ORIGINAL ARTICLE

MrgX2 is a promiscuous receptor for basic peptides causing mast cell pseudo-allergic and anaphylactoid reactions

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Abstract

Activation of MrgX2, an orphan G protein-coupled receptor expressed on mast cells, leads to degranulation and histamine release. Human MrgX2 binds promiscuously to structurally diverse peptides and small molecules that tend to have basic properties (basic secretagogues), resulting in acute histamine-like adverse drug reactions of injected therapeutic agents. We set out to identify MrgX2 orthologues from other mammalian species used in nonclinical stages of drug development. Previously, the only known orthologue of human MrgX2 was from mouse, encoded by *Mrgprb2*. MrgX2 genes of rat, dog (beagle), minipig, pig, and Rhesus and cynomolgus monkey were identified by bioinformatic approaches and verified by their ability to mediate calcium mobilization in transfected cells in response to the classical MrgX2 agonist, compound 48/80. The peptide GSK3212448 is an inhibitor of the PRC2 epigenetic regulator that caused profound anaphylactoid reactions upon intravenous infusion to rat. We showed GSK3212448 to be a potent MrgX2 agonist particularly at rat MrgX2. We screened sets of drug-like molecules and peptides to confirm the highly promiscuous nature of MrgX2. Approximately 20% of drug-like molecules activated MrgX2 (pEC_{50} ranging from 4.5 to 6), with the principle determinant being basicity. All peptides tested of net charge +3 or greater exhibited agonist activity, including the cell penetrating peptides polyarginine (acetyl-Arg_9-amide) and TAT (49-60), a fragment of HIV-1 TAT protein. Finally, we showed that the glycopeptide antibiotic vancomycin, which is associated with clinical pseudo-allergic reactions known as red man syndrome, is an agonist of MrgX2.

1 | INTRODUCTION

For more than a decade, it has been appreciated that MrgX2, a G protein-coupled receptor expressed by human mast cells,[1] is probably responsible for the histamine release evoked by 48/80, a synthetic polymer commonly used as a degranulating agent. However, the clinical relevance of this receptor was uncovered only recently, with conclusive identification of the previously elusive mouse MrgX2 homologue, encoded by *Mrgprb2*.[2] Mice genetically deleted for *Mrgprb2* were resistant to local histamine-like effects upon injection with several agents that cause injection-site reactions in man. Previous studies highlight the promiscuous nature of MrgX2, which can be activated by many synthetic small-molecules and peptides, including US Food and Drug Administration (FDA)-approved drugs, as well as endogenous antimicrobial peptides and neuropeptides.[2-5] We sought to investigate whether assets in nonclinical drug development, that cause unexplained histamine-like and/or anaphylactoid effects in animals, interact with MrgX2. Based on our results, we considered whether MrgX2 should be included during assessment of secondary (off-target) pharmacology in the early stages of drug development.[6]

Human MrgX2 is encoded by *MRGPRX2* (for Mas-related G protein-coupled receptor), a member of a large gene family divided into subclasses A to H, and X. *MrgprD*, *MrgprE*, *MrgprF*, and *MrgprG* subclasses contain a single gene per mammalian species with high sequence homology, and so are readily identified as an orthologous group.[4] In contrast the *MrgprA*, *MrgprB*, and *MrgprC* subclasses are rodent-specific, and the *MRGPRX* subclass is absent from rodents.[7] A clue to evolution of these receptors is that the large *MrgprA* and *MrgprB* subclasses (with at least 26 members in rat and mouse) and the smaller *MRGPRX* subclass (4 members in human) lie at syntenic chromosomal loci (containing *SAAL1*, *PTPN5*, and *ZDHHC13* genes), suggesting a common origin.[8] Human *MRGPRX2* and mouse *MrgprB2* are orthologous, as defined by common function (they encode receptors responsive to compound 48/80 and cortistatin-14) and expression (in subsets of mast cells), even though their sequence identity is relatively low (52%). Low similarity led to their original classification as nominally separate Mrg subclasses.[2] We now understand that ligand specificity, G-protein signaling, and transcriptional regulation are conserved in *MRGPRX2/MrgprB2* despite divergence in overall sequence. A plausible explanation for these features, as well as the differences between rat and mouse loci, is that in rodent this locus has been subject to frequent retrotransposon insertion, resulting in duplicative expansion and coordinate divergence.[2,7] Families A to C and X may be more appropriately regarded as a single subclass of the Mrg family, since human MrgX1 (encoded by *MRGPRX1*) similarly shares ligands and patterns of expression in common with mouse A and C receptors.[2]

Activation of mast cell MrgX2 triggers dual intracellular signaling cascades attributed to G-proteins of the pertussis-toxin sensitive G_i-family and the calcium mobilizing $G_{q/11}$-family.[8] Typically, both pathways are required to mediate the ultimate mast cell responses of degranulation, chemotaxis, and cytokine release. Ligand activation of MrgX2 may be conveniently studied by measuring intracellular calcium release in transfected cultured cells (eg HEK293). This approach relies on either endogenous G-proteins present in the host cells used, or additional co-transfected G-proteins that confer calcium responses, such as $G_{\alpha15}$ or $G_{\alpha16}$. Here, we have used this approach in U2 osteosarcoma (U2OS) and other cells to confirm the ligand profile of mouse MrgX2 (encoded by *MrgprB2*),[2] and to characterize predicted orthologues of MrgX2 from species relevant to nonclinical drug testing. MrgX2 agonists also induce association of the receptor with β-arrestin, a property used previously to identify the -opioid agonist TAN-67 as an agonist of MrgX2.[5] We used receptor association with β-arrestin as an orthogonal assay to confirm newly described agonists of human MrgX2. Our findings show that MrgX2 is conserved in mammals. We characterize a peptide that caused unexpected anaphylactoid symptoms during development, and show it activates MrgX2. Finally, we show that the antibiotic vancomycin is also a MrgX2 agonist. Vancomycin causes clinical histamine-like effects typified by itching, rash and sometimes headache, dizziness, agitation, fever and even chest pain and breathing difficulties. These symptoms, known as red man syndrome, may be associated with activation of MrgX2.

2 | MATERIALS AND METHODS

2.1 | Compounds & peptides

Compound 48/80 & Lys-Bk (Kallidin) were from Sigma Aldrich, UK. Mastoparan, Icatibant (HOE-140), BAM 8-22 & BAM 22-P peptides were from Tocris BioSciences (Bristol, UK). Cortistatin-14 was obtained from GenScript (USA) and Tocris BioSciences (Bristol, UK). Other peptides were either obtained from the GSK compound library or synthesized using automated solid-phase peptide synthesis via the Fmoc/tBu strategy. Their identity was confirmed by HPLC-MS. Carbachol was from Alfa Aesar, USA. Vancomycin was from Sigma Aldrich (Cat#: V1130).

2.2 | Cell culture

U2OS (ATCC Cat# HTB-96, RRID:CVCL_0042) cells were maintained in DMEM-F12 (Sigma, UK), supplemented with 10% FBS & 1% Glutamax at 37°C in a humidified atmosphere of 5% CO_2. Cells

were maintained between $0.3-2 \times 10^6$ viable cells/ml. HEK293-MSRII cells [9] were used from frozen stocks and plated in DMEM with high glucose, L-glutamine, pyruvate HCl, 0.5x sodium bicarbonate, 10 mM HEPES supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 1% Glutamax, and 1% Penicillin-Streptomycin (supplements from Life Technologies). Sf9 insect cells (Super9; ECACC Cat# 89070101, RRID:CVCL_0549) were routinely cultured with HyClone SFX media (Fisher, UK), incubated at 27°C with 125 rpm shaking, and maintained between $0.4-8 \times 10^6$ viable cells/ml.

2.3 | BacMam Generation

Genes encoding human *MRGPRX1* and MrgX2 orthologues (Table S3) were synthesized at GenScript (China). Receptors or $G_{\alpha 16}$ were cloned into pHTBV1mcs3, a modified version of the pHTBV1 BacMam expression vector[10] using EcoRI and HindIII cloning sites. Recombinant baculoviruses were generated according to manufacturer's protocols using the Bac-to-Bac system (Invitrogen, UK). All BacMam stocks were sterility checked, confirmed to be mycoplasma-free, and stored at 4°C in the dark to increase viral stability.

2.4 | Ca^{2+} mobilization assays

U2OS or HEK293-MSRII cells were washed, resuspended in fresh media and transduced in suspension with virus at optimized multiplicity of infection (MOI) prior to assay ($G_{\alpha 16}$: MOI 45; MrgX2: MOI 25). Cells were seeded into black clear-bottomed 384-well plates at 1.5×10^4 cells/well and incubated for 24 or 48 hours (37°C, 5% CO_2). Prior to assay, media was removed (BlueWasher, BluCatBio) and plates were incubated (1 hours, 37°C, 5% CO_2) with 20 or 30 µL Hank's buffered saline solution containing 25 mmol/L HEPES, pH 7.2 (assay buffer) supplemented with 30 µmol/L probenecid (Sigma), 3 µmol/L Fluo-4 (Molecular Probes), & 750 µmol/L Brilliant Black (MP Biomedicals). On the FLIPR^{TETRA} (Molecular Devices), 10 µL compound was added to dye-loaded cells (final test concentration 10-100 µmol/L), and fluorescence (488 nm excitation/530 nm emission) read for 10 seconds to set background followed by 120-150 seconds to capture response. Raw data were processed as maximum-minimum and compounds were tested in quadruplet on each assay occasion.

2.5 | ß-arrestin recruitment assay in hMrgX2^{ProLink}-CHO cells

5000 cells/well (Eurofins Pharma Discovery Services PathHunter®; Cat # 93-0309C2; RRID:CVCL_KY49) seeded with 20 µL AssayComplete Cell Plating 2 reagent into white 384-well plates (Corning) were incubated overnight (37°C/ 5% CO_2). Test agents were serially diluted in DMSO, added to OptiMEM/0.1% BSA (to 5x final), and 5 µL incubated with cells (90 minutes at 37°C, then

30 minutes at room temperature). Luminescence was detected after addition of 12.5 µL PathHunter® Detection reagent (Cat # 93-0001; room temperature; 2 hours) using an Envision platereader (Perkin Elmer).[11]

2.6 | Data Analysis

Data were analysed in GraphPad PRISM 6 as maximum-minimum values normalized against the background average. pEC$_{50}$ values were determined using a four-parameter curve fitting equation, and statistical comparisons were performed using unpaired t-test with Welch's correction (*$P < .05$; **$P < .01$).

2.7 | Assessment of intravenous pharmacokinetics

Animal studies were ethically reviewed and carried out in accordance with the Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. Detailed study procedures are provided in Supplementary Methods.

3 | RESULTS

3.1 | A pan-mammalian family of MrgX2 receptors

We investigated whether inadvertent activation of mast cells via their MrgX2 receptor could be the cause of histamine-like and/or anaphylactoid symptoms, where observed in the past during preclinical in vivo testing for certain drugs in development. Typically, species affected by such symptoms are rat, dog and occasionally minipig or nonhuman primate. We therefore sought to profile putative MrgX2 agonists across orthologous receptors spanning a range of mammalian species.

We first identified a cell host suitable for expression of MrgX2. Human U2OS cells released intracellular calcium upon treatment with histamine which activates an endogenously-expressed receptor (Figure 1B). No U2OS calcium response was detected upon treatment with the MrgX2 agonists cortistatin-14, 48/80, BAM22P, or icatibant. U2OS cells therefore were presumed to lack endogenous MrgX2 (Figure 1A).

Next, we introduced human MrgX2 (hMrgX2) by using a modified baculovirus, BacMam, that can transduce exogenous genes and express them under control of a mammalian promoter. Titration of hMrgX2 BacMam up to a multiplicity of infection (MOI) of 100 conferred calcium responses to cortistatin-14 and 48/80 (Figure 1C&D). Half-maximal effects at different MOI ranged from pEC50 = 6.1 to 6.5 for cortistatin-14, and pEC50 = 5.4 to 5.6 for 48/80. These potencies were lower than reported values,[2] therefore we included BacMam viruses encoding $G_{\alpha 16}$ to enhance coupling to calcium-mobilization pathways. $G_{\alpha 16}$ potentiated hMrgX2 responses to 48/80, and resulting half-maximal effects were consistent with reported
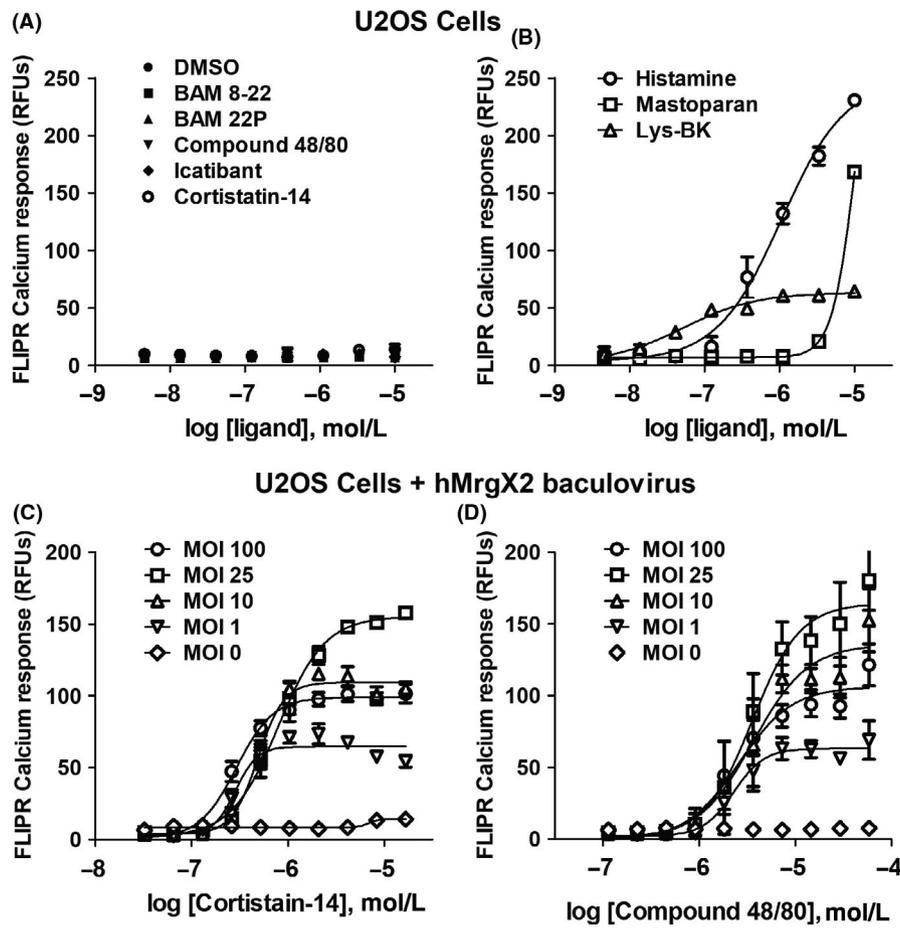
FIGURE 1 Baculovirus-mediated expression of MrgX2 in U2OS cells. (A) U2OS cells (untransfected) were treated with MrgX2 agonists (Cortistatin-14, 48/80, icatibant and BAM 22P) and MrgX1 agonists (BAM8-22 and BAM 22P). (B) U2OS cells were treated with histamine, which activates an endogenously expressed receptor, kallidin (Lys-Bk; a bradykinin agonist) and mastoparan. Kallidin has been described as an MrgX2 agonist[2] but the potency of response in U2OS cells suggested an endogenously expressed bradykinin receptor. U2OS cell calcium responses to mastoparan were unaffected by transfection of MrgX2 (data not shown), consistent with literature reports of receptor-independent effects of mastoparan.[31] (C & D) Baculovirus containing *Mrgprx2*, the gene encoding human MrgX2, adjacent to the CMV promoter was transduced into U2OS cells at multiplicities of infection (MOI) up to 100, followed by treatment with cortistatin-14 (C) or 48/80 (D). Cells were loaded with calcium-sensitive Fluo-4 dye and intracellular calcium release measured in real-time using the microplate-based FLIPR^{TETRA} apparatus. Single representative experiments are shown

values (pEC50 = 5.9 ± 0.18; n = 3 for 48/80). U2OS cells transduced with $G_{\alpha16}$ in the absence of hMrgX2 were unresponsive (Figure 2A). We also expressed human MrgX1 (hMrgX1), using BAM-22P and BAM 8-22 as reference agonists. hMrgX1 agonist responses were also potentiated by co-transduction with $G_{\alpha16}$. BAM-22P activated hMrgX1-$G_{\alpha16}$ and hMrgX2-$G_{\alpha16}$ cells at similar potencies (data not shown), whereas BAM 8-22 had a higher potency at hMrgX1-$G_{\alpha16}$ cells, as expected (Figure 2B). 48/80 was specific for hMrgX2, since no calcium responses of hMrgX1-$G_{\alpha16}$ cells were observed (data not shown).

Mouse MrgX2 (mMrgX2) is the product of the *Mrgprb2* gene.[2] We observed that transduction of U2OS cells with *Mrgprb2* BacMam at MOI comparable to those used for hMrgX2 was detrimental to health of cells. This effect was apparent morphologically as rounded cells and by marked reduction in calcium release in response to histamine (data not shown). Reducing the MOI of *Mrgprb2* restored potency of endogenous histamine responses

and conferred calcium responses to 48/80 at potencies consistent with previously reported values (Figure 2C).[2] Genes encoding MrgX2 orthologues in other species have not yet been described. The human chromosome 11 region that contains genes encoding MrgX1 and MrgX2 is syntenic to regions of rat chromosome 1 and mouse chromosome 7. In rodent, these loci have undergone gene duplication and at least eight candidate rat orthologues exhibit homology to MrgX2.[12] Unambiguous prediction of the rat MrgX2 orthologue based on sequence similarity to mMrgX2 and hMrgX2 was not possible (see Supplementary Methods), therefore we expressed the four most likely candidate genes (*Mrgprx1*, *Mrgprx2*, *Mrgprx2l*, and *Mrgprb3*) in U2OS cells. Only *Mrgprb3* conferred a response to 48/80 and cortistatin-14 (Figure 2D and Figure S1). Based on this and other evidence[8] we ascribed *Mrgprb3* to encode rat MrgX2 protein (rMrgX2) and to be orthologous to human *MRGPRX2* and mouse *Mrgprb2*. We attempted to express mMrgX2 and rMrgX2 under the same conditions as hMrgX2, *ie*
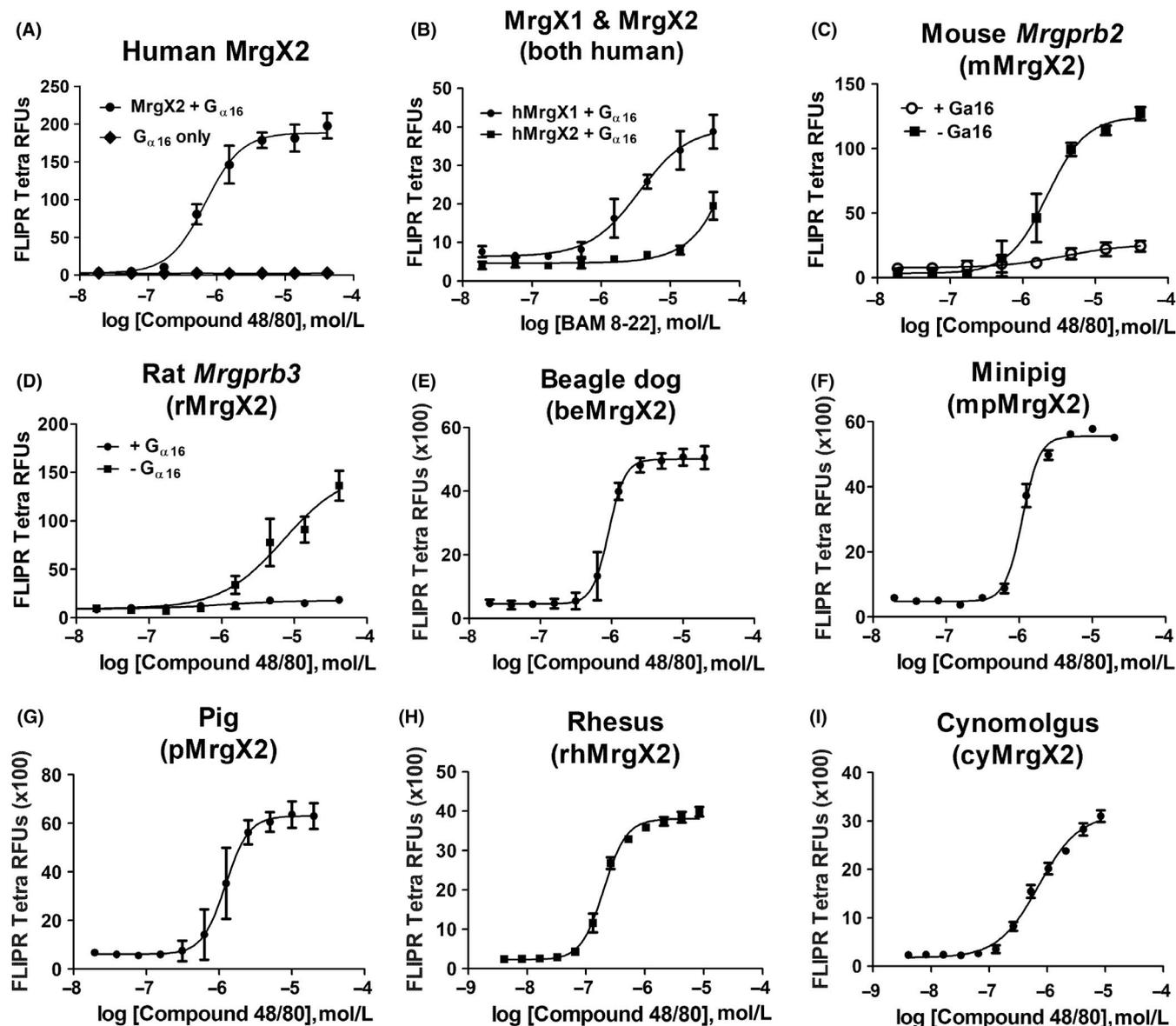
FIGURE 2 A pan-mammalian family of MrgX2 receptors. (A) U2OS cells expressing human MrgX2 plus $G_{\alpha16}$ or $G_{\alpha16}$ alone. (B) U2OS cells expressing $G_{\alpha16}$ in combination with hMrgX1 or hMrgX2. In other panels, U2OS cells expressed MrgX2 orthologues of mouse (C), rat (D), beagle dog (E), minipig (F), pig (G), Rhesus monkey (H), or cynomolgus monkey (I). In E-I, experiments were performed in the absence of $G_{\alpha16}$. Genes were introduced by baculovirus-mediated transfection, and calcium responses were determined (FLIPR^{TETRA}) after addition of agonists BAM 8-22 (B) or 48/80 (all other panels). Panels show single representative experiments, with each data point corresponding to mean ± SD of 3-16 technical replicates. For experiment replication, see Results

in the presence of $G_{\alpha16}$. Introduction of $G_{\alpha16}$ into U2OS cells in combination with either mMrgX2 or rMrgX2 potentiated calcium responses of the endogenous histamine receptor as expected (Fig. S2). However, $G_{\alpha16}$ reduced the efficacy of mMrgX2 responses to 48/80, and ablated rMrgX2 responses to 48/80 (Figure 2C & D). This effect was titratable (Fig. S2). Subsequent experiments were performed using U2OS cells transduced with mMrgX2 or rMrgX2 in the absence of additional G_{α}.

Unambiguous predictions of MrgX2 orthologue-encoding genes were made in the genomic sequence of canine (beagle), porcine (minipig and pig) and primate (Rhesus and cynomolgus monkey) species (see Table S3 for gene identities). Orthologous receptors were

termed beMrgX2 (beagle dog), mpMrgX2 (minipig), pMrgX2 (pig), rhMrgX2 (Rhesus monkey), and cyMrgX2 (cynomolgus monkey). Predicted full-length beMrgX2 conferred responses to cortistatin-14, but potency was low compared to human (Fig. S3). Predicted open reading-frames for canine and porcine MrgX2 have distinctive elongated N-terminal domains, compared to primate and rodent sequences (Fig. S4). To investigate the influence of the N-terminal domain, we truncated beMrgX2, removing the section of N-terminus not common to primate MrgX2 (human, Rhesus and cynomolgus sequences have contiguous N-termini in the alignment; Fig. S4). This truncation increased potency of cortistatin-14 (pEC50 = 7.1), comparable to that observed at hMrgX2 (Fig S3). Truncated beMrgX2

was also responsive to 48/80 (Figure 2E). For consistency and to allow cross-species comparison, we expressed equivalent truncated versions of pig and minipig MrgX2 (Fig. S4), which were also responsive to 48/80 (Figure 2F & G). Finally, rhMrgX2 and cyMrgX2 were also shown to be responsive to 48/80 (Figure 2H & I). For all MrgX2 orthologues except human, co-expression of $G_{\alpha16}$ provided no consistent improvement in sensitivity and efficacy compared to expression of receptor alone, indicating that the complement of G_α present in U2OS cells is sufficient to support calcium release downstream of MrgX2. To investigate whether alternate host cells might also be suitable for MrgX2 expression, we transfected mpMrgX2 into HEK293-MSRII-$G_{\alpha16}$ cells. Consistent with observations in U2OS, mpMrgX2 conferred calcium responses to cortistatin-14 and 48/80, both of which failed to activate untransfected host cells (data not shown). Histamine treatment did not cause calcium release in HEK293-MSRII-$G_{\alpha16}$ cells (data not shown). This indicates that the endogenous histamine receptor in U2OS cells is absent in HEK293-MSRII-$G_{\alpha16}$ cells, and that agonist-stimulated intracellular calcium release by MrgX2-expressing U2OS cells is not a secondary consequence of receptor-evoked histamine release. For subsequent evaluation of compound activity, U2OS cells were used. hMrgX2 was expressed in combination with $G_{\alpha16}$; other orthologues were expressed in the absence of $G_{\alpha16}$. Beagle, pig, and minipig MrgX2 were expressed as N-terminal truncates. Compound 48/80 was used as a reference agonist. Half-maximal responses to 48/80 of different orthologues (n independent experiments) were: mMrgX2: 5.5 ± 0.19 (n = 3), rMrgX2: 5.4 ± 0.1 (n = 4), beMrgX2: 6.1 ± 0.08 (n = 3), mpMrgX2: 5.9 ± 0.10 (n = 3), pMrgX2: 5.9 (n = 1), rhMrgX2: 6.7 (n = 1), cyMrgX2: 6.1 (n = 1) (mean pEC_{50} ± SEM, based on a notional molecular weight for 48/80 of 500).

3.2 | Cell-penetrant peptides with MrgX2 agonist activity

GSK3212448 (peptide 1) and peptide 4 are stabilized peptides with related sequences (Table S1). Both caused unexpected histamine-like symptoms in vivo. These peptides were designed to penetrate cells and disrupt the interaction between EZH2 (enhancer of zeste homolog 2) and EED (embryonic ectoderm development), which are components of the epigenetic regulator PRC2 (Polycomb repressive complex 2). In common with similar published peptides that reduce trimethylation activity of PRC2 at histone H3 K_{27},[13] GSK3212448 and peptide 4 bind EED with high affinity (IC_{50} = 20 nmol/L, for GSK3212448). Predicted net charge at pH 7 is +5 for GSK3212448 and +3 for peptide 4. To determine pharmacokinetic properties of the peptides, rats were treated intravenously by bolus dose (5 mg/kg) given over approximately 1 minute. Both peptides were tested as part of the same study. For each peptide, the first dosed animal showed potential signs of anaphylaxis, with blue-colored extremities, gasping, and limpness. Symptoms were observed within 3 minutes for peptide 4 and within 2 minutes for GSK3212448; animals were immediately
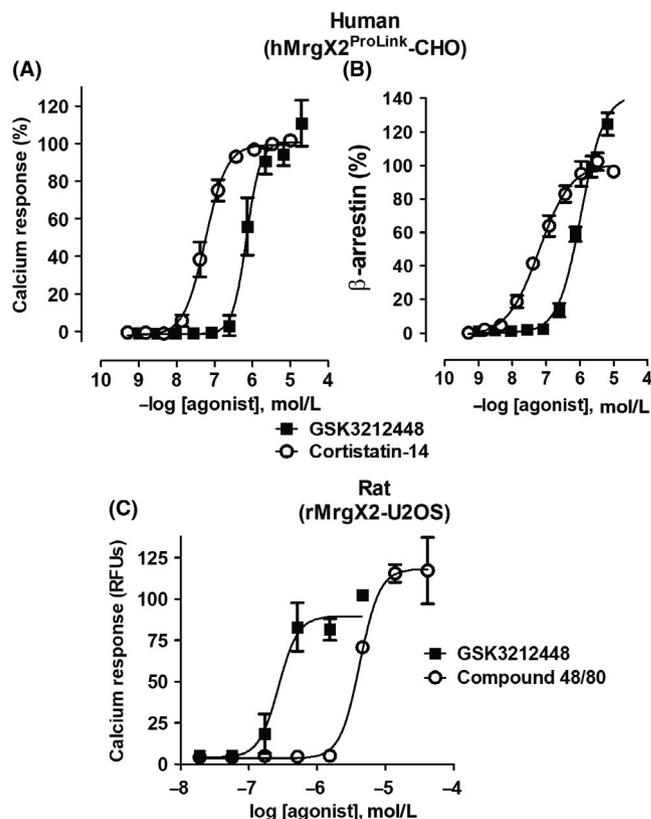
FIGURE 3 Peptide GSK3212448 is an agonist of MrgX2. (A & B) hMrgX2^{ProLink}-CHO cells were challenged with peptides GSK3212448 (Ac-*-Nle-FS*NRQKILRRT*ILN*EWKQR-NH$_2$; filled squares) or cortistatin-14 (open circles). Agonist effects on intracellular calcium were determined using FLIPR^{TETRA} (A), and agonist-induced association of hMrgX2 with β-arrestin$_2$ was measured using an enzyme fragment complementation (EFC) assay (B). Bars show mean ± SD across four independent experiment occasions, normalizing within each experiment to the effect of cortistatin-14. (C) rMrgX2-U2OS cells were challenged with GSK3212448 or 48/80. Data show mean ± SD raw FLIPR^{TETRA} counts across replicates in a single representative experiment; across multiple experiments, pEC_{50} of GSK3212448 at rMrgX2 was 6.5 ± 0.21 (n = 4)

euthanized by cervical dislocation and death was confirmed. No further rats were dosed. Subsequently, we found GSK3212448 to activate hMrgX2. Preliminary data suggested that GSK3212448 could activate hMrgX2-$G_{\alpha16}$-U2OS but not $G_{\alpha16}$-U2OS cells (not shown). Using CHO-K1 cells stably expressing C-terminally tagged hMrgX2 (hMrgX2^{ProLink}-CHO), GSK3212448 caused robust, concentration-dependent calcium responses (pEC_{50} = 6.1 ± 0.14 (n = 4); Figure 3A). hMrgX2^{ProLink}-CHO cells also express a tagged version of β-arrestin$_2$, allowing measurement of agonist-induced association between receptor and β-arrestin$_2$. GSK3212448 induced association of hMrgX2 with β-arrestin$_2$ (pEC_{50} = 6.0 ± 0.10 (n = 4); Figure 3B). In U2OS cells, GSK3212448 activated the rat orthologue (rMrgX2) 18-fold more potently than the reference agonist 48/80 (pEC_{50} = 6.5 ± 0.21 and 5.2 ± 0.17 (both n = 4) for GSK3212448 and 48/80, respectively; Figure 3C). GSK3212448 also activated beMrgX2, mpMrgX2, and pMrgX2 (data not shown).

In the pharmacokinetic study, GSK3212448 was injected at a concentration > 1000-fold greater than its pEC_{50} at rMrgX2, potentially sufficient to cause systemic degranulation of connective-tissue mast cells after dose dilution into circulation. Peptide 4 also activates hMrgX2 (pEC_{50} = 5.4 ± 0.05 (n = 4; calcium) and 5.2 ± 0.06 (n = 3; β-arrestin$_2$); Table S1). These findings offer a mechanistic explanation of anaphylactoid symptoms and illustrate how prior knowledge of MrgX2 activity and agonist profile across species orthologues could mitigate risk of anaphylactoid symptoms by enabling a knowledge-based choice of species and dosing regimen.

3.3 | Diverse basic peptides activate MrgX2

We next investigated the structure-activity relationship (SAR) of peptides for agonism of MrgX2. Like GSK3212448, many known MrgX2 peptide agonists have net basicity. Cell penetrating peptides are often also basic, since positive charge can aid cell penetration, with little additional requirement for specific amino acids or sequence motifs.[14] We tested 21 peptides of varying length, sequence and chemical modification, and of various predicted negative, neutral or positive net charge, using hMrgX2^{ProLink}-CHO cells to measure calcium mobilization and receptor association with β-arrestin$_2$ (Table S1). All highly basic peptides in this set (net positive charge ≥ +3) activated hMrgX2 (pEC50 ≥ 4.9 in at least one assay), whereas no neutral or acidic peptide tested (net charge ≤ 0) activated hMrgX2. At net charge + 1 or + 2, peptides had either MrgX2-active or -inactive properties. There was little apparent relationship between MrgX2 activity and specific amino acid sequence characteristics or length. Our set of peptides included TAT (49-60), the basic domain of HIV-1 TAT protein (Ac-RKKRRQRRRPPQ-NH$_2$), a well-studied cell penetrating peptide of net charge +8.[14] Consistent with our SAR, TAT (49-60) activated hMrgX2 (pEC_{50} = 6.2 ± 0.18, n = 4 (calcium mobilization); pEC_{50} = 5.3 ± 0.05, n = 4 (β-arrestin$_2$ association)). To illustrate that MrgX2 activation is not sequence-specific, we also tested a peptide containing only arginine residues (polyarginine; Ac-Arg$_9$-amide), showing that basic residues alone are sufficient to activate MrgX2 (pEC_{50} = 6.9 ± 0.10, n = 4 (calcium mobilization); pEC_{50} = 5.8 ± 0.10, n = 4 (β-arrestin$_2$ association)).

Next, we reviewed literature reports of peptides tested in recombinant hMrgX2 calcium and/or β-arrestin assays. These peptides have diverse origins and include various hormones, fragments of albumin isolated from blood plasma, anti-bacterial peptides, and pharmaceutical agents. A compilation of agonist pEC_{50} values from these reports is presented in Table S2. In all cases, the relationship between net charge and hMrgX2 agonist activity described above holds. Figure 4 shows a scatter plot of hMrgX2 potency vs predicted charge at neutral pH for peptides tested in this study and literature peptides, including also the peptidomimetic polymer 48/80 (data from the combination of Tables S1 and S2). In summary, peptides of charge ≥+3 commonly activate MrgX2, whereas peptides of
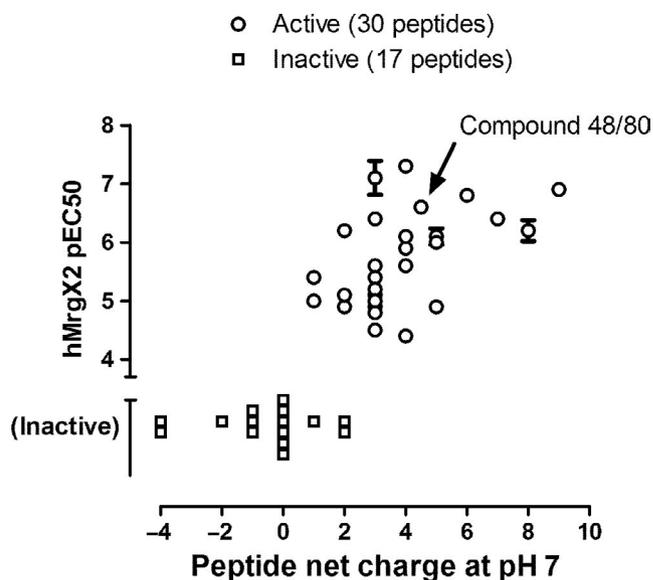

FIGURE 4 Relationship between hMrgX2 agonist potency and charge amongst peptides. Forty-six peptides are included, plus compound 48/80 whose polymeric structure is analogous to a basic peptide, with a repeating unit that presents a basic secondary amine side-chain (48/80 is given a notional net charge at pH 7.0 of 4.5 units, here). hMrgX2 agonist data were either generated within this study (Table S1, which also shows experimental replication) or are extracted from literature reports (Table S2) and show pEC_{50} values (mean ± SD for data generated within this study) for calcium mobilization, or, where no pEC_{50} for calcium mobilization was available, for β-arrestin association assays

charge ≤ 0 rarely activate MrgX2. This relationship is consistent with the numerous peptide mast cell degranulating agents described in the literature.

3.4 | Diverse basic small-molecules activate MrgX2

We investigated whether MrgX2 was similarly promiscuous toward activation by small molecules. We tested 368 structurally diverse drug-like compounds. Roughly one-third of compounds tested (137) induced concentration-dependent calcium mobilization in HEK293-MSRII cells expressing hMrgX2 plus $G_{\alpha16}$, measured using FLIPR. To distinguish hMrgX2-selective agonists from compounds acting at other receptors endogenously expressed in HEK293-MSRII cells, active compounds were retested using cells transduced either with hMrgX2 plus $G_{\alpha16}$ or $G_{\alpha16}$ alone (both tested in duplicate). Sixty-eight compounds (≈18% of the initial set) exhibited hMrgX2-specific activation with pEC50 > 4.5 (criteria for defining hits are described in Figure 5 Legend). The initial compound set reflected diverse properties, comprising 47 weak or strong acids, 87 weak or strong bases, 19 zwitterions and 198 compounds predicted to be neutral at pH 7.0 (pKa and pK_B predicted using Chemaxon tools). hMrgX2-actives amongst this set were highly enriched in basic and zwitterionic compounds (55 of 68 hits; Figure 5A,B). However, pEC_{50} did not correlate with predicted pK_B (Figure 5C).
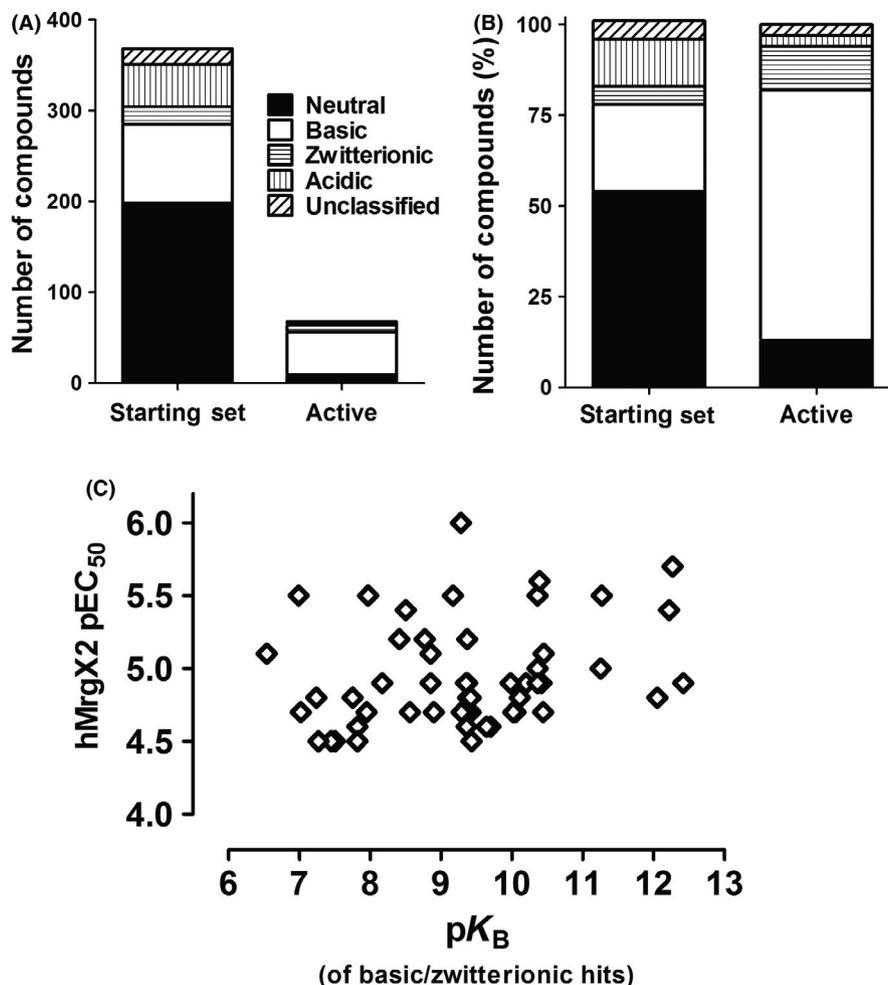
FIGURE 5 Frequency of MrgX2-active agonists amongst drug-like small molecules. Structurally diverse drug-like compounds (structures not disclosed) were tested for hMrgX2 agonism using HEK293-MSRII cells. Bargraphs show (A) number and (B) proportion of neutral, acidic or basic compounds in the 368-compound starting set and amongst MrgX2-active hits. Calcium mobilization assays were performed in Poly-D-lysine coated 384-well plates (Greiner Bio-One), and buffer contained 2.5 mmol/L probenecid, 2 µmol/L Fluo-4, and 500 µmol/L Brilliant Black (otherwise as described in Methods). Test compounds were serially diluted in DMSO, transferred to 384-well polypropylene V-bottom plates (Greiner Bio-One) using an ECHO 555 liquid handler (Labcyte), then further diluted into assay buffer supplemented with 0.03% Pluronic acid (Sigma) and 0.03% CHAPS (Sigma), before test in duplicate. Curve-fitting (Activitybase XE, IDBS) was performed where fold increase over basal was ≥ 1.3. Cortistatin-14 and carbachol (to activate endogenous muscarinic receptor) were included to ensure consistency between plates. MrgX2-active hits were defined as having $pEC_{50} > 4.5$ at hMrgX2, >0.5 log unit greater pEC_{50} at hMrgX2-$G_{\alpha16}$ compared to cells transfected with $G_{\alpha16}$ alone, and > 50% efficacy compared to Cortistatin-14. (C) Correlation of basicity (calculated pK_B) with hMrgX2 mean pEC_{50} (n = 2) for 68 hMrgX2-active hits. Acid and base properties were categorized using in silico Chemaxon tools

3.5 | Activation of MrgX2 by vancomycin as a potential cause of Red Man Syndrome

Ciprofloxacin and other fluoroquinolone antibiotics are reported to activate MrgX2.[2] These drugs are associated with clinical pseudo-allergic reactions, and ciprofloxacin caused mMrgX2-dependent degranulation of mouse mast cells in vitro, and mMrgX2-dependent reduction in body-temperature in vivo.[2] Antibiotics are often dosed intravenously, rapidly achieving high exposures both systemically and in tissue. We reviewed the literature for other antibiotics associated with clinical anaphylactoid or allergic-type reactions, possibly mediated via hMrgX2. Vancomycin induces characteristic pruritus, flushing and rash which may be accompanied by dizziness

and agitation, headache, chills, and fever; symptoms collectively termed red man syndrome.[15] Red man syndrome has been linked to vancomycin treatment for more than 30 years,[16] and may be caused by oral, intraperitoneal, or intravenous routes of administration.[17,18] Vancomycin is a glycopeptide with several exposed basic centers, reminiscent of GSK3212448 and other peptides shown here to activate hMrgX2. In hMrgX2-transfected U2OS cells, vancomycin elicited calcium responses with similar efficacy to 48/80 (pEC_{50} = 4.4 ± 0.04 and E_{max} = 87 ± 8% (n = 2) on hMrgX2-$G_{\alpha16}$-U2OS cells; Figure 6). Negligible calcium responses were observed in untransfected U2OS parent cells challenged with either vancomycin or 48/80 whereas robust U2OS calcium responses were evoked by histamine (data not shown), consistent with activation of MrgX2 by vancomycin. Activation of hMrgX2ProLink-CHO cells did not reach

FIGURE 6 Vancomycin activates MrgX2. Calcium mobilization assays were performed as described in Figure 2, expressing MrgX2 orthologues in U2OS cells (beMrgX2, pMrgX2, and mpMrgX2 were expressed truncated of their elongated N-terminal domains, and hMrgX2 was expressed in combination with $G_{\alpha16}$). Data were normalized to the effect of 48/80 (100%). Representative experiments are shown; each data point is mean ± SD of 3-16 determinations. For experimental replication, see Results

log [agonist], mol/L
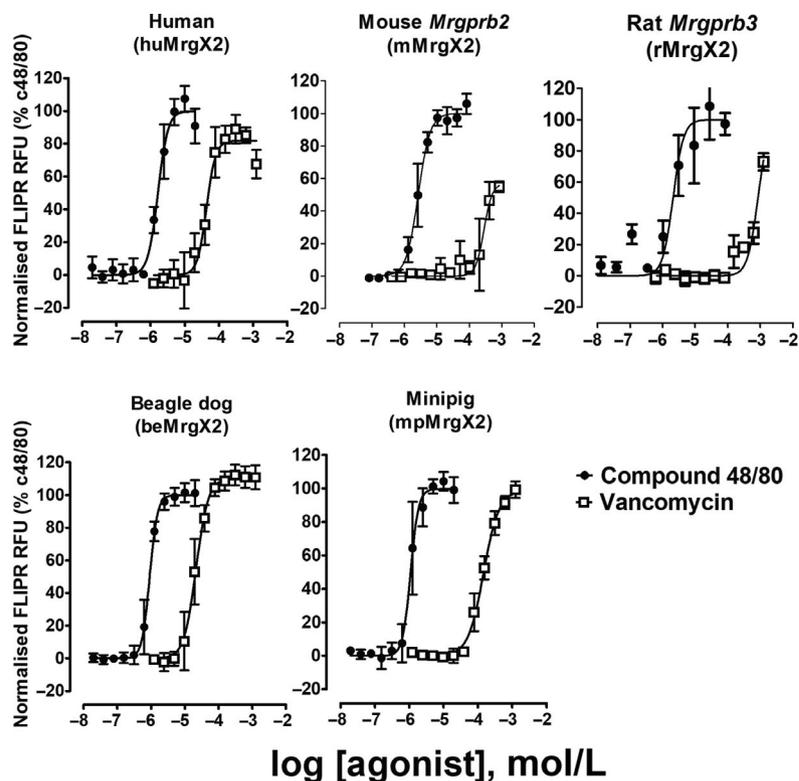
maximal effect and curve-fitting was not attempted. However, vancomycin (0.3mM) caused approximately 50% of maximal effect of cortistatin-14 (Fig. S5). Potency of vancomycin to activate hMrgX2 is low compared with other MrgX2 agonists associated with pseudo-allergic reactions. However, trough (lowest) free serum concentrations in vancomycin-treated patients are ≈5 ± 2 μmol/L (13.6 ± 5.9 mg/L; 50% protein binding) and C_{max} may exceed 30 μmol/L.[19] Therefore, vancomycin activity at hMrgX2 may be sufficient to cause red man syndrome and other histamine-like or pseudo-allergic reactions at clinical exposures. Vancomycin induces degranulation of isolated rat mast cells,[20] so we tested whether rodent MrgX2 was also sensitive to the drug. Vancomycin-evoked calcium release in mMrgX2- and rMrgX2-expressing U2OS cells did not reach maximal effect at 1mM (Figure 6) consistent with the high concentrations of vancomycin required for full degranulation of rat mast cells (4.55 mg/ml or 3 mmol/L).[20] Finally, we showed that vancomycin behaved as a full or near-full agonist at beMrgX2 and mpMrgX2 (pEC_{50} = 4.6 ± 0.2 and E_{max} = 97 ± 12% (n = 3) at beMrgX2 and 3.8 ± 0.07 and E_{max} = 84 ± 13% (n = 3) at mpMrgX2; Figure 6). In summary, activation of MrgX2 by vancomycin is sufficient to mediate mast cell degranulation and the clinical adverse reactions termed red man syndrome.

4 | DISCUSSION

Histamine-like symptoms and anaphylaxis are sometimes encountered during in vivo testing of novel chemical entities in mammalian species. This category of adverse event occurs more frequently for injected or intravenously infused agents, though examples of oral administration leading to similar symptoms are known. In the scenario of drug development, encountering such symptoms necessitates complex risk assessment for translation from nonclinical species to human, and is especially challenging in the absence of known or suspected causative mechanisms. The landmark work of McNeil et al (2015) suggested that some histamine-like symptoms caused by drugs in development might be associated with IgE-independent mast cell degranulation caused by agonism of the MrgX2 receptor. By identifying mouse MrgX2, the authors showed conclusively that medicines causing anaphylactoid reactions, such as injection site reactions, mediated these effects via agonism of MrgX2. Reactions like those seen in human could be replicated in wild-type mice but were absent in animals genetically deleted for MrgX2.[2]

We set out to show that MrgX2 orthologues occur not only in human and mouse but also in other mammalian species corresponding to those commonly used in preclinical studies: rat, dog, minipig, and nonhuman primate. Other authors have termed MrgX2 "primate-exclusive",[3] but we were able to unambiguously predict candidate MrgX2 orthologues of nonprimate species from publicly-available genomic sequence. Expression of candidate MrgX2 orthologues in U2OS cells conferred calcium responses to the archetypal MrgX2 ligand, 48/80. In rat, no single likely orthologue could be predicted amongst the multiple homologues that are present in the rat syntenic locus due to gene duplication and divergence events in the rodent lineage.[7] Instead, four potential orthologues were tested. Only one rat gene, *Mrgprb3*, conferred calcium responses to compound 48/80 upon transfection into U2OS cells. We therefore ascribed *Mrgprb3* to encode rat MrgX2 (rMrgX2) and

to be orthologous to human *Mrgprx2* and mouse *Mrgprb2* (encoding hMrgX2 and mMrgX2 respectively). Findings reported by other authors support this.[8]

Additional G_α subunits substantially reduced responses of rat and mouse MrgX2. Incompatibility with $G_{\alpha16}$ may be specific to the U2OS cells used here, because agonist responses were observed for mMrgX2 (Mrgrpb2) expressed in HEK293 cells in combination with $G_{\alpha15}$.[2] Further studies will be required to understand the underlying cause, but our data establish a key difference between rodent and human MrgX2 and illustrate that expression of rodent MrgX2 under the same conditions as used for hMrgX2 may not support functional agonist responses. Conceivably, this difference may underlie the difficulty encountered by other authors in establishing functional expression of mMrgX2/Mrgprb2.[3]

Subramanian et al, (2016) reported that rMrgX2 (*MrgprB3*) was highly expressed in rat peritoneal mast cells, detected by RT-PCR, whereas several other Mrg-homologues were expressed at only low levels.[8] Here, we have not examined mast cells expression, but rather we show an association of a novel MrgX2 agonist, peptide GSK3212448, with systemic anaphylactoid symptoms in rat. We have also identified small-molecule development drugs for which histamine-like symptoms had been observed during preclinical in vivo studies, using text-mining searches of study reports or anecdotal accounts. These agents also activate MrgX2 across species and will be the subject of a separate report. We speculate that species-specific histamine-like effects of development drugs might result, at least in part, from greater potency at specific MrgX2 orthologues. This would be unsurprising given the relatively low sequence homology amongst orthologues (substance P is 360-fold less potent at mouse than human, whereas icatibant is only twofold less potent at mouse[2]). Strikingly, GSK3212448 had greater potency at rMrgX2 than hMrgX2, and is the most potent agonist of rMrgX2 yet observed.

We tested further peptides and small-molecules to understand the SAR of MrgX2. Known peptides with MrgX2 agonist activity are remarkably numerous, and include several ligands of other GPCRs, such as Substance P and cortistatin-14. Our findings show a lack of sequence-specificity amongst active MrgX2 agonist peptides, which instead have in common net positive charge at pH7.0. Polyarginine (Ac-Arg_9-amide) was close to equipotent to cortistatin-14 (Table S1), illustrating that basic amino acids alone are sufficient to activate MrgX2 and that hydrophobic amino acids or other sequence features are not required. Combining data from this study and from literature reports suggests a relationship between charge and activity as follows: firstly, peptides with net charge +3 or greater show MrgX2 agonist activity; secondly, peptides with MrgX2 agonist activity have net charge +1 or greater. Further studies on a larger array of peptides will be required to understand the quantitative relationship between MrgX2 potency and structure, especially charge presentation (eg positive charge in or near an amphipathic helix) and the minimum peptide length for MrgX2 activity.

The possibility that endogenously occurring peptides and proteins represent physiologically-relevant ligands for MrgX2 has been explored in depth for cortistatin-14,[21] Substance P,[22] endorphins,[3] β-defensins,[23] and others.[24] However, our finding that activation of MrgX2 may not be sequence-specific but is instead driven by charge raises the possibility that no physiologically relevant cognate endogenous MrgX2 agonist exists. Activation of MrgX2 by peptides such as Substance P and cortistatin-14 at concentrations in the range 100–1000 nmol/L may be coincidental due to their high basic charge. MrgX2 may operate as a sentinel, analogous to pathogen pattern-sensors such as FPR receptors, activating elements of the innate immune system in response to local pathogen-derived features. FPR1 and FPR2 are expressed on leucocytes and respond to N-formyl motifs characteristic of bacteria, or to other bacterially-derived peptides.[25] By analogy, MrgX2 enables mast cells to respond to pathogen-derived basic peptides including those that have cell-penetrating properties. As well as the cell-penetrant fragment of HIV TAT protein, TAT (49-60), shown here to activate hMrgX2, *S aureus* -toxin and competence-stimulating peptide (CSP)-1 secreted by Gram-positive bacteria also activate hMrgX2.[26,27] In this way MrgX2 may detect both viral and bacterial xenobiotic features.

The promiscuous nature of MrgX2 is also reflected in our finding of frequent weak agonist activity amongst drug-like small molecules (≈18% of compounds tested). As with peptides, many novel MrgX2 agonists were basic. Based on our understanding of MrgX2 SAR, we hypothesized that vancomycin might also activate MrgX2, and confirmed this through testing. Vancomycin is a basic glycopeptide that achieves high exposures in treated patients, and is associated with histamine-like symptoms termed red-man syndrome.[15] During preparation of this manuscript, Azimi et al (2017) also showed vancomycin to activate hMrgX2, inducing calcium mobilization in HEK293 cells expressing hMrgX2 and degranulation of LAD-2 mast cells. Both effects could be blocked by an MrgX2 antagonist.[26] We showed vancomycin to activate all mammalian MrgX2 orthologues tested. It is somewhat surprising that no histamine-like symptoms were observed in literature reports of vancomycin administration to dog (free C_{max} ≈35 µmol/L; ≈100 µg/ml, assuming 50% protein binding)[28] and minipig (free C_{max} ≈23 µmol/L; 34 µg/mL).[29] Possibly these studies did not cause sufficient (or sufficiently rapid) exposure to vancomycin to induce the symptoms. Other antibiotics have also been associated with red man syndrome, including ciprofloxacin,[15] which has also been shown to activate MrgX2.[2] We speculate that agents and/or dosing regimens that lead to high C_{max} and fast onset of C_{max} (rapid, high exposure) in combination with MrgX2 agonism, would be the most liable to result in systemic anaphylactoid symptoms. Oral vancomycin can also cause symptoms.[30] Red man syndrome is often associated with the first dose of vancomycin and can by mitigated or avoided by reduced infusion rates, whereas combining vancomycin with other antibiotics, contrast dyes or opioid analgesics can amplify red man syndrome.[15] We speculate that slower onset of C_{max} desensitizes MrgX2 to limit histamine release, whereas combination of vancomycin with other potential MrgX2 agonists could have additive effects. Basic peptides as therapeutics may be particularly liable for MrgX2-mediated histamine-like

reactions, though subcutaneous and intramuscular administration may lead to local injection-site reactions,[2] rather than the systemic effects we observed with GSK3212448 in rat.

In conclusion, our data extend the association between agonism of the mast cell MrgX2 receptor and histamine-like anaphylactoid effects into both clinical and preclinical drug-development settings. Antagonism of MrgX2 could potentially negate side-effects experienced by patients reliant on vancomycin or other medicines with cross-reactivity at MrgX2. Anaphylactoid effects of unknown mechanism are not usually the cause to cease drug development, but can complicate and extend the time and cost of nonclinical development for new agents. Evaluation of MrgX2 agonist activity at both human and mammalian orthologues generates valuable information prior to in vivo safety or efficacy testing, and has the potential to inform choice of model species and dosing regimen to avoid unintended histamine-like symptoms.

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DISCLOSURES

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

Participated in research design: SD, NWC, RMS, AIL, AMM, ZW, LAW, AG, AMB, DADW, & AJB. Conducted experiments: JG, JL, RMS, LAW, & LA. Performed data analysis: JG, SD, JL, RMS, LAW, LA, AMB, & AJB. Wrote or contributed to the writing of manuscript: JG, SD, RMS, AMB, DADW, & AJB.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.