Published in final edited form as:

J Med Chem. 2019 March 14; 62(5): 2738–2749. doi:10.1021/acs.jmedchem.9b00053.

Discovery of Polypharmacological Melanocortin-3 and –4 Receptor Probes and Identification of a 100-Fold Selective nM MC3R Agonist versus a μ M MC4R Partial Agonist

Katlyn A. Fleming¹, Katie T. Freeman¹, Mike D. Powers¹, Radleigh G. Santos², Ginamarie Debevec², Marc A. Giulianotti², Richard A. Houghten², Skye R. Doering¹, Clemencia Pinilla^{**}, and Carrie Haskell-Luevano^{*,1}

¹University of Minnesota, Department of Medicinal Chemistry, Minneapolis, Minnesota 55455, United States

²Torrey Pines Institute for Molecular Studies, Port St. Lucie, Florida 34987, United States

Abstract

The centrally expressed melanocortin-3 and melanocortin-4 receptors (MC3R, MC4R) are established targets to treat diseases of positive and negative energy homeostasis. We previously reported (*J. Med. Chem.* 60:4342 2017) mixture-based positional scanning approaches to identify dual MC3R agonist and MC4R antagonist tetrapeptides. Herein, 46 tetrapeptides were chosen for MC3R agonist screening selectivity profiles, synthesized, and pharmacologically characterized at the mouse melanocortin-1, –3, –4, and –5 receptors. Substitutions to the tetrapeptide template were selected solely based on MC3R agonist potency from the mixture-based screen. This study resulted in the discovery of compound **42** (Ac-Val-Gln-(pI)DPhe-DTic-NH₂), a full MC3R agonist that is 100-fold selective for the MC3R over the μ M MC4R partial agonist pharmacology. This compound represents a first-in-class MC3R-selective agonist. This ligand will serve as a useful *in vivo* molecular probe for the investigation of the roles of the MC3R and MC4R in diseases of dysregulated energy homeostasis.

Graphical Abstract

Corresponding Author.** chaskell@umn.edu; Phone: 612-626-9262; Fax: 612-626-3114; Street Address: Department of Medicinal Chemistry, University of Minnesota, 308 Harvard Street SE, Minneapolis, MN, 55455, USA. *To whom correspondence about the use of positional scanning libraries** should be addressed at pinilla@tpims.org at Torrey Pines Institute for Molecular Studies. **Author Contributions.** K.A.F. and C.H.-L. designed the research. K.A.F., K.T.F., and M.D.P. performed experiments. K.A.F., R.G.S., C.P., and C.H.-L. analyzed the data. K.A.F. wrote the manuscript with the help of C.H.-L., R.G.S., and C.P.

Conflict of Interest. The authors declare no competing financial interests.

Keywords

structure-activity relationships; melanocortin receptors; mixture-based positional scanning; melanocortin-3 receptor; melanocortin-1 receptor

Introduction

There are five reported melanocortin G protein-coupled receptors (GPCRs, MC1–5R).^{1–8} Of the melanocortin receptors, the MC3R^{9–12} and MC4R^{13–15} expressed in the central nervous system (CNS) have been shown to regulate food intake and the maintenance of energy homeostasis, and have been investigated as promising targets for anti-obesity drugs.^{9–11, 13–14} These melanocortin receptors signal through stimulatory G_{αs} protein subunits and induce β-Arrestin recruitment.^{16–18} Agonist stimulation of the MCRs increases intracellular cyclic AMP (cAMP) levels. Posttranslational processing of the proopiomelanocortin (POMC) gene transcript results in α-, β-, and γ-melanocyte stimulating hormones (MSHs), endogenous agonists for the MC3R and MC4R, which have been implicated in the maintenance of energy homeostasis.^{19–23} There are two endogenous antagonists for these receptors: agouti signaling protein (ASP) and agouti-related protein (AGRP), and administration of MC3R and MC4R antagonists to the brain results in an increase in food intake.^{11, 13, 24–29}

Disruption of the MC4R has been shown to result in obesity in mice and humans, with both mice and humans displaying a hyperphagic phenotype.^{14–15} The role of the MC3R is more enigmatic. The MC3R has been linked with multiple different functions; it may play an autoregulatory role in the melanocortin signaling, and has also been linked with feeding-related behaviors such as food anticipatory activity and gorging, as well as food motivational responses in mice.^{9, 11, 30–33} Melanocortin receptor agonists have been pursued for the treatment of diseases of positive energy balance (such as obesity), while melanocortin receptor antagonists have been investigated as potential therapeutics for the treatment of disease of negative energy balance (such as anorexia, cachexia, and failure to thrive).^{29–30, 32–34}

One limitation to targeting both the MC3R and MC4R is that these two receptors have different, non-redundant roles in the maintenance of energy homeostasis.³⁴ Selective ligands for the MC3R may be used as probes to clarify the biological role(s) of this receptor. In rodents, intracerebroventricular injection of the dual MC3R/MC4R melanocortin antagonist AGRP increase food intake and alleviates the effects of self-starvation.^{11, 29, 35–36}

Antagonist ligands at the MC4R have been proposed as treatments for diseases of negative energy balance like eating disorders, cachexia, and failure to thrive in children.^{35–39} If the fasting-related behavioral phenotypes exhibited by mice in regards to the MC3R (including gorging, increased motivation for food reward) are also relevant in humans, it is possible that compounds possessing MC3R agonist activity and MC4R antagonist activity could be used in order to increase food intake via the MC4R, without causing potentially harmful side effects like gorging or food motivational responses in patients with eating disorders via the MC3R. Additionally, MC3R-selective probes will be extremely useful in the *in vivo*

clarification of MC3R function; to date, the precise mechanisms of action of this receptor remain elusive.

There have been numerous ligands developed for the study of the melanocortin system (reviewed by Ericson *et al*),⁴⁰ none of which to date possess potent activity at the MC3R and no activity at the MC4R. There have been some ligands discovered that possess some selectivity for the MC3R or MC4R over the other receptor.^{41–52} These studies have primarily centered on rational design principles based on endogenous melanocortin ligands or through the derivatization of small molecule scaffolds.^{41–45} Our laboratories have utilized data derived from unbiased screening approaches like mixture-based positional scanning libraries to develop melanocortin ligands.^{53–54} In this technique, peptides with a single fixed amino acid in one position and equimolar mixtures of amino acids in all other positions are assayed. Mixtures possessing activity will do so both because of structural redundancies among multiple peptides in the mixtures, and because a mixture's activity is the harmonic mean of that of its constituents.⁵⁵ One study performed a mixture-based positional screen with a tetrapeptide scaffold possessing an acetylated N-terminus and an amidated C-terminus with the goal of discovering mouse (m) mMC3R-selective tetrapeptides that possessed no activity at the mMC4R.⁵³ This study found that the substitution pattern Ac-His-Arg-(pI)DPhe-Tic-NH₂ possessed a unique polypharmacology (MC3R agonist/MC4R antagonist).⁵³ A library of individual peptides based on the Ac-Xxx-Arg-(pI)DPhe-Xxx-NH₂ scaffold was then synthesized, the most potent of which at the both mMC3R and mMC4R was the peptide Ac-Arg-Arg-(pI)DPhe-Tic-NH₂.⁵³

The study reported herein carries out a more comprehensive deconvolution using the defined amino acids of the most active mixtures at each position of the published screening results for MC3R potency.⁵³ Forty-eight individual tetrapeptides possessing Val, Arg, or His in the first position, Arg or Gln in the second position, (pI)DPhe or (pCl)DPhe in the third position, and Tic, DTic, Cha, or DPro in the fourth position were synthesized.⁵³ The aim of this study was two-fold: 1) to identify a tetrapeptide that possesses a selective pharmacological profile for the MC3R over the MC4R, and 2) to identify tetrapeptides with increased potencies that possess the same polypharmacology (MC3R agonist and MC4R antagonist) as the ligands previously reported.⁵³

Results and Discussion

Peptide Synthesis and Characterization.

The tetrapeptides described in this manuscript were synthesized manually in parallel using fluorenylmethoxycarbonyl (Fmoc) methodology.^{56–57} The tetrapeptides were then purified using semi-preparative reverse phase high-pressure liquid chromatography (RP-HPLC) to be >95% pure via analytical RP-HPLC using two diverse solvent systems (see Table 1), with purity confirmed using HPLC at wavelength 214 nm. The analytical HPLC traces for these compounds can be found in the supplemental Figure S3 through Figure S48. Additionally, the correct peptide masses were confirmed using electrospray ionization (ESI) time of flight (TOF) mass spectrometry (University of Minnesota LeClaire-Dow Instrumentation Facility). Representative mass spectra can be observed in supplemental Figure S49.

Compounds were first assayed as agonists in HEK293 cells stably expressing the mMC1R, mMC3R, mMC4R, or mMC5R using the β -Galactosidase reporter gene assay previously developed.⁵⁸ Compounds that activated the receptor >90% were considered full agonist compounds. Compounds that did not produce >90% receptor activation were assayed as antagonists using a Schild analysis with NDP-MSH as the agonist, for which a pA_2 value is reported [$pA_2 = -\log(K_i)$].⁵⁹ Fold-change calculations for antagonist compounds were performed using the K_i values derived from the Schild analysis [$pA_2 = -\log(K_i)$]. To characterize agonist activity, the effective concentration of compound that produces a 50% assay signal (EC_{50}) is provided. To characterize antagonist activity, the log concentration of antagonist that requires a two-fold concentration of agonist to be administered to yield the same assay response as the agonist compound when administered alone (pA_2) is provided.

Deconvolution of Tetrapeptide Positional Scanning Library.

Individual peptides were derived from the combinations of the defined amino acids of the most active mMC3R agonist mixtures at each position from a mixture-based positional scanning library (TPI924) screen performed previously.⁵³ These tetrapeptides contained Val, Arg, or His in the first position, Arg or Gln in the second position, (pI)DPhe or (pCl)DPhe in the third position, and Tic, DTic, Cha, or DPro in the fourth position. The structures of these amino acids can be found in the supplemental Figure S1. The tetrapeptides were acetylated at the N-termini, and amidated at the C-termini. The amino acid substitutions were chosen based on potency at the MC3R alone, irrespective of potency at the other melanocortin receptors assayed in this study. Two tetrapeptides designed could not be purified (Ac-Val-Gln-(pI)DPhe-Cha-NH₂ and Ac-Val-Gln-(pCl)DPhe-Cha-NH₂), and therefore are not included in this manuscript.

Structure Activity Relationships.

It is important to note that while the deconvolution was done solely on the agonist activity of the tetrapeptide positional scanning library at the mMC3R, the subsequent determination of the activity of these compounds in other mouse melanocortin receptors provides further characterization of the compounds herein. This characterization allows further exploration of the relationships between structural analogs with different peptides at different positions and contributes to the overall structure-activity relationship of melanocortin ligands in the literature. All results for agonist and antagonist activity at the mMC3R and mMC4R are summarized in Table 2 and the corresponding Figure 1. Additional agonist results for mMC1R and mMC5R are summarized in Table 3. The proportions of peptides at varying levels of activity for the single fixed amino acids studied at each position (R1, R2, R3, and R4), which visually represent the SAR observed in this study, are presented in Figure S50, Figure S51, and Figure S52.

mMC3R Structure Activity Relationships.

Most of the compounds possessed agonist activity at the mMC3R with various degrees of potency. Three out of four of the most potent compounds, with EC_{50} values 10 nM or less (**33**, **34**, **18**, **21**) possess an Ac-Xxx-Arg-(pI)DPhe -Xxx-NH₂ motif. Among these ligands, both Val and Arg are equally represented in R1, and both Tic and DTic are equally

represented in R4. The pharmacology at the mMC3R and mMC4R of compound **18** is shown in Figure 2. The peptide that diverges from this motif is **21** which possesses.⁵³

a (pCl)DPhe in R3. Eighteen compounds with EC₅₀ values between 10 and 100 nM were identified. Among these compounds are the two previously identified compounds and reported in Doering *et al*⁵³ (compounds **1** and **17**). These results here, determined using the reporter gene assay described herein, are consistent with what was previously reported using an AlphaScreen assay platform.⁵³

mMC4R Structure Activity Relationships.

Equally striking results were apparent in the determination of mMC4R agonist activity for this set of compounds. Five of the tetrapeptides examined in this study (**13**, **21**, **29**, **37**, and **44**) possessed full agonist activity at the mMC4R, and a sixth (**5**) produced 80% receptor activation. All of these peptides contain the Ac-Xxx-Xxx-(pCl)DPhe-Tic-NH₂ motif. Twenty-three partial agonists were identified, which in this study was defined as between 20% and 90% receptor activation. Dose response curves for these compounds can be observed in Figure S2. Twelve of these compounds possess partial agonist efficacies between 50–80% (B), and seven of those twelve possess Gln in R2. Of the remaining eleven partial agonists with efficacies below 50% (A), three possessed a Gln in R2. Six compounds in this study (that did not possess a DPro in R4) resulted in some agonist activity at the mMC4R at up to 100 μ M concentrations. Three possessed His in R1, four possessed an Arg in R2, four of these compounds possessed a (pI)DPhe in R3, three possessed DTic and three possessed Cha in R4.

Analogous patterns to those observed when studying the full and partial agonists above are present in the analysis of mMC4R antagonist activity. The seven most potent antagonists of the compounds tested possessed pA₂ values between 7 and 7.6. All of these compounds possessed Arg in R2, five possessed Arg in R1, most possessed (pI)DPhe in R3, three possessed DTic in R4, and three possessed Cha in R4. The two most potent mMC4R antagonists in this study (**18**, **34**) possessed an Ac-Xxx-Arg-(pI)DPhe-DTic-NH₂ motif, with Arg and Val in R1. Fifteen additional compounds possessed less potent but still evident antagonist activity, with pA₂ values between 5.9 and 7. This included all the compounds with the Ac-His-Arg-(pI)DPhe-Xxx-NH₂ motif (**1**, **2**, **3**), all of the compounds possessing an Ac-Arg-Gln-(pI)DPhe-Xxx-NH₂ motif (**25**, **26**, **27**), two of the compounds possessing the Ac-His-Arg-(pCl)DPhe-Xxx-NH₂ motif (**6**, **7**), and two of the compounds possessing the Ac-Val-Arg-(pCl)DPhe-Xxx-NH₂ motif (**38**, **39**). Five members of this group possessed the sequence Ac-Xxx-Gln-(pI)DPhe-Xxx-NH₂ (**26**, **27**, **25**, **11**, **41**). An additional member of this group possessed the structure Ac-Val-Arg-(pI)DPhe-Tic-NH₂ (**33**), and another possessed the structure Ac-Arg-Gln-(pCl)DPhe-DTic-NH₂ (**30**). Overall, it appears that the Ac-Xxx-Arg-(pI)DPhe-Xxx-NH₂ motif results in decreased partial agonist efficacy, and that antagonist potency can be increased using the Ac-Arg-Arg-Xxx-Xxx-NH₂ motif.

mMC1R and mMC5R Structure Activity Relationships.

Most of the compounds without DPro in R4 possessed nanomolar agonist EC₅₀ values when assayed at both the mMC1R and mMC5R (see Table 3). The eighteen most potent

compounds ($EC_{50} < 10$ nM) at the mMC1R all possessed an Arg in R2 rather than a Gln, which was similar to the SAR observed for agonist activity at the mMC3R, and the most potent compound at the mMC1R at this receptor was **37** ($EC_{50} = 0.18$ nM). Of the top thirteen most potent compounds ($EC_{50} < 5$ nM) at the mMC5R, nine possess an Arg in R1, ten possess an Arg in R2, and ten possess a (pI)DPhe in R3, which was similar to the pattern observed for mMC4R agonist potency. The most potent compounds in this set against the mMC5R receptor (**33**, **26**, **18**), were equipotent to NDP-MSH. Three compounds were identified that possessed a selective mMC1R pharmacological profile. Compounds **5** and **37** (sequences Ac-His-Arg-(pCl)DPhe-Tic-NH₂ and Ac-Val-Arg-(pCl)DPhe-Tic-NH₂ respectively) were both >100-fold more potent at the mMC1R than the other receptors examined in this study. Compound **38** (sequence Ac-Val-Arg-(pCl)DPhe-DTic-NH₂) was nearly 100-fold selective for the mMC1R over the other melanocortin receptors examined in this study, and possesses micromolar antagonist activity at the mMC4R ($pA_2 = 6.2$) as opposed to agonist activity, which the other ligands possess. Most notably, compound **37** possesses a potency of 0.18 nM at the mMC1R, is 170-fold selective against the mMC3R, is 830-fold selective against the mMC4R, and 220-fold selective against the mMC5R. Previous studies have found that capping of the N-terminus of the tetrapeptide His-DPhe-Arg-Trp-NH₂ with a 4-phenylbutyryl group was able to enhance MC1R binding potency and selectivity,^{60–61} so future studies may be performed with different capping groups of **37** to further enhance the potency and selectivity of this tetrapeptide. The MC1R has been a highly investigated target in skin cancer, particularly in the area of imaging, and is involved in skin pigmentation.^{62–64}

mMC3R/mMC4R Polypharmacology.

In the optimization of mMC3R/mMC4R polypharmacology (a visual representation of which may be observed in Figure 3), the Ac-Xxx-Arg-(pI)DPhe-DTic-NH₂ sequence was favored as the unique motif possessing both the most potent mMC3R agonists and the most potent mMC4R antagonists. Both Arg and Val substituents in R1 were equipotent at the mMC3R and possessed similar activities at the MC4R (**18** and **34** respectively). Compound **18** is a mMC4R partial agonist with an EC_{50} value of 30 nM and results in 20% partial agonist efficacy relative to NDP-MSH. The SAR of this compound compared to previously discovered compounds (and other ligands in this study) can be observed in Figure 4A and 4B. The pharmacology of this compound at the mMC3R and mMC4R can be observed in Figure 2. Compound **34** is a less potent partial agonist at the mMC4R than **18** with an EC_{50} value of 160 nM, and results in 30% receptor activation relative to NDP-MSH.

The SAR of compounds optimized for an MC3R agonist/MC4R antagonist pharmacology is similar to the SAR of mMC3R agonist activity. At the mMC3R Arg and Val are favored in R1, Arg in R2, and (pI)DPhe in R3. Where this SAR differs is that Tic and DTic in R4 are equally favored for mMC3R potency, while only DTic is favored for mMC3R/mMC4R polypharmacology at R4. It can be hypothesized that this is due to the increase in receptor activation and agonist activity for compounds possessing a Tic in R4 relative to compounds possessing a DTic in R4 at the mMC4R. For MC4R antagonist potency, Val and Arg are favored in R1, Arg in R2, (pI)DPhe in R3, and DTic or Cha in R4. The SAR for MC4R antagonist potency is similar to the SAR of compounds possessing the most potent MC3R

agonist/MC4R antagonist polypharmacology, with the exception that Cha in R4 is not favored for mMC3R potency.

MC3R vs. MC4R Selectivity.

None of the compounds examined in this study were “cleanly” selective, in that they possessed agonist activity at the MC3R and no activity at the MC4R (agonist or antagonist). The analysis of selectivity for a set of compounds that possesses agonist activity at one receptor and a mixture of pharmacological activities at another receptor (i.e. agonist, partial agonist, and antagonist) is complicated. The analysis of compounds for MC3R selectivity depicted visually can be observed in Figure 5. For the purposes of developing an MC3R selective ligand devoid of MC4R activity, MC3R potency is desired. Additionally, the ideal selective probe at the mMC4R would possess a high EC₅₀ value (if the ligand possesses partial or full agonist activity) compared to other ligands in this study, result in a low percent receptor activation relative to NDP-MSH, and have a low pA₂ (if the ligand possesses antagonist activity). In Figure 5, compounds with these desired traits are summarized in a heat map ranging from blue (generally more desired) to purple (generally less desired). In this figure, the compounds are arranged so that the most selective mMC3R agonists are at the top (calculated using the EC₅₀ values at the MC3R and MC4R), and the least selective mMC3R ligands are at the bottom. Compound **42** (sequence Ac-Val-Gln-(pI)DPhe-DTic-NH₂) is the most potent tetrapeptide at the mMC3R with the desired traits at the mMC4R compared to the other ligands in this study. This compound possesses an EC₅₀ value of 50 nM at the mMC3R, is a partial agonist at the mMC4R with an EC₅₀ value of 5,000 nM and receptor activation of 50%, and does not possess antagonist activity at the concentrations examined in this study. The pharmacology of this peptide at the mMC3R and mMC4R can be observed in Figure 2. The pharmacological activity of this compound relative to other compounds at the melanocortin receptors can be observed in Figure 4A and 4B. Compound **42** is 100-fold selective for the mMC3R over the mMC4R.

Conclusions.

This study reports the synthesis and melanocortin receptor pharmacology of a 46-member library of individual compounds derived from a mixture-based positional scanning library screen previously reported at the mMC3R.⁵³ Two compounds discovered in this study possess four-fold increased agonist potency at the mMC3R and three-fold increased antagonist potency at the mMC4R compared to previously discovered compounds (compounds **18** and **34** compared to compounds **1** and **17**).⁵³ This study also resulted in the identification of an MC3R agonist that is 100-fold selective for the MC3R over the MC4R, is a full agonist at the MC3R (EC₅₀ = 50 nM), and is a partial (50%) agonist at the MC4R (**42**, EC₅₀ = 5,000 nM). Additionally, one mMC1R-selective ligand was discovered (**37**) that possessed subnanomolar potency and was >100-fold selective for the mMC1R over all the other receptors examined in this study. The ligands and SAR obtained from these studies will be useful in developing potent and selective probes for the MC3R and MC1R, as well as developing probes with a more potent polypharmacology at the MC3R and MC4R. These compounds will make useful *in vivo* probes to study the functional roles of the central melanocortin receptors in the maintenance of energy homeostasis, and to study melanoma via the mMC1R.

Experimental Section

Peptide Synthesis.

Tetrapeptides peptides were synthesized using flourenyl-9-methoxycarbonyl (Fmoc) methodology on a RINK-Amide-MBHA (0.51 mequiv/g substitution) purchased from Peptides International (Louisville, KY).^{56–57} Coupling reagent O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU) was purchased from Peptides International (Louisville, KY). Amino acids Fmoc-DPro, Fmoc-cyclohexylalanine (Fmoc-Cha), Fmoc-Arg(Pbf), Fmoc-His(Trt), Fmoc-Val, Fmoc-Gln, and Fmoc-(pCl)DPhe were purchased from Peptides International (Louisville, KY). The Fmoc-tetrahydroisoquinoline-3-carboxylic acid (Fmoc-Tic) and Fmoc-(pI)DPhe were purchased from Synthetech (Albany, OR). Additional Fmoc-(pI)DPhe was purchased from Anspec (Thornleigh, Australia). Dichloromethane (DCM), N,N-dimethylformamide (DMF), methanol, acetonitrile, and anhydrous ethyl ether were purchased from Fisher (Fair Lawn, NJ). Trifluoroacetic acid (TFA), piperidine, dimethyl sulfoxide (DMSO), N,N-diisopropylethylamine (DIEA), and triisopropylsilane (TIS) were purchased from Sigma-Aldrich (St. Louis, MO). All reagents were ACS grade or better, and were used without purification.

Peptides were synthesized in parallel at a 0.05 mmol scale on a RINK-Amide MBHA resin using a semi-automated synthesizer (LabTech 1, Advanced Chemtech, Louisville, KY). Resin was swelled in dichloromethane (DCM) for at least one hour prior to the first coupling. Synthesis was performed in a 40-well Teflon reaction block. The Fmoc-protected resin was deprotected using 20% piperidine in DMF (2 mL/well, 1 X 2 min, 1 X 18 min), then rinsed with DMF. Fmoc protected amino acid (3.1 equiv), HBTU (3 equiv), and DIEA (5 equiv) were added, and the solution was agitated for 1 hour. The presence of a free secondary amine was monitored using a chloranil test, and the presence of a primary amine was monitored using a Kaiser test.^{65–66} The coupling reaction was performed a second time if necessary, and the reaction was monitored for completeness using a chloranil or Kaiser test. Next, the amino acid was deprotected with 20% piperidine in DMF. Deprotection was monitored again using a chloranil or Kaiser test. The second, third, and fourth amino acids were coupled using the same procedure as described above. Following synthesis, the N-terminal Fmoc amines were deprotected with 20% piperidine in DMF and dried *in vacuo* after washing with methanol.

Peptides were cleaved from solid support using a cleavage cocktail containing 91% trifluoroacetic acid (Sigma-Aldrich), 3% H₂O, 3% triisopropylsilane (Aldrich), and 3% thioanisole (Fluka). Approximately 4 mL of cocktail was added to the resin and the solution was agitated for 2 hours. Peptides were precipitated with cold ether and pelleted via centrifugation (4,000 rpm at 4°C for 4 minutes, ThermoScientific Sorvall Legend XTR). The supernatant was decanted, and the peptides were then dried *in vacuo* in the presence of desiccant overnight.

Peptides were purified via reverse phase high pressure liquid chromatography (RP-HPLC, Shimadzu) on a semi-preparative C₁₈ column (Vydac 218TP1010, 1 cm X 25 cm) using acetonitrile and 0.1% TFA in H₂O. Analytical data was then collected using an analytical

C₁₈ column (Vydac 218TP, 4.6 mm X 250 mm) on a Shimadzu chromatography system equipped with a photodiode array detector in two different solvent systems: acetonitrile/0.1% TFA in H₂O, and methanol/0.1% TFA in H₂O to confirm purity > 95%. The peptide purity was determined by HPLC at a wavelength of 214 nm. Molecular weights were then confirmed using electrospray ionization (ESI) and time of flight (TOF) mass spectrometry analysis (Bruker BioTOF II ESI/TOF-MS, LeClaire-Dow Instrumentation Facility, University of Minnesota). Peptides were assayed as TFA salts and not corrected for peptide content.

β-Galactosidase cAMP Assay.

Compounds were stored at 10⁻² M concentrations in DMSO and stored at -20°C until the day of the assay. HEK293 cells were grown in 10 cm plates with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% bovine serum and 1% penicillin-streptomycin. Cells stably expressing either the mMC1R, mMC3R, mMC4R, or mMC5R were transiently transfected with 4 μg of CRE/β-Galactosidase reporter gene per plate as reported previously, using a calcium phosphate methodology.^{53-54, 58, 67} Forty-eight hours post transfection, approximately 10,000 cells were plated into each well in a collagen-treated Nunclon Delta Surface 96-well plate (Thermo Fischer Scientific) and incubated overnight at 37°C and 5% CO₂. The following morning, cells were stimulated with 50 μL peptide (10⁻⁴ M to 10⁻¹⁰ M for tetrapeptide compounds, 10⁻⁶ M to 10⁻¹² M for the NDP-MSH control) diluted in assay medium, forskolin (10⁻⁴ M) control diluted in assay medium, or only assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 M isobutylmethylxanthine) control for 6 hours. Following aspiration of the assay media, 50 μL of lysis buffer (250 mM Tris-HCl pH = 8.0 and 0.1% Triton X-100) was added to each well in the 96 well plate. The plates were then stored in the -80°C freezer for up to a week. On the day the assay was measured, the plates containing the lysate were thawed. To determine protein content, 10 μL was taken from each well, which was transferred to another 96 well plate containing 200 μL of a 1:5 dilution of Bio Rad G250 protein dye solution diluted with water in each well. The OD₅₉₅ was then measured on a 96-well plate reader (Molecular Devices) within one hour of adding the lysate. To determine β-galactosidase gene expression, 40 μL of a phosphate-buffered saline solution containing 0.5% BSA was added to each well in the plates containing just the cell lysate. Next, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM MgCl₂, 10 mM KCl, 5 mM β-mercaptoethanol, 2 mg/ml ONPG) was added to each well. The plates were incubated at 37°C, and the OD₄₀₅ absorbance was measured using a 96-well plate reader (Molecular Devices). Plates were incubated until the both the forskolin and 10⁻⁶M NDP-MSH wells possessed absorbance values between 1.0 and 1.5 relative absorbance units. Data were then normalized to protein content, and subsequently normalized to the maximum response of the positive controls.

Cell Data Analysis.

The data collected were analyzed using PRISM software (v4.0, GraphPad Inc.). Antagonist potency was determined using a Schild analysis [$pA_2 = -\log(K_i)$] and the agonist ligand NDP-MSH.⁵⁹ Agonist potency was evaluated by calculating the EC₅₀ values using non-linear regression analysis with the PRISM software. The values reported herein represent the

mean of three or more independent experiments performed in duplicate $\pm$ the standard error of the mean (SEM).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources. This work has been supported by the NIH Grant R01DK091906 (C.H.-L.). K.A.F. is the recipient of a 2018 Bighley Fellowship, a 2018 Rowell Fellowship, and 2019 Rowell Fellowship. We also acknowledge the receipt of a 2017 Wallin Neuroscience Discovery Fund Award.

Abbreviations

ACTH	adrenocorticotropin hormone
Fmoc	9-fluorenylmethoxycarbonyl
AGRP	agouti-related protein
GPCR	G-protein coupled receptor
cAMP	cyclic 5'-adenosine monophosphate
MC1R	melanocortin-1 receptor
MC2R	melanocortin-2 receptor
MC3R	melanocortin-3 receptor
MC4R	melanocortin-4 receptor
MC5R	melanocortin-5 receptor
MSH	melanocyte stimulating hormone
POMC	proopiomelanocortin
α-MSH	alpha-melanocyte stimulating hormone
β-MSH	beta-melanocyte stimulating hormone
γ-MSH	gamma-melanocyte stimulating hormone
μM	micromolar
NDP-MSH	(4-Norleucine-7-D-Phenylalanine) Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH ₂
Cha	cyclohexylalanine
RP-HPLC	reverse-phase high-pressure liquid chromatography

References

1. Chhajlani V; Muceniece R; Wikberg JES, Molecular Cloning of a Novel Human Melanocortin Receptor. *Biochem. Biophys. Res. Commun* 1993, 195, 866–873. [PubMed: 8396929]
2. Chhajlani V; Wikberg JES, Molecular Cloning and Expression of the Human Melanocyte Stimulating Hormone Receptor cDNA. *FEBS Lett* 1992, 309, 417–420. [PubMed: 1516719]
3. Gantz I; Konda Y; Tashiro T; Shimoto Y; Miwa H; Munzert G; Watson SJ; DelValle J; Yamada T, Molecular Cloning of a Novel Melanocortin Receptor. *J. Biol. Chem* 1993, 268, 8246–8250. [PubMed: 8463333]
4. Gantz I; Miwa H; Konda Y; Shimoto Y; Tashiro T; Watson SJ; DelValle J; Yamada T, Molecular Cloning, Expression, and Gene Localization of a Fourth Melanocortin Receptor. *J. Biol. Chem* 1993, 268, 15174–15179. [PubMed: 8392067]
5. Gantz I; Shimoto Y; Konda Y; Miwa H; Dickinson CJ; Yamada T, Molecular Cloning, Expression, and Characterization of a Fifth Melanocortin Receptor. *Biochem. Biophys. Res. Commun* 1994, 200, 1214–1220. [PubMed: 8185570]
6. Griffon N; Mignon V; Facchinetti P; Diaz J; Schwartz JC; Sokoloff P, Molecular Cloning and Characterisation of the Rat Fifth Melanocortin Receptor. *Biochem. Biophys. Res. Commun* 1994, 200, 1007–1014. [PubMed: 8179577]
7. Mountjoy K; Robbins L; Mortrud M; Cone R, The Cloning of a Family of Genes that Encode the Melanocortin Receptors. *Science* 1992, 257, 1248–1251. [PubMed: 1325670]
8. Roselli-Rehfuß L; Mountjoy KG; Robbins LS; Mortrud MT; Low MJ; Tatro JB; Entwistle ML; Simerly RB; Cone RD, Identification of a Receptor for γ Melanotropin and Other Proopiomelanocortin Peptides in the Hypothalamus and Limbic System. *Proc. Natl. Acad. Sci* 1993, 90, 8856–8860. [PubMed: 8415620]
9. Butler AA; Kesterson RA; Khong K; Cullen MJ; Pelleymounter MA; Dekoning J; Baetscher M; Cone RD, A Unique Metabolic Syndrome Causes Obesity in the Melanocortin-3 Receptor-Deficient Mouse. *Endocrinology* 2000, 141, 3518–3521. [PubMed: 10965927]
10. Chen AS; Marsh DJ; Trumbauer ME; Frazier EG; Guan X-M; Yu H; Rosenblum CI; Vongs A; Feng Y; Cao L; Metzger JM; Strack AM; Camacho RE; Mellin TN; Nunes CN; Min W; Fisher J; Gopal-Truter S; MacIntyre DE; Chen HY; Van der Ploeg LHT, Inactivation of the Mouse Melanocortin-3 Receptor Results in Increased Fat Mass and Reduced Lean Body Mass. *Nature Genetics* 2000, 26, 97–102. [PubMed: 10973258]
11. Irani BG; Xiang Z; Yarandi HN; Holder JR; Moore MC; Bauzo RM; Proneth B; Shaw AM; Millard WJ; Chambers JB; Benoit SC; Clegg DJ; Haskell-Luevano C, Implication of the Melanocortin-3 Receptor in the Regulation of Food Intake. *European journal of pharmacology* 2011, 660, 80–87. [PubMed: 21199647]
12. Rowland NE; Schaub JW; Robertson KL; Andreassen A; Haskell-Luevano C, Effect of MTII on Food Intake and Brain c-fos in Melanocortin-3, Melanocortin-4, and Double MC3 and MC4 Receptor Knockout Mice. *Peptides* 2010, 31, 2314–2317. [PubMed: 20800636]
13. Fan W; Boston BA; Kesterson RA; Hruby VJ; Cone RD, Role of Melanocortinergic Neurons in Feeding and the Agouti Obesity Syndrome. *Nature* 1997, 385, 165–168. [PubMed: 8990120]
14. Huszar D; Lynch CA; Fairchild-Huntress V; Dunmore JH; Fang Q; Berkemeier LR; Gu W; Kesterson RA; Boston BA; Cone RD; Smith FJ; Campfield LA; Burn P; Lee F, Targeted Disruption of the Melanocortin-4 Receptor Results in Obesity in Mice. *Cell* 1997, 88, 131–141. [PubMed: 9019399]
15. Farooqi IS; Keogh JM; Yeo GSH; Lank EJ; Cheetham T; O'Rahilly S, Clinical Spectrum of Obesity and Mutations in the Melanocortin 4 Receptor Gene. *The New England Journal of Medicine* 2003, 348, 1085–1095. [PubMed: 12646665]
16. Shinyama H; Masuzaki H; Fang H; Flier JS, Regulation of Melanocortin-4 Receptor Signaling: Agonist-Mediated Desensitization and Internalization. *Endocrinology* 2003, 144, 1301–1314. [PubMed: 12639913]
17. Kroeze WK; Sassano MF; Huang XP; Lansu K; McCorvy JD; Giguere PM; Sciaky N; Roth BL, PRESTO-TANGO: An Open-Source Resource for Interrogation of the Druggable Human GPCR-ome. *Nature Structural & Molecular Biology* 2015, 22, 362–369.

18. Cai M; Stankova M; Pond SJK; Mayorov AV; Perry JW; Yamamura HI; Trivedi D; Hruby VJ, Real Time Differentiation of G-Protein Coupled Receptor (GPCR) Agonist and Antagonist by Two Photon Fluorescence Laser Microscopy. *J. Am. Chem. Soc* 2004, 126, 7160–7161. [PubMed: 15186137]
19. Nakanishi S; Inoue A; Kita T; Inoue A; Nakamura M; Chang ACY; Cohen SN; Numa S, Nucleotide Sequence of Cloned cDNA for Bovine Corticotropin- β -Lipotropin Precursor. *Nature* 1979, 278, 423–427. [PubMed: 221818]
20. Eipper BA; Mains RE, Structure and Biosynthesis of Pro-Adrenocorticotropin/Endorphin and Related Peptides. *Endocr. Rev* 1980, 1, 1–27. [PubMed: 6262069]
21. Smith AI; Funder JW, Proopiomelanocortin Processing in the Pituitary, Central Nervous System, and Peripheral Tissues. *Endocr. Rev* 1988, 9, 159–179. [PubMed: 3286233]
22. Krude H; Biebermann H; Luck W; Horn R; Brabant G; Gruters A, Severe Early-Onset Obesity, Adrenal Insufficiency and Red Hair Pigmentation Caused by POMC Mutations in Humans. *Nature Genetics* 1998, 19, 155–157. [PubMed: 9620771]
23. Yaswen L; Diehl N; Brennan MB; Hochgeschwender U, Obesity in the Mouse Model of Pro-Opiomelanocortin Deficiency Responds to Peripheral Melanocortin. *Nature Medicine* 1999, 5, 1066–1070.
24. Bultman SJ; Michaud EJ; Woychik RP, Molecular Characterization of the Mouse Agouti Locus. *Cell* 1992, 71, 1195–1204. [PubMed: 1473152]
25. Ollmann MM; Wilson BD; Yang Y-K; Kerns JA; Chen Y; Gantz I; Barsh GS, Antagonism of Central Melanocortin Receptors in Vitro and in Vivo by Agouti-Related Protein. *Science* 1997, 278, 135–138. [PubMed: 9311920]
26. Blanchard SG; Harris CO; Ittoop O; Nichols JS; Parks DJ; Truesdale AT; Wilkison WO, Agouti Antagonism of Melanocortin Binding and Action in the B16F10 Murine Melanoma Cell Line. *Biochemistry* 1995, 34, 10406–10411. [PubMed: 7544613]
27. Fong TM; Mao C; MacNeil T; Kalyani R; Smith T; Weinberg D; Tota MR; Van der Ploeg LHT, ART (Protein Product of Agouti-Related Transcript) as an Antagonist of MC-3 and MC-4 Receptors. *Biochem. Biophys. Res. Commun* 1997, 237, 629–631. [PubMed: 9299416]
28. Miller MW; Duhl DM; Vrieling H; Cordes SP; Ollmann MM; Winkes BM; Barsh GS, Cloning of the Mouse Agouti Gene Predicts a Secreted Protein Ubiquitously Expressed in Mice Carrying the Lethal Yellow Mutation. *Genes & Development* 1993, 7, 454–467. [PubMed: 8449404]
29. Ebihara K; Ogawa Y; Katsuura G; Numata Y; Masuzaki H; Satoh N; Tamaki M; Yoshioka T; Hayase M; Matsuoka N; Aizawa-Abe M; Yoshimasa Y; Nakao K, Involvement of Agouti-Related Protein, an Endogenous Antagonist of Hypothalamic Melanocortin Receptor, in Leptin Action. *Diabetes* 1999, 48, 2028–2033. [PubMed: 10512369]
30. Renquist BJ; Murphy JG; Larson EA; Olsen D; Klein RF; Ellacott KLJ; Cone RD, Melanocortin-3 Receptor Regulates the Normal Fasting Response. *Proc. Natl. Acad. Sci* 2012, 109, E1489–E1498. [PubMed: 22573815]
31. Girardet C; Mavrikaki MM; Stevens JR; Miller CA; Marks DL; Butler AA, Melanocortin-3 Receptors Expressed in Nkx2.1(+ve) Neurons are Sufficient for Controlling Appetitive Responses to Hypocaloric Conditioning. *Scientific Reports* 2017, 7, 44444. [PubMed: 28294152]
32. Mavrikaki M; Girardet C; Kern A; Faruzzi Brantley A.; Miller CA; Macarthur H; Marks DL; Butler AA, Melanocortin-3 Receptors in the Limbic System Mediate Feeding-Related Motivational Responses During Weight Loss. *Molecular Metabolism* 2016, 5, 566–579. [PubMed: 27408780]
33. Lippert RN; Ellacott KLJ; Cone RD, Gender-Specific Roles for the Melanocortin-3 Receptor in the Regulation of the Mesolimbic Dopamine System in Mice. *Endocrinology* 2014, 155, 1718–1727. [PubMed: 24605830]
34. Atalayer D; Robertson KL; Haskell-Luevano C; Andreasen A; Rowland NE, Food Demand and Meal Size in Mice with Single or Combined Disruption of Melanocortin Type 3 and 4 Receptors. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 2010, 298, R1667–R1674.
35. Kas MJH; van Dijk G; Scheurink AJW; Adan RAH, Agouti-Related Protein Prevents Self-Starvation. *Mol. Psychiatry* 2003, 8, 235–240. [PubMed: 12610657]

36. Adan RAH; Hillebrand JIG; De Rijke C; Nijenhuis W; Vink TOM; Garner KM; Kas MJH, Melanocortin System and Eating Disorders. *Annals of the New York Academy of Sciences* 2003, 994, 267–274. [PubMed: 12851325]
37. Ge Y-L; Ohta T; Driscoll DJ; Nicholls RD; Kalra SP, Anorexigenic Melanocortin Signaling in the Hypothalamus is Augmented in Association with Failure-to-Thrive in a Transgenic Mouse Model for Prader–Willi Syndrome. *Brain Research* 2002, 957, 42–45. [PubMed: 12443978]
38. Marks DL; Butler AA; Turner R; Brookhart G; Cone RD, Differential Role of Melanocortin Receptor Subtypes in Cachexia. *Endocrinology* 2003, 144, 1513–1523. [PubMed: 12639936]
39. Marks DL; Ling N; Cone RD, Role of the Central Melanocortin System in Cachexia. *Cancer Research* 2001, 61, 1432–1438. [PubMed: 11245447]
40. Ericson MD; Lensing CJ; Fleming KA; Schlasner KN; Doering SR; Haskell-Luevano C, Bench-Top to Clinical Therapies: A Review of Melanocortin Ligands from 1954 to 2016. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 2017, 1863, 2414–2435. [PubMed: 28363699]
41. Bednarek MA; MacNeil T; Tang R; Fong TM; Cabello MA; Maroto M; Teran A, Potent and Selective Agonists of α -Melanotropin (α MSH) Action at Human Melanocortin Receptor 5; Linear Analogs of α -Melanotropin. *Peptides* 2007, 28, 1020–1028. [PubMed: 17376561]
42. Todorovic A; Joseph CG; Sorensen NB; Wood MS; Haskell-Luevano C, Structure–Activity Relationships of Melanocortin Agonists Containing the Benzimidazole Scaffold. *Chemical Biology & Drug Design* 2007, 69, 338–349. [PubMed: 17539826]
43. Carotenuto A; Merlino F; Cai M; Brancaccio D; Yousif AM; Novellino E; Hruby VJ; Grieco P, Discovery of Novel Potent and Selective Agonists at the Melanocortin-3 Receptor. *Journal of Medicinal Chemistry* 2015, 58, 9773–9778. [PubMed: 26599352]
44. Hong Q; Bakshi RK; Dellufeficio J; He S; Ye Z; Dobbelaar PH; Sebat IK; Guo L; Liu J; Jian T; Tang R; Kalyani RN; MacNeil T; Vongs A; Rosenblum CI; Weinberg DH; Peng Q; Tamvakopoulos C; Miller RR; Stearns RA; Cashen D; Martin WJ; Chen AS; Metzger JM; Chen HY; Strack AM; Fong TM; MacIntyre E; Van der Ploeg LHT; Wyvratt MJ; Nargund RP, Optimization of Privileged Structures for Selective and Potent Melanocortin Subtype-4 Receptor Ligands. *Bioorganic & Medicinal Chemistry Letters* 2010, 20, 4483–4486. [PubMed: 20598533]
45. Hess S; Linde Y; Ovadia O; Safrai E; Shalev DE; Swed A; Halbfinger E; Lapidot T; Winkler I; Gabinet Y; Faier A; Yarden D; Xiang Z; Portillo FP; Haskell-Luevano C; Gilon C; Hoffman A, Backbone Cyclic Peptidomimetic Melanocortin-4 Receptor Agonist as a Novel Orally Administrated Drug Lead for Treating Obesity. *Journal of Medicinal Chemistry* 2008, 51, 1026–1034. [PubMed: 18220330]
46. Fleming KA; Ericson MD; Freeman KT; Adank DN; Lunzer MM; Wilber SL; Haskell-Luevano C, Structure–Activity Relationship Studies of a Macrocyclic AGRP-Mimetic Scaffold c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro] Yield Potent and Selective Melanocortin-4 Receptor Antagonists and Melanocortin-5 Receptor Inverse Agonists That Increase Food Intake in Mice. *ACS Chemical Neuroscience* 2018, 9, 1141–1151. [PubMed: 29363944]
47. Fleming KA; Freeman KT; Ericson MD; Haskell-Luevano C, Synergistic Multiresidue Substitutions of a Macrocyclic c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-dPro] Agouti-Related Protein (AGRP) Scaffold Yield Potent and >600-Fold MC4R versus MC3R Selective Melanocortin Receptor Antagonists. *Journal of Medicinal Chemistry* 2018, 61, 7729–7740. [PubMed: 30035543]
48. Tala SR; Singh A; Lensing CJ; Schnell SM; Freeman KT; Rocca JR; Haskell-Luevano C, 1,2,3-Triazole Rings as a Disulfide Bond Mimetic in Chimeric AGRP-Melanocortin Peptides: Design, Synthesis, and Functional Characterization. *ACS Chemical Neuroscience* 2018, 9, 1001–1013. [PubMed: 29257879]
49. Gonçalves JPL; Palmer D; Meldal M, MC4R Agonists: Structural Overview on Antiobesity Therapeutics. *Trends in Pharmacological Sciences* 2018, 39, 402–423. [PubMed: 29478721]
50. Palmer D; Gonçalves JPL; Hansen LV; Wu B; Hald H; Schoffelen S; Diness F; Le Quement ST; Nielsen TE; Meldal M, Click-Chemistry-Mediated Synthesis of Selective Melanocortin Receptor 4 Agonists. *Journal of Medicinal Chemistry* 2017, 60, 8716–8730. [PubMed: 28972753]
51. Testa C; Scrima M; Grimaldi M; D’Ursi AM; Dirain ML; Lubin-Germain N; Singh A; Haskell-Luevano C; Chorev M; Rovero P; Papini AM, 1,4-Disubstituted-[1,2,3]triazolyl-Containing

Analogues of MT-II: Design, Synthesis, Conformational Analysis, and Biological Activity. *Journal of Medicinal Chemistry* 2014, 57, 9424–9434. [PubMed: 25347033]

52. Lensing CJ; Adank DN; Doering SR; Wilber SL; Andreasen A; Schaub JW; Xiang Z; Haskell-Luevano C, Ac-Trp-DPhe(p-I)-Arg-Trp-NH₂, a 250-Fold Selective Melanocortin-4 Receptor (MC4R) Antagonist over the Melanocortin-3 Receptor (MC3R), Affects Energy Homeostasis in Male and Female Mice Differently. *ACS Chemical Neuroscience* 2016, 7, 1283–1291. [PubMed: 27385405]
53. Doering SR; Freeman KT; Schnell SM; Haslach EM; Dirain M; Debevec G; Geer P; Santos RG; Giulianotti MA; Pinilla C; Appel JR; Speth RC; Houghten RA; Haskell-Luevano C, Discovery of Mixed Pharmacology Melanocortin-3 Agonists and Melanocortin-4 Receptor Tetrapeptide Antagonist Compounds (TACOs) Based on the Sequence Ac-Xaa1-Arg-(pI)DPhe-Xaa4-NH₂. *Journal of Medicinal Chemistry* 2017, 60, 4342–4357. [PubMed: 28453292]
54. Haslach EM; Huang H; Dirain M; Debevec G; Geer P; Santos RG; Giulianotti MA; Pinilla C; Appel JR; Doering SR; Walters MA; Houghten RA; Haskell-Luevano C, Identification of Tetrapeptides from a Mixture Based Positional Scanning Library That Can Restore nM Full Agonist Function of the L106P, I69T, I102S, A219V, C271Y, and C271R Human Melanocortin-4 Polymorphic Receptors (hMC4Rs). *Journal of Medicinal Chemistry* 2014, 57, 4615–4628. [PubMed: 24517312]
55. Santos RG; Appel JR; Giulianotti MA; Edwards BS; Sklar LA; Houghten RA; Pinilla C, The Mathematics of a Successful Deconvolution: A Quantitative Assessment of Mixture-Based Combinatorial Libraries Screened Against Two Formylpeptide Receptors. *Molecules* 2013, 18, 6408–6424. [PubMed: 23722730]
56. Carpino LA, The 9-Fluorenylmethoxycarbonyl Function, a New Base-Sensitive Amino-Protecting Group. *Journal of the American Chemical Society* 1970, 92, 5748–5749.
57. Carpino LAH, G. Y., The 9-Fluorenylmethoxycarbonyl Amino-Protecting Group. *Journal of Organic Chemistry* 1972, 37, 3404–3409.
58. Chen WB; Shields TS; Stork PJS; Cone RD, A Colorimetric Assay for Measuring Activation of Gs- and Gq-Coupled Signaling Pathways. *Analytical Biochemistry* 1995, 226, 349–354. [PubMed: 7793637]
59. Schild HO, pA A New Scale for the Measurement of Drug Antagonism. *British Journal of Pharmacology and Chemotherapy* 1947, 120, 29–46.
60. Koikov LN; Ebetino FH; Solinsky MG; Cross-Doersen D; Knittel JJ, Sub-Nanomolar hMC1R Agonists by End-Capping of the Melanocortin Tetrapeptide His-d-Phe-Arg-Trp-NH₂. *Bioorganic & Medicinal Chemistry Letters* 2003, 13, 2647–2650. [PubMed: 12873485]
61. Koikov LN; Ebetino FH; Solinsky MG; Cross-Doersen D; Knittel JJ, Analogs of Sub-Nanomolar hMC1R Agonist LK-184 [Ph(CH₂)₃CO-His-d-Phe-Arg-Trp-NH₂]. An Additional Binding Site Within the Human Melanocortin Receptor 1? *Bioorganic & Medicinal Chemistry Letters* 2004, 14, 3997–4000. [PubMed: 15225714]
62. Lerner AB; McGuire JS, Effect of α - and β -Melanocyte Stimulating Hormones on the Skin Colour of Man. *Nature* 1961, 189, 176–179. [PubMed: 13761067]
63. Barkey NM; Tafreshi NK; Josan JS; De Silva CR; Sill KN; Hruby VJ; Gillies RJ; Morse DL; Vagner J, Development of Melanoma-Targeted Polymer Micelles by Conjugation of a Melanocortin 1 Receptor (MC1R) Specific Ligand. *Journal of Medicinal Chemistry* 2011, 54, 8078–8084. [PubMed: 22011200]
64. Barkey NM; Preihs C; Cornell HH; Martinez G; Carie A; Vagner J; Xu L; Lloyd MC; Lynch VM; Hruby VJ; Sessler JL; Sill KN; Gillies RJ; Morse DL, Development and in vivo Quantitative Magnetic Resonance Imaging of Polymer Micelles Targeted to the Melanocortin 1 Receptor. *Journal of medicinal chemistry* 2013, 56, 10.1021/jm4005576.
65. Christensen T, Qualitative Test for Monitoring Coupling Completeness in Solid-Phase Peptide-Synthesis Using Chloranil. *Acta Chemica Scandinavica, Series B: Organic Chemistry and Biochemistry* 1979, 33, 763–766.
66. Kaiser E; Colescott RL; Bossinger CD; Cook PI, Color Test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. *Analytical Biochemistry* 1970, 34, 595–598. [PubMed: 5443684]

67. Chen CA; Okayama H, Calcium Phosphate-Mediated Gene Transfer: A Highly Efficient Transfection System for Stably Transforming Cells with Plasmid DNA. *BioTechniques* 1988, 6, 632–8. [PubMed: 3273409]

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Figure 1.

Illustration of the partial agonist activities described in Table 2. “A” indicates that a partial agonist resulted in percent receptor activation from 20–50%, and “B” indicates that the partial agonist resulted in percent receptor activation from 50–90%.

Figure 2.

Illustrations of the *in vitro* agonist (mMC3R) and antagonist (mMC4R) pharmacology of compounds **18** and **42**. A Schild experimental strategy was used to determine antagonist activity at the mMC4R, using NDP-MSH as the agonist.⁵⁹ Above, it can be observed that compound **18** possesses a potent mMC3R agonist/mMC4R antagonist polypharmacology. Below, it can be observed that compound **42** is a potent mMC3R agonist, does not possess mMC4R antagonist activity, and is a partial agonist with 50% efficacy at the mMC4R.

MC3R Agonist/MC4R Antagonist Polypharmacology

CMPD ID	Ac	R1	R2	R3	R4	NH ₂	mMC3R EC ₅₀ (nM)	mMC4R pA ₂
18	Ac	Arg	Arg	(pI)DPhe	DTic	NH ₂	8	7.6
34	Ac	Val	Arg	(pI)DPhe	DTic	NH ₂	6	7.6
17	Ac	Arg	Arg	(pI)DPhe	Tic	NH ₂	21	7.1
23	Ac	Arg	Arg	(pCl)DPhe	Cha	NH ₂	60	7.1
19	Ac	Arg	Arg	(pI)DPhe	Cha	NH ₂	26	7
22	Ac	Arg	Arg	(pCl)DPhe	DTic	NH ₂	21	7
35	Ac	Val	Arg	(pI)DPhe	Cha	NH ₂	60	7
2	Ac	His	Arg	(pI)DPhe	DTic	NH ₂	51	6.9
26	Ac	Arg	Gln	(pI)DPhe	DTic	NH ₂	15	6.8
27	Ac	Arg	Gln	(pI)DPhe	Cha	NH ₂	100	6.7
33	Ac	Val	Arg	(pI)DPhe	Tic	NH ₂	4	6.7
1	Ac	His	Arg	(pI)DPhe	Tic	NH ₂	30	6.6
3	Ac	His	Arg	(pI)DPhe	Cha	NH ₂	50	6.6
6	Ac	His	Arg	(pCl)DPhe	DTic	NH ₂	210	6.5
7	Ac	His	Arg	(pCl)DPhe	Cha	NH ₂	60	6.4
39	Ac	Val	Arg	(pCl)DPhe	Cha	NH ₂	40	6.4
25	Ac	Arg	Gln	(pI)DPhe	Tic	NH ₂	40	6.2
31	Ac	Arg	Gln	(pCl)DPhe	Cha	NH ₂	210	6.2
38	Ac	Val	Arg	(pCl)DPhe	DTic	NH ₂	50	6.2
11	Ac	His	Gln	(pI)DPhe	Cha	NH ₂	350	6.1
41	Ac	Val	Gln	(pI)DPhe	Tic	NH ₂	100	6.1
30	Ac	Arg	Gln	(pCl)DPhe	DTic	NH ₂	105	5.9
4	Ac	His	Arg	(pI)DPhe	DPro	NH ₂	10,000	<5.5
5	Ac	His	Arg	(pCl)DPhe	Tic	NH ₂	110	<5.5
8	Ac	His	Arg	(pCl)DPhe	DPro	NH ₂	50,000	<5.5
9	Ac	His	Gln	(pI)DPhe	Tic	NH ₂	800	<5.5
10	Ac	His	Gln	(pI)DPhe	DTic	NH ₂	270	<5.5
12	Ac	His	Gln	(pI)DPhe	DPro	NH ₂	none	<5.5
14	Ac	His	Gln	(pCl)DPhe	DTic	NH ₂	1,400	<5.5
15	Ac	His	Gln	(pCl)DPhe	Cha	NH ₂	720	<5.5
16	Ac	His	Gln	(pCl)DPhe	DPro	NH ₂	none	<5.5
20	Ac	Arg	Arg	(pI)DPhe	DPro	NH ₂	3,000	<5.5
24	Ac	Arg	Arg	(pCl)DPhe	DPro	NH ₂	44,000	<5.5
28	Ac	Arg	Gln	(pI)DPhe	DPro	NH ₂	18,000	<5.5
29	Ac	Arg	Gln	(pCl)DPhe	Tic	NH ₂	500	<5.5
32	Ac	Arg	Gln	(pCl)DPhe	DPro	NH ₂	30,000	<5.5
36	Ac	Val	Arg	(pI)DPhe	DPro	NH ₂	4,400	<5.5
40	Ac	Val	Arg	(pCl)DPhe	DPro	NH ₂	19,000	<5.5
42	Ac	Val	Gln	(pI)DPhe	DTic	NH ₂	50	<5.5
43	Ac	Val	Gln	(pI)DPhe	DPro	NH ₂	none	<5.5
45	Ac	Val	Gln	(pCl)DPhe	DTic	NH ₂	700	<5.5
46	Ac	Val	Gln	(pCl)DPhe	DPro	NH ₂	16,000	<5.5
13	Ac	His	Gln	(pCl)DPhe	Tic	NH ₂	2,000	agonist
21	Ac	Arg	Arg	(pCl)DPhe	Tic	NH ₂	10	agonist
37	Ac	Val	Arg	(pCl)DPhe	Tic	NH ₂	30	agonist
44	Ac	Val	Gln	(pCl)DPhe	Tic	NH ₂	1,100	agonist

Figure 3.

Pseudo heat-map depicting the identification of compounds with the most potent mMC3R/mMC4R polypharmacology. Compounds are shown with a heat map ranging from green (more potent) to red (less potent). The desired polypharmacological probe in this study is a potent mMC3R agonist and an mMC4R antagonist, so the EC₅₀ (nM) values are provided for the mMC3R and the pA₂ values are provided for the mMC4R. Compounds that possess potency values colored in green in both columns were considered to be more desired polypharmacological probes than the other compounds in this study. In this figure, compounds are arranged by antagonist pA₂ values at the mMC4R from most potent at the top, to least potent (and agonist compounds) at the bottom. The most potent polypharmacological probe discovered in this study, **18**, is on top of this list. Antagonist mMC4R SAR trends discussed in the text are boxed in the sequences, such as the presence of the “Ac-Val/Arg-Arg-Xxx-Xxx-NH₂” motif and the “Ac-Xxx-Xxx-(pI)DPhe-DTic-NH₂”

motif among the most potent mMC4R antagonist compounds, and the prevalence of the “Ac-Xxx-Xxx-(pCl)DPhe-Tic-NH₂” motif among all of the agonist compounds. The compound identifiers of previously discovered peptides are identified in grey, and the compound identifiers for the two lead compounds (**18**, **42**) are highlighted in red and blue respectively.

Figure 4.

Radar plots depicting the pharmacological activities of key compounds identified in this study and highlighted in the text (**18**, **42**), as well as reference compounds identified in previous studies (**1**, **17**). The closer to the outside of the radar plot the compound is, the more potent that compound is at that particular receptor. **A**) Potency values of key compounds at the mMC1R, mMC3R, mMC4R, and mMC5R, expressed as pEC₅₀ (agonist values) or pA₂ (antagonist values; for MC3R agonist/MC4R antagonist polypharmacological probes only). The values that correspond to pEC₅₀ values are expressed in filled dots, while the mMC4R antagonist pA₂ values for the polypharmacological probes are expressed as empty dots. It can be observed that the most potent polypharmacological probe (**18**, red) is the farthest out of the plot for both mMC3R and mMC4R potency. It can also be observed that the most mMC3R vs. mMC4R selective compound discovered in this study (**42**, blue) is present at the innermost part of the radar plot for mMC4R potency. **B**) Potency values at the mMC3R and mMC4R and percent mMC4R activation observed at 100 μ M relative to NDP-MSH (divided by a factor of 10 to keep these values on the same plot) for key mMC3R and mMC4R compounds, using the same color scale as described in **A**. Here, it can be observed that **18** (red) is an improvement upon previous polypharmacological probes as it is more potent at the mMC3R and mMC4R, and possesses decreased partial agonist efficacy. Additionally, the reduction in mMC4R potency and percent receptor activation while maintaining mMC3R potency can be observed for the most selective compound discovered in this study, compound **42** (blue).

MC3R vs. MC4R Selectivity

CMPD ID	Ac	R1	R2	R3	R4	NH ₂	mMC3R EC ₅₀ (nM)	mMC4R EC ₅₀ (nM)	% Activation	pA ₂	Fold Selectivity (MC4R/MC3R)
42	Ac	Val	Gln	(pI)DPhe	DTic	NH ₂	50	5,000	A	<5.5	100
26	Ac	Arg	Gln	(pI)DPhe	DTic	NH ₂	15	1,300	B	6.8	87
30	Ac	Arg	Gln	(pCl)DPhe	DTic	NH ₂	105	6,000	B	5.9	57
1	Ac	His	Arg	(pI)DPhe	Tic	NH ₂	30	1,200	B	6.6	40
39	Ac	Val	Arg	(pCl)DPhe	Cha	NH ₂	40	1,600	B	6.4	40
41	Ac	Val	Gln	(pI)DPhe	Tic	NH ₂	100	3,000	B	6.1	30
34	Ac	Val	Arg	(pI)DPhe	DTic	NH ₂	6	160	A	7.6	27
10	Ac	His	Gln	(pI)DPhe	DTic	NH ₂	270	6,500	A	<5.5	24
27	Ac	Arg	Gln	(pI)DPhe	Cha	NH ₂	100	2,300	A	6.7	23
33	Ac	Val	Arg	(pI)DPhe	Tic	NH ₂	4	90	B	6.7	23
6	Ac	His	Arg	(pCl)DPhe	DTic	NH ₂	210	3,100	A	6.5	15
9	Ac	His	Gln	(pI)DPhe	Tic	NH ₂	800	9000	B	<5.5	11
25	Ac	Arg	Gln	(pI)DPhe	Tic	NH ₂	40	410	B	6.2	10
44	Ac	Val	Gln	(pCl)DPhe	Tic	NH ₂	1,100	9,000	full agonist		8
38	Ac	Val	Arg	(pCl)DPhe	DTic	NH ₂	50	400	A	6.2	8
22	Ac	Arg	Arg	(pCl)DPhe	DTic	NH ₂	21	160	A	7	8
15	Ac	His	Gln	(pCl)DPhe	Cha	NH ₂	720	5,000	B	<5.5	7
7	Ac	His	Arg	(pCl)DPhe	Cha	NH ₂	60	380	A	6.4	6
31	Ac	Arg	Gln	(pCl)DPhe	Cha	NH ₂	210	1,100	B	6.2	5
37	Ac	Val	Arg	(pCl)DPhe	Tic	NH ₂	30	150	full agonist		5
23	Ac	Arg	Arg	(pCl)DPhe	Cha	NH ₂	60	260	A	7.1	4
17	Ac	Arg	Arg	(pI)DPhe	Tic	NH ₂	21	90	B	7.1	4
13	Ac	His	Gln	(pCl)DPhe	Tic	NH ₂	2,000	8,000	full agonist		4
18	Ac	Arg	Arg	(pI)DPhe	DTic	NH ₂	8	30	A	7.6	4
21	Ac	Arg	Arg	(pCl)DPhe	Tic	NH ₂	10	26	full agonist		3
29	Ac	Arg	Gln	(pCl)DPhe	Tic	NH ₂	500	1,100	full agonist		2
20	Ac	Arg	Arg	(pI)DPhe	DPro	NH ₂	3,000	6,000	A	<5.5	2
5	Ac	His	Arg	(pCl)DPhe	Tic	NH ₂	110	140	B	<5.5	1
19	Ac	Arg	Arg	(pI)DPhe	Cha	NH ₂	26	20% @ 100μM		7	
3	Ac	His	Arg	(pI)DPhe	Cha	NH ₂	50	> 100,000		6.6	
2	Ac	His	Arg	(pI)DPhe	DTic	NH ₂	51	20% @ 100μM		6.9	
35	Ac	Val	Arg	(pI)DPhe	Cha	NH ₂	60	30% @ 100μM		7	
11	Ac	His	Gln	(pI)DPhe	Cha	NH ₂	350	20% @ 100μM		6.1	
45	Ac	Val	Gln	(pCl)DPhe	DTic	NH ₂	700	60% @ 100μM		<5.5	
14	Ac	His	Gln	(pCl)DPhe	DTic	NH ₂	1,400	75% @ 100μM		<5.5	
36	Ac	Val	Arg	(pI)DPhe	DPro	NH ₂	4,400	45% @ 100μM		<5.5	
4	Ac	His	Arg	(pI)DPhe	DPro	NH ₂	10,000	20% @ 100μM		<5.5	
46	Ac	Val	Gln	(pCl)DPhe	DPro	NH ₂	16,000	20% @ 100μM		<5.5	
28	Ac	Arg	Gln	(pI)DPhe	DPro	NH ₂	18,000	> 100,000		<5.5	
40	Ac	Val	Arg	(pCl)DPhe	DPro	NH ₂	19,000	> 100,000		<5.5	
32	Ac	Arg	Gln	(pCl)DPhe	DPro	NH ₂	30,000	> 100,000		<5.5	
24	Ac	Arg	Arg	(pCl)DPhe	DPro	NH ₂	44,000	45% @ 100μM		<5.5	
8	Ac	His	Arg	(pCl)DPhe	DPro	NH ₂	50,000	> 100,000		<5.5	
12	Ac	His	Gln	(pI)DPhe	DPro	NH ₂	85% @ 100μM	> 100,000		<5.5	
16	Ac	His	Gln	(pCl)DPhe	DPro	NH ₂	45% @ 100μM	40% @ 100μM		<5.5	
43	Ac	Val	Gln	(pI)DPhe	DPro	NH ₂	70% @ 100μM	> 100,000		<5.5	

Figure 5.

Pseudo heat-map depicting the identification of MC3R versus MC4R selective compounds. Compounds are shown with a heat map ranging from blue (more MC3R selective vs. MC4R) to purple (less MC3R selective vs. MC4R). Pharmacological parameters considered more MC3R selective vs. MC4R (shown in blue) include a low MC3R EC₅₀, high MC4R EC₅₀, low MC4R percent receptor activation, and low MC4R pA₂. Pharmacological parameters considered less MC3R selective vs. MC4R (shown in purple) include a high MC3R EC₅₀, low MC4R EC₅₀, high MC4R percent receptor activation, and a high MC4R pA₂. There were no MC3R agonist ligands discovered in this study that did not possess any activity (agonist or antagonist) at the MC4R. Therefore, the MC3R agonist EC₅₀ (nM) values are provided along with the different mMC4R pharmacological parameters determined in this study: EC₅₀ (nM) and percent receptor activation for full or partial mMC4R agonists (percent receptor activation depicted as either A, if the compound resulted

in a partial agonist efficacy between 20–50%, or B, if the compound resulted in a partial agonist efficacy between 50–90%), percent receptor activation at 100 μ M for compounds that resulted in some receptor activation but did not produce a sigmoidal dose response, and antagonist pA_2 values. Fold selectivity determined using the EC_{50} values at both receptors is also provided. The desired MC3R vs. MC4R selective agonist should possess a low MC3R EC_{50} (nM) value, possess a high (or no) MC4R EC_{50} (nM) value, result in a low percent MC4R activation, and possess a low antagonist pA_2 value. Compounds that possess potency values colored in blue in all the columns were considered to be more MC3R vs. MC4R selective than the other compounds in this study. In this figure, compounds are arranged by fold MC3R vs. MC4R selectivity, determined using only the EC_{50} values at both receptors. The most selective MC3R vs. MC4R ligand discovered in this study, **42**, is also is the most potent MC3R ligand with blue bars in each column, and is highlighted in blue. The most selective compounds possessed an Ac-Xxx-Gln-Xxx-DTic-NH₂ motif. The compound identifiers of previously discovered peptides are identified in grey, and the compound identifiers for the two lead compounds (**18**, **42**) are highlighted in red and blue respectively.

Table 1:

Analytical Characterization Data for Peptides

Compound Number	Compound ID	Sequence				Mass Information (M)		HPLC Analysis ^a		Purity % ^b		
		Ac	R1	R2	R3	R4	NH ₂	Calculated	Observed		MeCN RT	MeOH RT
1 [*]	KAF4098-1	Ac	His	Arg	(pI)DPhe	Tic	NH ₂	784.2	783.8	15.2	23.6	>98
2	KAF4098-2	Ac	His	Arg	(pI)DPhe	DTic	NH ₂	784.2	784.3	14.3	22.4	>98
3	KAF4026-1	Ac	His	Arg	(pI)DPhe	Cha	NH ₂	778.3	778.4	16.7	27.2	>98
4	KAF4098-3	Ac	His	Arg	(pI)DPhe	DPro	NH ₂	722.2	722.6	10.5	16.5	>98
5	KAF4098-4	Ac	His	Arg	(pCl)DPhe	Tic	NH ₂	692.3	692.7	14.5	22.4	>95
6	KAF4098-5	Ac	His	Arg	(pCl)DPhe	DTic	NH ₂	692.3	692.4	13.6	21.2	>98
7	KAF4026-2	Ac	His	Arg	(pCl)DPhe	Cha	NH ₂	686.3	686.4	15.9	26.0	>98
8	KAF4098-6	Ac	His	Arg	(pCl)DPhe	DPro	NH ₂	630.3	630.5	9.6	15.0	>95
9	KAF4098-7	Ac	His	Gln	(pI)DPhe	Tic	NH ₂	756.2	756.3	15.9	25.2	>98
10	KAF4098-8	Ac	His	Gln	(pI)DPhe	DTic	NH ₂	756.2	756.3	15.1	24.1	>95
11	KAF4026-3	Ac	His	Gln	(pI)DPhe	Cha	NH ₂	750.2	749.9	17.5	28.9	>98
12	KAF4098-9	Ac	His	Gln	(pI)DPhe	DPro	NH ₂	694.8	694.3	10.8	17.4	>98
13	KAF4098-10	Ac	His	Gln	(pCl)DPhe	Tic	NH ₂	664.3	664.4	15.1	23.8	>95
14	KAF4098-11	Ac	His	Gln	(pCl)DPhe	DTic	NH ₂	664.3	663.9	14.2	22.5	>95
15	KAF4026-4	Ac	His	Gln	(pCl)DPhe	Cha	NH ₂	658.3	658.4	16.7	27.7	>98
16	KAF4098-12	Ac	His	Gln	(pCl)DPhe	DPro	NH ₂	602.2	601.8	9.6	15.4	>95
17 [*]	KAF4098-13	Ac	Arg	Arg	(pI)DPhe	Tic	NH ₂	803.3	803.2	15.2	23.8	>98
18	KAF4098-14	Ac	Arg	Arg	(pI)DPhe	DTic	NH ₂	803.3	803.1	14.4	22.5	>98
19	KAF4026-5	Ac	Arg	Arg	(pI)DPhe	Cha	NH ₂	797.3	797.6	16.5	27.7	>98
20	KAF4098-15	Ac	Arg	Arg	(pI)DPhe	DPro	NH ₂	741.3	741.2	10.7	17.0	>98
21	KAF4098-16	Ac	Arg	Arg	(pCl)DPhe	Tic	NH ₂	711.3	711.4	14.4	22.5	>95
22	KAF4098-17	Ac	Arg	Arg	(pCl)DPhe	DTic	NH ₂	711.3	711.3	13.4	20.9	>98
23	KAF4026-6	Ac	Arg	Arg	(pCl)DPhe	Cha	NH ₂	705.4	705.7	16.0	26.2	>98
24	KAF4098-18	Ac	Arg	Arg	(pCl)DPhe	DPro	NH ₂	649.3	649.6	9.6	15.3	>98
25	KAF4098-19	Ac	Arg	Gln	(pI)DPhe	Tic	NH ₂	775.2	775.7	15.9	25.1	>98

Compound Number	Compound ID	Sequence				Mass Information (M)		HPLC Analysis ^a		Purity % ^b		
		Ac	R1	R2	R3	R4	NH ₂	Calculated	Observed		MeCN RT	MeOH RT
26	KAF4098-20	Ac	Arg	Gln	(pI)DPhe	D ⁺ Tic	NH ₂	775.2	775.5	15.1	24	>98
27	KAF4026-7	Ac	Arg	Gln	(pI)DPhe	Cha	NH ₂	769.3	769.4	17.4	28.7	>98
28	KAF4098-21	Ac	Arg	Gln	(pI)DPhe	D ⁺ Pro	NH ₂	713.2	712.9	11	17.8	>95
29	KAF4098-22	Ac	Arg	Gln	(pCI)DPhe	Tic	NH ₂	683.3	683.4	15.2	24	>95
30	KAF4098-23	Ac	Arg	Gln	(pCI)DPhe	D ⁺ Tic	NH ₂	683.3	683.2	14.2	22.5	>98
31	KAF4026-8	Ac	Arg	Gln	(pCI)DPhe	Cha	NH ₂	677.3	677.6	16.6	27.4	>95
32	KAF4098-24	Ac	Arg	Gln	(pCI)DPhe	D ⁺ Pro	NH ₂	621.3	621.2	9.9	15.8	>95
33	KAF4098-25	Ac	Val	Arg	(pI)DPhe	Tic	NH ₂	746.2	746.1	17.7	27.6	>95
34	KAF4098-26	Ac	Val	Arg	(pI)DPhe	D ⁺ Tic	NH ₂	746.2	746.6	16.9	26.6	>98
35	KAF4026-9	Ac	Val	Arg	(pI)DPhe	Cha	NH ₂	740.3	740.5	19.3	30.1	>98
36	KAF4098-27	Ac	Val	Arg	(pI)DPhe	D ⁺ Pro	NH ₂	684.2	684.2	13.1	21.5	>98
37	KAF4098-28	Ac	Val	Arg	(pCI)DPhe	Tic	NH ₂	654.3	654.1	16.9	26.5	>95
38	KAF4098-29	Ac	Val	Arg	(pCI)DPhe	D ⁺ Tic	NH ₂	654.3	654	16.1	25.4	>98
39	KAF4026-10	Ac	Val	Arg	(pCI)DPhe	Cha	NH ₂	648.4	648.4	18.2	29.4	>98
40	KAF4098-30	Ac	Val	Arg	(pCI)DPhe	D ⁺ Pro	NH ₂	592.3	592.1	12.1	19.7	>95
41	KAF4098-31	Ac	Val	Gln	(pI)DPhe	Tic	NH ₂	718.2	718.3	18.1	28.7	>95
42	KAF4098-32	Ac	Val	Gln	(pI)DPhe	D ⁺ Tic	NH ₂	718.2	718.3	17.3	27.6	>98
43	KAF4098-33	Ac	Val	Gln	(pI)DPhe	D ⁺ Pro	NH ₂	656.2	656.3	13.2	22.2	>98
44	KAF4098-34	Ac	Val	Gln	(pCI)DPhe	Tic	NH ₂	626.3	626.4	17.3	27.5	>95
45	KAF4098-35	Ac	Val	Gln	(pCI)DPhe	D ⁺ Tic	NH ₂	626.3	626.3	16.3	26.3	>98
46	KAF4098-36	Ac	Val	Gln	(pCI)DPhe	D ⁺ Pro	NH ₂	564.3	564.4	12.2	20.4	>95

^aHPLC Analysis indicates the retention time of the peptide in either solvent system 1 (10% acetonitrile in 0.1% trifluoroacetic acid in water and a gradient of 90% acetonitrile over 35 minutes) or solvent system 2 (10% methanol to 0.1% trifluoroacetic acid in water and a gradient of 90% methanol over 35 minutes). An analytical Vydac C18 column (Vydac 218TP104) was used to obtain these values with a flow rate of 1.5 mL/min.

^bPurity of the peptides was determined through analysis of the HPLC chromatographs at a wavelength of 214 nm.

* These compounds have been previously reported.⁵³

Table 2:

Pharmacology of Tetrapeptides at the mMC3R and mMC4R

Compound Number	Sequence						mMC3R	mMC4R	
	Ac	R1	R2	R3	R4	NH ₂	Agonist EC ₅₀ (nM)	Agonist EC ₅₀ (nM)	Antagonist pA ₂
NDP-MSH							0.414 ± 0.009	0.279 ± 0.004	
1 *	Ac	His	Arg	(pI)DPhe	Tic	NH ₂	30 ± 10	1,200 ± 800 (B)	6.6 ± 0.2
2	Ac	His	Arg	(pI)DPhe	DTic	NH ₂	51 ± 5	20% @ 100 μM	6.9 ± 0.2
3	Ac	His	Arg	(pI)DPhe	Cha	NH ₂	50 ± 30	>100,000	6.6 ± 0.2
4	Ac	His	Arg	(pI)DPhe	DPro	NH ₂	10,000 ± 5,000	20% @ 100 μM	<5.5
5	Ac	His	Arg	(pCl)DPhe	Tic	NH ₂	110 ± 30	140 ± 20 (B)	<5.5
6	Ac	His	Arg	(pCl)DPhe	DTic	NH ₂	210 ± 70	3,100 ± 2,200 (A)	6.5 ± 0.1
7	Ac	His	Arg	(pCl)DPhe	Cha	NH ₂	60 ± 10	380 ± 150 (A)	6.4 ± 0.1
8	Ac	His	Arg	(pCl)DPhe	DPro	NH ₂	50,000 ± 10,000	>100,000	<5.5
9	Ac	His	Gln	(pI)DPhe	Tic	NH ₂	800 ± 200	9,000 ± 3,000 (B)	<5.5
10	Ac	His	Gln	(pI)DPhe	DTic	NH ₂	270 ± 60	6,500 ± 2,200 (A)	<5.5
11	Ac	His	Gln	(pI)DPhe	Cha	NH ₂	350 ± 60	20% @ 100 μM	6.1 ± 0.2
12	Ac	His	Gln	(pI)DPhe	DPro	NH ₂	85% @ 100 μM	>100,000	<5.5
13	Ac	His	Gln	(pCl)DPhe	Tic	NH ₂	2,000 ± 500	8,000 ± 1,000	
14	Ac	His	Gln	(pCl)DPhe	DTic	NH ₂	1,400 ± 400	75% @ 100 μM	<5.5
15	Ac	His	Gln	(pCl)DPhe	Cha	NH ₂	720 ± 30	5,000 ± 1,000 (B)	<5.5
16	Ac	His	Gln	(pCl)DPhe	DPro	NH ₂	45% @ 100 μM	40% @ 100 μM	<5.5
17 *	Ac	Arg	Arg	(pI)DPhe	Tic	NH ₂	21 ± 14	90 ± 40 (B)	7.1 ± 0.2
18	Ac	Arg	Arg	(pI)DPhe	DTic	NH ₂	8 ± 5	30 ± 10 (A)	7.6 ± 0.3
19	Ac	Arg	Arg	(pI)DPhe	Cha	NH ₂	26 ± 9	20% @ 100 μM	7 ± 0.1
20	Ac	Arg	Arg	(pI)DPhe	DPro	NH ₂	3,000 ± 300	6,000 ± 2,000 (A)	<5.5
21	Ac	Arg	Arg	(pCl)DPhe	Tic	NH ₂	10 ± 3	26 ± 3	
22	Ac	Arg	Arg	(pCl)DPhe	DTic	NH ₂	21 ± 7	160 ± 60 (A)	7 ± 0.2
23	Ac	Arg	Arg	(pCl)DPhe	Cha	NH ₂	60 ± 20	260 ± 90 (A)	7.1 ± 0.3
24	Ac	Arg	Arg	(pCl)DPhe	DPro	NH ₂	44,000 ± 34,000	45% @ 100 μM	<5.5
25	Ac	Arg	Gln	(pI)DPhe	Tic	NH ₂	40 ± 10	410 ± 50 (B)	6.2 ± 0.3
26	Ac	Arg	Gln	(pI)DPhe	DTic	NH ₂	15 ± 5	1,300 ± 400 (B)	6.8 ± 0.4
27	Ac	Arg	Gln	(pI)DPhe	Cha	NH ₂	100 ± 30	2,300 ± 2,100 (A)	6.7 ± 0.2
28	Ac	Arg	Gln	(pI)DPhe	DPro	NH ₂	18,000 ± 4,000	>100,000	<5.5
29	Ac	Arg	Gln	(pCl)DPhe	Tic	NH ₂	500 ± 200	1,100 ± 200	
30	Ac	Arg	Gln	(pCl)DPhe	DTic	NH ₂	105 ± 6	6,000 ± 1,000 (B)	5.9 ± 0.2
31	Ac	Arg	Gln	(pCl)DPhe	Cha	NH ₂	210 ± 40	1,100 ± 300 (B)	6.2 ± 0.1
32	Ac	Arg	Gln	(pCl)DPhe	DPro	NH ₂	30,000 ± 10,000	>100,000	<5.5
33	Ac	Val	Arg	(pI)DPhe	Tic	NH ₂	4 ± 1	90 ± 20 (B)	6.7 ± 0.1
34	Ac	Val	Arg	(pI)DPhe	DTic	NH ₂	6 ± 2	160 ± 60 (A)	7.6 ± 0.5

Compound Number	Sequence					mMC3R		mMC4R	
	Ac	R1	R2	R3	R4	NH ₂	Agonist EC ₅₀ (nM)	Agonist EC ₅₀ (nM)	Antagonist pA ₂
35	Ac	Val	Arg	(pI)DPhe	Cha	NH ₂	60 ± 30	30% @ 100 μM	7 ± 0.2
36	Ac	Val	Arg	(pI)DPhe	DPro	NH ₂	4,400 ± 800	45% @ 100 μM	<5.5
37	Ac	Val	Arg	(pCl)DPhe	Tic	NH ₂	30 ± 10	150 ± 40	
38	Ac	Val	Arg	(pCl)DPhe	DTic	NH ₂	50 ± 20	400 ± 100 (A)	6.2 ± 0.2
39	Ac	Val	Arg	(pCl)DPhe	Cha	NH ₂	40 ± 10	1,600 ± 1,200 (B)	6.4 ± 0.1
40	Ac	Val	Arg	(pCl)DPhe	DPro	NH ₂	19,000 ± 7,000	>100,000	<5.5
41	Ac	Val	Gln	(pI)DPhe	Tic	NH ₂	100 ± 10	3,000 ± 800 (B)	6.1 ± 0.3
42	Ac	Val	Gln	(pI)DPhe	DTic	NH ₂	50 ± 10	5,000 ± 2,000 (A)	<5.5
43	Ac	Val	Gln	(pI)DPhe	DPro	NH ₂	70% @ 100 μM	>100,000	<5.5
44	Ac	Val	Gln	(pCl)DPhe	Tic	NH ₂	1,100 ± 200	9,000 ± 900	
45	Ac	Val	Gln	(pCl)DPhe	DTic	NH ₂	700 ± 100	60% @ 100 μM	<5.5
46	Ac	Val	Gln	(pCl)DPhe	DPro	NH ₂	16,000 ± 4,000	20% @ 100 μM	<5.5

The values expressed here as the mean ± the standard error of the mean (SEM) of a minimum of three independent experiments consisting of duplicate replicates. Compounds that activated the receptor >90% of the NDP-MSH control were considered full agonist compounds. Compounds that did not produce >90% receptor activation were assayed as antagonists using a Schild analysis with NDP-MSH as the agonist, for which a pA₂ value is reported [pA₂ = -log(K_i)].⁵⁹ Compounds that resulted in receptor activation of less than 20% at 100 μM relative to NDP-MSH are listed as "> 100,000". Compounds that resulted in some receptor activation at 100 μM but did not produce a sigmoidal dose-response are listed by the percent receptor activation observed at 100 μM (%X @ 100 μM). For partial agonist compounds EC₅₀ (nM) potency values are provided. "A" indicates that a partial agonist resulted in percent receptor activation from 20–50%, and "B" indicates that the partial agonist resulted in percent receptor activation from 50–90% (see Figure 1). The use of "<5.5" indicates that no antagonist potency was observed at up to 10 μM concentrations.

* These compounds have been previously reported

Table 3:

Agonist Pharmacology of Tetrapeptides at the mMC1R and mMC5R

Compound Number	Sequence						mMC1R EC ₅₀ (nM)	mMC5R EC ₅₀ (nM)
	Ac	R1	R2	R3	R4	NH ₂		
NDP-MSH							0.051 ± 0.003	1.98 ± 0.08
1 *	Ac	His	Arg	(pI)DPhe	Tic	NH ₂	1.3 ± 0.9	9 ± 3
2	Ac	His	Arg	(pI)DPhe	DTic	NH ₂	1.0 ± 0.6	18 ± 6
3	Ac	His	Arg	(pI)DPhe	Cha	NH ₂	2.4 ± 1	5 ± 1
4	Ac	His	Arg	(pI)DPhe	DPro	NH ₂	300 ± 100	4,200 ± 2,800
5	Ac	His	Arg	(pCI)DPhe	Tic	NH ₂	0.3 ± 0.05	50 ± 20
6	Ac	His	Arg	(pCI)DPhe	DTic	NH ₂	2.0 ± 0.9	80 ± 70
7	Ac	His	Arg	(pCI)DPhe	Cha	NH ₂	5 ± 2	11 ± 4
8	Ac	His	Arg	(pCI)DPhe	DPro	NH ₂	1,800 ± 700	28,000 ± 16,000
9	Ac	His	Gln	(pI)DPhe	Tic	NH ₂	250 ± 120	50 ± 30
10	Ac	His	Gln	(pI)DPhe	DTic	NH ₂	400 ± 100	55 ± 37
11	Ac	His	Gln	(pI)DPhe	Cha	NH ₂	500 ± 200	50 ± 40
12	Ac	His	Gln	(pI)DPhe	DPro	NH ₂	11,000 ± 6,000	9,000 ± 3,000
13	Ac	His	Gln	(pCI)DPhe	Tic	NH ₂	80 ± 10	2,400 ± 1,700
14	Ac	His	Gln	(pCI)DPhe	DTic	NH ₂	400 ± 200	140 ± 70
15	Ac	His	Gln	(pCI)DPhe	Cha	NH ₂	400 ± 100	40 ± 10
16	Ac	His	Gln	(pCI)DPhe	DPro	NH ₂	15,000 ± 4,000	27,000 ± 7,000
17 *	Ac	Arg	Arg	(pI)DPhe	Tic	NH ₂	0.3 ± 0.1	2.5 ± 1.3
18	Ac	Arg	Arg	(pI)DPhe	DTic	NH ₂	0.6 ± 0.2	2.2 ± 1.1
19	Ac	Arg	Arg	(pI)DPhe	Cha	NH ₂	4 ± 2	3 ± 1
20	Ac	Arg	Arg	(pI)DPhe	DPro	NH ₂	24 ± 5	1,200 ± 300
21	Ac	Arg	Arg	(pCI)DPhe	Tic	NH ₂	0.4 ± 0.1	3 ± 0.7
22	Ac	Arg	Arg	(pCI)DPhe	DTic	NH ₂	0.3 ± 0.1	5 ± 2
23	Ac	Arg	Arg	(pCI)DPhe	Cha	NH ₂	2 ± 1	4 ± 1
24	Ac	Arg	Arg	(pCI)DPhe	DPro	NH ₂	70 ± 20	1,300 ± 400
25	Ac	Arg	Gln	(pI)DPhe	Tic	NH ₂	15 ± 2	3 ± 1
26	Ac	Arg	Gln	(pI)DPhe	DTic	NH ₂	10 ± 3	1.6 ± 0.2
27	Ac	Arg	Gln	(pI)DPhe	Cha	NH ₂	80 ± 30	4.6 ± 0.8
28	Ac	Arg	Gln	(pI)DPhe	DPro	NH ₂	3,000 ± 1,000	1,800 ± 200
29	Ac	Arg	Gln	(pCI)DPhe	Tic	NH ₂	40 ± 20	13 ± 6
30	Ac	Arg	Gln	(pCI)DPhe	DTic	NH ₂	40 ± 20	30 ± 20
31	Ac	Arg	Gln	(pCI)DPhe	Cha	NH ₂	100 ± 60	18 ± 6
32	Ac	Arg	Gln	(pCI)DPhe	DPro	NH ₂	3,600 ± 900	9,000 ± 3,000
33	Ac	Val	Arg	(pI)DPhe	Tic	NH ₂	0.3 ± 0.1	1.4 ± 0.4
34	Ac	Val	Arg	(pI)DPhe	DTic	NH ₂	0.2 ± 0.06	3.4 ± 0.6

Compound Number	Sequence						mMC1R EC ₅₀ (nM)	mMC5R EC ₅₀ (nM)
	Ac	R1	R2	R3	R4	NH ₂		
35	Ac	Val	Arg	(pI)DPhe	Cha	NH ₂	2 ± 1	5 ± 3
36	Ac	Val	Arg	(pI)DPhe	DPro	NH ₂	30 ± 10	600 ± 200
37	Ac	Val	Arg	(pCl)DPhe	Tic	NH ₂	0.2 ± 0.06	40 ± 20
38	Ac	Val	Arg	(pCl)DPhe	DTic	NH ₂	0.6 ± 0.1	52 ± 2
39	Ac	Val	Arg	(pCl)DPhe	Cha	NH ₂	0.7 ± 0.3	8 ± 3
40	Ac	Val	Arg	(pCl)DPhe	DPro	NH ₂	130 ± 70	10,000 ± 1,000
41	Ac	Val	Gln	(pI)DPhe	Tic	NH ₂	30 ± 10	240 ± 170
42	Ac	Val	Gln	(pI)DPhe	DTic	NH ₂	40 ± 0	13 ± 4
43	Ac	Val	Gln	(pI)DPhe	DPro	NH ₂	2,000 ± 900	4,000 ± 2,000
44	Ac	Val	Gln	(pCl)DPhe	Tic	NH ₂	21 ± 7	820 ± 760
45	Ac	Val	Gln	(pCl)DPhe	DTic	NH ₂	170 ± 60	90 ± 40
46	Ac	Val	Gln	(pCl)DPhe	DPro	NH ₂	1,100 ± 600	7,400 ± 700

The values expressed here as the mean ± the standard error of the mean (SEM) of a minimum of three independent experiments consisting of duplicate replicates. All of these compounds are full agonists.

* These compounds have been previously reported.⁵³