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Inhibitory effects of Gamichungsangbohatang on chemokines related asthma in A549 human epithelial cells

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SUMMARY

Recently a major goal in asthma therapy is to reduce or prevent the inflammatory response of airway. Eosinophilic accumulation in the tissue is a prominent feature of allergic diseases including asthma. Production of chemokines by bronchial epithelial cells may contribute to the allergic inflammation by recruiting eosinophils. In this study we evaluated the inhibitory effect of Gamichungsangbohatang (GMCSBHT), used traditionally in treating asthma, on secretion of chemokines for eosinophils in human A549 epithelial cells. Chemokines such as eotaxin, RANTES, IL-8 were inhibited in a dose-dependent manner, but IL-16 showed no inhibition by GMCSBHT. These findings indicate that GMCSBHT might be a therapeutic value in treating asthma by suppression of chemokines secretion associated with local accumulation of eosinophils.

Key words: Gamichungsangbohatang; Eosinophil migration; Asthma; Chemokine

INTRODUCTION

Asthma is a chronic inflammatory disorder of the airways in which many cells and cellular elements play a role. Airway inflammation is associated with airway hyperresponsiveness, airflow limitation, and respiratory symptoms. The key inflammatory cells involved in asthma are lymphocytes, eosinophils, mast cells, antigen presenting cells and, in a percentage of asthmatic patients, neutrophils (Bateman *et al.*, 2008). Although numerous inflammatory cells contribute to various aspects of disease pathogenesis, enough evidence has shown that the localization and activation of eosinophils within the tissue may be an important factor. Eosinophils are often considered to play a major role in inducing airway inflammation and hyperresponsiveness, potentially by means of the secretion of their toxic granule proteins, which might damage the airway mucosa. Accordingly elucidating the mechanisms that regulate the selective tissue accumulation of eosinophils is a topic of intense research focus.

Eosinophil recruitment from peripheral blood into the airways is controlled by adhesion molecules and chemokines. Chemokines are a group of structurally related small proteins with a common biological activity of inducing directional migration (chemotaxis) of various cell types. Chemokines have been widely viewed as pathogenic mediators of acute and chronic inflammation and tissue damage in allergies and asthma by selective eosinophil recruitment to sites of inflammation. Suppression of chemokines may interrupt the sequence of signals in an allergic response (Gangur

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and Oppenheim, 2000).

Ga-Mi-Chung-Sang-Bo-Ha-Tang (GMCSBHT) has been used to treat asthma in Korea for centuries. Recently several clinical findings indicated that GMCSBHT improved the Quality of Live Questionnaire for Adult Korean Asthmatics scores and pulmonary function test parameters (Jung *et al.*, 2002, 2004). According to the Roh's study (2005), CSBHT inhibited airway inflammation and remodeling in a murine model of chronic asthma. However the therapeutic mechanisms of this GMCSBHT are still remains unclear.

The aim of this study was therefore to investigate the effect of GMCSBHT on the secretion of chemokines like eotaxin, regulated upon activation in normal T-cell expressed and secreted (RANTES), Interleukin-8 (IL-8) and IL-16 from airway epithelial cells.

MATERIALS AND METHODS

Cell culture

A549 cells, human type II-like epithelial lung cells, were obtained from Korean Cell Line Bank (Cancer Research Institute, Seoul, Korea). The cells were cultured in tissue flasks in 100% humidity and 5% CO_2 at 37 in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum and penicillin-streptomycin, at 1×10^6 cells/ml. A549 cells were then plated onto 12-well, flat-bottom tissue culture plates at a density of 5×10^5 cells/ well in hormonally defined RPMI media as described previously. The medium was changed 2 days until the cells became confluent and then the cells were used for the experiments.

Preparation of GMCSBHT

GMCSBHT, which contains 18 species of medicinal plants (Table 1), was purchased from Korean

Table 1. Contents of GMCSBHT and the amounts of standard materials

Contents	Quantity (%)	Standard materials (SM)	SM quantities (mg/GMCSBHT, ex. 1g)
Rehmanniae Radix Vaporata	11.94	5-HMF	1.81 ± 0.06
Discoreae Radix	8.96	Allantoin	5.08 ± 0.91
Corni Fructus	8.96	Loganin	2.78 ± 0.04
Hoelen	5.97		
Moutan Cortex Radicis	5.97	Paeonol	0.05 ± 0.00
		Paeoniflorin	0.94 ± 0.09
Alismatis Radix	5.97	Alisol B acetate	0.015 ± 0.001
Maximowicziae Fructus	4.48	Schizandrin	0.22 ± 0.00
Ponciri Fructus	4.48	Poncirin	8.77 ± 0.12
Liriopis Tuber	4.48		
Asparagi Radix	4.48		
Fritillariae Roylei Bulbus	4.48		
Platycodi Radix	4.48		
Coptidis Rhizoma	4.48	Berberine	1.17 ± 0.36
Ansu Semen	4.48	Amygdalin	23.30 ± 1.40
Pinelliae Rhizoma	4.48	Homogentisic acid	3.09 ± 0.23
Tricosanthis Semen	4.48		
Scutellariae Radix	4.48	Baicalin	1.30 ± 0.03
		Baicalein	0.15 ± 0.00
		Wogonin	0.09 ± 0.00
Glycyrrhizae Radix	2.99	Glycyrrhizic acid	1.17 ± 0.00

Association of Crude Medicinal Herbs (Seoul, Korea) and washed with distilled water. Every herb of GMCSBHT was ground to a fine powder and mixed with amount of 335 g as the ratio in the Table 1. These were extracted with 70% ethanol (Duksan Phanrmaceutical Co., Ltd. (Ahn-san, Korea); technical reagent) (v/v) by using an ultra-sonicator (Branson, Danbury, CT, USA) for 10 min at room temperature and then gradually extracted with 80%, 90% and 100% ethanol using the same method. The alcoholic solution extract was evaporated at 60 and then freeze-dried. The yield was 81.54 g (yield: 21.4%). GMCSBHT extracts were dissolved in ultra-pure distilled water and then sterilized by passing through 0.22 um syringe filter.

The HPLC analysis of standard materials to test samples

The dried ethanol extract, about 240 mg of GMCSBHT was accurately weighed, then put in a test tube, and dissolved in 4 ml of 50% methanol (HPLC reagent, J.T. Baker Co., Ltd., USA) followed by filtering using 0.45 μm syringe filter (PVDF, Waters, USA). Each marker substances (standard materials) used for the quantitative analysis to GMCSBHT was purchased; 5-HMF (Sigma, USA), allantoin (Sigma), loganin (Wako, Japan), paeonol (Wako), paeoniflorin (Wako), alisol B acetate (Wako), schizandrin (Wako), poncirin (Sigma), berberine (Sigma), amygdalin (Sigma), homogentisic acid (Sigma), baicalin (Sigma), baicalein (Wako), wogonin (Wako), glycyrrhizic acid (Sigma). The standard materials were weighed 10 mg, and were dissolved according to the analysis condition of standard materials. The dissolved standard solution was diluted to 0.1, 0.5, 1.0, 1.5, 2.0 mg/ml respectively, and then the standard HPLC chromatogram was obtained. The relationship between the concentration and the peakarea was measured using the minimum square method (R^2 value). The HPLC apparatus was a Water Breeze System (717 + Autosampler, 2487 dual γ absorbance detector, 2996 photodiode array system, 1525 binary HPLC pump, Waters Co., USA), and Waters Empower System (Ver. 5.00, Waters Co., USA) was used for data aquistion and integration.

The quantity of standard materials contained in GMCSBHT was calculated by the following foumula:
The amount (g) of standard materials
= [{the quantitative amount (mg) of standard materials × the purity of standard material} × AT/AS × 1/(the dilution concentration rate of test sample×the amount of test sample)]/n (n = -3)

Where AT is the peakarea of test sample containing standard material and AS is the peakarea of standard material.

The standard materials for quantitative analysis of each herb a medicine contained in GMCSBHT are shown in Table 1. From the results of the standard calibration curve, R^2 values of all marker substances were between 0.991 and 0.999. The amount of standard materials in GMCSBHT is indicated in Table 1.

Cytotoxicity assays

To determine the cytotoxicity capacity of the A549 cells, the Cell Titer 96 non-radioactive cell proliferation assay (Promega, USA.) was used following the directions provided by the manufacturer. Cells were plated at 2×10^4 cells/well in a total volume of 100 ul. The epithelial cell layers (A549) were then washed two times and cultured in GMCSBHT extract (0, 0.1, 1, 10, 100, 1000 ug/ml) containing medium. Cells were incubated for 48 h at 37 in 5% CO_2 incubator.

Chemokine assays

Cells were plated at 5×10^5 cells/well in a total volume of 1 ml. After the cells became confluent, the medium changed to serum-free RPMI medium for 24 h. A549 cells were exposed to increasing concentration of TNF-α (25 ng/ml) and IL-4 (50 ng/ml) for 24 h. The cells were stimulated again with a combination of TNF-α (25 ng/ml) and IL-1β

(10 ng/ml) for 24 h. The A549 cells layers were then washed two times with PBS and cultured in GMCSBHT extract (0, 0.1, 1, 10, 100, 1000 ug/ml) containing medium. Cells were incubated for 48 h at 37 °C in 5% CO_2 incubator. Cell free culture supernatants were collected. Eotaxin were assayed using enzyme-linked immunosorbent assay (ELISA) kits according to the instructions of the manufacturers. Assay kits for eotaxin were purchased form BD Biosciences (San Diego, CA, USA), and the minimum detectable concentration of eotaxin was 6.3 pg/ml. Assay kits for RANTES, IL-8, IL-16 were purchased form BioSource International (Camarillo, CA, USA) and the minimum detectable concentration of RANTES, IL-8, IL-16 was 31.2, 15.6, 23.4 pg/ml respectively.

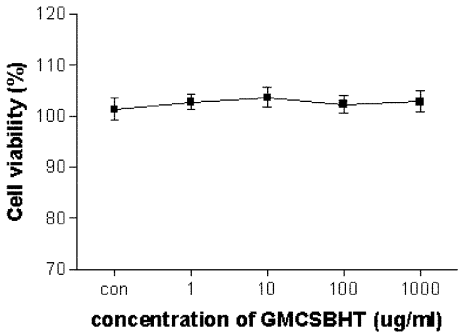

Fig. 1. Cell viability of A549 cell for GMCSBHT. After incubate A549 cells for 24 h, change the culture-medium to RPMI medium containing GMCSBHT extract at the concentration of 0, 0.1, 1, 10, 100, 1000 ug/ml and culture for 24 h. Add MTS solution and activate for 30 min. And measure cell proliferation by reading optical density at 490 nm. Data normalization was performed by control.

Statistical analysis

Statistical analysis of the data was carried out using the Prism 3.02 software (GraphicPad Software Inc. CA, USA). Data were presented as mean ± S.E.M. and statistical analysis was performed by one-way ANOVA for multiple comparisons. Results with $P < 0.05$ were considered statistically significant.

RESULTS

Cytotoxicity evaluation

To determine the cytotoxicity of GMCSBHT, we treated A549 cells with GMCSBHT at a concentration of 0, 0.1, 1, 10, 100, 1000 ug/ml. Increasing concentrations of GMCSBHT did not affect cell viability, so we thought that GMCSBHT was not cytotoxic to A549 cells (Fig. 1).

Effects of GMCSBHT on eotaxin, RANTES, IL-8 and IL-16 secretion levels by ELISA analysis

TNF-α, IL-4 and IL-1β induced the accumulation of chemokines in A549 pulmonary epithelial cell line. To study the effect of GMCSBHT on A549 cells stimulated with TNF-α, IL-4 and IL-1β, we assayed eotaxin, RANTES, IL-8 and IL-16 secretion levels using ELISA kits according to the manufacturer's instructions.

Eotaxin secretion was decreased significantly by 16% after 100 ug/ml GMCSBHT treatment, and by 44% after 1000 ug/ml treatment (Fig. 2A). We already confirmed that GMCSBHT has no effect on cell viability by a cytotoxicity test. Thus we concluded that GMCSBHT inhibits eotaxin secretion.

RANTES secretion was significantly inhibited by 44.8% after 1,000 ug/ml of GMCSBHT treatment (Fig. 2B).

And on IL-8, GMCSBHT at 0.1, 1, 10, 100 ug/ml showed around 20% inhibition effects. Especially in the presence of GMCSBHT at 1000ug/ml, the secretion of IL-8 was decreased significantly by 39.3% (Fig. 2C).

In contrast, IL-16 secretion was not diminished significantly at all concentration of GMCSBHT (Fig. 2D).

DISCUSSION

Asthma is a chronic inflammatory disorder of the airways and the chronic inflammation is associated with airway hyperresponsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing (Bateman *et al.*,

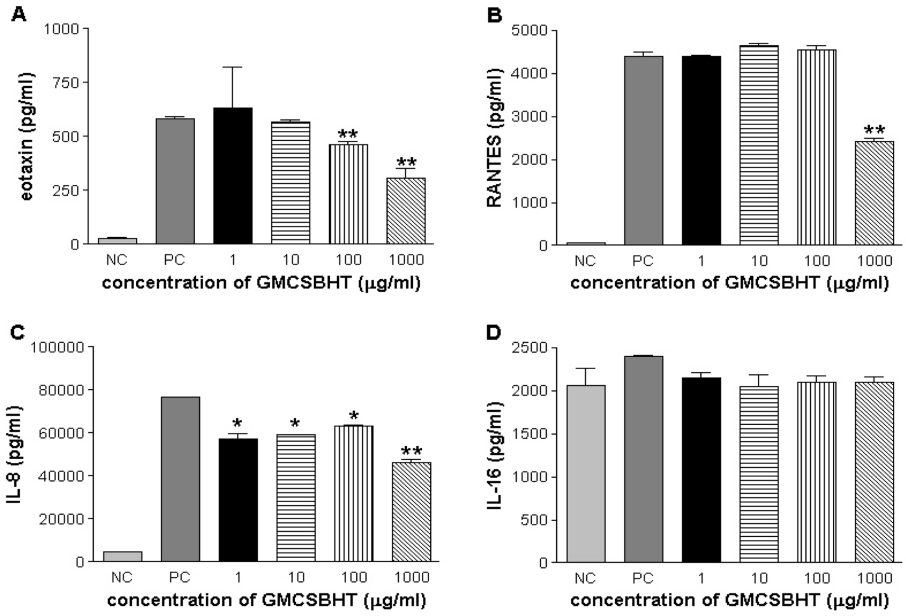

Fig. 2. Inhibitory effects of GMCSBHT on eotaxin (A), RANTES (B), IL-8 (C), IL-16 (D) secretion from A549 cells stimulated with cytokines by ELISA analysis. NC: negative control (untreated A549 cells, only medium), PC: positive control (TNF-α, IL-4 and IL-1β stimulated A549 cells). Data was expressed as the mean ± S.E.M. $^{*}P < 0.05$, $^{**}P < 0.01$ analyzed by the one-way ANOVA.

2008). A characteristic and histologically striking feature of allergic diseases such as asthma is the up to 100-fold increase in the accumulation of eosinophils at inflammatory sites (Wardlaw, 2004). A study by Justice *et al.* (2003) showed that the ablation of virtually all pulmonary eosinophils in OVA-treated mice resulted in a significant decrease in mucus accumulation and abolished allergen-induced airway hyperresponsiveness. That supported the concept eosinophilic infiltration of the lung is a definig component of chronic inflammation that distinguishes asthma from other chronic inflammatory diseases of the airway. Eosinophils can release proinflammatory mediators, such as platelet activating factor and eicosanoids, and eosinophil granule proteins are toxic for airway epithelium. In addition, eosinophils amplify the inflammatory cascade by producing their own chemoattractants which accelerate the recruitment of eosinophils into the inflammatory focus (Rothenberg, 1998). These facts have led to a consensus that eosinophils are major effector cells for tissue inflammation in various allergic diseases. Preferential eosinophil accumulation at the site of allergic inflammation is believed to be the result of several events, such as selective adhesion interaction with endothelial cells and specific chemotactic factors. Numerous chemotactic substances act on eosinophils, including derivatives of arachidonic acid such as leukotriene B4, other lipid mediators such as platelet-activating factor, bacterial products, interleukins, and various chemokines (Rotheberg, 1998).

Chemokines are a family of low-molecular-weight (8 kd to 12 kd) chemotactic cytokines that regulate leukocyte movement (Ono *et al.*, 2003). Chemokines induce circulating leukocyte subsets (eosinophils, basophils, and Th2 lymphocytes and to lesser extent monocytes) to leave the blood stream and migrate to the inflammatory foci by a process of chemotaxis. Subsequent activation of these cells by the locally prevalent cytokines and

chemokines leads to their degranulation and release of histamine, lipid mediators, leukopeptides and reactive oxygen species that mediate tissue damage resulting in the clinical late phase reaction (Gangur and Oppenheim, 2000). On the basis of role of chemokines in allergic inflammation, novel therapeutic approaches for allergies and asthma are being evaluated by targeting chemokines and their receptors.

The alveolar epithelium, which has been traditionally regarded as a target of the inflammatory response, may play an important role by its capacity to modify the development and resolution of the inflammatory reaction into the alveolar space. Productions of chemokines by alveolar epithelial cells may contribute to the local accumulation of inflammatory cells in asthmatic patients. A549 cells used in this study, synthesized the mRNA and immunoreactive proteins for several chemokines, including IL-8, RANTES, and eotaxin, which directly or indirectly influence the activity of eosinophils (Cheng *et al.,* 2001).

Several studies have demonstrated that certain traditional herbal medicine formulas have theraputic benefits in allergic asthma. Among several traditional herbal medicines, GMCSBHT is frequently prescribed herbal medicine for treating asthma. The effects of GMCSBHT have been investigated in vivo and at the clinical level (Jung *et al.,* 2002, 2004; Hwang *et al.,* 2003; Choi *et al.,* 2004; Roh *et al.,* 2005; Heo *et al.,* 2006; Min *et al.,* 2006). But, the therapeutic mechanisms of this GMCSBHT are not certificated so far. We assessed whether GMCSBHT inhibits chemokines that contribute to the local accumulation of eosinophils. We measured eotaxin, RANTES, IL-8, IL-16 secretion after GMCSBHT treatment, and confirmed GMCSBHT inhibitied eotaxin, RANTES, IL-8 in dose-dependent manner. But IL-16 was not suppressd by GMCSBHT.

In the present study GMSCSBHT reduced eotaxin secretion significantly by 16% at 100 ug/ml and 44% at 1000 ug/ml. Eotaxin, a CC chemokine, is a potent and selective chemotactic factor for eosinophils. It is expressed by structural cells-mainly epithelial cells in normal subjects-and inflammatory cells, and it acts via CC Chemokine receptor 3 highly expressed on eosinophils (Rothenberg, 1998). Many studies investigated the role of eotaxin in the recruitment of eosinophils. Eotaxin's chemotactic effect was greater with eosinophils from asthmatic subjects than with eosinophils from normal subjects (Ferland *et al.,* 2001). The blocking of eotaxin with antibodies to eotaxin caused an inhibition of eosinophil chemotactic activity in A549 cells stimulated with TNF-α and IL-4 by approximately 70% (Cheng *et al.,* 2001). And the generation of eotaxin null mice by targeted gene disruption revealed a definitive role for eotaxin in enhacing antigen-induced tissue eosinophilia and the specificity of eotaxin for eosinophil (Rothenberg *et al.,* 1997). Besides it is widely accepted that corticosteroids effectively reduce eotaxin expression and lung eosinophilia in asthmatic patients and that this is usually associated with favorable clinical response (Fukakusa *et al.,* 2005).

According to our results, RANTES was not inhibited after 0.1, 1, 10, 100 ug/ml GMCSBHT treatment, but the secretion of RANTES decreased by 44.8% at 1,000 ug/ml, high concentration. RANTES belongs to CC chemokine family and induces leukocyte migration to sites of inflammation. RANTES production is generated predominantly by CD8 + T cells, epithelial cells, fibroblasts and platelets and is a particular feature of inflammation. It mediates the trafficking and homing of classical lymphoid cells such as T cells and monocytes, but also acts on a range of other cells, including basophils, eosinophils, natural killer cells, dendritic cells and mast cells (Appray and Rowland-Jones, 2001). Several studies demonstrated the presence of increased amounts of RSNTES in bronchoalveolar lavege (BAL) fluid from asthmatic patients (Alam *et al.,* 1996; Sur *et al.,* 1996; Teran *et al.,* 1996; Tillie-Lebond *et al.,* 2000). Measurements of the chemokines in BAL fluid showed that levels of

RANTES were 12-fold greater in asthmatic children than in normal children. Compared with the other eosinophil-activating chemokines, RANTES was the chemokine released in the greatest concentrations (Rojas-Ramos *et al.,* 2003). In another study an endobronchial allergen challenge increased the concentration of RANTES in BAL fluid, which correlated with the number of eosinophils. Further, and anti-RANTES antibody abolished the eosinophil chemotactic activity of the BAL fluid (Samson *et al.,* 1996; Teran *et al.,* 1996).

IL-8 has been shown to have chemotactic activity for neutrophils and causes airway neutrophilia. On the other hand IL-8 may potentiate eosinophil recruitment and activation, and evidence suggests that IL-8 can function as a chemotactic factor for cytokine-primed eosinophils (Shute, 1994). Dose-dependent migration of eosinophils in response to IL-8 has also been demonstrated (Erger and Casale, 1995). Neutrophils are increased in a very small proportion of asthmatic children while eosinophils are consistently elevated in the BAL fluid. But in recent years, there has been increasing evidence that the number of neutrophils and the IL-8 levels increase prominently in patients with acute exacerbations of asthma by viral infection, acute fatal asthma that requires intubation or mechanical ventilation and severe persistent asthma (Norzila *et al.,* 2000; Rojas-Ramos, 2003; Kikuchi *et al.,* 2005). These studies suggest that in addition to eosinophils and lymphocytes, neutrophils are also present and may play a contributing role in more severe asthma (Bateman *et al.,* 2008) and IL-8 is an important mediator of neutrophilia (Ordonez *et al.,* 2000). Moreover, neutrophils are capable of producing mediators which may modify the movement or activation status of eosinophils. A positive correlation between the percentages of neutrophils and eosinophils in induced sputum from severe persistent asthmatics was found (Kikuchi *et al.,* 2005). Corticosteroids are known to effectively abolish eosinophilia in asthmatic patients, but they have been shown to increase neutrophilia in the serum and tissue of these patients. The failure of oral corticosteroids to inhibit IL-8 mRNA expression might contribute to persistent airway neutrophilia observed in patients with moderate-to-severe asthma, despite treatment with corticosteroids (Fukakusa *et al.,* 2005). On the other hand, in this study GMCSBHT had significant inhibitory effects on IL-8 production. So GMCSBHT is thought as having different therapeutic mechanism from corticosteroids. Further we could expect a theraputic effect of GMCSBHT on neutrophilia in acute sever ashtma or persistent asthma state.

IL-16 is secreted by airway epithelial cells after stimulation with histamine, a mast cell–derived mediator released after IgE cross-linking and contribute to allergen-driven airway inflammation. IL-16 has chemotactic activities for CD4 + cells, including CD4 + T cells and CD4-bearing eosinophils and provides a synergistic signal for proliferation of CD4 + T cells (Laberge *et al.,* 2000; Yoshimoto *et al.,* 2000). Downregulation of IL-16 expression by dexamethasone suggests that glucocorticoids may inhibit airway inflammation partly by suppressing the synthesis of inflammatory cytokines including IL-16 (Arima *et al.,* 1999). In another study, anti–IL-16 antibodies inhibit eosinophil chemotactic activity strongly and this blocking effect is the same as that of the eotaxin antibody (Cheng *et al.,* 2001). These observations indicate that IL-16 is responsible for part of chemoattractant activity for eosinophils and may play an important role in airway inflammation in subjects with allergic asthma. But our study could not show significant inhibition IL-16 by GMCSBHT.

In summary, we found that GMCSBHT suppressed the secretion of some chemokines associated with eosinophil accumulation such as eotaxin, RANTES, IL-8 in A549 cells. It indicates that GMCSBHT may inhibit the inflammatory process by eosinophils. Certainly more studies on other chemokines and in vivo model should be needed to support the anti-inflammatory effect of GMCSBHT. Besides we presented the possibility of GMCSBHT to decease

neutrophilia by inhibiting secretion of IL-8. Additional studies are needed to elucidate the effect of GMCSBHT on neutrophil and its mechanism. Therefore, we suggested that GMCSBHT could be beneficial for treating asthma.

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