ORIGINAL ARTICLE

2,4,6,-trihydroxy-3-geranylacetophenone (tHGA) Suppresses Chronic Allergic Airway Inflammation in Ovalbumin-Sensitized Mice via Intraperitoneal Route

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ABSTRACT

Introduction: Asthma is a condition characterized by eosinophilic airway inflammation and remodelling that involves several pathological changes, including subepithelial fibrosis, mucus hypersecretion, smooth muscle growth, and vascular changes. The present study aimed to determine the effect of tHGA administered intraperitoneally in a chronic asthma mouse model that closely mimics the human asthma. **Methods:** Ovalbumin-sensitized and challenged BALB/c mice were i.p. administered with tHGA at different doses (20 and 2 mg/kg). Respiratory function was measured, and brochoalveolar lavage, blood and lung samples were then obtained and analyzed. **Results:** The airways of OVA-induced mice developed increased pulmonary inflammation with increased levels of cytokines, chemokines, and changes in vascular permeability. Intraperitoneal administration of tHGA in OVA-induced mice significantly and dose-dependently inhibited the airway inflammation, production of immunoglobulin E, Th2-type cytokines and chemokines, and inflammatory mediators. Treatment with tHGA also significantly reduced the airway hyperresposiveness in response to increased methacholine doses. **Conclusion:** This study demonstrates that the efficacy of tHGA in alleviating chronic asthmatic symptoms in mouse model improved significantly when administered intraperitoneally compared to oral route. Furthermore, this study also supports that tHGA has a therapeutic potential in chronic asthma management by acting as a cysteinyl leukotrienes (CysLT) inhibitor.

Keywords: Asthma, Airway hyperresponsiveness, Airway remodelling, Cysteinyl leukotrienes, tHGA

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INTRODUCTION

Chronic asthma is a complex pulmonary disease exacerbated by the persistent airway inflammation, hyperresponsiveness, and irreversible airway obstruction, which cause a deterioration of lung function leading towards pathological airway remodelling (1,2). The structural remodelling in the asthmatic airways include the thickening of airway walls, increased smooth muscle cell hyperplasia/hypertrophy, mucus hypersecretion, and sub epithelial fibrosis (3-5). These pathological changes which characterize chronic asthma are driven by various inflammatory mediators secreted as a result of airway inflammation. For example, Th2 cytokines such as interleukin-4 (IL-4), interleukin-5 (IL-5), and interleukin-13 (IL-13) elicit the production of immunoglobulin E (IgE) and eosinophil recruitment. IL-13 also induces mucus hypersecretion and airway hyperresponsiveness (6, 7). These cytokines also induce the inflammatory and structural cells to secrete several proinflammatory molecules including lipid-derived mediators, chemokines such as RANTES (CCL5), and growth factors which collectively promote subsequent infiltration of inflammatory cells into the airways (8). While Th2 cytokines promote asthmatic airway inflammation, evidence suggests that Th2 response is shifted to a Th1 response in a chronic allergic airway inflammation. The activation of Th1 response leads to the production of interferon gamma (IFN-γ). IFN- γ is involved in cancelling the effects of Th2 activation including bronchial hyperresponsiveness, mucus goblet

hyperplasia, and eosinophilia (9, 10, 11).

In particular, a fibrotic cytokine, TGF-β, is thought to be one of the key sentinels in eliciting airway remodelling in asthma (12). Kiwamoto et al. has demonstrated that the expression of TGF-β in Th2-dominant GATA-3-tg mice increased after being repetitively challenged to OVA and its expression can be suppressed further by a leukotriene receptor antagonist, montelukast (13). There is also evidence showing that the recruitment and migration of leukocytes to the inflammatory sites require adhesion molecules whose expression can be induced by cytokines (14, 15). A previous study demonstrated that toluene diisocyanate (TDI)-induced asthma resulted in elevated expression of intracellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM) and matrix metalloproteinase-9 (MMP-9), as well as increased numbers of inflammatory cells (16).

We have previously reported that a bioactive component found in *Melecopia ptelefolia*, identified as 2,4,6,-trihydroxy-3-geranyl acetophenone (tHGA), inhibited CysLT synthesis, one of the mediators of asthma via the inhibition of 5-lipoxygenase (5-LOX) (19). CysLT synthesis requires the activation of arachidonic acid by 5-LOX catalysis, which was facilitated by 5-LOX activating protein (FLAP), the key protein needed for the synthesis of the leukotrienes. CysLT possesses proinflammatory features associated in promoting airway eosinophilia, increasing mucus hypersecretion, and also acting as a potent bronchoconstrictor, which can lead to asthma attack (17, 18). We have also shown in the previous work that tHGA was able to prevent allergic airway inflammation in ovalbumin-sensitized mice by targeting CysLT synthesis (19). Previously, we have reported that at least 40 mg/kg tHGA was able to attenuate airway inflammation in a murine model of chronic asthma when administered via the oral route. Furthermore, a higher dose (80 mg/kg) attenuated both airway inflammation and remodelling in that study (20). The present study aimed to determine whether lower doses of tHGA are capable of suppressing the airway inflammation and remodelling upon chronic allergic airway inflammation when administered via intraperitoneal route.

MATERIALS AND METHODS

Animals

BALB/c mice (8 - 10 weeks old, male) purchased from the Institute of Medical Research, Kuala Lumpur, were housed at the Physiology Lab Experimentation Room located at the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. The mice were acclimatized at 22 + 2°C with 12 h dark/light cycle and handled in line with the standard guidelines for the care and use of laboratory animals (National Research, 1996). This study was approved by the Animal Experimentation Ethics Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

Ovalbumin (OVA) sensitization, airway challenge and tHGA administration

The mice were OVA-sensitized intraperitoneally on day 0, 7 and 14, as previously described (19). At day 21, the mice were exposed for 30 minutes to aerosolized 2% OVA (w/v) in PBS (pH 7.4) by an ultrasonic nebulizer (Omron, Japan) for three days per week, for six consecutive weeks. tHGA (2 and 20 mg/kg body weight) was dissolved in PBS with 1% w/v Tween 80 and 1% w/v DMSO and were administered intraperitoneally 10 days before the last challenge (day 49-58), 1 hour prior to OVA aerosolization (Figure 1).

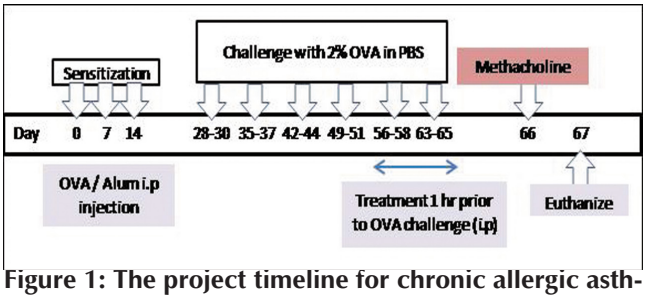

Figure 1: The project timeline for chronic allergic asthma

The bronchoalveolar lavage fluid (BALF) analysis

Mice were culled following the methacholine challenge. The lungs were flushed four times with 0.9 ml cold PBS using 22G feeding needle cannulated from the trachea of the mice to recover the bronchoalveolar lavage fluid (BALF). The BALF samples were then centrifuged at 400 xg at 4°C for 10 minutes. The supernatant was collected and stored at -80°C until required, whereas the cell pellet was resuspended in PBS for slide preparation. The cells were loaded onto the cytospin (450 µl) and spun at 500 xg for 5 minutes at 4°C. For differential cell count, fixed cells were stained with the Wright's Stain and 10 µl of the remaining cell suspension were used for cell quantification by the trypan blue exclusion method.

ELISA analysis of BALF

The concentrations of IL-4, IL-5, IL-10, IL-13, RANTES, and TGF-β were measured using commercial immunoassay kit according to the manufacturer's instruction. IL-4, IL-5 and IL-10 concentrations were measured with ELISA kits specific for mouse IL-4, IL-5, and IL-10 (BD Pharmingen, San Diego, CA, USA), whereas IL-13, RANTES, and TGF-β concentrations were measured using mouse DuoSet specific for each factor (R&D Systems). CysLT levels were measured using an enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, USA).

Total and OVA-Specific serum IgE measurement

Blood from each mouse was drawn through cardiac puncture and subjected to centrifugation to obtain serum. The levels of total (BD Pharmingen, San Diego, CA, USA) and OVA-specific serum IgE (Bio-Rad) were

measured by commercial ELISA kits following the manufacturer's instruction.

Lung Histology

The lungs collected were fixed in 10% formalin and processed accordingly to obtain sections which were stained with either hematoxylin and eosin (H&E), periodic acid shift (PAS), or Masson trichrome (MT). Airway inflammation of the lung was examined in the peribronchiol and perivascular regions. The total numbers of inflammatory cells in peribronchiol and perivascular regions were counted and averaged with the total number of airways per section to obtain the mean number of airway-infiltrated inflammatory cells. The goblet cell hyperplasia was determined by dividing PAS-positive cells by the total number of airways for the average number of goblet cells per slide. For the collagen deposition, the areas of the MT-positive stained were determined and divided by the area of each section to get the percentage of collagen deposition in the airways.

Airway hyperresponsiveness (AHR) Measurement to Methacholine (Mch)

Post 24 hours of the final OVA challenge, each mice was placed in an unrestrained whole body plethysmograph (Buxco Electronics, Inc. Troy, NY) to assess the AHR in response to methacholine (Acetyl-β-methylcholine chloride, catalogue # A2251, Sigma-Aldrich). Responses to graded doses of methacholine (6, 12, 25, and 50 mg/ml) were assessed by recording Penh value for 5 minutes to each dose. Penh values were then averaged and used for comparison between the experimental groups.

Statistical analysis

Data analysis was performed using a commercial statistical software, Graphpad Prism (version 5.01). Each data set was statistically analyzed by performing one-way ANOVA, followed by Dunnett's post hoc test to confirm significant differences between experimental treatments. Data was presented as the mean + SEM and were considered statistically significant when $P < 0.05$.

RESULTS

tHGA Effects on the Total and Differential Cell Count

The total and differential cell counts were performed on BALF samples to determine the effects of tHGA on the inflammatory cell infiltration into the airway. Fig.2 shows that OVA-induced mice displayed a significant influx of leukocytes with increased total cell count and number of eosinophils compared to the naive group. The total cell count and number of eosinophils were significantly reduced upon treatment with tHGA at 20 mg/kg, in contrast to untreated OVA-sensitized/challenged mice (Fig.2).

Effects of tHGA on Th2 cytokines, chemokines, TGF-β and CysLT concentrations in BALF

The levels of IL-4, IL-5, IL-10, IL-13, RANTES, TGF-β, and CysLT in BALF samples were measured to examine the tHGA effect on the secretion of proinflammatory mediators as common indicators of airway inflammation. Fig.3 shows that the levels of IL-4, IL-5, IL-10, IL-13, RANTES, TGF-β, and CysLT in OVA-induced mice were significantly elevated compared to the naive group, and the levels were significantly reduced with 20 mg/kg tHGA treatment. tHGA treatment at 2 mg/kg was able to reduce the levels of some mediators but failed to show an inhibitory effect on IL-4, TGF-β, RANTES, and CysLT (Fig.3).

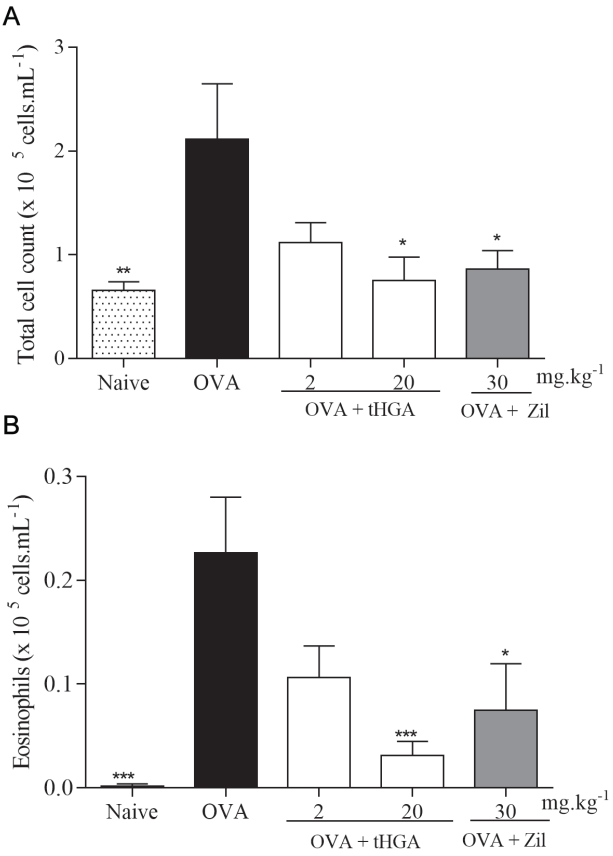

Figure 2: The effects of tHGA on total cell count (A) and eosinophil count (B) in BALF. Trypan blue-stained cell pellets were counted using a hemacytometer and differential cell counts of eosinophil were obtained by examining of Wright's stained cytosmear. Data is expressed as mean + SEM. Significant difference from OVA-sensitized/challenged mice, *P<0.05, **P<0.01, ***P<0.001.OVA + Zil = zileuton-treated group.

tHGA treatment effects on the total and OVA-specific serum IgE

The levels of total serum IgE and OVA-specific IgE were measured to determine the tHGA effect on OVA sensitization. Fig.4 shows that serum total IgE and OVA-specific IgE in OVA-sensitized/challenged mice were significantly higher than naive mice, and both were significantly reduced when treated with tHGA at 20 mg/kg, but not at 2 mg/kg.

Effects of tHGA on airway cell infiltration, goblet cell hyperplasia and deposition of collagen

Further histopathological studies were conducted on

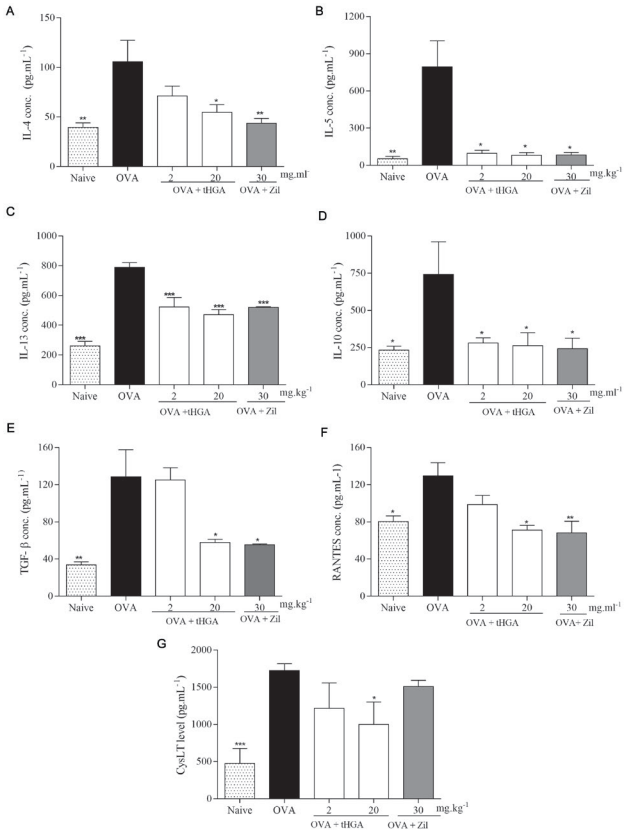

Figure 3: The effects of tHGA on Th2 cytokines, chemokines, TGF-β, and CysLT levels in BALF. Concentrations of (A) IL-4, (B) IL-5, (C) IL-13, (D) IL-10, (E) TGF-β, (F) RANTES, and (G) CysLT were measured by ELISA. Data is expressed as mean + SEM. Significant difference from OVA-sensitized/challenged mice, **P*<0.05, ***P*<0.01, ****P*<0.001. OVA + Zil = zileuton-treated group.

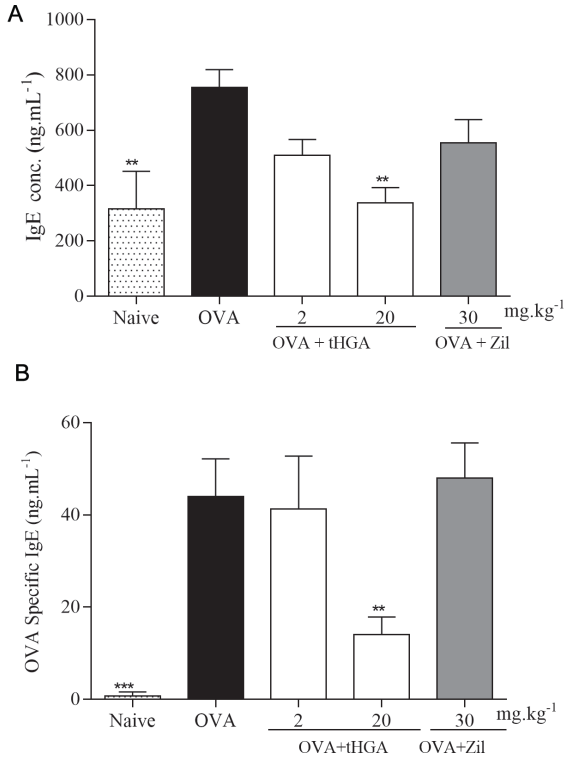

Figure 4: The effects of tHGA on (A) total IgE and (B) OVA-Specific IgE in serum. Values are expressed as mean + SEM. Significant difference from OVA-sensitized/challenged mice, ***P*<0.01, ****P*<0.001. OVA + Zil = zileuton-treated group.

the lung tissue to assess the antiasthmatic effect of tHGA. H & E staining of the lung tissue revealed greater influx of inflammatory cells within the perivascular and peribronchiol connective tissue in OVA-sensitized/challenged mice (Fig.5d) than that of naive mice (Fig.5a). The infiltrating inflammatory cells in OVA-induced mice was significantly reduced when treated with 20 mg/kg tHGA (Fig.5j). Mucus hypersecretion, an airway remodelling feature in a chronic asthmatic model is commonly indicated by goblet cell hyperplasia, as determined by PAS staining. Goblet cell hyperplasia observed in the airway lamina propria was more pronounced in OVA-induced mice (Fig.5e) than that of the PBS group (Fig.5b). In contrast, 20 mg/kg tHGA significantly diminished the number of goblet cell hyperplasia in OVA-induced mice (Fig.5k). Heightened deposition of airway collagen, another characteristic of airway remodelling in chronic asthma, was observed in OVA-induced murine lung tissue when assessed by the Masson Trichrome stain. Compared to the PBS-treated group (Fig.5c), dense collagen deposition surrounding the airways and blood vessels was visible in the OVA-induced group (Fig.5f). Collagen deposition was markedly reduced by tHGA treatment at 20 mg/kg (Fig.5l).

Effect of tHGA on airway function

Airway function in conscious mice was measured as Penh 24 hours post the final OVA challenge. Penh was used as a measurement unit to indicate the airflow obstruction and the increase value of Penh was observed with increasing doses of the methacholine, an AHR promoter. OVA-induced mice showed elevated Penh value compared to those showed in the unrestrained naive mice (Fig.6). This might be due to the narrowing of the airways in response to allergen including ovalbumin that cause the bronchoconstriction which lead to respiratory dysfunction. Treatment of OVA-induced mice with tHGA at 2 mg/kg and 20 mg/kg showed significant reduction of the Penh compared to the untreated OVA-induced mice at the 25 and 50 mg/mL methacholine challenge doses. The positive control, Zileuton, a 5-LOX inhibitor also showed significantly reducted respiratory changes at the same doses.

DISCUSSION

In our previous study, we reported that oral administration of tHGA in a murine model of chronic asthma attenuates airway inflammation and airway remodelling at the highest dose (80 mg/kg). This study aimed to determine whether lower doses of tHGA treatment (2 and 20 mg/kg) via parenteral route have therapeutic effect on airway inflammation and remodelling in the same animal model. This murine model of chronic asthma has been shown to reproduce important hallmarks of the human asthma condition (21). Warner *et al*. has defined airway remodelling as alterations in the quantity, composition, and organization of cells and extracellular components

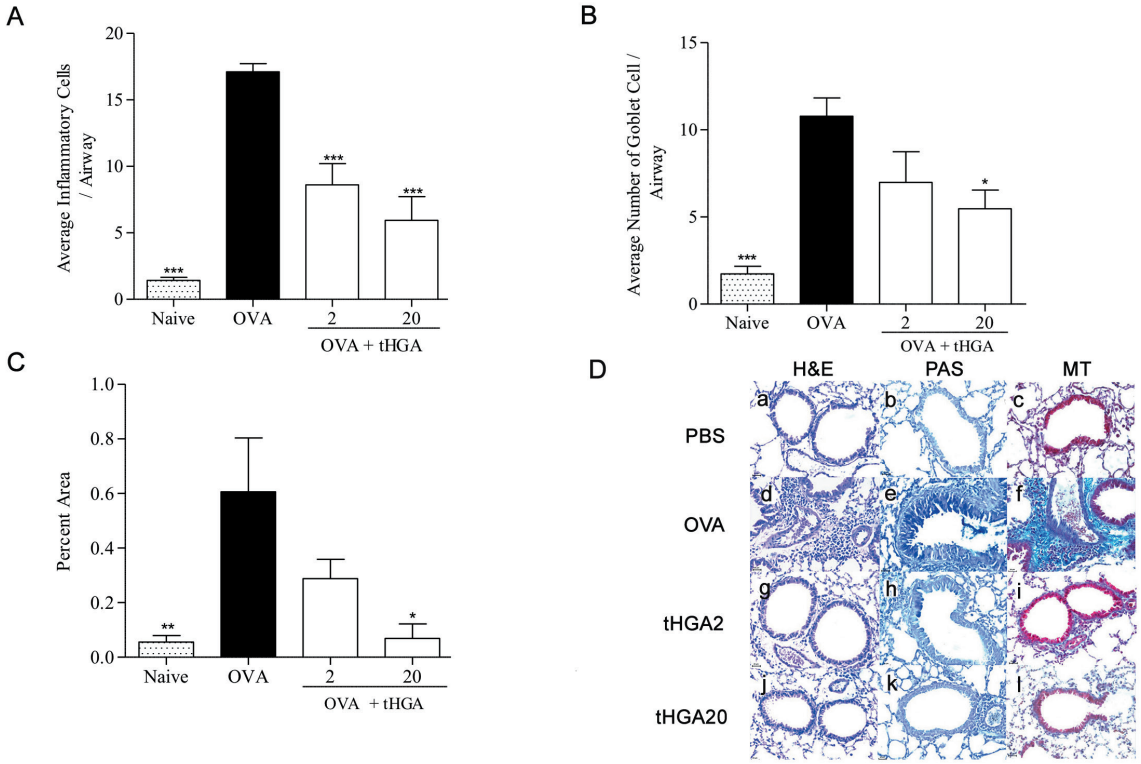

Figure 5: Quantitative analysis of inflammatory cell infiltration (A), goblet cell metaplasia (B), and area of collagen deposition surrounding airways (C). H and E staining for PBS control group (a), OVA-sensitized/challenged mice (d), tHGA treatment at 2 mg/kg (g), and tHGA treatment at 20 mg/kg (j) for airway inflammation. PAS staining for PBS control group (b), OVA-sensitized/challenged mice (e), tHGA treatment at 2 mg/kg (h), and tHGA treatment at 20 mg/kg (k) for goblet cell hyperplasia. Masson-trichrome staining for PBS control group (c), OVA-sensitized/challenged mice (f), tHGA treatment at 2 mg/kg (i), and tHGA treatment at 20 mg/kg (l) for collagen deposition. Data is expressed as mean + SEM. Significant difference from OVA-sensitized/challenged mice, *P<0.05, **P<0.01, ***P<0.001.OVA + Zil = zileuton-treated group.

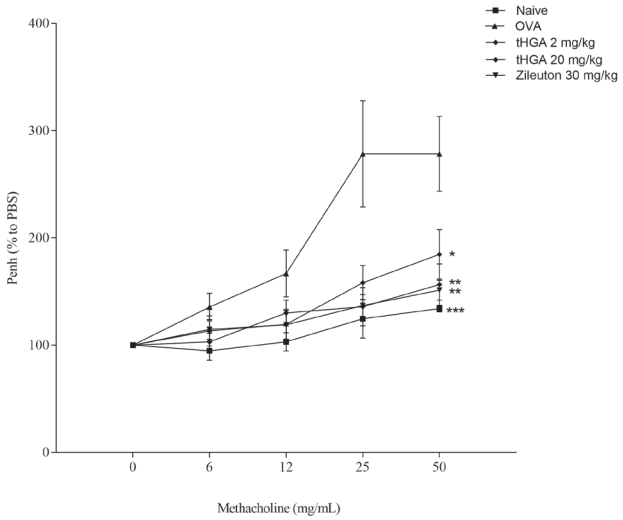

Figure 6: The effect of tHGA on airway hyperresponsiveness to aerosolized methacholine. Values are expressed as mean + SEM. Significant difference from OVA-sensitized/challenged mice, *P<0.05, **P<0.01, ***P<0.001.OVA + Zil = zileuton-treated group.

of the airway wall (22). Accumulating evidence alludes that airway remodelling results in the deterioration of lung function and by preventing the airway remodelling, the severity of the disease may be attenuated (23, 24). Current treatments, which heavily depend on glucocorticosteroid as a reliever to asthma symptoms, have been successful in treating airway inflammation but unable to reverse the remodelling of the airways. New treatments that can attenuate both airway inflammation and remodelling are in need to replace these current treatments.

Our recent work showed that oral administration of tHGA significantly supressed both airway inflammation and remodelling at the highest dose tested (80 mg/kg). While its anti-inflammatory effect was still achieved at a lower dose, 40 mg/kg, the lowest dose tested (20 mg/kg) failed to show any signifcant effects (20). In contrast, the present study shows that tHGA was able to significantly suppress both airway inflammation and remodelling at 20 mg/kg when it was administered intraperitoneally. Although its anti-inflammatory effects were observed at 2 mg/kg, this dose however was not sufficient to suppress airway remodelling. These findings suggest that administration of tHGA via the parenteral route significantly improves the efficacy of tHGA and may be a better alternative for the treatment of chronic asthma. The lower efficacy of tHGA via oral administration is likely attributed to its low bioavailability due incomplete absorption or high first pass effect. In the present study, OVA-sensitized/challenged mice showed greater airway inflammation and remodelling in comparison to the PBS control group. In particular, OVA-sensitized/challenged mice displayed increased airway hyperresponsiveness, greater eosinophil infiltration, hypersecretion of mucus, and collagen deposition in the airways. Such remodelling of the airway was also associated with increased levels of Th2 cytokines, chemoattractant molecules, and

growth factors. Th2 cytokines such as IL-4, IL-5, and IL-13 are secreted by activated T helper type 2 cells. tHGA, a nonmetal chelator compound has been reported to alleviate the synthesis of Th2 cytokines in the acute allergic asthma mouse model. The present study demonstrates that similar effects of tHGA were observed in a murine model of chronic allergic asthma. As previously mentioned, reduced levels of Th2 cytokines may possibly be due to tHGA's ability to supress 5-LO/CysLT synthesis. Th2 cytokines and CysLT has been suggested to positively and mutually modulate the activation of one another in which CysLT production was regulated by the Th2 cytokines via the coordination of 5-LOX nuclear import and LTC4 synthase expression (13, 25).

CysLT, however, is known to take part in the remodelling of airways via processes unrelated to inflammation (13). It directly promotes airway smooth muscle hyperplasia and hyperthrophy and increases collagen synthesis by lung fibroblasts (26). Th2 cytokines, in particular IL-4 and IL-13, secreted by infiltrating inflammatory cells can induce TGF-β1 production by bronchial epithelial cells, a factor that is able to directly convert subepithelial fibroblasts into myofibroblasts or through arginase (27). Blocking TGF-β activity was shown to inhibit remodelling phenotypes that include epithelial cell shedding, airway smooth muscle cell hypertrophy, and hyperplasia, hypersecretion of mucus, and angiogenesis in a murine asthmatic model (28). Espinosa *et al*. has demonstrated a synergism correlation between CysLT and TGF-β, which suggests that LTD4 alone was incapable of supporting smooth muscle cell proliferation in the absence of TGF-β1 or IL-13, two factors shown to increase the expression of CysLT1 receptor (26). Thus, we believe that the downregulation of CysLT and TGF-β expression may partly mediate the inhibitory effect of tHGA on sub epithelial fibrosis .

The effects of tHGA on chemoattractant molecules may also be related to the inhibition of CysLT synthesis. Mariko et al. proved that the effector functions of eosinophils were effectively induced by joint exposure to ICAM-1 or VCAM-1 and CysLT. This combinatory exposure significantly induced eosinophil O2 – generation that also activate the respiratory burst or degranulation (29). Findings from Hemelaers *et al*. further suggest active participation of CysLT in the chemotaxis of eosinophils in the asthmatic airway as they observed the inhibition of chemotactic activity of sputum eosinophils of both corticosteroid-naive and corticosteroid-treated asthmatic patients when treated with a leukotriene receptor antagonist, montelukast (30). Nagata *et al*. meanwhile reported that LTD4 was shown to increase the CD11b and CD18 expression, which enhances eosinophil adhesion to ICAM-1. Interaction of IL-4 with very late antigen-4 (VLA-4) upregulated the expression of VCAM-1, thus promoted the recruitment of eosinophils (31, 32). RANTES (CCL5) is among the chemokines that can be produced in substantial amounts by structural cells such as fibroblasts, epithelial, and endothelial cells that can contribute to airway remodelling once activated. RANTES, a chemotactic factor for T cells, eosinophils, basophils, is a key player in the leukocyte recruitment into the sites of inflammation. This study demonstrates a significant decrease in the RANTES levels in OVA-induced mice when treated with tHGA compared to untreated OVA-induced group. This may be due to tHGA ability to supress Th2 cytokines secretion which subsequently inhibit the activation of RANTES expression.

In this study, we reported that tHGA was able to reduce the CysLT, IgE, and OVA-specific serum IgE levels in OVA-sensitized/challenged mice in a dose dependent manner when administered via intraperitoneal route. In contrast, the positive control Zileuton did not show such inhibitory effect on these parameters in serum. Although zileuton is a well-known oral active inhibitor of 5-LOX which inhibits CysLT (33), findings from this study suggest that Zileuton may be less effective at reducing IgE levels when administered via intraperitoneal route. In comparison to Zileuton, our findings suggest that while tHGA may be an orally active inhibitor of 5-LOX, the efficacy of tHGA in reducing the cysLT, IgE and OVA-specific serum IgE levels was improved when it was administered to the mice intraperitoneally.

CONCLUSION

This study demonstrates the therapeutic effects of tHGA in chronic allergic airway inflammation when administered via intraperitoneal route, mainly through the inhibition of eosinophil infiltration, airway hyperresponsiveness, goblet cell hyperplasia, Th2 cytokines expression, and subepithelial fibrosis. This study also highlights that different administration routes indeed affect the doses of tHGA required to attenuate the chronic asthmatic symptoms. Specifically, lower dose of tHGA is sufficient to attenuate airway inflammation and remodeling in murine model of chronic asthma with parenteral administration compared to oral administration. While this study supports the therapeutic potential of tHGA in treating chronic asthma, further studies are adamant in elucidating the mechanism by which tHGA exerts its effects on airway inflammation and remodelling.

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