

Novel small molecule MRGPRX2 antagonists inhibit a murine model of allergic reaction



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Background: Activation of Mas-related G protein-coupled receptor X2 (MRGPRX2) is a crucial non-IgE pathway for mast cell activation associated with allergic reactions and inflammation. Only a few peptides and small compounds targeting MRGPRX2 have been reported, with limited information on their pharmacologic activity.

Objective: We sought to develop novel small molecule MRGPRX2 antagonists to treat MRGPRX2-mediated allergies and inflammation.

Methods: A computational approach was used to design novel small molecules as MRGPRX2 antagonists. The short-listed molecules were synthesized and characterized by liquid chromatography and mass spectrometry as well as nuclear magnetic resonance. Inhibitory activity on MRGPRX2 signaling was assessed *in vitro* by using functional bioassays (β -hexosaminidase, calcium flux, and chemokine synthesis) and receptor activation assays (β -arrestin recruitment and Western blot analysis) in human LAD-2 mast cells and HTLA cells.

In vivo effects of the novel MRGPRX2 antagonists were assessed using a mouse model of acute allergy and systemic anaphylaxis. **Results:** The novel small molecules demonstrated higher binding affinity with MRGPRX2 in the docking study. The half-maximal inhibitory concentration is in the low micromolar range (5–21 μ M). The small molecules inhibited not only the early phase of mast cell activation but also the late phase, associated with chemokine and prostaglandin release. Further, Western blot analysis revealed inhibition of downstream phospholipase C- γ , extracellular signal-regulated protein kinase 1/2, and Akt signaling pathway. Moreover, in the mouse models of allergies, small molecule administration effectively blocks acute, systemic allergic reactions and inflammation and prevents systemic anaphylaxis.

Conclusion: The small molecules might hold a significant therapeutic promise to treat MRGPRX2-mediated allergies and inflammation. (*J Allergy Clin Immunol* 2023;151:1110–22.)

Key words: Inflammation, mast cell activation, non-IgE-mediated allergies, skin, structure aided drug designing

Allergies have become prevalent worldwide and common in all age groups. In the United States, allergies are the sixth leading cause of chronic illness, affecting over 50 million Americans at a cost of \$18 billion a year.^{1,2} Allergies not only reduce quality of life and cause an economic burden but also deprive people from receiving life-saving vaccines and drugs.^{3,4} Mas-related G protein-coupled receptor X2 (MRGPRX2) is a novel G protein-coupled receptor (GPCR) that provokes a prominent non-IgE-mediated immune reaction in skin-related diseases, asthma, and even coronavirus disease 2019 vaccine-induced allergic reactions.^{4,6} Several endogenous peptides, allergens, infectious agents, toxins, and US Food and Drug Administration (FDA)-approved drugs such as fluoroquinolone antibiotics, neuromuscular blockers, opioids, and diagnostic agents have been reported to cause allergic and inflammatory reactions via MRGPRX2-mediated mast cell (MC) activation.^{7,8}

Critical evidence, including functional expression of MRGPRX2 on skin MCs, involvement of the murine homolog Mas-related G protein-coupled receptor B2 (MRGPRB2) in allergic conditions in mice, and upregulation of MRGPRX2 expression in the skin of patients with skin diseases, strongly suggests the existence of a pathophysiologic role of MRGPRX2 in allergic diseases.^{9,10} Despite the availability of different class of antiallergic drugs, most are for symptomatic use. Moreover, patient disease may be refractory to current medications.¹¹ Therefore, it is crucial to develop novel treatment strategies for MC-related allergic diseases.

Recently, MRGPRX2 has been established as a potential therapeutic target through observations that MRGPRX2's (and its mouse ortholog, MRGPRB2's) knockdown in human MCs or mice resulted in a significant abolishment of MC degranulation,¹² allergic reactions,^{12,13} atopic dermatitis,¹⁴ and nonhistaminergic itch.¹⁵ In addition, MRGPRX2 transcript and protein levels are increased in patients with chronic urticaria and rocuronium-induced allergic reactions.¹⁶ Therefore, developing novel antagonists of MRGPRX2 possesses tremendous therapeutic and social impact. In line with this, recent efforts have been invested in the peptide-, small compound-, or natural compound-based antagonist development of MRGPRX2, although none has been approved for clinical use to date.⁷

Two recent findings on the ligand-binding site of MRGPRX2 have improved our understanding of MRGPRX2 and will expedite the development of strategies.^{17,18} We previously identified the natural compound genistein as a lead molecule for MRGPRX2 antagonist development.¹⁹ In the present study, we integrated structure-aided drug design, docking, synthetic, and

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Abbreviations used

DRC:	Dose–response curve
EC ₅₀ :	Half-maximal effective concentration
E _{max} :	Maximum efficiency
ERK1/2:	Extracellular signal-regulated protein kinase 1/2
FDA:	US Food and Drug Administration
GPCR:	G protein–coupled receptor
IC ₅₀ :	Half-maximal inhibitory concentration
LAD-2:	Laboratory of Allergic Diseases 2
MC:	Mast cell
MCP-1:	Monocyte chemoattractant protein 1
MRGPRB2/X2:	Mas-related G protein–coupled receptor B2/X2
PDB:	Protein Data Bank (http://www.wwpdb.org/)
PGD ₂ :	Prostaglandin D ₂
PLC-γ:	Phospholipase C-γ
PMC:	Peritoneum-derived MC
STIM1:	Sensor stromal interaction molecule 1
Tango:	Transcriptional output, with transcriptional activation following arrestin translocation screening system (aka PRESTO-Tango)

experimental pharmacologic approaches to design and develop novel small molecule MRGPRX2 antagonists from the lead molecule. By using a battery of *in vitro* and *in vivo* assays, we found that these novel small molecules can inhibit MRGPRX2-mediated MC degranulation, release of chemokine, prostaglandin, and downstream signaling pathway. In addition, parenteral administration of small molecules significantly reduced tissue permeability, inflammation, and anaphylaxis symptoms in mice models of allergies. This indicates that the novel small molecules can be further developed as a therapeutic drug candidate to resolve MRGPRX2/MRGPRB2-mediated allergic reactions and other inflammatory diseases.

METHODS

Molecular docking and pharmacophore identification

We used our previously developed homology model of MRGPRX2,²⁰ which was compared and verified with the recently identified 3-dimensional crystal structure of MRGPRX2 (Protein Data Bank [PDB] 7S8N; www.wwpdb.org).¹⁸ Molecular docking simulations were performed by Auto Dock Vina,²¹ and pharmacophore regions of lead molecule genistein were identified by Pharmit.²²

In silico designing and screening of potential novel small molecules

On the basis of the pharmacophore and amino acid interaction map of the lead molecule with MRGPRX2, we used the strategy of structure extension and variation in ring size. This involved introducing the functional group and aromatic rings to the lead structure to probe for extra binding interactions with the MRGPRX2. As a result, we designed a library of novel small molecules by introducing different functional groups at different carbon positions of genistein. The novel small molecules were screened, short-listed, and further optimized for affinity through a cycle of structural derivatization and docking.

Small molecule synthesis and characterization

Synthesis, purification, and characterization of novel small molecules were performed as described in the Methods in this article's Online Repository at www.jacionline.org.

Mice

Wild-type C57BL/6 adult male mice, 6 to 8 weeks old, were purchased from the Laboratory Animal Unit of the University of Hong Kong (accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International). Mice were housed under a 24-hour light/dark cycle, with food and water *ad libitum*. The experimental protocols for the mouse experiment were approved by the Committee on the Use of Live Animals in Teaching and Research of the university (approval 5125-19).

Statistical analysis

All comparisons were performed by GraphPad Prism (GraphPad Software, La Jolla, Calif) or Microsoft Excel (Microsoft, Redmond, Wash) software. The graphs were plotted by GraphPad Prism 9.3.1; data are presented as means ± SEMs of at least 3 independent experiments unless otherwise specified. *P* values are shown on top of the corresponding columns, as determined by 1-way ANOVA followed by the Dunnett multiple comparisons test, Mann-Whitney test, Kruskal-Wallis test, or unpaired 2-sided *t* test, as appropriate and as indicated in the figures.

Detailed methodology is provided in the Methods in the Online Repository at www.jacionline.org.

RESULTS

Molecular docking and pharmacophore identification of the lead molecule

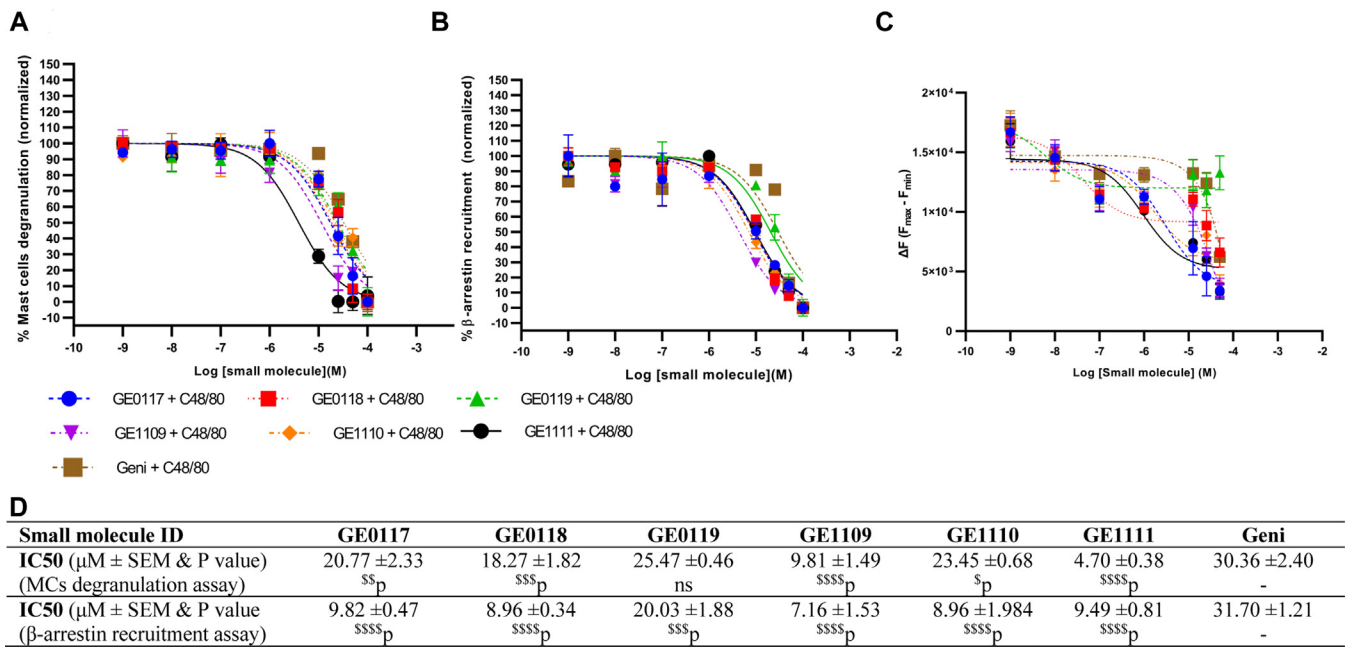
Using molecular docking with MRGPRX2 (PDB 7S8N), we showed that genistein bound at the shallow, negatively charged pocket of MRGPRX2 (see Fig E1, A and B, in the Online Repository at www.jacionline.org). The negatively charged pocket comprises Trp248, Phe244, Glu164, Asp184, Trp243, and Leu247,¹⁸ and ionic interaction with Glu164 and Asp184 has been reported to be crucial for ligand recognition and MRGPRX2 activation^{18,23} (see Fig E2, A and B, in the Online Repository).

Designing and screening of novel small molecules

We postulated that introducing diverse functional groups at different positions or coupling the chromone and phenol ring with other heterocyclic scaffolds may increase the binding interaction.^{24,25} We have designed a series of novel small molecules by substituting the hydrogen atom of the naphthoate ring at C17/C21 (Fig E2, D) and C18/C22 (Fig E2, E) with methyl acetate and carbamide; we found a significant increase in the binding affinity and amino acid interaction. The *in silico* results showed that 6 short-listed novel small molecules (GE0117, GE0118, GE0119, GE1109, GE1110, and GE1111) are binding at the same binding site as genistein with comparatively high affinity at the MRGPRX2 binding pocket via several hydrogen bonds, π -sigma, π - π stacking, π -alkyl, and π -sulfur bonds (Fig E1, D-I, and Fig E2, G-L). The short-listed molecules were reviewed for synthesis feasibility and drug-like properties²⁶ (see Table E2 in the Online Repository at www.jacionline.org), then short-listed for chemical synthesis.

Synthesis and characterization of novel target molecules

All the novel small molecules were synthesized via Suzuki coupling between naphthalimide/phenalenone derivatives and naphthoate ring core.²⁷ The synthesized novel small molecules were characterized by nuclear magnetic resonance, infrared radiation, and high resolution mass spectrometry (HRMS); purity (>95% for all compounds) was determined by



liquid chromatography and mass spectrometry as well as high-performance liquid chromatography. Complete data are available in the Methods in the Online Repository at www.jacionline.org.

Novel small molecules inhibit *in vitro* MC degranulation and MRGPRX2 receptor activation

MRGPRX2 mediate MC degranulation to release their stored and *de novo* synthesized inflammatory mediators to surrounding tissues.²⁸ To evaluate the antagonistic potential of novel small molecules, we measured their effects on human Laboratory of Allergic Diseases 2 (LAD-2) MC degranulation (functional assay) and on MRGPRX2 Tango- (ie, transcriptional output, with transcriptional activation following arrestin translocation screening system, aka PRESTO-Tango) transfected HTLA cells via β -arrestin recruitment assay. In the preliminary screening, the novel small molecules demonstrated significant concentration-dependent inhibition (50 and 100 μM) of C48/80 (5 μM)-induced MC degranulation and β -arrestin recruitment ($^{****}P < .001$) (see Fig E3, A and B, in the Online Repository at www.jacionline.org). The novel small molecules did not show cell toxicity up to 100 μM concentration in MCs and HTLA cells (Fig E3, C and D). To get preliminary insights into the selectivity of novel small molecules, we screened some potential target GPCRs, particularly other subtypes of MRGPRs and GPCRs expressed on MCs (see Table E4 in the Online Repository). There was no significant difference in the small molecule-treated cells compared to basal activation (without inhibitors), which showed preliminary evidence of MRGPRX2 selectivity (Fig E3, E). Furthermore, we measured the half-maximal inhibitory concentration (IC₅₀) of novel small molecules and observed a concentration-dependent inhibition with an IC₅₀ in the

micromolar range (Fig 1, A, B, and D). In the MC degranulation assay, GE1109 and GE1111 have shown significant ($^{\circ\circ\circ\circ}P < .0001$) inhibitory activity compared to the lead molecule. However, comparatively, GE0117 ($^{\circ\circ}P < .01$), GE0118 ($^{\circ\circ\circ}P < .001$), and GE1110 ($^{\circ}P < .05$) showed a weak and GE0119 a nonsignificant inhibitory activity in MC degranulation (Fig 1, A and D). In alignment with the functional assay results, in the MRGPRX2 activation assay, GE0117, GE0118, GE1109, GE1110, GE1111 ($^{\circ\circ\circ\circ}P < .0001$), and GE0119 ($^{\circ\circ\circ}P < .001$) showed significant inhibition compared to the lead molecule (Fig 1, B and D). There was no intrinsic agonistic activity by a graded concentration of novel small molecules when treated without C48/80 (see Fig E4, A and B, in the Online Repository at www.jacionline.org). Further, to verify the MRGPRX2 inhibitory activity of our novel small molecules, we performed C48/80-induced Ca^{2+} flux assay on human LAD-2 MCs.^{29,30} MCs pretreated with GE0117, GE1109, GE1110, and GE1111 showed a concentration-dependent inhibition of C48/80 (5 μM)-induced Ca^{2+} flux. However, the GE0118-, GE0119-, and lead molecule-treated MCs showed Ca^{2+} flux inhibitory response only at higher concentrations (50 and 100 μM), suggesting a lower potency than other small molecules (Fig 1, C).

Efficacy of novel small molecules on dose-response curve of C48/80 and late phase of MC activation shows MRGPRX2 antagonism

We tested the antagonistic efficacy of the novel small molecules by treating cells with or without small molecules 30 minutes before C48/80 stimulation (10 μM for 30 minutes) and evaluated the effect on the dose-response curve (DRC) of

C48/80 (Fig 2, A and B). In the MC degranulation assay, we found that GE0117, GE1109, and GE1111 increased the half-maximal effective concentration (EC_{50}) and decreased the maximum efficiency (E_{max}) response of C48/80 (Fig 2, A and C). GE1111 demonstrated the most prominent effect on C48/80 DRC, which almost flattened the DRC and changed the EC_{50} from $3.09 \pm 0.16 \mu\text{M}$ to $282.5 \pm 7.85 \mu\text{M}$ and decreased E_{max} by 75.21%. GE0117 ($^{$$$}P < .001$) and GE1111 ($^{$$$$}P < .0001$) showed potent activity compared to the lead molecule (genistein) and significantly changed the EC_{50} and E_{max} response. However, compared to the lead molecule, there was no significant change in the EC_{50} of C48/80 in the presence of the novel small molecules GE0118, GE0119, GE1109, and GE1110 (Fig 2, C).

Likewise, in the β -arrestin recruitment assay, treatment with GE0117, GE0118, GE1110, and GE1111 changed the EC_{50} and E_{max} of C48/80 (Fig 2, B). Among all these, GE1111 showed the most prominent effect on C48/80 DRC; it increased the EC_{50} of C48/80 from $2.975 \pm 0.535 \mu\text{M}$ to $200.4 \pm 3.159 \mu\text{M}$, and it decreased E_{max} by 58.441%. However, GE0119, GE1109, and the lead molecule showed a moderate effect on C48/80 DRC. Compared to the lead molecule, GE0117 ($^{$$$$}P < .0001$), GE0118 ($^{$$$$}P < .0001$), GE1110 ($^{$$$$}P < .0001$), and GE1111 ($^{$$$$}P < .0001$) showed a significant effect in changing the EC_{50} and E_{max} responses (Fig 2, C). There was no significant change in the EC_{50} of C48/80 compared to the lead molecule.

On the bases of IC_{50} and the above results on its efficacy and potency, we short-listed 3 novel small molecules (GE0117, GE1109, and GE1111) for further experiments. To test whether the potent novel small molecules regulate the delayed phase response of MC activation, we examined the production and release of monocyte chemoattractant protein 1 (MCP-1) and prostaglandin D_2 (PGD_2) in MCs. For MCP-1, MCs were pretreated with small molecules, the lead molecule, and vehicle for 30 minutes before C48/80 stimulation (100 ng/mL for 6 hours). C48/80-stimulated MCs showed a significant increase ($^{####}P < .0001$) in the production of MCP-1 compared to vehicle control cells (Fig 2, D). MCs pretreated with GE0117 and GE1111 significantly inhibited the release of MCP-1 at all the tested concentrations, while GE1109 showed significant activity only at 25 and 50 μM compared to C48/80 control. However, compared to the lead molecule, only GE1111 12.5 μM concentration showed a significant difference ($^*P < .05$) in the inhibition of MCP-1 release. At the same time, there was no significant difference with other small molecules at any concentration.

MRGPRX2 agonists such as C48/80³¹ and morphine³² have been reported to increase PGD_2 in MCs. For PGD_2 , MCs were pretreated with small molecules, the lead molecule, and vehicle for 1 hour before C48/80 stimulation (30 $\mu\text{g/mL}$ for 24 hours). Likewise, we found a significant increase in PGD_2 ($^{####}P < .0001$) on stimulation with C48/80. However, novel small molecule treatment showed significant inhibition of PGD_2 release compared to C48/80 control (Fig 2, E). Compared to the lead molecule (individual concentration), GE1111 treatment showed a significant difference ($^{$$$}P < .0001$) in PGD_2 release at all tested concentrations. However, GE0117 and GE1109 showed significant differences at higher concentrations (Fig 2, E). The experimental results demonstrate the potent activity of novel small molecules (compared to the lead molecule) in inhibiting the MC degranulation, MRGPRX2 activation, and release of inflammatory mediators.

Novel small molecules attenuated C48/80-induced mouse peritoneal MC degranulation, paw edema, and tissue extravasation in allergic mice models

The isolated peritoneum-derived MCs (PMCs) from wild-type C57BL/6 adult male mice, 6 to 8 weeks old, were treated with 2 different concentrations (25 and 50 μM) of small molecules and stimulated by C48/80 (30 $\mu\text{g/mL}$). Fig 3, A, shows the inhibitory effect of novel small molecules on PMC degranulation. C48/80 induced a significant PMC degranulation compared to basal level ($^{####}P < .0001$); however, this response was significantly inhibited by both concentrations of small molecules and the lead molecule. Compared to the lead molecule genistein (50 μM), only GE0117 (50 μM , $^*P < .05$), GE1109 (50 μM , $^{**}P < .01$), and GE1111 (25 μM and 50 μM , $^{**}P < .01$) showed significant effect in the inhibition of PMC degranulation. Consistent with previous findings, this indicates a higher efficacy of GE0117, GE1109, and GE1111 than the lead molecule.

A C48/80-induced allergic mice model represents the immediate phase of MC response (inflammation and vascular leakage) because of its short duration and acute nature.^{12,19} Wild-type C57BL/6 mice were treated with small molecules, the lead molecule, and vehicle intraperitoneally at a dose of 5 mg/kg body weight, followed by a tail vein injection of Evans blue dye. After 30 minutes, C48/80 (10 μL of 30 $\mu\text{g/mL}$) was injected. After 15 minutes, we observed a significant increase ($^{###}P < .001$) in the Evans blue dye extravasation in the ear (30.05 ± 4.02) and paw (11.18 ± 1.24) of C48/80-treated mice compared to saline-treated mouse ear (9.19 ± 2.24) and paw (4.55 ± 0.89 ; Fig 3, B-D (i-ii)). These responses, however, were significantly reduced in mice pretreated with GE0117 (ear 14.2 ± 1.88 ; paw 5.00 ± 0.69), GE1109 (ear 15.55 ± 2.49 ; paw 8.47 ± 1.34), and GE1111 (ear 10.67 ± 2.96 ; paw 4.82 ± 0.84). Compared to the lead molecule, only GE0117 ($^*P < .05$ ear and paw) and GE1111 ($^{$$$}P < .001$ ear; $^*P < .05$ paw) showed significant inhibition of Evans blue extravasation, while GE1109 showed no significant difference.

Moreover, C48/80-treated mice ($70.84 \pm 6.67\%$) showed a significant increase in paw thickness compared to saline-treated mice ($25.47 \pm 9.32\%$) (Fig 3, D (iii)). However, paw thickness was significantly attenuated in mice pretreated with GE0117 ($24.58 \pm 6.62\%$) and GE1111 ($28.28 \pm 8.79\%$). Comparatively, GE1109 and the lead molecule showed less efficacy in reversing inflammation and Evans blue dye extravasation. However, there was no significant difference in the attenuation of paw thickness among the small molecule-treated group and those treated with the lead molecule. The accumulation of vascular fluids in the paw tissue is degranulation of MC mediators,¹² which causes vasodilation and vascular leakage; thus, the observed decrease in paw edema may likely be through inhibition of MC activation.

The ear sections of the vehicle control groups appeared normal in the epidermal cell layer, without any apparent changes (Fig 3, E and F). However, ear sections of the C48/80 mice (10 μL of 30 $\mu\text{g/mL}$ for 15 minutes) showed a significant increase ($^{####}P < .0001$) in epidermal thickness and cellular infiltration compared to vehicle control mice. In contrast, all small molecule pretreated mice showed a significant decrease (GE11017 $^{****}P < .0001$, GE1109 $^{***}P < .001$, and GE1111 $^{****}P < .0001$) in the epidermal thickness.

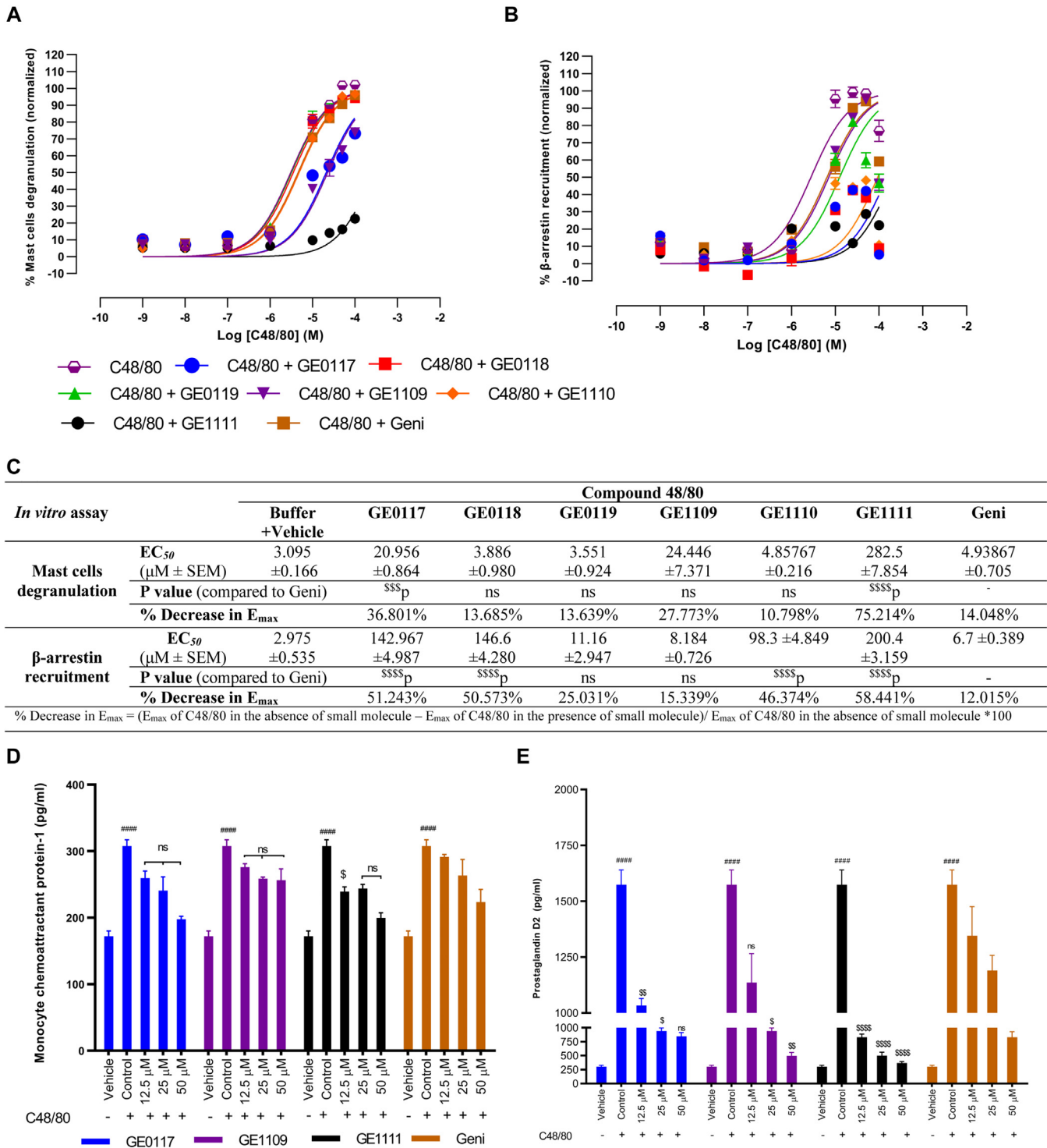


FIG 2. Effect of a single concentration (10 μM) of novel small molecules on DRC of C48/80. EC₅₀ and E_{max} response were measured via (A) MC degranulation on human LAD-2 MCs and (B) β-arrestin recruitment assay on MRGPRX2 Tango–transfected HTLA cells. (C) Summary of EC₅₀ and E_{max} changes. (D and E) Effect of potent novel small molecules (GE0117, GE1109, and GE1111) on C48/80 induced MCP-1 and PGD₂ release from human LAD-2 MCs. Data are presented as means ± SEMs of 3 independent experiments (n = 3). Statistical significance was determined by 1-way ANOVA and multiple comparisons; ns, nonsignificant. ^{\$}P < .05, ^{\$\$}P < .01, ^{\$\$\$}P < .001, ^{\$\$\$\$}P < .0001 compared to genistein (lead molecule) and ^{####}P < .0001 compared to vehicle control. For statistical analysis of MCP-1 and PGD₂ release, the individual concentration of genistein was compared to the individual concentration of small molecules.

Further, we performed toluidine blue staining to evaluate the *in vivo* inhibitory effect on MC degranulation. Ear sections of C48/80-treated mice showed a significant number of degranulated MCs compared to vehicle control mice (Fig 3, G and H), while there was no significant difference in the total numbers of MCs between mice treated with vehicle and the small molecule (data not shown). However, pretreatment with GE1107 (** $P < .01$), GE1109 (** $P < .01$), and GE1111 (**** $P < .0001$) significantly reduced MC degranulation. These histologic findings supported the *in vitro* and *ex vivo* MC degranulation results and demonstrated the potential of the novel small molecule in inhibiting MC-induced inflammation associated with allergic reactions.

Novel small molecules inhibited systemic anaphylaxis *in vivo*

Systemic anaphylaxis is a sudden-onset, life-threatening systemic allergic reaction that manifests as hypothermia, scratching, edema, and respiratory distress.³³ To test the effect of small molecules on systemic anaphylaxis, we treated wild-type C57BL/6 mice with small molecules, the lead molecule, and vehicle intraperitoneally at a dose of 5 mg/kg body weight 30 minutes before C48/80 intraperitoneal injection (8 mg/kg). Fig 4, A, shows the drop in body surface temperature in experimental groups. The body core temperature of vehicle + C48/80-treated mice started decreasing significantly after 10 minutes (^{##} $P < .01$) and remained significantly low at all time points (^{####} $P < .0001$) compared to vehicle control mice. We observed a maximum temperature drop at between 50 and 80 minutes of the period. However, compared to the lead molecule, only GE1111-treated mice showed a significant effect in preventing a reduction in body temperature. There was no significant difference in the lead molecule and vehicle + C48/80-treated mice at any time point. In addition, in a semiquantitative analysis of anaphylaxis symptoms (see Table E3 in the Online Repository at www.jacionline.org) and vehicle + C48/80-treated mice showed severe anaphylaxis symptoms (Fig 4, B). Mice pretreated with the small molecule GE1111 showed significant inhibition of their anaphylaxis symptoms score compared to lead molecule-treated mice. Further, we evaluated the systemic MCP-1 level from the serum of the experimental mice. Vehicle + C48/80-treated mice showed a significantly high (^{####} $P < .0001$) serum MCP-1 level compared to vehicle control mice. Mice pretreated with small molecules or the lead molecule at 5 mg/kg body weight demonstrated significant inhibition of serum MCP-1 (Fig 4, C). Compared to the lead molecule, GE1109 (^{\$} $P < .05$) and GE1111 (^{\$} $P < .01$) showed a significant difference in the inhibition of serum MCP-1. The significant statistical difference between GE1111- and lead molecule-treated mice indicates a higher efficacy and potency of GE1111 over the lead molecule and other small molecules. Therefore, for our further experiments, we short-listed the small molecule GE1111.

Novel small molecule GE1111 inhibits the response of different MRGPRX2 agonists

Several peptides (CST-14, ant venom peptide P17, and Ala 4 P17) and drugs (ciprofloxacin) have been reported to activate MRGPRX2.^{7,12,34} We therefore thought it interesting to explore the antagonistic activity of potent small molecule GE1111 on these MRGPRX2 agonists. The IC₅₀ of GE1111 against these agonists ranged 7 to 10 μ M (data not shown), so we used a fixed

concentration of 10 μ M of GE1111 and evaluated its effect on the DRC of the abovementioned agonists. CST-14 is a potent MRGPRX2 agonist¹⁸; GE1111 demonstrated a significant increase in EC₅₀ and decreased E_{max} response of CST-14-induced MC degranulation (Fig 5, A (i) and C) and β -arrestin recruitment assay (Fig 5, B (i) and C). Also, we observed a similar effect of GE1111 on EC₅₀ and E_{max} of our recently reported MRGPRX2 agonist peptide P17 (Fig 5, A (ii) and B (ii)) and its potent alanine analog Ala 4 P17²⁰ (Fig 5, A (iii), B (iii), and C) in both assays.

In addition, we evaluated the effect of GE1111 on ciprofloxacin, an FDA-approved fluoroquinolone antibiotic.^{13,35} Consistent with previous observations, ciprofloxacin induced MC degranulation (Fig 5, A (iv) and C) and MRGPRX2 activation (Fig 5, B (iv) and C) at a higher concentration compared to other MRGPRX2 agonists,¹² which was significantly changed by GE1111 treatment. Our findings demonstrated a broad-spectrum antagonistic activity of GE1111 against diverse agonists, indicating good potential for its use as a drug candidate to treat MRGPRX2-mediated diseases.

Novel small molecule GE1111 obstructs MRGPRX2 signal transduction pathway

Activation of MRGPRX2 provokes the downstream phospholipase C- γ (PLC- γ), extracellular signal-regulated protein kinase 1/2 (ERK1/2), and Akt signaling pathways.³⁶ A 15-minute C48/80 stimulation (30 μ g/mL) of MCs showed a significant increase of PLC- γ (^{##} $P < .01$), ERK1/2 (^{##} $P < .01$), and Akt ([#] $P < .05$) phosphorylation compared to vehicle control MCs (Fig 6, A and B (i-iii)). GE1111 pretreatment showed a significant decrease in the expression of p-PLC- γ at higher concentrations (^{*} $P < .05$ at 25 μ M and ^{**} $P < .01$ at 50 μ M). Also, the expression of p-ERK1/2 was significantly inhibited by GE1111 at 25 μ M (^{***} $P < .001$) and 50 μ M (^{****} $P < .0001$) concentrations. Similarly, GE1111 significantly inhibited the expression of p-Akt at 25 μ M (^{*} $P < .05$) and 50 μ M (^{**} $P < .01$) concentrations (Fig 6, A and B (i-iii)).

A recent study has shown the crucial role of store-operated Ca²⁺ entry (aka SOCE) via the Ca²⁺ sensor stromal interaction molecule 1 (STIM1) in MRGPRX2 activation.³⁰ To gain more insight into the underlying mechanism of GE1111 on Ca²⁺ flux response, we measured the protein expression of Ca²⁺ channel STIM1 in human LAD-2 MCs. GE1111-treated MCs showed a significant inhibition of C48/80-induced STIM1 expression (^{*} $P < .05$ at 12.5 and 25 μ M and ^{**} $P < .01$ at 50 μ M) (Fig 6, A and B (iv)). Collectively our data showed the antagonistic activity of GE1111 on the downstream signaling pathway of MRGPRX2.

DISCUSSION

MRGPRX2 is a family member of Mas-related gene receptors emerging as a prominent receptor involved in non-IgE-mediated allergic reactions,⁷ itch,¹⁵ rosacea,³⁷ urticaria,³⁸ adverse drug reactions,³⁵ and atopic dermatitis.³⁹ The multiple ligand binding puzzles of MRGPRX2⁴⁰ have recently been solved via the determination of the 3-D crystal structure of MRGPRX2.^{17,18} As we note above, recently, several molecules (mostly natural compounds) have been reported to inhibit MRGPRX2 activation. Ogasawara et al⁴¹ recently reported the *in vitro* MRGPRX2 antagonistic activity and mechanism of 2 heterocyclic small compounds (molecular weight of 223-268 g/mol) on human cord

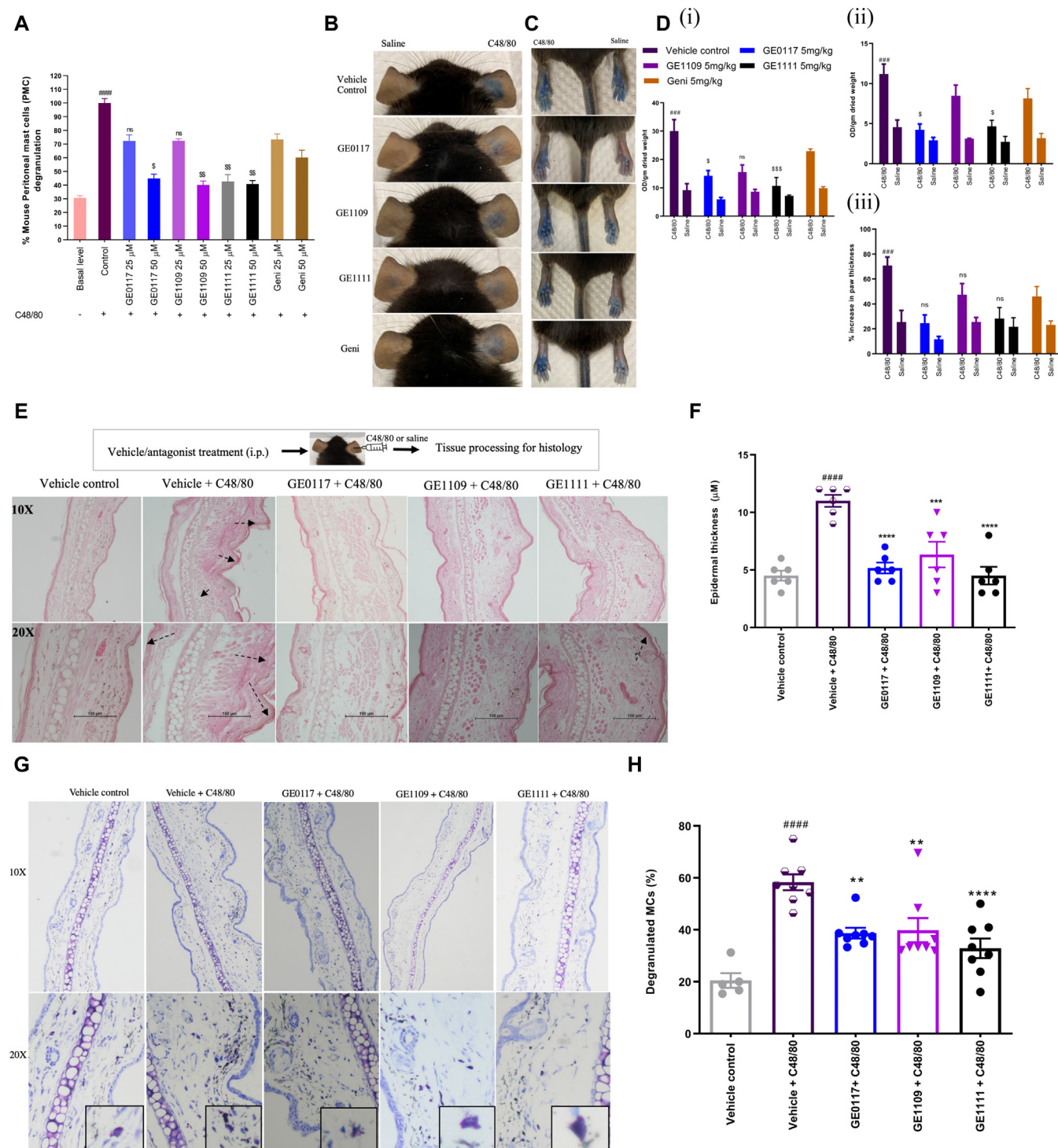


FIG 3. *Ex vivo* and *in vivo* evaluation of the effect of the novel small molecules on C48/80 (30 µg/mL) induced mouse PMC degranulation, local allergic reactions, and histologic changes. **(A)** Novel small molecules showed concentration-dependent inhibition of C48/80-induced PMC degranulation. **(B)** Representative images of C48/80-induced Evans blue-stained extravasation in mouse ear. **(C)** Representative paw images of C48/80-induced Evans blue-stained extravasation in mouse paw. **(D)** (i) Quantification of Evans blue leakage into the ear after 15 minutes of C48/80 injection. (ii) Quantification of Evans blue leakage into the paw after 15 minutes of C48/80 injection. (iii) Quantification of percentage increase in paw thickness after 15 minutes of C48/80 injection. Data are presented as means $\pm$ SEMs (for *ex vivo* PMCs, $n = 3$ per group; for *in vivo* evaluation, $n = 6-8$ mice per group). Statistical significance was determined by 1-way ANOVA and multiple comparisons; *ns*, nonsignificant, $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$ compared to genistein (lead molecule) and $^{####}P < .001$ compared to saline. **(E)** Representative images of hematoxylin and eosin-stained ear sections of mice of experimental groups; dotted arrows represent change in tissue morphology. **(F)** Quantitative

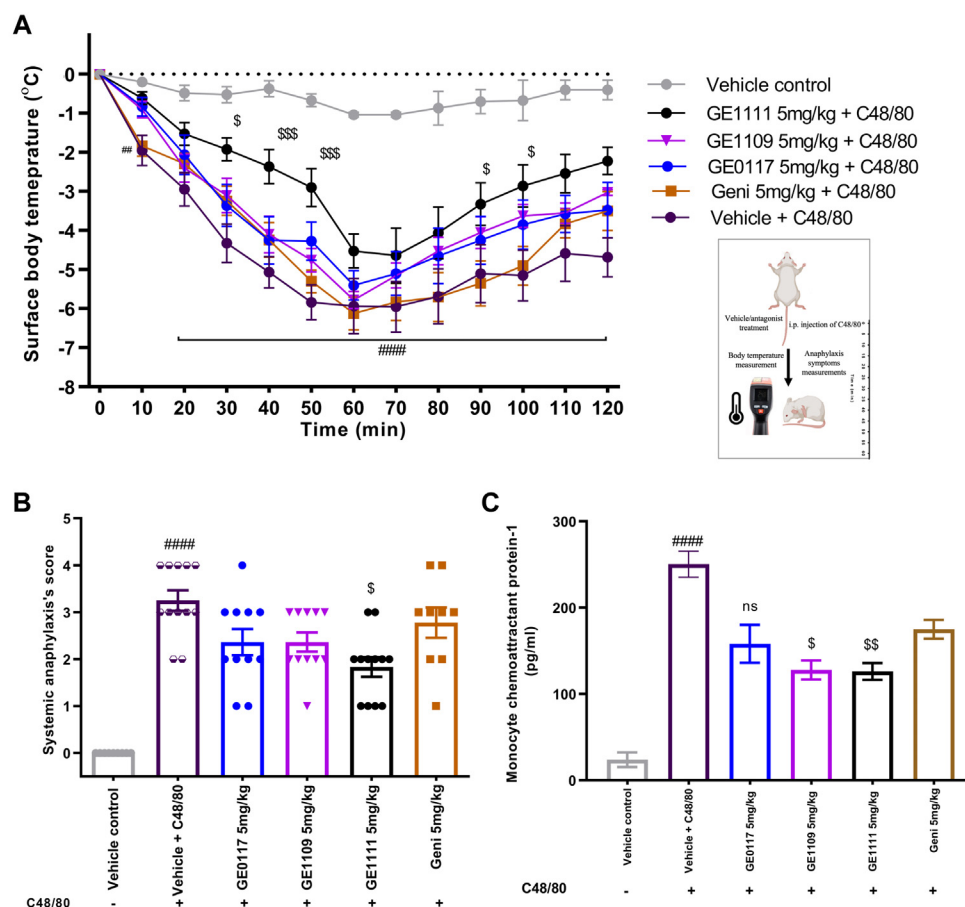


FIG 4. *In vivo* evaluation of 5 mg/kg body weight dose of GE0117, GE1109, and GE1111 on C48/80 (8 mg/kg body weight, intraperitoneal injection) induced systemic anaphylaxis (decrease in body temperature and anaphylaxis symptoms). Effect on (A) changes in body temperature and (B) quantitative systemic anaphylaxis symptoms in experimental mice. (C) Effect of small molecule treatment on serum MCP-1 of mice after 120 minutes of C48/80 injection. Data are presented as means $\pm$ SEMs from 6 to 12 mice per group. Statistical significance was determined by 1- or 2-way ANOVA and multiple comparisons; ns, nonsignificant, $^{\circ}P < .05$, $^{\circ\circ}P < .01$, $^{\circ\circ\circ}P < .001$ compared to genistein (lead molecule) and $^{\circ\circ\circ\circ}P < .0001$ compared to vehicle control.

blood-derived MCs. However, these compounds did not show antagonistic activity on *ex vivo* mouse MC activation, so they provide only limited information on *in vivo* translation. One possible reason might be the difference in similarity between human MRGPRX2 and its mouse ortholog, MRGPRB2, which leads to differences in ligand response.^{12,42} In the present study, we designed novel small molecules using genistein as the lead molecule, then tested their efficacy on *in vitro* human LAD-2 MCs, *ex vivo* mouse PMC, and *in vivo* mouse model of allergies. The novel antagonists possess small molecule-like properties (molecular weight of 422-466 g/mol) and inhibited the MRGPRX2-mediated *in vitro*, *ex vivo*, and *in vivo* allergic and inflammatory responses.

Previously, several novel drug molecules have been designed and developed by quantifying the binding affinities between small molecules and macromolecular targets.^{24,25} We used a similar *in silico* method of structure-based screening to identify potential hits out of thousands of compounds.^{43,44} This computational method has been used successfully in determining novel and potent molecules in several drug discovery projects, such as peroxisome proliferator-activated receptor gamma (aka PPAR γ) partial agonists,⁴⁵ 5-HT1A agonist,⁴⁶ and novel nonnucleoside DNA alkyl transferase inhibitors.⁴⁷

We used a structure-based drug-designing approach to improve the potency of lead molecule genistein. A higher potency can be achieved by improving the magnitude of ligand binding affinity to

evaluation of epidermal thickness in different experimental groups' tissue sections (original magnification $20\times$). (G) Representative images of toluidine blue-stained ear sections of experimental groups; bold arrows indicate intact nondegranulated MCs and dotted arrows degranulated MCs. (H) Quantitative evaluation of degranulated MCs in different experimental groups' tissue sections (original magnification $20\times$). Data represent epidermal thickness (in μM ; mean $\pm$ SEM) and percentage of degranulated MCs (mean $\pm$ SEM) from 6 to 8 mice per group. $^{\circ\circ\circ\circ}P < .0001$ compared to vehicle control, $^{\circ\circ\circ}P < .001$, $^{\circ\circ}P < .001$ compared to vehicle + C48/80.

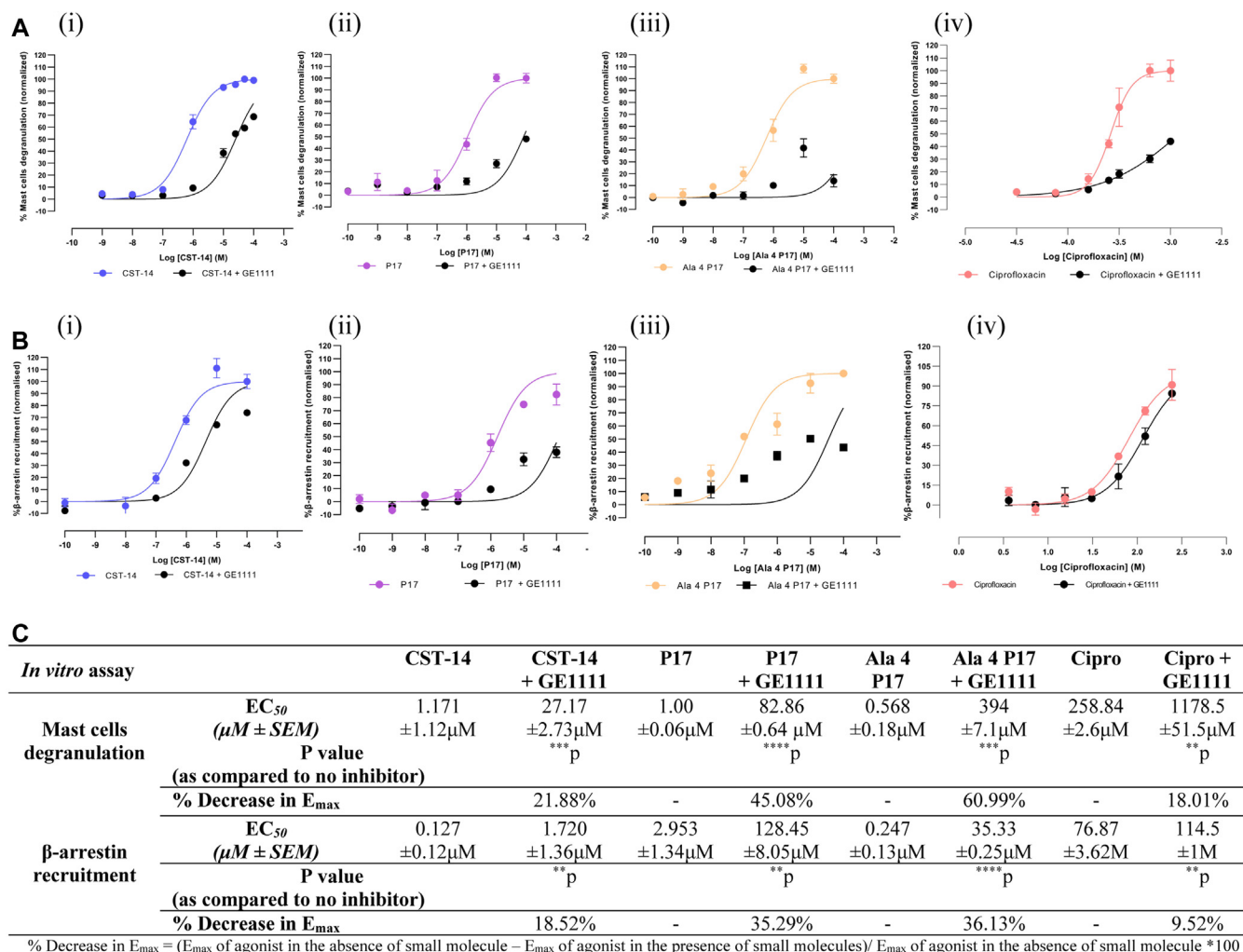


FIG 5. Effect of a single concentration (10 μ M) of the potent novel small molecule GE1111 on the DRC of different classes of MRGPRX2 agonists. **(A)** (i) DRC of CST-14, (ii) ant venom peptide P17, (iii) potent alanine analog Ala 4 P17, and (iv) fluoroquinolone antibiotic ciprofloxacin via MC degranulation assay. **(B)** (i) DRC of CST-14, (ii) ant venom peptide P17, (iii) potent alanine analog Ala 4 P17, and (iv) fluoroquinolone antibiotic ciprofloxacin via β -arrestin recruitment assay. **(C)** Summary of the effect of GE1111 on the EC_{50} and E_{max} of MRGPRX2 agonists. Each data point represents the mean $\pm$ SEM of 2 or 3 independent experiments. P values are determined by unpaired 2-tailed t test; ** $P < .01$, *** $P < .001$, **** $P < .0001$ compared to agonists without inhibitor.

the target receptor. We designed a library of novel small molecules by introducing different functional groups and structure extensions of genistein. By extending the chromone ring of genistein to naphthalimide/phenalen-1-one and combining it with the naphthoate ring, novel molecules demonstrated a significant increase in the binding affinity with MRGPRX2. On the basis of the binding affinity with MRGPRX2 and synthesis feasibility, we short-listed 6 hybrid molecules for chemical synthesis. As a result of the unprecedented chemistry of these novel molecules, we designed a novel chemical synthesis approach to synthesize the key intermediates, which were later coupled via Suzuki coupling reaction to obtain the final compounds. The small molecules were fully characterized and obtained at >95% purity.

After ascertaining the *in vitro* safety of all novel small molecules via (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (aka MTT) assay on human LAD-2 MCs and HTLA cells, we evaluated the preliminary target selectivity via

screening potential GPCRs. There was no receptor activation by these small molecules, which can be considered as MRGPRX2 selectivity of novel small molecules. However, with the available assay, we could only measure the receptor's activation. We tested the antagonistic effect of these novel small molecules in several cell models. Small molecules GE0118, GE1109, GE1109, and GE1111 demonstrated significant inhibition of MC degranulation and MRGPRX2 activation with IC_{50} of 5 to 21 μ M. This is particularly important because MRGPRX2-mediated MC degranulation is increasingly implicated in various pathologies.^{7,15,48} However, compared to the lead molecule, only GE1111 showed more potent inhibitory activity, with IC_{50} below 10 μ M in both assays, indicating the necessity of further efforts to increase the potency of these small molecules. Consistent with previous findings on slow decay of intracellular Ca^{2+} signal in the MRGPRX2-mediated MC degranulation,^{29,30} we also found a sustained Ca^{2+} flux signal on activation by

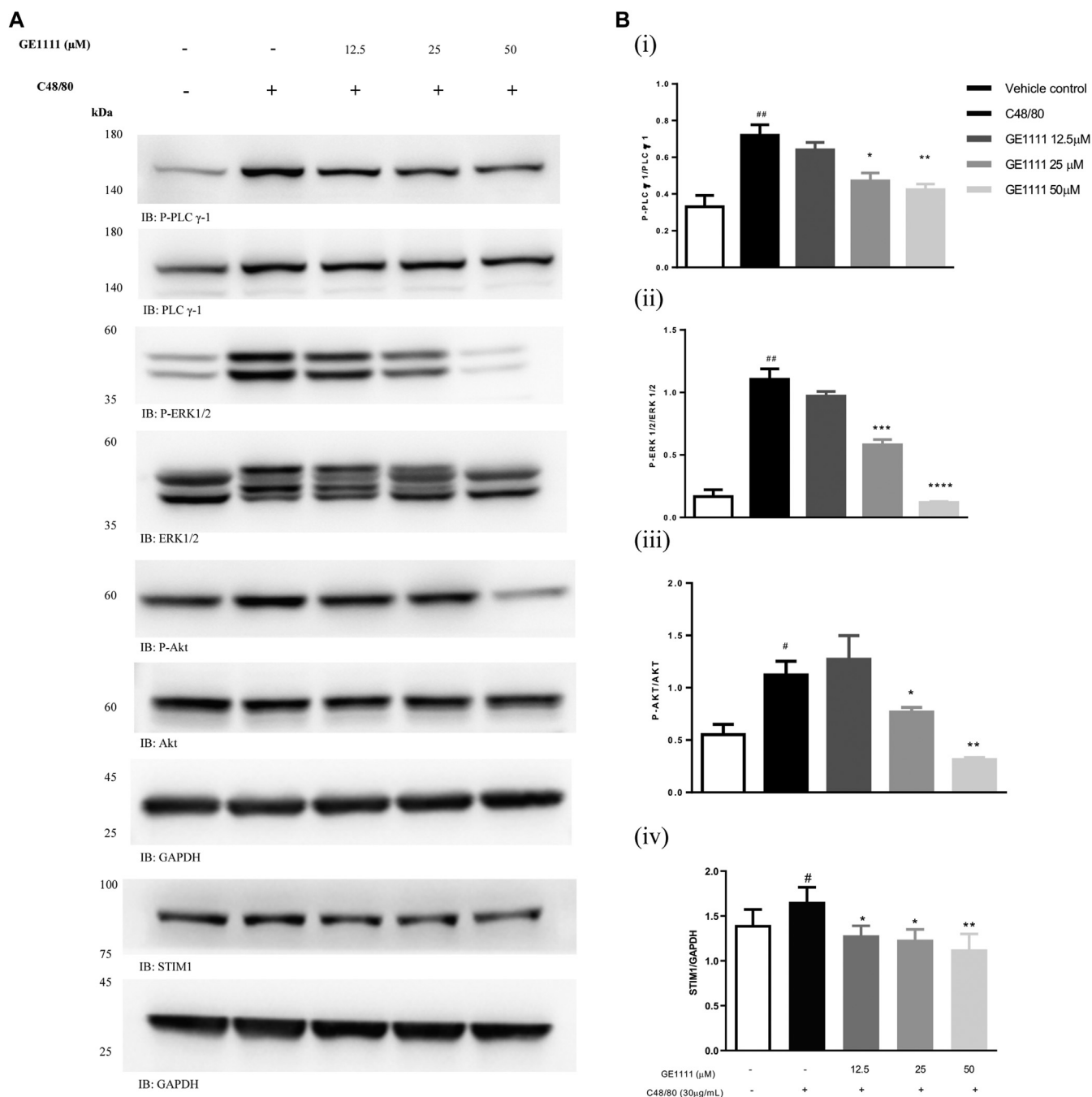


FIG 6. Effect of the potent novel small molecule GE1111 on the expression of the downstream signaling pathway of MRGPRX2 in human LAD-2 MCs. Human LAD-2 MCs were treated with vehicle or graded concentration GE1111 and then stimulated by 30 μg/mL of C48/80 for 15 minutes. **(A)** Images of representative blots of PLC γ-1 (P-PLC γ-1), ERK1/2 (P-ERK1/2), Akt (P-Akt), and STIM1 protein expression. **(B)** Bar graph showing the relative intensities of bands for (i) PLC γ-1, (ii) ERK1/2, (iii) Akt, and (iv) STIM1. Phosphorylated levels of protein were normalized to the total level of the respective proteins. Data from 3 independent experiments are shown as means ± SEMs. Statistical significance was determined by unpaired Student *t* test; #*P* < .05, ##*P* < .01 compared to control (vehicle + buffer), and **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001 compared to C48/80 + vehicle.

C48/80.^{29,49} MCs treated with the novel small molecules demonstrated a significant decrease in the Ca²⁺ flux signal, which indicates that our small molecules can inhibit the early phase of MC activation.

The small molecule-treated MCs and MRGPRX2-transfected HTLA cells (10 μM concentration) showed an increase in the EC₅₀ and a decrease in the E_{max} response of C48/80. Furthermore, the molecular docking study observed a similar binding pocket of

C48/80 and small molecules with several cysteine amino acid residues (Fig E1, J, and Fig E2). Therefore, the change in both EC_{50} and inhibition of E_{max} might be due to the irreversible competitive antagonistic nature of small molecules.^{50,51}

MC mediators can be classified as presynthesized mediators,⁵² gene expression-induced chemokines/cytokines,⁵³ and *de novo* synthesized phospholipid metabolite mediators⁵⁴ on the basis of the generation-and-release pattern. Considering this, we sought to evaluate the effect of small molecules on the chemokine MCP-1,^{55,56} and we *de novo* synthesized phospholipid PGD_2 ⁵⁷ release from C48/80-stimulated MCs. MCP-1 plays a crucial role in the migration and infiltration of immune cells such as monocytes, microglia, and memory T lymphocytes to the site of injury or infection.⁵⁶ In addition, membrane phospholipid-derived PGD_2 play a critical role in inducing vasodilatation and increased permeability of blood vessels in several allergic diseases.⁵⁸ The release of MCP-1 and PGD_2 from MCs on C48/80 stimulation in our study was consistent with previous observations.^{20,59,60} Small molecule treatment significantly decreased MCP-1 and PGD_2 release, suggesting that our novel small molecules can also inhibit the late phase of MC activation as well as reduce ongoing allergic and inflammatory reactions. Interestingly, the PGD_2 inhibition response was more significant than MCP-1, which may indicate a divergent signaling pathway of MRGPRX2,^{23,36} which can be addressed in future studies.

The *in vitro* data firmly establish the MRGPRX2 antagonistic activity of our small molecules. To corroborate the physiologic relevance *in vivo*, first, we investigated the effect of these antagonists on C48/80-induced MC degranulation of mouse PMC. Second, we investigated the antiallergic activity of GE0117, GE1109, and GE1111 in C48/80-induced allergy^{12,61} and an anaphylaxis mouse model.¹² Mouse MRGPRB2 receptor demonstrated a similar agonistic response to C48/80 as its human counterpart, MRGPRX2.¹² In addition to the considerable difference in sequence similarity (~53%), there are also several similar crucial similar features between MRGPRB2 and MRGPRX2, such as showing selective expression in connective tissue MCs and exhibiting the same ligand activation profiles.¹² Therefore, PMC and mouse models of allergies play an indispensable role in evaluating MRGPRX2/MRGPRB2 activity at the physiologic level. The *ex vivo* evaluation of MC degranulation on PMC demonstrated a similar effect to human LAD-2 MCs, which validated our *in vitro* findings. In the mouse model of acute allergy, we examined the 2 crucial immediate symptoms of allergies: tissue extravasation and edema. Our novel small molecules showed promising inhibitory activities against C48/80-induced tissue extravasation and paw edema, indicating *in vivo* MRGPRB2 inhibition.⁶²

Furthermore, the epidermal thickness of mouse ear as an indicator of inflammation and *in vivo* MC degranulation was examined via histology. As expected,⁵⁹ the ear sections of C48/80-treated mice demonstrated significantly increased epidermal thickness and MC degranulation, which was significantly abated in mice pretreated with our small molecules. In alignment with the acute allergy results, in the systemic anaphylaxis mice model, we further demonstrated that systemic anaphylaxis induced by C48/80 was reversed by small molecule treatment. This was important because systemic anaphylaxis causes a rapid drop in body temperature and also induces severe bronchoconstriction and allergies.⁶³ The potent small molecule GE1111 demonstrated a significant effect in preventing hypothermia and anaphylaxis

symptoms severity. However, the other 2 small molecules did not significantly impact body temperature but prevented the anaphylaxis severity. In systemic anaphylaxis mice, we found a high level of serum MCP-1 level, which is consistent with previous studies.^{31,64} All the small molecule-treated mice showed a lower serum MCP-1 level, indicating inhibition of systemic cytokine release from MCs to the bloodstream, which supports our *in vitro* findings.

Unlike other GPCRs, MRGPRX2 is activated by structurally diverse chemical compounds, including endogenous and exogenous peptides (CST-14, P17, and Ala 4 P17)^{7,20} and drugs (ciprofloxacin).⁷ Interestingly, we have shown that the potent small molecule GE1111 inhibited MC degranulation and MRGPRX2 activation response of these agonists to the same extent as C48/80 (except ciprofloxacin). The difference in the MRGPRX2 activation response of ciprofloxacin might be due to its nature as a ligand (balanced or biased agonist)⁶⁵ or a divergent signaling pathway.⁶⁶ It is worth noting that MRGPRX2-mediated MCs response activation and signaling can be different depending on ligand type.¹⁸ For instance, C48/80 is a balanced agonist; it activates both G proteins and β -arrestins.⁶⁷ Not much information is available for the other ligands tested here (CST-14, P17, Ala 4 P17, and ciprofloxacin), and this should be addressed in future studies.

MRGPRX2 activation on MCs provokes the downstream PLC/ERK/Akt signal transduction pathways,^{30,36,48,68} which further contribute to ongoing allergic and inflammatory reactions. Consistent with previous studies,^{48,69} we showed that C48/80-stimulated human LAD-2 MCs trigger the downstream PLC- γ , ERK1/2, and Akt signaling pathways, while GE1111 treatment significantly inhibited the expression of p-PLC- γ , p-ERK1/2, and p-Akt at different concentrations. In addition, we showed the effect of GE1111 on the Ca^{2+} flux mechanism of MRGPRX2. MRGPRX2 utilizes STIM1 for store-operated Ca^{2+} entry³⁰ and regulates its activity. GE1111-treated human LAD-2 MCs demonstrated significant inhibition of C48/80-induced STIM1 expression and thus inhibited the Ca^{2+} flux response and downstream signaling pathway. This finding is consistent with our *in vitro* Ca^{2+} flux results.

Taken together, the *in vitro* and *in vivo* studies corroborated the MRGPRX2 antagonistic activity of naphthalimide/phenalenone and the naphthoate ring-based small molecules. Further efforts in structure optimization may enhance the potency of small molecules, from a micromolar to nanomolar scale. We used a human MC line and a mouse allergy model to evaluate MRGPRX2's antagonistic activity of novel small molecules. Considering the difference in sequence similarity between human MRGPRX2 and its homologs, mouse MRGPRB2, use of humanized allergic models, and *ex vivo* human MCs or basophils will strengthen our findings. Also, *in vitro* binding and comprehensive target selectivity assays of these molecules will support the current findings. In addition, pharmacokinetic and *in vivo* toxicity studies remain to be addressed in further studies to extrapolate these findings to clinical applications.

MCs' MRGPRX2 activation plays a crucial role in the etiology of non-IgE-mediated allergic reactions to several endogenous, exogenous peptides, FDA-approved drugs, and chronic inflammation associated with rosacea, atopic dermatitis, and asthma. Our encouraging results provide a strong rationale for using novel small molecule MRGPRX2 antagonists to treat these chronic allergic diseases.

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Clinical implication: The small molecule MRGPRX2 antagonists can be developed as novel therapeutic drug candidates to treat refractory allergies, drug-induced allergies, itch, and inflammation.

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