Murepavadin, a Small Molecule Host Defense Peptide Mimetic, Activates Mast Cells via MRGPRX2 and MrgprB2

Aetas Amponnawarat1,2, Chalatip Chompunud Na Ayudhya1,3 and Hydar Ali1*

1 Department of Basic and Translational Sciences, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, United States, 2 Department of Family and Community Dentistry, Faculty of Dentistry, Chiang Mai University, Chiang Mai, Thailand, 3 Department of Oral Diagnosis, Faculty of Dentistry, Naresuan University, Phitsanulok, Thailand

Pseudomonas aeruginosa is a frequent cause of hospital-acquired wound infection and is difficult to treat because it forms biofilms and displays antibiotic resistance. Previous studies in mice demonstrated that mast cells (MCs) not only contribute to P. aeruginosa eradication but also promote wound healing via an unknown mechanism. We recently reported that host defense peptides (HDPs) induce human MC degranulation via Mas-related G protein-coupled receptor-X2 (MRGPRX2). Small molecule HDP mimetics have distinct advantages over HDPs because they are inexpensive to synthesize and display high stability, bioavailability, and low toxicity. Murepavadin is a lipidated HDP mimetic, (also known as POL7080), which displays antibacterial activity against a broad panel of multi-drug-resistant P. aeruginosa. We found that murepavadin induces Ca2+ mobilization, degranulation, chemokine IL-8 and CCL3 production in a human MC line (LAD2 cells) endogenously expressing MRGPRX2. Murepavadin also caused degranulation in RBL-2H3 cells expressing MRGPRX2 but this response was significantly reduced in cells expressing missense variants within the receptor’s ligand binding (G165E) or G protein coupling (V282M) domains. Compound 48/80 induced β-arrestin recruitment and promoted receptor internalization, which resulted in substantial decrease in the subsequent responsiveness to the MRGPRX2 agonist. By contrast, murepavadin did not cause β-arrestin-mediated MRGPRX2 regulation. Murepavadin induced degranulation in mouse peritoneal MCs via MrgprB2 (ortholog of human MRGPRX2) and caused increased vascular permeability in wild-type mice but not in MrgprB2−/− mice. The data presented herein demonstrate that murepavadin activates human MCs via MRGPRX2 and murine MCs via MrgprB2 and that MRGPRX2 is resistant to β-arrestin-mediated receptor regulation. Thus, besides its direct activity against P. aeruginosa, murepavadin may contribute to bacterial clearance and promote wound healing by harnessing MC’s immunomodulatory property via the activation of MRGPRX2.

Keywords: murepavadin, mast cells, MrgprB2, MRGPRX2, host defense peptides, antimicrobial peptides
INTRODUCTION

The emergence of multidrug-resistant bacterial infections poses a global public health threat that warrants urgent need for alternative therapeutic approaches (1). Host defense peptides (HDPs), previously known as antimicrobial peptides (AMPs), such as the cathelicidin LL-37 and human β-defensins are considered as promising antimicrobial agents (2–4). Recent evidence demonstrated that in addition to their direct antimicrobial activity, HDPs promote the recruitment and activation of various immune cells including mast cells (MCs), neutrophils, monocytes and lymphocytes (5–8). These HDPs also display angiogenic activity and contribute to wound healing (8, 9). However, many HDPs cause the lysis of erythrocytes and cause cytotoxicity against a variety of cells (10, 11). In recent years, great strides have been made in optimizing HDPs to minimize their toxicity and to improve their stability, which can also modulate the immune system for therapeutic benefits (12, 13).

Mast cells (MCs) are multifunctional immune cells of hematopoietic origin that are found in vascularized tissues such as the oral mucosa, intestine and the skin. MCs play an important role in host defense and promote wound healing (14–16). In addition to high affinity IgE receptor (FccRII), a subtype of human MCs (MCCTG contain both tryptase and chymase) expresses a G protein-coupled receptor (GPCR) known as Mas-related GPCR-X2 (MRGPRX2) (17, 18). This receptor is highly expressed in human skin MCs but is also present in lung and gut MCs but at lower levels (17, 19, 20). Mouse connective tissue MCs (CTMC; skin, nasopharynx and peritoneal) express MrgprB2 (ortholog of human MRGPRX2) (16, 21). Both receptors are activated by human and mouse HDPs (22–24). Studies with human MCs expressing MRGPRX2 and MrgprB2-/- mice have strongly implicated these receptors in innate immunity and wound healing (15, 16). The major portal of entry for pathogen is the interface between host and external microenvironment such as the skin, nasopharynx, and peritoneum. MrgprB2-expressing CTMCs are found abundantly at these sites and contribute to host defense against bacterial infection through the release of MC-derived mediators and the subsequent recruitment of neutrophils (16). Furthermore, pharmacological activation of MgrpB2 at these sites results in decreased bacterial count and reduced disease severity in vivo (16).

Pseudomonas aeruginosa is a Gram-negative bacterium that often presents a therapeutic challenge due to its ability to form biofilms and to display antibiotic resistance (25, 26). Zimmerman et al. (27), utilized a topical P. aeruginosa infection model and demonstrated that MCs contribute to both bacterial elimination and promote wound healing. However, they found that culturing MCs infected with P. aeruginosa in vitro is insufficient to eliminate bacteria unless they are co-cultured with keratinocytes. MC mediators released in response to P. aeruginosa infection results in the secretion of HDPs such as mouse β-defensin-14 (Defb14, ortholog of human β-defensin-3) from keratinocytes (27). These findings suggest that MC-derived mediators confer protective immunity through the promotion of endogenous HDP secretion, which in turn, directly kill the bacteria and restrain the infection (27).

Protegrin-1 is a HDP that was originally purified from porcine leukocytes (28). We have recently shown that protegrin-1 activates human MCs via MRGPRX2 (29). However, small molecule HDP mimetics have a number of advantages over natural HDPs because of their superior stability, bioavailability, and reduced toxicity (30, 31). Moreover, several approaches have been used to increase hydrophobicity and membrane activity of HDP mimetics (32). Murepavadin is a lipided protegrin-1 mimetic, (also known as POL7080), which specifically targets P. aeruginosa, including multidrug-resistant clinical isolates (33). Thus, it could be used for the treatment of antibiotic-resistant P. aeruginosa skin infection (34, 35). Given that HDPs including protegrin-1 activate human MCs via MRGPRX2 (22, 23, 29), raises the interesting possibility that potential therapeutic action of murepavadin for P. aeruginosa skin infection likely reflects both MRGPRX2-mediated MC activation and its direct antimicrobial activity. However, the possibility that murepavadin activates MCs has not been tested.

Besides G proteins, most GPCR agonists activate another signaling pathway that requires the recruitment of adapter proteins known as β-arrestins. This β-arrestin-mediated pathway was first identified for its role in receptor desensitization (uncoupling of receptor/G protein interaction) and internalization (36). Agonists that prefer to activate G proteins over β-arrestins are known as G protein-biased agonists, whereas agonists that selectively activate β-arrestins are known as β-arrestin-biased agonists. By contrast, agonists that activate both pathways are designated as balanced agonists. We have recently shown that while compound 48/80 (C48/80) acts as a balanced agonist for MRGPRX2, an angiogenic host defense peptide serves as a G protein-biased agonist (37). The purpose of this study was to test if murepavadin activates human MCs via MRGPRX2 and to determine if it serves as a balanced or biased agonist for the receptor. The data presented herein demonstrate that murepavadin activates human and murine MCs via MRGPRX2 and MgrpB2, respectively and that it serves as a G protein-biased agonist for MRGPRX2 without the involvement of β-arrestin-mediated receptor regulation. These findings have important implications for the potential utilization of murepavadin in modulating antibiotic-resistant cutaneous infections.

MATERIALS AND METHODS

Materials

All reagents used for cell culture were purchased from Invitrogen (Gaithersburg, MD, USA). Recombinant mouse interleukin-3 (IL-3), mouse stem cell factor (SCF), and recombinant human SCF (thSCF) were obtained from Peprotech (Rocky Hill, NJ, USA). Compound 48/80 (C48/80) was obtained from AnaSpec (Fremont, CA, USA). Murepavadin (Catalog HY-P1674A) was from MedChem Express. P-nitrophenyl-N-acetyl-β-D-glucosamine (PNAG) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pertussis toxin (PTx) was from List Biological Laboratories.
Fura-2 acetoxyethyl ester was from Abcam (Cambridge, MA, USA). Bright-Glo Luciferase was from Promega (Madison, WI, USA). Phycocyanin (PE)-conjugated anti-human MRGPRX2 antibody was from BioLegend (San Diego, CA, USA). Amaxa Nucleofector Kit V was from Lonza (Gaithersburg, MD, USA). DuoSet ELISA kits were from R&D Systems (Minneapolis, MN, USA). Hemagglutinin (HA)-tagged MRGPRX2 plasmid in pReceiver-M06 vector was obtained from GeneCopoeia (Rockville, MD, USA). MRGPRX2-Tango plasmid (Addgene no. 66440) was a gift from Dr. Bryan Roth.

**Mice**

C57BL/6 (wild-type; WT) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed in pathogen-free cages. WT mice with the deletion of *MrgrBP2* transcript (*MrgrpB2/−/−* mice) were generated as previously described (38). Eight-to-twelve-week-old male and female mice were used. All animal experiments performed in this study were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

**Cell Culture**

The human MC line (LAD2 cell) was kindly provided by Drs. Kirshenbaum and Metcalfe (Laboratory of Allergic Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH), USA). LAD2 cells were cultured in complete StemPro-34 medium supplemented with l-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL), and rhSCF (100 ng/mL), and the medium was hemi-depleted weekly (39).

Rat basophilic leukemia (RBL-2H3) cells were cultured as monolayers in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/mL) and streptomycin (100 µg/mL) (40). RBL-2H3 cells stably expressing human MRGPRX2 were maintained similarly in the presence of 1 ng/mL G418. HTLA cells (HEK-293T cells stably expressing a Tα-dependent luciferase reporter and a β-arrestin2-TEV protease fusion gene) were cultured in DMEM supplemented with 10% FBS, L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 µg/mL), hygromycin (200 µg/mL), puromycin (5 µg/mL), and G418 (500 µg/mL) (41). All cell cultures were kept at 37°C incubator with 5% CO2.

Peritoneal mast cells (PMCs) were established from peritoneal lavages of WT and *MrgrpB2/−/−* mice and were cultured for 4–8 weeks in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% FCS, and recombinant mouse IL-3 (10 ng/mL) and SCF (30 ng/mL). Cells were then determined for MC receptor expression and function, and were used within 4–8 weeks (38, 42).

**Degranulation**

Human LAD2 (1 × 10⁴ cells/well), RBL-2H3, RBL-MRGPRX2 (5 × 10⁴ cells/well), or murine peritoneal MCs (5 × 10⁵ cells/well) were washed and plated in a total volume of 50 µL HEPES buffer in 96-well plates. Cells were then stimulated with murepavadin for 30 min at 37°C. Total level of β-hexosaminidase release were assessed by lysing the cells with 0.1% Triton X-100, whereas cells without any stimulation were designated as controls. Aliquots (20 µL) of supernatants were incubated with 1 mM PNAG (20 µL) for 1 h at 37°C. The reaction was then stopped by adding stop buffer (250 µL; 0.1 M Na₂CO₃/0.1 M NaHCO₃). Quantification of β-hexosaminidase level was determined by measuring the absorbance at 405 nm using a Versamax microplate spectrophotometer (Molecular Devices, San Jose, CA, USA) (40).

In some experiments, cells were pretreated with PTx (100 ng/mL, 16 h) prior to any stimulation to assess the inhibitory effect of PTx on MC degranulation.

**Calcium Mobilization**

Human LAD2 cells (3 × 10⁵), RBL-2H3 or RBL-MRGPRX2 (2 × 10⁶) were loaded with Fura-2 acetoxyethyl ester (1 µM for 30 min at 37°C) in 1.5 mL of HEPES buffer containing 0.1% BSA. Cells were then washed and allowed complete de-esterification for 15 min at room temperature. Cells were then resuspended in buffer and stimulated with Murepavadin or C48/80. Calcium mobilization was determined by the ratio between dual excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm using a Hitachi F-2700 Fluorescence Spectrophotometer.

**Cytokine and Chemokine Production and Measurement**

LAD2 cells (3 × 10⁵ cells/mL) were washed with medium, resuspended in fresh medium, and stimulated with indicated concentrations of Murepavadin for 24 h at 37°C with 5% CO₂. Cell-free supernatants were collected and kept at -80°C until further analyses. Similarly, RBL-2H3 or RBL-MRGPRX2 cells (2 × 10⁵ cells/mL) were seeded in a 24-well plate and cultured overnight in a 37°C incubator with 5% CO₂. The next day, the medium was aspirated, fresh medium was added to the cells, and the cells were stimulated with indicated concentrations of Murepavadin for 24 h at 37°C with 5% CO₂. Cell-free supernatants were collected and kept at -80°C until further analyses. The cytokine and chemokine production were measured using human CCL3/MIP-1 alpha, human IL-8/ CXCL8, rat JE/MCP-1/CCL2 and rat TNF-alpha DuoSet ELISA kits (R&D Systems) following the manufacturer’s protocols.

To determine the inhibitory effect of PTx on cytokine and chemokine production, pretreatment with PTx (100 ng/mL, 16 h) was performed prior to any stimulation.

**Generation of Cells Transiently Expressing MRGPRX2 and Its Variants**

Transient transfections in RBL-2H3 cells expressing WT-MRGPRX2 and its naturally occurring missense variants within MRGPRX2’s ligand binding cradle (G165E) or G protein-coupling region (V282M) were performed as described previously (43, 44). RBL-2H3 cells (2 × 10⁶) were transfected with 2 µg of HA-tagged plasmid using the Amaxa Nucleofector Device and Amaxa Kit V and were used within 16 – 20 h post-transfection.

Flow cytometry was used to determine cell surface expression of transiently transfecants. Cells (5 × 10⁵) were incubated with PE-
anti-MRGPRX2 antibody for 30 min, washed in ice-cold FACS buffer (PBS containing 2% FCS and 0.09% NaN₃), followed by fixation with 1.5% paraformaldehyde. Expression level of MRGPRX2 and its variants were analyzed using a BD LSR II flow cytometer (San Jose, CA, USA) with WinList software, version 8.

Transcriptional Activation Following Arrestin Translocation (Tango) Assay
HTLA-MRGPRX2 cells (5 × 10⁵ cells/well, 96-well plate) were cultured overnight in a 37°C incubator. The next day, the medium was removed and cells were exposed to Murepavadin in an antibiotic-free medium (160 µL) for 16 h at 37°C. The medium was then replaced with 100 µL of Bright-Glo solution and relative luminescence was analyzed using a Thermo Luminoskan Ascent 392 Microplate Luminometer (41).

Receptor Internalization
RBL-MRGPRX2 or HTLA-MRGPRX2 cells (5 × 10⁵) were treated with C48/80 or Murepavadin for 30 min, 3, 6, and 16 h to induce cell surface receptor internalization. After indicated time, cell surface expression was determined by flow cytometry as described above.

Evan’s Blue Dye Extravasation
Mice (WT and MrgrpB2⁻/⁻) were intravenously injected with 1% Evan’s blue followed by intradermal injection of 20 µL Murepavadin (30 µM) in the right paw and PBS (vehicle) in the left paw. After 30 min, the mice were euthanized and the paws were removed, weighed, dissolved in 500 µL formamide and incubated at 56°C overnight. The Evan’s blue dye extravasation was measured by collecting the supernatants and the absorbance was measured at 650 nm using microplate spectrophotometer.

Statistical Analysis
Data shown represent mean ± SEM value derived from at least three independent experiments. Statistical significance was calculated using t-test and one-way or two-way ANOVA analyzed by GraphPad Prism version 9.0.1. Significant differences were set at *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

RESULTS
Murepavadin Induces Intracellular Ca²⁺ Mobilization, Degranulation and Chemokine Generation in Human MCs
We previously demonstrated that LL-37, human β-defensins and protegrin-1 activate human MCs via MRGPRX2 at concentrations ranging from 1 - 10 µM (22, 23, 29). To determine if murepavadin activates MCs, we initially utilized a human MC line, LAD2 cells and tested the ability of 10 µM murepavadin to induce Ca²⁺ mobilization. As shown in Figure 1A, murepavadin at this concentration induced a robust and sustained Ca²⁺ response. Next, we asked if murepavadin induces MC degranulation. We found that murepavadin induced a dose-dependent degranulation as measured by β-hexosaminidase release, reaching ~80% at a concentration of 10 µM with an EC₅₀ value of ~3 µM (Figure 1B). While MC degranulation promotes increased vascular permeability in vivo, its innate and adaptive immune function require the generation of chemokines. We therefore investigated the ability of murepavadin to stimulate chemokine IL-8 and CCL3 production in LAD2 cells. At a concentration of 10 µM, murepavadin induced substantial IL-8 (Figure 1C) and CCL3 (Figure 1D).

To explore the underlying mechanism via which murepavadin activates MCs, we utilized RBL-2H3 cells that were transfected to stably express human MRGPRX2 (RBL-MRGPRX2). We found that murepavadin induced Ca²⁺ mobilization, β-hexosaminidase as well as TNF-α and CCL2 production from RBL-MRGPRX2, but not from untransfected cells (Figures 2A-E). Moreover, we found that Pertussis toxin (PTx), an inhibitor of Gαi/o family of G proteins, caused significant inhibition of murepavadin-induced degranulation, TNF-α and CCL2 production (Figures 2C-E). Taken together, these findings demonstrate that murepavadin causes the activation of human MCs specifically via MRGPRX2 and that these responses are G protein-dependent.

Naturally Occurring MRGPRX2 Missense Variants Are Hypo-Responsive to Murepavadin for MC Degranulation
We have previously identified naturally occurring loss-of-function MRGPRX2 missense variants within the receptor’s ligand binding (G165E) and G protein coupling (V282M) domains (Figures 3A, B) (43, 44). To further validate
murepavadin’s specificity for MRGPRX2, we transiently transfected RBL-2H3 cells with cDNAs encoding each of these variants. Flow cytometry analyses confirmed cell surface expression of MRGPRX2 (WT) and its missense variants (G165E and V282M) (Figure 3C). While murepavadin (10 µM) induced β-hexosaminidase release in cells expressing WT-MRGPRX2, this response was significantly inhibited in cells transiently expressing the G165E or V282M variant (Figure 3D). These findings substantiate the notion that murepavadin utilizes MRGPRX2 to activate MCs.

Murepavadin Does Not Induce MRGPRX2 Internalization or Desensitization

We have previously shown that C48/80 not only induces β-arrestin recruitment but also causes MRGPRX2 internalization (37). We found that incubation of HTLA-MRGPRX2 cells with C48/80 (3 µg/mL; 0.5 h -16 h) induced substantial receptor internalization but murepavadin had no effect (Figures 4C, D). The data presented in Figures 4E, F show representative histograms of cell surface MRGPRX2 following incubation of HTLA-MRGPRX2 cells with C48/80 (3 µg/mL) and murepavadin (10 µM) for 16 h. These data clearly demonstrate that while C48/80 promotes β-arrestin recruitment and caused MRGPRX2 internalization, murepavadin does not induce these responses.

To confirm the biological relevance of the findings described above, we performed receptor internalization studies in RBL-MRGPRX2 cells by flow cytometry. Similar to the situation in HTLA-MRGPRX2 cells, C48/80 induced substantial receptor internalization in RBL-MRGPRX2 cells but murepavadin had no effect (Figures 5A, B). As for β-arrestin recruitment (Figure 4A) and receptor internalization (Figures 5A, B), incubation of cells with C48/80 (3 µg/mL) 16 h) resulted in substantial inhibition of Ca2+ mobilization response to the stimulation by the same agonist (Figure 5C). Conversely, cells preincubated with murepavadin (10 µM, 16 h) had little to no effect on Ca2+ response to stimulation by the same agonist (Figure 5D).
Murepavadin Activates Murine MCs 
In Vitro and In Vivo via MrgprB2

It has been previously shown that C48/80 causes substantial degranulation in mouse peritoneal MCs (PMCs) via MrgprB2 (21). To test if murepavadin induces degranulation in murine PMCs and to determine the role of MrgprB2 in this response, we cultured PMCs from peritoneal lavage of wild-type (WT) and MrgprB2−/− mice (38, 42). We found that murepavadin induced degranulation in PMCs cultured from WT mice but this response was not observed in cells cultured from MrgprB2−/− mice.

FIGURE 3 | Naturally occurring MRGPRX2 missense variants are hypo-responsive to murepavadin for MC degranulation. (A) Snake diagram of MRGPRX2 indicating the two amino acid residues to be investigated. (B) Single amino acid substitution for each of the MRGPRX2 variant is shown in the table. (C) Cell surface receptor expression of the wild-type MRGPRX2 (WT) and its missense variants (G165E and V282M) was confirmed using flow cytometry. (D) RBL cells expressing MRGPRX2 (WT) and its variants (G165E and V282M) were exposed to 10 µM murepavadin (30 min) and degranulation was assayed by measuring the release of β-hexosaminidase. Statistical significance was determined by two-way ANOVA with Tukey’s multiple comparisons at a value ****p < 0.0001 and ns denotes “not significant”.

FIGURE 4 | Murepavadin does not promote β-arrestin recruitment following MRGPRX2 activation. (A, B) HTLA-MRGPRX2 cells were exposed to indicated concentrations of C48/80 or murepavadin for 16 h. Medium was removed and Bright-Glo solution (100 µL) was added into each well (96-well plate) and β-arrestin gene expression (in relative luminescence unit) was measured. (C, D) HTLA-MRGPRX2 cells were exposed to MRGPRX2 ligand (C48/80 or murepavadin, 16 h) and cell surface receptor expression of MRGPRX2 was confirmed using flow cytometry and quantified using a mean fluorescent intensity (MFI) in comparison to the untreated control. (E, F) Representative histograms of MRGPRX2 cell surface receptor expression of HTLA-MRGPRX2 cells. Statistical significance was determined by one-way ANOVA with Dunnett’s multiple comparisons at a value ****p < 0.0001 and ns denotes “not significant”.

| Protein Change | Number | GPCRdb | Functional Annotation | Rs Number |
|----------------|--------|--------|-----------------------|-----------|
| G165E          | 4.61x10^6 | Ligand binding                  | rs141744602 |
| V282M          | 7.56x10^6 | G protein coupling               | rs719141680 |
To determine if murepavadin induces degranulation of cutaneous MCs in vivo, we performed intradermal injection of murepavadin (30 µM, 20 µL) or PBS into the paw after intravenous injection of Evan’s blue dye. Consistent with peritoneal MC degranulation in vitro, murepavadin caused a significant increase in vascular permeability when compared to PBS. However, this vascular permeability response was abolished in MrgprB2−/− mice (Figures 6B, C). Together, these data demonstrate that murepavadin induces degranulation in murine MCs to cause increased vascular permeability via the activation of MrgprB2.

**DISCUSSION**

Murepavadin is a synthetic cyclic β-hairpin HDP mimetic that targets an outer membrane protein transporter LptD of the Gram-
negative bacterium P. aeruginosa, which makes it highly specific to this pathogen (45, 46). In *in vitro* studies, murepavadin is very effective against a wide-ranging species of multi-drug resistant *Pseudomonas* bacteria and demonstrates exceptional efficacy in sepsis, lung, and thigh infection models *in vivo* (47). However, intravenous administration of murepavadin for treating nosocomial pneumonia has been temporarily halted due to reports of kidney injury. Despite this, an inhaled formulation of murepavadin is under investigation for its potential effectiveness in treating *Pseudomonas* infection in patients with cystic fibrosis (33, 48). We made the novel observation that murepavadin is able to induce human MC activation through MRGPRX2 and murine MC activation through MrgrpB2. Because human skin MCs express MRGPRX2 at high levels (17, 19, 49), these findings suggest that murepavadin can be utilized for treating *P. aeruginosa* skin infection through harnessing MCs’ host defense and wound healing properties.

*P. aeruginosa* skin infection is associated with high morbidity and mortality rates mainly because of its ability to form biofilms and to resist multiple antibiotics (25, 26). Therefore, novel treatment approaches together with rapid wound closure are critical to control this type of infection. Weller et al. (50), showed that skin wounding associated with reinfection (15). Thus, it is highly likely that activation of murine MCs by mastoparan through MrgrpB2 confers both innate and adaptive immunity to provide protection against bacterial infection and reinfection. Given that murepavadin activates human MCs via MRGPRX2, it should be possible to use this HDP mimic for the modulation of *S. aureus* skin infection. Because murepavadin appears to have a lower EC50 value for MC degranulation than mastoparan (15), our prediction is that lower concentration of the drug will be required to treat *S. aureus* infection. Also, since murepavadin is an HDP mimic and is less susceptible to degradation than mastoparan, its effectiveness is likely to be greater than that of mastoparan, thus requiring fewer treatments to clear infection and to promote healing.

It is generally accepted that most GPCRs activate two parallel but independent signaling pathways; one involving G proteins while second pathway is independent of G proteins but requires the recruitment of β-arrestins (53–56). For certain GPCRs, agonists can activate only G proteins (G protein biased) or only β-arrestin (β-arrestin biased) or both (balanced). Agonists that induce both pathways not only activate G protein-mediated signaling but also result in dissociation of G proteins from the receptor (desensitization) and promote the receptor internalization. We have previously shown that while C48/80 acts as balanced agonist for MRGPRX2, an HDP angiogenic peptide, AG-30/5C acts as a G protein-biased agonist for the receptor. Thus, C48/80 caused substantial β-arrestin recruitment while AG-30/5C had little to no effect. Most importantly, preincubation of cells with C48/80 resulted in a significant reduction of cell surface receptor expression and loss of cell responsiveness to all MRGPRX2 agonists tested. By contrast, AG-30/5C had little to no effect on cell surface receptor expression or MC degranulation to any of the MRGPRX2 agonists tested (37). The data presented herein demonstrate that similar to AG-30/5C, murepavadin acts as a G protein-biased agonist for MRGPRX2. Thus, it is possible that the resistance MRGPRX2 to undergo desensitization and internalization by murepavadin could enhance its therapeutic potential.
In summary, although HDPs have therapeutic potential for the treatment of multi-drug-resistant bacterial infections, their relative instability and cytotoxicity have limited their usefulness as prospective antimicrobial agents. Small molecule HDP mimetics do not display these limitations and murepavadin was synthesized to specifically target *P. aeruginosa*. However, the possibility that murepavadin could activate MCs has not been suspected. In the present study, we have shown that murepavadin induces degranulation, TNF-α, IL-8 and CCL3 production via MRGPRX2, a receptor that is primarily expressed in human skin MCs. Thus, the ability of murepavadin to exploit MC’s immunomodulatory functions could form the basis for the treatment of skin infection caused by bacterial species such as *P. aeruginosa* and *S. aureus*. Furthermore, the findings presented herein that MCs expressing missense MRGPRX2 variants G165E (rs141744602) and V282M (rs779414608) were resistant to murepavadin-induced degranulation likely has important clinical implications. It is possible that murepavadin may not effectively clear microbial infection in individuals harboring these MRGPRX2 polymorphisms because of their inability to support MC degranulation.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

**ETHICS STATEMENT**

The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of the University of Pennsylvania.

**AUTHOR CONTRIBUTIONS**

HA contributed to conception, supervision and funding acquisition of the study. AA and CC performed the experiments and analyzed the data. AA and HA wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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