Original Research

SG-HQ2 inhibits mast cell-mediated allergic inflammation through suppression of histamine release and pro-inflammatory cytokines

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Abstract

In this study, we investigated the effect of 3,4,5-trihydroxy-N-(8-hydroxyquinolin-2-yl)benzamide) (SG-HQ2), a synthetic analogue of gallic acid (3,4,5-trihydroxybenzoic acid), on the mast cell-mediated allergic inflammation and the possible mechanism of action. Mast cells play major roles in immunoglobulin E-mediated allergic responses by the release of histamine, lipid-derived mediators, and pro-inflammatory cytokines. We previously reported the potential effects of gallic acid using allergic inflammation models. For incremental research, we synthesized the SG-HQ2 by the modification of functional groups from gallic acid. SG-HQ2 attenuated histamine release by the reduction of intracellular calcium in human mast cells and primary peritoneal mast cells. The inhibitory efficacy of SG-HQ2 was similar with gallic acid. Enhanced expression of pro-inflammatory cytokines such as tumor necrosis factor-α, interleukin-1β, interleukin-4, and interleukin-6 in activated mast cells was significantly diminished by SG-HQ2 100 times lower concentration of gallic acid. This inhibitory effect was mediated by the reduction of nuclear factor-κB. In animal models, SG-HQ2 inhibited compound 48/80-induced serum histamine release and immunoglobulin E-mediated local allergic reaction, passive cutaneous anaphylaxis. Our results indicate that SG-HQ2, an analogue of gallic acid, might be a possible therapeutic candidate for mast cell-mediated allergic inflammatory diseases through suppression of histamine release and pro-inflammatory cytokines.

Keywords: Allergic inflammation, mast cells, histamine, pro-inflammatory cytokine

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Introduction

Allergy such as allergic rhinitis, asthma, atopic dermatitis, and food allergy is defined as hypersensitivity, which is caused by normally harmless substances. However, allergic disorders are prevalent diseases and result in lethal problems called anaphylaxis, which occurs within a few minutes.[1] Correlations between allergy and inflammation have been determined, which was classified into three phases: early-phase, late-phase, and chronic allergic inflammation.[2] In early-phase reactions, histamine, a major factor on the allergic response, is released from mast cells and induces vasodilation, increasing of vascular permeability, and recruitment of leukocytes. Repetitive allergen exposure changes organic function affecting structural cells and increases production of cytokines, as a result, chronic allergic inflammation occurs.[3]

Mast cells play key roles in immunoglobulin E (IgE)-mediated allergic reactions through secretion of preformed or newly synthesized mediators such as histamine, lipid-derived mediators, chemokines, cytokines, and growth factors.[4] The signaling pathway of mast cells triggered by antigen cross-linking of IgE bound to FcεRI has been previously described. After stimulation of FcεRI, it increases degranulation, production of lipid-derived mediators, and expression of cytokines.[5] Therefore, suppression of histamine release and pro-inflammatory cytokine might be proper therapeutic targets to reduce allergic inflammation. Human mast cells (HMC-1) are known as an appropriate instrument for studying allergic reactions through histamine release and expression of pro-inflammatory cytokines.[6]

Gallic acid (3,4,5-trihydroxybenzoic acid), a polyphenyl natural product obtained from red wine, green tea, and

roots of various herbs, is known to have various biological effects like antioxidation, anti-inflammation, and anticancer.[7–9] In our previous research, we showed the antiallergic inflammatory effects of gallic acid through the inhibition of histamine release and pro-inflammatory cytokine production in mast cells.[10] To increase the efficiency of gallic acid, the amide derivative SG-HQ2 (3,4,5-trihydroxy-N-(8-hydroxyquinolin-2-yl)benzamide) was synthesized through modification of functional groups. Carboxyl group of gallic acid was substituted with amide derivative. In this study, we demonstrated the antiallergic inflammatory effects of SG-HQ2 compared with gallic acid using various *in vitro* and *in vivo* models.

Materials and methods

Animals

Imprinting Control Region mice (male, 6 weeks) and Sprague–Dawley (SD) rats (male, 10 weeks) were purchased from Dae-Han Biolink (Daejeon, Korea). Throughout the study, the animals were housed five per cage in a laminar airflow room maintained at temperature of $22 \pm 2\,^{\circ}C$ and relative humidity of $55 \pm 5\,^{\circ}C$. The care and treatment of the mice were 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

Compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), phorbol 12-mystate 13-acetate (PMA), and calcium ionophore A23187 were purchased from Sigma-Aldrich (St Louis, MO). HMC-1 and rat peritoneal mast cells (RPMC) were grown in Iscove's modified Dulbecco's medium and α-minimum essential medium (GIBCO, Grand Island, NY), respectively, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B at 37 °C in 5% CO_2. The passage ranging 4–8 of HMC-1 cells were used throughout the study.

Preparation of RPMC

The peritoneal cells were isolated from SD rats as previously described.[11] In brief, rats were anesthetized with CO_2 and injected with 50 mL of Tyrode buffer A (137 mmol/L NaCl, 5.6 mmol/L glucose, 12 mmol/L $NaHCO_3$, 2.7 mmol/L KCl, 0.3 mmol/L NaH_2PO_4, and 0.1% gelatin) into the peritoneal cavity, and the abdomen was gently massaged for approximately 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated using a Pasteur pipette. The peritoneal cells were sedimented at 150 g for 10 min at room temperature and resuspended in Tyrode buffer A. Mast cells were separated from the major components of rat peritoneal cells, i.e. macrophages and small lymphocytes. Peritoneal cells were suspended in 1 mL of Tyrode buffer A, layered on 2 mL of Histodenz (Sigma-Aldrich) solution, and centrifuged at 400 g for 10 min at room temperature. The cells remaining at the buffer–Histodenz interface were aspirated and discarded, and the cells in the pellet were washed and resuspended. Mast cell preparations were approximately 95% pure based on toluidine blue staining. More than 97% of the cells were viable based on trypan blue exclusion.

Preparation of SG-HQ2

To a DMF solution (2.0 mL) of 3,4,5-tris(benzyloxy)benzoic acid (220 mg, 0.5 mmol) were added 2-amino-8-quinolinol (81 mg, 0.51 mmol), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (0.12 mg, 0.60 mmol), 1-hydroxybenzotriazole hydrate (11 mg, 81 μmol), and *N,N*-diisopropylethylamine (0.18 mL, 1.0 mmol). After stirring for 24 h, the reaction mixture was diluted with ethyl acetate and washed with water and brine, dried over $MgSO_4$, and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel (ethyl acetate/*n*-hexane = 1:1) to afford SG-HQ1 (170 mg, 58%). ^{1}H-NMR (600 MHz, $CDCl_3$) δ 7.65 (m, 1H), 7.44 (s, 2H), 7.38 (d, 1H, $J = 7.2$ Hz), 7.37–7.32 (m, 6H), 7.29–7.26 (m, 10H), 6.51–6.63 (m, 1H), 5.17 (s, 2H), 5.13 (s, 4H); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 165.0, 157.1, 152.5, 145.1, 140.4, 137.8, 137.5, 136.7, 128.5, 128.5, 128.2, 128.0, 127.6, 125.6, 125.6, 125.0, 124.9, 121.9, 121.8, 112.4, 109.6, 75.1, 71.1; LRMS (ESI) *m/z* 583 (M+H) and 605 (M+Na). A methanol solution (3.0 mL) of SG-HQ1 (59 mg, 0.10 mmol) and 10% Pd/C (16 mg) was placed under an atmosphere of hydrogen. After stirring for 24 h, the reaction mixture was diluted with ethyl acetate, filtered through a short pad of silica gel, and concentrated *in vacuo*. The residue was purified by flash column chromatography on silica gel (dichloromethane/methanol = 10:1) to afforded SG-HQ2 (30 mg, 96%). ^{1}H-NMR (600 MHz, CD_3OD) δ 7.93 (d, 1H, $J = 9.0$ Hz), 7.52 (dd, 1H, $J = 1.2$ and 7.8 Hz), 7.30–7.27 (m, 3H), 7.20 (t, 1H, $J = 7.8$ Hz), 6.81 (d, 1H, $J = 9.0$ Hz); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 166.1, 158.5, 145.2, 145.1, 144.5, 140.5, 139.0, 138.3, 137.6, 124.6, 122.3, 121.9, 120.9, 119.4, 117.6, 112.7, 112.2, 111.4, 109.5, 108.6; LRMS (ESI) *m/z* 313 (M+H). SG-HQ2 and gallic acid were dissolved in dimethyl sulfoxide (DMSO) and then diluted with phosphate-buffered saline (PBS). Proportion of DMSO in the treated samples was regulated to be less than 1% to minimize the toxic effects on the mast cells and mice.

Histamine assay

To determinate histamine level in serum and mast cells, *o*-phthaldialdehyde spectrofluorometric procedure was carried out, and the fluorescence intensity was detected using fluorescent plate reader (Molecular Devices, Sunnyvale, CA) at an excitation of 360 nm and an emission of 440 nm, as previously described.[12] The blood from the mice was centrifuged at 400 g for 15 min at 4 °C, and the serum was withdrawn to measure histamine content. HMC-1 cells (1×10^6/well in 24-well plates) were pretreated with or without SG-HQ2 for 30 min and then stimulated with PMA (40 nmol/L) plus A23187 (1 μmol/L) for 8 h. RPMC (2×10^4/well in 24-well plates) were pretreated with or without SG-HQ2 for 30 min and then stimulated with compound 48/80 (5 μg/mL) for 10 min. The cells were

separated from the released histamine by centrifugation at 400 g for 5 min at 4 °C.

Intracellular calcium

The intracellular calcium was measured with the use of the fluorescence indicator Fluo-3/AM (Invitrogen, Carlsbad, CA).[12] HMC-1 cells (1×10^5/well in 96-well plates) and RPMC (1×10^4/well in 96-well plates) were preincubated with Fluo-3/AM for 1 h at 37 °C. After washing the dye from the cell surface with Tyrode buffer B (137 mmol/L NaCl, 5.5 mmol/L glucose, 12 mmol/L $NaHCO_3$, 2.7 mmol/L KCl, 0.2 mmol/L NaH_2PO_4, 1 mmol/L $MgCl_2$, and 1.8 mmol/L $CaCl_2$), the cells were pretreated with or without SG-HQ2 for 30 min and then stimulated with PMA (40 nmol/L) plus A23187 (1 μmol/L) or compound 48/80 (5 μg/mL). The fluorescence intensity was detected using fluorescent plate reader at an excitation of 485 nm and an emission of 520 nm. Intracellular calcium was calculated using relative absorbance of control value compared with the control and expressed.

RNA extraction and real-time polymerase chain reaction

Prior to isolation of total cellular RNA, HMC-1 cells (1×10^6/well in 24-well plates) were pretreated with or without SG-HQ2 for 30 min and stimulated with PMA (40 nmol/L) plus A23187 (1 μmol/L) for 2 h. RNAiso Plus reagent (Takarabio Inc., Shiga, Japan) was used for extraction, according to the manufacturer's protocol. The complementary DNA (cDNA) was synthesized from 2 μg of total RNA using the Maxime RT-Pre Mix Kit (iNtRON Biotechnology, Daejeon, Korea). The quantitative real-time polymerase chain reaction (PCR) was carried out using the Thermal Cycler Dice TP850 (Takarabio Inc.), according to the manufacturer's protocol. The 25 μL reaction mixture was made up of: 2 μL of cDNA (200 ng), 1 μL of each forward and reverse primer (0.4 μmol/L), 12.5 μL of SYBR Premix Ex Taq (Takarabio Inc.), and 8.5 μL of dH_2O. The conditions for the PCR steps were similar to our previous research.[13]

Protein extraction and Western blot

Nuclear and cytosol proteins were extracted as described previously.[14] Before protein extraction, HMC-1 cells (2×10^6/well in 6-well plates) were pretreated with or without SG-HQ2 for 30 min and stimulated with PMA (40 nmol/L) plus A23187 (1 μmol/L) for 2 h. After suspension in 100 μL of cell lysis buffer A (0.5% Triton X-100, 150 mmol/L NaCl, 10 mmol/L (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) [HEPES], 1 mmol/L ethylenediaminetetraacetic acid [EDTA]/Na_3VO_4, 0.5 mmol/L phenylmethanesulfonylfluoride/dithiothreitol [PMSF/DTT], and 5 μg/mL leupeptin/aprotinin), cells were voltexed, incubated for 5 min on ice, centrifuged 400 g for 5 min at 4 °C, and then the supernatant was gathered as cytosol protein extracts. Pellets washed thrice with 1 mL of PBS were suspended in 25 μL of cell lysis buffer B (25% glycerol, 420 mmol/L NaCl, 20 mmol/L HEPES, 1.2 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 1 mmol/L Na_3VO_4, 0.5 mmol/L PMSF/DTT, and 5 μg/mL leupeptin/aprotinin), voltexed, sonicated for 30 s, incubated for 20 min on ice, centrifuged 15,000 g for 15 min at 4 °C, and then supernatant was gathered as nuclear protein extracts. Samples of protein were electrophoresed using 8% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Immunodetection was carried out using chemiluminescent substrate (Thermo scientific, Waltham, MA).

Compound 48/80-induced systemic anaphylaxis

Mice ($n = 5$/group) were given an intraperitoneal injection of 8 mg/kg body weight (BW) of the mast cell degranulator compound 48/80. SG-HQ2 was administered intraperitoneally at doses of 0.1–10 mg/kg BW 1 h before the injection of compound 48/80. One hour after the injection of degranulator, blood was obtained from the abdominal artery of each mouse to measure serum histamine level.[11]

IgE-mediated passive cutaneous anaphylaxis

An IgE-mediated passive cutaneous anaphylaxis (PCA) was carried out as described previously.[15] To induce the PCA reaction, ear skins of mice ($n = 5$/group) were sensitized with an intradermal injection of anti-DNP IgE (0.5 μg/site) for 48 h. SG-HQ2 was administered intraperitoneally at doses of 0.1–10 mg/kg BW 1 h before the intravenous injection of DNP-HSA (1 μg/mouse) and 4% Evans blue (1:1) mixture. Thirty minutes after the challenge, the mice were euthanized, and both ear skins were collected for measurement of the pigment dye. The amount of dye was determined colorimetrically after extraction with 1 mL of 1 mol/L KOH and 9 mL of acetone and phosphoric acid (5:13) mixture. The absorbent intensity of the extract was detected by spectrophotometry at 620 nm.

Statistical analysis

Each data represents the mean ± SE of three independent experiments, and 5 mice per group were used in the *in vivo* experiments. Statistical analyses were performed using Prism5 (GraphPad Software, San Diego, CA), and treatment effects were analysed using a one-way ANOVA and then Dunnett's test. $P < 0.05$ was used to indicate significant differences throughout the study.

Results

Effects of SG-HQ2 on the degranulation of mast cells

Histamine released from mast cells is one of the most important mediators in allergic reactions and the therapeutic target for allergic disorders.[3] We evaluated the inhibitory effect of SG-HQ2 on the histamine release from HMC-1 and RPMC. Histamine release was increased after stimulation with PMA plus A23187 on HMC-1 cells and with compound 48/80 on RPMC. Pretreatment of SG-HQ2 dose-dependently suppressed histamine release from both mast cells (Figure 1). Equal inhibitory effect was shown between SG-HQ2 and gallic acid at the same

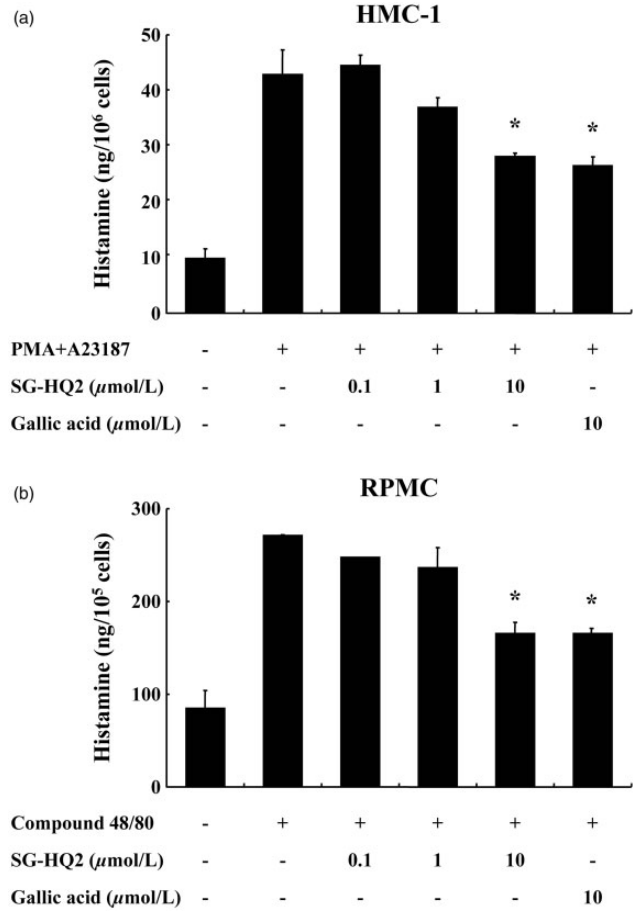

Figure 1 Effects of SG-HQ2 on the histamine release from mast cells: (a) HMC-1 cells (1×10^6/well) were pretreated with or without SG-HQ2 for 30 min and then stimulated with PMA (40 nmol/L) plus A23187 (1 μmol/L) and (b) RPMC (2×10^4/well) were pretreated with or without SG-HQ2 for 30 min and then stimulated with compound 48/80 (5 μg/mL). Histamine level was detected by fluorescent plate reader. Each data represents the mean ± SE of three independent experiments. *Significant difference at $P < 0.05$.
SG-HQ2: (3,4,5-trihydroxy-N-(8-hydroxyquinolin-2-yl)benzamide); HMC-1: human mast cells; PMA: phorbol 12-mystate 13-acetate; RPMC: rat peritoneal mast cells; SE: standard error

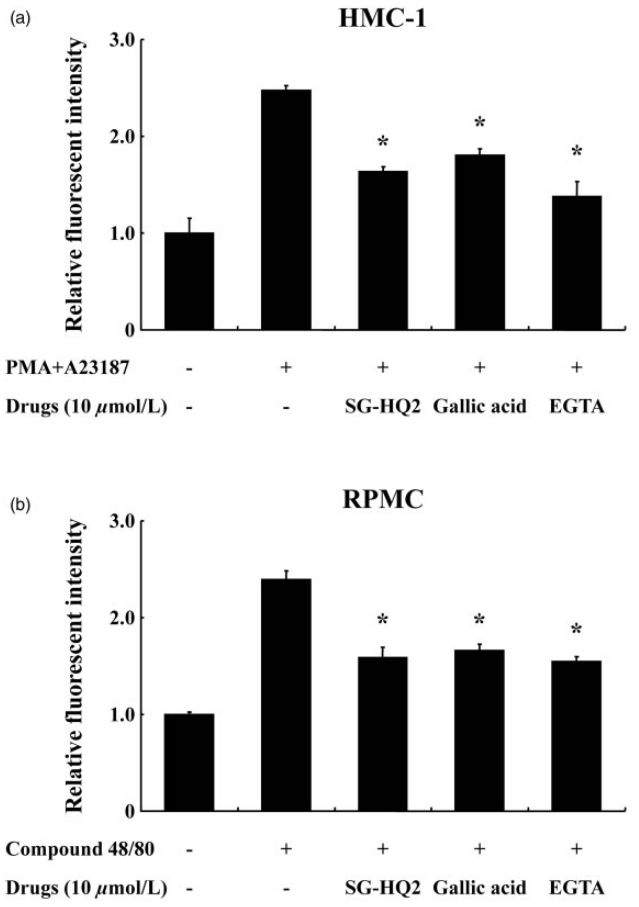

Figure 2 Effects of SG-HQ2 on the intracellular calcium in mast cells: (a) HMC-1 cells (1×10^5/well) were pretreated with or without SG-HQ2 for 30 min and then stimulated with PMA (40 nmol/L) plus A23187 (1 μmol/L) and (b) RPMC (1×10^4/well) were pretreated with or without SG-HQ2 for 30 min and then stimulated with compound 48/80 (5 μg/mL). EGTA, a calcium chelator, was used as a positive control. Intracellular calcium was detected using fluorescent plate reader. Each data represents the mean ± SE of three independent experiments. *Significant difference at $P < 0.05$.
SG-HQ2: (3,4,5-trihydroxy-N-(8-hydroxyquinolin-2-yl)benzamide); HMC-1: human mast cells; PMA: phorbol 12-mystate 13-acetate; RPMC: rat peritoneal mast cells; EGTA: Ethylene glycol tetraacetic acid; SE: standard error

concentration (10 μmol/L). β-Hexosaminidase assay is also widely used to detect degranulation of mast cells.[16] SG-HQ2 and gallic acid showed similar suppressive effects (Supplementary Figure 1A). To confirm the cytotoxicity of SG-HQ2, XTT assay FACS analysis were carried out. SG-HQ2 did not induce cytotoxicity up to 100 μmol/L during 24 h (Supplementary Figure 1B, C). As a result, SG-HQ2 similarly inhibited the degranulation of mast cells in comparison with gallic acid without cytotoxicity.

Effects of SG-HQ2 on the intracellular calcium in mast cells

Calcium is a key messenger in the mast cell activation signaling.[17] Stimulation of PLCγ triggered by antigen cross-linking of IgE bound to FcεRI causes calcium release from endoplasmic reticulum through the generation of IP_3 and finally extracellular calcium flows in the cytoplasm.[18] Calcium influx is essential for degranulation through binding of synaptotagmin to the SNARE complex, which results in granule fusion-to-cell membranes.[19] To study the mechanism of the inhibitory effect on the histamine release by SG-HQ2, we assayed the intracellular calcium level. Intracellular calcium was increased after stimulation with PMA plus A23187 on HMC-1 cells and with compound 48/80 on RPMC. SG-HQ2 decreased intracellular calcium in both stimulated mast cells, and the reduction was similar with gallic acid (Figure 2). Ethylene glycol tetraacetic acid (EGTA), a calcium chelator, was used as a positive control.

Effects of SG-HQ2 on the expression of pro-inflammatory cytokines

Pro-inflammatory cytokines are concerned with various inflammatory responses such as recruitment and activation of immune cells, as well as tissue remodeling in chronic

inflammation.[20] To assess the influences of SG-HQ2 on the expression of pro-inflammatory cytokines in HMC-1 cells, real-time PCR was carried out. Gene expression of these cytokines was induced by stimulation with PMA plus A23187 after pretreatment of SG-HQ2 for 30 min. The results showed the concentration-dependent suppression of tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β, IL-4, and IL-6 by SG-HQ2 (Figure 3). SG-HQ2 indicated similar or more powerful inhibition in comparison with gallic acid, i.e. TNF-α: 64% inhibition at 100 nmol/L of SG-HQ2, 55% inhibition at 10 μmol/L of gallic acid.

Effects of SG-HQ2 on the activation of nuclear factor-κB

It is formerly informed that translocation of nuclear factor (NF)-κB from cytoplasm to nucleus following degradation of IκBα induces transcription of pro-inflammatory cytokines.[21] To determine the suppressive mechanism on the pro-inflammatory cytokines, activation of NF-κB was evaluated. In our results, nuclear translocation of NF-κB and degradation of IκBα was shown on the stimulated HMC-1 cells with PMA plus A23187, and then SG-HQ2 significantly hindered both translocation and degradation even 100 times lower concentration of gallic acid (Figure 4).

Effects of SG-HQ2 on the compound 48/80-induced systemic anaphylaxis

The animal model for the systemic anaphylaxis was used to examine immediate-type allergic reaction. Compound 48/80, a degranulator of mast cells, was used to induce lethal systemic anaphylaxis.[11] Intraperitoneal injection of compound 48/80 caused sudden increasing of histamine concentrations in the mice serum, eventually it resulted in lethal effects on the mice. After administration of compound 48/80 (8 mg/kg BW), we monitored mice for 1 h. Serum histamine level was dramatically increased by compound 48/80 and decreased in case of dose-dependent injection of SG-HQ2 (Figure 5). Similar to the results of *in vitro* histamine assay using mast cells, SG-HQ2 and gallic acid showed suppressive effects on the serum histamine release.

Effects of SG-HQ2 on the IgE-mediated PCA

Another animal model, IgE-mediated PCA, was used to confirm the antiallergic effects of SG-HQ2.[15] Anti-DNP IgE (0.5 μg/site) was intradermally injected prior to the

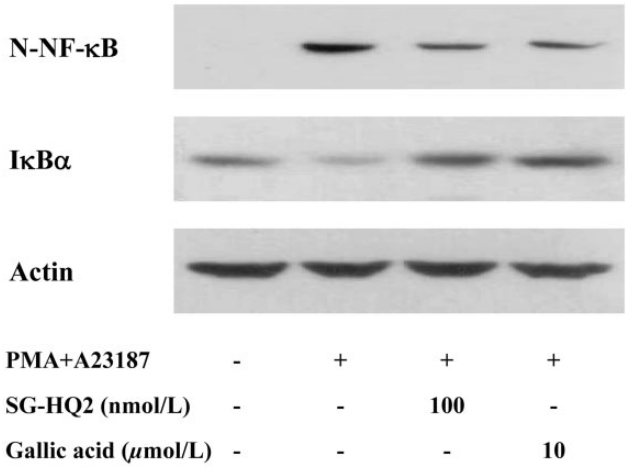

Figure 4 Effects of SG-HQ2 on the activation of NF-κB. Effects of SG-HQ2 on the expression of pro-inflammatory cytokines. HMC-1 cells (2×10^6/well) were pretreated with or without SG-HQ2 for 30 min and then stimulated with PMA (40 nmol/L) plus A23187 (1 μmol/L). Nuclear translocation of NF-κB was assayed by Western blot (N-NF-κB, nuclear NF-κB). The band is a representative of three independent experiments.
SG-HQ2: (3,4,5-trihydroxy-N-(8-hydroxyquinolin-2-yl)benzamide); HMC-1: human mast cells; PMA: phorbol 12-mystate 13-acetate; NF-κB: nuclear factor-kappa B

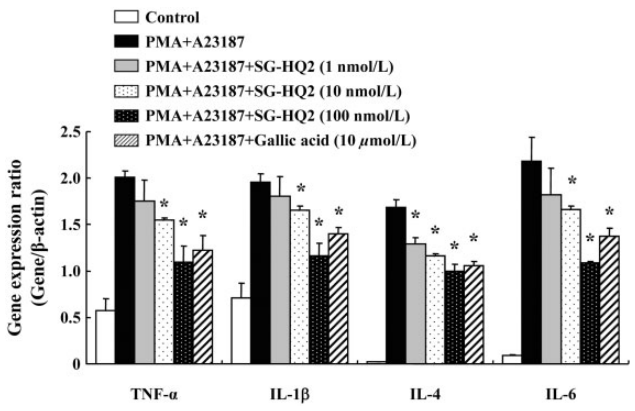

Figure 3 Effects of SG-HQ2 on the expression of pro-inflammatory cytokines. HMC-1 cells (1×10^6/well) were pretreated with or without SG-HQ2 for 30 min and then stimulated with PMA (40 nmol/L) plus A23187 (1 μmol/L). Extraction and analysis of mRNA was performed as described in Materials and methods. The gene expression of pro-inflammatory cytokines was determined by real-time PCR. Each data represents the mean ± SE of three independent experiments. *Significant difference at $P < 0.05$.
SG-HQ2: (3,4,5-trihydroxy-N-(8-hydroxyquinolin-2-yl)benzamide); HMC-1: human mast cells; PMA: phorbol 12-mystate 13-acetate; PCR: polymerase chain reaction; SE: standard error

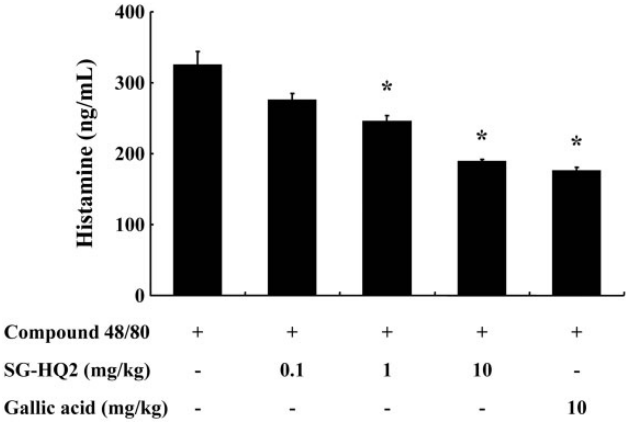

Figure 5 Effects of SG-HQ2 on the compound 48/80-induced systemic anaphylaxis. Mice ($n = 5$/group) were given an intraperitoneal injection of 8 mg/kg BW of the mast cell degranulator compound 48/80. SG-HQ2 was administered intraperitoneally at doses of 0.1–10 mg/kg BW 1 h before the injection of compound 48/80. The blood was obtained from the abdominal artery of each mouse to measure serum histamine level. Serum histamine level was detected by fluorescent plate reader. Each data represents the mean ± SE of three independent experiments. *Significant difference at $P < 0.05$.
SG-HQ2: (3,4,5-trihydroxy-N-(8-hydroxyquinolin-2-yl)benzamide); BW: body weight; SE: standard error

intravenous injection of DNP-HSA (1 μg/mouse) and 4% Evans blue (1:1) mixture to induce PCA reaction in mice ears. After challenge of antigen, blue spot was shown in sensitized site because of increased vascular permeability through histamine release from mast cells. The administration of SG-HQ2 reduced the size of the spot and attenuated the blue color in a dose-dependent manner (Figure 6B). SG-HQ2 and gallic acid presented similar reductions on the pigment absorbance. These reductions of absorbance are conclusively related to the inhibition of histamine release from mast cells.

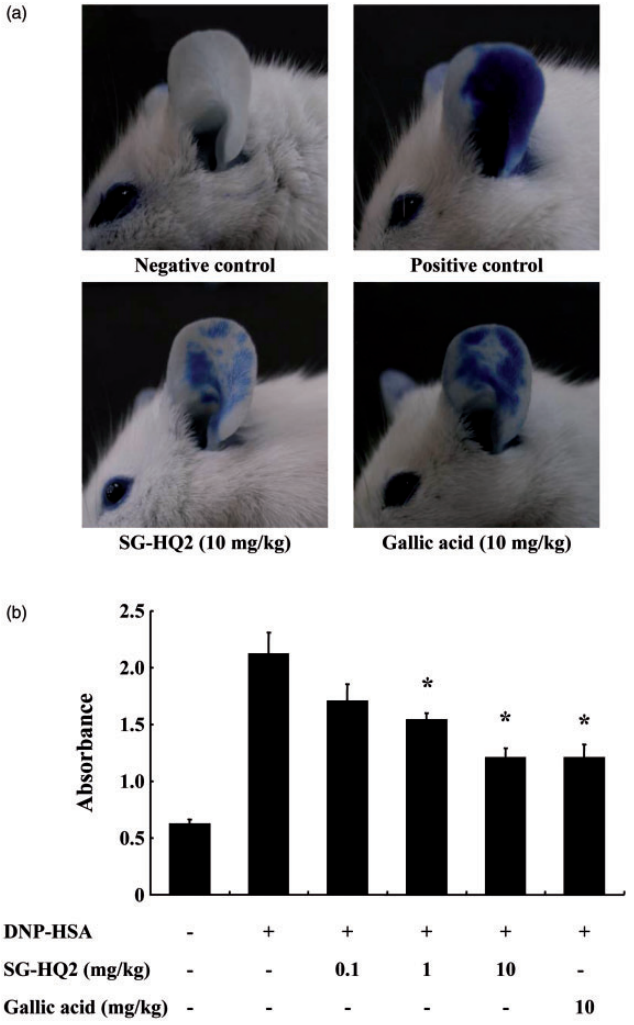

Figure 6 Effects of SG-HQ2 on the IgE-mediated PCA. Ear skins of mice ($n = 5$/group) were sensitized with an intradermal injection of anti-DNP IgE (0.5 μg/site) for 48 h. SG-HQ2 was administered intraperitoneally at doses of 0.1–10 mg/kg BW 1 h before the intravenous injection of DNP-HSA (1 μg/mouse) and 4% Evans blue (1:1) mixture. Thirty minutes after the challenge, both ear skins were collected for measurement of the pigment dye. Each amount of dye was extracted as described in Materials and methods and detected by spectrophotometry. Each data represents the mean ± SE of three independent experiments. *Significant difference at $P < 0.05$. (A color version of this figure is available in the online journal.)
SG-HQ2: (3,4,5-trihydroxy-N-(8-hydroxyquinolin-2-yl)benzamide); IgE: immunoglobulin E; PCA: passive cutaneous anaphylaxis; anti-DNP: anti-dinitrophenyl; DNP: dinitrophenyl; HSA: human serum albumin; SE: standard error

Discussion

Development of therapeutic candidates directly working on mast cells, major producers of histamine, is important because it is widely known that histamine plays a crucial role in allergic responses. In our previous reports, epigallocatechin gallate (EGCG) and gallic acid, a part of EGCG, attenuated mast cell-mediated allergic responses.[10,22] The interesting biological roles of gallic acid led us to explore synthesis of gallic acid derivatives. To increase therapeutic efficiency of gallic acid, ester and amide derivatives were investigated and their inhibitory effects on allergic inflammation were examined.

In the present study, we demonstrated that SG-HQ2 has antiallergic inflammatory effect through regulation of degranulation and expression of pro-inflammatory cytokines on mast cells. First of all, release of histamine and β-hexosaminidase was measured to assess degranulation of mast cells. Typical allergic symptoms such as edema, warmth, and erythema are caused by histamine, which results in vasodilation, increase of vascular permeability, and recruitment of leukocytes.[3] As a result, suppression of mast cell degranulation, especially release of histamine, is critical to reduce allergic disorders. There are numerous researches about mast cell degranulation using compound 48/80 and PMA plus A23187. Increased histamine and β-hexosaminidase release on stimulated HMC-1 cells with PMA plus A23187 were decreased by treatment of SG-HQ2 in a dose-dependent manner. To complement the defect of HMC-1 cells as modified cell line, RPMC, fully matured mast cells, were used to confirm the inhibitory effect of SG-HQ2 on the histamine release. SG-HQ2 showed similar result in both HMC-1 cells and RPMC. The compound 48/80-induced systemic anaphylaxis and the IgE-mediated PCA model are used extensively to assess the *in vivo* antiallergic effect, and it is well known that histamine is a key molecule in both anaphylaxis. SG-HQ2 had a suppressive effect on both the animal models, which might be caused by reduction of histamine release from mast cells.

Calcium is known as a major factor in the signaling pathway of mast cell degranulation.[17] Increase of intracellular calcium level in activated mast cells affects SNARE complex, which makes exocytosis more effective through granule fusion-to-cell membranes.[19] Accordingly, obstruction of calcium influx across plasma membranes could be a proper target to inhibit mast cell degranulation. In our study, calcium ionophore A23187 caused calcium influx in mast cells, and SG-HQ2 has an effect on the reduction of intracellular calcium. Similar tendency was shown in RPMC stimulated with compound 48/80, and SG-HQ2 and gallic acid reduced the intracellular calcium as much as EGTA. These results indicate that SG-HQ2 inhibits mast cell degranulation by blocking calcium movement across the cell membrane and eventually acute-phase allergic reaction caused by histamine might be suppressed.

Another function of mast cells is a main provider of pro-inflammatory cytokines. Various cytokines from mast cells are more important in chronic-phase allergic reactions. To investigate antiallergic inflammatory effect of SG-HQ2,

expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-4, and IL-6 was measured. TNF-α, a major cytokine released from mast cells, plays a key role in the adaptive immunity by activating NF-κB and regulates adhesion molecules and transendothelial migration of immune cells.[23,24] IL-1β is activated in inflammasome of mast cells and macrophages.[13,25] Inflammasome-dependent IL-1β affects various allergic diseases such as contact hypersensitivity, atopic dermatitis, and bronchial asthma.[26] IL-4 related to the production of IgE and the recruitment of eosinophil is well known as one of the Th2-type cytokines that is associated with allergic reaction.[27] IL-6 is known to maintain $CD4^+$ T cell survival and promote Th2 modulation.[28] Local accumulation of IL-6 produced from mast cells is concerned with PCA reaction.[29] These reports suggest that the inhibition of pro-inflammatory cytokines is also important to remedy allergic disorders. In our study, transcription of pro-inflammatory cytokines was increased on HMC-1 cells stimulated with PMA plus A23197, which was suppressed by SG-HQ2 in a dose dependent manner.

Intracellular calcium has been known to regulate not only histamine release but also gene expression of pro-inflammatory cytokines. There are several reports regarding the correlations between intracellular calcium and gene expression of inflammatory cytokines such as TNF-α and IL-6 on mast cells.[30,31] In our results, SG-HQ2 suppressed expression of pro-inflammatory cytokines more effectively at 100 times lower concentration compared with gallic acid although both drugs similarly reduced intracellular calcium. To find the reason for the higher suppression of pro-inflammatory cytokines by the SG-HQ2, activation of NF-κB was measured. NF-κB is a major mediator to regulate gene transcription on mast cells.[21] NF-κB moves into the nucleus after degradation of IκBα. In the nucleus, NF-κB plays a transcription factor of various pro-inflammatory cytokines. Our results showed that considerably increased nuclear NF-κB on stimulated HMC-1 cells was suppressed by SG-HQ2 at even 100 times lower concentration than gallic acid. Accordingly, from these results, we could speculate that inhibition of pro-inflammatory cytokines by SG-HQ2 might be more closely related to NF-κB than intracellular calcium. The mitogen-activated protein kinase (MAPK) cascade is also known to be associated with the expression of pro-inflammatory cytokines.[32] In our previous research, gallic acid inhibited the activation of p38 MAPK.[10] Therefore, it is possible that SG-HQ2 attenuates expression of pro-inflammatory cytokines by working on the activation of MAPK as well as NF-κB.

In this study, we showed the antiallergic inflammatory effect of SG-HQ2. Our data showed that SG-HQ2 inhibited histamine release and expression of pro-inflammatory cytokines, and it was associated with the reduction of intracellular calcium and NF-κB. The results give an insight about the antiallergic inflammatory mechanism of SG-HQ2 on mast cells. Conclusively, we expect that SG-HQ2 might be a candidate to remedy allergic inflammatory disorders by working on mast cells.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the data, and review of the manuscript. IGJ and HHK performed the major experiments and wrote the manuscript. PHP and TKK have made substantial contributions to the conception and design of the study. SYS, TYS, and SHK supervised the research and co-wrote the manuscript.

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