio, Inc.). Reverse transcriptase was inactivated by heating to 85 °C for 5 s. The resulting cDNA from cultured HNEpCs was used as a template for quantitative real-time polymerase chain reaction (qPCR).

2.5. Analysis of mRNA expression using quantitative real-time polymerase chain reaction

HDC, H1R, H3R, H4R, TSLP, and MUC5AC mRNA expression levels were assessed using qPCR. Following the addition of a stimulant (10⁻³ or 10⁻⁴ mol/L HA, 1 μg/mL lipopolysaccharide (LPS) from Escherichia coli (Sigma–Aldrich), or 25 μg/mL polyinosinic-polycytidylic acid [poly(I:C)]}, alone or in combination with SYN (100 μg/mL), HNEpCs were cultured in a 35-mm plastic plate at a density of 2 × 10⁵ cells/plate for 1 or 8 h. Total RNA was then extracted from the cells using the TRIzol® reagent. Using the PrimeScript™ RT master mix (Takara Bio, Inc.), the extracted RNA was reverse-transcribed into cDNA, which was then amplified with SYBR® Premix EX Taq II (Takara Bio, Inc.). The reactions were performed in a final volume of 50 μL, containing 25 μL of SYBR® Premix (2×), 1 μL of cDNA, 2 μL of each 5 pmol/μL primer, and 22 μL of water. Each qPCR amplification was performed under the following conditions: 95 °C for 30 s, followed by 50 cycles at 95 °C for 5 s and 60 °C for 30 s. The relative gene expression was calculated using the 2⁻ΔΔCt method. The PCR primers used for all target genes and for the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), are shown in Table 2. A ΔΔCt value ≥ 2 was considered to represent a significant difference in gene expression.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sense</th>
<th>Antisense</th>
<th>References</th>
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<td>H1R</td>
<td>F:5’-TGG TCA CAG TAG GGC TCA AC-3’</td>
<td>R:5’-CAA GGT GGG CAG GTA GAA GT-3’</td>
<td>[27]</td>
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<tr>
<td>H4R</td>
<td>F:5’-GGC TCA CTA CTG ACT ATC TG-3’</td>
<td>R:5’-CCT TCA TCC TTC CAA GAC TC-3’</td>
<td>[28]</td>
</tr>
<tr>
<td>HDC</td>
<td>F:5’-ATG ATG GAG CCT GAG TAC AG-3’</td>
<td>R:5’-CCT GAG TTG TCA GCA TGC CTG AGG TAG-3’</td>
<td>[29]</td>
</tr>
<tr>
<td>TSLP</td>
<td>F:5’-CAT GGA AGT GCT GTC GAA GA-3’</td>
<td>R:5’-TTT CCG TGA CCA ATC CTT TC-3’</td>
<td>[30]</td>
</tr>
<tr>
<td>MUC5AC</td>
<td>F:5’-TCC GGC CTC ATC TTC TCC-3’</td>
<td>R:5’-ACT TGG GCA CTG GTG CTG-3’</td>
<td>[31]</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F:5’-TGA AGG TCG GAG TCA ACG GAT TTG GT-3’</td>
<td>R:5’-CAT GTG GGC CAT GAG GTC CAC CAC-3’</td>
<td>[28]</td>
</tr>
</tbody>
</table>
2.6. Quantification of TSLP protein with enzyme-linked immunosorbent assay (ELISA)
We conducted the assay according to the human TSLP DuoSet ELISA (R&D Systems, Inc.) protocol, as described below. Following 24-h incubation in a 96-well plate of (a) the mixtures of TSLP (reference standard) and the 55 components of Kampo medicines (crude drug: 1 μg/mL), the mixtures were collected, or (b) HNEpCs treated with 25 μg/mL poly(I:C) and the 55 components of Kampo medicines (crude drug) and the culture supernatants were collected after 24 h. The levels of TSLP in the culture media were measured with ELISA. Based on the results, three components of Kampo medicines, which yielded the greatest inhibition of TSLP in the respective culture, were selected for further experiments. To assess the concentration dependencies of the effects of these herbal inhibitors, HNEpCs were co-stimulated with poly (I:C) and different concentrations (1:200, 1:800, 1:3,200, and 1:12,800 dilutions) of the inhibitors, and TSLP levels were measured with ELISA.

2.7. Tissue specimens
After obtaining an approval from the Institutional Review Boards of the Keio University Hospital and its associated hospital, as well as written consent from patients, we obtained nasal mucosal tissue from four patients (aged 37–73 years) at the Saiseikai Yokohama City Eastern Hospital. The patients had been diagnosed with chronic hypertrophic rhinitis or allergic rhinitis and had undergone surgery. Some of the tissues were preserved within several hours at—80 °C for mRNA expression analysis, and the remaining tissues were fixed in 10% neutral buffered formalin for immunohistochemical staining.

2.8. Immunohistochemical staining of HDC
Immunohistochemical analysis of HDC was performed with a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). Formalin-fixed human nasal mucosal tissue was dehydrated in alcohol, embedded in paraffin, thinly sliced into sequential 4-μm thick sections, and mounted on slides. Antigen retrieval was performed by heating the sections at 120 °C for 15 min in an autoclave. Endogenous peroxidases were inactivated with a 3% hydrogen peroxide solution. The sections were blocked with 1% bovine serum albumin for 60 min at room temperature to permeabilize the plasma membrane and block nonspecific binding sites. Then, the sections were incubated with a rabbit anti-human HDC antibody (1:1,000; ab37291, Abcam, Cambridge, UK) for 24 h at 4 °C, washed with TPBS [PBS containing 0.1% (w/v) Triton X-100], and incubated with a biotinylated anti-rabbit secondary antibody. The signals were visualized with the Vectastatin ABC reagent and diaminobenzidine and viewed under a microscope (Olympus BX51, 200 × magnification).

2.9. Detection of HDC by western blotting
Protein lysates (20 μg per lane) were resolved by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the separated proteins were transferred to a nitrocellulose membrane using an iBlot® dry blotting system (Invitrogen, Carlsbad, CA). The membrane was blocked with 5% skim milk in TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) and then incubated with a primary antibody at 4 °C for 12 h. A rabbit anti- human HDC polyclonal antibody (ab37291; Abcam) and a rabbit anti-human β-actin antibody (ab16039; Abcam) were used as primary antibodies. After being washed with TBST, the membrane was incubated with a goat polyclonal anti-rabbit IgG peroxidase-conjugated secondary antibody (0.1 μg/mL; Sigma-Aldrich) for 60 min at room temperature and washed again. Signals were visualized using ImmunoStar reagents (Wako Pure Chemical Industries, Ltd.) and an ImageQuant™ LAS 500 imaging system (GE Healthcare, Buckinghamshire, UK). The intensity of each band was quantified using the ImageJ image analysis software (National Institutes of Health, USA).

2.10. Statistical analysis
All experiments were performed in triplicate, and the results are shown as the mean ± standard deviation. Significant differences between groups were determined using one-way analysis of
variance. A value of \( p < 0.05 \) was deemed statistically significant, whereas that in the range of \( 0.05 < p < 0.1 \) was considered to show a statistically significant trend.

3. Results

3.1. Selection of candidates as therapeutic agents for allergies

Direct degradation effects on exogenously added TSLP (reference standard) were examined for 55 different components of Kampo medicines (crude drug: 1 \( \mu \)g/mL; Table 1), and the TSLP levels were measured using ELISA after 24 h of incubation. The three Kampo medicine components associated with the lowest levels of TSLP were (in a decreasing order): SYN, amygdalin, and alisol A (Figure 1 (a)). The 55 components of Kampo medicines (drugs) were also examined in co-stimulation assays, wherein endogenous TSLP secretion was measured after co-stimulation of HNEpCs with 25 \( \mu \)g/mL poly(I:C) and a Kampo medicine. TSLP levels were measured 24 h later, and the three components that demonstrated the strongest inhibition of TSLP were (in a decreasing order): SYN, limonin, and the evodiamine standard (Figure 1 (b)). Therefore, the Kampo medicine components selected after screening with ELISA (i.e., those that demonstrated strong effects in at least one experiment) were SYN, amygdalin, alisol A, limonin, and the evodiamine standard.

Of these five Kampo medicine components, the one that demonstrated the strongest effect was SYN. Further, we examined these five types of Kampo medicines (1 mg/mL) in a concentration-response experiment with 25 \( \mu \)g/mL poly(I:C)-stimulated HNEpCs using serial dilutions of 1:200, 1:800, 1:3,200, and 1:12,800. SYN was the only candidate that demonstrated concentration-
dependent effects (Figure 2). Based on the screening results, SYN was selected as a potential novel therapeutic agent for allergic rhinitis, and we performed experiments to examine the site of action of SYN.

3.2. Inhibitory effects of SYN on histamine receptors

After 1 h of stimulation with $10^{-4}$ mol/L HA alone, the level of H1R mRNA significantly increased, while co-stimulation with HA and SYN resulted in a significant decrease in the H1R mRNA level ($p < 0.05$). Significant decreases in H1R mRNA expression were also observed after co-stimulation with FEX, LCC, and Pran ($p < 0.05$; Figure 3).

The H4R mRNA level significantly increased after 1 h of stimulation with $10^{-4}$ mol/L HA alone ($p < 0.05$). After co-stimulation with $10^{-4}$ mol/L HA and SYN, the H4R mRNA level did not significantly differ between HA stimulation and co-stimulation with HA and SYN, although a tendency toward reduced expression was observed ($0.05 < p < 0.1$). No significant differences were observed in the H4R mRNA levels between HA stimulation and co-stimulation with HA and any of the comparators (FEX, XQLT, LCC, or Pran; Figure 4).

3.3. Inhibitory effect of SYN on histamine expression

Regarding the HDC protein expression in the inferior turbinate mucosa of patients with allergic rhinitis, immunostaining did not detect HDC. There is no clear difference in HDC expression could be observed between the patients with and without allergic rhinitis in this experiment (Figure 5).

Western blot analysis showed that the HDC protein was not expressed in the patients without allergic rhinitis, whereas HDC protein expression was observed in those with allergic rhinitis (Figure 6).

After 8 h of stimulation of HNEpCs with LPS (1 μg/mL) alone, the HDC mRNA expression level significantly increased ($p < 0.05$), while co-stimulation with LPS and SYN significantly reduced the HDC mRNA expression level ($p < 0.05$). A significant decrease in the HDC mRNA level was also observed after co-stimulation with FEX ($p < 0.05$; Figure 7).
These results suggest that although HDC protein expression was observed in allergic rhinitis, distinguishable macroscopic changes were not noted.

### 3.4. Anti-inflammatory effect of SYN

After 1 h of stimulation of HNEpCs with $10^{-4}$ mol/L HA alone, the TSLP mRNA level significantly increased ($p < 0.05$), while co-stimulation with HA and SYN resulted in a significant decrease in the TSLP mRNA level ($p < 0.05$). No significant differences were noted between stimulation with $10^{-4}$ mol/L HA alone and co-stimulation with HA and any of the comparators (FEX, XQLT, LCC, or Pran; Figure 8).

### 3.5. Inhibitory effects of SYN on mucin expression

Following 1 h of stimulation of HNEpCs with $10^{-3}$ mol/L HA alone, the MUC5AC mRNA expression significantly increased ($p < 0.05$), while co-stimulation with HA and SYN resulted in a significant decrease in the MUC5AC mRNA level ($p < 0.05$). A significant decrease was also observed after co-stimulation with HA and Pran ($p < 0.05$; Figure 9).

These results suggest that although HDC protein expression was observed in allergic rhinitis, distinguishable macroscopic changes were not noted.

### 3.4. Anti-inflammatory effect of SYN

After 1 h of stimulation of HNEpCs with $10^{-4}$ mol/L HA alone, the TSLP mRNA level significantly increased ($p < 0.05$), while co-stimulation with HA and SYN resulted in a significant decrease in the TSLP mRNA level ($p < 0.05$). No significant differences were noted between stimulation with $10^{-4}$ mol/L HA alone and co-stimulation with HA and any of the comparators (FEX, XQLT, LCC, or Pran; Figure 8).

### 3.5. Inhibitory effects of SYN on mucin expression

Following 1 h of stimulation of HNEpCs with $10^{-3}$ mol/L HA alone, the MUC5AC mRNA expression significantly increased ($p < 0.05$), while co-stimulation with HA and SYN resulted in a significant decrease in the MUC5AC mRNA level ($p < 0.05$). A significant decrease was also observed after co-stimulation with HA and Pran ($p < 0.05$; Figure 9).
After 8 h of stimulation of HNEpCs with LPS (1 μg/mL) alone, the MUC5B mRNA expression level significantly increased ($p < 0.05$). No significant difference was observed between the MUC5B mRNA levels after LPS stimulation and co-stimulation with LPS and SYN, whereas a tendency toward reduced mRNA expression was observed after co-stimulation with FEX ($0.05 < p < 0.1$; Figure 10).

4. Discussion

In this study, we aimed to identify novel potential therapeutic agents for allergic rhinitis. We screened crude drugs for their inhibitory activity against TSLP secretion as an indicator and found candidate substances that caused degradation of TSLP, as well as candidates that inhibited TSLP secretion in stimulated HNEpC cultures. The candidate that demonstrated the strongest inhibitory effect using both criteria was SYN.

SYN, which is present in Chen Pi (the dried peel of oranges and other citrus fruits), has a benzene ring with one less hydroxyl group than in adrenaline, and it demonstrates similar activities to those of adrenaline. A previous study has reported that immature *Citrus unshiu* exhibits anti-allergic...
In addition, SYN has been reported to significantly inhibit the IL-4-induced upregulation of eosinophils and eotaxin-1 in mouse fibroblast cultures (Roh et al., 2014). Furthermore, phenylephrine (neo-synephrine) has been reported to stimulate nasal ciliary movement in a normal human nose (Phillips, McCaffrey, & Kern, 1990).

Figure 11 shows a hypothetical signal transduction cascade that may be activated in vivo in response to allergen-induced priming. In the early-phase reaction, HDC activity leads to the production of HA, which activates its receptor and leads to TSLP secretion. Mucin is thought to subsequently respond in the late-phase reaction. We examined the action of SYN at each phase in mono-stimulation and co-stimulation assays.

In the present study, the HDC protein was expressed in the nasal mucosae of patients with allergic rhinitis but not in those without allergic rhinitis. In addition, priming of nasal mucosa cell activity (Kubo, Yano, & Matsuda, 1989). In addition, SYN has been reported to significantly inhibit the IL-4-induced upregulation of eosinophils and eotaxin-1 in mouse fibroblast cultures (Roh et al., 2014). Furthermore, phenylephrine (neo-synephrine) has been reported to stimulate nasal ciliary movement in a normal human nose (Phillips, McCaffrey, & Kern, 1990).

Figure 7. Expression of histidine decarboxylase (HDC) mRNA expression after treatment with a single stimulant [1 μg/mL lipopolysaccharide (LPS)] in human nasal epithelial cells (HNEpCs) for 8 h or co-treatment with LPS and synephrine (SYN) or fexofenadine (FEX) for 8 h. *Statistical significance in the expression levels of HDC between non-stimulated (untreated control) and LPS-stimulated cells (P < 0.05). The comparison between a single LPS-stimulant and co-stimulant LPS and SYN or FEX with respect to mRNA expression of HDC in HNEpCs is indicated by * = p < 0.05. RFC = relative fold change.

Figure 8. Expression of thymic stromal lymphopoietin (TSLP) mRNA after treatment with a single stimulant [10^{-4} mol/L histamine (HA)] in human nasal epithelial cells (HNEpCs) for 1 h or co-treatment with HA and synephrine or control drugs [fexofenadine (FEX), Xiao-qing-long-tang (XQLT), L-carbocisteine (LCC), or pranlukast (Pran)] for 1 h. *Statistical significance in the expression levels of TSLP between non-stimulated (untreated control) and HA-stimulated cells (P < 0.05). The comparison between a single HA-stimulant and co-stimulant HA and SYN with respect to mRNA expression of TSLP in HNEpCs is indicated by * = p < 0.05. RFC = relative fold change.
cultures increased the HDC mRNA expression levels. Based on these results, it was suggested that HDC production is activated in the nasal mucosa in allergic rhinitis. SYN not only inhibited the H1R expression at the same level as FEX did but also reduced the HDC protein expression. These findings suggest that SYN reduces HA production by inhibiting HDC. We confirmed that the H1, H2, H3, and H4 receptors were expressed in HNEpCs, used in this experiment. While no significant inhibitory effect of SYN on H4R was obvious, there was a tendency toward significant inhibition.

The expression levels of TSLP mRNA were significantly increased by priming and significantly reduced by co-stimulation with SYN. These results suggest that SYN exerts an inhibitory effect on TSLP mRNA expression. Based on the results shown in Figure 1, TSLP disintegration directly into SYN or suppression of TSLP secretion, due to a direct action of SYN on cells, is considered a possible underlying mechanism. A schematic representation of the pathway, presented in Figure 11, indicates a possibility that reduced TSLP expression suppresses the expression of other factors.
As stated earlier, H4R is known to have a greater capacity for binding HA than H1R does. H4R is considered to be associated with itching in skin diseases. Schaper et al. (2016) have reported that H4R regulates the TSLP expression in human and mouse keratinocytes, and inhibition of H4R may help control the inflammatory response and itching, both of which involve TSLP. In the future, additional trials may reveal that SYN inhibits TSLP via H4R and is superior to the existing therapeutic agents.

*MUC5AC* mRNA expression was significantly reduced by co-stimulation with SYN or Pran, suggesting that both SYN and Pran exert inhibitory effects on *MUC5AC* mRNA expression. It has been reported that *MUC5AC* expression increases in allergic rhinitis (Shah, Ishinaga, Hou, Okano, & Takeuchi, 2013; Voynow, Selby, & Rose, 1998) and exacerbates asthma (Ordonez et al., 2001). SYN reduced the *MUC5AC* expression as much as Pran did. These findings suggest that SYN exerts a therapeutic effect similar to those of leukotriene antagonists. Although SYN did not affect the *MUC5B* expression in the present study, there was a greater tendency toward a lower *MUC5B* expression with SYN treatment than with FEX treatment, indicating that *MUC5B* expression may be regulated via H1R.

Based on the above findings, it can be suggested that the natural pharmaceutical product SYN can inhibit the HA production, HR expression, and tissue inflammation. In addition, SYN was demonstrated to potentially act not only in the early phase of allergic reactions but also in the late-phase reaction. Thus, SYN may contribute to the improvement of clinical symptoms of allergies, including sneezing and rhinorrhea (early-phase reactions) and nasal congestion (a late-phase symptom). Moreover, the finding that SYN inhibited the expression of *MUC5AC* mRNA, which encodes the core protein in mucin, indicates that SYN may be able to inhibit tissue remodeling. Tissue remodeling elicits mucus production, aggravates the severity of allergies, and makes allergies difficult to treat. Thus, SYN was found to be potentially useful as a novel therapeutic agent for allergic rhinitis.

We cultured commercially available HNEpCs and stimulated these cells with poly(I:C), LPS, or HA. However, it is questionable whether this *in vitro* system closely mimics the *in vivo* situation. In the future, culturing cells abraded from the nasal mucous membrane, because of allergic rhinitis, may help evaluate a response that more closely resembles the actual *in vivo* response of these cells. Additional experiments with *HR* and *MUC* genes and cytokines may also help elucidate the pathological mechanism.

5. Conclusion
SYN showed inhibitory effects on both early- and late-phase allergic reactions. In particular, in the early-phase reaction, SYN is considered to act from the HA production stage, which is close to the initiation of the allergic reaction pathway. The site of action of SYN differs from that of H1R antagonists. Therefore, SYN may serve as a novel therapeutic agent.
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The authors declare no competing interest.

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Correction
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References


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