Inhibition of mast cell degranulation by novel small molecule MRGPRX2 antagonists

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Background: Mas-related G protein–coupled receptor X2 (MRGPRX2) is a promiscuous receptor on mast cells that mediates IgE-independent degranulation and has been implicated in multiple mast cell–mediated disorders, including chronic urticaria, atopic dermatitis, and pain disorders. Although it is a promising therapeutic target, few potent, selective, small molecule antagonists have been identified, and functional effects of human MRGPRX2 inhibition have not been evaluated *in vivo*.

Objective: We sought to identify and characterize novel, potent, and selective orally active small molecule MRGPRX2 antagonists for potential treatment of mast cell–mediated disease.

Methods: Antagonists were identified using multiple functional assays in cell lines overexpressing human MRGPRX2, LAD2 mast cells, human peripheral stem cell–derived mast cells, and isolated skin mast cells. Skin mast cell degranulation was evaluated in *Mrgprb2*$^{em(-/-)}$ knockout and *Mrgprb2*$^{em(MRGPRX2)}$ transgenic human MRGPRX2 knock-in mice by assessment of agonist-induced skin vascular permeability. *Ex vivo* skin mast cell degranulation and associated histamine release was evaluated by microdialysis of human skin tissue samples.

Results: MRGPRX2 antagonists potently inhibited agonist-induced MRGPRX2 activation and mast cell degranulation in all mast cell types tested in an IgE-independent manner. Orally administered MRGPRX2 antagonists also inhibited agonist-induced degranulation and resulting vascular permeability in MRGPRX2 knock-in mice. In addition, antagonist treatment dose dependently inhibited agonist-induced degranulation in *ex vivo* human skin.

Conclusions: MRGPRX2 small molecule antagonists potently inhibited agonist-induced mast cell degranulation *in vitro* and *in vivo* as well as *ex vivo* in human skin, supporting potential therapeutic utility as a novel treatment for multiple human diseases involving clinically relevant mast cell activation. (J Allergy Clin Immunol 2024;■■■:■■■-■■■.)

Key words: *Inflammation, mast cells, degranulation, skin, neuropeptides, sensory neurons, IgE independent, urticaria*

Mast cells are granulocytes found in connective tissues and mucous membranes of barrier tissues including the skin, meninges, and respiratory and gastrointestinal tracts that mediate inflammatory responses, such as hypersensitivity, allergic, and inflammatory reactions.[1,2] Mast cells are activated via IgE-mediated allergic pathways (typically activated by environmental allergens) and IgE-independent pathways.[3,4] A defining feature of these cells is the presence of dense cytoplasmic granules that contain histamine, heparin, and a variety of proteases and proinflammatory mediators that are released upon cell activation in a process called degranulation.[5] Release of granule contents results in increased vascular permeability, inflammation, swelling, itch, and pain.[6]

Mas-related G protein-coupled receptors (MRGPRs) are class A rhodopsin-like G protein–coupled receptors that are expressed on sensory neurons and innate immune cells, which reside in close proximity within barrier tissues.[2,7] In humans, the MRGPR family comprises 8 receptors (MRGPRX1-MRGPRX4 and MRGPRD-MRGPRG) that can sense noxious stimuli and appear to have a role in pruritus, pain, and innate immunity. Of these receptors, MRGPRX2 is expressed on mast cells and is associated with IgE-independent mast cell activation and degranulation.[6,8] MRGPRX2 is a highly promiscuous receptor that is activated by numerous endogenous peptide agonists including neuropeptides, eosinophil granule proteins, and antimicrobial peptides, as well as many exogenous drugs including fluoroquinolone antibiotics, phenothiazines, neuromuscular blocking agents, hormone receptor modulators, and natural remedies.[6,7,9-11] Similarly, the mouse MRGPRB2 receptor, which is recognized as the functional ortholog of the human MRGPRX2 receptor, is also expressed on mast cells and activated by MRGPRX2 agonists.[12] Knockdown of MRGPRX2 in human mast cells or MRGPRB2 in murine mast cells reduces mast cell activation and degranulation in response to MRGPRX2 agonists, but does not reduce responses to IgE-induced activation.[3,10,12,13] Additionally, MRGPRB2 knockout mice display attenuated phenotypes, including reduced responses to MRGPRX2 agonist-induced vascular permeability, pseudoallergic and anaphylactic responses, lower itch and immune cell

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Abbreviations used
CRISPR: Clustered regularly interspaced short palindromic repeats
CSU: Chronic spontaneous urticaria
EC_{50}: Half-maximal effective concentration
IC_{50}: Half-maximal inhibitory concentration
IP-1: Inositol monophosphate
KI: Knock-in
KO: Knockout
LAD2: Laboratory of Allergic Diseases (mast cells)
MBP: Major basic protein
MRGPR: Mas-related G protein–coupled receptor
NK1R: Neurokinin-1 receptor
PACAP: Pituitary adenylate cyclase–activating peptide
PSCMC: Peripheral stem cell-derived mast cell

infiltration in allergic contact dermatitis, rosacea and atopic dermatitis, and reduced hyperalgesia in pain models.[3,6,14,15] Furthermore, several gain- and loss-of-function gene polymorphisms in *MRGPRX2* have been identified as associated with inflammatory or autoreactive diseases, and the presence of multiple loss-of-function polymorphisms leads to reduced mast cell activation with MRGPRX2 agonists.[16,17]

Activation of MRGPRX2 triggers release of proinflammatory mediators and multicellular signaling cascades that likely play a key role in multiple human diseases that exhibit underlying clinically relevant mast cell activation.[18,19] MRGPRX2 has been proposed to play an important role in mast cell–driven diseases such as chronic spontaneous urticaria (CSU).[10,12,20-25] Patients with CSU have been reported to have elevated plasma/serum levels of substance P, an agonist of MRGPRX2,[26,27] and a greater number of MRGPRX2-positive mast cells in skin biopsy specimens.[28] Enhanced skin reactivity to intradermally injected MRGPRX2 ligands has also been observed in CSU, consistent with increased MRGPRX2 expression and activity.[24,29] Current treatment options in CSU, including anti-IgE therapeutics, do not adequately control disease in many patients, consistent with a non-IgE mechanism driving disease in many of these patients.[30,31] Increased expression of MRGPRX2 and substance P has also been reported in lesional atopic dermatitis skin samples compared with nonlesional skin samples from patients.[32-34] In addition, MRGRPX2 has been implicated in pain conditions such as migraine, interstitial cystitis, and postoperative pain as well as numerous chronic inflammatory conditions such as asthma, rosacea, and ulcerative colitis.[14,34-40] Finally, MRGPRX2 has been proposed to play an important role in acute drug/idiopathic pseudoallergic reactions, given that multiple drugs activate this receptor.[12,20,21] Thus, accumulating evidence supports the role of MRGPRX2 in IgE-independent mast cell activation and makes it a potential therapeutic target for non-IgE–driven inflammatory and pain conditions that are precipitated by degranulating mast cells and are less amenable to current standard treatments. Inhibition of MRGPRX2-mediated mast cell activation and degranulation could potentially provide a novel treatment for a wide range of pain, allergic, and inflammatory conditions.

Overall, we demonstrate the identification of novel, orally active MRGPRX2 antagonists that inhibit mast cell degranulation with potential utility for treatment of mast cell mediated diseases. We screened potential small molecule antagonists *in vitro* and identified selective MRGPRX2 antagonists that blocked MRGPRX2-mediated mast cell degranulation. The *in vivo* efficacy of MRGPRX2 antagonists and ablation of MRGPRB2 on skin mast cell degranulation was evaluated in custom-generated MRGPRX2 knock-in (KI) and MRGPRB2 knockout (KO) transgenic mice by assessment of agonist-induced skin vascular permeability. Finally, MRGPRX2 antagonism was confirmed in *ex vivo* human skin via assessment of histamine release.

METHODS

A complete description of all methods used in this study is provided in this article's Methods section in the Online Repository at www.jacionline.org.

Cell culture and *in vitro* assays

Chinese hamster ovary cells stably expressing human MRGPRX2 and human embryonic kidney cells stably expressing mouse MRGPRB2 were used in agonist-induced inositol monophosphate (IP-1) accumulation and β-arrestin recruitment assays. Laboratory of Allergic Diseases (LAD2) mast cells (National Institutes of Health, Bethesda, Md), human peripheral stem cell–derived mast cells (PSCMCs), and isolated human skin mast cells were evaluated for agonist-induced β-hexosaminidase, tryptase, or cytokine release.

Generation and housing of mice

Clustered regularly interspaced short palindromic repeats (CRISPR)–mediated *Mrgprb2*$^{em(-/-)}$ KO and *Mrgprb2*$^{em(MRGPRX2)}$ transgenic MRGPRX2 KI mice in C57BL/6J background were custom generated). Gene expression of *MRGPRX2* in KI mice was confirmed by PCR and protein levels confirmed by flow cytometry analysis of MRGPRX2^{+} peritoneal mast cells. Mice were housed in pathogen-free conditions on a 12-hour light/dark cycle and provided with *ad libitum* access to food and water at Explora Biolabs (San Diego, Calif). Animal housing and procedures were performed according to Institutional Animal Care and Use Committee–approved protocols and conformed to the National Research Council *Guide for Care and Use of Laboratory Animals*.

Evans blue extravasation

For Evans blue extravasation (vascular permeability) assays, 8- to 10-week-old male C57BL/6J wild-type, MRGPRB2 KO or MRGPRX2 KI mice were restrained and injected intravenously with 1% Evans blue before intradermal treatment with vehicle PBS, MRGPRX2 agonists, or goat anti-mouse IgE. In some experiments, animals were orally gavaged with EP262 or vehicle 3 hours before intradermal injections. Evans blue dye was extracted from the skin tissue and quantified. Results are expressed as micrograms of dye per milligrams of tissue and normalized to vehicle.

Microdialysis in *ex vivo* human skin

Fresh human skin tissue from patients undergoing elective surgery was received 1 day after surgery. Microdialysis was carried out using a 4-Channel Microdialysis Syringe Pump with CMA 30 Linear Microdialysis Probes (CMA Microdialysis AB, Kista, Sweden), 6 kDa molecular weight cut-off. Antagonists

TABLE I. Endogenous and exogenous agonists activate MRGPRX2 to induce mast cell degranulation

Agonist tested	β-Hexosaminidase in LAD2 cells average EC_{50} ± SD (nM)	IP-1 in MRGPRX2 CHO cells average EC_{50} ± SD (nM)	β-Arrestin in MRGPRX2 CHO cells average EC_{50} ± SD (nM)
PACAP 1-27	27 ± 19	52 ± 48	78 ± 98
Cortistatin-14	51 ± 40	120 ± 100	350 ± 98
PACAP 1-38	120 ± 8	520 ± 260	ND
MBP-19	120 ± 83	1600 ± 300	ND
Compound 48/80	320 ± 170	460 ± 210	1,600 ± 0
Cetrorelix	320 ± 140	5,300 ± 2,300	1,500 ± 95
Substance P	500 ± 350	2,900 ± 2,000	1,600 ± 0
Chlorpromazine	4,600 ± 640	3,000 ± 1,500	2,700 ± 1,600
Dextromethorphan	51,000 ± 0	3,600 ± 1,500	3,400 ± 230
Icatibant	17,000 ± 13,000	17,000 ± 15,000	34,000 ± 7,600

n = 2-64 per condition.
CHO, Chinese hamster ovary; *ND,* not determined.

were diluted in vehicle perfusate and intradermally injected flanking the probe. After 1 hour, baseline perfusate was collected for 10 minutes, followed by intradermal injection of MRGPRX2 agonists flanking the probe. Perfusate samples were then collected over 5- to 10-minute intervals for 40 minutes and stored at −20°C before histamine quantification.

Statistical analysis

Data were analyzed with GraphPad Prism version 10.3.0 (GraphPad Software, Boston, Mass). Data are presented as mean ± SD or SEM unless otherwise specified.

RESULTS

A wide range of endogenous and exogenous agonists activate MRGPRX2 to induce mast cell degranulation

Using cell lines overexpressing human MRGPRX2 or the mouse functional ortholog MRGPRB2 and measuring IP-1 accumulation as a readout of Gq-coupled G protein–coupled receptor signaling, we confirmed that many structurally diverse agonists activate both MRGPRX2 and MRGPRB2. These include endogenous neuropeptides such as cortistatin-14, pituitary adenylate cyclase–activating peptide (PACAP), and substance P; immune mediators such as eosinophil major basic protein (MBP); and exogenous drugs such as icatibant (Table I; Table E1 in the Online Repository at www.jacionline.org). Treatment of the LAD2 human mast cell line with MRGPRX2 agonists induced mast cell degranulation as measured by β-hexosaminidase release (Table I), which was dependent on MRGPRX2, as CRISPR-mediated knockout of MRGPRX2 completely inhibited this effect (Fig 1, *A*). These studies demonstrated that mast cell degranulation induced by these agonists was fully mediated by MRGPRX2 and not via other receptors. MRGPRX2 agonists also induced degranulation across multiple mast cell types, including PSCMCs and isolated human skin mast cells (Fig 1, *B*; Fig E1 in the Online Repository at www.jacionline.org).

In addition to *in vitro* assays, we examined agonist-induced mast cell degranulation *in vivo* using a mouse model of vascular permeability as a measure of mast cell degranulation. Intradermal injection of MRGPRX2 agonists increased vascular permeability in wild-type mice, whereas MRGPRB2 KO mice showed reduced responses to all agonists tested (Fig 1, *C*), confirming previous reports.[3,12] Substance P–induced vascular permeability showed only partial dependence on MRGPRB2, likely due to activation of other receptors.

Potent small molecule MRGRPX2 antagonists inhibit MRGPRX2-mediated mast cell degranulation

After validating the efficacy of agonists to induce MRGPRX2 activation and mast cell degranulation, we optimized small molecules for potency against MRGPRX2. After iterative screening and compound modification to improve potency of potential antagonists, compounds EP262 and closely related analog EP9907 were identified with a half-maximal inhibitory concentration (IC_{50}) of 2.5 nM and 3.0 nM, respectively, versus cortistatin-14 in LAD2 mast cell degranulation assays (Fig 2, *A*). Further characterization revealed that EP262 and EP9907 completely inhibited MRGPRX2 activation in MRGPRX2-overexpressing Chinese hamster ovary cells in response to all agonists tested (Fig 2, *B*; Table II; Fig E2, *A*, in the Online Repository at www.jacionline.org) and acted as noncompetitive inverse agonists as evaluated by Schild plot analysis[41] and inhibition of basal receptor activity (Fig E3 in the Online Repository at www.jacionline.org). In addition, we evaluated EP262- and EP9907-mediated inhibition of other human MRGPR receptors as well as mouse and cynomolgus monkey MRGPRX2 orthologs to determine specificity and cross-species activity. EP262 and EP9907 failed to block activation of human MRGPRX1, MRGPRX3, and MRGPRX4 and weakly inhibited MRGPRD with potencies of 5.2 μM and 13 μM, respectively (Table E2 in the Online Repository at www.jacionline.org). Compared with potent inhibition of human MRGPRX2, EP262 and EP9907 exhibited lower potency at mouse MRGPRB2 (9-9.5 μM) and cynomolgus monkey MRGPRX2 (0.9-1.2 μM) (Table E3 in the Online Repository at www.jacionline.org). Furthermore, comprehensive *in vitro* off-target profiling EP262 versus a wide range of targets associated with clinical safety risk, including many other receptors, revealed a favorable selectivity profile with minimal off-target effects (data not shown). This indicated that EP262 is highly selective for MRGPRX2 with low potential for adverse effects related to off-target receptor interactions. No agonist or antagonist activities were observed at these targets at concentrations up to 1000-fold higher than its potency against the MRGPRX2 receptor.

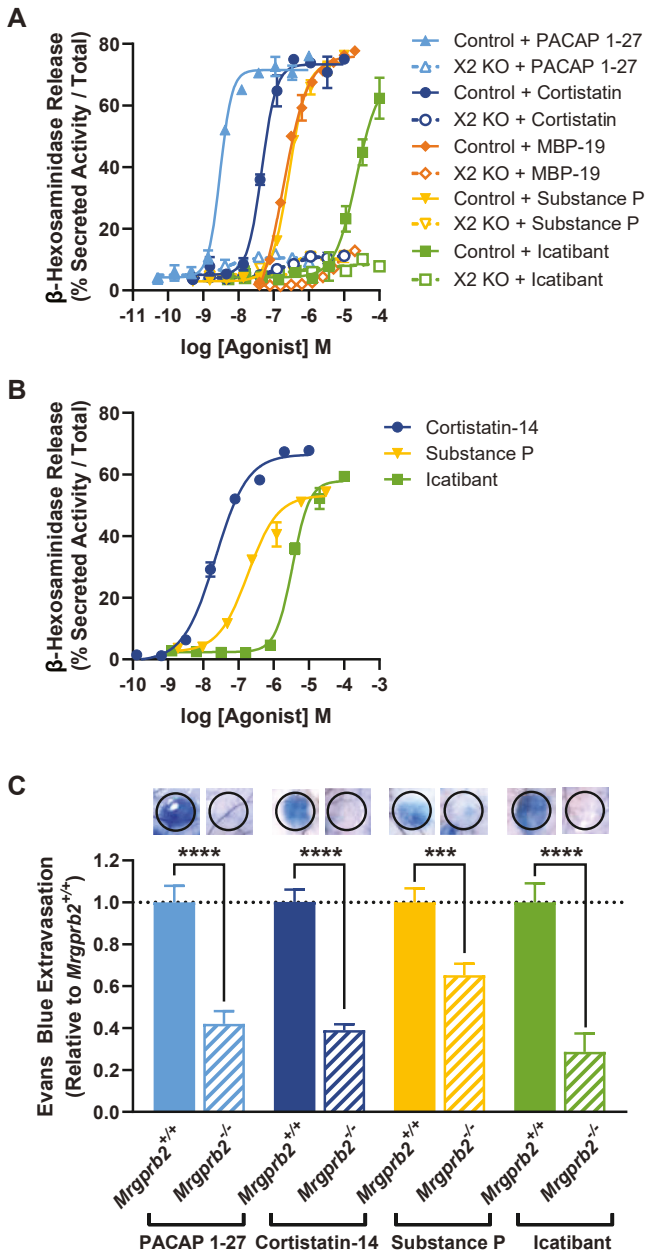

FIG 1. A wide range of endogenous and exogenous agonists activated MRGRPX2 to induce mast cell degranulation. **(A)** Degranulation in LAD2 human mast cells as measured by β-hexosaminidase release, which was blocked by knockout of MRGPRX2 (X2 KO) (n = 3 experiments). **(B)** Degranulation of PSCMCs as measured by β-hexosaminidase release (n = 5 experiments). **(C)** Intradermal injection of agonists induced mast cell degranulation and vascular permeability in *Mrgprb2*$^{+/+}$ wild-type mice, but not in *Mrgprb2*$^{-/-}$ animals. Data are mean ± SEM (n = 14-69 animals per group). ***$P < .001$, ****$P < .0001$ (unpaired *t* test).

Importantly, EP262 and EP9907 potently inhibited agonist-induced mast cell degranulation in multiple human mast cell types, including LAD2 mast cells (Fig 2, *C*; Table II; Fig E2, *B*), PSCMCs (Fig 2, *D*; Fig E2, *C*), and isolated human skin mast cells (Fig E4 in the Online Repository at www.jacionline.org). In addition to β-hexosaminidase, MRGPRX2 antagonists inhibited numerous other inflammatory mediators released upon agonist-induced mast cell degranulation, including tryptase release from PSCMCs (Fig 2, *E*; Fig E2, *D*), histamine release from LAD2 mast cells (Fig E5 in the Online Repository at www.jacionline.org), and secretion of a wide range of late-phase cytokines and chemokines from LAD2 mast cells (Fig 2, *F*; Fig E2, *E*), demonstrating complete inhibition of multiple measures of mast cell degranulation. The ability of antagonists to inhibit agonist-induced mast cell activation and degranulation was also observed via single cell time-lapse confocal microscopy in PSCMCs (Fig E6, *A*, in the Online Repository at www.jacionline.org). Cells were loaded with the calcium indicator dye Fluo-4 (Thermo Fisher Scientific, Waltham, Mass) and stimulated in the presence of the granule-binding glycoprotein, Av.SRho (Sigma-Aldrich, St Louis, Mo), to monitor early calcium signaling and degranulation dynamics at the single-cell level.[42] The MRGPRX2 agonist, substance P, rapidly induced robust calcium mobilization followed by degranulation (Fig E6, *B* and *C*), which was blocked by pretreatment with nanomolar concentrations of the MRGPRX2 antagonist, EP9907. This provided visual confirmation of inhibited degranulation in live mast cells (Fig E6, *D-K*).

EP262 inhibits agonist-induced skin mast cell degranulation *in vivo*

To evaluate human MRGPRX2 receptor function in mouse models, we developed proprietary transgenic MRGPRX2 KI mice by targeted CRISPR-mediated gene editing, in which the human *MRGPRX2* gene was inserted into the mouse *Mrgprb2* loci (Fig 3, *A*). We confirmed expression of human MRGPRX2 on mast cells by flow cytometry analysis of isolated peritoneal mast cells (Fig 3, *B*). Intradermal injection of MRGPRX2 agonists, including cortistatin-14, induced mast cell degranulation in the skin as measured by Evans blue vascular permeability assays (Fig E7 in the Online Repository at www.jacionline.org). Agonist potency was consistent with *in vitro* experiments, confirming functional MRGPRX2 in skin mast cells.

Oral treatment of MRGPRX2 KI animals with EP262 led to a dose-dependent inhibition of cortistatin-14–induced mast cell degranulation and skin vascular permeability (Fig 3, *C*). Oral pharmacokinetics experiments in mice informed dose selection for these *in vivo* studies (Fig E8 in the Online Repository at www.jacionline.org). EP262 significantly reduced degranulation and vascular permeability at free plasma concentrations as low as 1.6 nM with complete inhibition achieved at approximately 10 nM concentrations. In addition, oral treatment with EP262 (at approximately 20 nM free plasma concentrations) led to inhibition of MRGPRX2 activation by all agonists tested, including endogenous neuropeptides, eosinophil granule peptide MBP-19, and exogenous drug agonists (Fig 3, *D*). This is consistent with the ability of the antagonist to block mast cell degranulation induced by all MRGPRX2 agonists *in vitro*. Similarly, EP9907 also blocked agonist-induced degranulation *in vivo* (Fig E9 in the Online Repository at www.jacionline.org). There were no other phenotypic effects or safety-related observations in animals dosed with MRGPRX2 antagonists at any concentration tested (data not shown).

MRGPRX2 antagonists block mast cell degranulation by MRGPRX2 agonists, but not IgE

Previous studies reported that MRGPRX2 mediates mast cell degranulation independently of the IgE-induced pathway, making

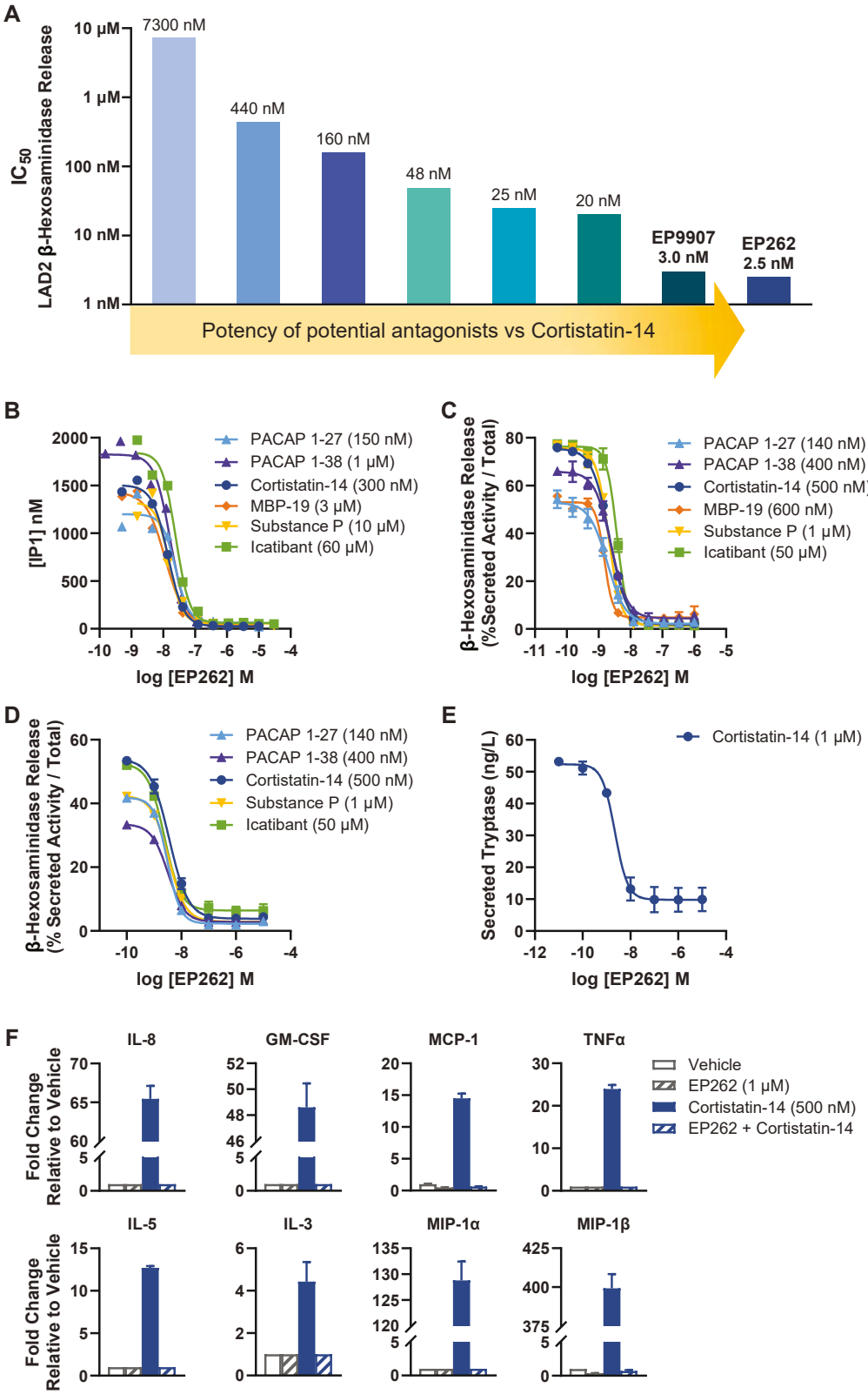

FIG 2. Identification of MRGPRX2 antagonists that potently inhibit MRGPRX2 activity. **(A)** Small molecule optimization in the development of the potent MRGPRX2 antagonists, with low nM potency, as assessed by β-hexosaminidase release in LAD2 mast cells. EP262 inhibited activation of MRGPRX2 in overexpressing cells by all agonists tested, as measured by **(B)** IP-1 levels (representative values shown, n = 3-17 experiments), **(C)** β-Hexosaminidase release in LAD2 mast cells (n = 5 experiments), and **(D)** PSCMCs (n = 3 experiments). EP262 also inhibited **(E)** agonist-induced tryptase secretion from PSCMCs (n = 2 experiments) and **(F)** secretion of cytokines and chemokines from LAD2 mast cells. Data are mean ± SEM (n = 2-3 experiments). *MCP-1,* Monocyte chemoattractant protein-1; *MIP-1α,* macrophage inflammatory protein-1 alpha; *MIP-1β,* macrophage inflammatory protein-1 beta

TABLE II. MRGPRX2 antagonist EP262 inhibits activation by endogenous and exogenous agonists

Agonist tested	β-Hexosaminidase in LAD2 cells average IC_{50} ± SD (nM)	IP-1 in MRGPRX2 CHO cells average IC_{50} ± SD (nM)	β-Arrestin in MRGPRX2 CHO cells average IC_{50} ± SD (nM)
Cetrorelix	0.67 ± 0.17	17 ± 3.9	3.5 ± 0.3
Chlorpromazine	0.76 ± 0.085	13 ± 0.3	4.3 ± 1.4
Dextromethorphan	1.4 ± 0.14	27 ± 13	3.4 ± 0.9
MBP-19	1.4 ± 0.11	11 ± 0.98	ND
PACAP 1-27	1.8 ± 0.091	22 ± 6.4	3.9 ± 2.8
Substance P	2.2 ± 0.15	15 ± 0.95	1.5 ± 0.6
Cortistatin-14	2.5 ± 0.38	14 ± 6.0	2.4 ± 0.2
PACAP 1-38	2.6 ± 0.18	16 ± 1.4	ND
Compound 48/80	3.3 ± 0.39	15 ± 3.1	4.7 ± 3.8
Icatibant	3.4 ± 0.52	24 ± 4	1.2 ± 0.2

Average IC_{50} of antagonist versus agonist at approximately EC_{80} concentrations (concentration that gives 80% of maximal response). n = 2-17 per condition.
CHO, Chinese hamster ovary; *ND,* not determined.

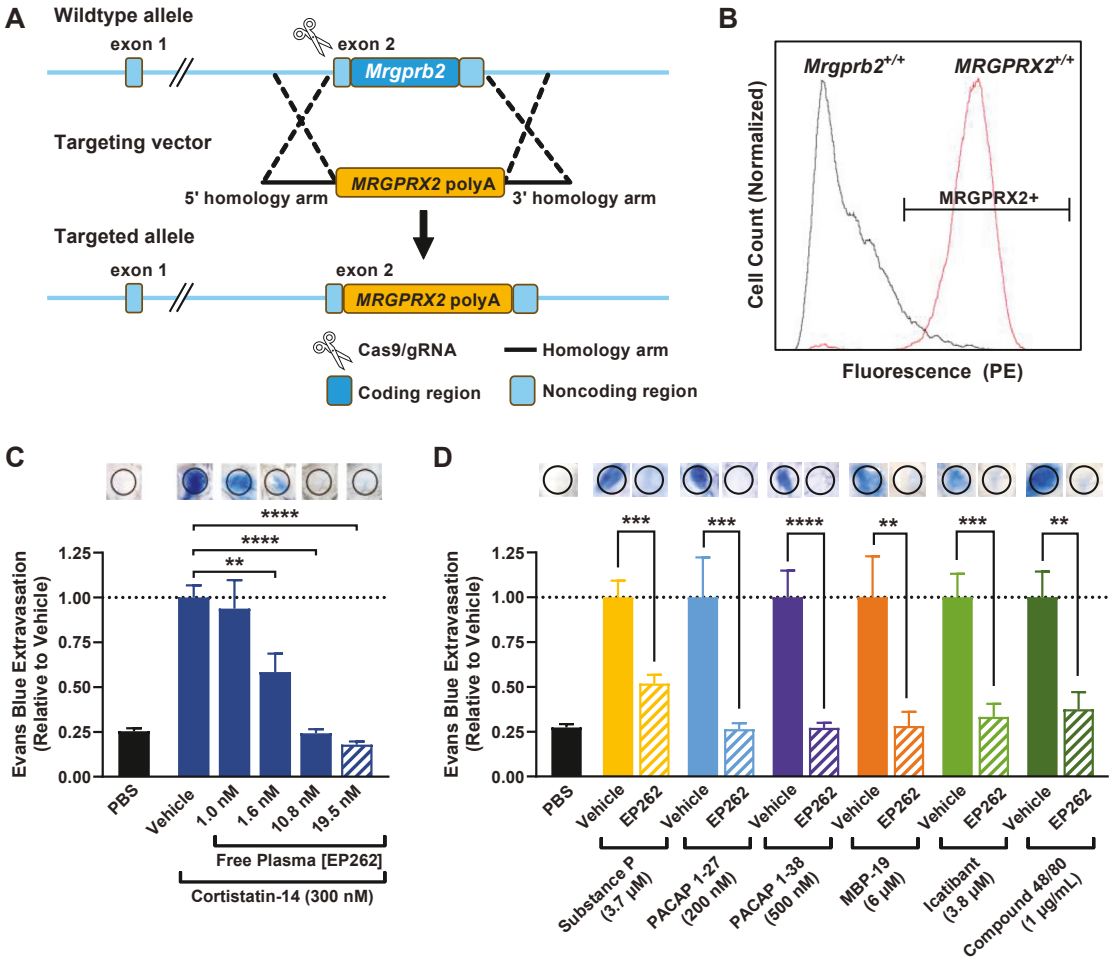

FIG 3. EP262 inhibited *in vivo* mast cell degranulation. **(A)** Generation of the *Mrgprb2*$^{em(MRGPRX2)}$ transgenic KI mouse, replacing mouse *Mrgprb2* with human *MRGPRX2* behind the endogenous *Mrgprb2* promoter. **(B)** Representative histogram demonstrating confirmation of human MRGPRX2 expression in peritoneal mast cells isolated from KI mice (shown as *MRGPRX2*$^{+/+}$ in *red*) compared with *Mrgprb2*$^{+/+}$ wild-type mice (*black*) by flow cytometry. **(C)** Oral treatment of KI mice with EP262 inhibited mast cell degranulation and vascular permeability induced by the MRGPRX2 agonist cortistatin-14, with increased inhibition correlating with increased plasma levels of EP262. Data are mean ± SEM (n = 10-21 animals per group). $^{**}P < .01$, $^{***}P < 0.01$, $^{****}P < .0001$ for EP262 versus cortistatin-14 (one-way ANOVA with post hoc Tukey multiple comparisons test). **(D)** EP262 (19.5 nM free plasma concentrations) inhibited mast cell degranulation and vascular permeability *in vivo* induced by all agonists tested, including substance P, PACAP 1-27, PACAP 1-38, MBP-19, icatibant, and compound 48/80. Data are mean ± SEM (n = 6-11 animals per group). $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$ for EP262 versus Vehicle (unpaired *t* test). *gRNA,* Guide RNA; *PE,* phycoerythrin.

MRGPRX2 an attractive target for therapeutic treatment of non–IgE-driven conditions.[3,4,42-44] To better understand the relationship between these 2 pathways of mast cell degranulation, we knocked out MRGPRX2 or the high-affinity IgE receptor, FCεRIα, in LAD2 mast cells by CRISPR-mediated gene editing and evaluated degranulation induced by either IgE or the MRGPRX2 agonist, cortistatin-14 (Fig 4, *A*). Whereas knockout of MRGPRX2 completely inhibited cortistatin-14–induced degranulation, it did not reduce response to IgE. Conversely, knockout of the IgE receptor inhibited degranulation induced by IgE, but not cortistatin-14. Accordingly, treatment with EP262 did not inhibit IgE-induced degranulation in either LAD2 mast cells or PSCMCs, consistent with a non–IgE-mediated mechanism of action (Fig 4, *B* and *C*). Similar to *in vitro* experiments, we found that although knockout of the MRGPRB2 receptor (Fig 4, *D*) or oral EP262 administration to MRGPRX2 KI mice (Fig 4, *E*) completely inhibited cortistatin-14–induced mast cell degranulation and vascular permeability, IgE-induced mast cell degranulation was unchanged.

Ex vivo human skin mast cell degranulation is inhibited by treatment with EP262

To further validate inhibition of mast cell degranulation in intact human tissue, we examined the effect of EP262 in human skin explants by microdialysis, which enables real-time monitoring of select degranulation products. Intradermal injections of MRGPRX2 agonists, cortistatin-14 and icatibant, led to robust dose-dependent increases in histamine release across multiple donors (Fig E10 in the Online Repository at www.jacionline.org), indicative of mast cell degranulation. Pretreatment with intradermal injection of EP262 led to dose-dependent inhibition of both cortistatin-14 and icatibant-induced histamine release (Fig 5, *A* and *B*).

DISCUSSION

An increasing amount of evidence shows that MRGPRX2 mediates IgE-independent mast cell activation in response to a wide range of agonists, triggering release of proinflammatory mediators and a multicellular signaling cascade that likely plays a key role in multiple mast cell–mediated human diseases.[6,18,30,35] Here, we describe the identification of novel small molecule antagonists that specifically and potently inhibit the human MRGPRX2 receptor *in vitro* and *in vivo*.

Several studies have shown that expression of MRGPRX2 and its agonists are upregulated in patients with chronic urticaria, allergic contact dermatitis, atopic dermatitis, chronic prurigo, asthma, and other mast cell–associated diseases.[28,32,34,45] However, the ability to interrogate the function and potential of MRGPRX2 has been limited by the lack of potent bioactive antagonists. Several lower-affinity compounds have been reported to be inhibitors of MRGPRX2 in the literature.[46-53] However, they do not display potent activity across various functional assays *in vitro* and, in many cases, are not bioactive *in vivo*. The molecules we identified via systematic high-throughput screening and subsequent medicinal chemistry optimization showed high potency in multiple functional assays in a variety of mast cells. As exemplified by EP262 and EP9907, these molecules blocked activation of MRGPRX2 against all agonists tested, functioned as noncompetitive inverse agonists, and displayed high selectivity over other MRGPRs and many other receptors. Importantly, treatment with EP262 and EP9907 potently inhibited MRGPRX2 agonist–induced activation and mast cell degranulation in all assays tested, including inhibition of both early-phase degranulation products such as histamine and late-phase degranulation products such as cytokines. This contrasts with reports of other molecules that demonstrate high potency in specific assays involving MRGPRX2 overexpression, but do not block endogenously expressed MRGPRX2-mediated mast cell degranulation with equal potency and efficacy.[46-53] Also, the previously published reports describe only *in vivo* evaluation of antagonists at the murine ortholog Mrgprb2, which does not show the utility of these molecules at the human MRGPRX2 receptor.[46,47,50,51] In our study, EP262 and EP9907 blocked activation of LAD2 mast cells and primary human mast cells derived from multiple tissues, as well as inhibited the human MRGPRX2 receptor *in vivo* using transgenic KI mice. Finally, these MRGPRX2 antagonists potently inhibited agonist-induced mast cell degranulation in intact human tissue *ex vivo*, providing proof-of-concept in physiologically complex intact human tissue.

The MRGPR receptors are quite variable with relatively diverse primary amino acid sequences across different species, and members of the MRGPRX family are found only in primates and several other nonrodent species.[11] Even though human MRGPRX2 and mouse MRGPRB2 are recognized to be functional orthologs, they exhibit significant differences in sequence similarity (approximately 53% overall sequence similarity).[8] It is therefore challenging to identify antagonists that are equally efficacious at blocking human MRGPRX2 and ortholog receptors in other species, including mouse MRGPRB2. To enable *in vivo* pharmacological evaluation of MRGPRX2 antagonists, we generated transgenic MRGPRX2 KI mice, in which the human *MRGPRX2* gene was inserted into the mouse *Mrgprb2* loci. We confirmed expression of human MRGPRX2 in mast cells and confirmed functionality *in vivo* by demonstrating that multiple MRGPRX2 agonists increased skin vascular permeability, a measure of mast cell degranulation.[25] Mast cell degranulation induced by MRGPRX2 agonists, but not IgE stimulation, was reversed by oral EP262 in MRGPRX2 KI animals, consistent with the IgE-independent role of MRGPRX2. Furthermore, EP262 was highly potent, resulting in a 90% inhibitory EP262 free plasma concentration of 2.3 nM against cortistatin-14 in the vascular permeability model.[3,12] Inhibition of vascular permeability by all MRGPRX2 agonists tested was observed at free plasma EP262 concentrations of approximately 20 nM or less in MRGPRX2 KI mice. Importantly, this is the first report demonstrating *in vivo* inhibition of human MRGPRX2 using an orally active small molecule antagonist.

MRGPRX2 is a highly promiscuous receptor that is activated by numerous structurally diverse agonists that also activate other canonical receptors.[6,9,10] Confirming previous studies, loss of either human MRGPRX2 or mouse MRGPRB2 in mast cells led to reduced mast cell activation and degranulation in response to MRGPRX2 agonists. This demonstrated that mast cell activation induced by these agonists is driven by MRGPRX2 and not through other receptors. For example, the neuropeptide substance P activates mast cells via MRGPRX2 and not via the neurokinin-1 receptor (NK1R), as shown by complete inhibition of substance P–induced degranulation *in vitro* and *in vivo* when MRGPRX2 and MRGPRB2 are absent. Interestingly, NK1R antagonists

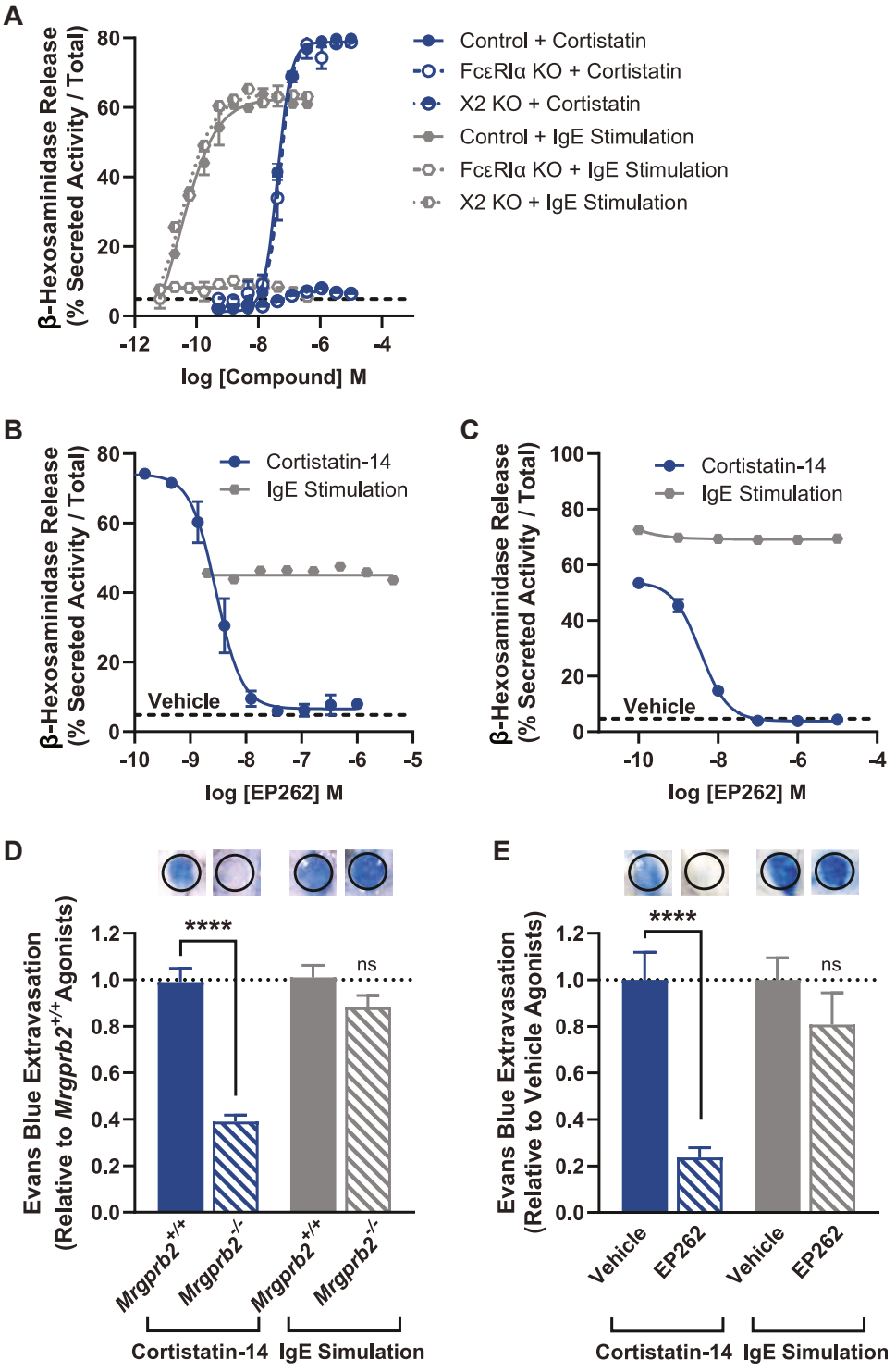

FIG 4. MRGPRX2-mediated mast cell degranulation is IgE independent. **(A)** KO of the high-affinity IgE receptor FCεRIα blocked LAD2 mast cell degranulation in response to IgE stimulation, but did not alter response to MRGPRX2 agonists, whereas KO of MRGPRX2 completely inhibited degranulation in response to MRGPRX2 agonists but not IgE stimulation. Similarly, EP262 potently inhibited degranulation induced by MRGPRX2 agonist cortistatin-14 but not IgE stimulation in LAD2 cells **(B)** as well as in peripheral stem cell–derived mast cells **(C)**. **(D)** Loss of Mrgprb2 in mice prevented mast cell degranulation and vascular permeability induced by MRGPRB2/X2 agonists including cortistatin-14 (3 μM) but not IgE stimulation (n = 66-69 animals per group). **(E)** Oral EP262 treatment (19.5 nM free plasma concentrations) in MRGPRX2 KI mice also inhibited mast cell degranulation induced by cortistatin-14 (300 nM) but not IgE stimulation (n = 10 animals per group). Data shown in *(D)* and *(E)* are mean ± SEM. ****$P < .0001$, ns = not significant (unpaired *t* test).

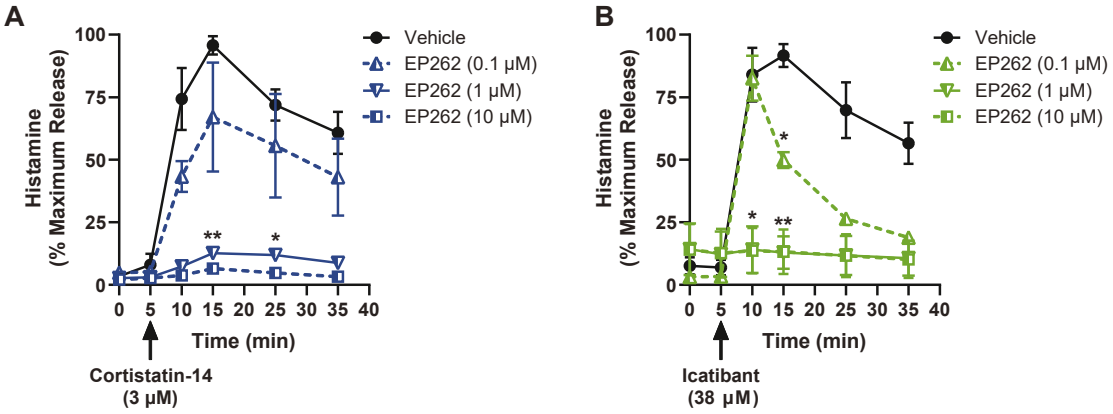

FIG 5. EP262 inhibited agonist-induced mast cell degranulation in *ex vivo* human skin. Treatment with EP262 dose-dependently inhibited mast cell degranulation and histamine release induced by both cortistatin-14 **(A)** and icatibant **(B)**. Data are mean ± SEM (n = 3) and are normalized to maximal agonist-induced histamine response. $*P < .05$, $**P < .01$ for EP262 treatments versus vehicle (two-way ANOVA with post hoc Tukey multiple comparisons test).

have been shown to have off-target activity at mouse MRGPRB2, but not human MRGPRX2. This likely explains the lack of translation of mouse studies to clinical investigation of NK1R antagonists.[54]

Recent structural understanding of MRGPRX2 has revealed a distinct extracellular ligand-binding pocket, which is described as wide open and shallow and is made up of 2 different subpockets, with charge interactions at acidic residues D184 and E164 shown to be critical for receptor activation.[53,55] Additional studies demonstrated that substance P analogs devoid of key residues failed to induce mast cell degranulation, confirming ligand-MRGPRX2 interactions.[56,57] The large binding pocket also accounts for receptor promiscuity in binding a large variety of structurally diverse cationic ligands, and the lower potency observed for receptor agonists. For example, PACAP peptides are highly potent at their canonical PAC1R (half-maximal effective concentration [EC_{50}] = approximately 2 nM), but activate MRGPRX2 only at higher concentrations (EC_{50} = 50-500 nM).[58] This supports the hypothesis that MRGPRX2 activation occurs when ligand concentrations reach higher pathological levels, eg, when homeostasis is disrupted. Accordingly, ablation of Mrgprb2 or inhibition of MRGPRX2 did not show any phenotypic effects under normal conditions, and there were no safety observations in animals dosed with high concentrations of MRGPRX2 antagonists.[12] Elevated levels of MRGPRX2 agonists such as substance P and eosinophil cationic protein have been demonstrated in the serum of patients with chronic urticaria, atopic dermatitis, and other inflammatory conditions.[27,59-61] Additionally, increased expression of cortistatin-14 and substance P has been observed in the skin of patients with chronic prurigo and atopic dermatitis.[32,45] However, the endogenous tissue concentrations of these ligands in normal or pathological conditions are unknown. It is tempting to speculate that, under pathological conditions, locally high concentrations of neuropeptides released by activated sensory neurons induce degranulation in nearby mast cells. Release of proinflammatory mediators can then initiate and maintain downstream chronic inflammation and disease.

In mast cell–mediated diseases such as chronic urticaria, both IgE-dependent and independent pathways are implicated.[30] Given that patients with CSU show enhanced skin reactivity to intradermally injected MRGPRX2 agonists, MRGPRX2 may play a critical role in mast cell sensitivity.[24,29] Here, we characterize the IgE-independent mechanism of MRGPRX2 in acute mast cell degranulation using both pharmacological and genetic methods. To our knowledge, the molecules we describe are the most potent and druglike MRGPRX2 antagonists identified to date. Potent and orally active MRGPRX2 antagonists are a potential novel mechanism for treating non–IgE-mediated mast cell–dependent diseases. Accordingly, EP262 is currently being investigated in clinical trials as a once-daily oral therapy for chronic spontaneous urticaria (NCT06077773), chronic inducible urticaria (NCT06050928), and atopic dermatitis (NCT06144424). The results of these studies will illuminate the potential contribution of MRGPRX2 in human health and disease.

DISCLOSURE STATEMENT

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Disclosure of potential conflicts of interest: J. Wollam, M. Solomon, C. Villescaz, M. Lanier, A. Vest, J. Napora, B. Charlot, C. Cavarlez, S. Evans, C. Bacon, A. Vasquez, D. Freeman, A. Kim, L. Dvorak, B. Selfridge, L. Huang, A. Nevarez, H. Dedman, J. Brooks, G. Timony, E. Martinborough, M. F. Boehm, and V. Viswanath are employees of Escient Pharmaceuticals and hold stock in the company. S. Frischbutter has received research funding from Escient Pharmaceuticals. M. Metz has received honoraria as a consultant for Escient Pharmaceuticals. N. Gaudenzio has a patent entitled “Method to treat type 2 inflammation or mast-cell dependent disease” (WO2020229648A1), acts as a scientific consultant for Escient Pharmaceuticals, and acts as Chief Scientific Officer and is a shareholder at Genoskin. N. Serhan declares that they have no relevant conflicts of interest.

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Key messages

- **Novel small molecule MRGPRX2 antagonists potently inhibited agonist-induced mast cell degranulation in cultured mast cells, in mice, and in *ex vivo* human skin in an IgE-independent manner.**
- **Potent orally active MRGPRX2 antagonists have potential for management of mast cell–mediated disorders including inflammatory and pain conditions that are less amenable to current standard treatments.**

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