Incorporation of Indoylated Phenylalanine Yields a Sub-Micromolar Selective Melanocortin-4 Receptor Antagonist Tetrapeptide

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ABSTRACT: The melanocortin family is involved in many physiological functions, including pigmentation, steroidogenesis, and appetite. The centrally expressed melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R) possess overlapping but distinct roles in energy homeostasis. Herein, the third and fourth positions of a tetrapeptide lead compound [Ac-Arg-Arg-(pI)DPhe-Tic-NH$_2$], previously reported to possess MC3R agonist and MC4R antagonist activities, were substituted with indoylated phenylalanine (Wsf/Wrf) residues in an attempt to generate receptor subtype selective compounds. At the third position, D-amino acids were required for melanocortin agonist activity, while both L- and D-residues resulted in MC4R antagonist activity. These results indicate that L-indoylated phenylalanine residues at the third position of the scaffold can generate MC4R over MC3R selective antagonist ligands, resulting in a substitution pattern that may be exploited for novel MC4R ligands that can be used to probe the in vivo activity of the MC4R without involvement of the MC3R.

INTRODUCTION

The five known melanocortin receptors are involved in numerous biological pathways, including skin and hair pigmentation, steroidogenesis, and exocrine gland function in rodents. These receptors are stimulated by endogenous agonists derived from the proopiomelanocortin gene transcript and are inhibited by two naturally occurring agonists, agouti and agouti-related protein. The centrally expressed melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R, respectively) are both involved in energy homeostasis, with agonist ligands to these receptors suppressing appetite, while administration of antagonist compounds increases food intake. Due to the increased food consumption and body weight observed following insufficient MC4R signaling in rodents and humans, potent and selective MC4R compounds have been developed, including IMCIVREE, an MC4R agonist peptide approved to treat obesity resulting from select genetic signaling deficiencies upstream of the MC4R. The role of the MC3R is less clear. Whereas MC4R knockout (KO) mice were shown to be hyperphagic and possessed increased weight compared to littermate controls, MC3R KO mice possess similar body weight to littermate controls. Although of similar weight, MC3R KO mice possess higher fat mass and lower lean mass, compared to wildtype littermates, the MC3R also plays a role in appetite.

Administration of the MC3R/MC4R antagonist AGRP into wildtype (intact MC3R/MC4R), MC4R KO (intact MC3R), and MC3R KO (intact MC4R) mice resulted in a dose-dependent increase in food intake, with significant differences compared to saline injection out to at least 7 days post-administration. Thus, although much research focus has been on the MC4R, the MC3R remains a viable target for altering appetite and fat storage as a potential treatment strategy to assist in weight-loss treatments. As the MC3R has also been postulated to be involved in growth and onset of puberty, developing selective ligands for the melanocortin receptors will help to probe the contributions of the different receptor subtypes to a variety of physiological effects.

An unbiased method for identifying novel MC3R agonist ligands via a mixture-based positional scan observed that compared to the canonical melanocortin agonist tetrapeptide sequence Ac-His-DPhe-Arg-Trp-NH$_2$, inverting DPhe and Arg resulted in MC3R agonist ligands with MC4R antagonist pharmacology. Among the most potent of these tetrapeptide
agonist compounds (TACO) was Ac-Arg-Arg-(pL)DPhe-Tic-NH₂27, possessing 16 nM agonist potency at the MC3R and an antagonist pA₂ value of 7.8 (corresponding to an antagonist Kᵢ value of 16 nM) at the MC4R.27 Another group found that intracerebroventricular (icv) administration of this compound (5 μg) in male mice resulted in a statistically significant increase in food intake at 4, 6, and 24 h compared to the artificial cerebrospinal fluid vehicle control.25 Administration of this compound in MC3R KO mice resulted in no significant difference in food intake at 1, 2, 4, and 24 h compared to the vehicle.25 As MC3R KO mice have intact MC4R, these data may suggest that the MC3R agonism is responsible for the increase in food intake compared to the MC4R antagonism. However, administration of the melanocortin agonist ligands Ac-His-DPhe-Arg-Trp-NH₂ and MTII in MC4R KO mice (with intact MC3R) resulted in a decrease in food intake, implying that administration of MC3R agonists decreases food intake. As compensatory mechanisms for feeding develop in mice, the development of selective ligands that modulate one receptor member within a closely related family such as the melanocortin receptors would help elucidate the contributions of the individual receptors.

One way to improve the pharmacological activity and/or selectivity of a peptide is to restrict the rotational flexibility of the amino acid side chains.30 Exploring χ-space may indicate the orientation of a sidechain required for receptor interaction. While this can be done by adding methyl groups to the β-carbon of amino acid side chains, recent advancements have installed indole moieties on the side chain of aromatic phenyl groups, which both restricts the rotational flexibility of both aromatic groups and adds steric bulk that may generate new interactions with the receptors.33–35 The Fmoc-protected versions of these amino acids (Fmoc-Wsf-OH, Fmoc-Wrf-OH, Fmoc-wsf-OH, and Fmoc-wrf-OH) were obtained on a gram scale, according to published protocols.33 For these amino acids, the first letter denotes L- or D-tryptophan (W or w, respectively), the second denotes either R- or S-stereochemistry of Cβ, and the third letter denotes phenylalanine, as previously published.33,35 The lead TACO compound, previously identified to have pharmacologically relevant in vivo results, has two phenyl-derived groups [(pL)DPhe and Tic]. Due to the different interactions of this compound with the MC3R (agonist) and MC4R (antagonist), it was hypothesized that introduction of rotationally restricted Phe derivatives at the (pL)DPhe and Tic positions may lead to ligands with enhanced selectivity for one receptor over the other due to the side-chain orientation and added bulk of the indole group.

Herein, eight peptides derived from the TACO scaffold [Ac-Arg-Arg-(pL)DPhe-Tic-NH₂] were synthesized, individually substituting indoylated Phe amino acids (Figure 1 and Table 1) at the third and fourth positions. Compounds were synthesized with standard Fmoc chemistries,30,35 purified to greater than 95% purity, and characterized by mass spectral analysis (Table 1, University of Minnesota’s Mass Spectrometry Lab). Purified peptides were pharmacologically characterized at the mouse (m)MC1R, mMC3R, mMC4R, and mMC5R, utilizing the commercially available AlphaScreen cAMP assay. Since the MC2R is only stimulated by ACTH,38 it was excluded from this study. Ligands that did not stimulate the receptors to at least 20% of the maximal NDP-MSH signal were considered inactive. Compounds that did not stimulate the receptor to greater than 50% activity of NDP-MSH were then assayed as antagonists, utilizing NDP-MSH as the agonist in a Schild antagonist paradigm.39 Since the AlphaScreen kit is a loss-of-signal assay, the pharmacology curves were normalized for illustrative purposes.

### RESULTS AND DISCUSSION

The parent compound in this series (1) was a full agonist at the mMC1R, mMC3R, and mMC5R (EC₅₀ = 0.18, 10, and 7 nM, respectively; Table 2 and Figure 2) and was a nanomolar potent mMC4R antagonist (pA₂ = 8.1). These values are similar to the original pharmacological characterization values for this peptide, where agonist EC₅₀ values of 0.51, 16, and 8.8 nM were reported for the mMC1R, mMC3R, and mMC5R, respectively.27 This compound was also reported to be an mMC4R antagonist, with a pA₂ value 7.8,21 similar to the 8.1 value reported herein.

Ligands possessing an indoylated L-phenylalanine stereochemistry at the fourth position were partial (2) or full (3, Figure 2) agonists at the mMC1R, mMC3R, and mMC5R and were micromolar to sub-micromolar potent antagonists at the mMC4R. Compound 2, with 50% efficacy at the mMC3R, was also a sub-micromolar potent antagonist at this receptor (pA₂ = 6.2). Tetrapeptides with an indoylated D-phenylalanine substitution at the fourth position (4 and 5) possessed functional activities similar to the L derivatives, with full to partial agonist activity at the mMC1R and mMC3R. While compound 4 was a full agonist at the mMC5R, tetrapeptide 5 partially activated the receptor at the highest concentration assayed (60% maximal NDP-MSH signal at 100 μM concentrations, Figure 2). Both 4 and 5 also possessed micromolar mMC4R antagonist potency (pA₂ = 5.5 and 5.2, respectively). Previous studies examining similar tetrapeptides reported similar pharmacological activities at the mMC1R,
mMC3R, mMC4R, and mMC5R when the aminos Tic or DTic were substituted at the fourth position. Another study showed that substitution of the α-amino acids NaI(2’) or DNal(2’) at the fourth position of the Ac-Arg-Arg-(pI)DPhe-XXX scaffold resulted in similar agonist potencies at the mMC1R and mMC5R and similar antagonist potencies at the mMC4R, and both could interact with MC3R, although a DNal(2’) substitution resulted in agonist activity and NaI(2’) substitution possessed antagonist activity. The results support the present study that indicates that the fourth position within the scaffold can tolerate both L and D isomers, although the decreased potencies without corresponding gains in selectivity suggest that additional stereochemical bulk was not beneficial in ligand design.

Compared to the fourth position, the stereochemistry of the third substitution was critical for agonist activity. Tetrapeptide 6, possessing the indolyated L-phenylalanine Wsf, possessed minimal partial agonism at the mMC1R (20% maximal NDP-MSH signal), did not stimulate the mMC3R, mMC4R, and mMC5R at concentrations up to 100 μM, and was a micromolar potent mMC1R and mMC4R antagonist (pA2 = 5.9 and 5.2, respectively). The other indolyated L-phenylalanine derivative at the third position (7) did not stimulate the melanocortin receptors assayed at the concentrations assayed (Figure 2), possessed micromolar to sub-micromolar antagonist potencies at the mMC1R (pA2 = 5.4 and mMC4R (pA2 = 6.1), and did not antagonize the mMC3R and mMC5R (Figure 3). Compounds substituted with indolyated L-phenylalanine at the third position (8 and 9) possessed partial agonist activity at the mMC1R, mMC3R, and mMC5R (with compound 8 possessing full agonist efficacy at the mMC5R), and both were micromolar potent antagonists at the mMC4R (pA2 = 5.7 and 5.5 for 8 and 9, respectively). The stereochemistry at this position within the scaffold has not previously been explored, with prior reports always utilizing a D-amino acid. The original mixture-based positional scan identified (pCl)DPhe and (pI)DPhe as the substitutions at the third position with the highest percentage activity in the initial screening (20 and 26%, respectively) for mMC3R activity, with minimal MC3R activity reported for the (pCl)Phe and (pI)Phe L-isomers (2 and −1%, respectively). The data herein supports the mixture-based positional scan results of the importance of D-stereochemistry at the third position for melanocortin agonism within this scaffold.

Although D-stereochemistry is critical for agonism, both L- and D-isomers are tolerated at the third position for MC4R antagonism. Compound 7, containing the Wsf substitution at the third position, possessed sub-micromolar potent mMC4R antagonism (pA2 = 6.1) and was inactive at the mMC3R, at the assayed agonist and antagonist concentrations, resulting in at least a 10-fold mMC4R over mMC3R selective ligand. Compared to the lead compound 1, 7 was 100-fold less potent as an mMC4R antagonist and more than 10,000-fold less potent as an agonist at the mMC3R, suggesting that this substitution may be useful in the development of MC4R antagonists from this scaffold that lacks MC3R activity. Increased MC4R antagonist potency may be gained by additional structure–activity studies, potentially resulting in useful in vivo probe compounds.

While the indolyated phenylalanine amino acids were incorporated to fix the axis of rotation of the phenylalanine side-chain, the incorporation of the indoyl group also added additional stereochemical bulk. Further studies are needed to explore if the resulting potency decreases were the result of the fixed side-chain rotation or the incorporation of the bulky indole group. Incorporating amino acids with methyl groups added to the β-carbon of Phe and Trp could be used to address this question.

### CONCLUSIONS

In conclusion, L- and D-indolyated phenylalanine amino acids were substituted at the third and fourth positions of a previously identified tetrapeptide scaffold [Ac-Arg-Arg-(pI)DPhe-Tic-NH2] possessing mMC3R agonism and mMC4R antagonism in an attempt to generate ligands with receptor sub-type selectivity. In agreement with prior reports, both L- and D-amino acids were tolerated at the fourth position, albeit with decreased potency relative to the parent ligand. At the third position, D-amino acids were required for agonist activity at the mMC1R, mMC3R, and mMC5R. In contrast, both L- and D-indolyated phenylalanine resulted in mMC4R antagonism, indicating a potential motif that can generate mMC4R antagonist ligands within the parent scaffold. Such MC4R-selective ligands may be useful as in vivo probe ligands to investigate the physiological functions of the different melanocortin receptors.

### METHODS

**Peptide Synthesis.** Peptides were synthesized by standard Fmoc chemistry. Unless otherwise specified, amino acids, 4-(2′,4′-dimethoxyphenyl)-Fmoc-aminoethyl) phenoxyacetyl MBHA (Rink Amide MBHA) resin, and coupling reagent 2-

### Table 1. Analytical Characterization Data for the Tetrapeptides Synthesized in This Study

| Peptide | Compound ID | Sequence | k’ (MeCN) | k’ (MeOH) | M (Calc) | M + H (Obs) | Purity |
|---------|-------------|----------|-----------|-----------|----------|-------------|--------|
| DMD-40 | Ac-Arg-Arg-(pI)DPhe-Tic-NH2 | 7.0 | 11.4 | 803.3 | 804.3 | >98% |
| COR7-11 | Ac-Arg-Arg-(pI)DPhe-Wsf-NH2 | 7.7 | 11.4 | 906.3 | 907.7 | >97% |
| COR7-25 | Ac-Arg-Arg-(pI)DPhe-Wrf-NH2 | 8.5 | 12.4 | 906.3 | 907.7 | >98% |
| COR7-18 | Ac-Arg-Arg-(pI)DPhe-wsf-NH2 | 6.9 | 11.1 | 906.3 | 907.7 | >96% |
| COR7-32 | Ac-Arg-Arg-(pI)DPhe-wrf-NH2 | 6.9 | 11.3 | 906.3 | 907.7 | >96% |
| COR7-39 | Ac-Arg-Arg-Wsf-Tic-NH2 | 6.5 | 10.3 | 792.4 | 793.5 | >95% |
| COR7-119 | Ac-Arg-Arg-Wsf-Tic-NH2 | 6.7 | 10.6 | 792.4 | 793.5 | >96% |
| COR7-126 | Ac-Arg-Arg-wsf-Tic-NH2 | 7.0 | 10.6 | 792.4 | 793.5 | >96% |
| COR7-133 | Ac-Arg-Arg-wrf-Tic-NH2 | 7.9 | 12.7 | 792.4 | 793.5 | >95% |

“HPLC k’ = [(peptide retention time—solvent retention time)/solvent retention time] in MeCN (10% acetonitrile in 0.1% trifluoroacetic acid and a gradient to 90% acetonitrile over 35 min) or MeOH (10% methanol in 0.1% trifluoroacetic acid/water and a gradient to 90% methanol over 35 min). An analytical Vydac C18 column (Vydac 218TP104) was used with a flow rate of 1.5 mL/min. The peptide purity was determined by HPLC at a wavelength of 214 nm.”

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Table 2. Tetrapeptide Pharmacology at the Mouse Melanocortin Receptors

| Peptide       | Compound ID | Sequence                  | mMC1R  | pA2     | mMC3R  | pA2     | mMC4R  | pA2     | mMC5R  | pA2     |
|---------------|-------------|---------------------------|--------|---------|--------|---------|--------|---------|--------|---------|
| NDP-MSH       |             |                           | EC₅₀ (nM) | pA₂     | EC₅₀ (nM) | pA₂     | EC₅₀ (nM) | pA₂     | EC₅₀ (nM) | pA₂     |
| 1             | MDE7-40     | Ac-Arg-Arg-(pI)DPhe-Tic-NH₂ | 0.013 ± 0.002 | 0.16 ± 0.02 | 0.68 ± 0.06 | 0.26 ± 0.06 |
| 2             | COR7-11     | Ac-Arg-Arg-(pI)DPhe-Wsf-NH₂ | 0.18 ± 0.04 | 10 ± 2 | 25% @ 100 μM | 8.1 ± 0.1 | 7 ± 1 |
| 3             | COR7-25     | Ac-Arg-Arg-(pI)DPhe-Wrf-NH₂ | 380 ± 90 | partial agonist 260 ± 50 80% NDP-MSH | partial agonist 2400 ± 500 50% NDP-MSH | 6.2 ± 0.1 |
| 4             | COR7-18     | Ac-Arg-Arg-(pI)DPhe-wsf-NH₂ | 210 ± 40 | 570 ± 80 | partial agonist 900 ± 100 75% NDP-MSH | 40% @ 100 μM | 6.0 ± 0.1 | 240 ± 60 |
| 5             | COR7-32     | Ac-Arg-Arg-(pI)DPhe-wrf-NH₂ | partial agonist 1400 ± 200 65% NDP-MSH | partial agonist 5700 ± 200 70% NDP-MSH | >100,000 | 5.2 ± 0.2 | 60% @ 100 μM |
| 6             | COR7-39     | Ac-Arg-Arg-Wsf-Tic-NH₂ | partial agonist 6700 ± 1500 20% NDP-MSH | 5.9 ± 0.2 | >100,000 | <5.0 | >100,000 | 5.2 ± 0.1 | >100,000 | <5.0 |
| 7             | COR7-119    | Ac-Arg-Arg-Wrf-Tic-NH₂ | >100,000 | 5.4 ± 0.1 | >100,000 | <5.0 | >100,000 | 6.1 ± 0.1 | >100,000 | <5.0 |
| 8             | COR7-126    | Ac-Arg-Arg-wsf-Tic-NH₂ | partial agonist 900 ± 200 60% NDP-MSH | partial agonist 800 ± 100 80% NDP-MSH | >100,000 | 5.7 ± 0.2 | 350 ± 40 |
| 9             | COR7-133    | Ac-Arg-Arg-wrf-Tic-NH₂ | partial agonist 210 ± 30 80% NDP-MSH | partial agonist 2700 ± 500 60% NDP-MSH | 30% @ 100 μM | 5.5 ± 0.1 | partial agonist 3200 ± 400 55% NDP-MSH |

The indicated errors represent the standard error of the mean determined from at least three independent experiments. The percentage denotes the percentage maximal stimulatory response (compared to NDP-MSH) observed at 100 μM, but not enough stimulation was observed to determine an EC₅₀ value. The use of >100,000 indicates that the compound was examined but lacked agonist activity at concentrations up to 100 μM in at least two independent experiments. Antagonist pA₂ values were determined using a Schild analysis and the agonist NDP-MSH. The use of <5.0 indicates that no antagonist potency was observed in the highest concentration range assayed (10,000, 5,000, 1,000, and 500 nM). Tetrapeptides-labeled partial agonists were observed to possess partial agonist activity (an observable sigmoidal dose–response curve that plateaus at an efficacy below that of the NDP-MSH standard), with the apparent EC₅₀ values and percentage of receptor activation relative to NDP-MSH.
(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were purchased from Peptides International (Louisville, KY). The indoylated amino acids were synthesized as previously described.\textsuperscript{33,35} The Fmoc-Tic-OH amino acid was purchased from Synthetech (Albany, OR), and the Fmoc-(pI)DPhe-OH residue was purchased from Alfa Aesar (Tewksbury, MA). Dichloromethane (DCM), methanol (MeOH), acetonitrile, dimethylformamide (DMF), and anhydrous ethyl ether were obtained from Fisher (Fair Lawn, NJ). Trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), triisopropylsilane (TIS), N,N-diisopropylethylamine (DIEA), and 1-[bis(dimethylamino)methylene]1H-1,2,3-triazol[4,5-b]pyridinium 3-oxide hexafluorophosphate piperidine (HATU) were purchased from Sigma-Aldrich (St. Louis, MO). All reagents and chemicals were ACS grade or better and were used without further purification.

The syntheses of Fmoc-Wsf-OH and Fmoc-Wrf-OH have previously been described.\textsuperscript{33} Fmoc-wrf-OH was obtained as described in Scheme 4 in ref 33. Fmoc-wsf-OH was obtained as described in Scheme 5 in ref 33.

\((2R,3R)-2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-(1-(tert-butoxycarbonyl)-1H-indol-3-yl)-3-phenylpropanoic Acid (Fmoc-wrf-OH).\) \textsuperscript{1}H-NMR (250 MHz, DMSO-\(d_6\)) : \(\delta\) 12.52 (s, 1H), 8.14 (d, \(J = 8.6\) Hz, 1H), 7.97 (d, \(J = 7.8\) Hz, 1H), 7.85 (dd, \(J = 7.2, 2.9\) Hz, 2H), 7.75 (s, 1H), 7.56 (d, \(J = 7.2\) Hz, 1H), 7.47–7.13 (m, 13H), 7.04 (t, \(J = 7.5\) Hz, 1H), 4.89 (t, \(J = 9.9\) Hz, 1H), 4.67 (d, \(J = 10.5\) Hz, 1H), 4.48–4.34 (m, 1H), 4.23 (t, \(J = 6.9\) Hz, 1H), 1.44 (s, 9H) ppm.

\((2R,3S)-2-(((9H-Fluoren-9-yl)methoxy)carbonyl)amino)-3-(1-(tert-butoxycarbonyl)-1H-indol-3-yl)-3-phenylpropanoic Acid (Fmoc-wsf-OH).\) \textsuperscript{1}H-NMR (250

Figure 2. Illustration of the agonist pharmacology of NDP-MSH, 1, 3, 5, and 7 at the mMC1R, mMC3R, mMC4R, and mMC5R.

Figure 3. Illustration of the antagonist pharmacology of 7 at the mMC1R, mMC3R, mMC4R, and mMC5R.
MHz, DMSO-d$_6$): δ 12.69 (s, 1H), 7.99 (d, J = 8.2 Hz, 1H), 7.87 (d, J = 7.1 Hz, 3H), 7.79 (s, 1H), 7.57 (d, J = 7.6 Hz, 2H), 7.48–7.08 (m, 11H), 4.91 (t, J = 9.7, 1H), 4.65 (d, J = 10.4 Hz, 1H), 4.29–4.01 (m, 3H), 1.63 (s, 9H) ppm.

Peptides were synthesized on a Rink Amide MBHA resin (0.51 meq/g) with a manual microwave synthesizer (CEM Discover SPS). The syntheses consisted of two repeated steps: (i) removal of the resin-bound Fmoc group by a solution of 20% piperidine in DMF (1 × 2 min, rt and 1 × 4 min, 75 °C, 30 W for microwave) and (ii) coupling of the desired Fmoc-protected amino acid (3.1 equiv) using HBTU (3 equiv) and DIEA (5.1 equiv) in DMF (1 × 5 min, 75 °C, 30 W for microwave). A longer irradiation time (10 min) with increased eq of Arg (5.1), HBTU (5), and DIEA (7.1) was utilized for Arg coupling. Coupling of indoylated amino acids was performed with lower eq of the amino acid (2.1 equiv) and the coupling reagent HATU (2.0 equiv) for longer periods (1 h) at room temperature. Deprotection and coupling reactions were monitored by the ninhydrin and chloranil colorometric assays$^{44,44}$ and repeated if necessary. Following the removal of the terminal Fmoc group, the N-terminal amine was capped using a 3:1 acetic anhydride/pyridine solution at room temperature for 30 min. Tetrapeptides were side-chain deprotected and cleaved from resin with a solution of 91:3:3:3 (TFA/TIPS/H$_2$O/thioanisole) for 2 h. The peptides were precipitated with ice-cold ethyl ether.

Crude peptides were purified using a Shimadzu RP-HPLC system with a UV detector and a semi-preparative RP-HPLC C18-bonded silica column (Vydac 218TP1010, 1.0 × 25 cm). Analytical RP-HPLC using a Shimadzu system equipped with a photodiode array detector and C18 silica column (Vydac 218TP104, 0.46 × 25 cm) in two unique solvent systems (acetonitrile and methanol) was used to assess peptide purity (≥95% pure). The correct average molecular mass was verified by ESI-TOF MS (Bruker BioTOF II, University of Minnesota Mass Spectrometry Lab).

cAMP AlphaScreen Bioassay. Peptide ligands were dissolved in DMSO at a stock concentration of 10$^{-2}$ M and were characterized pharmacologically using HEK293 cells stably expressing the mouse MC1R, MC3R, MC4R, and MC5R by the cAMP AlphaScreen assay (PerkinElmer), according to the manufacturer’s instructions and as previously described.$^{46,48}$

Briefly, cells 70–90% confluent were dislodged with Versene (Gibco) at 37 °C and plated 10,000 cells/well in a 384-well plate (Optiplate) with 10 μL freshly prepared stimulation buffer (1X HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH = 7.4) with 0.5 μg anti-cAMP acceptor beads per well. The cells were stimulated with the addition of 5 μL stimulation buffer containing peptide (a seven-point dose–response curve was used, starting at 10$^{-4}$ to 10$^{-7}$ M, determined by ligand potency) or forskolin (10$^{-4}$ M) and incubated in the dark at room temperature for 2 h.

Following stimulation, streptavidin donor beads (0.5 μg) and biotinylated-cAMP (0.62 μmol) were added to the wells in a subdued light environment with 10 μL lysis buffer (5 mM HEPES, 0.3% Tween-20, 0.1% BSA, pH = 7.4), and the plates were incubated in the dark at room temperature for an additional 2 h. Plates were read on an Enspire (PerkinElmer) Alpha-plate reader using a pre-normalized assay protocol (set by the manufacturer).

Data Analysis. The EC$_{50}$ values represent the mean of duplicate replicates obtained in three independent experiments. The EC$_{50}$ estimates and the associated standard errors (SEM) were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (version 4.0, GraphPad Inc.). The peptides were assayed as TFA salts and not corrected for the peptide content.

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### Notes

The authors declare no competing financial interest.

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