Published in final edited form as:

J Med Chem. 2018 September 13; 61(17): 7729–7740. doi:10.1021/acs.jmedchem.8b00684.

Synergistic Multi-Residue 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

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Abstract

Antagonist ligands of the melanocortin-3 and -4 receptors (MC3R, MC4R), including agouti-related protein (AGRP), are postulated to be targets for the treatment of diseases of negative energy balance. Previous studies reported the macrocyclic MC3R/MC4R antagonist c[Pro¹-Arg²-Phe³-Phe⁴-Asn⁵-Ala⁶-Phe⁷-DPro⁸], which is 250-fold less potent at the mouse (m) mMC3R and 3-fold less potent at the mMC4R than AGRP. Previous studies explored the structure-activity relationships around individual positions in this template. Herein, a multiresidue substitution strategy is utilized, combining the lead sequence with hPhe⁴, Dap⁵, Arg⁵, Ser⁶, and Nle⁷ substitutions previously reported. Two compounds from this study (**16**, **20**) contain an hPhe⁴/Ser⁶/Nle⁷ substitution pattern, are 3–6 fold more potent than AGRP at the mMC4R, and are 600–800 fold selective for the mMC4R over the mMC3R. Another lead compound (**21**), possessing the hPhe⁴/Arg⁵ substitutions, is only 5-fold less potent than AGRP at the mMC3R and is equipotent to AGRP at the mMC4R.

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Keywords

AGRP; structure-activity relationships; melanocortin receptors; macrocycles

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Author Contributions. K.A.F., M.D.E., and C.H.-L. designed the research. K.A.F. and K.T.F. performed experiments. K.A.F. and C.H.-L. analyzed the data. K.A.F. wrote the manuscript with the help of C.H.-L.

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

Introduction

There have been five G protein-coupled melanocortin receptors (GPCRs, MC1–5R) discovered to date. Following stimulation by agonist ligands, these receptors couple to stimulatory G-proteins (producing an increase in intracellular cyclic AMP [cAMP]) and to β -Arrestin (resulting in receptor internalization). Naturally occurring agonists for these receptors, produced after posttranslational processing of the proopiomelanocortin (POMC) gene transcript, include the adrenocorticotrophic hormone (ACTH) and the α -, β -, and γ -melanocyte stimulating hormones (MSHs). Endogenous antagonists for this receptor include agouti signaling protein (ASP) and agouti-related protein (AGRP). Many components of the melanocortin system have been found to play roles in energy homeostasis, including the MC3R, MC4R, POMC-derived agonists, ASP, and AGRP. There have been numerous peptide and small molecule melanocortin ligands discovered to date, as reviewed by Ericson *et al.* Agonist ligands for these receptors have made attractive targets for diseases like metabolic syndrome, while antagonist ligands are postulated to be treatments for diseases of negative energy balance due to their ability to increase food intake.

In rat models of anorexia, self-starvation can be induced by restriction of food in the presence of running wheels. It has been proposed that insufficient central melanocortin receptor suppression may be the cause of this phenotype, which can be alleviated by central administration of AGRP. In transgenic mouse models, mice overexpressing AGRP weigh more than wild type control mice. Additionally, administration of AGRP increases food intake in mice. It has been thought that melanocortin receptor antagonist ligands may be viable therapeutics for the treatment of diseases in which an increase in food intake is desired, such as anorexia nervosa, cachexia, and failure to thrive in children.

Studies using recombinant human (h)AGRP demonstrated that AGRP is an antagonist of the endogenous agonist α -MSH at the hMC3R and hMC4R, and does not have antagonist activity at the hMC1R. At the constitutively active hMC4R and at a mutated mouse (m)MC4R that is constitutively active, the active C-terminal domain of AGRP [AGRP(83–132)] has been reported to possess inverse agonist activity. The active loop of AGRP possesses the sequence c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys], containing the hypothesized Arg-Phe-Phe tripeptide antagonist sequence. Structure-activity relationship (SAR) studies have investigated this core scaffold. Notably, the decapeptide containing the active loop sequence H-Tyr[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-OH (AGRP[109–118]) is a micromolar agonist at the MC1R ($EC_{50} = 3 \mu M$), and does not possess quantifiable antagonist potency at the mMC3R at up to $10 \mu M$ concentrations. However, Schild analysis of antagonist potency has indicated that AGRP(109–118) possesses submicromolar antagonist potency at the MC4R. Many subsequent studies have observed similar melanocortin receptor pharmacology, including agonist activity at the mMC1R, micromolar potency at the mMC3R, and a range of potencies at the mMC4R (generally ranging from micromolar to nanomolar).

NMR structural studies performed on AGRP have demonstrated that the active loop sequence of AGRP forms a β -hairpin, stabilized by five disulfide bonds.[–] In macrocyclic peptides, it has been previously established that heterochiral proline residues can induce a β -hairpin turn conformation.[–] This rationale led to the development of the macrocyclic octapeptide c[Pro¹–Arg²–Phe³–Phe⁴–Asn⁵–Ala⁶–Phe⁷–DPro⁸], containing the active loop sequence of AGRP cyclized through a DPro-Pro motif rather than through a disulfide bond via the endogenous Cys residues. This motif was hypothesized to better allow the macrocycle to adopt a β -hairpin loop structure, allowing it to better recapitulate the structure observed in the full-length hormone of the naturally occurring tripeptide antagonist sequence, and therefore increase potency. In comparison to the decapeptide discussed above, which possesses a pA₂ at the mMC4R of 6.8, this macrocyclic octapeptide (peptide **1** in this study) possesses a pA₂ of 7.7–8.1, depending on the cAMP assay utilized.[•]

Although peptide **1** possessed increased potency compared to the disulfide cyclized peptide at the mMC4R, it still does not match the potency of the native hormone AGRP (pA₂ = 8.7–9.1). Therefore, the SAR around this octapeptide scaffold has been explored in order to generate more potent and/or selective MC4R antagonists.[–] These SAR analyses have been performed on the Phe³, Phe⁴, Asn⁵, Ala⁶, and Phe⁷ positions. The results of these previous studies at the mMC4R are summarized in Figure 1.[–] One substitution in the Phe position (hPhe⁴) possessed increased potency compared to the lead at the mMC4R. Numerous substitutions were found to increase potency at the Asn⁵ position.[–] One substitution at the Ala⁶ position (Ser⁶) was found to be equipotent to the lead compound. Two types of amino acids maintained MC4R antagonist potency at the Phe⁷ position: aromatic (Trp⁷, Tyr⁷), and linear aliphatic (Ala⁷, Nle⁷).[–] In particular, it was noted that peptides possessing linear aliphatic amino acids in the 7 position displayed increased selectivity for the mMC4R over the mMC3R compared to the lead. While the mMC4R possesses a varied SAR based on the substitutions examined in these studies, with pA₂ values ranging from micromolar to nanomolar, most of these ligands possess micromolar antagonist activity at the mMC3R or no activity at all. Three substitutions examined possessed potency values of 7.0 or greater at the mMC3R, and these ligands possessed a Gly⁵ substitution (pA₂ = 7.6), Dab⁵ substitution (pA₂ = 7.0), and Lys⁵ substitution (pA₂ = 7.0). Some peptides examined in these studies have appeared to possess inverse agonist activity at the mMC5R, and contain a variety of substitutions to the Asn⁵, Ala⁶, and Phe⁷ positions.[–]

Selective antagonist ligands for the mMC4R will make useful pharmacological probes, and can be used to understand the role of the melanocortin system in the etiology of diseases like anorexia, cachexia, and failure-to-thrive. Therefore, the development of potent and selective pharmacological probes is desired. Furthermore, selective mMC4R antagonist activity could potentially be desirable from a therapeutic perspective for certain patient populations. Mice deficient in the mMC3R have been shown to possess dysregulated fasted-related feeding behaviors such as food anticipatory activity, gorging, and motivational responses.^{••} It is currently unknown if these behaviors in mice translate to behaviors in humans. It could be speculated that an mMC4R-selective antagonist may be a useful therapeutic for patients where an increase in food intake is desired, but the modulation of such MC3R-mediated feeding behaviors may be harmful and/or reduce patient compliance, such as in patients with

anorexia. The current study expands upon previous reports by utilizing a multiresidue-substitution strategy in this core octapeptide macrocyclic template. By combining substitutions which increase mMC4R potency (hPhe⁴, Dap⁵, Arg⁵), substitutions that are contained in ASP (Arg⁵, Ser⁶), and substitutions that increase selectivity for the mMC4R (Nle⁷), it was hypothesized that the pharmacological properties of this peptide could be fine-tuned to generate potent and selective probes for the mMC4R.

Results

Peptide Synthesis and Characterization.

The peptides used in this study were synthesized with a semi-automated synthesizer using standard fluorenylmethoxycarbonyl (Fmoc) methodology. The singly-substituted macrocyclic octapeptides that have been previously reported were resynthesized, characterized, and assayed for these studies. The structures of the amino acids used are provided in Figure 2. The peptides reported herein were purified using semi-preparative reverse phase high-pressure liquid chromatography (RP-HPLC). The purity of the peptides was confirmed to be >95% via analytical RP-HPLC (RP-HPLC traces may be found in supplementary Figure S1 through Figure S24) using two different solvent systems. The masses of the peptides were confirmed using matrix-assisted laser desorption ionization (MALDI) time of flight (TOF) mass spectrometry or electrospray ionization (ESI) time of flight (TOF) mass spectrometry (University of Minnesota LeClaire-Dow Instrumentation Facility). Analytical characterization data for these peptides may be found in Supplemental Table S1.

Initially, compounds were assayed as agonists in HEK293 cells stably expressing the mMC1R, mMC3R, mMC4R, or mMC5R using the AlphaScreen cAMP Assay. The results of these experiments for agonist activity at the mMC1R and inverse agonist activity at the mMC5R are summarized in Table 1. None of the library compounds possessed a greater than 20% agonist response at 100 μ M concentrations at the mMC3R or mMC4R, so antagonist potencies were measured for these compounds. Competitive antagonist potencies of the macrocyclic peptides were measured using Schild analyses at the mMC3R and mMC4R with the synthetic, non-selective, potent melanocortin agonist NDP-MSH. The results of these antagonist experiments at the mMC3R and mMC4R are summarized in Table 2. The fold-changes in potency discussed herein were calculated using the K_i values derived from the Schild analysis [$pA_2 = -\log(K_i)$].

Library Design.

In this study, individual substitutions to the macrocyclic octapeptide template were identified from previous reports. The hPhe⁴, Ser⁶, and Nle⁷ substitutions were chosen because of their decreased antagonist activity at the mMC3R while maintaining mMC4R antagonist potency. The Dap⁵ residue was chosen because it increased mMC4R antagonist potency (Dap⁵). Additionally, the Arg⁵ and Ser⁶ substitutions were chosen because these amino acids are present in the active loop of ASP, not AGRP, and it has been previously observed that the substitution of ASP active-loop residues into the AGRP-based macrocyclic template maintain mMC4R antagonist potency (Arg⁵, Ser⁶). In the current study, it was hypothesized

that combining multiple substitutions and perhaps a synergistic outcome of multiple substitutions would result in increased potency and selectivity at the mMC4R.

MC1R Activity.

Twenty-one of the 24 peptides examined in this study did not possess full agonist potency at the mMC1R. Of these peptides, seven possessed partial agonist activity wherein they did not stimulate the receptor up to 100% of NDP-MSH but were able to generate a sigmoidal dose response, five resulted in some agonist activity at 100 μ M concentrations, and nine did not possess any agonist activity at up to 100 μ M. The remaining three peptides resulted in full agonist activity at the mMC1R.

MC5R Activity.

Twenty-one of the 24 peptides examined in this study did not result in any receptor activation at up to 100 μ M. Two peptides appeared to result in inverse agonist activity with an apparent sigmoidal dose-response, and one peptide resulted in some decrease in receptor activation at up to 100 μ M relative to basal.

MC3R Activity.

All the peptides synthesized in these studies possessed antagonist activity at the mMC3R against agonist ligand NDP-MSH. One of these peptides possessed an antagonist pA₂ potency value between 5.5–6.0. Eleven of these peptides possessed an antagonist pA₂ potency value between 6.0–7.0. Eleven of these peptides possessed an antagonist pA₂ potency value between 7.0–8.0. One peptide possessed an antagonist pA₂ potency value of 8.0 or greater.

MC4R Activity.

All the peptides synthesized in these studies possessed antagonist activity at the mMC4R against agonist ligand NDP-MSH. Notably, none of the peptides synthesized and investigated as part of these studies possessed an antagonist pA₂ potency value of less than 8.0. Three of the peptides possessed an antagonist pA₂ potency value between 8.0–8.5. Nine of the peptides possessed an antagonist pA₂ potency value between 8.5–9.0. Nine of the peptides possessed an antagonist pA₂ potency value between 9.0–9.5. Three of the peptides possessed an antagonist pA₂ potency value of 9.5 or greater.

Discussion and Conclusions

MC1R Structure-Activity Relationship.

As has been previously reported, many of these peptides possessed micromolar agonist activity at the mMC1R.[–] Most of the peptides that generated a sigmoidal dose-response and thus possessed quantifiable EC₅₀ values did not possess the hPhe⁴ or Arg⁵ substitutions. Generally, peptides possessing the hPhe⁴ substitution did not possess agonist activity at the mMC1R at up to 100 μ M concentrations (**14**, which possessed hPhe⁴ in combination with Nle⁷; **16**, which possessed the hPhe⁴, Ser⁶, and Nle⁷ substitutions; **22**, which possessed the hPhe⁴, Arg⁵, and Nle⁷ substitutions; **18**, which possessed hPhe⁴, Dap⁵, and Nle⁷

substitutions; and **20**, which possessed hPhe⁴, Dap⁵, Ser⁶, and Nle⁷ substitutions). Two of the hPhe⁴-substituted peptides resulted in partial MC1R activation between 20–30% at 100 μ M concentrations (**23**, which possessed hPhe⁴, Arg⁵, and Ser⁶ substitutions; and **21**, which possessed hPhe⁴ and Arg⁵ substitutions), and three hPhe⁴-substituted peptides produced an assay response between 30%–40% at 100 μ M concentrations (**13**, possessing only the hPhe⁴ substitution; **15**, possessing the hPhe⁴ and Ser⁶ substitution; **19**, possessing the hPhe⁴, Dap⁵, and Ser⁶ substitutions). Two of the hPhe⁴-substituted peptides were partial agonists: **17**, possessing the hPhe⁴ and Dap⁵ substitutions (EC_{50} = 580 nM, 70% receptor activation), and **24**, which possessed the hPhe⁴, Arg⁵, Ser⁶, and Nle⁷ substitutions (EC_{50} = 400 nM, 25% receptor activation).

MC5R Structure-Activity Relationship.

It has previously been observed that some peptides derived from this macrocyclic template may result in some inverse agonist activity at the mMC5R.[–] In this library, inverse agonist activity was observed for three peptides. One of these compounds possessed the Nle⁷-substitution (**2**), one of these possessed the Ser⁶-substitution (**3**), and the last one possessed both the Ser⁶ and Nle⁷ substitutions (**4**). For the remainder of these peptides, inverse agonist activity was not observed at the mMC5R. Both the Nle⁷-substituted peptide and the Ser⁶-substituted peptide have been previously reported to possess inverse agonist activity at the mMC5R.[–]

mMC3R Structure Activity Relationship.

Nearly all the compounds, except for **13** (the hPhe⁴-substituted compound), were either approximately equipotent to or more potent than the lead compound **1** at the mMC3R. Notably, 6 compounds possessed increased potencies of greater than 10-fold at the mMC3R compared to **1**. These compounds possessed the following substitutions: Arg⁵ (**9**), Arg⁵ and Nle⁷ (**10**), or a combination of the hPhe⁴ and Arg⁵ substitution (**21**, **22**, **23**, and **24**). Although by itself the hPhe⁴-substitution reduced potency at the mMC3R 5-fold compared to **1**, the macrocycles that possess this substitution combined with the Arg⁵ substitution all possessed increased potencies ranging from 16-fold to 47-fold. The combination of the hPhe⁴ and Arg⁵ substitutions appeared to result in a synergistic increase in mMC3R potency. The pharmacological results from combining these two substitutions were unexpected, as the singly-substituted hPhe⁴ compound decreased mMC3R potency, and showed that the utilization of a multiresidue substitution strategy may lead to results that are counterintuitive to what may initially be speculated.

mMC4R Structure-Activity Relationship.

Nine peptides possessed 10-fold or greater increased potency at the mMC4R compared to **1**. Many of these peptides possessed the Nle⁷ substitution in combination with another substitution, including Dap⁵, Ser⁶, and Nle⁷ (**8**), Arg⁵ and Nle⁷ (**10**), Arg⁵, Ser⁶, and Nle⁷ (**12**), or hPhe⁴, Ser⁶, and Nle⁷ (**16**). Neither the Nle⁷ or Ser⁶ individual (**2** or **3**) substitutions or the Nle⁷/Ser⁶ combination (**4**) increased potency greater than 10-fold. This Ser⁶/Nle⁷ motif appeared to have worked best when combined with other substitutions to synergistically result in at least a 10-fold increase in mMC4R activity compared to **1**.

mMC4R Selectivity Structure-Activity Relationship.

Many of the individual substitutions were selected for their ability to potentiate selectivity for the mMC4R over the mMC3R. The structure-activity relationship of these compounds at the mMC3R and mMC4R can be found in Figure 3. Interestingly, one of the peptides in this study displayed a 7-fold decrease in mMC4R selectivity compared to **1**. This peptide was **21**, which possessed hPhe⁴ and Arg⁵ substitutions. The dose response curves of this compound at the mMC3R and mMC4R can be observed in Figure 4. This peptide possessed the highest fold-increase in potency at the mMC3R (47-fold) and a 6-fold increase in potency at the mMC4R. As a result, it was 8-fold selective for the mMC4R over the mMC3R. It also possessed nanomolar antagonist potency at the mMC3R, which is unusual for peptides of this scaffold, as peptides containing the DPro-Pro macrocyclic scaffold used in this study have decreased mMC4R selectivity via maintaining micromolar antagonist activity at the mMC3R and possessing a decreased potency at the mMC4R compared to the lead scaffold. Instead, this peptide possessed pA₂ values of 8.0±0.3 and 8.9±0.3 at the mMC3R and mMC4R respectively. Additionally, this peptide nearly recapitulated the antagonist pharmacology observed in AGRP (despite its reduced size), which has previously been reported to possess pA₂ of 8.7 at both the mMC3R and mMC4R. This indicates a promising direction in the development of peptides utilizing this scaffold that possess the same antagonist potency at both the MC3R and MC4R as the native hormone, despite the size difference between the C-terminal domain of AGRP and the 8-residue macrocycle.

Previous studies using a β-galactosidase reporter gene assay (compared to the cAMP accumulation assay utilized in this study) discovered that the hPhe⁴-substituted peptide did not possess antagonist activity at the mMC3R and was 5-fold less potent than **1** at the mMC4R, therefore making it selective for the mMC4R. Other reports, using the same assay as this study, found that the Nle⁷-substituted peptide increased selectivity for the mMC4R over the mMC3R (albeit to a lesser extent than the hPhe⁴-substituted peptide) while maintaining mMC4R antagonist potency. In the present study, the hPhe⁴-substituted peptide (**13**) possessed antagonist potency at the mMC3R (pA₂ = 5.6±0.2) and was equipotent to the lead peptide at the mMC4R, making it approximately 500-fold selective for the mMC4R over the mMC3R. Overall, peptides that possessed the hPhe⁴-substitution were more selective for the mMC4R over the mMC3R (excluding peptides that possessed both hPhe⁴ and Arg⁵ substitutions, which increased mMC3R antagonist potency, as discussed above). Interestingly, although hPhe⁴ and Nle⁷ substitutions were chosen due to their ability to increase mMC4R selectivity, the peptide that possessed both the hPhe⁴ and Nle⁷ substitution (**14**) resulted in decreased selectivity for the mMC4R as compared to the hPhe⁴ substitution alone (**13**). Notably, this trend did not remain throughout the set of peptides, as the most selective peptides in this study possessed the combination of the two substitutions, including hPhe⁴/Ser⁶/Nle⁷ (**16**, 800-fold more potent at the mMC4R than the mMC3R) and hPhe⁴/Dap⁵/Ser⁶/Nle⁷ (**20**, 600-fold more potent at the mMC4R than the mMC3R). The dose response curves of these two compounds are provided in Figure 4. These peptides differed in the amino acid side chain at the 5 position (Asn for **16**, and Dap for **20**). As expected from previous studies, the Dap⁵ substitution increased potency at the mMC4R compared to the Asn⁵ substitution. The peptide **16** was 13-fold more potent than the lead compound at the mMC4R, and **20** was 25-fold more potent than the lead compound at the mMC4R. Other

than the peptides discussed above, three other peptides possessed >200-fold selectivity for the MC4R over the MC3R. The substitution pattern in these peptides were: Dap⁵/Ser⁶/Nle⁷ (**8**, 430-fold selectivity), Arg⁵/Ser⁶/Nle⁷ (**12**, 320-fold selectivity), and hPhe⁴/Dap⁵/Nle⁷ (**18**, 250-fold selective). As discussed above, while the Ser⁶/Nle⁷ motif did not increase selectivity, the combination of this motif with many other substitutions utilized in this study resulted in a synergistic increase in selectivity compared to the individual substitutions alone. The exception to this is the combination of the Ser⁶/Nle⁷ motif with the hPhe⁴/Arg⁵ motif (**24**), which possessed a reduced selectivity for the mMC4R. This may be expected, given that the hPhe⁴/Arg⁵ motif resulted in a synergistic 47-fold increase in mMC3R potency, and therefore resulted in a reduced MC4R selectivity.

In conclusion, this study utilized a multiresidue substitution strategy to test the hypothesis that the pharmacological properties possessed by singly-substituted macrocyclic AGRP mimetics could be combined and amplified in order to generate ligands with increased potency and/or selectivity for the mMC4R over the mMC3R. Herein, it was found that peptides containing the Ser⁶/Nle⁷ motif, in combination with the hPhe⁴ and/or Dap⁵ substitutions, possessed increased potency at the mMC4R. It was also found that this Ser⁶/Nle⁷ motif appeared to work synergistically to generate peptides with enhanced selectivity for the mMC4R over the mMC3R, especially when used in combination with substitutions like hPhe⁴, Asn⁵, or Dap⁵. Unexpectedly, one of the motifs examined in this study (hPhe⁴/Arg⁵) resulted in a synergistic increase in mMC3R potency, despite the hPhe⁴-substituted peptide possessing the lowest pA₂ value at the mMC3R of all the peptides examined in this study (**13**, pA₂ = 5.6). As discussed above, peptides possessing the Arg⁵ substitution largely increased mMC3R potency and thus decreased mMC4R selectivity, especially when used in combination with the hPhe⁴ substitution.

This study produced three lead ligands with interesting pharmacological profiles. One of these ligands, **16** (containing hPhe⁴, Ser⁶, and Nle⁷ substitutions), did not result in mMC1R stimulation, had a pA₂ of 6.33 at the mMC3R, had a pA₂ of 9.23 at the mMC4R, did not result in mMC5R stimulation, and was 800-fold selective for the mMC4R over the mMC3R. Another of these ligands, **20** (containing hPhe⁴, Dap⁵, Ser⁶, and Nle⁷ substitutions), did not result in mMC1R stimulation, had a pA₂ of 6.7 at the mMC3R, had a pA₂ of 9.5 at the mMC4R, did not result in mMC5R stimulation, and was 630-fold selective for the mMC4R over the mMC3R. While this ligand was less selective for the mMC4R than **16**, it was also more potent at the mMC4R than **16**. The peptide **20** possesses potency greater than that of AGRP at the mMC4R (pA₂=8.7) and another lead antagonist ligand in the field, SHU9119 (pA₂=9.2) in the cAMP assay used in the present study. Finally, **21** (containing hPhe and Arg substitutions), resulted in 25% receptor stimulation at the mMC1R, had a pA₂ of 8.0 at the mMC3R, had a pA₂ of 8.9 at the mMC4R, and did not result in mMC5R stimulation. The octapeptide **21** possessed nearly equipotent antagonist activity to the active form of the native hormone AGRP at both receptors (pA₂ of AGRP at the mMC3R and mMC4R = 8.7), and represents a promising start for the generation of dual antagonist ligands that are equipotent at both the mMC3R and mMC4R compared to AGRP.

These three peptides possess dual antagonist pharmacology or selective antagonist pharmacology for the mMC3R and mMC4R. Importantly, they possess the same core

scaffold, which will allow them to serve as more ideal candidate probes for future comparative *in vivo* studies as they should theoretically possess more similar pharmacokinetic properties than, for example, **20** (an octapeptide) as compared to AGRP (a 50-residue protein in the active form). Herein, three macrocyclic octapeptides were discovered using a multiresidue substitution strategy that will make useful pharmacological probes for understanding the role of the melanocortin system in diseases of negative energy balance such as anorexia, cachexia, and failure to thrive in children.

Experimental Section

Peptide Synthesis.

The peptides investigated in this study were synthesized using fluorenyl-9-methoxycarbonyl (Fmoc) chemistry using a H-Pro-CTC Resin (0.67 mequiv/g substitution) purchased from Peptides International (Louisville, KY).⁷ The amino acids Fmoc-DPro, Fmoc-Phe, Fmoc-Nle, Fmoc-Ser(tBu), Fmoc-Ala, Fmoc-Asn(Trt), Fmoc-Dap(Boc), and Fmoc-Arg(Pbf) were purchased from Peptides International (Louisville, KY). The amino acid Fmoc-homophenylalanine (Fmoc-hPhe) was purchased from Synthetech (Albany, OR). The coupling reagents used in this study, O-benzotriazol-1-yl-N,N,N',N'-tetramethyluronium hexafluorophosphate (HBTU), (benzotriazolyl-oxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP), and 1-hydroxybenzotriazole (HOBt), were purchased from Peptides International (Louisville, KY). The solvents N,N-dimethylformamide (DMF), anhydrous ethyl ether, dichloromethane (DCM), methanol, and acetonitrile 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.

The peptides investigated in this study were synthesized in parallel using a semiautomated synthesizer (LabTech 1, Advanced Chemtech, Louisville, KY) at a 0.05 mmol scale on a H-Pro-CTC-resin. The H-Pro-CTC-resin was swelled in dimethylformamide (DMF) for at least one hour prior to the first coupling. The amino acid in the 8 position (DPro⁸) and subsequent amino acids were coupled using the semi-automated synthesizer in a 40-well Teflon reaction block. For natural amino acids the following coupling conditions were used: Fmoc protected amino acid (3.1 equiv), HBTU (3 equiv), and DIEA (5 equiv) were added, and the solution was agitated for 1 hour. For unnatural amino acids (e.g. DPro, Nle, Dap), the following coupling conditions were used: Fmoc protected amino acid (1.56 equiv), HBTU (1.5 equiv), HOBt (1.5 equiv), and DIEA (2.5 equiv) were added, and the solution was agitated for 1 hour. All amino acids were coupled using a double-couple strategy, so each well was rinsed with DMF and then the coupling procedure was repeated. The amino acids were Fmoc-deprotected with 20% piperidine in DMF (1 × 2 min, 1 × 20 min). Coupling and deprotection completeness was monitored via colorimetric assays, and the procedures repeated if necessary. A chloranil test was used to test for the presence of free secondary amines. A Kaiser test was used to test for the presence of free primary amines. Following synthesis, the N-terminal Fmoc amines were deprotected with 20% piperidine in DMF (4 mL/well, 1 × 2 min, 1 × 20 min) and dried *in vacuo* after washing with methanol.

Peptides were cleaved from solid support using a cleavage cocktail composed of 1% trifluoroacetic acid (TFA) in dichloromethane (DCM). The peptides were washed three times with 2 mL of cleavage solution for 2 minutes. The peptide solutions were concentrated using nitrogen, 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*. For the coupling of the C-terminus (Pro¹) to the N-terminus (Arg²) to create the macrocycles, the peptides were dissolved in DCM to a concentration of approximately 0.5 mM, and cyclized using BOP (3 equiv), HOBt (3 equiv), and DIEA (6 equiv), stirring the solution overnight at room temperature. The peptides were dried under reduced pressure for a minimum of 1 hour, and 5 mL of a deprotection solution composed of 95:2.5:2.5 TFA:H₂O:TIS were added at room temperature for 2 hours. The peptides were then concentrated using nitrogen and precipitated with cold ether. Peptides were pelleted via centrifugation at 4,000 rpm at 4°C for 4 minutes, and the supernatant was decanted. The final products were dried *in vacuo*.

Peptides were purified via reverse phase high pressure liquid chromatography (RPHPLC, Shimadzu) on a semi-preparative C₁₈ column (Vydac 218TP1010, 1 cm × 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 × 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. The molecular weights of **1** through **8** were then confirmed using matrix-assisted laser desorption ionization (MALDI) and time of flight (TOF) mass spectrometry analysis (AB-Sciex 5800 MALDI/TOF-MS, LeClaire-Dow Instrumentation Facility, University of Minnesota), using a cyano-4-hydroxycinnamic acid (CCA) matrix. The molecular weights of remaining peptides were confirmed using electrospray ionization (ESI) time of flight (TOF) mass spectrometry (University of Minnesota LeClaire-Dow Instrumentation Facility). Peptides were assayed as TFA salts and not corrected for peptide content.

AlphaScreen™ cAMP Functional Assay.

The functional cAMP potencies of peptides at HEK293 cells stably expressing the individual melanocortin receptors (mMC1R, mMC3R, mMC4R, and mMC5R) were measured using the AlphaScreen™ cAMP assay (PerkinElmer Life Sciences, Cat #6760625M). The assay was performed according to manufacturer instructions, and as previously described.[–]

Cells were approximately 90% confluent at the start of the assay. Growth medium was aspirated, and cells were rinsed with 1 mL Gibco® Versene solution, then 1 mL fresh Versene solution was added. Cells were incubated at 37°C until cells had detached from the plate, then pelleted via centrifugation (800 rpm, 5 minutes; Sorvall™ Legend™ XTR centrifuge, swinging bucket rotor). The medium was aspirated, and the cell pellet was resuspended a second time in Dulbecco's phosphate buffered saline solution (DPBS 1× [–] without calcium and magnesium chloride, Gibco® Cat#14190–144). A 10 µL aliquot of each cell line was removed for counting, during which the remaining cells were centrifuged a second time as described above. Cells were counted by adding 10 µL Trypan blue dye

(BioRad) and counted manually using a hemocytometer. After centrifugation, the DPBS was aspirated and the pellet was resuspended in a solution of freshly-made stimulation buffer [Hank's Balanced Salt Solution (HBSS 10× (–) sodium bicarbonate) and (–) phenol red, Gibco®), 0.5 mM isobutylmethylxanthine (IBMX), 5 mM HEPES buffer solution (1M, Gibco®), 0.1% bovine serum albumin (BSA) in Milli-Q water, pH = 7.4] to a concentration of 10,000 cells per μL . An acceptor bead solution was created by diluting the acceptor bead stock solution (5 mg/mL anti-cAMP acceptor beads in stimulation buffer) to a concentration of 0.1 mg/mL in stimulation buffer. The acceptor bead solution was added to the cells such that there were 0.5 μg anti-cAMP acceptor beads in each cell line.

Next, 10 μL of the cell/acceptor bead solution per well was added to an Opti-384 plate using a 16 channel pipettor. To each well 5 μL of compound was added. Compounds were run in duplicate replicates, and each cell line had a positive control (10^{-4} M forskolin) and negative control (plain stimulation buffer,). The 384-well plate was sealed with a cover slip, covered with aluminum foil, and incubated at room temperature in a dark desk drawer for 2 hours. A biotinylated cAMP/streptavidin-coated donor bead working solution was prepared by diluting the stock solution of donor beads (5 mg/mL) and cAMP biotinylated tracer (1 μL with $1\times$ PBS 14190–144) in a freshly prepared lysis buffer [10% Tween-20, 5 mM HEPES buffer solution (1M, Gibco®), 0.1% bovine serum albumin (BSA) in Milli-Q water, pH = 7.4] such that the final solution contained 0.5 μg of donor beads and 0.62 μmol biotinylated cAMP. This donor bead/biotinylated cAMP solution was allowed to incubate in a dark desk drawer at room temperature for a minimum of 30 minutes.

After the incubation period for the cells/acceptor beads/compound solution was completed, 10 μL of the donor bead/biotinylated cAMP mixture was added to each well in a room containing a green light using a multichannel pipettor, mixing well. The plate was then re-sealed with the cover slip, covered in aluminum foil, and allowed to incubate in a dark desk drawer at room temperature for another 2 hours. Following the second incubation period, the plate was read by an Enspire™ Alpha plate reader using a pre-normalized assay protocol set by the manufacturer. Assays were run in duplicate wells in at least three independent experiments.

Cell Data Analysis.

The data collected were analyzed using PRISM software (v4.0, GraphPad Inc.). Agonist potency was evaluated by calculating the EC_{50} values using non-linear regression analysis with the PRISM software. Antagonist potency was determined using a Schild analysis [$\text{pA}_2 = -\log(\text{K}_i)$] and the agonist ligand NDP-MSH. Because this assay is loss-of-function mMC3R, and mMC4R data were normalized to represent a percent response relative to control ligand (NDP-MSH) for illustrations, as has been reported previously.[–] Inverse agonist activity for the mMC5R was calculated by normalizing signal to a basal response, thus yielding a percent difference from basal activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources. This work has been supported in part by NIH Grant R01DK091906. The Haskell-Luevano laboratory is a recipient of a 2017 Wallin Neuroscience Discovery Fund Award through the University of Minnesota. M.D.E. is a recipient of an NIH F32 Postdoctoral Fellowship (F32DK108402). K.A.F. is a recipient of a 2018 Bighley Fellowship and a 2018 Rowell Fellowship.

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

 μ M

micromolar

NDP-MSH (4-Norleucine-7-D-Phenylalanine)Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH₂**Nle**

norleucine

Dap

diaminopropionic acid

hPhe

homophenylalanine

RP-HPLC

reverse-phase high-pressure liquid chromatography

SAR

structure-activity relationships

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Figure 1.

Summary of the SAR observed for this macrocyclic peptide at the mMC4R. The lead compound is shown in the grey bar, possessing the native AGRP active-loop sequence cyclized through a DPro-Pro motif. Below each amino acid in the template is listed the individual substitutions examined in previous studies. Amino acid substitutions colored in green indicate that peptide containing this single substitution was more potent than the lead scaffold at the mMC4R, amino acid substitutions colored in black indicate that the peptide containing this single substitution was equipotent to the lead scaffold, amino acid substitutions colored in blue indicate that the peptide containing this single substitution possessed decreased potency compared to the lead scaffold, and amino acids shown in red indicate that the peptide containing this single substitution did not possess antagonist potency that could be observed at the highest concentrations used in the assay. The studies that examine the Phe³, Phe⁴, Asn⁵, and Phe⁷ positions utilize many unnatural amino acids, and the structures of the amino acids are provided and colored as described above. The substitutions utilized in this study are highlighted in yellow.

Figure 2.

A) Structure of the core molecular scaffold used in these studies, c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro]. The positions modified in this study are highlighted by coloring the side chains. **B)** Structures of the amino acids used in these studies. The abbreviations are listed below the structures of their corresponding amino acids, and the three letter abbreviations of unnatural amino acids are shown in red. Amino acid side chains that are used as part of these substitution studies are highlighted with the color of the corresponding amino acid side chain they replace in **2A**.

Figure 3.

Structure activity relationship trends observed in the library discussed herein. **A)** Compound sequences and heat map of mMC3R potency. Changes from the lead compound are shown in grey. The most potent compounds are listed on top, and the least potent compounds are listed on the bottom. Trends observed in this set include the hPhe⁴/Arg⁵ motif, which increases mMC3R potency. **B)** Compound sequences and heat map of mMC4R potency. Changes from the lead compound are shown in grey. The most potent compounds are listed on top, and the least potent compounds are listed on the bottom. Trends observed in this set include the high abundance of the Ser⁶/Nle⁷ motif in the most potent compounds in this set, and the increased prevalence of hPhe⁴ in the most potent mMC4R compounds. Notably, the hPhe⁴/Arg⁵ motif is not grouped in this data set, indicating that while this motif increases mMC3R potency, it likely does not do so at the expense of mMC4R potency. **C)** Compound sequences and heat map of mMC4R selectivity (calculated using antagonist K_i values; pA₂ = -log[K_i]). Changes from the lead compound are shown in grey. The most selective compounds are listed on top, and the least selective compounds are listed on the bottom. Trends observed in this data set are that the Nle⁷ substitution causes an increase in mMC4R selectivity, especially when combined with Ser⁶. The compounds possessing the Arg⁵ substitution are almost entirely grouped in the least selective compounds, as are compounds possessing the hPhe⁴/Arg⁵ motif, as discussed in the text. **D)** Radar plot depicting the pharmacological profiles of the three lead ligands discussed in this manuscript (compounds **16**, **20**, and **21**) compared to **1** at the melanocortin receptors. Compound **1** is depicted in grey, compound **16** is depicted in purple, compound **20** is depicted in blue, and compound

21 is depicted in red. The compound IDs of these four compounds are also shaded in **A–C** in the corresponding color. To fit these data on one chart, a log scale was used for potency values and the fold-selectivity for the mMC4R over the mMC3R was divided by 100. **E**) Radar plot depicting the pharmacological activity of the three lead ligands discussed in this manuscript compared to **1** at the mMC3R and mMC4R, using the same color scale as above. In this graph, pA_2 values were converted to nanomolar antagonist K_i values ($pA_2 = -\log[K_i]$), and fold-selectivity for the mMC4R over the mMC3R is calculated using the K_i values.

Figure 4.

Illustrations of the *in vitro* antagonist (mMC3R, mMC4R) pharmacology of **16**, **20**, and **21**.

A Schild antagonist experimental strategy was implemented, using the agonist NDPMSH.

Data were normalized to NDP-MSH response, as has been reported previously.⁷

Table 1:

Pharmacology of the macrocyclic AGRP-based peptides at the mMC1R and mMC5R.

Compound ID	Sequence	Agonist mMC1R EC50 (nM)	Inverse Agonist mMC5R EC50 (nM)
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Tyr-Gly-Lys-Pro-Val-NH ₂	0.010±0.001	EC50 = 0.14±0.01
1*	c[Pro Arg Phe Phe Asn Ala Phe DPro]	2800±600 (80%)	>100,000
2*	c[Pro Arg Phe Phe Asn Ala Nle DPro]	1700±300 (50%)	10±6 (-20%)
3*	c[Pro Arg Phe Phe Asn Ser Phe DPro]	1200±300	6±3 (-14%)
4	c[Pro Arg Phe Phe Asn Ser Nle DPro]	2600±300 (75%)	-35% @ 100µM
5*	c[Pro Arg Phe Phe Dap Ala Phe DPro]	1600±400 (80%)	>100,000
6	c[Pro Arg Phe Phe Dap Ala Nle DPro]	190±60	>100,000
7	c[Pro Arg Phe Phe Dap Ser Phe DPro]	600±100 (85%)	>100,000
8	c[Pro Arg Phe Phe Dap Ser Nle DPro]	>100,000	>100,000
9*	c[Pro Arg Phe Phe Arg Ala Phe DPro]	600±200 (25%)	>100,000
10	c[Pro Arg Phe Phe Arg Ala Nle DPro]	>100,000	>100,000
11	c[Pro Arg Phe Phe Arg Ser Phe DPro]	>100,000	>100,000
12	c[Pro Arg Phe Phe Arg Ser Nle DPro]	>100,000	>100,000
13*	c[Pro Arg Phe Phe hPhe Asn Ala Phe DPro]	35% @ 100µM	>100,000
14	c[Pro Arg Phe Phe hPhe Asn Ala Nle DPro]	>100,000	>100,000
15	c[Pro Arg Phe Phe hPhe Asn Ser Phe DPro]	40% @ 100µM	>100,000
16	c[Pro Arg Phe Phe hPhe Asn Ser Nle DPro]	>100,000	>100,000
17	c[Pro Arg Phe Phe hPhe Dap Ala Phe DPro]	560±80 (70%)	>100,000
18	c[Pro Arg Phe Phe hPhe Dap Ala Nle DPro]	>100,000	>100,000
19	c[Pro Arg Phe Phe hPhe Dap Ser Phe DPro]	40% @ 100µM	>100,000
20	c[Pro Arg Phe Phe hPhe Dap Ser Nle DPro]	>100,000	>100,000
21	c[Pro Arg Phe Phe hPhe Arg Ala Phe DPro]	25% @ 100µM	>100,000
22	c[Pro Arg Phe Phe hPhe Arg Ala Nle DPro]	>100,000	>100,000
23	c[Pro Arg Phe Phe hPhe Arg Ser Phe DPro]	30% @ 100µM	>100,000
24	c[Pro Arg Phe Phe hPhe Arg Ser Nle DPro]	400±200	>100,000

Compounds were assayed in duplicate replicates and values are expressed as the mean ± the standard error of the mean (SEM) of at least 3 independent experiments. The use of "(X%)" indicates that the compound possessed partial agonist activity and was able to stimulate the receptor to a given percent. The use of "X% @ 100 µM" indicates that the compound partially stimulated the receptor to a given percent, but did not generate a sigmoidal dose-response at 100 µM. The use of ">100,000" indicates that the compound did not stimulate the receptor greater than 20% at 100 µM. For mMC5R inverse

agonist studies, the use of "X (-X%)" indicates that the compound displayed inverse agonist efficacy of X% relative to basal and appeared to generate a sigmoidal dose-response of potency X. The use of "X-X% @ 100 μ M" indicates that the compound displayed inverse agonist efficacy of X% at 100 μ M, but did not generate a sigmoidal dose-response at up to 100 μ M concentrations.

* These singly substituted macrocyclic octapeptides that have been previously reported²¹ and were resynthesized, re-characterized, and re-assayed herein as controls.

Table 2.

Pharmacology of the macrocyclic AGRP-based peptides at the mMC3R and mMC4R.

Compound ID	Sequence	Antagonist mMC3R pA ₂	Antagonist mMC4R pA ₂
AGRP^d		8.7±0.1	8.7±0.2
SHU9119^d	Ac-Nle-c[Asp-His-DNaI(2')-Arg-Trp-Lys]-NH ₂	8.7±0.3 (partial agonist)	9.2±0.1
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH ₂	EC ₅₀ = 0.09±0.01	EC ₅₀ = 0.64±0.06
hAGRP^d (109–118)	Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr	No activity (Agonist or Antagonist)	6.8±0.4
1[*]	c[Pro Arg Phe Phe Asn Ala Phe DPro]	6.3±0.03	8.1±0.2
2[*]	c[Pro Arg Phe Phe Asn Ala Nle DPro]	6.6±0.03	8.9±0.1
3[*]	c[Pro Arg Phe Phe Asn Ser Phe DPro]	6.1±0.3	8.1±0.2
4	c[Pro Arg Phe Phe Asn Ser Nle DPro]	6.6±0.2	8.5±0.2
5[*]	c[Pro Arg Phe Phe Dap Ala Phe DPro]	7.2±0.1	8.9±0.1
6	c[Pro Arg Phe Phe Dap Ala Nle DPro]	7.1±0.2	9.0±0.2
7	c[Pro Arg Phe Phe Dap Ser Phe DPro]	6.8±0.03	9.1±0.1
8	c[Pro Arg Phe Phe Dap Ser Nle DPro]	6.5±0.1	9.1±0.03
9[*]	c[Pro Arg Phe Phe Arg Ala Phe DPro]	7.4±0.1	8.7±0.1
10	c[Pro Arg Phe Phe Arg Ala Nle DPro]	7.5±0.1	9.1±0.03
11	c[Pro Arg Phe Phe Arg Ser Phe DPro]	7.1±0.1	9.0±0.1
12	c[Pro Arg Phe Phe Arg Ser Nle DPro]	7.1±0.03	9.6±0.1
13[*]	c[Pro Arg Phe hPhe Asn Ala Phe DPro]	5.6±0.02	8.3±0.2
14	c[Pro Arg Phe hPhe Asn Ala Nle DPro]	6.2±0.06	8.6±0.1
15	c[Pro Arg Phe hPhe Asn Ser Phe DPro]	6.5±0.2	8.6±0.2
16	c[Pro Arg Phe hPhe Asn Ser Nle DPro]	6.3±0.1	9.2±0.1
17	c[Pro Arg Phe hPhe Dap Ala Phe DPro]	7.1±0.2	9.1±0.1
18	c[Pro Arg Phe hPhe Dap Ala Nle DPro]	6.7±0.1	9.1±0.1
19	c[Pro Arg Phe hPhe Dap Ser Phe DPro]	7.2±0.03	9.2±0.03
20	c[Pro Arg Phe hPhe Dap Ser Nle DPro]	6.7±0.2	9.5±0.2
21	c[Pro Arg Phe hPhe Arg Ala Phe DPro]	8.0±0.3	8.9±0.3
22	c[Pro Arg Phe hPhe Arg Ala Nle DPro]	7.5±0.1	8.8±0.1

Compound ID	Sequence								Antagonist	Antagonist
									mMC3R pA ₂	mMC4R pA ₂

23	c[	Pro	Arg	Phe	hPhe	Arg	Ser	Phe	DPro]	7.7±0.1	8.8±0.03
24	c[	Pro	Arg	Phe	hPhe	Arg	Ser	Nle	DPro]	7.9±0.1	9.5±0.2

Compounds were assayed in duplicate replicates and values are expressed as the mean ± the standard error of the mean (SEM) of at least 3 independent experiments. The pA₂ values were determined using the Schild analysis with agonist NDP-MSH (pA₂ = -log[K₁]).

²Pharmacological data for these peptides have been previously published.^{1,2} The data for hAGRP(109–118) is from a cAMP β-galactosidase reporter gene bioassay and different from the ALPHA assay reported herein.

^{*}These singly substituted macrocyclic octapeptides that have been previously reported³ and were resynthesized, re-characterized, and re-assayed herein as controls.