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Towards a design rule for peptides that activate human mast cells *via* MRGPRX2

Lei Lu[1, 2], Shammy Raj[1], Narcy Arizmendi[3], Jie Ding[4], Gary Eitzen[5], Peter Kwan[4], Marianna Kulka[3, 6] and Larry D. Unsworth[1]*

1. Department of Chemical and Materials Engineering, Donadeo Innovation Centre for Engineering, 9211-116 Street NW, University of Alberta, Edmonton, AB, Canada, T6G1H9
2. School of Life Science and Engineering, Southwest Jiaotong University, Chengdu, Sichuan, China, 610031
3. National Research Council (Canada), 11421 Saskatchewan Drive NW, Edmonton, AB, Canada, T6G2M9
4. Wound Healing Research Group, Division of Plastic and Reconstructive Surgery, University of Alberta, 2D2.28 WMC, 8440-112 Street, Edmonton, AB, Canada, T6G2B7
5. Department of Cell Biology, MSB 5-14, University of Alberta, Edmonton, AB, Canada, T6G2H7
6. Department of Medical Microbiology and Immunology, 6-020 Katz Group Centre, University of Alberta, Edmonton, AB, Canada, T6G2E1

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ABSTRACT

Mas-related G-protein coupled receptor member X2 (MRGPRX2) is a mast cell-specific receptor which can be activated by various unrelated endogenous peptides, basic secretagogues, and approved drugs, among others, to cause degranulation and release of inflammatory molecules in a non-IgE mediated manner. Proadrenomedullin N-terminal 12 (PAMP-12) is a well-known endogenous secretagogue which differentially activates mast cells *via* the MRGPRX2 receptor. However, binding domain, core motif, and hence the ligand-receptor binding mechanisms for peptide ligands are ill-defined. Using alanine scanning and terminal truncation techniques, we have shown that peptide sequences, FRKKW and WNKWAL are the two-core motifs that activate human LAD-2 mast cells with similar potency as PAMP-12. Based on our work and previous literature, a generalized physicochemical structure for artificial peptides with agonist activity towards MRGPRX2 could be reduced to X_a-$(Y)_{(n\geq 3)}$-X_b where: (i) X_a is a hydrophobic residue with an aromatic ring; (ii) X_b is any hydrophobic residue; and (iii) Y must have a minimum of one positively charged residue with the remainder being uncharged residues. In support of this initial design rule, artificial sequences WKKKW and FKKKF were tested and showed the same activity towards MRGPRX2 as PAMP-12; even though they were not derived from any known MRGPRX2 activating peptide sequences. Furthermore, all of these peptides were shown to specifically activate mast cells in human skin. The identification of both the biomimetic core-motifs for PAMP-12 as well as these extremely short artificial peptides provides fundamental insight into MRGPRX2 based pathways.

1. Introduction

Tissue resident mast cells play a significant role in innate and adaptive immune responses.[1] In immune-initiated allergic inflammation, mast cell activation is mediated through antigen crosslinking of FcεRI receptors that rapidly cause mast cell degranulation, releasing a plethora of immune-modulatory molecules including histamine, cytokines, and proteases.[2,3] However, various unrelated endogenous peptides, basic secretagogues, and clinically approved drugs have been shown to activate mast cells independent of the FcεRI pathway.[4] It was determined that the Mas-related G-protein coupled receptor X2 (MRGPRX2), first identified in sensory neurons, was involved in this non-IgE mediated activation of mast cells.[5]

MRGPRX2 has been identified to play a pivotal role in itch, allergy and other inflammatory diseases where its overexpression has been associated with asthma, atopic dermatitis, and psoriasis.[6,7] Various peptide stimuli, including peptide toxins, neuropeptides, antibacterial peptides and endogenous peptide fragments have been shown to activate mast cells *via* MRGPRX2.[8,9] Moreover, peptide drugs including icatibant, cetrorelix, octreotide and leuprolide have been shown to be potent MRGPRX2 activators; inducing mast cell activation and allergic-type responses, such as injection-site reactions (ISRs).[4,10–14] Thus, elucidating the peptide structure that induces mast cell activation *via* MRGPRX2 is fundamental to understanding the immune response which is crucial to the successful development of therapeutic peptide drugs.

To elucidate the molecular basis for peptide-MRGPRX2 interactions, a peptide library based on Proadrenomedullin N-terminal 12 (PAMP-12) was used to identify amino acid residues responsible for MRGPRX2 induced activation of mast cells. PAMP-12 is the C-terminal region of PAMP-20 (a hypotensive peptide expressed in the adrenal medulla) that contains only 12 amino

acids but has potent MRGPRX2 activity.[4,9] A PAMP-12 peptide library was developed through common peptide library screening techniques: N and C terminal truncations, and alanine scanning. MRGPRX2 expressing HEK-293 (HEK-X2) cells were used to screen these synthetic peptides, where binding was evidenced by calcium (Ca^{2+}) mobilization using ratiometric Fura-2 dye.[15] MRGPRX2 expressing LAD-2 human mast cell culture was used to confirm activation through monitoring the release of β-hexosaminidase upon exposure to these peptides. Selected peptides were further tested on epidermis-free human skin biopsies to test their efficacy in activating local mast cells. Overexpression of tryptase gene (TPSAB1) was studied through quantitative polymerase chain reaction (qPCR). Based on the screening results and other known MRGPRX2 peptide stimuli, with structural similarities[16], we surmised a generalized principle for the design and development of artificial peptides that can activate mast cells through MRGPRX2. The peptides were tested against MRGPRX2 expressing HEK cells, LAD-2 human mast cells and skin mast cells in tissue to prove the validity of the design principle. Finding the core motif of PAMP-12 is of fundamental importance, and shows that MRGPRX2 can be activated by peptides with as few as 5 amino acids. Further identification of peptide sequences that are both fully artificial and extremely short (5 mer) gives us our first step in defining a general rule for peptides that activate human mast cells *via* MRGPRX2.

2. Materials and methods

2.1. Transfection of HEK-293 cells with MRGPRX2 receptor

HEK-293 cells stably expressing Gα15 and the MRGPRX2 receptors was kindly provided by Dr. Xinzhong Dong (reference our Nature paper here) and were cultured according to established protocols.

2.2. Screening of peptide library with MRGPRX2 transfected HEK cells

In a typical experiment, wild type HEK-293 cells (HEK-WT) and MRGPRX2 transfected HEK-293 cells (HEK-X2) were seeded in the alternate rows of a 96 well, clear bottom, black polystyrene microplate (Corning®, Corning, NY at a density of 4×10^4 cells per well in 200 µL DMEM (high glucose, pyruvate, Gibco, Thermo Fisher) culture media supplemented with 10% fetal bovine serum (FBS, Gibco, Thermo Fischer), 2 mM L-Glutamine (Gibco, Thermo Fisher Scientific, Waltham, MA) and 100 U/mL of penicillin and 100 µg/mL of streptomycin (Pen Strep Gibco, Thermo Fisher Scientific, Waltham, MA) and incubated for 24 h at 37 ºC in 5% CO_2. Then, culture media was removed and replaced with fresh media loaded with 1 µM of Fura-2 AM dye (Gibco, Thermo Fisher Scientific, Waltham, MA) and incubated for 45 min at 37 ºC in 5% CO_2. After incubation, cells were washed with HTB buffer (25 mM HEPES buffer (Gibco, Thermo Fisher Scientific, Waltham, MA), 120 mM NaCl (Sigma Aldrich, Oakville, ON, Canada), 5 mM KCl (Sigma Aldrich, Oakville, ON, Canada), 1 mg/mL glucose (Sigma Aldrich, Oakville, ON, Canada), 1 mg/mL bovine serum albumin (BSA, Sigma Aldrich, Oakville, ON, Canada) and freshly added 1.8 mM $CaCl_2$ (Sigma Aldrich, Oakville, ON, Canada)); and re-suspended in 100 µL of HTB buffer. Fluorescence was measured using FlexStation 3 (Molecular Devices, San Jose, CA) at dual excitation wavelength of 340 and 380 nm and an emission wavelength of 520 nm. After generating a baseline, 50 µl of 3 µM peptide solution was added into the wells to make a final concentration of 1 µM. Data was represented as the ratio of fluorescence at 340 nm and 380 nm (F_{340} / F_{380}). Due to limitation of the machine, readings while dispensing the liquid into the assay plate was shown as saturated, and hence the values from the preceding readings was taken into consideration.

2.3. Determination of intracellular calcium concentration upon activation by peptides

Similar experimental protocol as mentioned above was followed to determine the intracellular calcium upon peptide activation. $[Ca^{+2}]$ was quantified using the equation:[15]

$$[Ca^{+2}] = K_d \frac{F_{380min}}{F_{380max}} \frac{(R - R_{min})}{(R_{max} - R)}$$

Where, K_d = dissociation constant of Fura 2 (135 nM (as per supplier), R is the ratio of fluorescence at 340 and 380 nm excitation wavelength for respective peptides. R_{max} is the maximum fluorescence ratio observed by the addition of 50 µL of 30 µM ionomycin and R_{min} is the minimum fluorescence observed by the addition of 50 µL of 100 mM, 2.5% EGTA (Sigma Aldrich, Oakville, ON, Canada) – Triton X-100 (Sigma Aldrich, Oakville, ON, Canada) solution. F_{380min} and F_{380max} are the fluorescence intensity of Fura 2 in bound and free state respectively. R_{max}, R_{min}, F_{380min} and F_{380max} was calculated for each column in a plate and was averaged over the entire plate. Data is represented after base line correction followed by blank correction.

2.4. Evaluation of LAD-2 degranulation – β-Hexosaminidase (β-hex) release assay

LAD2 human mast cells were cultured in StemPro-34 SFM medium (Life Technologies, Rockville, MD) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin, 100 ng/mL recombinant human stem cell factor (SCF, Peprotech, Rocky Hill, NJ). Cells were maintained at 0.1 $\times 10^6$ cells/mL at 37 ºC and 5% CO_2 fresh media was added to the cultures every 3-7 days. For the assay, 0.25 $\times$ 10^5 LAD2 cells per well were washed and resuspended in 90 µL prewarmed HEPES buffer (10 mM, with 0.4% BSA, pH 7.4), and activated by adding 10 µL peptide solutions in PBS (1x, pH 7.4) for 30 min at 37 ºC. β-hex release was quantified through analysis of the hydrolysis of p-nitrophenyl N-acetyl- b-D-glucosamide (Sigma Aldrich, Oakville, ON, Canada) in 0.1 M sodium citrate buffer (pH 4.5) for both the supernatant and in total cell

lysates solubilized with 0.01% Triton X-100 for 90 min at 37 ºC. The reaction was stopped by adding glycine buffer (pH 10.7). Read absorbance at 405 nm with reference filter at 620 nm. The percentage of β-hex released into the supernatant was calculated as a percentage of β-hex in the supernatant relative to the cell lysate and supernatant combined.

2.5. *Ex-vivo* activation of skin mast cell

To be written by NARCY/MK

Human skin was harvested from abdominoplasty surgical discard specimens obtained following written informed consent as approved by the University of Alberta research ethics board. In a typical experiment, 8 mm punches were made in epidermis removed human skin biopsies as described previously. Skin punches were immersed in 1.5 mL of 10 µM of peptides solutions in PBS and were incubated for 4, 12 and 24 h at 37 ºC in 5% CO_2. After incubation, tissue was washed with PBS and snap freezed in liquid nitrogen and stored at -80 ºC until further processing. Total RNA was isolated and purified using a RNeasy Plus Kit (Qiagen, Hilden Germany) according to the manufacturer's protocol, and 200 ng RNA were used to synthetized cDNA by M-MLV reverse transcriptase (Invitrogen, Waltham, MA) according to the manufacturer's instructions. The gene expression levels of tryptase (TSAPB1) enzyme were analyzed by a StepOnePlus real-time PCR system (Applied Biosystems, Thermofisher Scientific, Waltham, MA) with gene-specific primers and probes sets (Integrated DNA Technologies, Coralville IA). The PrimeTime qPCR oligonucleotides for TPSAB1 (Assay ID, Hs.PT.58.19121290.g) and a reference gene, HPRT1 (Assay ID, Hs.PT.58v.45621572) (Table 1), were obtained from Integrated DNA Technologies (Coralville, IA). The PCR mixture (20 µl total volume) consisted of the template, primers, and probe for each gene, and the PrimeTime® Gene expression Master Mix (Integrated

DNA Technologies, Coralville IA). Real-time PCR amplification was carried out as follows: initial denaturation cycle at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec, and annealing and elongation at 60°C for 1 min, with a final extension at 97°C for 10 min. The results were analyzed by the 2-ΔΔCT method using HPRT1 to normalize gene expression. Data were obtained from three independent measurements performed in duplicate.

Table 1: Primer and probes sequences for Tryptase and HPRT1.

ID	Sequence name
Hs.PT.58.19121290.g	TPSAB1, HOMO_SAPIENS
Sequence	
Primer	CAG TGG TGT TTT GGA CAG
Primer	CGG CCT GGC ATC TAC AC
Probe	/56-FAM/TGA CTC ACG /ZEN/GCT TTT TGG
ID	**Sequence name**
Hs.PT.58v.45621572	HPRT1, HOMO_SAPIENS
Sequence	
Primer	GCG ATG TCA ATA GGA CTC
Primer	TTG TTG TAG GAT ATG CCC TTG A
Probe	/5HEX/AGC CTA AGA /ZEN/TGA GAG TTC AAG

Trizol contaminated cDNAs were cleaned up using the QIAquick PCR purification Kit (Qiagen, Hiden, Germany) following manufacturer's protocol, followed by qPCR analysis. Please add some **details** for qPCR, the following is my example, you may not use ddPCR this time.

The ddPCR conditions comprised of an initial denaturation for 10 min at 95 ºC followed by 45 cycles of denaturation for 30 s at 94 ºC, and annealing and extension for 1 min at 60 ºC, and the final extension for 10 min at 98 ºC. Template cDNA was omitted from the ddPCR reaction for no template control (NTC). QuantaSoft Software (Bio-Rad, USA) was employed to analyze ddPCR

results, and the absolute concentration of TPSAB1 transcripts in each sample determined by ddPCR was divided by the ACTB transcripts and presented as percentage based on normal skin samples (NS average taken as 100).

2.6. Statistical analysis

All data were conducted in at least quadruplicate with independent repeats and presented as average ± standard error of the mean (SEM). The statistical significance of differences between mean values was determined using one-way ANOVA followed by two-tailed Student's t-test for analysis of variance, where significance was evaluated for $p<0.05$, $p<0.01$, $p<0.001$.

3. Results and discussion

3.1. Specificity of PAMP-12 towards MRGPRX2 receptor

MRGPRX2 is a low-affinity receptor, differentially expressed on mast cells depending on their site of origin; where skin, and synovial mast cells express high levels of MRGPRX2 receptor, mucosal mast cells in the lung and heart have limited receptor expression.[5,17] MRGPRX2 receptor binding of PAMP-12 is known to lead to the activation of human mast cells.[4,9] Calcium (Ca^{+2}) flux kinetics were monitored upon PAMP-12 incubation with MRGPRX2 transfected HEK cells (HEK-X2) or associated wild type HEK cells (HEK-WT) (Figure 1a, b). To understand the effect of PAMP-12 concentration on HEX-X2 activation, a range of PAMP-12 concentrations from 0.01 to 100 μM were evaluated in a similar manner (Figure 1a) and no significant differences in released Ca^{+2} with increasing PAMP-12 concentration was observed. However, on average, there was a stepwise increase in Ca^{+2} flux between 0.1 to 0.5 μM PAMP-12 concentration. A 1 μM peptide concentration was used to compare the activation potential for on MRGPRX2 transfected HEK cells (Figure 1b). This control study showed that HEK-X2 cells stimulated with 1 μM PAMP-12 yielded a substantial release of Ca^{+2}, whereas HEK-WT cells remained at basal levels.

LAD2 is a MRGPRX2 expressing human mast cell line and was used as a model human cell line to verify PAMP-12 functionality, while non-MRGPRX2 expressing BMMCs (murine) were used as controls. As with Ca^{+2} release from HEX-X2 cells, β-hexosaminidase release upon mast cell activation is indicative of the extent of degranulation. PAMP-12 was shown to have a concentration dependent β-hexosaminidase release from LAD2 cells (Figure 1c); a saturation of which was found at 10 μM PAMP-12 concentration. As a result, a 10 μM peptide concentration was used to evaluate the activation of LAD2s for all the peptides studied throughout this study. Similar to Ca^{+2} flux in HEK-X2 cells, it was observed that exposure to 10 μM of PAMP-12 yielded

a 68% β-hexosaminidase release in MRGPRX2 expressing LAD2 cells while BMMCs showed no β-hexosaminidase release upon activation by the same concentration of PAMP-12 (Figure 1d).

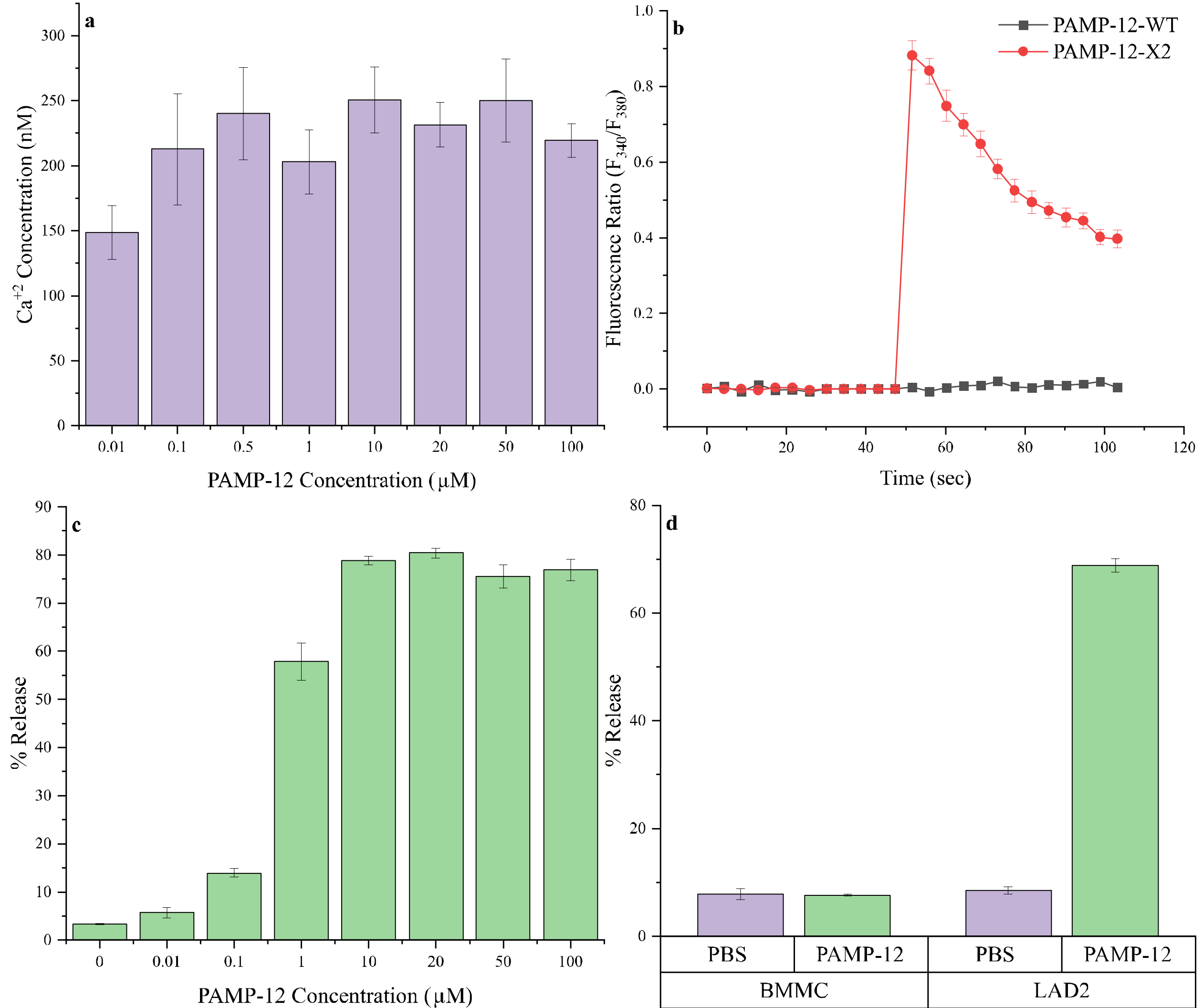


Figure 1: Specificity of PAMP-12 towards MRGPRX2 receptor. a) dose dependent Ca^{+2} release from HEK-X2 with increasing PAMP-12 concentration b) Ca^{+2} mobilization in HEK-X2 and HEK-WT cells upon incubation with 1 μM PAMP-12; c) concentration dependent β-hexosaminidase release in LAD-2 cells upon incubation with PAMP-12; and d) β-hexosaminidase release from LAD-2 cells versus BMMCs upon incubation with 10 μM PAMP-12

3.2. N and C truncation of PAMP-12

Calcium mobilization in MRGPRX2 transfected Chinese hamster ovary cells cells stimulated with PAMP-12 and PAMP yielded EC_{50} values of 41 and 223 nM respectively; suggesting PAMP-12 has a higher activating potential than PAMP.[9] A series of peptides derived from N-(TS-Nx), C-(TS-Cy), and N+C-truncated (TS-NxCy) peptides from PAMP-12 (Table 2) were screened using HEK-X2 and LAD2 cells so as to identify if a core motif exists with similar activation potential as PAMP-12. HEK-X2 activation, compared to HEK-WT, upon incubation with these peptides was conducted (Figure 2). Similar to PAMP-12 induced activation (Figure 1b) all derived peptides induced calcium mobilization in HEK-X2 and not in HEK-WT, confirming truncated peptides do bind the MRGPRX2 receptor.

Calcium released upon incubation with these truncated peptides was quantified using the Grynkiewicz equation.[15] As shown in Figure 3a, TS-N1 to TS-N5 showed a Ca^{+2} release of 231 ± 36, 177 ± 21, 152 ± 17, 203 ± 21, and 59 ± 07 nM, respectively. There was no significant difference between PAMP-12 (174 ± 7 nM) and TS-N1 to TS-N4, unlike TS-N5 that resulted in a significant decrease in Ca^{+2} flux. Calcium released by TS-N5 was only 34 ± 4 % of PAMP-12 and 29 ± 4 % of TS-N4, a decrease of 66% and 71% respectively. Similar results were observed using LAD2 cells (Figure 3b), viz., PAMP-12 and truncated sequence TSN1 to TS-N4 showed a respective β-hexosaminidase release of 68 ± 1, 64 ± 3, 51 ± 1, 40 ± 3 and 66 ± 2% respectively, while β-hexosaminidase release in TS-N5 was reduced to 5%. Results from the calcium mobilization and β-hexosaminidase release showed that in the library of N-Truncated sequences, TS-N4 is the minimum sequence, which can activate mast cell through MRGPRX2 receptor. Further removal of residue from N-terminal, resulting in TS-N5, very significantly reduced the activation potential of the peptide.

Table 2: Peptide sequences of the truncated peptides. The peptides were modified with an acetyl group and an amide group at the N-terminal and C-terminal, respectively.

Peptide	Peptide Sequence
PAMP-12	FRKKWNKWALSR
N-Truncated Peptides	
TS-N1	RKKWNKWALSR
TS-N2	KKWNKWALSR
TS-N3	KWNKWALSR
TS-N4	WNKWALSR
TS-N5	NKWALSR
C-Truncated Peptides	
TS-C1	FRKKWNKWALS
TS-C2	FRKKWNKWAL
TS-C3	FRKKWNKW
TS-C4	FRKKW
TS-C5	FRKK
TS-C6	FRK
TS-C7	FR
N+C-Truncated Peptides	
TS-N4C1	WNKWAL
TS-N4C2	WNKWA
TS-N4C3	WNKW
TS-N4C4	WNK

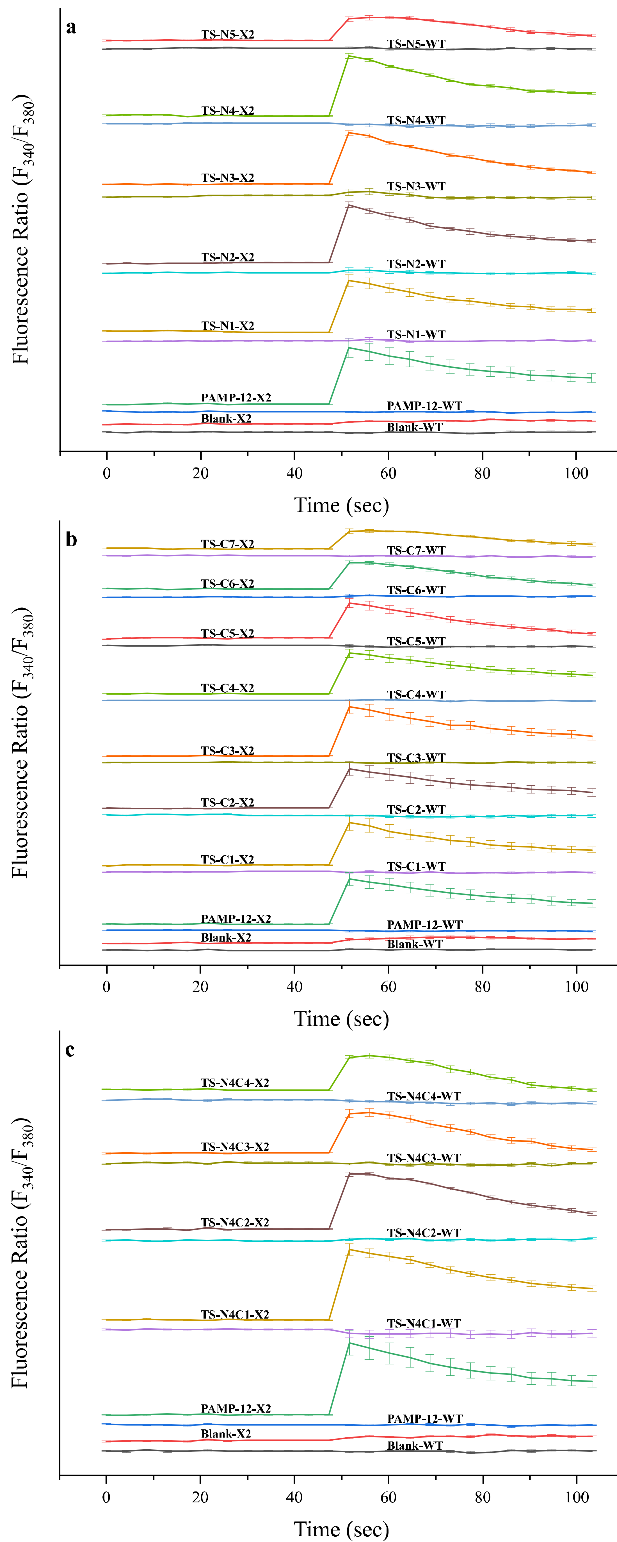


Figure 2: Truncated peptide activity towards MRGPRX2 receptor as confirmed using calcium mobilization in HEK-X2 and HEK-WT cells. a) N-Truncated peptides b) C-Truncated peptides and c) N+C-Truncated peptides.

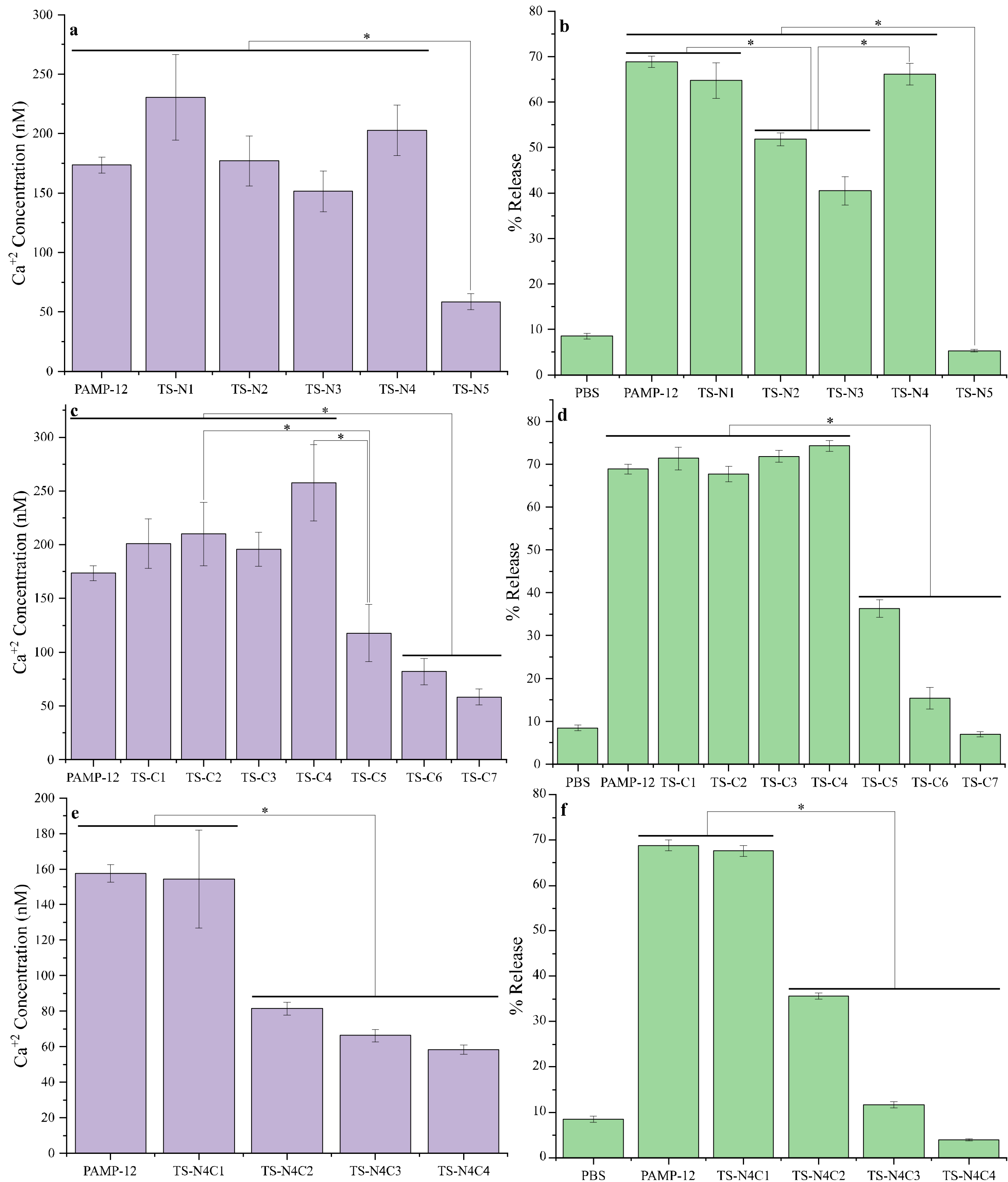


Figure 3: Calcium flux measured using ratiometric Fura-2 dye in HEK-X2 cells upon incubation with 1 μM concentration of (a) N-truncated peptides (c) C-truncated peptides and (e) N+C-truncated peptides. β-hexosaminidase release study on LAD-2 cells upon stimulation by 10 μM of (b) N-truncated peptides and (d) C-truncated peptides and (f) N+C-truncated peptides. One-way ANOVA was used to find the significant difference among different peptides at $p < 0.05$.

The effect of C-terminal truncation on PAMP-12 activity potential against HEK-X2 cells showed that TS-C1 to TS-C4 resulted in Ca+2 release of 201 ± 23, 210 ± 30, 196 ± 16 and 257 ± 35 nM, respectively (Figure 3c). TS-C1 to TS-C3 peptides induced similar released amounts of calcium as PAMP-12 (174 ± 7 nM); TS-C4 was significantly higher. However, HEK-X2 incubation with peptides TS-C5 through TS-C7 showed an on average stepwise decrease in released calcium. TS-C5 showed a Ca+2 concentration of 118 ± 27 nM, 46 ± 12 % of TS-C4 or 68 ± 16 % of PAMP-12. TS-C6 and TS-C7 showed a calcium release of 81 ± 12 and 58 ± 7 nM, respectively. The trend in LAD2 activation results was similar to that observed for HEK-X2 (Figure 3d). PAMP-12 and TS-C1 to TS-C4 showed β-hexosaminidase release in the range of 67 to 75 %; TS-C5 showed a significant decrease in activity to 36 ± 2%. Consistent with Ca+2 release data, TS-C6 and TS-C7 showed further decrease in β-hexosaminidase release both being at 15% and 6% respectively. These results suggest that TS-C4 is the minimum sequence in the library of C-truncated sequences that can activate mast cells with a potential comparable to that of PAMP-12.

Residues at both ends of PAMP-12 were sequentially truncated (TS-NxCy) and the activation potential for the resulting peptides evaluated using HEK-X2 or LAD2 cells. As shown in Figure 3e, PAMP-12 and TS-N4C1 showed a comparable calcium mobilization of 158 ± 5 nM and 154 ± 27 nM, respectively. Removal of one more C-terminal residue from TS-N4C1 resulted in a decreased Ca^{+2} concentration of 81.44 ± 3.55 (TS-N4C2). This concentration was at 52 ± 3 and 53 ± 10 % of that of PAMP-12 and TS-N4C1, respectively. Analogous to HEK-X2 activation, LAD2 cells showed that while PAMP-12 and TS-N4C1 each caused a release of 68 and 67% respectively, the β-hexosaminidase released by TS-N4C2 was restricted to 35%; a decrease of about 48% with respect to PAMP-12 and TS-N4C1 (Figure 3f).

These results show that synthetic peptide TS-C4 and TS-N4C1 having amino acid sequences FRKKW and WNKWAL respectively, are the smallest peptides that can activate mast cells with the same potency as that of PAMP-12. Ca^{+2} released by these two peptides in HEK-X2 cells were 117 ± 13 % and 98 ± 18 % of PAMP-12, respectively. The respective percentage for the β-hexosaminidase release caused by FRKKW and WNKWAL for LAD2 cells, with respect to PAMP-12, were 108 and 98%.

Results obtained here are comparable to earlier published report, which showed an activity value of 97 ± 5 % for WNKWALSR and 100 ± 12 % for PAMP-12 when compared to cortistatin-14.[18] Since, MRGPRX2 ligands have shown a concentration dependent activation profile, dose dependent response of FRKKW and WNKAWAL on β-hexosaminidase was as well studied. Peptide sequences FRKKW and WNKWAL showed a concentration-based release profile having comparable activation potential as that of PAMP-12 on LAD-2 cells (Figure 4). Using dose dependent results on LAD-2 cells, EC_{50} values for PAMP-12, FRKKW and WNKWAL were found to be 0.47, 0.87 and 1.26 μM, respectively.

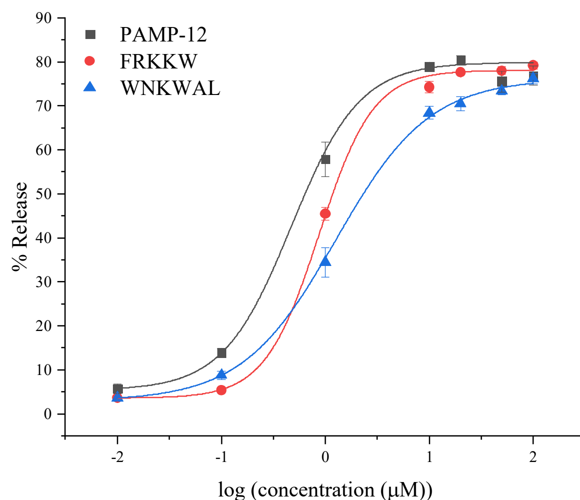


Figure 4: Dose dependent β-hexosaminidase response of the two identified core motifs, FRKKW and WNKWAL on LAD-2 mast cells.

Protein fragments have been reported as being more potent activators of the MRGPRX2 receptor compared to their precursors. As discussed above, PAMP-12 and its core motifs FRKKW and WNKWAL have comparable activity towards mast cell activation as their precursor. Similarly, Cortistatin-17 showed a calcium response in MRGPRX2 transfected HEK cells with an EC_{50} value of 0.099 μM while its smaller analog cortistatin-14 had an EC_{50} value of 0.025 μM.[10] Cathelicidin, LL-37 and its derived peptide FK-13 having a sequence FKRIVQRIKDFLR showed equivalent degranulation in LAD2 mast cells.[19] Following a similar trend, the fragments of albumin and chaperonin proteins have also shown a calcium response in MRGPRX2 expressing HEK cells and degranulation in mast cells.[8,20] These data suggests that size of the ligand molecule plays an important role in their activation potential towards the MRGPRX2 receptor. One plausible explanation could be the active domains are sterically hindered from interacting with the receptor when found in larger molecules. Based on our result, it can be argued that the receptor-binding domain of these molecules are a fraction of the overall size of PAMP, and still much smaller than even PAMP-12.

MRGPRX2 being low affinity entails a higher ligand concentration for activation and hence a dose dependent activation profile has been observed for various ligands which activate through MRGPRX2 receptor.[8,10,21] Among various ligands, which activate through MRGPRX2, neuropeptide Cortistatin-14 has been shown to be most potent with a reported EC_{50} value of 0.025 μM and 0.106 μM in two different findings.[4,10] EC_{50} value for other ligands including Compound 48/80, Substance P and the PAMP-12 have been reported to be 0.470 μg/ml, 0.152 μM to 8.02 μM and 0.166 μM respectively.[4,5,10] Several clinically administered antimicrobial drugs have also been reported to induce pseudo allergic reactions at higher dosage. Drugs like Terbinafine, Amorolfine, Ketoconazole, Sisomicin, Gentamicin, Micronomicin, Sulfamethoxazole, Sulfadoxin and

Mafenide acetate have showed an EC_{50} value in the range of 84.4 to 808.4 µg/ml towards MRGPRX2 activation with a dose dependent response.[22] Similarly, albumin fragments which showed activity towards MRGPRX2 were active only at micro molar concentration, showing an EC_{50} value in the range of 50-100 µM.[8]

3.3. Significance of amino acid residues in mast cell activation through MRGPRX2 receptor

Alanine scanning of PAMP-12 was used to understand the significance of individual amino acids in inducing mast cell degranulation by substituting individual amino acids with alanine (ASn) (Table 3). The interaction of these peptides with HEK-X2 and HEK-WT cells was measured (Figure 5a) and it was observed that all peptides caused calcium flux in HEK-X2 but not HEK-WT cells. Results showed that alanine scanning did not alter the receptor specificity of the peptides and they still activated the cell through MRGPRX2.

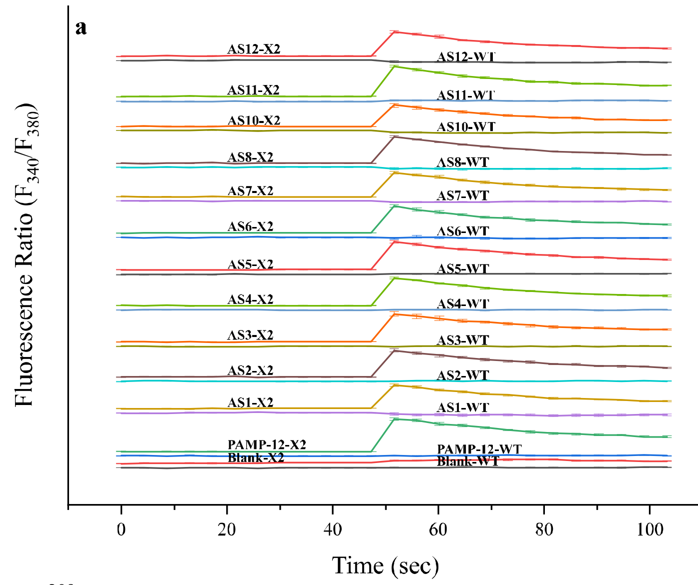
Table 3: Peptide library created through alanine scanning of PAMP-12. The peptides contained an acetyl group and an amide group at the N-terminal and C-terminal, respectively

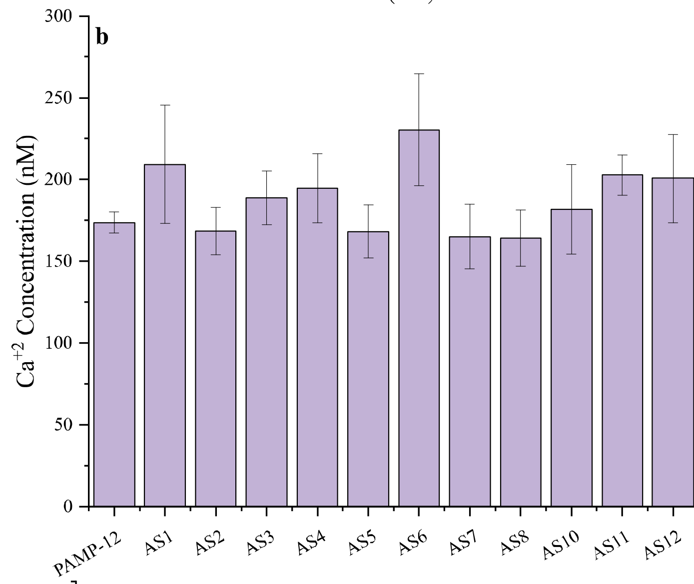
Peptide	Peptide Sequence
PAMP-12	FRKKWNKWALSR
AS1	**A**RKKWNKWALSR
AS2	F**A**KKWNKWALSR
AS3	FR**A**KWNKWALSR
AS4	FRK**A**WNKWALSR
AS5	FRKK**A**NKWALSR
AS6	FRKKW**A**KWALSR
AS7	FRKKWN**A**WALSR
AS8	FRKKWNK**A**ALSR
AS10	FRKKWNKWA**A**SR
AS11	FRKKWNKWAL**A**R
AS12	FRKKWNKWALS**A**

Though the quantification of calcium results from HEK-X2 cells and its One-Way ANOVA analysis did not show any significant difference among peptides (Figure 5b), β-hexosaminidase release from LAD-2 degranulation indicated the significance of specific residues (Figure 5c). Results showed that the replacement of tryptophan (W) with alanine at the 5th position from N-terminal in PAMP-12 (i.e. AS5) caused a significant decrease in degranulation. While PAMP-12 showed a β-hexosaminidase release of 68 %, AS5 caused a degranulation of only 50%, a decrease of 26.5% with respect to PAMP-12. The importance of tryptophan at 5th position was also evident in the results of truncated sequences. Both identified core sequences, FRKKW and WNKWAL include tryptophan in the sequence. Removal of W from FRKKW decreased calcium flux by around 54% with respect to FRKKW and the β-hexosaminidase release in LAD2 cells decreased to 36% as compared to 74% by FRKKW (TS-C5, Figure 3). Similarly, a comparison between WNKWALSR (TS-N4) and NKWALSR (TS-N5) showed a respective Ca^{+2} flux of 203 ± 21and 59 ± 7 nM in HEK-X2 cells. In addition, in LAD2 cells, β-hexosaminidase release by TS-N5 was 5% as compared to 66% released by TS-N4 (Figure 3).

These results suggest that tryptophan at 5th position from N-terminal in PAMP-12 may constitute the receptor binding domain for MRGPRX2 within the whole PAMP-12 sequence. Also, the presence of a hydrophobic residue bearing an aromatic group at the N terminal (F in PAMP-12 and TS-C4; W in TS-N4C1) was vital for MRGPRX2 activation. Results obtained in our study are in accordance with other published reports. It has been shown that activity of PAMP [10-20], lacking N-terminal hydrophobic residue decreased by 15% compared to PAMP-12. Similarly, cortistatin-14 with hydrophobic proline at N-terminal was 4 times more potent than cortistatin-17, which has aspartic acid (polar) at its N-terminal.[18] Additionally, albumin fragment [412-423] (TKKVPQVSTPTL) having threonine (polar) at the N-terminal failed to cause both Ca^{+2} release

in MRGPRX2 transfected HEK cells and β-hexosaminidase release in LAD2 cells. However, albumin fragment [407-423], [408-423] and [409-423] (having sequences – LLVRYTKKVPQVSTPTL, LVRYTKKVPQVSTPTL and VRYTKKVPQVSTPTL respectively) with a hydrophobic residue at N-terminal activated both MRGPRX2 transfected HEK cells and LAD-2 cells.[8] Falling in line, cathelicidin LL-37 and its derived peptide FK-13 (LL-37 [17-19]) having a sequence FKRIVQRIKDFLR showed equivalent degranulation in LAD2; both these peptides had hydrophobic N-terminal residues.[19]


a
Fluorescence Ratio (F_{340}/F_{380})
Time (sec)
AS12-X2
AS12-WT
AS11-X2
AS11-WT
AS10-X2
AS10-WT
AS8-X2
AS8-WT
AS7-X2
AS7-WT
AS6-X2
AS6-WT
AS5-X2
AS5-WT
AS4-X2
AS4-WT
AS3-X2
AS3-WT
AS2-X2
AS2-WT
AS1-X2
AS1-WT
PAMP-12-X2
PAMP-12-WT
Blank-X2
Blank-WT


b
Ca^{+2} Concentration (nM)
PAMP-12
AS1
AS2
AS3
AS4
AS5
AS6
AS7
AS8
AS10
AS11
AS12

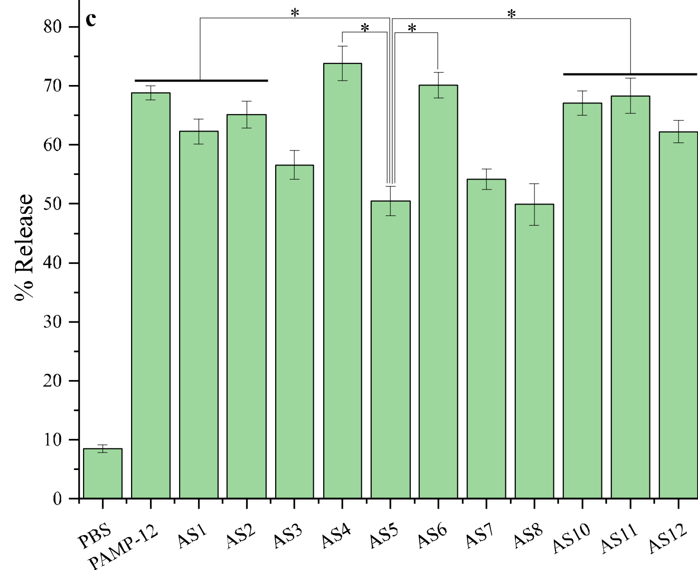

c
% Release
PBS
PAMP-12
AS1
AS2
AS3
AS4
AS5
AS6
AS7
AS8
AS10
AS11
AS12

Figure 5: a) Specificity of PAMP-12 derived peptides using alanine scanning towards MRGPRX2 receptor, confirmed by studying the calcium mobilization in HEK-X2 and HEK-WT cells b) Calcium flux measure using ratiometric Fura-2 dye in HEK-X2 cells upon incubation with 1 μM concentration of scanned peptides, and c) β-hexosaminidase release study on LAD-2 cells upon stimulation by 10 μM peptide concentration. One-way ANOVA was used to find the significant difference among different peptides at $p < 0.05$

3.4. Determination of a generalized motif for MRGPR-X2 activation

A keen observation of various peptide sequences that have been shown to activate MRGPRX2 bearing mast cells highlights a distinct motif that seems common to all of these studies, including our peptide sequences - FRKKW and WNKWAL. This generalized amino acid sequence that binds MRGPRX2 may follow a format of: X_a-$(Y)_{n\geq3}$-X_b; where (i) X_a is a hydrophobic residue bearing aromatic ring in its side chain (F/W); (ii) X_b is any hydrophobic residue, and (iii) X_a and X_b are flanked by a group ($n \geq 3$) of at least 1-3 basic polar amino acid (i.e. +vely charged side chain) with the remaining being uncharged residues.

To test this hypothesis, we synthesized a series of customized synthetic peptides (Table 4). These customized peptides (CSTx) induced activity in HEK-X2 cells but not on wild type cells (Figure 6a). Calcium concentration was measured (Figure 6b) for PAMP-12, CST5 and CST7 to CST10 as 186 ± 8, 144 ± 14. 158 ± 16, 255 ± 22, 213 ± 22 and 172 ± 22 nM, respectively. Sequences CST1 to CST4, CST6 and CST11 showed minimal activity. These results showed that peptide sequence CST8 (WKKKW) and CST9 (FKKKF) had mean value greater than PAMP-12: WKKKW was 138 ± 13 % and FKKKF was 115 ± 13 % of PAMP-12. These sequences satisfy the hypothesis, where N-terminal residue is hydrophobic group bearing an aromatic group as the C terminal and is flanked by polar basic lysine group. Replacing the terminal groups with tyrosine, CST7 (YKKKY) decreased the mean by 15 % of that of PAMP-12. Tyrosine ranks lower than both phenylalanine and tryptophan in the hydrophobicity scale.[23] This is due to the presence of -

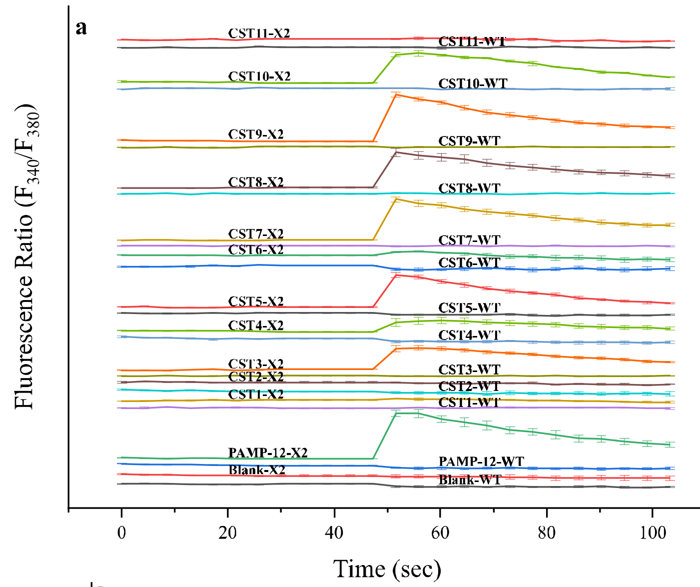
OH group in the side chain of tyrosine as compared to phenylalanine. –OH group present on tyrosine has a propensity to both accept and donate hydrogen atoms, which facilitates the formation of hydrogen bond, reducing the hydrophobicity of tyrosine. Peptide sequences (CST1, CST2, CST3, CST4 and CST6) and scrambled sequences (CST10 and CST11) which did not satisfy the hypothesis, showed reduced calcium mobilization. The results obtained from the calcium mobilization experiment was supported by degranulation assay (Figure 6c). β-hexosaminidase released by LAD2 cells upon activation by 10 µM peptide concentration of PAMP-12, and CST7 to CST10 was 68, 61, 76, 72 and 42% respectively. YKKKY having less polar tyrosine at 61% was less than PAMP-12, WKKKW and FKKKF.

Table 4: Custom peptides designed based on generalized motifs for MRGPRX2 receptor (both activating and non-activating). The peptides were modified with an acetyl group and an amide group at the N-terminal and C-terminal respectively

Peptide	Peptide Sequence
CST1	KKW
CST2	WKK
CST3	KWKW
CST4	WKKW
CST5	FKKF
CST6	YKKY
CST7	YKKKY
CST8	WKKKW
CST9	FKKKF
CST10	KWKWK
CST11	KYKYK

The presence of motif similarity in the ligands that activate MRGPRX2 has been discussed in detail in the review article by our group.[16] The amino acid sequence of PAMP-12 and cortisatin-14 has also been compared highlighting the similarities in their residues.[18] Various efforts have

been made to understand the ligand – receptor interactions. Molecular modelling portals like RaptorX and Phyre2 were used to study the binding pocket of the MRGPRX2 receptor.[24] It was reported that the binding pocket of MRGPRX2 consists of a large number of hydrophobic amino acid residues, with a very significant glutamine acid at 164th position such that mutation at 164th glutamine acid with arginine inhibited the activation by substance P and compound 48/80. In accordance with the above results, the presence of a terminal hydrophobic residue in the generalized motif, X1-(Y)n-X2, may indicate a hydrophobic effect that assists in the localization of the ligand within the binding pocket of the receptor. Polar basic groups, with positively charged side chain, within the generalized motif may facilitate an electrostatic interaction with negatively charged side chains of the glutamic residue at the 164th position. Previous work has employed an integrated approach, using homology models and structure based molecular docking to design opioid like synthetic agonists for MRGPRX2, which contains an aromatic ring structure with a free tertiary amine group. The aromatic ring could be hypothetically guiding the molecule towards the binding pocket, while free tertiary amine group facilitates the electrostatic interactions.[25] Similarly, non - peptide molecules that selectively activated MRGPRX2 receptor also contain an aromatic ring and amine groups in their structure.[26]


a
Fluorescence Ratio (F_{340}/F_{380})
Time (sec)
CST11-X2
CST11-WT
CST10-X2
CST10-WT
CST9-X2
CST9-WT
CST8-X2
CST8-WT
CST7-X2
CST7-WT
CST6-X2
CST6-WT
CST5-X2
CST5-WT
CST4-X2
CST4-WT
CST3-X2
CST3-WT
CST2-X2
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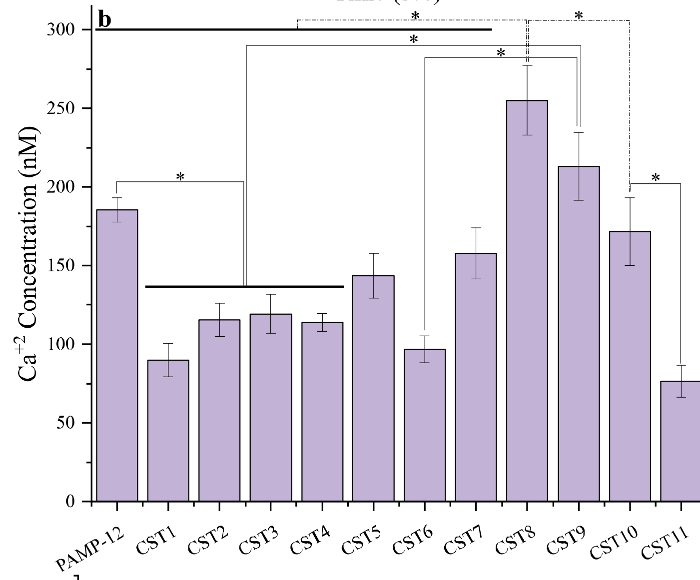
CST1-X2
CST1-WT
PAMP-12-X2
PAMP-12-WT
Blank-X2
Blank-WT


b
Ca^{+2} Concentration (nM)
PAMP-12
CST1
CST2
CST3
CST4
CST5
CST6
CST7
CST8
CST9
CST10
CST11

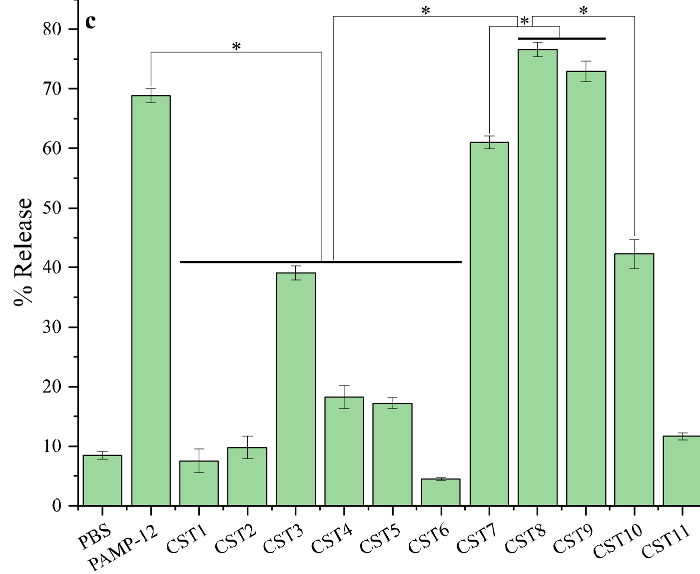

c
% Release
PBS
PAMP-12
CST1
CST2
CST3
CST4
CST5
CST6
CST7
CST8
CST9
CST10
CST11

Figure 6: a) Activation potential of customized peptides towards MRGPRX2 receptor, confirmed by studying the calcium mobilization in HEK-X2 and HEK-WT cells b) Calcium flux measured using ratiometric Fura-2 dye in HEK-X2 cells upon incubation with 1 µM concentration of designed peptides c) β-hexosaminidase release study on LAD-2 cells upon stimulation by 10 µM peptide concentration. One-way ANOVA was used to find the significant difference among different peptides at $p < 0.05$

3.5. Ex-vivo activation of human skin tissue mast cells

Skin tissue possess mast cells which belong to CT (chymase – tryptase) subclass, implying they secrete both the chymase and tryptase upon degranulation[27]. Skin mast cells express MRGPRX2 receptors on their surface.[17] Herein, we chose tryptase as a marker for mast cell activation in skin tissue. To test the activation potential for our peptide candidates towards tissue resident human mast cells, we removed the epidermis of human skin and incubated it with 10 µM solution concentrations of PAMP-12, core peptides, and synthesized peptides. RNA expression for the tryptase enzyme after 4, 12 and 24 hours of incubation was quantified using RT-qPCR and normalized against HPRT1 housekeeping gene (Figure 7). Our results showed that, compared to HPRT1, PAMP-12 showed a 1.5-fold increase in tryptase expression. Biomimetic peptides, FRKKW and WNKWAL, showed a 2- and 1.6-fold increase with respect to the house keeping gene. The artificial peptide, FKKKF, showed an expression level comparable to that of PAMP-12. Scramble sequences and synthesized peptides that did not adhere to this generalized peptide sequence had an expression level of half of the house keeping gene. Thus, these core motifs and designed peptides containing the generalize motif showed PAMP-12 comparable degranulation while the peptides lacking this motif showed reduced degranulation. These results support the generalized motif's ability to cause activation in mast cells.

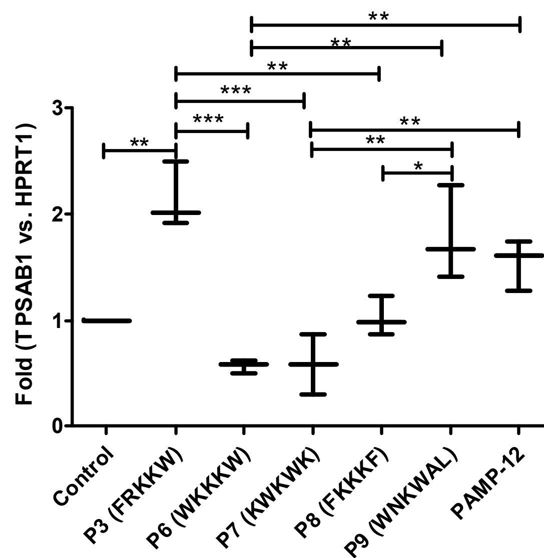


Figure 7: Effect of peptide administration to human skin tissue on tryptase (TPSAB1) expression HPRT1 was used to normalize gene expression (n = 3; *P < 0.05, **P < 0.01, and ***P < 0.001).

4. Conclusion

MRGPRX2 is an important mast cell receptor, which orchestrates the immune response of a body against various unrelated endogenous peptides, small molecular compounds and approved drugs. We, in here, have tried to elucidate the binding mechanism of this receptor. Using PAMP-12 as a model peptide, we have devised a design principle which can help in developing new peptidic molecules which can differentially activate mast cell through the MRGPRX2 receptor. Simultaneously, we have also identified the core motifs of endogenous peptide PAMP-12 which had similar activity as that of parent PAMP-12. The derived design rule for artificial peptide stimuli conforms to the structural similarity of known peptide ligands of MRGPRX2. These finding will help in further understanding of MRGPRX2 mediated mast cell activation, and design and development of new therapeutics to regulate the immunity of our body.

Acknowledgements

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