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Arg-Phe-Phe D-Amino Acid Stereochemistry Scan in the Macrocyclic Agouti-Related Protein Antagonist Scaffold c[Pro-Arg-Phe-Phe-Xaa-Ala-Phe-DPro] Results in Unanticipated Melanocortin-1 Receptor Agonist Profiles

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Abstract

The melanocortin-3 and melanocortin-4 receptors (MC3R and MC4R), endogenous agonists derived from the proopiomelanocortin gene transcript, and naturally-occurring antagonists agouti and agouti-related protein (AGRP) have been linked to biological pathways associated with energy homeostasis. The active tripeptide sequence of AGRP, Arg111-Phe112-Phe113, is located on a hypothesized β -hairpin loop. Herein, stereochemical modifications of the Arg-Phe-Phe sequence were examined in the octapeptide AGRP-derived macrocyclic scaffold c[Pro-Arg-Phe-Phe-Xxx-Ala-Phe-DPro], where Xxx was Asn or diaminopropionic acid (Dap). Macrocyclic peptides were synthesized with one, two, or three residues of the Arg-Phe-Phe sequence substituted with the corresponding D-isomer(s), generating a 14 compound library. While L-to-D inversions of the Arg-Phe-Phe sequence in a 20-residue AGRP-derived ligand previously resulted in agonist activity at the MC1R, MC3R, MC4R, and MC5R, only the MC1R was consistently stimulated by the macrocyclic ligands in the present study, with varying ligand potencies and efficacies observed at the MC1R. A general trend of increased MC4R antagonist potency was observed for Dap-containing compounds, while MC5R inverse agonist activity was observed for select ligands. It was observed that stereochemical modification of the Arg-Phe-Phe active tripeptide sequence was insufficient to convert melanocortin antagonist into agonists. Overall, these observations are important in the design of melanocortin ligands possessing potent and selective agonist and antagonist activities.

Graphical Abstract

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Keywords

AGRP; macrocycles; melanocortin receptors; D-amino acids

Introduction

The five melanocortin receptors (MCRs),^{1–8} discovered to date, are members of the G protein-coupled receptor (GPCR) superfamily. These receptors have been implicated in numerous physiological functions, including skin pigmentation (MC1R),^{1, 2} steroidogenesis (MC2R),^{1, 9} and energy homeostasis (MC3R and MC4R).^{10–13} The MCRs are stimulated by agonists derived from the proopiomelanocortin (POMC) gene transcript,¹⁴ and include α -MSH, β -MSH, γ -MSH, and ACTH. Common to these melanocortin agonists is a His-Phe-Arg-Trp tetrapeptide sequence. Due to the role of the MC3R and MC4R in energy homeostasis,^{10–13, 15} agonist compounds that decrease feeding have been advanced to clinical trials in humans, including several peptides and small molecules as previously reviewed.¹⁶ While MC4R-selective ligands decrease food intake, side-effects including increased blood pressure,¹⁷ increased erectile activity,¹⁸ and skin darkening^{19, 20} have been reported. New ligands that possess unique scaffolds may bypass some of these effects, illustrated by the use of setmelanotide in patients deficient in POMC to lower body weight without an accompanied increase in blood pressure.¹⁹

Unique to the melanocortin receptor family is the presence of two endogenous antagonists, agouti-signaling protein (ASP)^{21, 22} and agouti-related protein (AGRP).^{23–25} Common to both antagonists is a highly structured C-terminal domain possessing 5 disulfide bonds and a tripeptide Arg-Phe-Phe sequence that has been demonstrated to be critical for receptor affinity and antagonist potency.^{26, 27} While originally described as an antagonist at the MC1R and MC4R,²⁸ subsequent work with a synthetic derivative of ASP (ASP-YY) demonstrated that ASP may also possess antagonist pharmacology at the MC3R.²⁹ Pharmacological characterization of AGRP demonstrated that AGRP is an antagonist at the MC3R and MC4R,^{23, 24} as well as an inverse agonist at the MC4R.^{30, 31} Truncation studies involving AGRP indicated that the decapeptide H-Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-OH was the minimal sequence required for sub-micromolar functional potency at the MC4R,²⁷ and further extension of four terminal residues was required to achieve functional potency at the MC3R.³² Although α -MSH stimulation of the MC1R was not antagonized by AGRP,²⁴ the decapeptide H-Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-OH possessed micromolar affinity and functional agonist potency at the MC1R.³³ Replacing the disulfide bond with a lactam bridge by substituting Asp and diaminopropionic acid (Dap) for Cys resulted in a ligand (H-Tyr-c[Dap-Arg-Phe-Phe-Asn-Ala-Phe-Asp]-Tyr-NH₂)

that retained micromolar agonist potency at the MC1R.³⁴ These data suggest that although AGRP does not antagonize the MC1R, small peptides based upon the active loop of AGRP may be MC1R agonists.

Utilizing a 20 amino acid derivative of AGRP possessing the active loop sequence (AGRP[103–122]; H-Asp-Pro-Ala-Ala-Thr-Ala-Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-Ala-Arg-Lys-Leu-OH, with Cys105Ala, Cys108Ala, and Cys119Ala substitutions), Joseph *et al.* systemically replaced the Arg111, Phe112, and Phe113 residues with the corresponding D-isomers in single, double, and triple substitutions.³⁵ Although the all L-isomer was an antagonist at the MC3R and MC4R, a sub-micromolar agonist at the MC1R, and possessed no activity at the MC5R, agonist activity was observed for every D-substitution at the MC1R, MC3R, MC4R, and MC5R.³⁵ Additionally, replacing the DPhe-Arg-Trp tripeptide sequence of the potent, synthetic melanocortin agonists NDP-MSH³⁶ and MTII^{37, 38} with the Arg-Phe-Phe sequence also resulted in melanocortin agonist activity at the MC1R, MC3R, MC4R, and MC5R when the equivalent Phe112 was replaced with the D-isomer (Arg-DPhe-Phe) in both scaffolds.³⁹ Both reports indicate that MC3R and MC4R agonist activity may result from stereochemical modification of the Arg-Phe-Phe tripeptide sequence in the absence of the His-Phe-Arg-Trp tetrapeptide sequence common to the POMC-derived melanocortin agonists.

One approach to generate melanocortin antagonists has been to cyclize the Arg-Phe-Phe-Asn-Ala-Phe hexapeptide residues of AGRP (residues 111–116) head-to-tail through a DPro-Pro motif, resulting in the octapeptide c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro] macrocycle that was 50-fold less potent than AGRP at the MC4R.⁴⁰ Additional structure-activity relationship (SAR) studies at the Asn114 position indicated that substitution with Gly or basic amino acids increased antagonist potency relative to Asn.^{40, 41} Substitution of Asn with Dap resulted in the most potent macrocyclic ligand at the MC4R, which was equipotent to AGRP.^{40, 41} Further substitutions at the Ala115 and non-pharmacophore Phe116 positions indicated that Ser could replace Ala and norleucine (Nle), Trp, Tyr, and Ala could replace Phe and maintain potency relative to the native sequence at the MC4R.^{41, 42} These macrocyclic ligands possessed >100-fold decreased antagonist potency at the MC3R compared to AGRP, micromolar agonist potency or higher at the MC1R, and select compounds were apparent inverse agonists at the MC5R.^{40–42}

Prior studies have reported that AGRP-derived ligands possess MC1R agonist activity, and the stereochemical modification of Arg-Phe-Phe domain can generate MCR agonist activity across different receptors. It was hypothesized that stereochemical modifications of the Arg-Phe-Phe region in the potent c[Pro-Arg-Phe-Phe-Xxx-Ala-Phe-DPro] scaffold (where Xxx is Asn or Dap) would also generate MCR agonist activity. Furthermore, since this scaffold is more potent at the MC4R compared to the previously used 20 amino acid template, it was hypothesized that the resulting MCR agonist potency may also be increased, generating potent MCR agonists without the His-Phe-Arg-Trp pharmacophore. To test this hypothesis, systematic stereochemical modification of the Arg-Phe-Phe tripeptide sequence in both the Asn and Dap octapeptide macrocyclic scaffolds was performed, generating a 14 compound library that was assessed for activity at the MC1R, MC3R, MC4R, and MC5R.

Results

Peptide Synthesis and Characterization:

Peptides were synthesized manually using a semi-automated synthesizer or a microwave-assisted synthesizer using standard fluorenylmethoxycarbonyl (Fmoc) techniques.^{43, 44} Following cleavage from the resin while retaining amino acid side chains, the amide bond between the Arg and Pro was formed using BOP and HOBt to generate the peptide macrocycle as previously described.^{40–42, 45} After side-chain deprotection, peptides were purified to >95% by semi-preparative reverse-phase high pressure liquid chromatography (RP-HPLC). Peptides were assessed for purity using 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:

Macrocyclic peptides were assayed using HEK293 stably expressing the mouse MC1R, MC3R, MC4R and MC5R, according to the manufacturer's instructions and as previously described.^{46, 47} The MC2R is only stimulated by the ACTH and was therefore not studied. Ligands were first examined for agonist activity at the MCRs. Compounds that did not result in a full agonist signal at the MC3R and MC4R were then assayed for antagonist activity using a Schild paradigm⁴⁸ and the synthetic melanocortin agonist NDP-MSH.³⁶ The AlphaScreen cAMP signal was normalized to NDP-MSH and basal activity for illustrative purposes as previously described since it is a competition assay and results in lower signal at higher concentrations.^{40, 49} Compounds that were within a 3-fold range were considered equipotent due to the inherent error in the assay.

Arg-Phe-Phe D-Amino Acid Scan, Asn-Substituted Macrocyclic:

Compound **1**, comprised of the native sequence of the hypothesized active loop of AGRP cyclized through a DPro-Pro motif (c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro]), was previously reported to partially stimulate the MC1R (25% of maximal NDP-MSH signal at 100 μ M concentrations; Table 2), not stimulate the MC3R or MC4R, and possessed inverse agonist activity at the MC5R (–10% signal from basal activity, with an apparent potency of $EC_{50} = 130$ nM corresponding to the inflection point of a sigmoidal dose-response curve).⁴¹ This peptide was an antagonist at the MC3R and MC4R, with pA_2 values of 6.3 and 8.2, respectively.⁴¹ Substitution of DArg into this scaffold (**5**) resulted in partial stimulation of the MC1R (50% at 100 μ M; Figure 1), and loss of agonist and antagonist activities in the concentrations assayed at the MC3R, MC4R, and MC5R. A DPhe substitution at the Phe112 position (**4**) produced a peptide with partial agonist efficacy at the MC1R (55% maximal NDP-MSH stimulation, $EC_{50} = 700$ nM; Figure 1), equipotent antagonist potency within experimental error at the MC3R and MC4R ($pA_2 = 5.8$ and 7.7, respectively) compared to **1**, and decreased cAMP production 25% from basal levels at the MC5R at 100 μ M concentrations (Figure 1). Replacement of the Phe113 position with DPhe, **5**, yielded a peptide that partially stimulated the MC1R (50% NDP-MSH maximal signal at 100 μ M) and possessed micromolar antagonist potency at the MC4R ($pA_2 = 5.9$). No activity was observed for **5** at the MC3R or MC5R.

Replacing both the Arg111 and Phe112 positions with the D-isomers resulted in peptide **6**, which was a partial agonist at the MC1R (90% NDP-MSH maximal signal, $EC_{50} = 500$ nM) and partially activated the MC5R at 100 μ M concentrations (55% NDP-MSH maximal signal). No activity was observed for **6** at the MC3R or MC4R. The peptide **7**, possessing DArg and DPhe at positions Arg111 and Phe113, was a full agonist at the MC1R (1,200 nM) and MC5R (10,000 nM; Figure 1), and partially stimulated the MC3R at 100 μ M concentrations (35%). The substitutions of DPhe at both positions Phe112 and Phe113 resulted in peptide **8**, a partial agonist at the MC1R (85% NDP-MSH maximal signal, $EC_{50} = 800$ nM) and micromolar antagonist at the MC4R ($pA_2 = 5.9$). The replacement of Arg111, Phe112, and Phe113 with their D-isomers, **9**, resulted in similar partial agonist efficacy at the MC1R (85% NDP-MSH signal, $EC_{50} = 1,300$ nM) compared to **8**, while partially activating the MC5R at 100 μ M concentrations (55% NDP-MSH signal; Figure 1). No agonist or antagonist activity was observed for **9** at the MC3R or MC4R at the concentrations assayed.

Arg-Phe-Phe D-Amino Acid Scan, Dap-Substituted Macrocyclic:

Replacement of the Asn residue with a diaminopropionic acid (Dap) has previously been shown to increase the potency of the octapeptide macrocyclic scaffold, and is an equipotent antagonist at the MC4R compared to AGRP.^{40, 41} This peptide (**2**, c[Pro-Arg-Phe-Phe-Dap-Ala-Phe-DPro]) has previously been shown to partially stimulate the MC1R at 100 μ M concentrations (30% NDP-MSH signal), possess antagonist activity at the MC3R and MC4R ($pA_2 = 6.5$ and 8.7 , respectively), and was an apparent inverse agonist at the MC5R (15% decreased cAMP levels from basal, $EC_{50} = 60$ nM).⁴¹ Replacement of Arg111 in the scaffold with DArg, **10**, resulted in a peptide that partially activated the MC1R, MC3R and MC4R at 100 μ M concentrations (25%) while no activity was observed at the MC5R. Peptide **10** also possessed antagonist activity at the MC3R and MC4R, with pA_2 values of 5.7 and 6.8 , respectively. The substitution of Phe112 with DPhe resulted in **11**, the most potent full agonist at the MC1R in this study ($EC_{50} = 230$ nM; Figure 1). No agonist activity was observed for **11** at the MC3R and MC5R up to 100 μ M concentrations, while this peptide partially activated the MC4R at 100 μ M (20% NDP-MSH maximal signal). This peptide possessed equipotent MC4R antagonist potency compared to **2** at the MC4R ($pA_2 = 8.4$; Figure 2), and was a micromolar antagonist at the MC3R ($pA_2 = 5.9$). Replacement of Phe113 with DPhe, **12**, resulted in a peptide that was a partial agonist at the MC1R (75% NDP-MSH maximum signal, $EC_{50} = 800$ nM) and was an antagonist at the MC4R ($pA_2 = 7.3$). No activity was observed for **12** at the MC3R or MC5R at the concentrations assayed.

Double substitution at the Arg111 and Phe112 positions with their D-isomers resulted in **13**, which possessed partial agonist efficacy at the MC1R (70% NDP-MSH, $EC_{50} = 800$ nM), antagonist activity at the MC3R and MC4R ($pA_2 = 6.0$ and 5.5 , respectively), and decreased cAMP at the MC5R by 30% from basal levels at the MC5R at 100 μ M concentrations. A similar activity trend to **13** was observed when positions Arg111 and Phe113 were substituted with the corresponding D-amino acids, resulting in peptide **14**. This peptide (**14**) was a partial agonist at the MC1R (70% NDP-MSH, $EC_{50} = 450$ nM), possessed antagonist potency at the MC3R and MC4R ($pA_2 = 5.7$ and 6.8 , respectively), and decreased cAMP signal by 20% from basal activity at the MC5R. Replacement of both Phe112 and Phe113

with DPhe, **15**, generated a full agonist at the MC1R ($EC_{50} = 700$ nM), an antagonist at the MC3R ($pA_2 = 5.7$) and MC4R ($pA_2 = 7.4$), and no activity was observed at the MC5R at concentrations up to 100 μ M. The triple substitution of Arg111, Phe112, and Phe113 with the corresponding D-isomers (**16**) resulted in a partial agonist at the MC1R (60% NDP-MSH maximal signal, $EC_{50} = 2,200$ nM), no activity at the MC3R, a micromolar potent MC4R antagonist ($pA_2 = 5.8$), and a peptide that decreased cAMP production 15% from basal activity at 100 μ M concentrations.

Discussion

Select stereochemical inversion combinations of the Arg-Phe-Phe tripeptide domain in a twenty residue AGRP-derived cyclic structure was previously shown to result in agonist activity at the MC1R, MC3R, MC4R, and MC5R, without the His-Phe-Arg-Trp agonist sequence.³⁵ The two octapeptide macrocyclic scaffolds utilized in this study, c[Pro-Arg-Phe-Phe-Xxx-Ala-Phe-DPro] (where Xxx is Asn or Dap), are more potent MC4R antagonists than the previously reported twenty residue fragments, with pA_2 values at the MC4R of 8.2 (Asn octapeptide)⁴¹ and 8.7 (Dap octapeptide)⁴¹ compared to 6.9 (20 residue peptide).³⁵ Due to the increased antagonist potency at the MC4R, it was hypothesized that stereochemical modifications of either macrocyclic scaffold may result in melanocortin agonist ligands with increased potency at the MC1R, MC3R, MC4R, and MC5R, compared to the twenty residue AGRP derivative previously reported. However, only three compounds (**7**, **11**, and **15**) possessed full agonist efficacy at the MC1R and one compound (**7**) was a full agonist at the MC5R (Table 2). The MC3R and MC4R were at most stimulated to 35% and 25% the maximal signal of NDP-MSH at 100 μ M concentrations, perhaps suggesting that additional AGRP residues outside the hexapeptide sequence are required for consistent MCR agonism when inverting the stereochemical of the Arg-Phe-Phe tripeptide sequence of AGRP-derived ligands.

While previously AGRP-derived peptides have been reported to possess agonist potencies of 2.89 μ M (H-Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-OH),³³ 28.1 μ M (H-Tyr-c[Dap-Arg-Phe-Phe-Asn-Ala-Phe-Asp]-Tyr-NH₂),³⁴ and 960 nM (H-Asp-Pro-Ala-Ala-Thr-Ala-Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-Ala-Arg-Lys-Leu-OH),³⁵ the lead macrocycles for the present study were only able to partially stimulate the MC1R at 100 μ M concentrations (25% and 30% for **1** and **2**, respectively). Two of the macrocycles (**11** and **15**) from the present study possess sub-micromolar agonist potency and full efficacy at the MC1R, representing > 1,000-fold potency increase compared to the starting ligand **2**. Several additional macrocyclic ligands possessed sub-maximal agonist efficacy or partially stimulated the MC1R at 100 μ M concentrations. The broad range of agonist activities suggests that the macrocyclic scaffold may be tunable for MC1R potency and efficacy. The two most potent ligands for MC4R antagonist potency (**2** and **11** with $pA_2 = 8.7$ and 8.4, respectively) possess MC1R agonist activities of 30% maximal NDP-MSH stimulation at 100 μ M (**2**) and full agonist efficacy with an $EC_{50} = 230$ nM (**11**). One off-target effect of MC4R agonist compounds in clinical development has been skin darkening/altered pigmentation in humans, presumably due to MC1R agonism.^{19, 20} Due to the differential activities at the MC1R while retaining MC4R potency, the present macrocyclic scaffold may

be able to modulate the potential risk of altering pigmentation, suggesting a benefit to the AGRP-derived macrocycles.

In the 20-residue AGRP fragment, the two most potent MC1R agonist involved the DPhe112 and DPhe112/DPhe113 stereochemical modifications,³⁵ the same inversion patterns found in the most potent MC1R full agonists in the present study (**11** and **15**). The equivalent DPhe112 substitution also resulted in the most potent MC1R agonists when the Arg-Phe-Phe tripeptide sequence replaced the DPhe-Arg-Trp of NDP-MSH and MTII.³⁹ While these substitutions increased potency with retained efficacy in different scaffolds, the same stereochemical inversions in the Asn-containing macrocycle resulted in partial agonists possessing 55% (**4**) and 85% (**8**) the maximal signal of NDP-MSH. These results indicate that DPhe112 and DPhe112/DPhe113 stereochemical inversions are not sufficient alone to generate full MC1R agonist ligands.

It has previously been reported that the Dap substitution increased MC4R antagonist potency compared to the native sequence Asn residue.^{40, 41} Stereochemically inverted residues within the Arg-Phe-Phe tripeptide sequence also resulted in a similar trend, with Dap-substituted macrocycles possessing increased MC4R antagonist potency compared to the Asn-containing peptides. The four macrocycles that did not possess antagonist activity at the highest assayed concentrations (**3**, **6**, **7**, and **9**) all contained an Asn substitution. The equivalent Dap substituted peptides (**10**, **13**, **14**, and **16**) possess antagonist potencies of 6.8, 5.5, 6.8, and 5.8, respectively. Two Asn-containing macrocycles possess micromolar MC4R antagonist potencies (**5** and **8**; $pA_2 = 5.9$ for both). The equivalent DPhe113 and DPhe112/DPhe113 Dap substituted peptides (**12** and **15**) possessed sub-micromolar MC4R antagonist potencies ($pA_2 = 7.3$ and 7.4 , respectively). The most potent Asn antagonist at the MC4R (**4**, $pA_2 = 7.7$) contained the DPhe112 stereo-inversion. The same DPhe112 substitution in the Dap scaffold (**11**) resulted in an antagonist ligand with a $pA_2 = 8.4$. These data reflect the trend that Dap-substituted macrocycles were more potent antagonists at the MC4R, mirroring the prior observation that Dap substitution results in a more potent MC4R antagonist compared to Asn.

Ligands **1** and **2** have previously been reported to possess inverse agonist activity at the MC5R,⁴¹ as have other compounds based upon the AGRP-derived macrocyclic scaffold.⁴² In this report, four additional compounds possessed apparent inverse agonist activity at the MC5R (decreased cAMP levels from basal at increased compound concentrations). These four peptides (**4**, **13**, **14**, and **16**) resulted in decreased cAMP signals at 100 μ M concentrations, although the signal did not plateau in a sigmoidal dose-response curve. There is not a clear structure-activity relationship trend to explain this activity, as one of the ligands is based upon the Asn scaffold (**4**) while three contain the Dap residue (**13**, **14**, and **16**), and different D-residues with the Arg-Phe-Phe tripeptide sequence are present in each macrocycle. Efforts to further develop MC5R inverse agonist probes may be useful to clarify the role of this receptor *in vivo*.

Conclusions

The present study examined the functional effects of stereochemically modifying the Arg-Phe-Phe antagonist active sequence of AGRP-derived octapeptide macrocyclic ligands possessing either an Asn or Dap substitution. Unlike a previously report using a 20-residue AGRP scaffold, stereochemical modification with the octapeptide scaffolds did not result in agonist activity at all the melanocortin receptors assayed. While all the ligands in the present study were able to partially or fully stimulate the MC1R, no ligands were able to fully stimulate the MC3R or MC4R, while one compound fully activated the MC5R. Thus, stereochemical inversion of the AGRP Arg-Phe-Phe tripeptide sequence does not necessarily result in melanocortin agonist activity. A tunable agonist response at the MC1R was observed for these macrocycles, from partial receptor stimulation at 100 μ M to full agonist efficacy and a potency of 230 nM. This study also demonstrated that ligands possessing Dap substitutions possessed increased MC4R antagonist potencies compared to the native Asn residue. Stereochemical modification of the Phe112 position was also found to retain the highest MC4R antagonist potency in both scaffolds examined. These observations will be important in the design of future ligands to possess increased MC4R antagonist potency for potential treatment of states of negative energy balance, including anorexia and cachexia with minimal effects on skin pigmentation.

Experimental

Peptide Synthesis

All peptides were synthesized using standard Fmoc chemistry.^{43, 44} 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, and amino acids were purchased from Peptides International (Louisville, KY). 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 (0.76 meq/g substitution) in parallel using a 96-well block (LabTech I; Advanced ChemTech, Louisville, KY) or individually with a manual microwave synthesizer (CEM Discover SPS, Matthews, NC). 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 from 45 min. Individual syntheses consisted of two repeated steps separated by DMF washes: (i) removal of the Fmoc group with 20% piperidine (1x at rt for 2 min, 1x using microwave irradiation for 4 min at 75°C with 30W), and (ii) single coupling of the incoming Fmoc-protected amino acid (3 eq) with HBTU (3 eq) and DIPEA (5 eq) in DMF using microwave irradiation (75°C, 5 min, 30W). The Arg coupling utilized more Arg (5 eq), HBTU (5 eq), and DIPEA (7 eq), and a longer

irradiation time (10 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 through amide bond formation between the Arg and Pro residues 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 side chain protecting groups of the cyclized peptides were removed with 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 in H₂O) at a stock concentration of 10⁻² M and were pharmacologically characterized using HEK293 cells stably expressing the mouse MC1R, MC3R, MC4R, and MC5R by the cAMP AlphaScreen® assay (PerkinElmer) according to the manufacturer's instructions and as previously described.^{46, 47}

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. Compounds that did not possess agonist activity in two independent experiments and did not produce a shift in the agonist curve when assayed as an antagonist at 10,000, 5,000, 1,000, and 500 nM concentrations in an initial experiments were not further assayed. 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. As no compound produced a sigmoidal dose-response curve, the percent inverse activity reported was the percent change from basal to signal at 100 μ M concentrations. The ligands were assayed as TFA salts and not corrected for peptide content.

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Abbreviations Used:

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

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

RP-HPLC

reverse-phase high-pressure liquid chromatography

SAR

structure-activity relationships

SEM

standard error of the mean

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Figure 1:
Illustration of the normalized pharmacology of NDP-MSH and macrocyclic peptides **3**, **4**, **7**, **9**, and **11** at the mMC1R and MC5R.

Figure 2:
Illustration of the normalized antagonist pharmacology of **11** at the mMC3R and mMC4R.

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			
3	c[Pro- DArg -Phe-Phe-Asn-Ala-Phe-DPro]	20.3	30.2	976.5	977.5	>97%
4	c[Pro-Arg- DPhe -Phe-Asn-Ala-Phe-DPro]	17.2	27.8	976.5	977.5	>99%
5	c[Pro-Arg-Phe- DPhe -Asn-Ala-Phe-DPro]	17.3	27.4	976.5	977.5	>99%
6	c[Pro- DArg - DPhe -Phe-Asn-Ala-Phe-DPro]	17.3	26.9	976.5	977.6	>96%
7	c[Pro- DArg -Phe- DPhe -Asn-Ala-Phe-DPro]	19.3	29.0	976.5	977.6	>99%
8	c[Pro-Arg- DPhe - DPhe -Asn-Ala-Phe-DPro]	16.8	26.4	976.5	977.5	>98%
9	c[Pro- DArg - DPhe - DPhe -Asn-Ala-Phe-DPro]	17.2	26.5	976.5	977.5	>99%
10	c[Pro- DArg -Phe-Phe- <i>Dap</i> -Ala-Phe-DPro]	19.0	28.5	948.5	949.6	>95%
11	c[Pro-Arg- DPhe -Phe- <i>Dap</i> -Ala-Phe-DPro]	17.2	27.9	948.5	949.5	>99%
12	c[Pro-Arg-Phe- DPhe - <i>Dap</i> -Ala-Phe-DPro]	16.8	26.8	948.5	949.6	>99%
13	c[Pro- DArg - DPhe -Phe- <i>Dap</i> -Ala-Phe-DPro]	16.4	25.5	948.5	949.6	>99%
14	c[Pro- DArg -Phe- DPhe - <i>Dap</i> -Ala-Phe-DPro]	17.9	27.1	948.5	949.5	>99%
15	c[Pro-Arg- DPhe - DPhe - <i>Dap</i> -Ala-Phe-DPro]	16.8	26.2	948.5	949.5	>99%
16	c[Pro- DArg - DPhe - DPhe - <i>Dap</i> -Ala-Phe-DPro]	16.4	25.3	948.5	949.6	>99%

^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 Macrocyclic Analogues at the Mouse Melanocortin Receptors.^a

Peptide	Sequence	mMC1R		mMC3R		mMC4R		mMC5R	
		EC ₅₀ (nM)	EC ₅₀ (nM)	EC ₅₀ (nM)	pA ₂	EC ₅₀ (nM)	pA ₂	EC ₅₀ (nM)	EC ₅₀ (nM)
NDP-MSH		0.007±0.001	0.069±0.009			0.9±0.1		0.056±0.009	
1 [*]	c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro]	25% @ 100 μM	>100,000	6.3 ± 0.1		>100,000	8.2 ± 0.1	Inverse Agonist, –10%, 130 nM	
3	c[Pro- D Arg-Phe-Phe-Asn-Ala-Phe-DPro]	50% @ 100 μM	>100,000	<5.5		>100,000	<5.5	>100,000	
4	c[Pro-Arg- D Phe-Phe-Asn-Ala-Phe-DPro]	Partial Agonist 55% NDP-MSH (700 ± 200)	>100,000	5.8 ± 0.2		>100,000	7.7 ± 0.2	Inverse Agonist –25% @ 100 μM	
5	c[Pro-Arg-Phe- D Phe-Asn-Ala-Phe-DPro]	50% @ 100 μM	>100,000	<5.5		>100,000	5.9 ± 0.1	>100,000	
6	c[Pro- D Arg- D Phe-Phe-Asn-Ala-Phe-DPro]	Partial Agonist 90% NDP-MSH (500 ± 100)	>100,000	<5.5		>100,000	<5.5	55% @ 100 μM	
7	c[Pro- D Arg-Phe- D Phe-Asn-Ala-Phe-DPro]	1200 ± 300	35% @ 100 μM	<5.5		>100,000	<5.5	10,000 ± 3,000	
8	c[Pro-Arg- D Phe- D Phe-Asn-Ala-Phe-DPro]	Partial Agonist 85% NDP-MSH (800 ± 200)	>100,000	<5.5		>100,000	5.9 ± 0.2	>100,000	
9	c[Pro- D Arg- D Phe- D Phe-Asn-Ala-Phe-DPro]	Partial Agonist 85% NDP-MSH (1300 ± 300)	>100,000	<5.5		>100,000	<5.5	55% @ 100 μM	
2 [*]	c[Pro-Arg-Phe-Phe- <i>Dap</i> -Ala-Phe-DPro]	30% @ 100 μM	>100,000	6.52 ± 0.09		>100,000	8.7 ± 0.1	Inverse Agonist –15%, 60 nM	
10	c[Pro- D Arg-Phe-Phe- <i>Dap</i> -Ala-Phe-DPro]	25% @ 100 μM	25% @ 100 μM	5.7 ± 0.2		25% @ 100 μM	6.77 ± 0.05	>100,000	
11	c[Pro-Arg- D Phe-Phe- <i>Dap</i> -Ala-Phe-DPro]	230 ± 80	>100,000	5.92 ± 0.05		20% @ 100 μM	8.4 ± 0.1	>100,000	
12	c[Pro-Arg-Phe- D Phe- <i>Dap</i> -Ala-Phe-DPro]	Partial Agonist 75% NDP-MSH (800 ± 200)	>100,000	<5.5		>100,000	7.34 ± 0.08	>100,000	
13	c[Pro- D Arg- D Phe-Phe- <i>Dap</i> -Ala-Phe-DPro]	Partial Agonist 70% NDP-MSH (800 ± 300)	>100,000	6.0 ± 0.1		>100,000	5.5 ± 0.2	Inverse Agonist –30% @ 100 μM	
14	c[Pro- D Arg-Phe- D Phe- <i>Dap</i> -Ala-Phe-DPro]	Partial Agonist 70% NDP-MSH (450 ± 60)	>100,000	5.7 ± 0.2		>100,000	6.82 ± 0.06	Inverse Agonist –20% @ 100 μM	
15	c[Pro-Arg- D Phe- D Phe- <i>Dap</i> -Ala-Phe-DPro]	700 ± 300	>100,000	5.7 ± 0.1		>100,000	7.4 ± 0.1	>100,000	
16	c[Pro- D Arg- D Phe- <i>Dap</i> -Ala-Phe-DPro]	Partial Agonist 60% NDP-MSH (2200 ± 800)	>100,000	<5.5		>100,000	5.8 ± 0.2	Inverse Agonist –15% @ 100 μM	

The 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 μ M concentrations. A percentage denotes the percent maximal stimulatory response observed at 100 μ M concentrations but not enough stimulation was observed to determine an EC₅₀ value. 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 (compounds were considered full agonist if >90% maximal NDP-MSH signal was observed). Inverse agonist indicates that inverse agonist pharmacology was observed with the percent decrease from basal indicated. For inverse agonists, if a decrease in cAMP signal was observed without a sigmoidal dose-response curve, the percent change from basal at 100 μ M concentrations is indicated.

*The pharmacology for **1** and **2** has previously been reported.⁴¹