



Anti-allergic activity of 2,4,6-trihydroxy-3-geranylacetophenone (tHGA) via attenuation of IgE-mediated mast cell activation and inhibition of passive systemic anaphylaxis

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ABSTRACT

tHGA, a geranyl acetophenone compound originally isolated from a local shrub called *Melicope ptelefolia*, has been previously reported to prevent ovalbumin-induced allergic airway inflammation in a murine model of allergic asthma by targeting cysteinyl leukotriene synthesis. Mast cells are immune effector cells involved in the pathogenesis of allergic diseases including asthma by releasing cysteinyl leukotrienes. The anti-asthmatic properties of tHGA could be attributed to its inhibitory effect on mast cell degranulation. As mast cell degranulation is an important event in allergic responses, this study aimed to investigate the anti-allergic effects of tHGA in cellular and animal models of IgE-mediated mast cell degranulation. For *in vitro* model of IgE-mediated mast cell degranulation, DNP-IgE-sensitized RBL-2H3 cells were pre-treated with tHGA before challenged with DNP-BSA to induce degranulation. For IgE-mediated passive systemic anaphylaxis, Sprague Dawley rats were sensitized by intraperitoneal injection of DNP-IgE before challenged with DNP-BSA. Both *in vitro* and *in vivo* models showed that tHGA significantly inhibited the release of preformed mediators (β -hexosaminidase and histamine) as well as *de novo* mediators (interleukin-4, tumour necrosis factor- α , prostaglandin D₂ and leukotriene C₄). Pre-treatment of tHGA also prevented IgE-challenged RBL-2H3 cells and peritoneal mast cells from undergoing morphological changes associated with mast cell degranulation. These findings indicate that tHGA possesses potent anti-allergic activity via attenuation of IgE-mediated mast cell degranulation and inhibition of IgE-mediated passive systemic anaphylaxis. Thus, tHGA may have the potential to be developed as a mast cell stabilizer for the treatment of allergic diseases in the future.

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1. Introduction

The prevalence of allergic diseases including allergic asthma, anaphylaxis and eczema is escalating dramatically over the last 20 years in developed and developing countries (Pawankar et al. 2013). These allergic diseases have caused many problems especially in children (Pawankar et al. 2013). In general, allergic reactions can be categorised into the early phase reaction and the late phase reaction (Amin 2012). The early phase of the allergic reaction occurs rapidly, typically within minutes, following allergen exposure (Locksley 2010). This reaction will result in a process called mast cell degranulation where histamine and other mast cell granule proteins are released into the surrounding environment (Amin 2012; Schroeder 2011). The late phase of the allergic reaction, which usually occurs hours after its activation, involves the

production of leukotrienes, prostaglandins and cytokines by activated mast cells (Locksley 2010).

Mast cell is an important effector cell of the innate immune system. Mast cells express high-affinity immunoglobulin E (IgE) receptor (FcεRI) on the cell surface which binds easily with IgE produced by plasma cells (Kawakami and Galli 2002; Metcalfe et al. 1997). Mast cells can be activated by crosslinking of the FcεRIs with multivalent antigens, causing secretion of preformed mediators such as histamine and proteases in the early phase, as well as newly synthesized inflammatory mediators including prostaglandins, leukotrienes and other proinflammatory cytokines in the late phase of allergic reaction (Yamaguchi et al. 1999; Stevens and Austen 1989). The release of these mediators evokes vasodilation, bronchoconstriction and potent immune allergic and inflammatory responses (Sakai et al. 2010). Therefore, mast cell activation is widely recognized as a critical event in many IgE-mediated immune responses including allergic asthma, allergic rhinitis, atopic dermatitis and anaphylaxis (Galli and Tsai 2012; Sheinkopf et al. 2008; Holgate et al. 2005).

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Currently, anti-histamine drugs and steroids remain the mainstay of therapies to combat allergic diseases (Kaliner 2009). Small-molecule inhibitors that target leukotrienes (zileuton and pranlukast) or histamine receptors (clemastine and loratadine) are also commonly prescribed, although adverse effects such as cardiac toxicity and angioedema have occasionally been reported (Van Hoeske et al. 2007). Another common treatment is mast cell stabilizers, such as cromolyn sodium and ketotifen fumarate, which act by preventing the release of mediators from activated mast cells (Finn and Walsh 2013). However, unwanted side effects such as drowsiness, upset stomach, chest congestion and dry mouth have been reported (Oppenheimer and Casale 2002). As such, many researchers are now shifting their attention towards natural compounds or other traditional medicinal herbs to control immune responses and combat allergic reactions with less side effects (Tang et al. 2015; Chung et al. 2013).

2,4,6-trihydroxy-3-geranylacetophenone (tHGA) (Fig. 1) is an active compound originated from a local shrub namely *Melicope ptelefolia* (Shaari et al. 2011). Due to its exceptional antipyretic, anti-bacterial, analgesic and anti-inflammatory activities, *M. ptelefolia* has been traditionally used by locals to treat a wide range of diseases (Shaari et al. 2006). Our research group has previously shown that systemic treatment of tHGA prevents ovalbumin-induced allergic airway inflammation in a murine model of allergic asthma by suppressing the synthesis of cysteinyl leukotrienes (cysLT) and Th2-related cytokines including IL-4 (Ismail et al. 2012). As mast cells are immune effector cells involved in the pathogenesis of allergic diseases by releasing cysLT and IL-4 (Chung et al. 2013; Lu et al. 2011), we propose that the anti-asthmatic properties of tHGA could be attributed to its inhibitory effect on mast cell degranulation. Therefore, the purpose of this study was to investigate the anti-allergic effects of tHGA in cellular and animal model of IgE-mediated mast cell degranulation.

RBL-2H3 cell line was chosen as the cellular model of mast cell degranulation in this study. This cell line is commonly used to study mast cell degranulation which is characterized by the release of preformed and newly synthesized mediators of immune allergic response, following crosslinking of their IgE-bound FcεRI by multivalent allergens (Ng et al. 2012; Tang et al. 2012; Passante and Frankish 2009). For the *in vivo* work, the anti-allergic activity of tHGA via IgE-mediated mast cell degranulation was investigated using the IgE-mediated passive systemic anaphylaxis model in Sprague Dawley rats.

2. Materials and methods

2.1. Compound synthesis and preparation

The compound, tHGA, was synthesized according to previously described method (Ismail et al. 2012). For the *in vitro* study, tHGA stock solution (20 mM) was prepared by dissolving tHGA in 100% DMSO and the final concentration of DMSO was adjusted to 0.1% (v/v) in tyrode's buffer (pH 7.4, NaCl 7.89 g, MgCl₂ 0.2033 g, KCl 0.373 g, CaCl₂ 0.199 g, glucose 1.0 g, HEPES 4.77 g, dissolved in 1000 mL deionized water). For the *in vivo* study, tHGA was freshly prepared by dissolving tHGA in a vehicle solution containing 5% DMSO, 5% Tween-20 and 90% distilled water. Prior to experiments, the activity of several batches of synthetic tHGA were tested *in vitro* using the β-hexosaminidase release assay. There was minimal variation between different batches of tHGA (<3% variation).

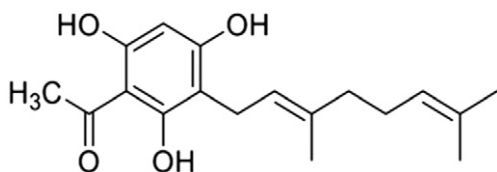


Fig. 1. The molecular structure of 2,4,6-trihydroxy-3-geranyl acetophenone (tHGA).

2.2. Antibodies and reagents

Mouse monoclonal anti-dinitrophenyl IgE (DNP-IgE) and 4-Nitrophenyl N-acetyl-β-D-glucosaminide (PNAG) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). WST-1 cell proliferation assay kit was purchased from Merck Millipore Co. (Temecula, CA, USA). DNP-conjugated bovine serum albumin (DNP-BSA) was purchased from Calbiochem (San Diego, CA, USA). The enzyme immunoassay analysis (EIA) kits for histamine, prostaglandins D₂ (PGD₂) and leukotrienes C₄ (LTC₄) were purchased from Cayman Chemicals (Ann Arbor, MI, USA). The enzyme-linked immunosorbent assay (ELISA) kits for interleukin-4 (IL-4) and tumour necrosis factor-α (TNF-α) were purchased from R&D systems (Minneapolis, MN, USA). Toluidine blue powder and ketotifen fumarate were purchased from MP Biomedicals (Santa Ana, California, USA). Alexa Fluor® 488 Phalloidin and Eagle's minimum essential medium (EMEM) were purchased from Life Technologies Inc. (Waltham, MA, USA). Percoll Plus was purchased from GE Healthcare Life Sciences (Little Chalfont, Bucks, UK). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Penicillin-Streptomycin solution was purchased from Biowest (Riverside, MO, USA).

2.3. Cell culture

RBL-2H3 cell line (rat basophilic leukaemia cell line) was purchased from the American Type Culture Collection (ATCC) and cultured in EMEM medium containing 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. RBL-2H3 cells were incubated at 37 °C in a 5% CO₂ humidified incubator and subcultured to new T25 tissue culture flask (3 × 10⁵ cells/flask) or used for assays when the confluency of cells reached 80%. Only RBL-2H3 cells of passage number ranging from 6 to 11 were used throughout this study.

2.4. Cytotoxicity assay

The cytotoxicity of various concentrations of tHGA on RBL-2H3 cells was determined using the WST-1 cell proliferation assay kit. The assay was performed according to the manufacturer's instructions. Briefly, RBL-2H3 cells (2.5 × 10⁵ cells/mL) were seeded in 96-well plate and sensitized with or without 1 µg/mL of DNP-IgE for 24 h. On the next day, the cells were treated with increasing concentrations of tHGA (1.25–40 µM) with or without 1 µg/mL of DNP-BSA for 24 h. The cells were then incubated with working solution of WST (10 µL) for 4 h and the readings were measured at 420 nm using a microplate reader (Molecular Devices Versa Max, Sunnyvale, CA, USA).

2.5. Toluidine blue staining

To visualize the release of granules by RBL-2H3 cells during degranulation, RBL-2H3 cells (5 × 10⁵ cells/mL) were seeded in 24-well plate sensitized with 1 µg/mL of DNP-IgE for 24 h. Next, the sensitized cells were rinsed with tyrode's buffer before pretreatment with tHGA (1.25, 5, 20 µM) for 20 min. The pretreated cells were then challenged with 1 µg/mL of DNP-BSA for 1 h. The cells were then stained with toluidine blue (0.1% w/v in 1% NaCl solution, pH 2.5) as previously described (Tang et al. 2012) with slight modifications. Briefly, cells were washed with PBS and then fixed with 300 µL of absolute methanol for 30 min at room temperature (RT). The fixative solution was then discarded and fixed cells were stained with 300 µL of toluidine blue dye (0.1% w/v in 1% NaCl solution, pH 2.5) for 20 min. Images of the stained cells were then examined and captured using an inverted microscope (Leica DM IL LED). Throughout this study, the internal drug control used was ketotifen fumarate, which is a clinically used anti-histamine drug for the treatment of general allergy by inhibiting mast cell degranulation (de Souza Santos et al. 2013).

2.6. F-actin microfilament staining

To visualize the microfilament cytoskeleton during membrane ruffling, RBL-2H3 cells (3×10^5 cells/mL) were seeded in 4-well chamber slide and sensitized with 1 $\mu\text{g/mL}$ of DNP-IgE for 24 h. On the next day, the sensitized cells were rinsed with tyrode's buffer before pre-treatment with tHGA (1.25, 5, 20 μM) for 20 min. The pretreated cells were then challenged with 1 $\mu\text{g/mL}$ of DNP-BSA for 1 h. At the end of DNP-BSA challenge, the cells were stained with Alexa Fluor-488 phalloidin as previously described (Chong et al., 2016) with slight modifications. Briefly, the treated cells were washed with PBS and then fixed with 4% paraformaldehyde for 10 min at RT. The fixed cells were then permeabilized with 0.2% Triton X-100 for 5 min followed by incubation with 1% BSA for 20 min at RT to reduce nonspecific binding. Afterwards, the cells were stained with 5 U/mL of Alexa Fluor® 488-labeled phalloidin diluted in 1% BSA (1:200) for 20 min at 37 °C in the dark. Finally, the slides were mounted using MOWIOL 4–88 mounting agent. The images were then examined and captured using a fluorescence imaging system (Leica DM2500) equipped with excitation (490 nm) and emission (520 nm) filters.

2.7. β -hexosaminidase and histamine release assay

To determine the effect of tHGA on the release of preformed mediators during mast cell degranulation, the release of β -hexosaminidase and histamine upon treatment with tHGA was examined. RBL-2H3 cells (2.5×10^5 cells/mL) were seeded in 96-well plate and sensitized with 1 $\mu\text{g/mL}$ of DNP-IgE for 24 h. On the next day, the sensitized cells were washed with tyrode's buffer before pretreatment with tHGA (1.25, 5, 20 μM) for 20 min. The pretreated cells were then challenged with 1 $\mu\text{g/mL}$ of DNP-BSA for 1 h. At the end of DNP-BSA challenge, the culture supernatant was transferred and centrifuged (17,000 g, 10 min) at 4 °C. The level of histamine in the culture supernatant was measured using the Histamine EIA kit according to the manufacturer's instructions.

Measurement of the activity of β -hexosaminidase released from the cells was carried out as previously described (Son et al. 2005) with slight modifications. Briefly, the culture supernatant from each group was transferred and centrifuged (17,000 g, 10 min) at 4 °C. Triton X-100 solution (1%) was added to lyse the cells and release intracellular β -hexosaminidase. Culture supernatant (50 μL) and cell lysate (50 μL) were transferred into separate wells in a non-tissue culture treated 96-well plate and mixed with 100 μL of substrate solution (1 mM p-nitrophenyl N-acetyl- β -D-glucosamine in 0.08 M citrate buffer, pH 4.5) respectively before 1 h incubation at 37 °C. The reaction was terminated by adding 50 μL of stop solution (0.4 M glycine, pH 10) to each well. The absorbance was recorded at 405 nm with an automated microplate reader (Molecular Devices Versa Max, Sunnyvale, CA, USA). The percentage of degranulation was calculated with the following formula:

$$\% \text{Degranulation} = (\text{OD supernatant}) / (\text{OD supernatant} + \text{OD Triton X}) \times 100\%$$

2.8. Analysis of TNF- α , IL-4, PGD₂ and LTC₄

To determine the effect of tHGA on the release of *de novo* mediators, RBL-2H3 cells (2×10^6 cells/mL) were seeded in 24-well plate and sensitized with 1 $\mu\text{g/mL}$ of DNP-IgE for 24 h. On the next day, the sensitized cells were rinsed with tyrode's buffer before pre-treatment with tHGA (1.25, 5, 20 μM) for 20 min. The pre-treated cells were then challenged with 1 $\mu\text{g/mL}$ of DNP-BSA for 6 h. At the end of DNP-BSA challenge, the culture supernatant was transferred and centrifuged (17,000 g, 10 min) at 4 °C. The levels of PGD₂ and LTC₄ were measured using PGD₂ and LTC₄

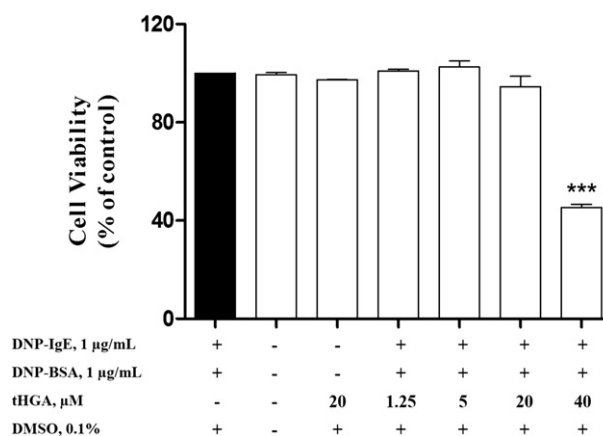


Fig. 2. The cytotoxic effects of tHGA on RBL-2H3 cells. The IgE-sensitized and challenged cells were incubated with increasing concentrations of tHGA (0–40 μM) for 24 h. The cytotoxicity of the treatments was determined by WST-1 cell proliferation assay. The results are expressed as the mean $\pm$ S.E.M values of three independent experiments. *** $p < 0.005$ as compared to the non-treated IgE-antigen complex-stimulated cells (black bar).

EIA kits, whereas the levels of IL-4 and TNF- α were measured using IL-4 and TNF- α ELISA assay kits according to the manufacturer's instructions.

2.9. Animals

Healthy male adult albino Sprague Dawley rats (aged 6–8 weeks old) were acclimatized in cages with the surrounding temperature of 24 ± 1 °C and a relative humidity of 55% and 12 h/12 h (light/dark) cycle for 7 days prior to the experiments. The rats were fed with standard laboratory chow and water *ad libitum*. The rats were divided into 7 groups as follow:

1. Sham group: sensitized and challenged with saline, no treatment.
2. IgE-BSA complex-stimulated group: sensitized with DNP-IgE and challenged with DNP-BSA, no treatment.
3. Vehicle control group: sensitized with DNP-IgE and challenged with DNP-BSA, pretreated with vehicle solution.
4. Drug control group: sensitized with DNP-IgE and challenged with DNP-BSA, pretreated with 1 mg/kg ketotifen fumarate.
5. tHGA-pretreated IgE-BSA complex-stimulated group 1: sensitized with DNP-IgE and challenged with DNP-BSA, pretreated with 20 mg/kg tHGA.
6. tHGA-pretreated IgE-BSA complex-stimulated group 2: sensitized with DNP-IgE and challenged with DNP-BSA, pretreated with 40 mg/kg tHGA.
7. tHGA-pretreated IgE-BSA complex-stimulated group 3: sensitized with DNP-IgE and challenged with DNP-BSA, pretreated with 80 mg/kg tHGA.

All animal experiments were performed in accordance with the institutional guidelines (The Institutional Animal Care and Use Committee, IACUC) of Universiti Putra Malaysia (UPM/IACUC/AUP-R071/2015).

2.10. Induction of IgE-mediated passive systemic anaphylaxis (PSA) in Sprague Dawley rats

The induction of IgE-mediated PSA was carried out as previously described (Lu et al. 2011) with slight modifications. Briefly, Sprague Dawley rats were sensitized by intraperitoneal injection of 10 $\mu\text{g/mL}$ DNP-IgE in saline or injected with saline alone. After 24 h, the rats were challenged with 1 mg/mL of DNP-BSA in saline intraperitoneally, after oral administration of tHGA (20, 40 and 80 mg/kg) for 1 h. Blood was collected by cardiac puncture 1 h after antigen challenge. Serum

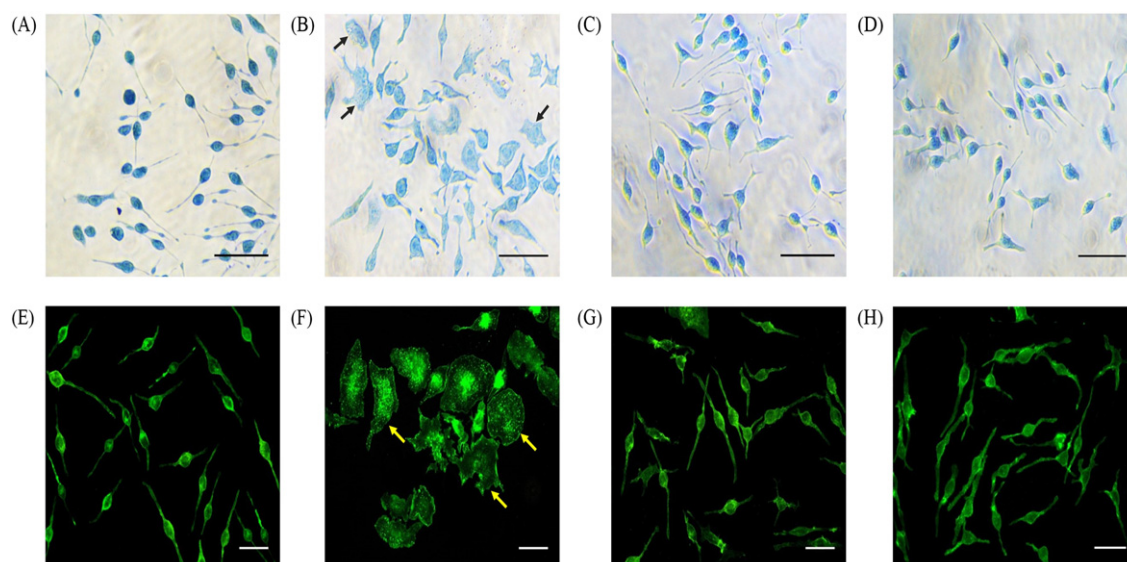


Fig. 3. The effects of tHGA on (A–D) toluidine blue staining and (E–H) F-actin staining in IgE-antigen complex-stimulated RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were preincubated in the presence or absence of tHGA (20 μ M) or ketotifen fumarate (300 μ M) for 20 min, and then stimulated with DNP-BSA (1 μ g/mL) for 1 h. Cells were then fixed for light or fluorescence microscopy examination of toluidine blue and F-actin respectively. A and E: Normal RBL-2H3 cells, B and F: IgE-antigen complex-stimulated RBL-2H3 cells, C and G: tHGA-pre-treated IgE-antigen complex-stimulated RBL-2H3 cells, D and H: ketotifen fumarate-pre-treated IgE-antigen complex-stimulated RBL-2H3 cells. Arrows in B indicate the presence of multiple granules extruded from the cells. Arrows in F indicate that the cells morphology became irregular and rounded due to disassembly of the F-actin cytoskeleton. Scale bar = 50 μ m.

histamine, IL-4, TNF- α , PGD₂ and LTC₄ concentrations were determined using respective assay kits according to the manufacturer's instructions.

2.11. Isolation and purification of peritoneal mast cells (PMC)

Mast cells were isolated from peritoneal lavage as previously described (Mousli et al. 1989) with slight modifications. In brief, rats were put under anaesthesia prior to injecting 20 mL of phosphate buffered saline (PBS) solution (6.7 mM Na₂HPO₄, 6.7 mM KH₂PO₄, 137 mM NaCl, 2.7 mM KCl, 0.8 mM CaCl₂, adjusted to pH 7.2) into the peritoneal cavity. The abdomen was gently massaged for about 2 min, carefully opened and the fluid containing peritoneal cells was aspirated with a Pasteur pipette. Peritoneal mast cells were then purified by using Percoll solution, according to previous study (Kovarova 2013). PMCs were separated from other cells through sedimentation at the bottom layer, whereas other cells formed a layer on top of the gradient and could easily be discarded by aspiration. Purified PMCs (1 $\times$ 10⁶ cells/mL) were

then stained with toluidine blue (0.1% w/v in 1% NaCl solution, pH 2.5) and quantified using a hemocytometer under an inverted microscope (Leica DM IL LED). The purity of mast cells was >93%. The viability of PMCs was determined by trypan blue dye exclusion test with a cell viability of $\geq$ 95%.

2.12. Light microscopy (toluidine blue staining)

Toluidine blue staining of PMCs was carried out as previously described (Penissi et al. 2009) with slight modifications. Briefly, PMCs (2 $\times$ 10⁵ cells/mL) were cytospun on a clean glass slide at 800 rpm for 4 min. The cells were fixed in 100% methanol for 30 min followed by staining with toluidine blue (0.1% w/v in 1% NaCl solution, pH 2.5) for 45 min. Stained PMCs were then viewed under an inverted microscope (Leica DM IL LED) to examine the cell morphology.

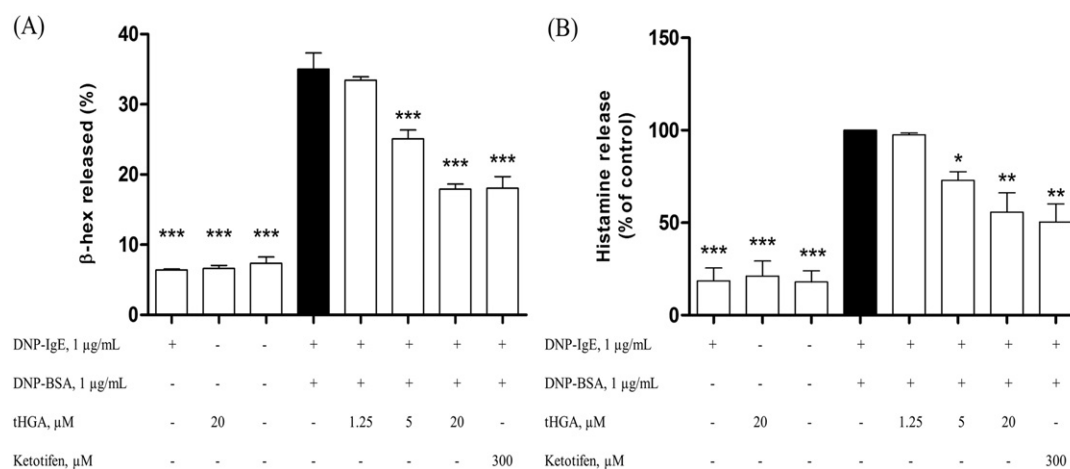


Fig. 4. The effects of tHGA on the levels of (A) β -hexosaminidase and (B) histamine release in IgE-antigen complex-stimulated RBL-2H3 cells. RBL-2H3 cells were preincubated with or without tHGA (1.25, 5, 20 μ M) for 20 min followed by the addition of DNP-BSA (1 μ g/mL) for 1 h to induce degranulation. The percentage of released β -hexosaminidase was calculated against the total amount of β -hexosaminidase whereas released histamine was calculated according to the EIA kit's protocol. Results are expressed as the mean $\pm$ S.E.M. of three independent experiments. * P < 0.05, ** P < 0.01 and *** P < 0.005 versus IgE-antigen complex-stimulated RBL-2H3 group (black bars).

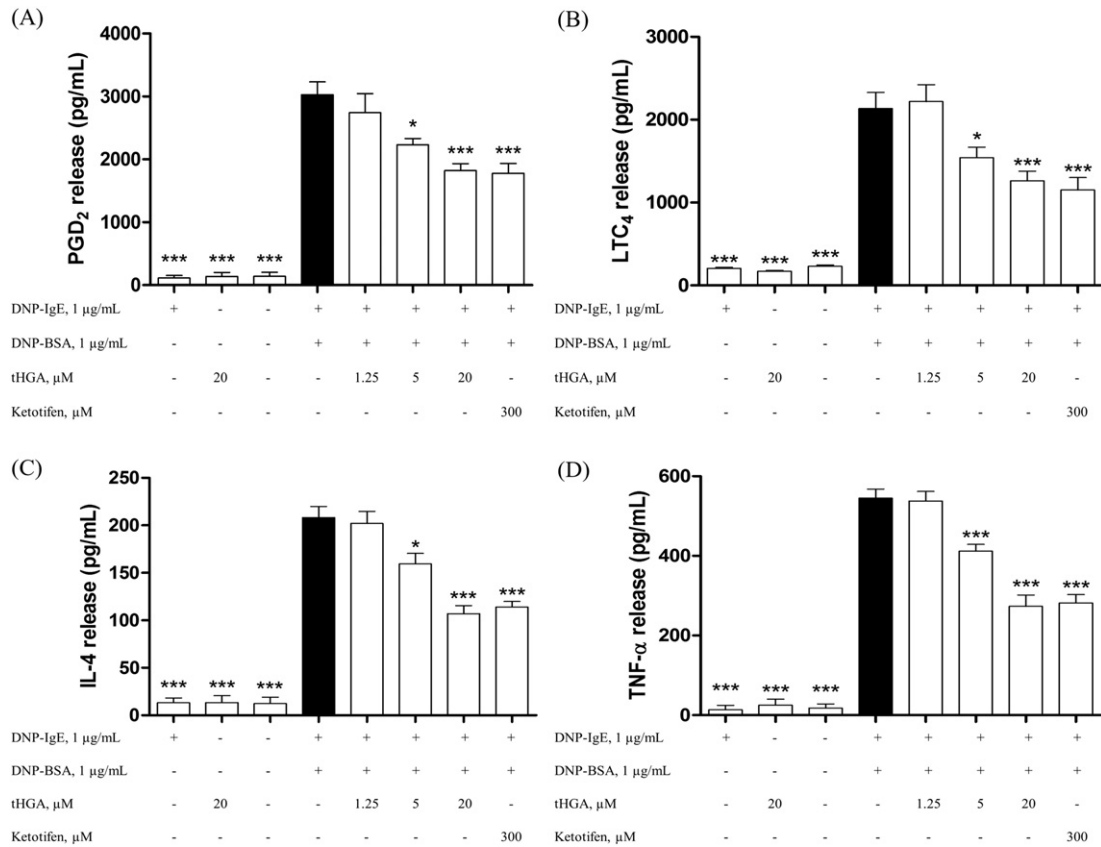


Fig. 5. The effects of tHGA on the levels of (A) PGD₂, (B) LTC₄, (C) IL-4 and (D) TNF-α released by IgE-antigen complex-stimulated RBL-2H3 cells. IgE-sensitized RBL-2H3 cells were pre-treated with tHGA (1.25, 5 and 20 μM) for 20 min, and then stimulated with DNP-BSA (1 μg/mL) for 6 h. The levels of lipid mediators and cytokines were determined using the EIA and ELISA kits according to the manufacturer's instructions. Results are expressed as the mean ± S.E. M values of three independent experiments. **P* < 0.05 and ****P* < 0.005 versus IgE-antigen complex-stimulated RBL-2H3 group (black bars).

2.13. Transmission electron microscopy (TEM)

TEM was carried out as previously described (Penissi et al. 2009) with slight modifications. Briefly, PMCs were fixed in 4% glutaraldehyde for 5 h at 4 °C. After fixation, PMCs were rinsed in 0.1 M sodium cacodylate buffer followed by addition of horse serum to clot the cells. The serum clot containing fixed PMCs was postfixed in 1% Osmium Tetroxide (OsO₄) for 2 h at 4 °C followed by a series of acetone dehydration. Then, the serum clots were embedded in a beam capsule. Semithin sections (1 μm) were cut with an automatic ultramicrotome (Leica Ultracut R, Austria) and stained with filtered 1% toluidine blue. Ultrathin sections (80 nm) were cut with diamond knives, stained with uranyl acetate and lead citrate, mounted on grids, and examined under a transmission electron microscope (Zeiss Libra® 120, Germany).

2.14. Scanning electron microscopy (SEM)

SEM was carried out as previously described (Penissi et al. 2009) with slight modifications. Briefly, PMCs were fixed in 4% glutaraldehyde for 5 h at 4 °C, rinsed in 0.1 M cacodylate buffer, postfixed in 1% OsO₄ for 2 h at 4 °C, and dehydrated through a series of acetone. Fixed PMCs were then subjected to CO₂ critical-point drying. Specimens were mounted on stubs and coated with gold in a sputtering device. Observations and micrographs were carried out under a scanning electron microscope (JEOL JSM 6400, Germany).

2.15. Statistical analysis

All experiments described were performed three times. The results were expressed as means ± S.E.M. Statistical analyses were performed using SPSS 19.0 (Chicago, IL, USA). One-way analysis of variance (ANOVA) followed by Tukey's test was used to compare the results from different treatment groups. *P* values of <0.05 were considered statistically significant.

3. Results

3.1. tHGA does not reduce RBL-2H3 cell viability at 20 μM and below

To ensure that the inhibitory effects of tHGA on mast cell degranulation was not due to its cytotoxicity on RBL-2H3 cells, the cytotoxic effect of tHGA on RBL-2H3 cells was examined using the WST-1 cell proliferation assay. Fig. 2 indicates that tHGA was not cytotoxic to RBL-2H3 cells at the concentration of 20 μM and below, in the presence or absence of DNP-IgE/DNP-BSA. In order to confirm the highest non-cytotoxic concentration of tHGA on RBL-2H3 cells, another round of cytotoxicity screening of tHGA's concentrations in a range of 10–40 μM was performed. The results show that tHGA significantly reduced RBL-2H3 cells viability at 25 μM (**P* < 0.05), 30 μM (***P* < 0.01), as well as 35 μM and 40 μM (****P* < 0.005) (data not shown). Thus, three non-cytotoxic concentrations of tHGA (1.25, 5, and 20 μM) were used for subsequent *in vitro* experiments in this study.

3.2. tHGA prevents the release of granules and membrane ruffling in IgE-antigen complex-stimulated RBL-2H3 cells

DNP-BSA is known to induce degranulation in IgE-sensitized RBL-2H3 cells (Wang et al. 2012). Mast cells can be easily identified by the presence of a cytoplasm dominated by distinctive secretory granules which stain metachromatically. Therefore, the effect of tHGA on the release of granules in IgE-BSA complex-stimulated RBL-2H3 cells was examined by toluidine blue staining. As shown in Fig. 3A, normal RBL-2H3 cells showed pleomorphic or spindle-shaped morphology with closely packed secretory granules in the cytoplasm. By contrast, the shape of IgE-antigen complex-stimulated RBL-2H3 cells were irregular with less staining of secretory granules in the cytoplasm due to extensive degranulation processes (Fig. 3B). This was also indicated by the presence of multiple granules extruded from the cells. Fig. 3C shows that 20 μ M tHGA-treated IgE-sensitized RBL-2H3 cells remained in regular spindle-shaped with closely packed secretory granules in the cytoplasm.

Membrane ruffling of activated mast cells is an early event that occurs following antigen challenge in order to facilitate the exocytosis of granules into the surrounding environment (Dr  ber et al. 2012). Hence, the effect of tHGA on membrane ruffling in IgE-BSA complex-stimulated RBL-2H3 cells was examined through staining of F-actin cytoskeleton with Alexa Fluor®-488. As shown in Fig. 3E, normal RBL-2H3 cells exhibited spindle shape with uniform distribution of F-actin at the cell periphery. However, after 1 h challenge of DNP-BSA, the cells became irregular, spreaded out and rounded due to disassembly of the F-actin cytoskeleton (Fig. 3F). Conversely, RBL-2H3 cells pre-treated with 20 μ M tHGA (Fig. 3G) exhibited regular spindle shape with uniform distribution of F-actin, indicating that F actin reorganization was inhibited and the polymerized F-actin cytoskeleton remained intact.

3.3. tHGA inhibits β -hexosaminidase and histamine release by IgE-antigen complex-stimulated RBL-2H3 cells

In this study, the degree of RBL-2H3 degranulation was determined by measuring the release of β -hexosaminidase and histamine. β -hexosaminidase and histamine are two important markers of mast cell degranulation. These two preformed mediators are released during the early stage of mast cell activation (Han et al. 2011). As shown in Fig. 4A and B, DNP-BSA challenge for 1 h significantly increased the release of both β -hexosaminidase and histamine by approximately 5.6 and 4.7-fold, respectively in IgE-antigen complex-stimulated cells. Conversely, pre-treatment with tHGA significantly suppressed degranulation in a concentration-dependent manner, as indicated by a decrease in the levels of β -hexosaminidase and histamine released. tHGA (20 μ M) significantly decreased the release of both β -hexosaminidase and histamine by approximately 49% and 44.3%, respectively compared to the IgE-antigen complex-stimulated RBL-2H3 cells. However, no significant inhibition was observed at 1.25 μ M of tHGA.

3.4. tHGA inhibits the release of *de novo* mediators by IgE-antigen complex-stimulated RBL-2H3 cells

Prolonged exposure of RBL-2H3 cells to antigen will lead to the late phase reaction which involves the production of *de novo* inflammatory mediators including lipid mediators and pro-inflammatory cytokines (Theoharides and Kalogeromitros 2006; Wang et al. 2012). Thus, the inhibitory effects of tHGA on the production of lipid mediators such as PGD₂ and LTC₄ and pro-inflammatory cytokines such as IL-4 and TNF- α were examined. As shown in Figs. 5A–D, DNP-BSA challenge for 6 h significantly increased the release of PGD₂, LTC₄, IL-4 and TNF- α by approximately 16–20-fold in IgE-antigen complex-stimulated cells. However, the levels of these *de novo* mediators released by IgE-antigen complex-stimulated RBL-2H3 cells were significantly reduced in the presence of tHGA. Pre-treatment of 20 μ M tHGA significantly reduced the release of all *de novo* mediators by approximately 40–50% in

comparison to the IgE-antigen complex-stimulated cells. However, no significant inhibition was observed at 1.25 μ M of tHGA.

3.5. tHGA reduces serum histamine, PGD₂, LTC₄, IL-4 and TNF- α levels in IgE-antigen complex-stimulated anaphylactic rats

The mast cell stabilizing effect of tHGA was further validated *in vivo* using the animal model of IgE-mediated passive systemic anaphylaxis (PSA) in rats. As tHGA has been reported to attenuate airway remodeling in a murine model of chronic asthma at 20, 40 and 80 mg/kg when administered orally (Lee et al. 2017), these doses were chosen for *in vivo* tHGA treatment in this study. Fig. 6 summarizes the effects of orally administrated tHGA on the levels of serum histamine, PGD₂, LTC₄, IL-4 and TNF- α in IgE-antigen complex-stimulated anaphylactic rats. According to Figs. 6A–E, DNP-BSA significantly induced the release of all selected key mediators by approximately 3.4–5.0-fold. On the other hand, tHGA significantly attenuated mast cell-dependent PSA reaction in a dose-dependent manner, evidenced by a decrease in the levels of serum histamine, PGD₂, LTC₄, IL-4 and TNF- α in anaphylactic rats pre-treated with tHGA. Notably, 40 and 80 mg/kg of tHGA were able to significantly decrease the levels of all mediators released by approximately 33–44% and 50–60%, respectively as compared to IgE-antigen complex-stimulated group.

3.6. tHGA prevents the extrusion of secretory granules in peritoneal mast cells from IgE-antigen complex-stimulated anaphylactic rats

PMCs were isolated from the peritoneal cavity of rats and identified by the presence of distinctive secretory granules which stained metachromatically with toluidine blue (representative figures of isolated PMCs are shown in Fig. 7). Fig. 7A shows that mast cells isolated from the sham group contain closely packed secretory granules within the cytoplasm. Fig. 7B shows that when challenged with DNP-BSA, mast cells underwent extensive degranulation with extrusion of multiple granules. Notably, pre-treatment with 80 mg/kg of tHGA (Fig. 7E) and ketotifen fumarate (Fig. 7F) show no obvious extrusion of granules from mast cells, with closely packed secretory granules within the cytoplasm, similar to that of the sham group. Quantitation of the percentage of cells that showed extrusion of secretory granules (Fig. 7G) shows that DNP-BSA significantly induced the granules extrusion by 10-fold. In contrast, pretreatment with 40 and 80 mg/kg of tHGA significantly and dose-dependently attenuated the extrusion of secretory granules in PMCs from IgE-antigen complex-stimulated anaphylactic rats by 37% and 56%, respectively as compared to IgE-antigen complex-stimulated group.

Figs. 8 shows the representative images of PMCs observed under scanning electron microscope (SEM). Normal mast cells from the sham group were characterized by round shape with smooth cell surface (Fig. 8A). When challenged with DNP-BSA, the shape of mast cells became irregular. The rupture-like cell membrane indicated the release of granules content into the surrounding environment (Fig. 8B). Fig. 8C to 8E shows that mast cells from anaphylactic rats pre-treated with tHGA. Notably, 80 mg/kg of tHGA (Fig. 8E) exhibited similar morphology and smooth cell surface to that of the sham group (Fig. 8A). Mast cells from rats pre-treated with 1 mg/kg ketotifen fumarate (Fig. 8F) and 40 mg/kg of tHGA (Fig. 8D) appeared to retain the normal round shape, however the cell surface did not appear as smooth as the normal mast cells surface (Fig. 8A).

Fig. 9 shows the representative images of PMCs observed under transmission electron microscope (TEM). As shown in Fig. 9A, numerous secretory granules were present and distributed evenly throughout the cytoplasm in normal mast cells isolated from the sham group. Almost all granules exhibited round or oval profiles and appeared homogeneously dense. Mast cells from the IgE-antigen complex-stimulated group displayed obvious morphological changes with evidence of enhanced granule release by exocytosis compared to the sham

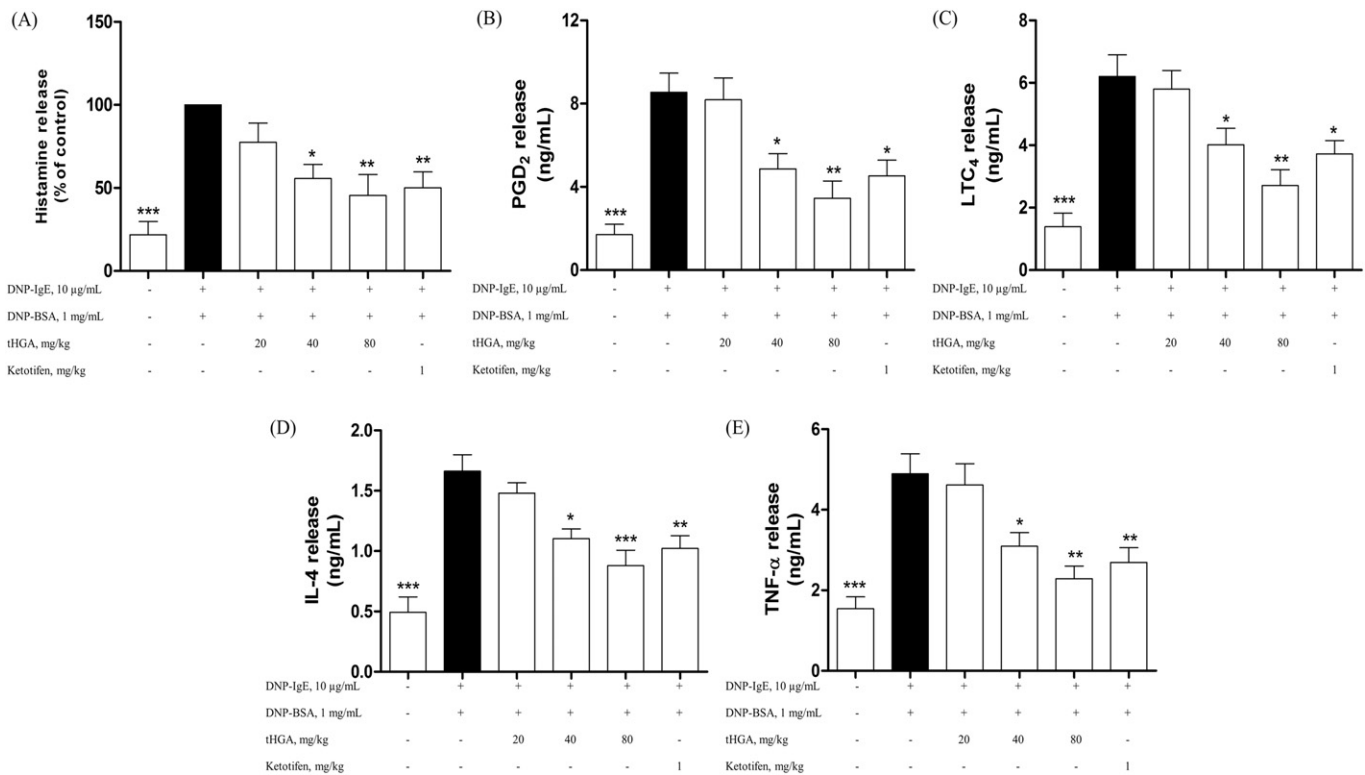


Fig. 6. The effects of tHGA on serum levels of (A) histamine, (B) PGD₂, (C) LTC₄, (D) IL-4 and (E) TNF-α in IgE-mediated PSA reactions in rats. Male Sprague Dawley adult rats were injected intraperitoneally with 10 μg/mL of anti-DNP. After 24 h, the rats were intraperitoneally injected with 1 mg/mL of DNP-BSA. Rats were given oral treatment of tHGA or ketotifen fumarate 1 h before DNP-BSA challenge. The levels of histamine, PGD₂, LTC₄, IL-4 and TNF-α were determined using EIA and ELISA kits, respectively according to the manufacturer's instructions. Results are expressed as the mean ± S.E.M. (n = 6 rats). *P < 0.05, **P < 0.01 and ***P < 0.005 versus IgE-antigen complex-stimulated rats (black bars).

group (Fig. 9B). These stimulated mast cells showed irregular secretory granules exhibiting different degrees of electron densities. Furthermore, the dense granular population within these mast cells appeared to be less and the granules were more separated compared to those from the sham group. Some granules were surrounded by dilated perigranular and electronlucent spaces whereas some of these spaces appeared fused, forming numerous cavities. Figs. 9C–E show mast cells from anaphylactic rats pre-treated with tHGA whereas Fig. 9F shows mast cells from anaphylactic rats pre-treated with ketotifen fumarate. Interestingly, 40 (Fig. 9D) and 80 mg/kg of tHGA (Fig. 9E) demonstrated minimal degranulation with no obvious morphological changes and enhanced granule release by exocytosis. Of note, the morphological characteristics and cytoplasmic distribution of the granules from 80 mg/kg of tHGA (Fig. 9E) and 1 mg/kg of ketotifen fumarate treatment groups (Fig. 9F) were comparable to those from the sham group (Fig. 9A).

4. Discussion

tHGA is a geranyl acetophenone originally found in the young leaves of *Melicope ptelefolia*. It is also a phloroglucinol compound that contains phloroglucinols as its common monomer unit (Chong et al., 2016). tHGA has been reported to demonstrate anti-inflammatory effects through dual LOX/COX inhibition (Shaari et al., 2011). In addition, our previous findings further demonstrated the anti-asthmatic effects of tHGA in a murine model of acute allergic airway inflammation by reducing airway hyperresponsiveness, eosinophilia, goblet cell hyperplasia, inflammatory cell infiltration, cystLT and Th2 cytokines synthesis including IL-4, IL-5 and IL-13 (Ismail et al., 2012). A recent study (Lee et al., 2017) also reported that oral tHGA treatment attenuated airway hyperresponsiveness and inhibited airway remodeling in a dose-dependent fashion by reducing inflammatory cell infiltration and decreased expression of cytokines associated with airway remodeling. However, these studies have not yet established the role of mast cell in the anti-

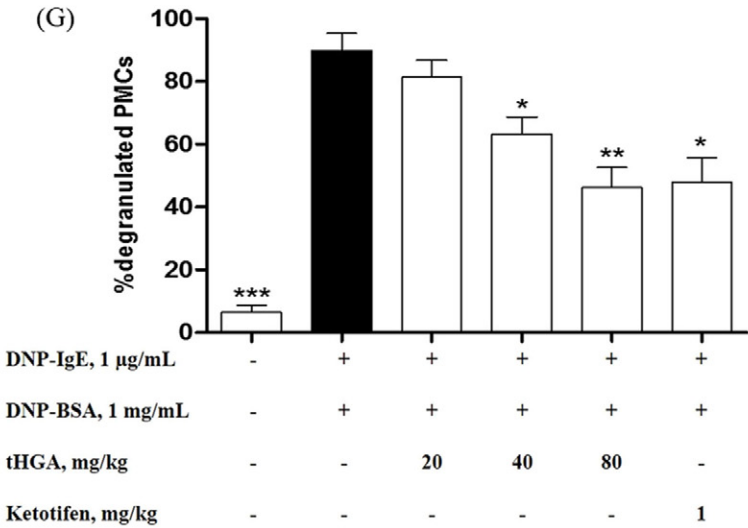
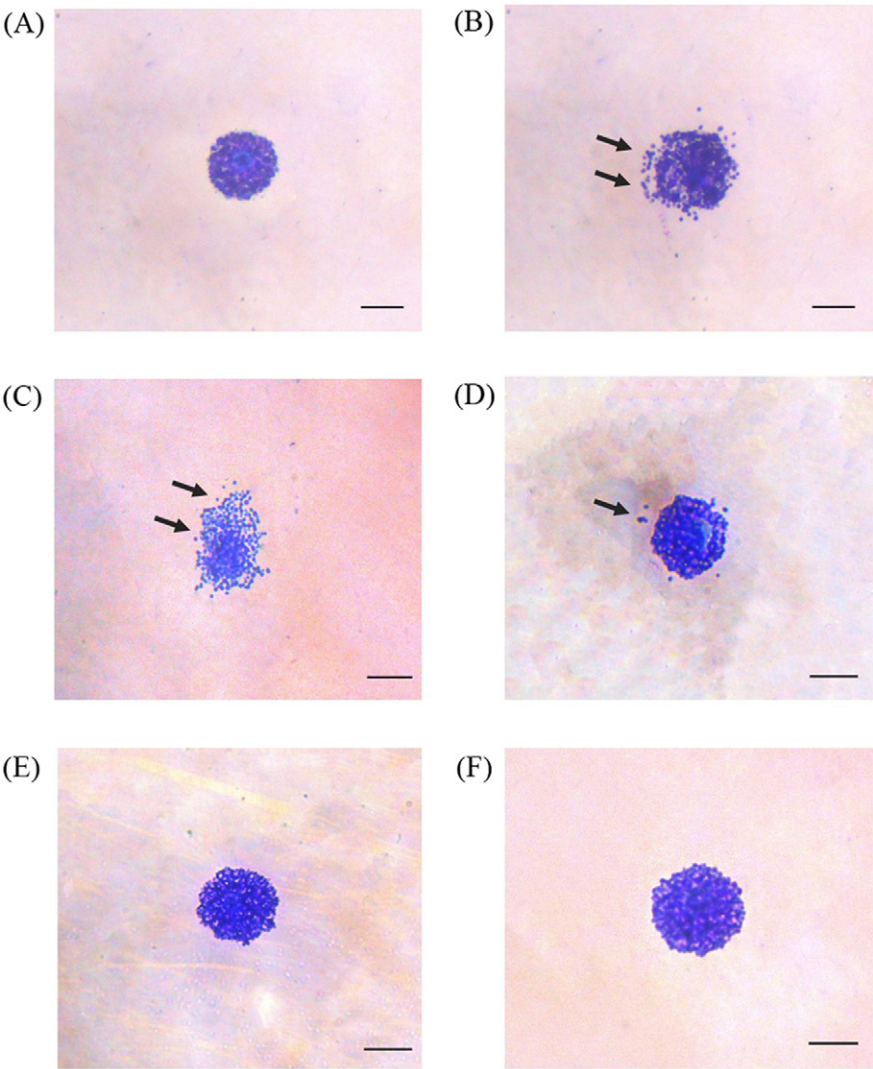
asthmatic effects of tHGA, which are considered as a key player in pathogenesis of allergic asthma (Bradding et al., 2006).

Allergic diseases including allergic asthma and anaphylaxis have been closely linked with the involvement of IgE. As mast cells highly express FcεRI receptors on their surface, they play a direct role in allergic diseases by readily forming cross-links between IgE and bivalent or multivalent antigen, resulting in the activation of mast cells (Barnes 2008; Kips 2001). Such reaction, also known as IgE-mediated mast cell degranulation, will result in the release of preformed mediators leading to various adverse events most commonly associated with allergy including increased vascular permeability, smooth muscle contraction, and mucus secretion (Stone et al., 2010). Since degranulation is a key event in IgE-mediated mast cell activation (Brown et al., 2008), the effect of tHGA on the degree of mast cell degranulation was investigated by measuring the release of preformed mediators. The results from degranulation assay indicated that tHGA attenuated the degranulation process, evidenced by the significant decrease of both β-hexosaminidase and histamine released by IgE-antigen complex-stimulated RBL-2H3 cells. A previous study demonstrated anti-allergic effects of phloroglucinol compounds on histamine release (Li et al., 2008). Therefore, it is hypothesized that mast cell stabilization properties of tHGA may be partially related to the anti-allergic properties of phloroglucinol compounds.

Activation of mast cells is highly associated with typical morphological changes of the cells (Levi-Schaffer et al., 2000). Microscopic examination of toluidine blue-stained cells confirmed that IgE-antigen complex-stimulated RBL-2H3 cells were able to preserve the cell morphology in spindle shape with less prominent granules in the cytoplasm upon pre-treatment with tHGA. Microfilaments, which play a central role in the regulation of cell morphology, are critical in both the early stages of mast cell activation and subsequent exocytosis process (Dráber et al., 2012). Mast cell exocytosis is accompanied by extensive reorganization of the actomyosin cortex and F-actin disassembly (Nishida et al., 2005). Cortical F-actin acts as a barrier to prevent the

access of granules to the plasma membrane during cell activation (Malacombe et al. 2006; Pendleton and Koffer 2001). Rearrangement of cortical F-actin allows the content inside the granules to be secreted out of the cells. In this study, fluorescence microscopic examination showed that IgE-antigen complex-stimulated RBL-2H3 cells had

decreased F-actin content with irregular cell morphology due to increased F-actin disassembly. The transformation of cell surface from a finely microvillus structure to a highly-spread surface structure upon DNP-BSA challenge was also evident. Thus, the mast cell-stabilizing properties of tHGA may be attributed to its ability to counteract the



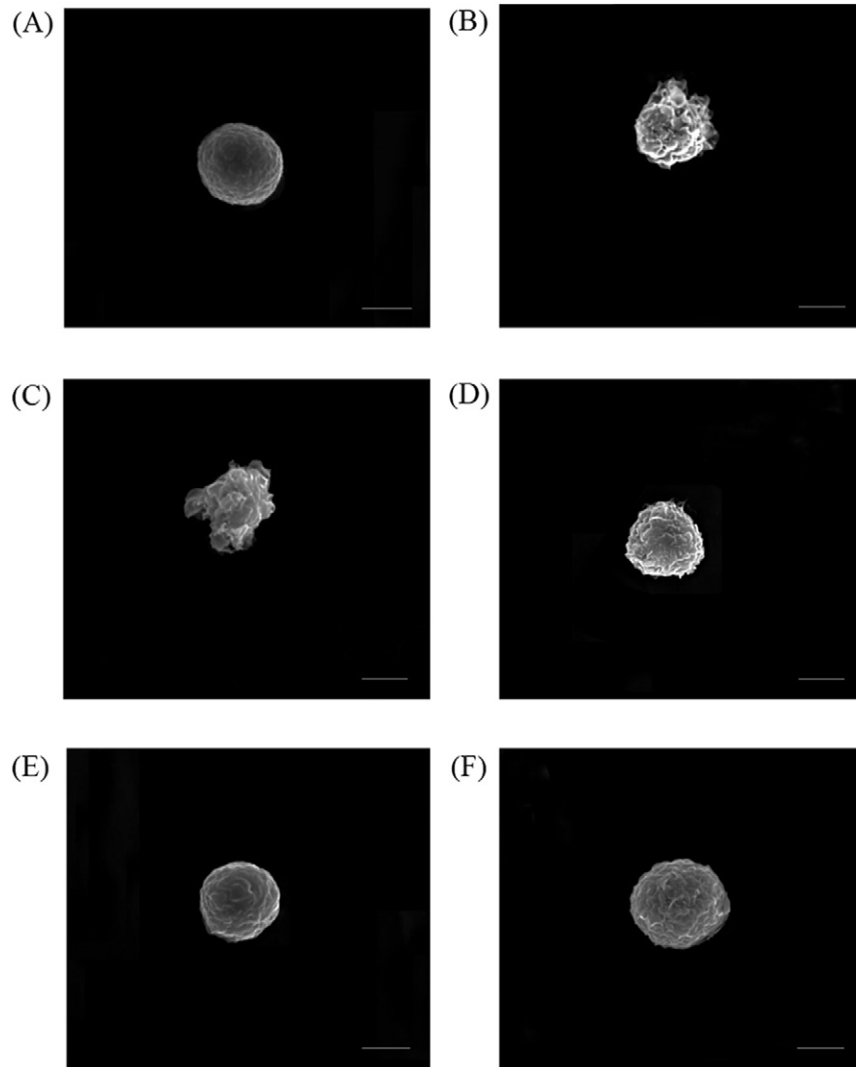


Fig. 8. Representative scanning electron-microscopic images of non-stimulated and IgE-antigen complex-stimulated rat peritoneal mast cells. Male Sprague Dawley adult rats were orally treated with tHGA or ketotifen fumarate for 1 h before challenged with 1 mg/mL BSA. After 1 h, the rats were sacrificed, peritoneal mast cells were isolated and purified using Percoll. Purified mast cells were fixed and prepared as described in methods for electron microscopy examination (scale bar = 50 μ m). A: sham group, B: IgE-antigen complex-stimulated group, C: IgE-antigen complex-stimulated group pre-treated with 20 mg/kg of tHGA, D: IgE-antigen complex-stimulated group pre-treated with 40 mg/kg of tHGA, E: IgE-antigen complex-stimulated group pre-treated with 80 mg/kg of tHGA, F: IgE-antigen complex-stimulated group pre-treated with 1 mg/kg of ketotifen fumarate.

plasma membrane deformation in degranulating mast cells by preventing the reorganization of actin cytoskeleton. It is believed that the mast cell-stabilizing properties of tHGA is very similar to that of ketotifen fumarate, which is the drug control of this study and a clinically used anti-allergic drug which acts as a mast cell stabilizer by counteracting plasma membrane deformation in degranulating mast cells (Baba et al. 2016).

In addition to the acute immediate events, prolonged activation by antigens causes mast cells to synthesize and release proinflammatory cytokines such as IL-4 and TNF- α and lipid mediators such as PGD₂ and LTC₄ (Chung et al. 2013; Giudice et al. 2007). Consequently, these events lead to leukocyte infiltration and the initiation of acquired

immune response, followed by a chronic phase that includes persistent inflammation, tissue remodeling and fibrosis (Overed-Sayer et al. 2014; Rao and Brown 2008). The late phase reaction involves the synthesis and release of *de novo* mediators within 4 to 6 h after the onset of early phase reaction (Yoo et al. 2013; Ng et al. 2012). As discussed earlier, tHGA was able to attenuate the release of preformed mediators in the early phase reaction of mast cell activation. Interestingly, tHGA also significantly inhibited the production and release of *de novo* lipid mediators such as PGD₂ and LTC₄ in the late phase reaction. Apart from the *de novo* lipid mediators, in response to antigen challenge, activated mast cells also produce and secrete TNF- α and IL-4, which are *de novo* pro-inflammatory cytokines that play critical roles in late phase

Fig. 7. Representative light-microscopic images of non-stimulated and IgE-antigen complex-stimulated rat peritoneal mast cells stained with toluidine blue. Male Sprague Dawley adult rats were orally treated with tHGA or ketotifen fumarate for 1 h before challenged with 1 μ g/mL BSA. After 1 h, the rats were sacrificed, peritoneal mast cells were isolated and purified using Percoll. Purified mast cells were fixed, stained with toluidine blue and examined by light microscopy (scale bar = 50 μ m). A: sham group. The cytoplasm was dominated by closely packed secretory granules. B: IgE-antigen complex-stimulated group. Extensive degranulation with the presence of multiple granules extruded from the cells (indicated by arrows). C: IgE-antigen complex-stimulated group pre-treated with 20 mg/kg of tHGA. D: IgE-antigen complex-stimulated group pre-treated with 40 mg/kg of tHGA. E: IgE-antigen complex-stimulated group pre-treated with 80 mg/kg of tHGA. F: IgE-antigen complex-stimulated group pre-treated with 1 mg/kg of ketotifen fumarate. The cell morphology of IgE-antigen complex-stimulated group pre-treated with 80 mg/kg of tHGA was comparable to that of the sham group. G: Quantitation of the percentage of cells that showed extrusion of secretory granules was carried out by counting the number of cells that either show extrusion or no extrusion of granules. Results are expressed as the mean $\pm$ S.E.M. (n = 6 rats). * P < 0.05, ** P < 0.01 and *** P < 0.005 versus IgE-antigen complex-stimulated rats (black bar).

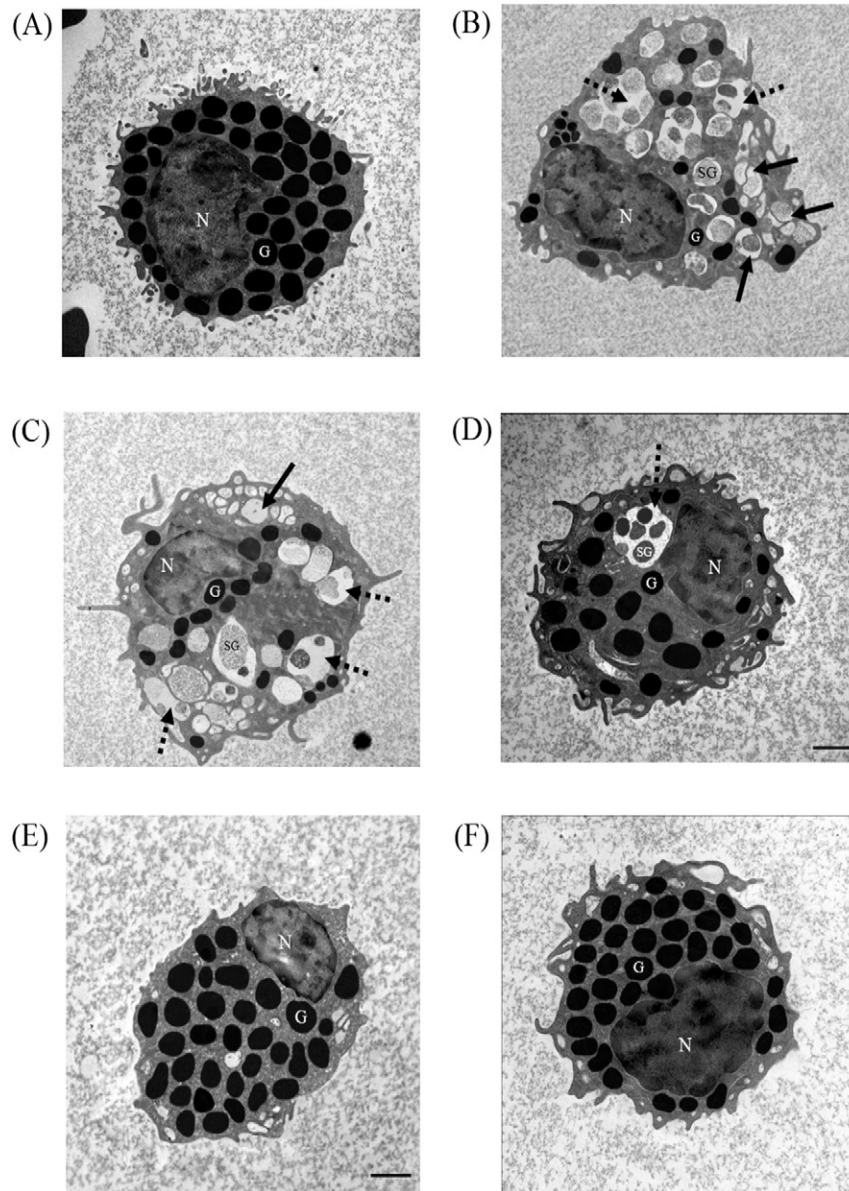


Fig. 9. Representative transmission electron-microscopic images of non-stimulated and IgE-antigen complex-stimulated rat peritoneal mast cells. Male Sprague Dawley adult rats were orally treated with tHGA or ketotifen fumarate for 1 h before challenged with 1 mg/mL BSA. After 1 h, the rats were sacrificed, peritoneal mast cells were isolated and purified using Percoll. Purified mast cells were fixed and prepared as described in methods for electron microscopy examination (Scale bar = 5 μ m). A: sham group, B: IgE-antigen complex-stimulated group, C: IgE-antigen complex-stimulated group pre-treated with 20 mg/kg of tHGA. D: IgE-antigen complex-stimulated group pre-treated with 40 mg/kg of tHGA. E: IgE-antigen complex-stimulated group pre-treated with 80 mg/kg of tHGA. F: IgE-antigen complex-stimulated group pre-treated with 1 mg/kg of ketotifen fumarate. N: nucleus, G: granules, SG: secreting granules. Black arrows indicate the dilated perigranular and electronlucent spaces within the cell cytoplasm. Dashed arrows indicate fused cavities within the cell cytoplasm.

hypersensitivity reactions (Huang et al. 2008; Wang et al. 2012). This study demonstrated that tHGA exhibited a concentration-dependent inhibitory effects against TNF- α and IL-4 secretion, somewhat similar to its effect on PGD₂ and LTC₄. These findings indicated that tHGA did not only attenuate the release of preformed mediators by stabilizing plasma membrane deformation in degranulating mast cells, but also decreased subsequent release of late phase mediators into the surrounding environment, most probably by targeting the signalling pathways that lead to the production of these mediators.

Since LTC₄ and PGD₂ are the metabolites of LOX and COX respectively, (Ricciotti and FitzGerald 2011; Church and Levi-Schaffer 1997), these findings are consistent with a previous report on the role of tHGA as a dual LOX/COX inhibitor (Shaari et al. 2011). Also, the inhibitory effect of tHGA on IL-4 secretion by activated mast cells could be associated with the anti-asthmatic effect of tHGA by reducing Th2 cytokines levels

in bronchoalveolar lavage fluid (BALF), as reported in another study (Ismail et al. 2012). Therefore, it is possible that the inhibitory effect of tHGA on allergic airway inflammation reported in previous study partly involves its inhibitory effect on mast cell activation and subsequent production and release of preformed and *de novo* mediators.

The inhibitory effect of tHGA on extensive reorganization of cytoskeleton that reduces exocytosis of the granules containing histamines and β -hexosaminidase *in vitro* were further examined *in vivo* using a rodent model of mast cell-mediated allergic reactions also known as IgE-mediated passive systemic anaphylaxis (PSA). Anaphylaxis is a serious allergic reaction characterized by rapid onset and may be lethal (Sampson et al. 2006). Upon exposure to allergen or other factors, activation of mast cells results in degranulation and instant release of preformed mediators (histamine), synthesis of arachidonic acid metabolites (prostaglandins, leukotrienes), and late-phase generation of

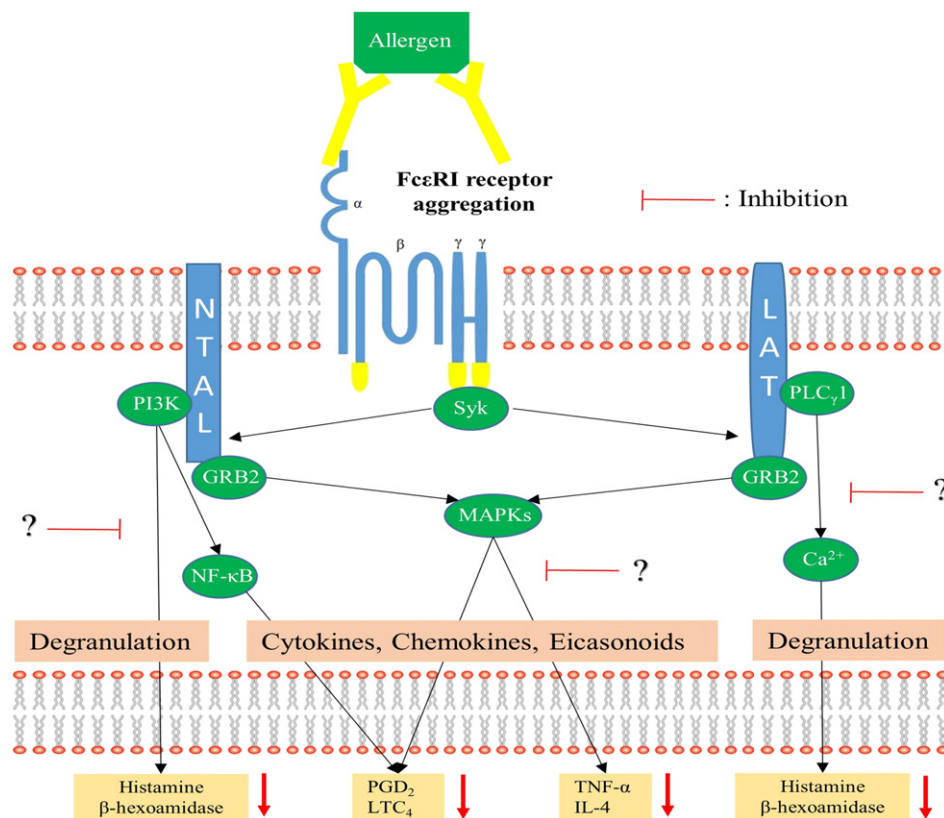


Fig. 10. Potential inhibitory mechanisms of tHGA on FcεRI-induced activation of mast cells. Future study on the mode of action and molecular target of tHGA in IgE-mediated mast cell degranulation may focus on both signalling pathways of LAT-PLCγ and NTAL-PI3K axis following FcεRI receptor activation.

cytokines (TNF-α) and chemokines (Metcalfe et al. 2009; Simons 2008) that mediate the signs and symptoms in anaphylaxis. Therefore, it is believed that the main factor in triggering anaphylaxis is the cellular events which result in the release of mediators from activated mast cells into the bloodstream throughout the body (Peavy and Metcalfe 2009). Previous studies consistently reported that isolated mast cells from animals underwent vigorous degranulation when sensitized with IgE and challenged with antigens (Lu et al. 2011; Penissi et al. 2009). tHGA pretreatment in murine model of IgE-mediated PSA demonstrated that tHGA was able to attenuate the serum levels of all selected proinflammatory mediators released, including histamine, PGD₂, LTC₄, IL-4 and TNF-α in anaphylactic rats. These findings corroborate our *in vitro* results, whereby tHGA was able to attenuate the release of proinflammatory mediators in the presence of antigen.

Microscopic examination of isolated PMCs from anaphylactic rats showed that activated PMCs underwent extensive changes in cell morphology, resulting in the extrusion of granules from the cells clearly identified by toluidine blue staining. In addition, electron microscopy examination also demonstrated changes on cell surface of PMCs as well as the reduction in the granules content due to their release into environment upon DNP-BSA challenge. These changes, however, were not apparent in PMCs isolated from rats pre-treated with tHGA, indicating that the presence of tHGA was able to prevent extensive morphological changes and also the release of granules from IgE-antigen complex-stimulated mast cells. Thus, these observations suggest that tHGA-mediated decrease in histamine and β-hexosaminidase release from activated mast cells may be due to its activity in reducing exocytosis and preserving granules within the cytoplasm of activated mast cells. Collectively, the *in vivo* results in this study further confirm our hypothesis that tHGA could be a potential mast cell stabilizer that ameliorates anaphylactic reactions by inhibiting mast cell activation.

Although tHGA have been demonstrated to inhibit the production of 5-lipoxygenase by human peripheral blood leukocytes and cysLT by

mouse peritoneal macrophages (Shaari et al. 2011) in inflammatory models, the present study is the first to demonstrate a direct inhibitory effect of tHGA on mast cells, which is a type of immune cells that play critical role in the pathogenesis of allergic asthma. The effects of tHGA on other immune cells especially eosinophils and T cells remain as our future interest due to their important roles in the pathogenesis of allergic diseases (Lloyd and Hessel, 2010; Walsh et al. 2010).

In summary, our present study demonstrated that tHGA inhibited degranulation, cytoskeleton rearrangement as well as the release of preformed and *de novo* mediators from IgE-antigen complex-stimulated mast cells. These inhibitory effects of tHGA was also demonstrated *in vivo*, where tHGA reduced the serum levels of preformed and *de novo* mediators by preventing mast cell degranulation and preserving the morphology of mast cells in IgE-mediated PSA reaction in rats. In conclusion, this study provides evidence on the anti-allergic properties of tHGA by inhibiting IgE-mediated mast cell degranulation. This study also suggests that tHGA may have the therapeutic potential for the prevention or treatment of allergic diseases mediated by mast cell activation. However, the mechanism of action by which tHGA act upon needs to be further explored. Based on previous studies which demonstrated important roles of adapter proteins, LAT and NTAL, in the release of preformed and *de novo* mediators by activated mast cells (Rivera 2005; Zhu et al. 2004), we speculate that tHGA may inhibit single or multiple signalling molecules located in the LAT-PLCγ or NTAL-PI3K axis pathway following FcεRI receptor activation (Fig. 10). Nevertheless, the molecular target of tHGA in the regulation of mast cell activation is yet to be confirmed and remains a future direction of this study.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Conflict of interest

The authors declare that there are no conflicts of interest.

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