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Structure-Activity Relationship Studies on a Macrocyclic Agouti-Related Protein (AGRP) Scaffold Reveal Agouti Signaling Protein (ASP) Residue Substitutions Maintain Melanocortin-4 Receptor Antagonist Potency and Result in Inverse Agonist Pharmacology at the Melanocortin-5 Receptor

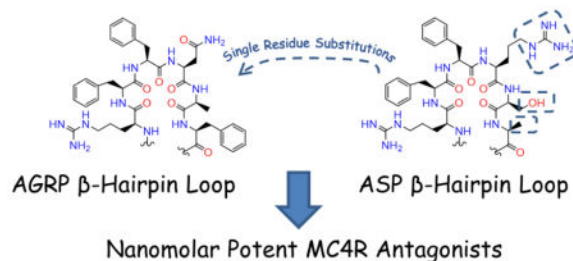
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Abstract

The melanocortin system consists of five reported receptors, agonists from the proopiomelanocortin gene transcript, and two antagonists, agouti-signaling protein (ASP) and agouti-related protein (AGRP). For both ASP and AGRP, the hypothesized Arg-Phe-Phe pharmacophores are on exposed β -hairpin loops. In this study, the Asn and Ala positions of a reported AGRP macrocyclic scaffold (c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro]) were explored with 14-compound and 8-compound libraries, respectively, to generate more potent, selective melanocortin receptor antagonists. Substituting diaminopropionic acid (Dap), DDap, and His at the Asn position yielded potent MC4R ligands, while replacing Ala with Ser maintained MC4R potency. Since these substitutions correlate to ASP loop residues, an additional Phe to Ala substitution was synthesized and observed to maintain MC4R potency. Seventeen compounds also possessed inverse agonist activity at the MC5R, the first report of this pharmacology. These findings are useful in developing molecular probes to study negative energy balance conditions and unidentified functions of the MC5R.

Graphical Abstract



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

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Keywords

AGRP; agouti; structure-activity relationships; melanocortin receptors; inverse agonist

Introduction

The melanocortin system has been implicated in many biological pathways. To date, five melanocortin G protein-coupled receptors (GPCRs) have been identified. The melanocortin-1 receptor (MC1R) plays an important role in pigmentation.^{1,2} The melanocortin-2 receptor (MC2R) is linked to steroidogenesis,² and is only stimulated by the endogenous adrenocorticotrophic hormone (ACTH).³ The centrally expressed melanocortin-3 and -4 receptors (MC3R and MC4R) have been associated with food intake and energy homeostasis.⁴⁻¹¹ Dysregulation or single nucleotide polymorphisms (SNPs) of the MC3R may predispose an individual to obesity,¹² while SNPs in the MC4R have been demonstrated to result in an obese phenotype.¹³ The melanocortin-5 receptor (MC5R) is expressed widely throughout the body with its physiological role less characterized,^{14,15} although it has been linked to exocrine function in mice.¹⁶ These melanocortin receptors (MCRs) are stimulated by endogenous agonists derived from the proopiomelanocortin gene transcript,¹⁷ which is processed into α -MSH, β -MSH, γ -MSH, and ACTH among other peptide products, as previously reviewed.^{18,19} The melanocortin system also possesses naturally occurring antagonists, agouti-signaling protein (ASP)^{20,21} and agouti-related protein (AGRP).²²⁻²⁴ Overexpression of ASP or AGRP in transgenic mice results in an obese phenotype, with the expression level of either protein correlating to weight gain.^{25,26} Transgenic mice overexpressing ASP recapitulate the lethal yellow (A^y) mouse strain, characterized by ectopic ASP expression,^{21,27} a yellow coat color (presumably due to MC1R antagonism),²⁸ and an obese phenotype (originally hypothesized to be due to MC4R antagonism, but may also involve MC3R antagonism).^{28,29} While a yellow coat color is not observed (implying a lack of MC1R antagonism), ectopic expression of AGRP increases weight gain,²⁶ similar to the dose-dependent increase in food intake with icv administration of AGRP.^{10,30} The AGRP-mediated increased consumption is observed in both MC3R and MC4R knockout mice, implying AGRP antagonism of both receptors may be responsible for the observed phenotype.¹⁰ Developing probes from ASP and AGRP may therefore be important in investigating the origins and potential therapeutics for obesity. Additionally, as potent orexigenic (appetite-inducing) compounds, ligands developed from ASP and AGRP may be useful in the treatment of negative energy balance disorders, including cachexia, anorexia, and failure to thrive in infants.³¹⁻³³

Full length ASP is 132 amino acids (Figure 1A), with the active form ASP(23-132) resulting from the cleavage of a 22-residue signal sequence.³⁴⁻³⁶ Recombinant mouse (m)ASP was reported to impair α -MSH-mediated cAMP production at the mMC1R^{28,37,38} and the human (h)MC4R,²⁸ but did not at the rat (r)MC3R.²⁸ The C-terminal domain of recombinant mASP is equipotent to ASP(23-132) in *Xenopus* melanophores³⁵ and in murine B16F10 cells (both presumably express the MC1R),³⁸ indicating this region possessing 5 disulfide bonds is responsible for the observed antagonist activity. Although recombinant mASP was purportedly not able to antagonize the rMC3R,²⁸ later work with the

recombinant hASP indicated this homolog was an antagonist at the hMC3R, with decreased potency relative to the hMC1R and hMC4R.³⁹ The authors reported seeing a similar trend for recombinant mASP at the human receptors, but did not show these data.³⁹ This functional activity correlated with a subsequent binding study which demonstrated that mASP was able to displace radiolabeled NDP-MSH at the mMC1R, mMC3R, and mMC4R with $K_{i\text{ app}}$ of 2.6, 190, and 54 nM, respectively.⁴⁰ In the same report, an alanine positional scan of the C-terminal domain of mASP indicated that replacing the Arg¹¹⁶ and Phe¹¹⁸ residues of mASP decreased affinity at the mMC1R, mMC3R, and mMC4R; additionally, substituting Ala at the Phe¹¹⁷ of mASP decreased affinity at the mMC4R.⁴⁰ To generate sufficient quantities of the hASP C-terminal domain for NMR analysis, a synthetic approach was explored.²⁹ Although synthesis of the 53 residue C-terminal domain of hASP resulted in poor yields due to misfolded products, substitution of two residues (Q115Y, S124Y, corresponding to equivalent residues in AGRP) resulted in one major observed product that possessed the correct molecular mass for the properly folded protein.²⁹ The synthetic hASP-YY possessed nanomolar antagonist potency at the hMC1R and hMC3R, and sub-nanomolar potency at the hMC4R.²⁹ Within the C-terminal domain of ASP-YY, the C-terminal loop (residues Arg¹²⁶-Asn¹³¹) has been implicated as necessary for potent MC1R binding.⁴¹

Agouti-related protein, AGRP, was first identified due to sequence homology and the Cys-spacing pattern with ASP (Figure 1A).²²⁻²⁴ Described as agouti-related protein,²³ agouti-related peptide,^{42,43} or agouti-related transcript,^{22,24,26} AGRP possesses nanomolar antagonist pharmacology at the centrally expressed MC3R and MC4R,^{22,23} as well as inverse agonist activity at the MC4R.^{44,45} The biologically active form of AGRP has been hypothesized to be AGRP(83-132) following processing by proprotein convertase 1.⁴⁶ The C-terminal domain of synthetic^{47,48} and recombinant AGRP²³ has been demonstrated to be equipotent to longer AGRP variants.^{23,48} Like ASP, the C-terminal domain of AGRP possesses an Arg-Phe-Phe tripeptide sequence important for binding and antagonist activity (Figure 1B).⁴⁹ Solution NMR structural studies of ASP-YY,²⁹ the C-terminal domain of AGRP,^{50,51} and a shortened “mini-AGRP”⁵² have all indicated that the hypothesized Arg-Phe-Phe pharmacophores are located on similar solvent-exposed β -hairpin loops (Figure 1C). Truncation studies of hAGRP, mASP, and hASP indicated that cyclic octapeptide fragments based upon the active loops of these molecules (c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys], c[Cys-Arg-Phe-Phe-Gly-Ser-Ala-Cys], and c[Cys-Arg-Phe-Phe-Arg-Ser-Ala-Cys], respectively) were the minimal required sequences for MC4R binding affinity, with the hASP octapeptide also possessing affinity at the MC3R.⁴⁹ While mASP was not further explored, elongation of hAGRP by two Tyr residues, H-Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-OH, was necessary for binding affinity at the MC3R and sub-micromolar functional activity at the MC4R.⁴⁹ An additional four residues were required for functional antagonism at the MC3R, indicating the importance of amino acids outside the β -hairpin loop for MC3R potency.⁵³ Although dodecapeptide and tetradecapeptides possess antagonist activity at the MC3R and MC4R, the potencies of truncated AGRP peptides are lower compared to AGRP.

Previously, it was hypothesized that inducing a β -hairpin structure in small AGRP-derived peptides containing the postulated Arg-Phe-Phe pharmacophore may result in more potent

antagonists.⁵⁴ A macrocyclic scaffold containing the active hexapeptide loop of ARGP cyclized through a DPro-Pro motif, c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro], was 50-fold less potent at the MC4R compared to AGRP.⁵⁴ Further structure-activity relationship studies at the Asn position indicated basic residues (diaminopropionic acid [Dap], diaminobutyric acid [Dab], Orn, Lys, Arg) or Gly increased MC4R potency. The most potent substitution, the Asn to Dap replacement, resulted in a sub-nanomolar potent MC4R antagonist that was 160-fold selective for the MC4R over the MC3R, induced partial MC1R activation at 100 μ M concentrations, and did not possess agonist activity at the MC5R.⁵⁴ To further investigate this scaffold, a series of truncated macrocycles were synthesized containing either the Asn or Dap residues and replacing the Arg-Phe-Phe tripeptide antagonist sequence with the melanocortin agonist tetrapeptide His-DPhe-Arg-Trp or tripeptide DPhe-Arg-Trp sequences.⁵⁵ This study examined if the AGRP DPro-Pro loop scaffold was amendable to substitutions that may result in melanocortin agonist activity as weight-management therapeutic leads. Two macrocycles were identified that possessed nanomolar agonist potencies at the MC4R, nanomolar to sub-nanomolar potencies at the MC1R and MC5R, and 30–40 nanomolar potencies at the MC3R, c[Pro-His-DPhe-Arg-Trp-Asn-Ala-Phe-DPro] and c[Pro-His-DPhe-Arg-Trp-Dap-Ala-DPro].⁵⁵

Due to the increased ligand potency for basic and Gly substitutions at the Asn position in the octapeptide macrocyclic scaffold, an additional SAR study was performed at the Asn position. To explore the ligand-receptor interface, substitutions at the Asn position with basic, acidic, polar, non-polar, and aromatic groups were assayed at the mouse MCRs. A similar series of substitutions explored the Ala position. From these two series, a substitution pattern was observed that substitution of the equivalent residue type in the exposed loop of ASP maintained MC4R potency in this AGRP scaffold while other substitutions decreased potency. Therefore, the non-pharmacophore Phe residue was replaced with an Ala found in the active loops of both hASP and mASP to examine if insertion of ASP residues into an AGRP scaffold would maintain MC4R potency.

Results & Discussion

Peptide Synthesis and Characterization

Peptides were synthesized manually or using an automated peptide synthesizer with standard fluorenylmethoxycarbonyl (Fmoc) chemistry.^{56,57} The macrocyclization chemistry to form the amide bond between the Arg and Pro residues using BOP and HOBT has previously been reported.^{54,55} Following cyclization and side-chain deprotection, peptides were purified by semi-preparative reverse-phase high pressure liquid chromatography (RP-HPLC). Peptides were assessed for purity (>95%) by analytical RP-HPLC in two solvent systems (Table 1), and the correct molecular mass was confirmed through matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS, University of Minnesota Mass Spectrometry Laboratory).

In vitro AlphaScreen cAMP Assay

The compounds were assayed using the AlphaScreen cAMP assay in HEK293 cells stably expressing the mouse melanocortin 1, 3, 4, and 5 receptors according to the manufacturer's

instructions and as previously reported.^{58–60} The MC2R is only stimulated by ACTH and was therefore excluded from this study. Compounds were first assayed for agonist activity at the MCRs; ligands that did not possess full agonist activity at the MC3R and MC4R were then assayed for antagonist activity using a Schild paradigm⁶¹ and NDP-MSH as the agonist. Since the AlphaScreen assay is a competition assay (higher concentrations of ligand result in lower signal), concentration-activity curves were normalized to baseline and maximal NDP-MSH signal for illustrative purposes as previously described.^{58,59} Due to the inherent error associated with the assay in our laboratory, compounds that were within a 3-fold potency range were considered equipotent.

Asn Position Substitutions

Previously, the AGRP-derived macrocyclic octapeptide c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro] was found to possess sub-micromolar antagonist potency at the MC4R ($pA_2 = 7.7$), and substitution at the Asn position with basic residues or Gly further increased potency (pA_2 values ranged from 8.3 to 9.1).⁵⁴ The most potent substitution, Asn to diaminopropionic acid (Dap), resulted in a ligand that was equipotent to the C-terminal domain of AGRP at the MC4R.⁵⁴ It was previously hypothesized that the active loop Asn residue in AGRP may be in close proximity to an Asp residue in the MC4R (Asp¹⁸⁹), and that incorporation of basic residues into AGRP analogues at this position might form a novel salt bridge with the MC4R.⁴⁷ While the increased potency observed for basic substitutions supported the postulated salt bridge, the increased potency for Gly suggested other residues might also be incorporated without diminishing potency. Therefore, an additional SAR study was performed at the Asn position to explore the side-chain requirements for antagonist potency and to further examine the hypothesized salt-bridge interaction. Short aliphatic residues (Ala and Abu) were utilized to mimic the steric space of Dap without the charge. Residues were included that could mimic the hydrogen bond donor and acceptor potential of Dap without the positive charge (Ser and Thr). The negatively charged Asp and Glu were incorporated to reverse the charge while varying side-chain length. Additional basic residues were also explored by inverting the stereochemistry (DDap) and incorporating a small charged heterocycle (His). Longer and/or branched aliphatic side chains Nle, Leu, and Val were integrated to examine the functional consequence of expanding the uncharged side chain, as were the aromatic side chains in Phe and Trp. The Dap substitution was synthesized as a control, and the C-terminal domain of AGRP [AGRP(86-132)] was run in parallel as an antagonist at the MC3R and MC4R using the AlphaScreen assay as an additional known ligand. All amino acid structures can be found in Figure 2.

In the present study, AGRP(86-132) possessed identical pA_2 values of 8.7 at both the MC3R and MC4R, similar to previous reported nanomolar potencies at these receptors (Table 2, Figure 3).^{22,23,41,46,48,53} The native sequence of the exposed β -hairpin loop (1) partially stimulated the MC1R at 100 μ M concentrations (25% of the maximal NDP-MSH signal) and did not possess agonist activity at the MC3R or MC4R up to 100 μ M. This peptide possessed sub-micromolar antagonist potency at the MC3R ($pA_2 = 6.3$) and nanomolar potency at the MC4R ($pA_2 = 8.2$; Figure 3). These values are comparable to a previous report where this peptide possessed micromolar agonist activity at the MC1R, and was an antagonist at the MC3R ($pA_2 = 6.4$) and MC4R ($pA_2 = 7.7$) using a β -galactosidase

reporter gene assay.⁵⁴ Insertion of the Dap residue (**2**) resulted in partial activation of the MC1R (30% NDP-MSH maximal signal) and did not stimulate cAMP production at the MC3R or MC4R up to 100 μ M concentrations. Peptide **2** possessed antagonist activity at the MC3R ($pA_2 = 6.5$) and MC4R ($pA_2 = 8.7$; Figure 3), similar to the previously reported pA_2 values of 6.9 and 9.1, respectively (**2** also was reported to partially activate the MC1R to 75% NDP-MSH at 100 μ M).⁵⁴ In both the previous and present study, **2** was an equipotent antagonist to AGRP(86-132) at the MC4R, and was over 100-fold selective for the MC4R over the MC3R despite using two different cAMP reporter assays.⁵⁴

While similar activity was observed for **1** and **2** at the MC1R, MC3R, and MC4R, a difference was observed at the MC5R. Previously, these compounds were reported to possess no activity at the MC5R at 100 μ M concentrations using a β -galactosidase assay.⁵⁴ In the present study, a decrease in normalized signal from basal activity was observed (Table 2, Figure 4), correlating to decreased levels of cAMP and suggesting an inverse agonist response for these ligands at the MC5R. From basal activity, a 10% and 15% dose-response difference in signal were observed for **1** (Figure 4) and **2**, respectively. Since a sigmoidal curve was observed, **1** and **2** possessed apparent potencies (the inflection point on the curve) of 130 nM and 60 nM, respectively. This activity was only observed at the MC5R, and not the other MCRs in the present study. Since the MCRs are all stably expressed in HEK293 cells, if another factor such as cellular toxicity or signaling through an alternative receptor was responsible, the decreased signal would be expected at additional melanocortin receptors. It therefore appears that the observed decreased levels of cAMP may be the result of inverse agonist activity at the MC5R. While many ligands displayed a similar sigmoidal dose-response compared to **1** and **2** (Figure 4, **4**, **5**, and **8** as examples), some ligands decreased cAMP levels at the MC5R without plateauing at 100 μ M concentrations (Figure 4, **21** and **24** as examples). The inverse agonist response of these compounds is reported as the percent decreased from basal at 100 μ M concentrations.

Substitution of the short aliphatic Ala (**3**) or Abu (**4**) residues at the Asn position resulted in ligands that were unable to stimulate any receptor tested. At the MC5R, **3** and **4** (Figure 4) decreased from basal signal 20% and had apparent potencies of 250 nM and 160 nM, respectively. Both peptides did not possess antagonist activity at the MC3R, and were 8-fold less potent at the MC4R compared to **1** ($pA_2 = 7.3$ for both). No agonist activity was observed when Asn was replaced with the polar amino acids Ser (**5**) or Thr (**6**) at the MC1R, MC3R, or MC4R. These peptides produced inverse agonist activity of 25% (with an apparent potency of 140 nM) and 20% (50 nM) for **5** (Figure 4) and **6**, respectively. Peptide **5** possessed antagonist potency at the MC3R ($pA_2 = 5.9$) and MC4R ($pA_2 = 7.7$), similar to the response of **6** ($pA_2 = 6.4$ and 7.8 at the MC3R and MC4R). When Asn was substituted with the acidic Asp residue (**7**), no agonist activity was observed for the MC1R, MC3R, and MC4R, while a 25% inverse agonist response was observed at 100 μ M concentrations at the MC5R. This peptide did not possess antagonist activity at the MC3R, and was 25-fold less potent at the MC4R compared to **1**. Elongating the acidic side chain by one methylene unit, **8**, resulted in the only peptide in this study with full MC1R agonist activity ($EC_{50} = 20.5$ μ M). This Glu substitution did not result in measurable activity at the MC3R, had a 10% inverse agonist response (apparent potency = 700 nM) at the MC5R, and was a 16-fold less

potent antagonist at the MC4R compared to **1**. When substituting the basic residues DDap or His (**9** or **10**, respectively), no agonist activity was observed at the MC1R, MC3R, and MC4R up to 100 μ M concentrations. Similar 10% inverse agonist responses were observed at the MC5R, with apparent potencies of 110 nM (**9**) and 10 nM (**10**). Both substitutions possessed sub-micromolar antagonist potencies at the MC3R (pA_2 values of 6.7 and 6.5) and nanomolar antagonist potencies at the MC4R (pA_2 values of 8.6, and 8.3). For the longer and/or branched aliphatic substitutions Nle, Leu, and Val (**11**, **12**, and **13**), the Val substituted **13** stimulated the MC1R to 55% the maximal signal of NDP-MSH at 100 μ M. These three ligands did not produce an agonist response at any other MCR. At the MC5R, **12** and **13** decreased the basal signal 20% and 25%, respectively, with apparent potencies of 110 nM and 510 nM. Peptide **12** was the only ligand of the three to possess micromolar antagonist potency at the MC3R ($pA_2 = 5.7$). These aliphatic substitutions possessed a range of potencies at the MC4R ($pA_2 = 6.9, 7.3, \text{ and } 5.9$ for **11**, **12**, and **13**, respectively), and were at least 8-fold less potent than **1**. When substituting the aromatic amino acid Phe (**14**), only antagonist activity at the MC4R was observed ($pA_2 = 6.6$). Incorporation of Trp, **15**, resulted in a ligand that partially stimulated the MC1R, MC3R, and MC4R (80%, 40%, and 50% maximal NDP-MSH signal) at 100 μ M concentrations. This substitution resulted in a 20% inverse agonist response with an apparent potency of 150 nM at the MC5R, and antagonist activity at the MC3R and MC4R ($pA_2 = 5.7$ and 6.8, respectively).

Certain potency trends at the MC4R were observed when grouping these AGRP-derived macrocycles varied at the Asn position by side-chain type. Residues containing basic side chains (Dap, DDap, and His) possessed the highest MC4R potency, similar to the previous report.⁵⁴ These results support a potential salt-bridge between this position and the acidic residue Asp¹⁸⁹ of the MC4R, as previously hypothesized.⁴⁷ Interestingly, the stereochemistry of the basic charge did not appear significant, as the Dap ($pA_2 = 8.7$) and DDap ($pA_2 = 8.6$) were equipotent at the MC4R. Amino acids with side chains capable of donating and accepting hydrogen bonds (Ser, Thr, and Asn) possessed slightly lower potency at the MC4R compared to basic substitutions. Aliphatic substitutions of similar size to Dap (Ala and Abu) or Asn (Leu) possessed similar potencies, less than the hydrogen bond donor capable side-chains. Substitution of Asn with Asp, replacing an amide with a carboxylic acid, decreased potency more than 10-fold, indicating a negative charge adversely impacted activity. The remaining acidic, aliphatic, and aromatic substitutions all possessed similar potency at the MC4R, with $pA_2 \approx 7$. The Val substituted ligand (**13**) was the only compound with micromolar or higher potency at the MC4R ($pA_2 = 5.9$). Although Val is similar in structure to Thr (both possess a branched side chain and differ by a methyl versus hydroxyl substitution), the Thr substituted **6** was 70-fold more potent compared to **13**. At the MC5R, no trends in percent inverse agonist activity of apparent potency were observed.

Ala Position Substitutions

While SAR studies for the Asn and two pharmacophore Phe positions have been reported on this scaffold,⁵⁴ the Ala position has not previously been studied. One structural model of AGRP interacting with the MC4R indicated that this Ala position of AGRP may be in close proximity to the His²⁶⁴ residue of the MC4R.⁶² Therefore, it was hypothesized that incorporating an acidic amino acid into the corresponding Ala position in the truncated

AGRP macrocycle may result in the formation of a new salt bridge and increase potency. A library of 8 compounds varied at the Ala position was synthesized and assayed at the mMCRs. To probe for a potential salt bridge with His²⁶⁴, the acidic residues Asp and Glu were included. Additional residues were incorporated to begin to probe the ligand requirements at this position for potent melanocortin receptor activity including the basic Lys and His, aromatic Phe, hydrogen bond donor and acceptor Ser, aliphatic Leu, and small Gly.

While the Ala position in the active loop of AGRP was previously hypothesized to be in close proximity to His²⁶⁴, substitution of Asp (**16**) or Glu (**17**) did not result in observable agonist or antagonist activity at the MC3R and MC4R. Both peptides partially stimulated the MC1R at 100 μ M concentrations (65% and 60% for **16** and **17**, respectively) and **16** produced an inverse agonist response at the MC5R (25% at 100 μ M). Incorporation of a basic Lys (**18**) resulted in a ligand that partially stimulated the MC1R (60%), MC3R (45%), and MC5R (45%; Figure 4), and possessed sub-micromolar antagonist potency at the MC4R ($pA_2 = 6.1$). Identical antagonist potency at the MC4R was observed for a His substitution (**19**, $pA_2 = 6.1$) compared to **18**, while a partial agonist response was observed at the MC1R (40% NDP-MSH efficacy with an $EC_{50} = 3,000$ nM). This compound did not possess activity at the MC5R (Figure 4). Substitution of an aromatic Phe (**20**) resulted in partial stimulation of all the MCRs assayed at 100 μ M concentrations (70%, 35%, 35%, and 25% of the maximal NDP-MSH signal at the MC1R, MC3R, MC4R, and MC5R, respectively), as well as micromolar antagonist potencies at the MC3R and MC4R ($pA_2 = 5.7$ for both receptors). Incorporating the polar Ser capable of donating and accepting hydrogen bonds resulted in peptide **21**, which did not possess agonist activity at the MC1R, MC3R, and MC4R up to 100 μ M concentrations, and generated an inverse agonist response at the MC5R (Figure 4, 30% at 100 μ M). Peptide **21** possessed sub-micromolar antagonist potency at the MC3R ($pA_2 = 6.2$), and was the only ligand in the Ala substitution series that was equipotent to **1** at the MC4R ($pA_2 = 8.2$; Figure 3). The aliphatic Leu substitution (**22**) induced a partial agonist response at the MC1R, MC3R, and MC4R (80%, 25%, and 25%, respectively), and was a sub-micromolar antagonist at the MC4R ($pA_2 = 6.2$). Incorporating the small Gly residue (**23**) resulted in a ligand that possessed inverse agonist activity at the MC5R (35% at 100 μ M concentrations) and sub-micromolar antagonist potency at the MC4R ($pA_2 = 6.1$).

The Ala position had previously been postulated to be near the His²⁶⁴ residue in the MC4R,⁶² and it was therefore hypothesized that incorporating a negatively charged residue may result in the formation of a novel salt bridge. If correct, replacement of the Ala with two acidic amino acids (Asp and Glu) might be expected to increase antagonist potency. However, both **16** and **17** did not possess antagonist activity at the MC3R or MC4R. While the current study does not support the postulated Ala-His²⁶⁴ proximity, it is possible that a longer form of AGRP orients this position in a different conformation that may result in a productive interaction. Altering the side chain stereochemistry, elongating/shortening a side chain possessing a negative charge, or utilizing longer AGRP substituted derivatives could be utilized to further examine this hypothesis.

Phe Position Substitution

Unlike substitutions at the Asn position, which resulted in a range of MC4R potencies, only one ligand substituted at the Ala position (Ser, **21**) was equipotent to the parent macrocycle **1** ($pA_2 = 8.2$ for both compounds). All other substitutions decreased potency more than 100-fold ($pA_2 < 6.2$) relative to **1**, perhaps indicating a more stringent structural requirement for ligand-receptor interactions at this position. While attempting to rationalize why Ser might be tolerated at the Ala position, it was observed that the equivalent loop position in both mouse and human ASP is a Ser (Figure 1B). Another compelling observation was that the equivalent position of Asn in the active loop of AGRP was either a basic Arg (human) or Gly (mouse) residue in ASP, a substitution pattern described herein and previously as possessing potent MC4R antagonism.⁵⁴ To extend these observations, it was hypothesized that substitution of the non-pharmacophore Phe position in the AGRP loop with Ala (found in the equivalent ASP position) would maintain MC4R antagonist potency. The resulting Phe to Ala substitution, **24**, possessed equipotent MC4R antagonist potency ($pA_2 = 8.2$; Figure 3) compared to the parent macrocycle **1**, supporting the observations that ASP loop residues substituted onto an AGRP macrocyclic template maintain MC4R antagonist potency. This peptide possessed sub-micromolar antagonist potency at the MC3R ($pA_2 = 6.1$), no agonist activity at the MC1R up to 100 μM concentrations, and resulted in inverse agonist activity at the MC5R (25% at 100 μM , Figure 4).

Thus, single substitutions of ASP active loop residues into a macrocyclic AGRP-derived peptide result in the maintenance of MC4R antagonist potency and approximately 100-fold decrease in MC3R antagonist potency, a similar decrease to the parent ligand. In the current study, AGRP was shown to be equipotent at the MC3R and MC4R ($pA_2 = 8.7$ at both receptors), in agreement with prior reports of nanomolar potencies at both receptors.^{22,23,53} The decreased MC3R potency has been observed in many AGRP truncated analogs, and was described by Tota *et al.* who found the minimal fragment needed to bind to the MC4R (H-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-OH) was unable to bind the MC3R.⁴⁹ Acetylating the N-terminal and amidating the C-terminal of this octapeptide was sufficient to achieve micromolar MC4R antagonist potency.⁴⁹ Further elongation by adding Tyr residues to both terminals was required to achieve MC3R binding and resulted in sub-micromolar potency at the MC4R.^{49,53} Two residues added to both the N- and C-terminals (Thr-Ala-Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-Ala-Arg-NH₂) or four residues added to the C-terminal (Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-Ala-Arg-Lys-Leu-NH₂) were required for sub-micromolar potency at the MC3R.⁵³ The prior study with DPro-Pro cyclized AGRP macrocycles also resulted in more potent antagonism at the MC4R relative to the MC3R.⁵⁴ Therefore, a common trend of truncated AGRP analogs is decreased potency at the MC3R, perhaps indicating that additional residues outside of the active loop are necessary for potent interaction at the MC3R.

While decreased MC3R potency is observed in many AGRP truncation studies, ASP was first described to interfere with α -MSH stimulation of the MC1R and MC4R, and not affect the MC3R and MC5R.²⁸ One interpretation of the current data, assuming ASP is an antagonist at the MC1R and MC4R while AGRP antagonizes the MC3R and MC4R, is that replacement of AGRP residues in the active loop sequence with ASP residues would be

expected to maintain MC4R antagonism while decreasing and/or minimizing MC3R potency. However, such an interpretation would ignore subsequent publications indicating ASP was capable of binding⁴⁰ and functionally antagonizing³⁹ the MC3R. Throughout all of these conflicting reports, a recombinant ASP (expressed using a baculovirus construct in insect cells) was utilized, potentially resulting in varying degrees of ASP purity and difficulty in accurately assessing the concentration of ASP.

To generate a pure ASP for functional characterization and NMR studies, McNulty *et al.* attempted to chemically synthesize the C-terminal domain of ASP,²⁹ previously shown to be as active as the full-length construct.³⁵ However, when attempting to cyclize the 53-residue fragment, less than 10% of the linear peptide folded with the correct disulfide bond pairing, with greater than 90% of the observed products resulting from incorrect disulfide bond formation.²⁹ This is unlike the chemical synthesis of the C-terminal domain of AGRP, which correctly folds to the proper disulfide bonding when put under oxidative conditions.^{48,50} In order to generate sufficient quantities of chemically synthesized ASP, two Tyr residues of AGRP were incorporated into the ASP synthesis (Gln115Tyr and Ser124Tyr) to generate a double-substituted form of ASP that properly folded under oxidative conditions.²⁹ This ASP-YY ligand was shown to possess potent antagonism at the MC3R (2.6 nM) as well as the MC1R (3.9 nM) and MC4R (0.5 nM), and incorporation of the two Tyr residues was crucial in yielding sufficient product for NMR studies. However, the addition of these Tyr amino acids confounds the activity of ASP at the MC3R, since it is impossible to discern if ASP-YY without the Tyr would retain the same activity, if the Tyr residues induce an altered conformation of the ligand, or if the Tyr amino acids result in novel ligand-receptor interactions. Despite these limitations, the *in vitro* pharmacological profile of ASP-YY at the MC1R, MC3R, and MC4R is similar to purified forms of recombinant ASP,^{39,40} supporting the hypothesis that ASP can interact at the MC3R.

In addition to being found in the active loop of ASP, select residue types in the current study are also found in species variants of AGRP. A PubMed BLASTp search of the sequence Cys-Arg-Phe-Phe-**Lys**-Ala-Phe-Cys (Asn to Lys) found this sequence to be in the AGRP precursor peptide of *Carassius auratus* (goldfish) and *Danio rerio* (zebrafish), indicating some species possess a basic residue in this position of the active loop of AGRP. Similarly, searching Cys-Arg-Phe-Phe-Asn-**Ser**-Phe-Cys (Ala to Ser) and Cys-Arg-Phe-Phe-Asn-**Thr**-Phe-Cys (Ala to Thr) found these sequences to be in the predicted AGRP of *Sarcophilus harrisi* (Tasmanian devil) and the precursor AGRP of *Rattus norvegicus* (rat), respectively. The observed residues types in the current study that maintained or increased MC4R potency (replacing Asn with basic residues, replacing Ala with hydroxyl-containing Ser) can also be found in naturally occurring AGRP orthologs, and may explain why these substitutions are tolerated at the MC4R.

An additional discovery from these structure-activity relationship studies was the apparent inverse agonism of several ligands at the MC5R. While the MC5R was previously reported to be constitutively active,⁴⁵ to the best of the authors' knowledge, no ligand to date has previously been shown to decrease cAMP through interaction with the MC5R. Twelve compounds (**1**, **2**, **3**, **4**, **5**, **6**, **8**, **9**, **10**, **12**, **13**, and **15**) decreased the basal-normalized signal in a sigmoidal dose-response curve. This set of compounds decreased the observed response

10–25% that of the basal response, with apparent potencies (the inflection point of the sigmoidal dose-response) ranging from 10 to 700 nM. All compounds with the sigmoidal dose-response MC5R inverse agonist activity were substituted at the Asn position within the scaffold. An additional 5 ligands (**7**, **16**, **21**, **23**, and **24**) decreased the response from basal 25–35%, although this activity did not plateau at high concentrations. This scaffold did not uniformly result in MC5R inverse agonism, as five ligands possessed no activity at the MC5R (**11**, **14**, **17**, **19**, and **22**), while two more compounds partially stimulated the MC5R (**18** and **20**). The variable MC5R activities suggest this is not an artifact due to the ligand scaffold, since a uniform response to all ligands was not observed. Additional structure-activity relationship studies on this scaffold at the MC5R, coupled with mutagenesis and docking studies, may help elucidate the putative ligand-receptor interactions between this class of ligands and the MC5R to explain the variable inverse agonist activity and how to improve the apparent potency and percent change from basal activity.

Conclusions

The present SAR study investigated the Asn and Ala positions within an AGRP-derived macrocyclic scaffold, c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro], in attempts to generate more potent and/or selective MCR antagonists. Similar to a prior SAR study, basic substitutions at the Asn position increased MC4R potency. Substitution of Ser at the Ala position maintained MC4R potency, while all other substitutions at the Ala position decreased MC4R antagonist potency. The same trends were observed at the MC3R, though ligands were approximately 100-fold less potent at this receptor compared to the MC4R. Observing that the potent MC4R substitutions were also found in the active loop of ASP, a final Phe to Ala substitution was synthesized and assayed, which was equipotent at the MC4R compared to the scaffold lead. These results indicated that the equivalent β -hairpin active loop positions of ASP could be inserted into this AGRP scaffold and resulted in similar or increased MC4R potency. From the increased knowledge of structural requirements for AGRP-derived ligand at the MC4R, this study may be important in the development of MC4R selective probes and lead molecules for potential weight gain therapeutics. Additionally, the observed MC5R inverse agonism from these SAR studies are useful in designing ligands and probes to clarify the role of the ubiquitously expressed MC5R *in vivo*.

Methods

Peptide Synthesis

All peptides were synthesized using standard Fmoc chemistry.^{56,57} The coupling reagents 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP) and 1-hydroxybenzotriazole (HOBt), the H-Pro-2-chlorotrityl resin, amino acids Fmoc-DPro, Fmoc-Phe, Fmoc-Ala, Fmoc-Abu (aminobutyric acid), Fmoc-Thr(tBu), Fmoc-Asp(tBu), Fmoc-Glu(tBu), Fmoc-Dap(Boc) (Diaminopropionic acid), Fmoc-Nle, Fmoc-Val, Fmoc-Leu, Fmoc-Trp(Boc), Fmoc-His(Trt), Fmoc-Gly, Fmoc-Ser(tBu), Fmoc-Lys(Boc), and Fmoc-Asn(Trt), and the agouti-related protein (AGRP86-132) were purchased from Peptides International (Louisville, KY). Amino acid Fmoc-D-Dap(Boc) was purchased from Bachem

(Torrance, CA). Dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), dimethylformamide (DMF) and anhydrous ethyl ether were purchased from Fisher (Fairlawn, NJ). Trifluoroacetic acid (TFA), dimethyl sulfoxide (DMSO), piperidine, triisopropylsilane (TIS), and *N,N*-diisopropylethylamine (DIEA) were purchased from Sigma-Aldrich (St. Louis, MO). All reagents and chemicals were ACS grade or better and were used without further purification.

Peptides were synthesized on a 0.05 mmol scale using H-Pro-2-chlorotrityl resin (0.68 to 0.76 meq/g substitution) in a 96-well block with an automated (Vantage Automated Parallel Peptide Synthesizer; Advanced ChemTech, Louisville, KY) or a semi-automated (LabTech I; Advanced ChemTech, Louisville, KY) instrument. Parallel syntheses consisted of two repeated steps separated by DMF washes: (i) removal of the Fmoc group with 20% piperidine in DMF (1x at rt for 5 min, 1x at rt for 20 min), and (ii) double coupling of the incoming Fmoc-protected amino acid (3.1 eq) with HBTU (3 eq) and DIEA (5 eq) in DMF at rt for 45 min. After completion of the syntheses, peptides were cleaved with a 99:1 DCM:TFA solution for 6 min. The cleavage solutions were concentrated and the side-chain protected linear peptides were precipitated using ice-cold ethyl ether. Peptides were cyclized in DCM with BOP (3 eq) and HOBt (3 eq) overnight using a peptide concentration of 1 mg/mL, and the DCM was removed under vacuum. Without further purification, the cyclized peptides were side-chain deprotected using a 95:2.5:2.5 TFA:TIS:H₂O solution for 2 h. The solution was concentrated and cyclic peptides precipitated with ice-cold ethyl ether.

Crude peptides were purified by RP-HPLC using a Shimadzu system with a UV detector and a semi-preparative RP-HPLC C18 bonded silica column (Vydac 218TP1010, 1 × 25 cm). Assayed peptides were at least 95% pure as assessed by analytical RP-HPLC utilizing a Shimadzu system with a photodiode array detector and an analytical C18 silica column (Vydac 218TP104, 0.46 × 25 cm) in two diverse solvent systems and had the correct average molecular mass by MALDI-MS (Applied Biosystems-Sciex 5800 MALDI/TOF/TOF-MS, University of Minnesota Mass Spectrometry Lab).

cAMP AlphaScreen® Bioassay

Cyclized peptides were dissolved in DMSO (NDP-MSH and AGRP in H₂O) at a stock concentration of 10⁻²M and were characterized pharmacologically using HEK293 cells stably expressing the mouse MC1R, MC3-5R by the cAMP AlphaScreen® assay (PerkinElmer) according to the manufacturer's instructions and as previously described.^{58,60}

Briefly, cells 70–90% confluent were dislodged with Versene (Gibco®) at 37 °C and plated 10,000 cells/well in a 384-well plate (Optiplate™) with 10 μL freshly prepared stimulation buffer (1X HBSS, 5 mM HEPES, 0.5 mM IBMX, 0.1% BSA, pH = 7.4) with 0.5 μg anti-cAMP acceptor beads per well. The cells were stimulated with the addition of 5 μL stimulation buffer containing peptide (concentrations from 10⁻⁴ to 10⁻¹³ M, determined by ligand potency) or forskolin (10⁻⁴ M) and incubated in the dark at room temperature for 2 hr.

Following stimulation, streptavidin donor beads (0.5 μg) and biotinylated-cAMP (0.62 μmol) were added to the wells in a green light environment with 10 μL lysis buffer (5 mM HEPES,

0.3% Tween-20, 0.1% BSA, pH = 7.4) and the plates were incubated in the dark at room temperature for an additional 2 hr. Plates were read on a Enspire (PerkinElmer) Alpha-plate reader using a pre-normalized assay protocol (set by the manufacturer).

Data Analysis

The EC₅₀ and pA₂ values represent the mean of duplicate replicates performed in at least three independent experiments. The EC₅₀ and pA₂ estimates and associated standard errors (SEM) were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (v4.0, GraphPad Inc.). When analyzing the inverse agonist activity at the MC5R, each replicate was normalized to the replicate signal at 10⁻¹⁰ M to observe change from basal activity. The percent inverse agonist activity was calculated from the normalized signal of three independent experiments. When a sigmoidal dose-response was observed, the percent inverse agonist activity reported was the change from basal to the plateau signal at high ligand concentrations; in these instances, the apparent potency was reported to be the inflection point of the sigmoidal curve. When inverse agonist activity was observed without a plateau at high concentrations, the percent inverse activity reported was the percent change from basal to signal at 100 μM concentrations. The ligands were assayed as TFA salts and not corrected for peptide context.

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Figure 1C was generated using the PDB files 1Y7K²⁹ and 1HYK,⁵⁰ and PyMOL Molecular Graphics System, version 1.7.0.5, Schrödinger, LLC, with the computing resources of University of Minnesota Supercomputing Institute.

Abbreviations

CTH	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

MCR

melanocortin 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

RP-HPLC

reverse-phase high-pressure liquid chromatography

SAR

structure-activity relationships

SNPs

single nucleotide polymorphisms

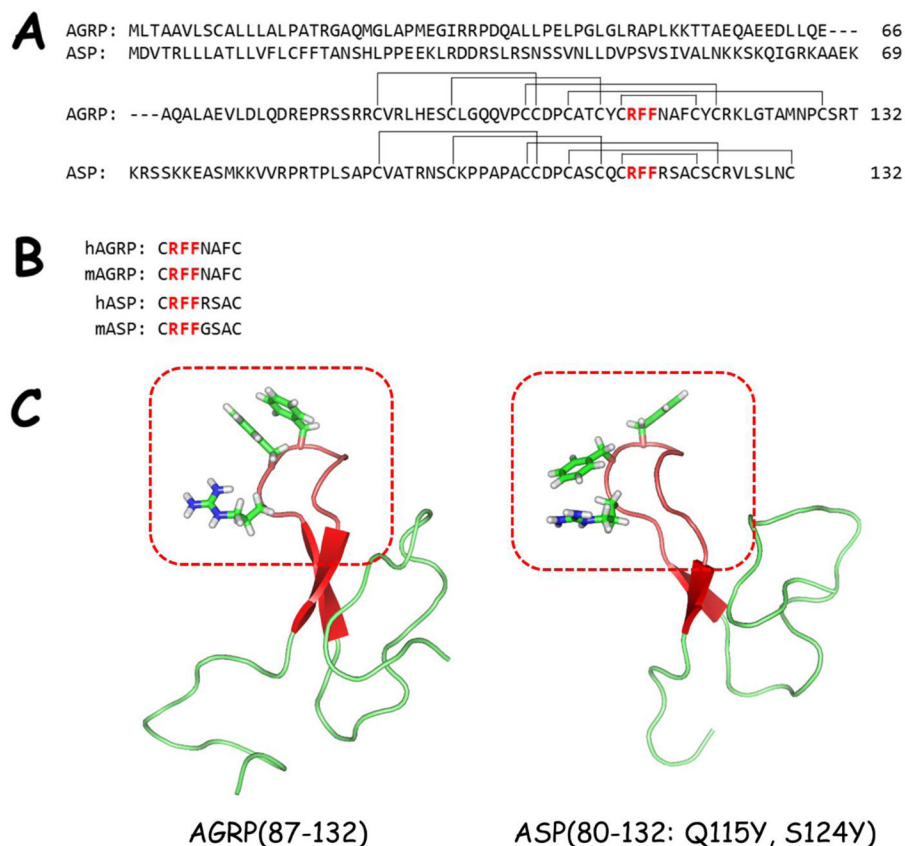
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**Figure 1.**

A) Sequence alignment of human ASP and AGRP. The hypothesized Arg-Phe-Phe pharmacophore region is highlighted in red. The disulfide pairing is also indicated.

B) Sequence alignment of the postulated active loop of human and mouse ASP and AGRP. The conserved Arg-Phe-Phe is highlighted in red.

C) NMR solution structures of human AGRP(87-132) (PDB = 1HYK)⁵⁰ and human ASP(80-132: Q115Y, S124Y) (PDB = 1Y7K).²⁹ The β -hairpin loops are colored red and highlighted in the red box. The Arg-Phe-Phe tripeptide side-chains are drawn to illustrate their similar positions within the structures.

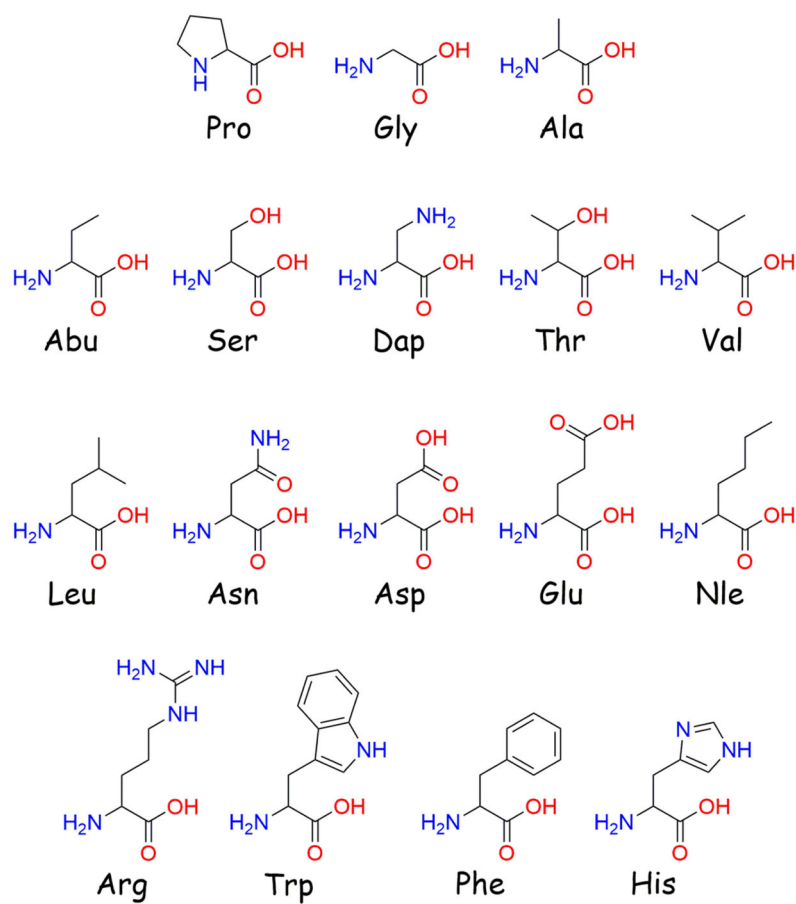


Figure 2.
Structures of the amino acids used in this study.

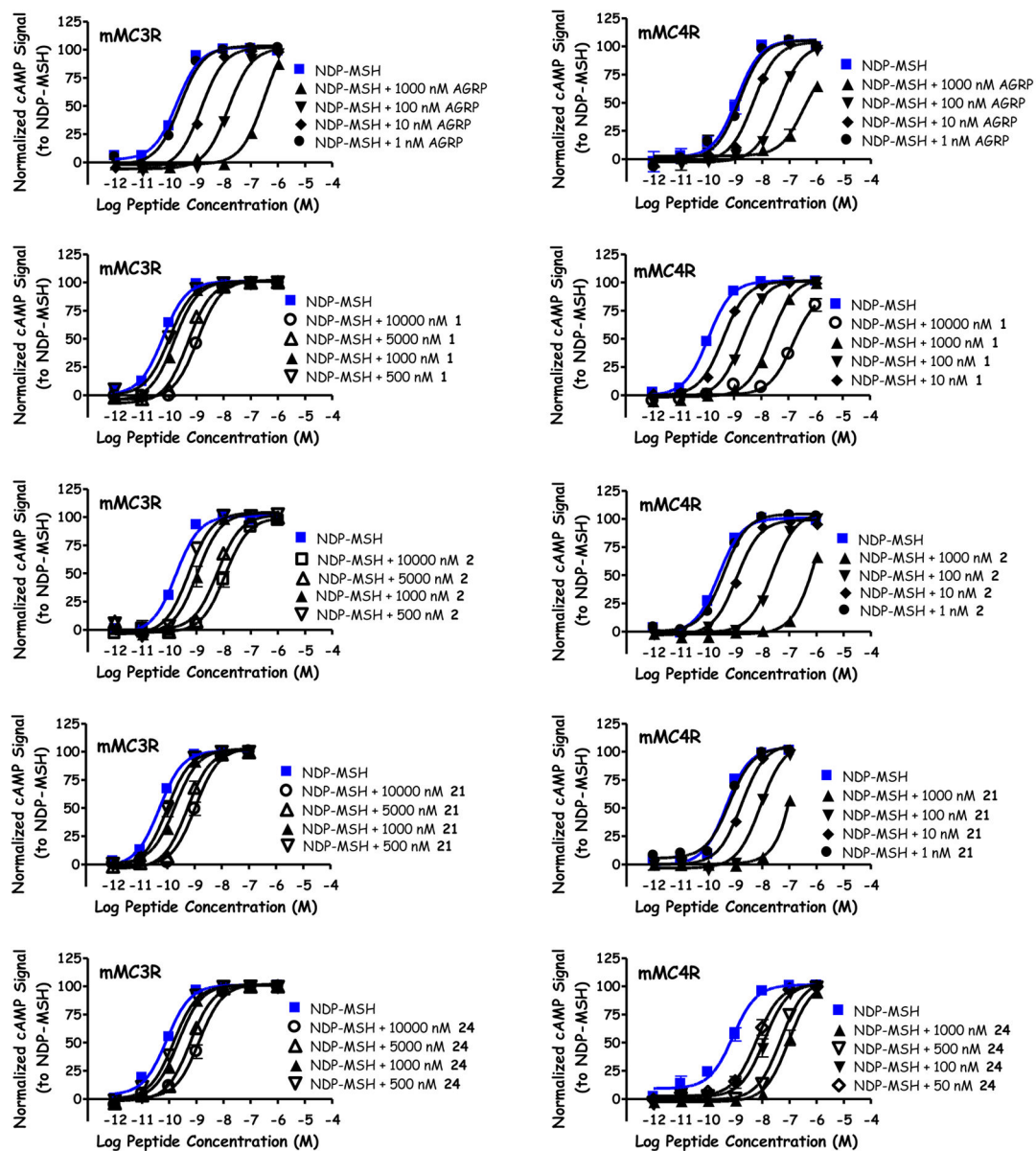


Figure 3.
Illustration of the antagonist pharmacology at the mMC3R and mMC4R for AGRP, 1, 2, 21, and 24.

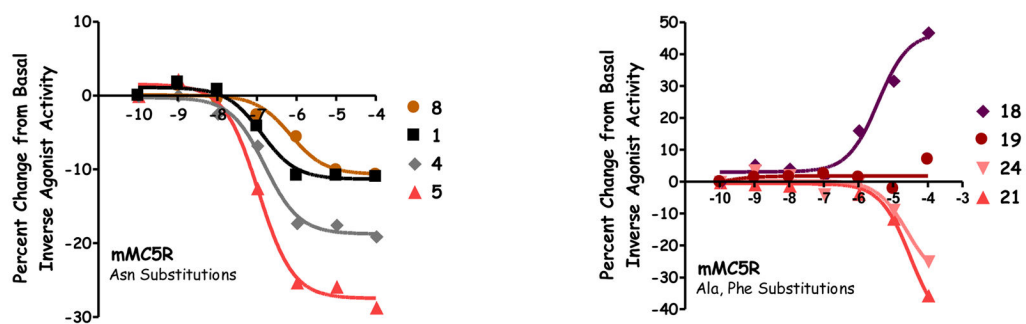


Figure 4.
Illustration of the pharmacology at the MC5R for **1, 4, 5, 8, 18, 19, 21, and 24.**

Table 1

Analytical Data for Peptides Synthesized in this Study.

Peptide	Sequence	Retention Time (min) ^a		M (calc)	mass spectral analysis (M+1)	purity %
		system 1	system 2			
1	c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro]	18.1	26.8	976.5	977.5	>99
2	c[Pro-Arg-Phe-Phe-Dap-Ala-Phe-DPro]	18.0	29.3	948.5	949.4	>98
3	c[Pro-Arg-Phe-Phe-Ala-Ala-Phe-DPro]	18.9	28.7	933.5	934.3	>99
4	c[Pro-Arg-Phe-Phe-Abu-Ala-Phe-DPro]	19.5	29.4	947.5	948.5	>98
5	c[Pro-Arg-Phe-Phe-Ser-Ala-Phe-DPro]	18.8	29.2	949.5	950.3	>99
6	c[Pro-Arg-Phe-Phe-Thr-Ala-Phe-DPro]	19.5	29.9	963.5	964.2	>99
7	c[Pro-Arg-Phe-Phe-Asp-Ala-Phe-DPro]	18.4	28.5	977.5	978.5	>99
8	c[Pro-Arg-Phe-Phe-Glu-Ala-Phe-DPro]	18.3	28.1	991.5	992.2	>99
9	c[Pro-Arg-Phe-Phe-DDap-Ala-Phe-DPro]	18.1	29.4	948.5	949.4	>95
10	c[Pro-Arg-Phe-Phe-His-Ala-Phe-DPro]	18.0	29.1	999.5	1000.2	>96
11	c[Pro-Arg-Phe-Phe-Nle-Ala-Phe-DPro]	21.2	31.0	975.5	976.3	>99
12	c[Pro-Arg-Phe-Phe-Leu-Ala-Phe-DPro]	21.0	30.8	975.5	976.3	>99
13	c[Pro-Arg-Phe-Phe-Val-Ala-Phe-DPro]	20.4	30.8	961.5	962.2	>99
14	c[Pro-Arg-Phe-Phe-Phe-Ala-Phe-DPro]	21.6	31.6	1009.5	1010.3	>98
15	c[Pro-Arg-Phe-Phe-Trp-Ala-Phe-DPro]	23.2	30.7	1048.5	1049.2	>97
16	c[Pro-Arg-Phe-Phe-Asn-Asp-Phe-DPro]	17.7	26.6	1020.5	1021.6	>99
17	c[Pro-Arg-Phe-Phe-Asn-Glu-Phe-DPro]	17.4	26.7	1034.5	1035.6	>98
18	c[Pro-Arg-Phe-Phe-Asn-Lys-Phe-DPro]	16.0	25.2	1033.6	1034.6	>99
19	c[Pro-Arg-Phe-Phe-Asn-His-Phe-DPro]	16.2	25.4	1042.5	1043.6	>99
20	c[Pro-Arg-Phe-Phe-Asn-Phe-Phe-DPro]	21.4	32.4	1052.5	1053.7	>97
21	c[Pro-Arg-Phe-Phe-Asn-Ser-Phe-DPro]	17.7	27.2	992.5	993.6	>96
22	c[Pro-Arg-Phe-Phe-Asn-Leu-Phe-DPro]	21.2	32.8	1018.5	1019.6	>98
23	c[Pro-Arg-Phe-Phe-Asn-Gly-Phe-DPro]	17.6	26.5	962.5	963.5	>96
24	c[Pro-Arg-Phe-Phe-Asn-Ala-Ala-DPro]	15.4	25.0	900.5	901.8	>97

^aPeptide retention times (min) are reported for solvent system 1 (10% acetonitrile in 0.1% trifluoroacetic acid/water and a gradient to 90% acetonitrile over 35 min) and solvent system 2 (10% methanol in 0.1% trifluoroacetic acid/water and a gradient to 90% methanol over 35 min). An analytical Vydac C18 column (Vydac 218TP104) was used with a flow rate of 1.5 mL/min. The peptide purity was determined by HPLC at a wavelength of 214 nm.

Table 2

Pharmacology of AGRP Loop Analogues at the Mouse Melanocortin Receptors.^a

Peptide	Sequence	mMC1R		mMC3R		mMC4R		mMC5R	
		EC ₅₀ (nM)	pA ₂	EC ₅₀ (nM)	pA ₂	EC ₅₀ (nM)	pA ₂	EC ₅₀ (nM)	pA ₂
NDP-MSH		0.021±0.002		0.19±0.02		0.41±0.03		0.18±0.02	
hAGRP(86-132)		N.D.		8.7±0.1		8.7±0.2		N.D.	
1	c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro]	25% @ 100µM		>100,000	6.3±0.1	>100,000	8.2±0.1	Inverse Agonist -10%, 130 nM	
2	c[Pro-Arg-Phe-Phe-Dap-Ala-Phe-DPro]	30% @ 100µM		>100,000	6.52±0.09	>100,000	8.7±0.1	Inverse Agonist -15%, 60 nM	
3	c[Pro-Arg-Phe-Phe-Ala-Ala-Phe-DPro]	>100,000		>100,000	<5.5	>100,000	7.32±0.09	Inverse Agonist -20%, 250 nM	
4	c[Pro-Arg-Phe-Phe-Abu-Ala-Phe-DPro]	>100,000		>100,000	<5.5	>100,000	7.3±0.2	Inverse Agonist -20%, 160 nM	
5	c[Pro-Arg-Phe-Phe-Ser-Ala-Phe-DPro]	>100,000		>100,000	5.9±0.2	>100,000	7.7±0.3	Inverse Agonist -25%, 140 nM	
6	c[Pro-Arg-Phe-Phe-Thr-Ala-Phe-DPro]	>100,000		>100,000	6.4±0.2	>100,000	7.76±0.07	Inverse Agonist -20%, 50 nM	
7	c[Pro-Arg-Phe-Phe-Asp-Ala-Phe-DPro]	>100,000		>100,000	<5.5	>100,000	6.79±0.07	Inverse Agonist -25% @ 100 µM	
8	c[Pro-Arg-Phe-Phe-Glu-Ala-Phe-DPro]	20,500±500		>100,000	<5.5	>100,000	7.00±0.09	Inverse Agonist -10%, 700 nM	
9	c[Pro-Arg-Phe-Phe-DDap-Ala-Phe-DPro]	>100,000		>100,000	6.7±0.2	>100,000	8.58±0.04	Inverse Agonist -10%, 110 nM	
10	c[Pro-Arg-Phe-Phe-His-Ala-Phe-DPro]	>100,000		>100,000	6.46±0.07	>100,000	8.3±0.1	Inverse Agonist -15%, 10 nM	
11	c[Pro-Arg-Phe-Phe-Nle-Ala-Phe-DPro]	>100,000		>100,000	<5.5	>100,000	6.9±0.1	>100,000	
12	c[Pro-Arg-Phe-Phe-Leu-Ala-Phe-DPro]	>100,000		>100,000	5.7±0.2	>100,000	7.34±0.04	Inverse Agonist -20%, 110 nM	
13	c[Pro-Arg-Phe-Phe-Val-Ala-Phe-DPro]	55% @ 100µM		>100,000	<5.5	>100,000	5.9±0.1	Inverse Agonist -25%, 510 nM	
14	c[Pro-Arg-Phe-Phe-Phe-Ala-Phe-DPro]	>100,000		>100,000	<5.5	>100,000	6.63±0.09	>100,000	
15	c[Pro-Arg-Phe-Phe-Trp-Ala-Phe-DPro]	80% @ 100µM		40% @ 100µM	5.70±0.08	50% @ 100µM	6.81±0.08	Inverse Agonist -20%, 150 nM	
16	c[Pro-Arg-Phe-Phe-Asn-Asp-Phe-DPro]	65% @ 100µM		>100,000	<5.5	>100,000	<5.5	Inverse Agonist -25% @ 100 µM	
17	c[Pro-Arg-Phe-Phe-Asn-Glu-Phe-DPro]	60% @ 100µM		>100,000	<5.5	>100,000	<5.5	>100,000	

Peptide	Sequence	mMC1R		mMC3R		mMC4R		mMCSR	
		EC ₅₀ (nM)	pA ₂	EC ₅₀ (nM)	pA ₂	EC ₅₀ (nM)	pA ₂	EC ₅₀ (nM)	pA ₂
18	c[Pro-Arg-Phe-Phe-Asn-Lys-Phe-DPro]	60% @ 100µM	<5.5	45% @ 100µM	<5.5	>100,000	6.11±0.01	>100,000	45% @ 100µM
19	c[Pro-Arg-Phe-Phe-Asn-His-Phe-DPro]	Partial Agonist 40% NDP (2,600±1,200)	<5.5	>100,000	<5.5	>100,000	6.07±0.09	>100,000	>100,000
20	c[Pro-Arg-Phe-Phe-Asn-Phe-Phe-DPro]	70% @ 100µM	5.72±0.08	35% @ 100µM	5.71±0.03	35% @ 100µM	5.71±0.03	35% @ 100µM	25% @ 100µM
21	c[Pro-Arg-Phe-Phe-Asn-Ser-Phe-DPro]	>100,000	6.2±0.1	>100,000	8.2±0.2	>100,000	8.2±0.2	>100,000	Inverse Agonist -30% @ 100 µM
22	c[Pro-Arg-Phe-Phe-Asn-Leu-Phe-DPro]	80% @ 100µM	<5.5	25% @ 100µM	6.18±0.05	25% @ 100µM	6.18±0.05	>100,000	>100,000
23	c[Pro-Arg-Phe-Phe-Asn-Gly-Phe-DPro]	>100,000	<5.5	>100,000	6.1±0.3	>100,000	6.1±0.3	>100,000	Inverse Agonist -35% @ 100 µM
24	c[Pro-Arg-Phe-Phe-Asn-Ala-Ala-DPro]	>100,000	6.1±0.2	>100,000	8.16±0.08	>100,000	8.16±0.08	>100,000	Inverse Agonist -25% @ 100 µM

²The indicated errors represent the standard error of the mean determined from at least three independent experiments. The antagonistic pA₂ values were determined using the Schild analysis and the agonist NDP-MSH. The use of >100,000 indicates that the compound was examined but lacked agonist activity at up to 100 µM concentrations. A percentage denotes the percent maximal stimulatory response observed at 100 µM concentrations but not enough stimulation was observed to determine an EC₅₀ value. N.D. indicates values not determined. The use of <5.5 indicates that no antagonist potency was observed in the highest concentration ranged assayed (10,000, 5,000, 1,000, and 500 nM). Partial agonist indicates partial agonist activity was observed, along with the percentage of activation relative to NDP-MSH and the EC₅₀. Inverse agonist indicates that inverse agonist pharmacology was observed with the percent decrease from basal indicated. For inverse agonists, an apparent potency is indicated for the inflection point on sigmoidal dose-response curves; if a decrease in cAMP signal was observed without a sigmoidal dose-response curve, the percent change from basal at 100 µM concentrations is indicated.