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Peptidomimetics of Arg-Phe-NH_2 as small molecule agonists of Mas-related gene C (MrgC) receptors

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ABSTRACT

A series of Arg-Phe-NH_2 peptidomimetics containing an Arg mimetic were synthesized and tested as agonists of human MrgX1, rat MrgC, and mouse MrgC11 receptors. As predicted from the previously established species specificity, these peptidomimetics were found to be devoid of MrgX1 agonist activity. In contrast, these compounds acted as agonists of MrgC and/or MrgC11 with varying degrees of potency. These new peptidomimetics should complement the existing small molecule human MrgX1 agonists and enhance our ability to assess the therapeutic utility of targeting Mrg receptors in rodent models.

1. Introduction

Mas-related gene (Mrg) receptors, also known as sensory-neuron specific receptors (SNSRs), represent a family of orphan GPCRs.[1] A subset of these receptors are expressed specifically in the sensory neurons of the dorsal root ganglion (DRG), implicating a role in modulating nociceptive signaling. Among them, human SNSR4 (also known as MrgX1) has gained increased interest as a therapeutic target partly owing to the known peptide-based agonists[2] as well as evidence for its role in nociception[3] and pruritus.[4]

Of the rodent Mrg receptors, mouse MrgC11[5] and rat MrgC[6] receptors are believed to be the closest orthologs of human MrgX1 because of their similar pattern of tissue expression in the DRG, substantial sequence homology,[5] and existence of common peptide-based ligands including BAM8–22 (Fig. 1). BAM8–22 is a proteolytic product (15-mer peptide) of proenkephalin A and is devoid of affinity for opioid receptors.[2] Because of its high potency for Mrg receptors and selectivity over opioid receptors, BAM8–22 has served as a valuable pharmacological tool for studying the physiological role of Mrg receptors. For example, intrathecal injection of BAM8–22 was found to attenuate both mechanical and thermal hyperalgesia in mice[7] and rats.[8] Skin administration of BAM8–22 was also reported to induce itch in mice[4] and humans[9] in a histamine-independent manner.

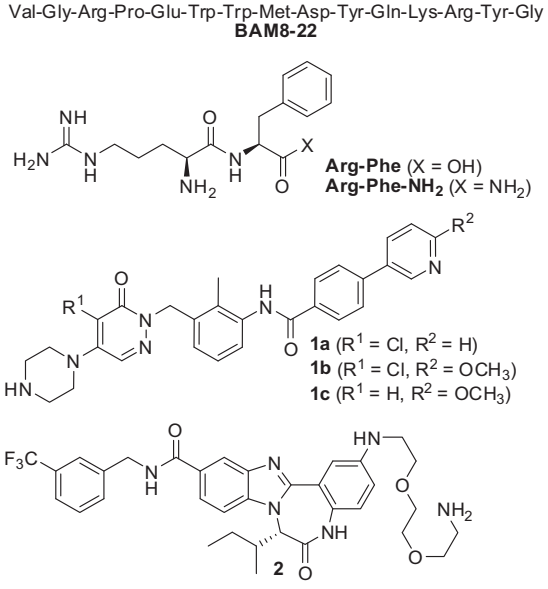

Figure 1. Representative Mrg receptor agonists.

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While BAM8–22 acts as a potent agonist for all three Mrg receptors, its truncated peptides appear to lose affinity to human MrgX1 while retaining activity at the rodent forms, mouse MrgC11 and rat MrgC. In a systematic in vitro study using HEK293 cells expressing human MrgX1, BAM8–22 exhibited agonist activity with an EC_{50} value of 14 nM while neither N-truncated (BAM15–22) nor C-truncated (BAM8–18) analogs showed activity.[2] In contrast, mouse MrgC11 was found to be activated by substantially smaller peptides containing a C-terminal -Arg-Phe(Tyr)-Gly or -Arg-Phe(Tyr)-NH_2 motif.[5] Even dipeptide Arg-Phe and its amide derivative Arg-Phe-NH_2 exhibited submicromolar EC_{50} values for activating MrgC11. Although only limited number of peptides were tested in rat MrgC assay, several Arg-Phe containing peptides substantially shorter than BAM8–22 displayed potency comparable to that of BAM8–22.[10]

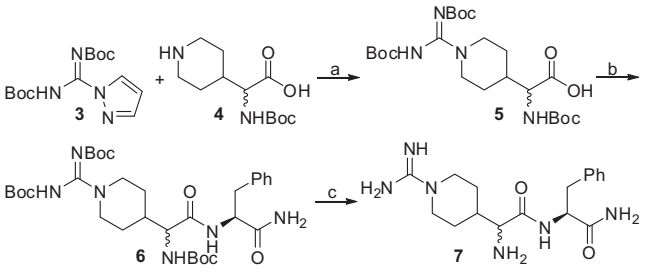
This raises the possibility of cross-species variation in potency for small molecule-based agonists particularly between human and rodent forms of Mrg receptors. Indeed, potent non-peptide human MrgX1 agonists **1a**–**c** (Fig. 1) discovered by GSK were found to have no detectable agonist activity against rat MrgC,[11] thus hindering their utility in rat preclinical models. A non-peptidergic agonist **2** reported by ACADIA[12] is selective to MrgX1 over MrgX2 receptors in both humans and rhesus monkeys though its activity in rodent MrgC receptors is unknown.

These findings have prompted us to explore the possibility of identifying a small molecule agonist with activity in rodent MrgC receptors. It is evident from the prior work that the key residues in these peptides are either Arg-Phe or Arg-Tyr, which can serve as a core template for the design of peptidomimetics with potent agonist activity for Mrg receptors. The presence of an arginine residue can be of particular advantage given the fact that a variety of arginine mimetics have been explored in efforts to design peptidomimetics derived from biologically active natural peptides containing an arginine residue.[13] In particular, development of fibrinogen receptor antagonists based on Arg-Gly-Asp motif represents one of the most relevant cases in which arginine mimetics were effectively utilized.[14,15] Herein we report synthesis of short Arg-Phe-NH_2 derivatives in which the arginine residue is substituted by an arginine mimetic. The resulting short peptide derivatives were tested in Mrg receptors of human, rat, and mouse for their agonist activity to examine the cross-species reactivity as well as their potential utility as pharmacological tools in rodent studies.

2. Results and discussion

2.1. Chemistry

Preparation of Arg-Phe-NH_2 analog **7** containing 1-guanyl-4-piperidineglycine as an arginine mimetic is illustrated in Scheme 1. *N*-Boc-4-piperidineglycine **4** was reacted with *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine **3** to form the protected 1-guanyl-4-piperidineglycine **5**. Coupling with L-phenylalanine amide provided **6**, which was subsequently deprotected with TFA to give the desired product **7**.



Scheme 1. Reagents and conditions: (a) methanol, rt; (b) L-phenylalanine amide·TFA, DIEA, HATU, DMF, rt, 5% from **4**; (c) TFA, dichloromethane, rt, 94%.

Synthesis of proline-containing derivatives **12** and **16** is shown in Scheme 2. *N*-Boc-4-azido-L-proline **8**[16] was coupled with L-phenylalanine amide to obtain **9**. Subsequent catalytic hydrogenation followed by reaction with *N*,*N*′-di-Boc-1*H*-pyrazole-1-carboxamidine **3** and deprotection with TFA afforded Arg-Phe-NH_2 analog **12** containing guanidino-L-proline as arginine mimic. Reaction of (4*R*)-*N*-Boc-4-(aminomethyl)-L-proline **13** with **3** provided fully Boc-protected derivative **14**. Coupling with L-phenylalanine amide followed by deprotection with TFA gave Arg-Phe-NH_2 analog **16** containing 4-guanidinomethyl-L-proline as an arginine mimic.

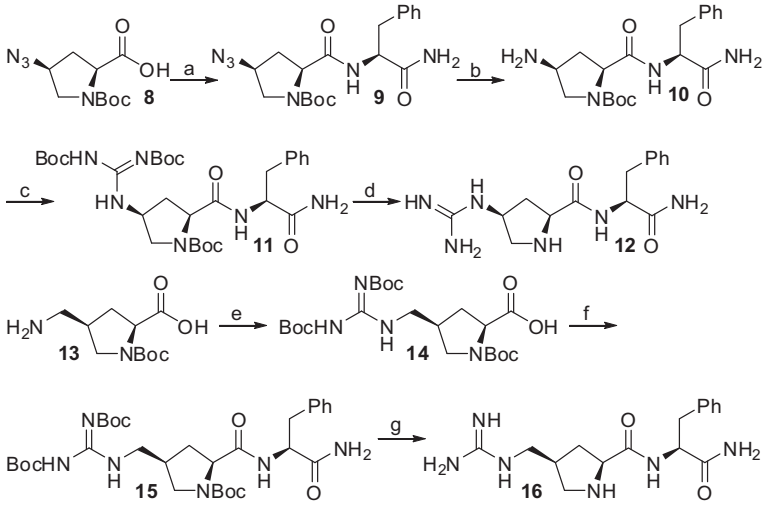
Preparation of Arg-Phe-NH_2 analogs **20** and **24** consisting of arginine mimics with a benzene ring backbone is illustrated in Scheme 3. *N*-Boc-DL-3-amino-phenylglycine **17**[17] was reacted with **3** to form **18**, which was subsequently coupled to L-phenylalanine amide to give **19**. Chromatographic purification of the crude material resulted in the separation of the two diastereomers **19a** and **19b**, which were deprotected with TFA to afford **20a** and **20b** (stereochemistry not assigned), respectively. A similar approach was taken for the synthesis of **24a** except that enantiomerically pure *N*-Boc-4-amino-phenylalanine **21a** (L-form) and **21b** (D-form) were used as starting materials to obtain **24a** and **24b**, respectively.

2.2. Biological evaluation

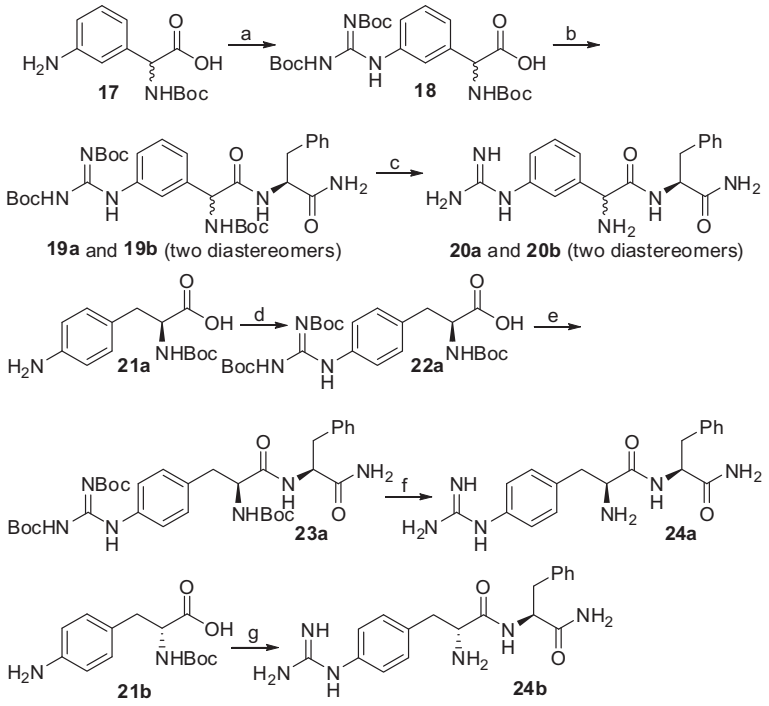
In vitro experiments measuring agonist activity against Mrg receptors were performed in a FLIPR assay using HEK293 cells stably transfected with human MrgX1. For rat MrgC and mouse MrgC11, transiently transfected HEK293 or KNRK cells were used.[11] As shown in Table 1, BAM8–22 exhibited agonist activity against Mrg receptors of all three species in our assays. Arg-Phe-NH_2 and adamantanecarbonyl-Arg-Phe-NH_2 were only active in rat MrgC and mouse MrgC11. In contrast, non-peptide agonist **1c** selectively activated human MrgX1. Compound **1c** had no measurable agonist activity at concentrations up to 2 μM (the highest concentration tested due to the limited solubility of **1c**). Our in vitro assay results clearly differentiate ligand specificity of human MrgX1 from rat and mouse Mrg receptors. These findings are in a good agreement with the cross-species variation known for Mrg receptors.[2,5]

Agonist activity of Arg-Phe-NH_2 derivatives containing Arg mimetics is summarized in Table 2. None of the peptidomimetics displayed agonist activity in human MrgX1. This is not surprising given the negligible agonist activity of Arg-Phe-NH_2 at human MrgX1. Several Arg-Phe-NH_2 peptidomimetics (compounds **12**, **16**, **20a**, and **20b**) failed to show agonist activity at rat MrgC while all of them showed full agonist activity (relative to BAM8–22) at mouse MrgC11 with varying degrees of potency. For instance, despite the lack of activity toward MrgC, compound **16** was the most potent MrgC11 agonist with submicromolar potency (pEC_{50} = 6.3). These findings highlight the significant species difference in ligand specificity that exists not only between human and rodents but also between rats and mice.

Compounds **7**, **24a**, and **24b** exhibited agonist activity at both MrgC and MrgC11. It is worth noting that 1-guanyl-4-piperidineglycine incorporated in **7** was also successfully used as an Arg mimetic in HLA-DR binding peptidomimetic ligands with improved plasma stability and slightly increased binding affinity.[18] Compound **24a** containing L-4-guanidinophenylalanine as an Arg mimetic showed weak yet appreciable agonist activity in both species. To our surprise, its diastereomer **24b** derived from D-4-guanidinophenylalanine was nearly 100- and 10-fold more potent than **24a** at rat MrgC and mouse MrgC11, respectively. In fact, compound **24b** represents the most potent rat MrgC agonist among the tested Arg-Phe-NH_2 peptidomimetics. This was unexpected



Scheme 2. Reagents and conditions: (a) L-phenylalanine amide·TFA, DIEA, HATU, DMF, rt, 98%; (b) H_2 (1 atm), 10% Pd/C, methanol, rt, 66%; (c) **3**, THF, rt, quant.; (d) TFA, dichloromethane, rt, 75%; (e) **3**, methanol, rt, 80%; (f) L-phenylalanine amide·TFA, DIEA, HATU, DMF, rt, 47%; (g) TFA, dichloromethane, rt, 79%.



Scheme 3. Reagents and conditions: (a) **3**, methanol, rt, 71%; (b) L-phenylalanine amide·TFA, DIEA, HATU, DMF, rt, 43% (**19a**), 45% (**19b**); (c) TFA, dichloromethane, rt, 94% for **20a**, 91% for **20b**; (d) **3**, methanol, rt, 97%; (e) L-phenylalanine amide·TFA, DIEA, HATU, DMF, rt, 94%; (f) TFA, dichloromethane, rt, 71%; (g) steps d–f, 29% for 3 steps.

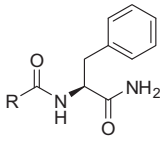
Table 1
Agonist activity of known Mrg receptor agonists[a]

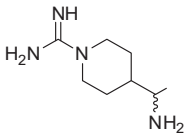
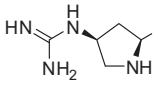
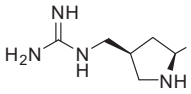
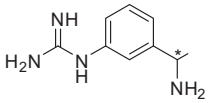
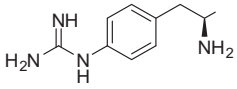
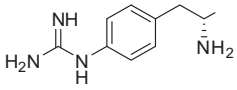
Compd	pEC_{50}[a]		
	Human MrgX1	Rat MrgC	Mouse MrgC11
BAM8–22	7.3 ± 0.1	6.5 ± 0.1	6.6 ± 0.1
Arg-Phe-NH_2	<4	5.2 ± 0.1	6.3 ± 0.4
1-Adamantanecarbonyl-Arg-Phe-NH_2	<4	5.2 ± 0.1	6.5 ± 0.2
1c	7.6 ± 0.2	<5.7	<5.7

[a] Negative logarithm of the concentration that produces half the maximal agonist response. Values are the average of at least three independent experiments ± SD.

as Phe-Met-Arg-Phe-NH_2 was reported to display submicromolar MrgC11 agonist activity while Phe-Met-D-Arg-Phe-NH_2 was found to be inactive toward MrgC11.[5,19] Moreover, an analog of cyclopentapeptide CXCR4 antagonist FC131, in which one of its two Arg residues were replaced by L-4-guanidinophenylalanine, was reported to be equipotent as a CXCR4 antagonist.[18] Three-dimensional structure predicted for the Phe-Met-Arg-Phe-NH_2–MrgC11 complex indicates that the Phe-NH_2 interacts favorably with Tyr110 (TM3) while the Arg makes salt bridges to Asp161 (TM4) and Asp179 (TM5).[19] Although speculative, it is conceivable that the peculiar constraint imposed by the 4-guanidinophenylalanine backbone resulted in a better retention of these key interactions by **24b** rather than **24a**. On the contrary, two diastereomers of

Table 2
Agonist activity of Arg-Phe-NH_2 derivatives containing arginine mimetics[a]



Compd	R	pEC_{50}[a]		
		Human MrgX1	Rat MrgC	Mouse MrgC11
7		<4	5.4 ± 0.2	6.1 ± 0.2
12		<4	<4	5.1 ± 0.2
16		<4	<4	6.3 ± 0.1
20a and **20b**		<4 <4	<4 <4	4.5 ± 0.1 4.5 ± 0.1
24a		<4	4.3 ± 0.3	4.8 ± 0.3
24b		<4	6.2 ± 0.3	5.9 ± 0.1

[a] Negative logarithm of the concentration that produces half the maximal agonist response. Values are the average of at least three independent experiments ± SD.

20 (**20a** and **20b**) containing 4-guanidinophenylglycine exhibited little difference in potency. Neither of the analogs showed agonist activity in MrgC, and both of them were equally weak agonists (pEC_{50} = 4.5) of MrgC11.

There is a possibility that Arg-Phe-NH_2 binds to the BAM8–22 binding site of human MrgX1 receptor without activating intracellular signaling and rather acts as an antagonist. To this end, we assessed antagonist activity of Arg-Phe-NH_2 and **24b** in human MrgX1 using BAM8–22 (200 nM) as an agonist and known MrgX1 antagonists **25**[20] and **26**[21] as positive controls (Fig. 2). In our assay, both **25** and **26** exhibited potent antagonist activity with IC_{50} values of 800 and 50 nM, respectively. Neither Arg-Phe-NH_2 nor **24b**, however, showed any antagonist activity at concentrations up to 200 μM. Thus, it appears that Arg-Phe-NH_2 motif alone is not sufficient to enable the binding to human MrgX1 as opposed to rodent Mrg receptors.

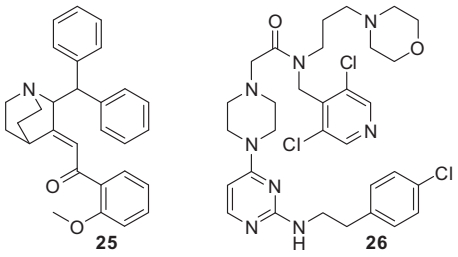


Figure 2. Known MrgX1 receptor antagonists.

3. Conclusions

Mrg receptors have gained increasing interest in connection with the development of novel therapeutic agents for pain and pruritus. While BAM8–22 has served as a valuable pharmacological tool in preclinical studies, development of smaller molecules should further facilitate understanding of the therapeutic utility of targeting Mrg receptors. The new peptidomimetics described in this paper have a preference for rodent Mrg receptors and should complement the existing human MrgX1 agonists such as compound **1c**. Indeed, it is encouraging that compound **24b** (termed JHU-58) attenuated neuropathic pain in both rats and mice but not in KO mice lacking a cluster of Mrg genes including MrgC11.[8] Collectively, these small molecule-based agonists should enhance our ability to assess the therapeutic utility of targeting Mrg receptors in rodent models.

4. Experimental

4.1. Chemistry

NMR spectra were recorded on a Bruker 400 instrument. Chemical shifts are reported in parts per million relative to TMS. Preparative HPLC was performed using a Jasco HPLC system equipped with a Jasco U-987 pump and a Jasco RI-2031 refractive index detector. The instrument was fitted with a Phenomenex Luna 10 μm Silica 100 Å (250 × 21.2 mm). An isocratic flow (10 mL/min) of 100% EtOAc was used unless otherwise specified. HPLC analyses were performed on a JASCO HPLC system fitted with a Phenomenex-Luna 5 μm C18 (250 × 4.6 mm) using a gradient of solvents A (0.1% TFA in water) and B (0.1% TFA in acetonitrile) at 1.0 mL/min flow rate. The gradient was 15% to 80% B over 40 min (detection at 210 nm). Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. BAM8–22 was purchased from Tocris Bioscience (Bristol, UK). Arg-Phe-NH_2 and 1-adamantanecarbonyl-Arg-Phe-NH_2 were purchased from Bachem Biosciences (King of Prussia, PA). Compounds **1c**,[11] **25**,[20] and **26**[21] were prepared according to the previously reported procedures.

4.1.1. 2-(1-(*N,N′*-Bis(*tert*-butoxycarbonyl)carbamimidoyl)piperidin-4-yl)-2-(*tert*-butoxycarbonylamino)acetic acid (5)

A milky mixture of 2-(*tert*-butoxycarbonylamino)-2-(piperidin-4-yl)acetic acid **4** (0.5 g, 1.94 mmol, 1 equiv) and *tert*-butyl(*1H*-pyrazol-1-yl)methanediylidenedicarbamate **3** (0.72 g, 2.32 mmol, 1.2 equiv) in methanol (12 mL) was stirred at rt over 2 days. As the reaction was not complete, excess methanol was added until the solution became colorless. Stirring continued for another day and the solvent was removed by rotary evaporation. The white residue was triturated in EtOAc and the resulting semi-solid **5** was used in the next step without further purification. ^{1}H NMR (CD_3OD) δ 1.46 (s, 27H), 1.4–2.07 (m, 5H), 2.95 (m, 2H), 3.80 (m, 2H), 4.02 (m, 1H).

4.1.2. 2-(1-(*N,N′*-Bis(*tert*-butoxycarbonyl)carbamimidoyl)piperidin-4-yl)-2-(*tert*-butoxycarbonylamino)acetyl-L-phenylglycine amide (6)

To a solution of **5** (from the above experiment) and phenylalanine amide·TFA salt (0.58 g, 2.09 mmol) in DMF (10 mL) diisopropylethylamine (1 mL, 5.70 mmol) and HATU (0.79 g, 2.08 mmol) were added at 0 °C. The bright yellow solution was stirred at 0 °C and gradually warmed to rt overnight. DMF was removed in vacuo and the residue was dissolved in EtOAc (50 mL). The organic solution was subsequently washed with 10% $KHSO_4$, satd $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated in vacuo. The resulting residue was purified by preparative HPLC followed by trituration with EtOAc to afford **6** (60 mg, 5% yield from **4**) as a white solid.

^{1}H NMR (CD_3OD) δ 0.63–0.80 (m, 2H), 1.12 (m, 1H), 1.42–1.50 (m, 27H), 1.58 (m, 2H), 2.59 (t, J = 12.1 Hz, 1H), 2.73 (t, J = 13.0 Hz, 2H), 3.42 (dd, J = 3.8, 13.9 Hz, 1H), 3.61 (m, 1H), 3.74 (br s, 1H), 3.98 (br s, 1H), 4.65 (dd, J = 3.8, 11.9 Hz, 1H), 7.22–7.29 (m, 5H).

4.1.3. (2-Amino-2-(1-carbamimidoylpiperidin-4-yl)acetyl-L-phenylglycine amide bis(trifluoroacetate) (7)

A solution of **6** (0.045 g, 0.07 mmol) in dichloromethane (1 mL) was treated with TFA (1 mL) for 1 h at rt. The solvents were removed and the excess of TFA was co-evaporated 3 times with dichloromethane. The residue was dried in vacuo, dissolved in water, and freeze-dried to give **7** (40 mg, 94%) as a white fluffy solid (bis-TFA salt). ^{1}H NMR (D_2O) δ 0.50–0.69 (m, 2H), 1.33 (d, J = 11.9 Hz, 2H), 1.79 (m, 1H), 2.78 (m, 3H), 3.34 (dd, J = 4.0, 14.4 Hz, 1H), 3.55 (t, J = 15.9 Hz, 2H), 3.75 (d, J = 4.6 Hz, 1H), 4.82 (dd, J = 4.0, 12.4 Hz, 1H), 7.28 (m, 5H); ^{13}C NMR (100 MHz, D_2O) δ 26.7, 27.9, 37.7, 38.1, 46.1, 46.3, 55.6, 57.9, 128.6, 130.0, 130.2, 137.9, 157.0, 169.2, 176.8. Anal. Calcd for $C_{17}H_{26}N_6O_2{\cdot}2TFA{\cdot}2\ H_2O$: C, 41.45; H, 4.97; N, 13.81. Found: C, 41.57; H, 5.08; N, 13.44. HPLC purity: 98%.

4.1.4. (2*S*,4*S*)-*tert*-Butyl 2-((*S*)-1-amino-1-oxo-3-phenylpropan-2-ylcarbamoyl)-4-azidopyrrolidine-1-carboxylate (9)

To a solution of **8** (0.30 g, 1.17 mmol, 1.0 equiv) and phenylalanine amide·TFA salt (0.39 g, 1.40 mmol, 1.2 equiv) in DMF (10 mL), diisopropylethylamine (0.82 mL, 4.64 mmol, 4.0 equiv) and HATU (0.54 g, 1.42 mmol, 1.2 equiv) were added at 0 °C. The bright yellow mixture was stirred at 0 °C and gradually warmed to rt overnight. DMF was removed in vacuo and the residue was dissolved in EtOAc (30 mL). The organic solution was subsequently washed with 10% $KHSO_4$, satd $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated in vacuo. The resulting residue was purified by preparative HPLC to afford **9** (0.46 g, 98% yield) as a white solid. ^{1}H NMR (CD_3OD) δ 1.34–1.43 (m, 9H), 1.99 (m, 2H), 2.46 (m, 1H), 2.96–3.17 (m, 2H), 3.64 (m, 1H), 4.42 (m, 2H), 4.65 (m, 1H), 7.27 (m, 5H).

4.1.5. (2*S*,4*S*)-*tert*-Butyl 4-amino-2-((*S*)-1-amino-1-oxo-3-phenylpropan-2-ylcarbamoyl)pyrrolidine-1-carboxylate (10)

A solution of **9** (0.42 g, 1.04 mmol) in methanol (10 mL) was hydrogenated overnight at balloon atmosphere in the presence of a catalytic amount of 10% Pd/C. The reaction was filtered through a pad of Celite and the filtrate was concentrated to afford **10** (0.26 g, 66%) as an off-white foam. ^{1}H NMR (CD_3OD) δ 1.28–1.44 (m, 9H), 1.56–1.68 (m, 1H), 2.0 (m, 1H), 2.32–2.44 (m, 1H), 2.80–3.20 (m, 2H), 3.42 (m, 1H), 3.66 (m, 1H), 4.10 (m, 1H), 4.57 (m, 1H), 7.28 (m, 5H).

4.1.6. (2*S*,4*S*)-*tert*-Butyl 2-((*S*)-1-amino-1-oxo-3-phenylpropan-2-ylcarbamoyl)-4-(2,3-bis(*tert*-butoxycarbonyl)guanidino) pyrrolidine-1-carboxylate (11)

A mixture of **10** (0.24 g, 0.64 mmol, 1.0 equiv) and **3** (0.30 g, 0.97 mmol, 1.5 equiv) in THF (7 mL) was stirred at rt over 3 days. The reaction mixture was concentrated and the resulting residue was filtered through a short pad of silica before being purified by preparative HPLC to afford **11** (^{1}H NMR yield: 97%) as colorless crystals containing 0.5 equiv (by NMR) of inseparable pyrazole. ^{1}H NMR ($CDCl_3$) δ 1.36–1.50 (m, 27H), 2.46 (m, 1H), 3.15 (m, 2H), 3.27 (m, 1H), 3.80 (m, 1H), 4.26 (m, 1H), 4.72 (m, 2H), 5.37 (m, 1H), 6.60 (br s, 1H), 6.82 (m, 1H), 7.21–7.31 (m, 6H), 8.60 (br s, 1H).

4.1.7. (2*S*,4*S*)-*N*-((*S*)-1-Amino-1-oxo-3-phenylpropan-2-yl)-4-guanidinopyrrolidine-2-carboxamide bis(trifluoroacetate) (12)

A solution of **11** (0.48 g, 0.78 mmol) in dichloromethane (5 mL) was treated with TFA (5 mL) for 2 h at rt. The solvents were removed and the excess of TFA was co-evaporated 3 times with dichloromethane. The residue was dried in vacuo, dissolved in water, and freeze-dried to give a fluffy solid foam which was purified by HPLC (isocratic 5% acetonitrile/water, 0.1% TFA) to give 0.22 g of **12** (containing 0.15 equiv of pyrazole) as a white fluffy solid (75% yield): ^{1}H NMR (D_2O) δ 2.16 (m, 1H), 2.73 (m, 1H), 2.98 (dd, J = 8.6, 13.9 Hz, 1H), 3.05 (dd, J = 6.8, 13.4 Hz, 1H), 3.45 (dd, J = 3.8, 12.6 Hz, 1H), 3.60 (dd, J = 6.3, 12.6 Hz, 1H), 4.34 (m, 1H), 4.41 (dd, J = 5.6, 8.8 Hz, 1H), 4.54 (t, J = 7.7 Hz, 1H), 7.23–7.30 (m, 5H). ^{13}C NMR (100 MHz, D2O) δ 35.6, 37.2, 50.4, 50.6, 55.6, 58.7, 127.6, 129.1, 129.5, 136.4, 156.5, 168.8, 175.5. Anal. Calcd for $C_{15}H_{22}N_6O_2{\cdot}2.5TFA{\cdot}1.2\ H_2O{\cdot}0.15$pyrazole: C, 38.51; H, 4.11; N, 13.22; F, 22.34. Found: C, 38.24; H, 3.93; N, 13.42; F, 22.08. HPLC purity: 93%.

4.1.8. (2*S*,4*R*)-4-((2,3-Bis(*tert*-butoxycarbonyl)guanidino)methyl)-1-(*tert*-butoxycarbonyl)pyrrolidine-2-carboxylic acid (14)

A mixture of **13** (0.45 g, 1.84 mmol, 1.0 equiv) and **3** (0.86 g, 2.77 mmol, 1.5 equiv) in methanol (8 mL) was stirred at rt over 5 days. The reaction mixture was concentrated and the resulting residue was purified by flash chromatography (eluent: hexanes/EtOAc/AcOH, 1:1:0.1%) to afford **14** (0.78 g, 80% ^{1}H NMR yield) as a white solid containing 1.3 equiv of inseparable pyrazole. ^{1}H NMR^{1}H NMR ($CDCl_3$) δ 1.40–1.50 (m, 27H), 1.96 (m, 1H), 2.39–2.53 (m, 2H), 3.15 (m, 1H), 3.33–3.48 (m, 2H), 3.69–3.80 (m, 1H), 4.26–4.36 (dt, J = 7.6, 38.9 Hz, 1H).

4.1.9. (2*S*,4*R*)-*tert*-Butyl 2-((*S*)-1-amino-1-oxo-3-phenylpropan-2-ylcarbamoyl)-4-((2,3-bis(*tert*-butoxycarbonyl)guanidino) methyl)pyrrolidine-1-carboxylate (15)

To a solution of **14** (0.33 g, 0.57 mmol, 1 equiv) and phenylalanine·TFA salt (0.19 g, 0.68 mmol, 1.2 equiv) in DMF (7 mL), diisopropylethylamine (0.40 mL, 2.27 mmol, 4.0 equiv) and HATU (0.26 g, 0.68 mmol, 1.2 equiv) were added at 0 °C. The bright yellow mixture was stirred at 0 °C and gradually warmed to rt over a period of 3 h. DMF was removed in vacuo and the residue was dissolved in EtOAc (20 mL). The organic solution was subsequently washed with 10% $KHSO_4$, satd $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated in vacuo. The resulting residue was purified by preparative HPLC to afford **15** (0.17 g, 47% yield) as a white solid. ^{1}H NMR (CD_3OD) δ 1.32–1.54 (m, 27H), 2.01 (m, 1H), 2.21–2.44 (m, 2H), 2.93–3.31 (m, 4H), 3.65 (m, 1H), 4.08 (m, 2H), 4.65 (m, 1H), 7.25 (m, 5H).

4.1.10. (2*S*,4*S*)-*N*-((*S*)-1-Amino-1-oxo-3-phenylpropan-2-yl)-4-(guanidinomethyl)pyrrolidine-2-carboxamide bis(trifluoroacetate) (16)

A solution of **15** (0.15 g, 0.24 mmol) in dichloromethane (4 mL) was treated with TFA (4 mL) for 2.5 h at rt. The solvents were removed and the excess of TFA was co-evaporated 3 times with dichloromethane. The residue was dried in vacuo, dissolved in water, and freeze-dried to give **16** (75 mg, 50%) as a fluffy solid foam (bis-TFA salt). ^{1}H NMR (D_2O) δ 1.68 (m, 1H), 2.57 (m, 1H), 2.70 (m, 1H), 3.00 (m, 1H), 3.08 (m, 2H), 3.21 (dd, J = 5.3, 7.1 Hz, 1H), 3.49 (dd, J = 7.6, 11.9 Hz, 1H), 4.32 (t, J = 8.3 Hz, 1H), 4.54 (d, J = 7.3 Hz, 1H), 4.54 (t, J = 7.7 Hz, 1H), 7.25 (m, 5H), ^{13}C NMR (100 MHz, D_2O) δ 33.3, 37.2, 37.8, 42.4, 48.8, 55.5, 59.7, 127.6, 129.1, 129.5, 136.5, 156.5, 169.1, 175.5. Anal. Calcd for $C_{16}H_{24}N_6O_2{\cdot}2.3TFA{\cdot}2\ H_2O$: C, 38.51; H, 4.11; N, 13.22; F, 22.34. Found: C, 38.24; H, 3.93; N, 13.42; F, 22.08. HPLC purity: >98%.

4.1.11. 2-(3-(2,3-Bis(*tert*-butoxycarbonyl)guanidino)phenyl)-2-(*tert*-butoxycarbonylamino)acetic acid (18)

A mixture of **17** (0.15 g, 0.56 mmol 1.0 equiv) and **3** (0.18 g, 0.58 mmol, 1.0 equiv) in methanol (5 mL) was stirred at rt for 1 day. The reaction mixture was concentrated and the resulting residue was subjected to purification by using a Biotage Isolera

One with the eluent hexanes/EtOAc (1:1 containing 1% AcOH) to give 0.21 g of **18** as a yellow foam (73%). ^{1}H NMR ($CDCl_3$) δ 1.42 (br s, 9H), 1.46 (br s, 9H), 1.50 (s, 9H), 5.20 (d, J = 7.1 Hz, 1H), 5.69 (d, J = 7.1 Hz, 1H), 7.12 (d, J = 7.3 Hz, 1H), 7.29 (m, 2H), 7.65 (d, J = 8.1 Hz, 1H), 11.24 (br s, 2H).

4.1.12. (2*S*)-2-(3-(2,3-Bis(*tert*-butoxycarbonyl)guanidino)phenyl)-2-(*tert*-butoxycarbonylamino)acetamido-3-phenylpropanamide bis(trifluoroacetate) (19a and 19b)

To a solution of **18** (0.10 g, 0.20 mmol, 1.0 equiv) and phenylalanine amide·TFA salt (0.066 g, 0.24 mmol, 1.2 equiv) in DMF (5 mL), diisopropylethylamine (0.14 mL, 0.80 mmol, 4.0 equiv) and HATU (0.090 g, 0.24 mmol, 1.2 equiv) were added at 0 °C. The bright yellow solution was stirred at 0 °C and gradually warmed to rt overnight. DMF was removed in vacuo and the residue was dissolved in EtOAc (30 mL). The organic solution was subsequently washed with 10% $KHSO_4$, satd $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated in vacuo. The resulting residue was purified by preparative HPLC to afford 55 mg (42%) of product **19a** as the less polar compound and 58 mg (44%) of product **19b** as the more polar compound. Compound **19a**: ^{1}H NMR ($CDCl_3$) δ 1.39–1.55 (m, 27H), 3.16 (d, J = 6.6 Hz, 2H), 4.72 (dd, J = 6.6, 14.4 Hz, 1H), 4.98 (d, J = 4.3 Hz, 1H), 5.35 (s, 1H), 5.57 (d, J = 4.6 Hz, 1H), 6.25 (s, 1H), 6.49 (d, J = 8.6 Hz, 1H), 6.92 (d, J = 7.6 Hz, 1H), 7.19–7.21 (m, 2H), 7.25–7.32 (m, 4H), 7.44 (m, 1H), 7.50 (m, 1H), 10.33 (s, 1H), 10.63 (s, 1H). Compound **19b**: ^{1}H NMR ($CDCl_3$) δ 1.40–1.55 (m, 27H), 3.03 (s, 2H), 4.67 (dd, J = 6.8, 14.7 Hz, 1H), 5.04 (d, J = 5.8 Hz, 1H), 5.49 (s, 1H), 5.73 (d, J = 6.1 Hz, 1H), 6.28 (s, 1H), 6.63 (d, J = 8.1 Hz, 1H), 6.97–7.03 (m, 3H), 7.17 (m, 3H), 7.29 (m, 1H), 7.43 (s, 1H), 7.64 (d, J = 7.8 Hz, 1H), 10.35 (s, 1H), 11.65 (s, 1H).

4.1.13. (2*S*)-2-(2-Amino-2-(3-guanidinophenyl)acetamido)-3-phenylpropanamide bis(trifluoroacetate (20a and 20b)

A solution of compound **19a** (0.050 g, 0.076 mmol) in dichloromethane (2 mL) was treated with TFA (2 mL) for 30 min at rt. The solvents were removed and the excess of TFA was co-evaporated 3 times with dichloromethane. The residue was dried in vacuo, dissolved in water, and freeze-dried to give product **20a** (0.045 g, 94%) as a white fluffy solid (bis-TFA salt). ^{1}H NMR (D_2O) δ 2.98 (dd, J = 3.3, 8.3 Hz, 2H), 4.53 (t, J = 7.6 Hz, 1H), 5.05 (s, 1H), 7.17 (d, J = 7.3 Hz, 2H), 7.27 (m, 4H), 7.37 (m, 2H), 7.52 (t, J = 7.8 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) 36.2, 54.9, 55.4, 124.7, 126.9, 127.2, 127.3, 128.4, 128.8, 131.0, 133.0, 135.1, 135.8, 156.1, 167.3, 174.6. Anal. Calcd for $C_{18}H_{22}N_6O_2$·2.3TFA·0.8 H_2O: C, 43.03; H, 4.09; N, 13.32. Found: C, 43.18; H, 4.18; N, 13.12. HPLC purity: >98%. Following the same experiment, compound **20b** (0.045 g, 91%) was obtained from **19b** as a bis-TFA salt; as a white fluffy solid. ^{1}H NMR (D_2O) δ 2.71 (dd, J = 10.9, 14.2 Hz, 1H), 3.14 (dd, J = 4.3, 14.2, Hz, 1H), 4.70 (dd, J = 4.6, 11.1 Hz, 1H), 5.02 (s, 1H), 6.90 (d, J = 14.9 Hz, 2H), 6.95 (s, 1H), 7.05–7.14 (m, 4H), 7.32 (m, 1H), 7.43 (t, J = 7.8 Hz, 1H); ^{13}C NMR (100 MHz, D_2O) 37.2, 54.6, 56.3, 123.7, 127.0, 127.2, 127.3, 128.8, 128.9, 131.6, 133.5, 135.7, 136.4, 156.1, 168.2, 175.8; Anal. Calcd for $C_{18}H_{22}N_6O_2$·2.5TFA·0.9 H_2O: C, 42.16; H, 3.97; N, 12.83. Found: C, 42.15; H, 4.03; N, 12.84. HPLC purity: >98%.

4.1.14. (*S*)-3-(4-(2,3-Bis(*tert*-butoxycarbonyl)guanidino)phenyl)-2-(*tert*-butoxycarbonylamino)propanoic acid (22a)

A mixture of **21a** (0.70 g, 2.50 mmol, 1.0 equiv) and **3** (0.64 g, 2.06 mmol, 0.83 equiv) in methanol (10 mL) was stirred at rt over weekend. The reaction mixture was concentrated and the residue was subjected to purification by Biotope Isolera One using EtOAc/hexanes (containing 2% AcOH) to give 1.04 g (97%) of product **22a**. ^{1}H NMR ($CDCl_3$) δ 1.44 (s, 9H), 1.50 (s, 9H), 1.53 (s, 9H), 3.06 (m, 2H), 4.58 (m, 1H), 4.99 (d, J = 7.6 Hz, 1H), 7.13 (d, J = 8.4 Hz, 2H), 7.50 (d, J = 8.6 Hz, 2H), 10.34 (br s, 1H).

4.1.15. (2*S*)-2-[(2*S*)-3-(4-(2,3-Bis(*tert*-butoxycarbonyl))guanidinophenyl)-2-(*tert*-butoxycarbonylamino)propanoyl]amino-3-phenylpropanamide (23a)

To a solution of **22a** (0.28 g, 0.54 mmol, 1.0 equiv) and phenylalanine amide·TFA salt (0.18 g, 0.63 mmol, 1.2 equiv) in DMF (10 mL), diisopropylethylamine (0.40 mL, 2.10 mmol, 4.0 equiv) and HATU (0.24 g, 0.63 mmol, 1.2 equiv) were added at 0 °C. The bright yellow solution was stirred at 0 °C and gradually warmed to rt overnight. DMF was removed in vacuo and the residue was dissolved in EtOAc (50 mL). The organic solution was subsequently washed with 10% $KHSO_4$, satd $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated in vacuo. The resulting residue was purified by Biotage Isolera One using EtOAc–hexanes to afford 0.33 g (92%) of product **23a** as a white solid cake. ^{1}H NMR (CD_3OD) δ 1.39 (s, 9H), 1.46 (br s, 9H), 1.65 (br s, 9H), 2.81 (m, 1H), 2.91 (m, 2H), 3.11 (m, 1H), 4.21 (m, 1H), 4.58 (m, 1H), 7.15 (m, 2H), 7.25 (m, 5H), 7.37 (m, 2H).

4.1.16. (2*S*)-2-Amino-*N*-((2*S*)-1-amino-1-oxo-3-phenylpropan-2-yl)-3-(4-guanidinophenyl)propanamide bis(trifluoroacetate) (24a)

A solution of compound **23a** (0.32 g, 0.48 mmol) in dichloromethane (6 mL) was treated with TFA (6 mL) for 4 h at rt. The solvents were removed and the excess of TFA was co-evaporated 3 times with dichloromethane. The residue was dried in vacuo, dissolved in water, and freeze-dried to give **24a** (0.25 g, 71%) as a white fluffy solid (bis-TFA salt). ^{1}H NMR (D_2O) δ 2.91 (m, 2H), 3.04 (dd, J = 8.8, 13.6 Hz, 1H), 3.18 (dd, J = 6.3, 13.9 Hz, 1H), 4.13 (dd, J = 6.6, 8.6 Hz, 1H), 4.51 (t, J = 7.6 Hz, 1H), 7.18 (m, 7H), 7.25 (m, 2H); ^{13}C NMR (100 MHz, D_2O) δ 36.3, 37.1, 53.9, 54.4, 126.3, 127.2, 128.7, 129.1, 130.8, 133.3, 133.8, 136.0, 156.2, 168.2, 174.1. Anal. Calcd for $C_{19}H_{24}N_6O_2$·2.65TFA·4.1 H_2O: C, 39.35; H, 4.38; N, 11.33. Found: C, 38.95; H, 3.98; N, 11.73. HPLC purity: >98%.

4.1.17. (*R*)-3-(4-(2,3-Bis(*tert*-butoxycarbonyl)guanidino)phenyl)-2-(*tert*-butoxycarbonylamino)propanoic acid (22b)

A mixture of **21b** (0.58 g, 2.07 mmol, 1.0 equiv) and **3** (0.53 g, 1.71 mmol, 0.83 equiv) in methanol (20 mL) was stirred at rt overnight. The reaction mixture was concentrated and the residue was subjected to purification by flash chromatography (eluent: 1:1:1% hexanes/EtOAc/AcOH) to give 0.64 g (72%) of product **22b.** ^{1}H NMR ($CDCl_3$) δ 1.46 (s, 9H), 1.51 (s, 9H), 1.54 (s, 9H), 3.13 (m, 2H), 4.56 (m, 1H), 4.94 (m, 1H), 7.14 (d, J = 8.6 Hz, 2H), 7.53 (d, J = 8.6 Hz, 2H), 10.34 (br s, 1H).

4.1.18. (2*S*)-2-[(2*R*)-3-(4-(2,3-Bis(*tert*-butoxycarbonyl))guanidinophenyl)-2-(*tert*-butoxycarbonylamino)propanoyl]amino-3-phenylpropanamide (23b)

To a solution of **22b** (0.64 g, 1.22 mmol, 1.0 equiv) and phenylalanine amide·TFA salt (0.41 g, 1.47 mmol, 1.2 equiv) in DMF (12 mL), diisopropylethylamine (0.85 mL, 4.90 mmol, 4.0 equiv) and HATU (0.56 g, 1.47 mmol, 1.2 equiv) were added at 0 °C. The bright yellow solution was stirred at 0 °C and gradually warmed to rt overnight. DMF was removed in vacuo and the residue was dissolved in EtOAc (50 mL). The organic solution was subsequently washed with 10% $KHSO_4$, satd $NaHCO_3$, brine, dried over Na_2SO_4, and concentrated in vacuo. The resulting residue was purified by Biotage Isolera One using EtOAc as eluent to afford 0.37 g (45%) of product **23b** as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.30 (s, 9H), 1.41 (s, 9H), 1.50 (s, 9H), 2.46 (m, 1H), 2.58 (dd, J = 3.3, 13.1 Hz, 1H), 2.75 (dd, J = 10.1, 13.4 Hz, 1H), 3.05 (dd, J = 4.0, 13.9 Hz, 1H), 4.10 (m, 1H), 4.44 (m, 1H), 6.80 (d, J = 8.1 Hz, 1H), 7.03 (d, J = 8.3 Hz, 2H), 7.17 (m, 2H), 7.25 (m, 2H), 7.36–7.41 (m, 3H), 8.30 (d, J = 8.8 Hz, 1H), 9.92 (s, 1H), 11.43 (s, 1H).

4.1.19. (2*R*)-2-Amino-*N*-((2*S*)-1-amino-1-oxo-3-phenylpropan-2-yl)-3-(4-guanidinophenyl)propanamide bis(trifluoroacetate) (24b)

A solution of compound **23b** (0.057 g, 0.085 mmol) in dichloromethane (3 mL) was treated with TFA (3 mL) for 30 min at rt. The solvents were removed and the excess of TFA was co-evaporated 3 times with dichloromethane. The residue was dried in vacuo, dissolved in water, and freeze-dried to give **24b** (0.051 g, 88%) as a white fluffy solid (bis-TFA salt). ^{1}H NMR (D_2O) δ 2.79 (dd, J = 10.1, 14.2 Hz, 1H), 2.91 (m, 2H), 3.09 (dd, J = 5.3, 14.2 Hz, 1H), 4.19 (t, J = 6.6 Hz, 1H),4.51 (dd, J = 5.3, 9.9 Hz, 1H), 6.85 (d, J = 8.3 Hz, 2H), 7.09 (d, J = 8.3 Hz, 2H), 7.22 (m, 3H), 7.31 (m, 2H); ^{13}C NMR (100 MHz, D_2O) 36.3, 37.4, 54.1, 55.3, 126.3, 127.7, 129.2, 129.4, 131.0, 133.1, 134.2, 136.7, 156.5, 169.3, 175.8. Anal. Calcd for $C_{19}H_{24}N_6O_2$·2.5TFA·1.6 H_2O: C, 42.28; H, 4.32; N, 12.33. Found: C, 42.09; H, 4.44; N, 12.23. HPLC purity: >98%.

4.2. In vitro Mrg receptor assays

HEK293 cells stably transfected with human MrgX1, HEK293 or KNRK cells transiently transfected with Mouse MrgC or Rat MrgC11 were plated in 96 well plates at 25,000 cell/well and incubated 2 days before imaging. On the day of imaging cells were incubated in 100 μL HBSS with 2 μM Fluo 4AM and 1% Trypan Red for 50 min at 37 °C. The cells were then equilibrated for 10 min at room temperature before imaging. Test compounds were dissolved in HBSS and diluted in a serial dilution. Test compounds, BAM8–22 (positive control) or HBSS (negative control) were added (50 μL into 100 μL) and cells were imaged on the FLIPR for 2 min. Data was exported as maximum–minimum fluorescent signal.

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