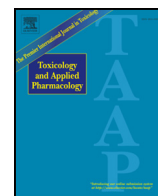




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Q1 Inhibitory effect of 1,2,4,5-tetramethoxybenzene on mast cell-mediated allergic inflammation through suppression of I κ B kinase complex

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ABSTRACT

As the importance of allergic disorders such as atopic dermatitis and allergic asthma, research on potential drug candidates becomes more necessary. Mast cells play an important role as initiators of allergic responses through the release of histamine; therefore, they should be the target of pharmaceutical development for the management of allergic inflammation. In our previous study, anti-allergic effect of extracts of *Amomum xanthioides* was demonstrated. To further investigate improved candidates, 1,2,4,5-tetramethoxybenzene (TMB) was isolated from methanol extracts of *A. xanthioides*. TMB dose-dependently attenuated the degranulation of mast cells without cytotoxicity by inhibiting calcium influx. TMB decreased the expression of pro-inflammatory cytokines such as tumor necrosis factor- α and interleukin (IL)-4 at both the transcriptional and translational levels. Increased expression of these cytokines was caused by translocation of nuclear factor- κ B into the nucleus, and it was hindered by suppressing activation of I κ B kinase complex. To confirm the effect of TMB *in vivo*, the ovalbumin (OVA)-induced active systemic anaphylaxis (ASA) and IgE-mediated passive cutaneous anaphylaxis (PCA) models were used. In the ASA model, hypothermia was decreased by oral administration of TMB, which attenuated serum histamine, OVA-specific IgE, and IL-4 levels. Increased pigmentation of Evans blue was reduced by TMB in a dose-dependent manner in the PCA model. Our results suggest that TMB is a possible therapeutic candidate for allergic inflammatory diseases that acts through the inhibition of mast cell degranulation and expression of pro-inflammatory cytokines.

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Introduction

Immune disorders have been considered worthy of research, especially allergic disorders such as anaphylaxis, allergic rhinitis, and atopic dermatitis. Mast cells play a key role in allergic disorders through the release of histamine, chemokines, cytokines, and growth factors. (Galli et al., 2008) Histamine, a major factor in the acute allergic response, causes vasodilation, increased vascular permeability, and consequently,

development of hypothermia and recruitment of leukocytes. (Galli and Tsai, 2012) Release of pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-4 initiates the chronic phase of allergy through enhancement of T cell activation or B cell survival. (Nakae et al., 2005; Acosta-Rodriguez et al., 2004) Thus, mast cells are suitable target for anti-allergic drug development.

Antigen cross-linking of IgE bound to Fc ϵ RI is necessary to trigger the activation of mast cells. B cells stimulated by antigen-presenting cells produce antigen-specific IgE, which binds to Fc ϵ RI on the mast cell surface. (Naik and Wala, 2013) Release and production of allergic mediators such as histamine, pro-inflammatory cytokines, and various enzymes are increased after activation of Fc ϵ RI from mast cells. (Sismanopoulos et al., 2012) Rat basophilic leukemia (RBL)-2H3 cells are widely used for *in vitro* studies of mast cell-mediated allergic inflammation, which occurs through degranulation and expression of pro-inflammatory cytokines. (Bae et al., 2011) IgE-mediated passive cutaneous anaphylaxis (PCA) and ovalbumin (OVA)-induced active systemic anaphylaxis (ASA) reactions are suitable *in vivo* models for investigating

Abbreviations: ASA, active systemic anaphylaxis; BW, body weight; DNP, dinitrophenyl; IP3, inositol 1,4,5-trisphosphate; MC, methylene chloride; MeOH, methanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OVA, ovalbumin; PCA, passive cutaneous anaphylaxis; RBL, rat basophilic leukemia; RPMCs, rat peritoneal mast cells; TMB, 1,2,4,5-tetramethoxybenzene.

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immediate hypersensitivity. (Kim et al., 2014) In both *in vivo* models, degranulation of mast cells after injection of an antigen induces a typical allergic response.

There have been many reports on the various biological effects of *Amomum xanthioides*. Extracts of *A. xanthioides* have been shown to attenuate cytokine-induced cell death through suppression of inducible nitric oxide synthase by the activation of NF- κ B, as well as liver damage by regulation of the antioxidation system. (Kwon et al., 2003; Wang et al., 2013) Our previous research demonstrated a suppressive effect of *A. xanthioides* on the mast cell-mediated allergic reaction. (Kim et al., 2007) To ascertain which compound produced this anti-allergic effect, preparative HPLC was carried out, and 1,2,4,5-tetramethoxybenzene (TMB) was isolated. In the present study, we aimed to evaluate the inhibitory effect of TMB on mast cell-mediated allergic inflammation and its underlying mechanism of action by using *in vitro* and *in vivo* models of mast cell-mediated allergic inflammation.

Materials and methods

Animals. Male Imprinting Control Region mice (aged 6 weeks) and Sprague–Dawley (SD) rats (aged 10 weeks) were purchased from the Dae-Han Biolink (Daejeon, Korea). Throughout the study, five animals per cage were housed in a room with laminar air flow, a temperature of 22 ± 2 °C, and relative humidity of 55 ± 5 °C. The care and treatment of the mice were carried out in accordance with the guidelines established by the Public Health Service Policy on the Humane Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of Kyungpook National University.

Reagents and cell culture. Anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), phorbol 12-myristate 13-acetate (PMA), OVA, 3,4,5-trihydroxybenzoic acid (gallic acid), dexamethasone, and histodenz were purchased from Sigma-Aldrich (St. Louis, MO). PP2, BAPTA-AM, and alum adjuvant were purchased from Calbiochem (La Jolla, CA), Invitrogen (Carlsbad, CA), and Thermo Scientific (Waltham, MA), respectively. RBL-2H3 cells and rat peritoneal mast cells (RPMCs) were grown in Dulbecco's modified Eagle's medium and α -minimum essential medium (GIBCO, Grand Island, NY) respectively supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin G, 100 μ g/ml streptomycin, and 250 ng/ml amphotericin B at 37 °C in 5% CO₂. For RBL-2H3 cells, passages 4 to 8 were used throughout the study.

Preparation of RPMCs. Peritoneal cells were isolated from SD rats as previously described. (Kim et al., 2014) In brief, the rats were anesthetized with CO₂ and injected with 20 ml of Tyrode's buffer A (137 mM NaCl, 5.6 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.3 mM NaH₂PO₄, and 0.1% gelatin) into the peritoneal cavity before gentle massage of the abdomen for approximately 90 s. The peritoneal cavity was carefully opened, and the fluid containing the peritoneal cells was aspirated using a Pasteur pipette. The cells were collected after centrifugation at 150 g for 10 min at room temperature and then resuspended in 1 ml of Tyrode's buffer A. To separate the mast cells from other major components of rat peritoneal cells, i.e., macrophages and small lymphocytes, the peritoneal cells suspended in Tyrode's buffer A were layered on 2 ml of 0.235 g/ml Histodenz solution and centrifuged at 400 g for 15 min at room temperature. The cells at the buffer–Histodenz interface were discarded, and the cells in the pellet were washed and resuspended. Mast cell preparations had the purity of approximately 95%, based on toluidine blue staining. More than 97% of the cells were viable, based on trypan blue staining.

Extraction from *A. xanthioides*. Fruits of *A. xanthioides* were purchased from Yak-Ryung-Si Market in Daegu, Korea and identified by Dr. Seung Ho Lee at College of Pharmacy, Yeungnam University, and a voucher

specimen (YU00194) was deposited in the Natural Product Laboratory at the College of Pharmacy, Yeungnam University, Korea.

Dried fruits of *A. xanthioides* (9.5 kg) were extracted with methanol (MeOH) (15 L $\times$ 3) at room temperature. The extracts were concentrated *in vacuo* to produce a black gum (440 g), which was dissolved in H₂O, continuously partitioned with solvents, and then respectively concentrated to give extracts of *n*-hexane (120 g), methylene chloride (MC) (32.7 g), ethyl acetate (9.8 g), and H₂O (275.1 g). The MC-soluble extract (32.7 g) eluted with a gradient mixture of 100% MC to 100% acetone was chromatographed on a silica gel liquid chromatography to obtain seven fractions (AF1–AF7).

Isolation of TMB. TMB was isolated from fraction AF3 (101.2 mg) using preparative HPLC (C₁₈, 250 $\times$ 21.2 mm, flow rate 6 ml/min) and eluted with MeOH:H₂O (gradient from 30:70 to 60:40). Column chromatography was carried out on Merck silica gel (70–230 mesh) and Merck Lichroprep RP-18 gel (40–63 μ m). HPLC separation was performed with the Gilson system, a UV detector, and a Luna C₁₈ column (250 $\times$ 21.20 mm, 10 micron). Thin-layer chromatography was performed using aluminum plates precoated with silica gel 60 F₂₅₄ and RP-18 F₂₅₄ (Merck, Darmstadt, Germany). Compounds were detected under UV light and then visualized by spraying the plates with vanillin-sulfuric acid reagent (1% ethanolic vanillin solution, 10% ethanolic sulfuric acid) and 10% sulfuric acid followed heating at 110 °C for 1 min. ¹H (250 MHz) and ¹³C (63 MHz) NMR spectra were recorded on a Bruker 250 MHz (DMX 250) with tetramethylsilane as an internal standard. NMR spectra were recorded on JEOL ECA-500 (¹H, 500 MHz, ¹³C, 125 MHz) spectrometers. All NMR experiments were performed at 294 K. The degree of purity of the TMB was over 97%.

TMB, gallic acid, and dexamethasone were dissolved in DMSO and then diluted with PBS. The proportion of DMSO in the treated samples was controlled to less than 0.1 % to minimize the interference.

Determination of cell viability. Cell viability was determined by colorimetric analysis based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). (Choi et al., 2014) Water-soluble MTT is converted into water-insoluble formazan by mitochondrial dehydrogenase. RBL-2H3 cells (3 $\times$ 10⁴/well in 96-well plates) were pretreated with various concentrations of TMB for 24 h and incubated with 1 mg/ml MTT at 37 °C. After 2 h, the formazan crystals were dissolved with DMSO, and then the absorbent intensity was detected using a spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 570 nm. The absorbent intensity of formazan formed in untreated control cells was calculated as 100% viability.

Determination of mast cell degranulation. To determine mast cell degranulation, levels of histamine in the serum and culture media were measured using the o-phthaldialdehyde spectrofluorometric procedure. The fluorescent intensity was detected using a fluorescent plate reader (Molecular Devices) at an excitation wavelength of 360 nm and an emission wavelength of 440 nm as previously described. (Kim et al., 2013) Release of β -hexosaminidase is also widely used as a marker of the mast cell degranulation. (Itoh et al., 2010) After incubation for 1 h at 37 °C with β -hexosaminidase substrate buffer (100 mM sodium citrate, 1 mM 4-nitrophenyl N-acetyl- β -D-galactosaminide, pH 4.5), the reaction was terminated using stop solution (0.1 M Na₂CO₃ and NaHCO₃), and the absorbent intensity was detected using a spectrophotometer at a wavelength of 405 nm. Blood from the mice was centrifuged at 400 g for 15 min at 4 °C, and the serum was drawn to measure histamine content. RBL-2H3 cells (5 $\times$ 10⁵/well in 12-well plates) were sensitized with anti-DNP IgE (50 ng/ml). After incubating overnight, the cells were pretreated with or without TMB for 1 h and then challenged with DNP-HSA (100 ng/ml) for 4 h. RPMCs (2 $\times$ 10⁴/well in 24-well plates) were handled similarly to RBL-2H3 cells; however, these were

challenged with DNP-HSA for only 30 min. The cells were separated from the media by centrifugation at 150 g for 5 min at 4 °C.

Determination of intracellular calcium level. The intracellular calcium level was measured using the fluorescence indicator Fluo-3/AM (Invitrogen). (Kim et al., 2013) RBL-2H3 cells (3×10^4 /well in 96-well plates) were sensitized with anti-DNP IgE (50 ng/ml). After incubating overnight, the cells were preincubated with Fluo-3/AM for 1 h at 37 °C and washed with Tyrode's buffer B (137 mM NaCl, 5.5 mM glucose, 12 mM NaHCO₃, 2.7 mM KCl, 0.2 mM NaH₂PO₄, 1 mM MgCl₂, and 1.8 mM CaCl₂) to remove the dye from the cell surface. The cells were pretreated with or without TMB for 1 h and then challenged with DNP-HSA (100 ng/ml). Fluorescent intensity was detected using a fluorescent plate reader at an excitation wavelength of 485 nm and an emission wavelength of 510 nm. Intracellular calcium level in untreated control cells was calculated as 1 relative absorbance.

RNA extraction and real-time PCR. Prior to isolation of total cellular RNA, RBL-2H3 cells (5×10^5 /well in 12-well plates) were sensitized with anti-DNP IgE (50 ng/ml). After incubating overnight, the cells were pretreated with or without TMB for 1 h and challenged with DNP-HSA (100 ng/ml) for 1 h. RNAiso Plus reagent (Takarabio Inc., Shiga, Japan) was used to extract total RNA according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2 µg of total RNA using the Maxime RT-Pre Mix Kit (iNtRON Biotechnology, Daejeon, Korea). Quantitative real-time PCR was carried out using the Thermal Cycler Dice TP850 (Takarabio Inc.) according to the manufacturer's protocol. The total 25 µl reaction mixture was composed as follows: 1.5 µl of cDNA (150 ng), 1 µl of each of the forward and reverse primers (0.4 µM), 12.5 µl of SYBR Premix Ex Taq (Takarabio Inc.), and 9 µl of dH₂O. The conditions for PCR were similar to the conditions in our previous research. (Bae et al., 2011)

ELISA. To measure the release of pro-inflammatory cytokines, ELISA was carried out using media from RBL-2H3 cells challenged with DNP-HSA and serum. ELISA was performed using an ELISA kit (BD Biosciences, San Diego, CA) on a 96-well Nunc immuno plate according to the manufacturer's protocol. Before the detection of OVA-specific IgE, the immune plate was coated with 20 µg of OVA instead of a capture antibody. After terminating the reaction with a substrate, the absorbent intensity was detected using a spectrophotometer at a wavelength of 450 nm.

Western blot. Nuclear and cytosolic proteins were extracted as previously described. (Choi et al., 2013) Before protein extraction, RBL-2H3 cells (2×10^6 /well in 6-well plates) were sensitized with anti-DNP IgE (50 ng/ml). After incubating overnight, the cells were pretreated with or without TMB for 1 h and challenged with DNP-HSA (100 ng/ml). After suspension in 100 µl of cell lysis buffer A (0.5% Triton X-100, 150 mM NaCl, 10 mM HEPES, 1 mM EDTA/Na₃VO₄, 0.5 mM PMSF/DTT, and 5 µg/ml leupeptin/aprotinin), the cells were vortexed, incubated for 5 min on ice, and centrifuged at 400 g for 5 min at 4 °C. The supernatant was collected as cytosolic protein extracts. The pellets were washed 3 times with 1 ml of PBS and then suspended in 25 µl of cell lysis buffer B (25% glycerol, 420 mM NaCl, 20 mM HEPES, 1.2 mM MgCl₂, 0.2 mM EDTA, 1 mM Na₃VO₄, 0.5 mM PMSF/DTT, and 5 µg/ml leupeptin/aprotinin), vortexed, sonicated for 30 s, incubated for 20 min on ice, and centrifuged at 15,000 g for 15 min at 4 °C. The supernatant was collected as nuclear protein extracts. Samples of the protein were electrophoresed using 8–12% SDS-PAGE and transferred to a nitrocellulose membrane. Immunodetection was carried out using a chemiluminescent substrate (Thermo scientific).

OVA-induced ASA. Mice ($n = 10$ /group) were sensitized with the OVA mixture (100 µg of OVA and 2 mg of alum adjuvant in 200 µl of PBS)

by intraperitoneal injection on day 0 and day 7. TMB was orally administered 3 times once every 2 days at doses of 0.1–10 mg/kg body weight (BW) after the second sensitization. On day 14, 200 µg of OVA was intraperitoneally injected, and then rectal temperature was measured every 10 min during 1 h. After 1 h, blood was obtained from the abdominal artery of each mouse to measure serum histamine, OVA-specific IgE, and IL-4 levels.

IgE-mediated PCA. An IgE-mediated PCA model was established as described previously. (Kim et al., 2014) To induce the PCA reaction, the skin on the ears of the mice ($n = 5$ /group) was sensitized with an intradermal injection of anti-DNP IgE (0.5 µg/site) for 48 h. TMB was administered orally at doses of 0.1–10 mg/kg BW 2 h before intravenous injection of DNP-HSA (1 mg/mouse) and 4% Evans blue (1:1) mixture. Thirty minutes later, the mice were euthanized, and both ears were collected to measure dye pigmentation and histology. The amount of dye was determined colorimetrically after extraction with 0.5 ml of 1 M KOH and 4.5 ml of an acetone and phosphoric acid (5:13) mixture. The absorbent intensity of the extract was detected using a spectrophotometer at a wavelength of 620 nm. Ear thickness was measured with a dial thickness gauge (Kori Seiki MFG. Co., Tokyo, Japan).

Histology and mast cell count. The ears were fixed with 4% formaldehyde and embedded in paraffin. Then 5 µm sections were stained with toluidine blue to count the number of mast cells at five sites randomly selected fields for each sample under 100× magnification.

Statistical analysis. Each data presented as a graph represents the mean ± SE of various independent experiments. Statistical analyses were performed using Prism5 (GraphPad Software, San Diego, CA), and treatment effects were analyzed using a one-way ANOVA and then Dunnett's test. $P < 0.05$ indicated a significant difference in the study.

Results

Determination of TMB

White amorphous powder; ¹H-NMR (CD₃OD, 250 MHz) δ_H 6.38 (2H, s, H-2,5), 3.87 (12H, s, H-CH₃O); ¹³C-NMR (CD₃OD, 63 MHz) δ_C 147.8 (C-1,3,4,6), 107.2 (C-2,5), 55.6 (C-CH₃O).

Effects of TMB on the degranulation of mast cells

Histamine is known as a key mediator in the allergic response and released through the degranulation of mast cells. (Galli and Tsai, 2012) To evaluate mast cell degranulation, we measured histamine release from both RBL-2H3 and RPMCs. TMB dose-dependently attenuated histamine release in both cells (Fig. 1C and D). Gallic acid and dexamethasone were used as positive controls throughout this research because they are known as effective drugs on mast cell-mediated allergic inflammation. (Kim et al., 2006; Wershil et al., 1995) At the 10 times lower concentration, TMB showed an inhibitory effect similar to that of gallic acid and dexamethasone. The β-hexosaminidase assay, another common method used to measure the degranulation of mast cells, was performed. (Itoh et al., 2010) TMB presented an equal effect on histamine and β-hexosaminidase release (Fig. 1E). MTT assay was carried out to detect cytotoxicity of TMB. TMB did not cause cytotoxicity up to 100 µM during 24 h (Fig. 1B). Therefore, TMB might have better suppressive effects on the degranulation of mast cells than those of gallic acid and dexamethasone without cytotoxicity.

Calcium is a key secondary messenger in mast cell signaling, especially during degranulation. After activation of PLCγ by antigen-antibody binding, extracellular calcium influx is increased following calcium release from the ER through the generation of IP₃. (Kalesnikoff

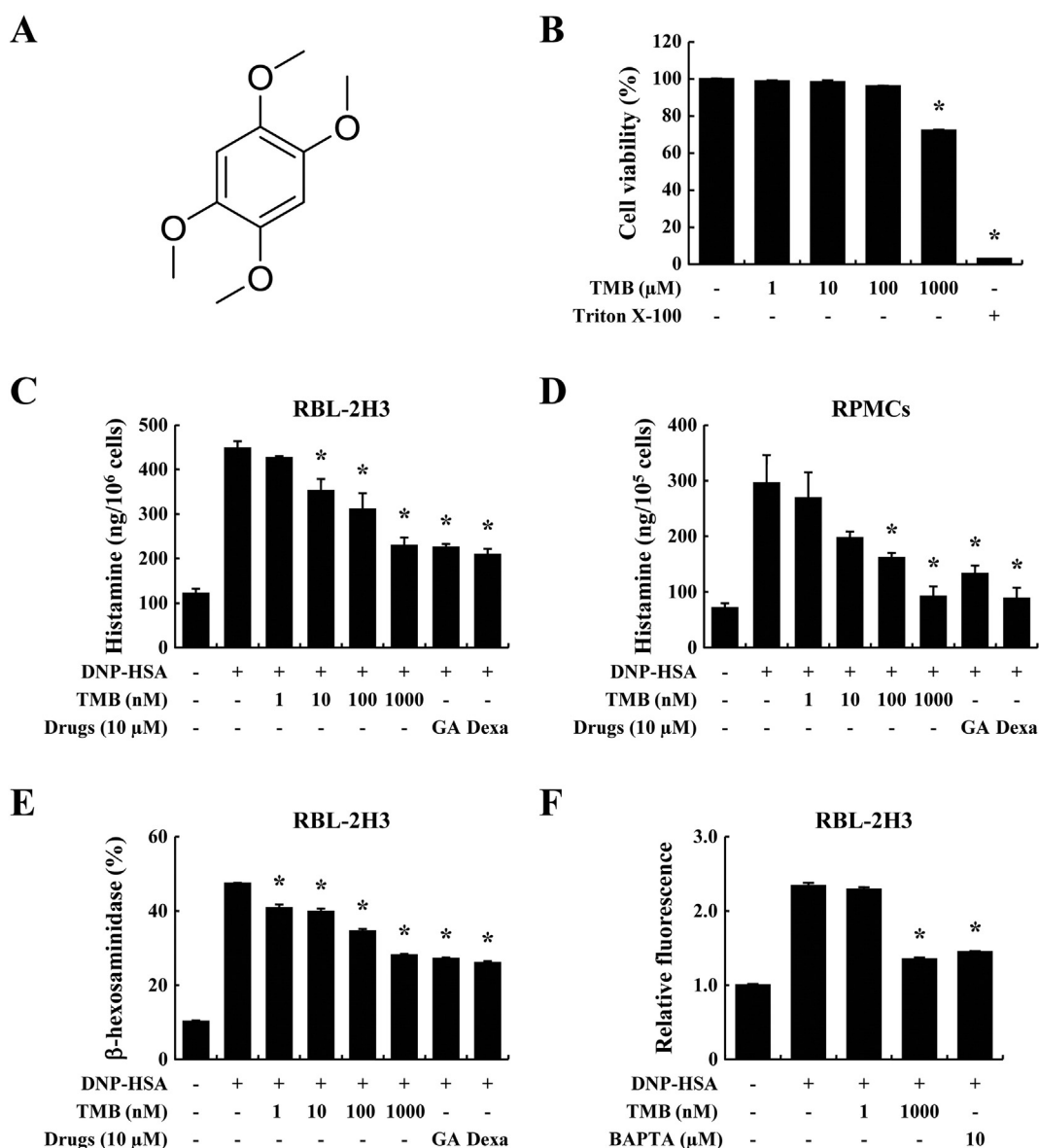


Fig. 1. Effects of TMB on the degranulation of mast cells. (A) Chemical structure of TMB. (B) RBL-2H3 cells were pretreated with or without TMB and then incubated with MTT. The absorbent intensity was detected using a spectrophotometer. (C and D) RBL-2H3 cells and RPMCs were pretreated with or without TMB. Histamine levels were detected using a fluorescent plate reader. (E) β -hexosaminidase levels were detected using a spectrophotometer. (F) RBL-2H3 cells were preincubated with Fluo-3/AM. Intracellular calcium was detected using a fluorescent plate reader. BAPTA, a calcium chelator, was used as a positive control. Each data represents the mean $\pm$ SE of three independent experiments. *Significant difference at $P < 0.05$. GA: gallic acid; Dexa: dexamethasone.

and Galli, 2008) The increased intracellular calcium level induces the degranulation of mast cells. (Kawakami and Galli, 2002) To investigate the action mechanism of TMB on the inhibition of histamine release, we measured the intracellular calcium level, which was enhanced by the stimulation with DNP-IgE binding, but attenuated by pretreatment with TMB. The suppressive effect of TMB was negligibly different from that of BAPTA-AM, a calcium chelator, at 10 times lower concentration (Fig. 1F).

Effects of TMB on the expression of pro-inflammatory cytokines

The production of various pro-inflammatory cytokines such as TNF- α , and IL-4 which results in activation and migration of leukocytes is also enhanced by antigen-IgE cross-linking. (Sismanopoulos et al., 2012) To assess the influence of TMB on the expression of pro-inflammatory cytokines in RBL-2H3 cells, real-time PCR and ELISA

were performed. Gene expression and production of pro-inflammatory cytokines, TNF- α and IL-4, was enhanced by the binding of DNP-HSA with anti-DNP IgE. However, this increase was suppressed by TMB in a dose-dependent manner (Fig. 2A and B). Gallic acid and dexamethasone were used as positive controls.

Translocation of NF- κ B into the nucleus after the degradation of I κ B α induces the expression of pro-inflammatory cytokines. (Metz et al., 2007) In our results, the translocation of NF- κ B was increased by antigen-IgE binding and hindered by TMB (Fig. 3). To anticipate the target point at which TMB inhibits the expression of pro-inflammatory cytokines, we investigated the effect of TMB on the activation of representative signaling proteins in mast cells such as Lyn, Syk, Akt, and IKK α / β (Fig. 4). TMB did not affect the activation of these proteins, but did hinder the activation of IKK α / β . PP2, a specific inhibitor for Src family kinases, was used as a positive control. We inferred that TMB inhibited the expression of pro-inflammatory cytokines through by

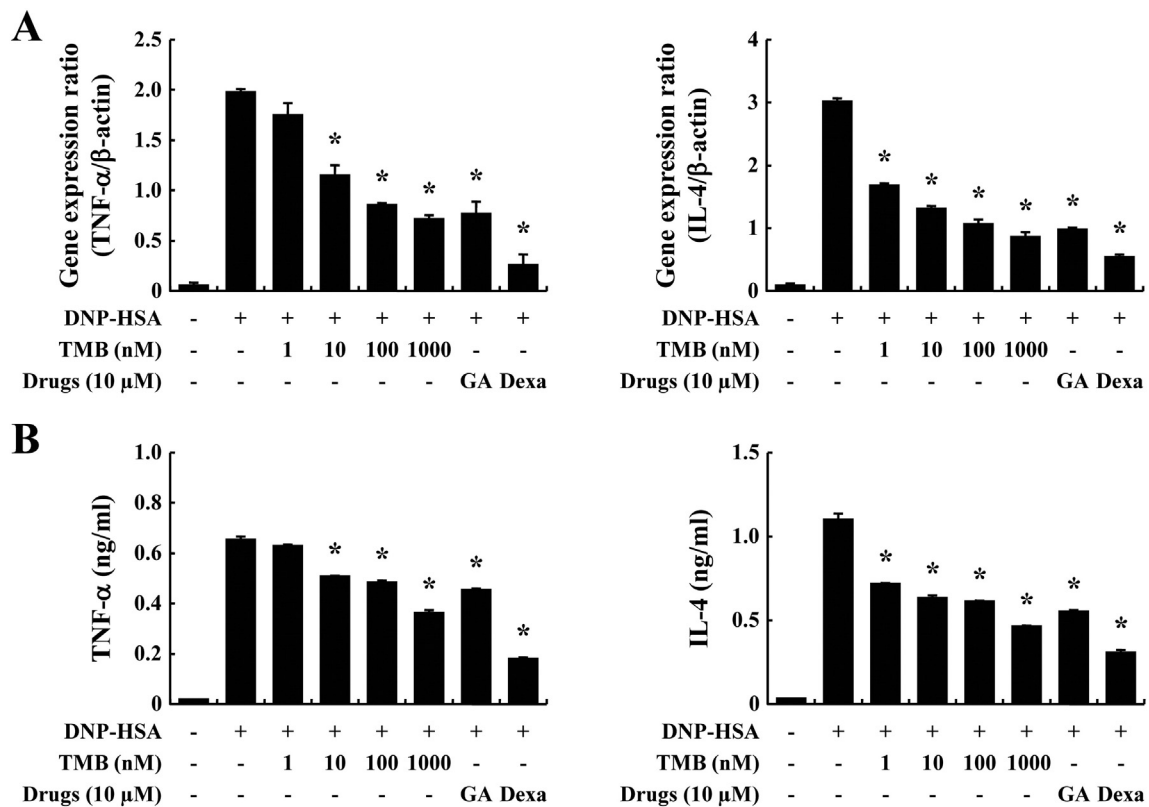


Fig. 2. Effects of TMB on the expression of pro-inflammatory cytokines. (A) RBL-2H3 cells were pretreated with or without TMB. Extraction and analysis of mRNA were performed as described in the Materials and methods section. The gene expression of pro-inflammatory cytokines was determined by real-time PCR. (B) The expression of pro-inflammatory cytokines at the translational level was measured by ELISA. Each data represents the mean $\pm$ SE of three independent experiments. *Significant difference at $P < 0.05$. GA: gallic acid; Dexa: dexamethasone.

blocking the activation of IKK α /β, not further upstream in the signaling pathway at Akt. To further confirm the inhibitory effect of TMB on IKK complex, RBL-2H3 cells were stimulated with PMA, an activator of protein kinase C which stimulates IKK complex. PMA-induced phosphorylation of IKK α /β was decreased by TMB (Supplementary Fig. 1A). In addition, TMB dose-dependently reduced PMA-induced secretion of IL-4 (Supplementary Fig. 1B).

Effects of TMB on OVA-induced ASA and IgE-mediated PCA

The systemic anaphylaxis model is widely used to examine immediate-type allergic responses, which are strongly associated with mast cells. (Marone et al., 2002) Mice sensitized through repetitive administrations of OVA with alum adjuvant were suffered from anaphylaxis through challenges of OVA. After an intraperitoneal injection of OVA, mice were monitored for 1 h. The body temperature of the mice decreased over 40–50 min, and the serum histamine level increased considerably. The decrease in rectal temperature was reduced by TMB, and the serum histamine level was also diminished, similar to results of the *in vitro* histamine assay (Fig. 5A–C). IgE plays a role in the allergic reaction, especially in the systemic anaphylaxis. (Finkelman et al., 2005) OVA-specific IgE and IL-4 in the serum were detected by ELISA. Both OVA-specific IgE and IL-4 levels were increased after a challenge with OVA, and decreased by administration of TMB (Fig. 5D and E). We suggest that TMB suppresses anaphylaxis by attenuating the release of histamine and the production of IgE.

The PCA model is also well known animal model used to demonstrate anti-allergic effects. (Kim et al., 2014) Anti-DNP IgE (0.5 μg/site) was intradermally injected prior to the intravenous injection of a DNP-HSA (1 mg/mouse) and 4% Evans blue (1:1) mixture to induce a PCA reaction in mice ears. We could see a blue spot at the sensitized site after an antigen challenge because of increased vascular permeability by histamine released from mast cells. The size and color of the blue spot were dose-dependently reduced by TMB (Fig. 6A and B). Ear thickness was increased by an injection of antigen and decreased by TMB (Fig. 6C). These reductions in absorbance and ear thickness might be due to the inhibitory effect of TMB on histamine release from mast cells. To confirm whether the reduction of vascular permeability was

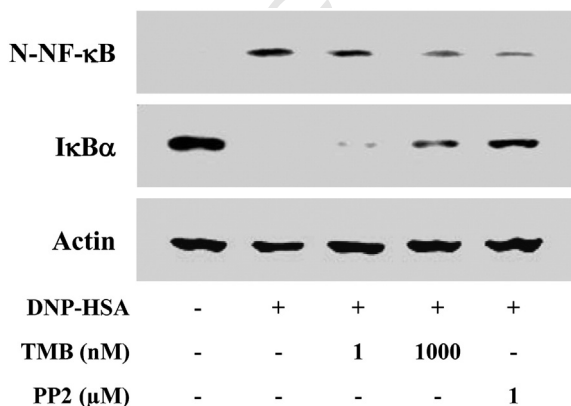


Fig. 3. Effects of TMB on the activation of NF-κB. RBL-2H3 cells were pretreated with or without TMB. Extraction of protein was performed as described in the Materials and methods section. Activation of NF-κB was assayed by Western blot (N-: nuclear). The band of actin was used as a loading control. PP2, a Src family inhibitor, was used as a positive control. The band is a representative of three independent experiments.

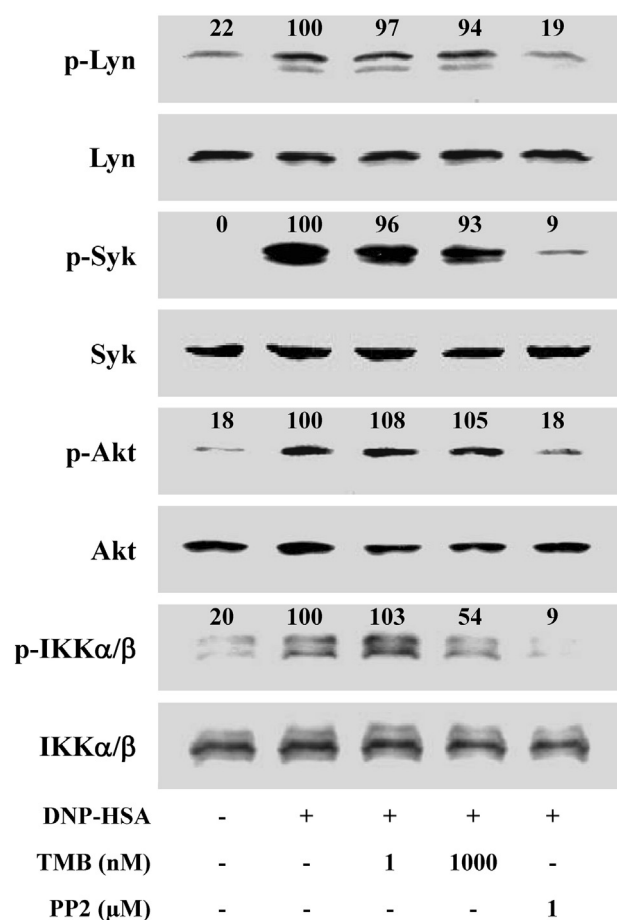


Fig. 4. Effects of TMB on the activation of signaling proteins. RBL-2H3 cells were pretreated with or without TMB. Extraction of protein was performed as described in the Materials and methods section. Activation of signaling proteins was assayed by Western blot (p-: phosphorylated). PP2, a Src family inhibitor, was used as a positive control. The band intensity was digitized and normalized by the relative ratio of phosphorylation/total. The band is a representative of three independent experiments.

preformed mediators, which play crucial roles in immediate-type hypersensitivity. (Kalesnikoff and Galli, 2008) Therefore, inhibiting mast cell degranulation is a key therapeutic target in allergic disorders. Histamine and β -hexosaminidase are well known as markers for mast cell degranulation. Histamine, mainly produced by mast cells, especially is one of the most important molecules in the allergic response. Allergic symptoms such as edema, warmth, and erythema are resulted from vasodilation and an increase of vascular permeability caused by histamine. (Galli and Tsai, 2012) In the histamine and β -hexosaminidase assay using cell line (RBL-2H3) and primary cells (RPMCs), TMB strongly inhibited mast cell degranulation. OVA-induced ASA and IgE-mediated PCA models are positively associated with histamine release from mast cells. (Finkelman et al., 2005; Sampson et al., 2005) In both animal models, increase of histamine release resulted in vasodilation, which eventually causes hypothermia and the Evans blue pigmentation. These symptoms were reduced thorough the oral administration of TMB. From these results, we inferred that TMB suppresses the immediate-type allergic reaction through the inhibition of mast cell degranulation, especially the release of histamine.

Correlations between degranulation and intracellular calcium levels in mast cells have already been proved through a number of studies, which also demonstrated the regulatory role of various calcium-dependent proteins. (Vennekens et al., 2007; Baba et al., 2008) After antigen-IgE binding to Fc ϵ RI, calcium stored in the ER is released into the cytoplasm through binding of inositol 1,4,5-trisphosphate (IP₃) with IP₃ receptor on the ER surface. Depletion of the calcium in the ER causes an influx of extracellular calcium, and then intracellular calcium level is rapidly elevated. (Kalesnikoff and Galli, 2008) Thus, attenuation of increasing intracellular calcium level is strongly associated with the reduction of mast cell degranulation. In our systems, increased intracellular calcium level was diminished by TMB as much as BAPTA-AM. Therefore the suppressive effect of TMB on mast cell degranulation might be due to reduction of intracellular calcium.

Pro-inflammatory cytokines are necessary in the progression to chronic allergic inflammation. Expression of pro-inflammatory cytokines such as TNF- α and IL-4 was increased through antigen-IgE cross-linking. TNF- α contained in the granules of mast cells plentifully stimulates adaptive immunity by activating NF- κ B and the transendothelial migration of immune cells. (Li et al., 2013; Galli and Tsai, 2010) IL-4, one of major Th2 cytokines which are essential in allergic reaction, promotes the production of IgE by working in B cells. (Brandt and Sivaprasad, 2011) According to these reports, suppressing expression of pro-inflammatory cytokines might be related to the inhibition of inflammation and allergic symptoms. TMB reduced the expression of these cytokines in both transcriptional and translational levels. IL-4 in particular was decreased in the serum of the OVA-induced ASA model after administration of TMB and the level of OVA-specific IgE was also decreased. We hypothesize that TMB decreases the magnitude of the allergic reaction by inhibiting the production of IgE through reduction of IL-4 expression, in addition to diminishing mast cell degranulation. TMB might regulate acute-phase allergic responses through the inhibition of mast cell degranulation, especially the release of histamine, and the progression to the chronic allergy through suppression of the production and release of pro-inflammatory cytokines.

NF- κ B is a key transcription factor that stimulates the expression of pro-inflammatory cytokines. (Newton and Dixit, 2012) Our results show that the degradation of I κ B α and translocation of NF- κ B into the nucleus stimulated by antigen-IgE cross-linking. This process was attenuated by TMB. As a result, TMB hinders the expression of pro-inflammatory cytokines by blocking the degradation of I κ B α and translocation of NF- κ B. Calcium also plays a role as an important secondary messenger in the expression of pro-inflammatory cytokines expression in mast cells. (Manikandan et al., 2012; Tanaka et al., 2005) It is possible that TMB suppresses the expression of pro-inflammatory cytokines by reducing the intracellular calcium level as well as the activation of NF- κ B.

caused by the damage of mast cells, the number of mast cells at the PCA site was counted (Fig. 6D and E). No difference of the number of mast cells was detected.

Discussion

Natural products have been considered valuable sources for drug development, and respectable amounts of newly approved therapeutic agents are derived from natural products. (Koehn and Carter, 2005; Newman and Cragg, 2012) There are innumerable reports that comment on the importance of natural medicines to evolve drug discovery. (Beghyn et al., 2008; Cragg and Newman, 2013) Amomi fructus, a traditional medicinal herb in Asia, has been used for digestive system disorders for two thousand years. (Wang et al., 2011) There is also a research on the anti-allergic inflammatory effects of extracts of *A. xanthioides*. (Kim et al., 2007) Even though intake of the natural medicines has been used as one of the most general therapeutic methods, ingestion of the potent single compound is better through increasing biological effects and alleviating side effects. To identify the specific efficacious compound, preparative HPLC was carried out, and TMB was isolated. Our study demonstrated the anti-allergic inflammatory effect of TMB using various *in vitro* and *in vivo* models.

In our results, TMB attenuated not only the degranulation and the expression of pro-inflammatory cytokines in mast cells, but systemic and cutaneous anaphylaxis. It is widely accepted that IgE-dependent mast cell activation leads to the secretion of various

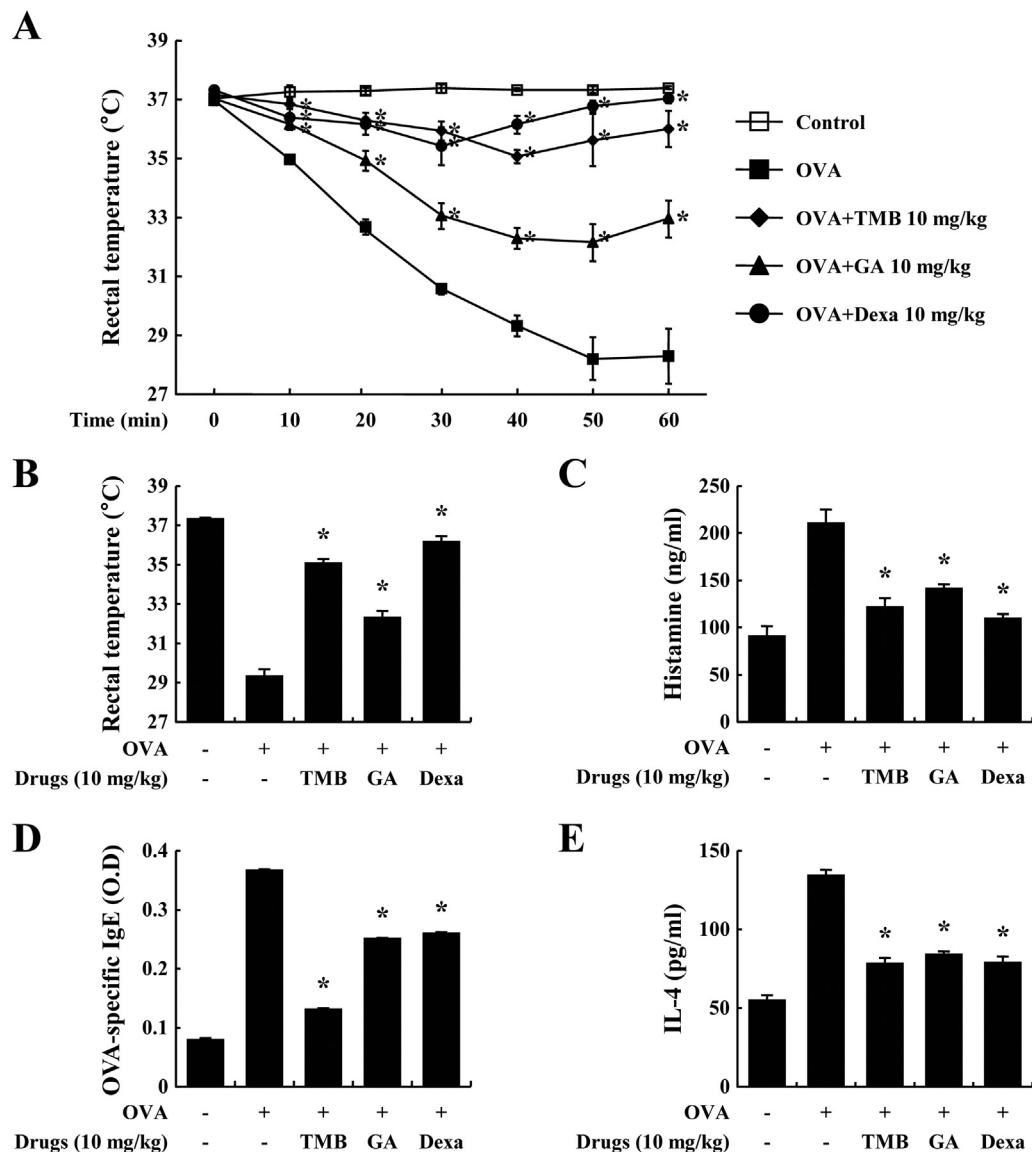


Fig. 5. Effects of TMB on OVA-induced ASA. Induction of systemic anaphylaxis and oral administration of TMB were described in the Materials and methods section. (A) Rectal temperature was measured every 10 min for 1 h. (B) Rectal temperature of the mice at 40 min. Blood was obtained from the abdominal artery of each mouse to measure serum histamine, OVA-specific IgE, and IL-4 levels. (C) Histamine levels were detected using a fluorescent plate reader. Serum histamine was suppressed by oral administration of TMB (D and E) OVA-specific IgE and IL-4 levels were detected by ELISA. Each data represents the mean $\pm$ SE ($n = 10$ per group). *Significant difference at $P < 0.05$. GA: gallic acid; Dexa: dexamethasone.

The intracellular signaling pathway of mast cells is well known, and the representative proteins such as Lyn, Syk, Akt, and IKK α/β were chosen in order to investigate possible targets of TMB. (Kalesnikoff and Galli, 2008) TMB did not have an effect on the activation of proteins from Lyn to Akt. However, TMB reduced the phosphorylation of IKK α/β . The IKK complex has been mainly reported to phosphorylate I κ B α and induce translocation of NF- κ B into the nucleus. (Kalesnikoff and Galli, 2008) In our study, blocking the IKK complex seemed to reduce the degradation of I κ B α and translocation of NF- κ B. The correlation between IKK and the degranulation of mast cells has been informed. IKK β activates SNAP-23, which has an essential role in the degranulation of mast cells and promotion of the anaphylactic reaction. (Hepp et al., 2005; Suzuki and Verma, 2008) Inhibition of the IKK complex by TMB might regulate the release of allergic mediators from mast cells and anaphylaxis. We speculate that inhibition of mast cell degranulation and pro-inflammatory cytokines expression by TMB occurs by blocking the activation of the IKK complex. TMB showed comparable or partially better anti-allergic inflammatory effects in mast cells compared to gallic

acid and dexamethasone which are known as useful medicines for allergic responses. While gallic acid has restrictions because of its poor bioavailability and rapid metabolism, and corticosteroids such as dexamethasone and hydrocortisone have various adverse effects, it is possible that TMB play a role as a therapeutic alternative. (Shahrzad et al., 2001; Zong et al., 1999; Ericson-Neilsen and Kaye, 2014)

In the present study, we aimed to assess the anti-allergic inflammatory effect of TMB using mast cells and animal models. TMB decreased degranulation of mast cells in a dose-dependent manner through attenuation of the intracellular calcium level. TMB also suppressed anaphylactic symptoms in the PCA and ASA models. The expression of pro-inflammatory cytokines was also hindered by TMB through the suppression of the activation of NF- κ B. We anticipate that anti-allergic inflammatory effects of TMB are due to the blockade of the IKK complex. To prove the role of TMB in mast cells more reliably, drug-protein direct binding assay is ongoing in our laboratory. Nevertheless, we assume that the target of TMB is an intermediate between Akt and IKK complex in mast cells. Conclusively, we suggest that TMB is a potential

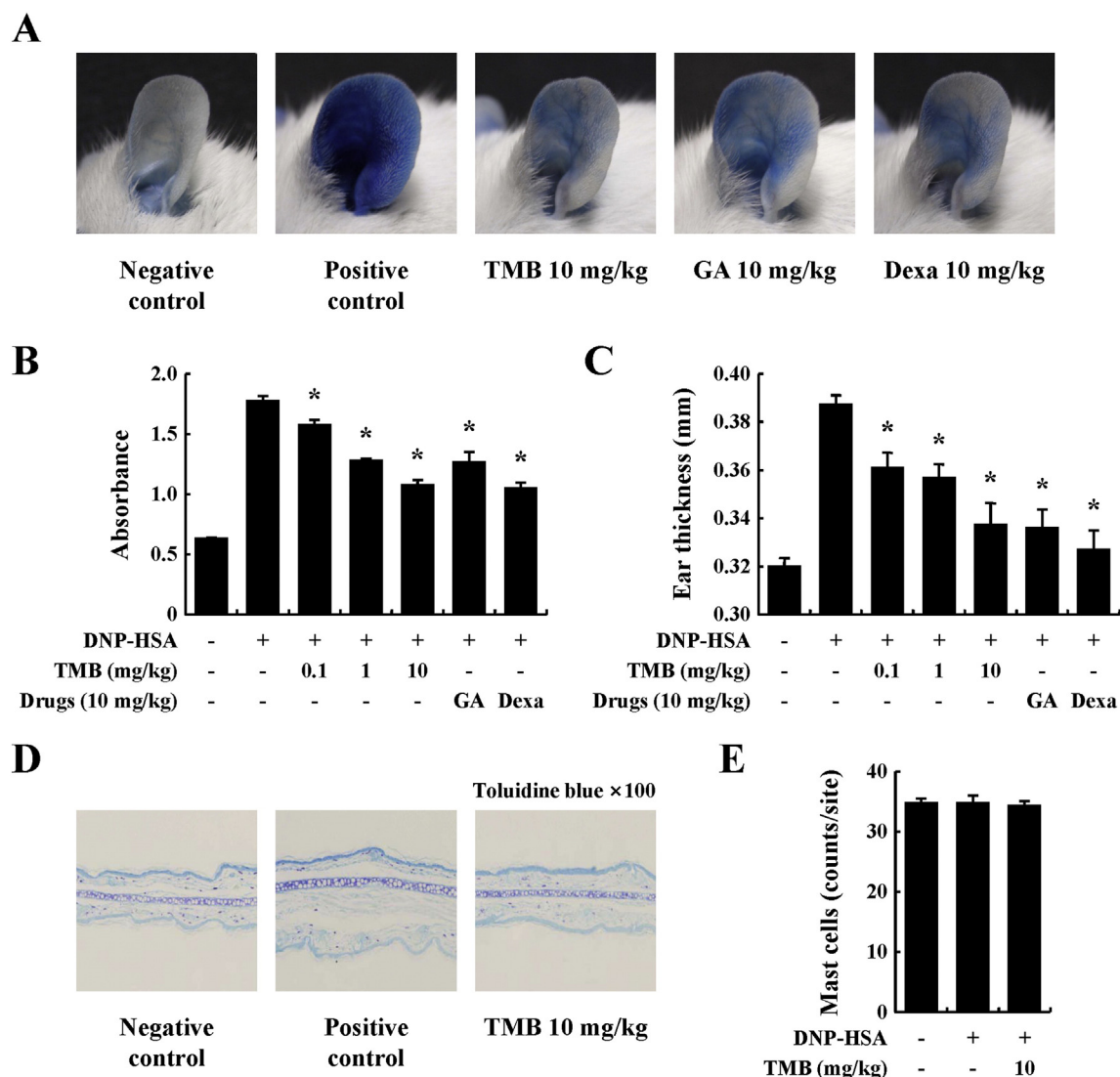


Fig. 6. Effects of TMB on the IgE-mediated PCA. The skin on the ears of the mice ($n = 5$ /group) were sensitized with an intradermal injection of anti-DNP IgE. TMB was administered orally at doses of 0.1–10 mg/kg BW 2 h before the intravenous injection of a DNP-HSA and 4% Evans blue (1:1) mixture. Thirty minutes later, the thickness of both ears was measured, and the ears were collected to measure the dye pigmentation and histology. (A and B) Each amount of the dye was extracted as described in the Materials and methods section and detected using a spectrophotometer. (C) Ear thickness was measured with a dial thickness gauge. (D) Representative photomicrographs of ear sections were stained with toluidine blue. (E) The number of mast cells was counted at the dermis. Each data represents the mean $\pm$ SE ($n = 5$ per group). *Significant difference at $P < 0.05$. GA: gallic acid; Dexa: dexamethasone.

therapeutic candidate for allergic disorders through inhibition of degranulation and pro-inflammatory cytokines expression in mast cells.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2015.05.006>.

Conflict of Interest

The authors have declared no conflict of interest.

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