Published in final edited form as:

J Med Chem. 2017 January 26; 60(2): 805–813. doi:10.1021/acs.jmedchem.6b01707.

A Macrocyclic Agouti-Related Protein/[Nle⁴, DPhe⁷] α -Melanocyte Stimulating Hormone Chimeric Scaffold Produces Sub-nanomolar Melanocortin Receptor Ligands

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Abstract

The melanocortin system consists of five receptor subtypes, endogenous agonists, and naturally occurring antagonists. These receptors and ligands have been implicated in numerous biological pathways including processes linked to obesity and food intake. Herein, a truncation structure-activity relationship study of chimeric agouti-related protein (AGRP)/[Nle⁴, DPhe⁷] α -Melanocyte Stimulating Hormone (NDP-MSH) ligands is reported. The tetrapeptide His-DPhe-Arg-Trp or tripeptide DPhe-Arg-Trp replaced the Arg-Phe-Phe sequence in the AGRP active loop derivative c[Pro-Arg-Phe-Phe-Xxx-Ala-Phe-DPro], where Xxx was the native Asn of AGRP or a diaminopropionic (Dap) acid residue previously shown to increase antagonist potency at the mMC4R. The Phe, Ala, and Dap/Asn residues were successively removed to generate a 14-member library that was assayed for agonist activity at the mouse MC1R, MC3R, MC4R, and MC5R. Two compounds possessed nanomolar agonist potency at the mMC4R, c[Pro-His-DPhe-Arg-Trp-Asn-Ala-Phe-DPro] and c[Pro-His-DPhe-Arg-Trp-Dap-Ala-DPro], and may be further developed to generate novel melanocortin probes and ligands for understanding and treating obesity.

TOC image

Keywords

AGRP; NDP-MSH; chimeric ligands; obesity; macrocycles

Introduction

The melanocortin system consists of five receptors, discovered to date, that are members of the family of G protein-coupled receptors (GPCRs),^{1–8} endogenous agonists including the

α -melanocyte stimulating hormone (MSH), β -MSH, γ -MSH, and adrenocorticotrophic hormone (ACTH) derived from the proopiomelanocortin (POMC) gene transcript,⁹ and the naturally occurring antagonists agouti and agouti-related protein (AGRP).^{10–12} These receptors and ligands are linked to numerous biological pathways, including pigmentation,^{13,14} steroidogenesis,¹⁵ and energy homeostasis.¹⁶ In particular, knock-out of the melanocortin 3- and 4-receptors (MC3R, MC4R) in mice alters metabolic phenotypes. Knockout MC3R mice possess normal body weight accompanied by an increase in fat mass, while disrupting the MC4R results in hyperphagia and obesity in mice.^{16–18} A review of MC3R human polymorphisms suggests this receptor may predispose an individual to obesity, although a pathogenic role of the MC3R in obesity is unclear.¹⁹ In contrast, mutations in the MC4R in humans have been shown to result in obesity; in one study, 5.8% of individuals with severe childhood obesity were found to have mutations in the MC4R.²⁰ Mice may therefore serve as a translation model for MC4R-related obesity in humans due to the similar hyperphagic, overweight phenotype observed. Central intracerebroventricular (i.c.v.) injection of the nonselective melanocortin agonists α -MSH,²¹ NDP-MSH,²² and MT-II^{23,24} decrease food intake in rodents, while i.c.v. administration of the synthetic SHU9119²³ and endogenous AGRP^{24,25} MC3R/MC4R antagonists increase food consumption. With estimated global obesity rates more than doubling from 1980 to 2014,²⁶ the development of novel probes to investigate the etiology of obesity and serve as potential therapeutic leads may be important in efforts to decrease this trend. While MC4R-selective agonists based upon the endogenous melanocortin agonists have previously been investigated, off-target effects including increased blood pressure and increased erectile activity have limited their clinical utility.^{27,28} New melanocortin ligands based upon novel scaffolds may bypass these side effects and may serve as probes in investigating MC4R-related obesity, as evidenced by the utilization of setmelanotide in POMC deficient humans to lower body weight without cardiovascular side effects.²⁹

It has previously been demonstrated that the endogenous melanocortin antagonist AGRP may be converted into an agonist by select modifications. The full-length AGRP is 132 amino acids in length, though the highly structured 46-residue C-terminal domain has been shown to be equipotent to the full-length protein.^{11,12} An Arg-Phe-Phe tripeptide sequence critical for activity is located on an exposed β -hairpin loop within the C-terminal domain.^{30–32} Further truncation of the C-terminal 12 amino acids and a Cys to Ala substitution has been shown to retain the β -hairpin loop structure with minimal loss in antagonist potency, resulting in “mini-AGRP.”³³ Substitution of the potent melanocortin agonist His-DPhe-Arg-Trp tetrapeptide sequence into the Arg-Phe-Phe residues of mini-AGRP resulted in the formation of a potent, nonselective melanocortin agonist, possessing nanomolar agonist potency at the MC3R and MC4R, and sub-nanomolar agonist potency at the MC1R and MC5R.^{34,35} The length, structural complexity, and relatively high synthetic cost of this chimeric mini-AGRP peptide hindered additional structure-activity relationship (SAR) studies. While further truncations beyond mini-AGRP traditionally result in diminished antagonist potencies, substitution of the His-DPhe-Arg-Trp tetrapeptide into the Tyr-flanked, lactam-cyclized β -hairpin active loop of AGRP (Tyr-c[Asp-His-DPhe-Arg-Trp-Asn-Ala-Phe-Dap]-Tyr-NH₂) resulted in a nonselective sub-nanomolar potent agonist at the MC1R, MC3R, MC4R, and MC5R.^{35,36} Similar nanomolar to sub-nanomolar potencies

were observed when a Tyr-flanked disulfide bridge was used to cyclize the active loop of AGRP (Tyr-c[Cys-His-DPhe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH₂).³⁷ Performing the opposite substitution by inserting the Arg-Phe-Phe tripeptide into the DPhe-Arg-Trp sequence of NDP-MSH resulted in sub-micromolar agonist potencies at the MCRs, while the same replacement in MTII only resulted in partial receptor stimulation at the MC1R and no activity at the MC3-5R at concentrations up to 100 μ M.³⁸

While truncation of AGRP classically results in diminished antagonist potency, one report demonstrated that the peptide c[Pro-Arg-Phe-Phe-Asn-Ala-Phe-DPro], the active loop of AGRP cyclized through a DPro-Pro motif, was only 50-fold less potent than AGRP(87–132) at the mMC4R despite the removal of 38 amino acids.³⁹ Further SAR studies demonstrated that replacement of the Asn with a Dap residue resulted in a cyclic octapeptide that was as potent an antagonist as AGRP at the MC4R, 160-fold selective for the MC4R over the MC3R, possessed minimal activity at the MC1R, and was unable to stimulate the MC5R at up to 100 μ M concentrations.³⁹ Due to the prior reported activity of AGRP chimeric peptides, and the potency and selectivity of the Dap-containing octapeptide, it was hypothesized that incorporating the melanocortin agonist His-DPhe-Arg-Trp sequence into the octapeptide scaffold may result in a potent and MC4R-selective agonist. Furthermore, since melanocortin agonists and AGRP have been shown to possess unique interactions with the MC4R⁴⁰ and the proposed macrocyclic scaffold is based upon the active loop of AGRP, it may be postulated that the resulting ligands may bypass the negative side effects of previously reported MC4R-selective compounds by interacting with unique MC4R residues compared to previous agonists.

In attempts to generate new potent, selective melanocortin agonists, a series of 14 cyclic peptides was synthesized and characterized at the mouse melanocortin receptors. The His-DPhe-Arg-Trp tetrapeptide and DPhe-Arg-Trp tripeptide sequences were substituted for the Arg-Phe-Phe tripeptide in the DPro-Pro cyclized antagonist scaffold. Peptides containing either the native Asn residue or the more potent MC4R Dap substitution were examined for differences in potency and/or selectivity. Additionally, truncations of the non-pharmacophore Phe, Ala, and Asn/Dap amino acids were examined to explore the optimal size of the cyclic agonists and the importance of these residues.

Results

Peptide Synthesis and Characterization

Peptides were manually synthesized with microwave-assisted deprotection and couplings steps using standard fluorenylmethoxycarbonyl (Fmoc) chemistry.⁴¹ Crude weight of the side-chain protected peptides following cleavage indicated initial yields of 47.5 – 71.3%. Peptides were cyclized as previously described,³⁹ but using DCM as the solvent rather than DMF. The use of DCM permitted solvent removal under vacuum instead of the use of a solid-phase extraction cartridge, simplifying the reaction scheme and resulted in quicker syntheses. Following side-chain deprotection, cyclic 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 distinct solvent systems (Table 1) and the correct molecular mass was determined by matrix-assisted laser desorption/

ionization mass spectrometry (MALDI-MS, University of Minnesota Mass Spectrometry Laboratory).

In Vitro AlphaScreen® cAMP Agonist Assay

The compounds were assayed for agonist activity using the AlphaScreen® cAMP assay using HEK293 cells stably transfected with the mouse melanocortin 1, 3, 4, and 5 receptors according to the manufacturer's instructions and as previously reported.^{42–44} The MC2R is only stimulated by ACTH and was therefore excluded from this study. The potent, nonselective melanocortin ligand NDP-MSH was used as a positive control;⁴⁵ the nonselective melanocortin agonists MTII and HfRW are included for reference. Since the AlphaScreen® cAMP assay is a competition assay resulting in a lower signal at higher concentrations of ligand, concentration-activity curves were normalized to baseline and maximal NDP-MSH signal for illustrative purposes as previously described.^{42,46,47} Due to the inherent error of the assay, compounds that were within a three-fold potency range were considered equipotent. Compounds that resulted in greater than 90% maximal NDP-MSH stimulation were considered to possess full agonist efficacy.

Table 2 summarizes the functional agonist pharmacology of the synthesized ligands. Substitution of the agonist His-DPhe-Arg-Trp sequence in the native antagonist loop in place of Arg-Phe-Phe resulted in compound **1**, possessing 0.35, 32, 1.4, and 0.45 nM agonist potencies at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively (Table 2, Figure 1). Removal of the Phe residue resulted in compound and decreased agonist potency relative to **1**, with potency losses of 34-, 21-, and 5-fold at the mMC1R, mMC4R, and mMC5R. Peptide **2** was a partial agonist at the mMC3R, stimulating to 85% maximal NDP-MSH signal with an EC₅₀ value of 300 nM. Removal of the Ala amino acid (**3**) further decreased agonist potency 140-, 120-, and 37-fold at the mMC1R, mMC4R and mMC5R, relative to **1**. Compound **3** was also a partial agonist at the mMC3R and possessed 65% the maximal efficacy of NDP-MSH with an EC₅₀ value of 510 nM.

Previously, it was shown that substitution of the Asn with a Dap residue could increase the antagonist potency of the resulting AGRP-derived octapeptide and resulted in a greater than 160-fold selectivity for the mMC4R over the mMC3R.³⁹ It was hypothesized that the same substitution with the agonist tetrapeptide His-DPhe-Arg-Trp sequence may create a potent and selective mMC4R agonist. This substitution was incorporated into peptide **4**, which possessed 24, 260, and 18 nM agonist potency at the mMC1R, mMC3R, and mMC5R. Compound **4** was a partial agonist at the mMC4R, possessed 70% of the maximal NDP-MSH response and 23 nM EC₅₀ value at this receptor. In contrast to the Asn-containing **1**, removal of the Phe residue increased agonist potency (Table 2, Figure 1). Compound **5** was able to fully stimulate the mMC4R (EC₅₀ = 1.6 nM), and possessed 1.1, 40, and 0.3 nM agonist potency at the mMC1R, mMC3R, and mMC5R. At all receptors assayed, **5** was equipotent to **1** (Table 2, Figure 1). Further truncation of the Ala residue (**6**) decreased agonist potency 420-, 590-, and 1,100-fold at the mMC1R, mMC4R, and mMC5R relative to **5**, and stimulated the mMC3R to 65% of the maximal NDP-MSH response at concentrations up to 100 μ M. Removal of the Asn/Dap position, resulting in the His-DPhe-Arg-Trp tetrapeptide sequence cyclized through a DPro-Pro motif in hexapeptide **7**, partially

stimulated the mMC3R and mMC4R relative to NDP-MSH (60% and 65% at 100 μ M). This peptide also possessed 900 and 6,000 nM agonist EC₅₀ values at the mMC1R and mMC5R, respectively.

It has previously been shown that the tripeptide Ac-DPhe-Arg-Trp-NH₂ possesses micromolar agonist potency at the mMC1R, mMC4R, and mMC5R,⁴⁸ and was the minimal fragment of NDP-MSH to possess agonist activity using the classic frog skin bioassay.⁴⁹ In the native loop sequence of AGRP, an Arg-Phe-Phe tripeptide is postulated to be the active pharmacophore. It was hypothesized that insertion of the DPhe-Arg-Trp tripeptide into the DPro-Pro cyclized loop mimetics of AGRP may result in increased potency and/or selectivity in the resulting chimeric peptides, since this agonist sequence is the same length as the postulated antagonist active sequence and would not alter peptide length and may result in less disruption of the loop structure. Insertion of the DPhe-Arg-Trp tripeptide into the native, Asn-containing, loop sequence cyclized through DPro-Pro residues resulted in peptide **8**, which was unable to fully stimulate the mMC3R (45% of maximal NDP-MSH at 100 μ M concentrations). This peptide was a weaker agonist relative to **1**, and possessed 400, 130, and 40 nM agonist potency at the mMC1R, mMC4R, and mMC5R, respectively (1140-, 90-, and 90-fold decreased potency relative to **1**). Similar potencies were observed at all the melanocortin receptors when the Phe amino acid was removed, generating **9**. The removal of the Ala residue (**10**) resulted in a peptide possessing partial agonist efficacy at the mMC1R and mMC4R (85% maximal NDP-MSH signal), with potencies of 40 and 1,100 nM, respectively. Peptide **10** partially stimulated the mMC5R (25% of NDP-MSH) and showed no agonist activity at the mMC3R at concentrations up to 100 μ M.

A similar trend of decreased agonist potency was observed when the DPhe-Arg-Trp was inserted into an AGRP active loop sequence where Asn was substituted with Dap (**11**). This compound was a partial agonist at the mMC1R and mMC3R (45% and 70% NDP-MSH maximum signal with EC₅₀ values of 190 and 3,000 nM, respectively), and possessed 100 and 46 nM potencies with full agonist efficacy at the mMC4R and mMC5R (Figure 1). Truncation of the Phe residue to generate **12** resulted in similar potency at the mMC4R (150 nM) and mMC5R (48 nM), full agonist efficacy at the mMC1R (600 nM), and partially stimulated the mMC3R (70% at 100 μ M concentrations). Further removal of the Ala residue to generate the hexapeptide **13** decreased agonist potencies at all receptors assayed, with no observable activity at the mMC3R or mMC5R at 100 μ M concentrations (Figure 1), 35% partial stimulation of the mMC4R (relative to NDP-MSH), and possessed partial agonist efficacy at the mMC1R (75% NDP-MSH, EC₅₀ = 110 nM). Peptide **14**, the result of cyclizing the agonist tripeptide DPhe-Arg-Trp with a DPro-Pro motif, possessed the lowest potency at the mMC1R (1,800 nM), no activity at the mMC3R or mMC5R with concentrations up to 100 μ M, and stimulated the mMC4R to 50% of the maximal level of NDP-MSH at 100 μ M.

Discussion

In the present study, peptides were synthesized on a chlorotrityl resin to permit cleavage from the resin while retaining the side-chain protecting groups. This was necessary to minimize side-chain cyclized byproducts. The syntheses were aided by microwave

irradiation, which has previously been shown to prematurely cleave peptides from chlorotrityl resins.⁵⁰ This was postulated to be due to direct thermal hydrolysis of the peptide from resin and was dependent on the amount of time the resin-bound peptide was exposed to elevated temperatures.⁵⁰ While longer peptides have been successfully synthesized using microwave irradiation on chlorotrityl resins, the first three amino acids were coupled without microwave irradiation due to the reported sensitivity of the resin.^{51,52} In the present study, all residues were coupled with microwave assistance, with reported crude yields of 48 – 72 % similar to previous syntheses from the same H-Pro-chlorotrityl resin at room temperature (52 – 99 %, unpublished results). It may be speculated that the relatively consistent yields may be a result of minimal time the peptide-resin was exposed to elevated temperatures (<10 min per coupling cycle) or the first amino acid (Pro) afforded some protection from premature cleavage, and may represent a unique opportunity to use microwave-assisted solid phase peptide synthesis when utilizing a chlorotrityl resin.

Melanocortin 1 Receptor Pharmacology

Prior chimeric ligands derived from the active loop of AGRP and the His-DPhe-Arg-Trp melanocortin agonist sequence resulted in sub-nanomolar agonist potency at the mMC1R, a receptor postulated to be involved in pigmentation.^{2,7} The disulfide bridged Tyr-c[Cys-His-DPhe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH₂ was reported to possess 0.4 nM agonist potency at the mMC1R.³⁷ Replacing the disulfide bridge with a lactam bridge, Tyr-c[Asp-His-DPhe-Arg-Trp-Asn-Ala-Phe-Dap]-Tyr-NH₂, resulted in a peptide ligand with 0.2 nM agonist potency.^{35,36} Insertion of the His-DPhe-Arg-Trp-Gly-Lys hexapeptide of the potent synthetic melanocortin agonist NDP-MSH into the active loop of the truncated mini-AGRP template resulted in cAMP stimulation when administered at 1 μ M concentrations to cells expressing the hMC1R; similar cAMP accumulation was observed when His-Phe-Arg-Trp was inserted in the same template.³⁴ A dose-response evaluation of this His-DPhe-Arg-Trp tetrapeptide inserted into the Arg-Phe-Phe sequence of mini-AGRP [Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂] demonstrated this peptide to possess 0.16 nM agonist potency at the mMC1R.³⁵ Compounds **1** and **5** from the present study possessed similar nanomolar or less potency at the mMC1R, with EC₅₀ values of 0.35 nM and 1.1 nM, respectively. It may be speculated that the MC1R agonist potency of **1** and Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂ may be due to a similar presentation of the His-DPhe-Arg-Trp on a loop structure. Conversion of **1** to **5**, which possesses similar potency, included removal of a Phe residue and Asn to Dap substitution. Either modification alone did not improve potency: truncation of the Phe (**2**) decreased agonist potency 34-fold relative to **1**; 70-fold decreased potency was observed for the Asn to Dap substitution (**4**). Compounds **10**, **11** and **13** were observed to be partial agonists at the mMC1R (Figure 1), stimulating the mMC1R 85%, 45%, and 75% of the maximal NDP-MSH response, with EC₅₀ values of 40, 190, and 110 nM, respectively. These three peptides did not possess a His residue in the agonist sequence, although other peptides lacking a His residue were full mMC1R agonists (**8**, **9**, **12**, and **14**).

Melanocortin 3 Receptor Pharmacology

The melanocortin 3 receptor has been reported to be expressed in many different tissues and expression in the central nervous system has been linked to pathways involving energy homeostasis and food intake.^{17,24} Previously reported AGRP/NDP-MSH chimeric ligands

Tyr-c[Cys-His-DPhe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH₂, Tyr-c[Asp-His-DPhe-Arg-Trp-Asn-Ala-Phe-Dap]-Tyr-NH₂, and Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂ have been reported to possess 2.0 nM, 1.0 nM, and 30 nM potencies, respectively, at the mMC3R.^{35–37} The most potent compounds from the present study, **1** and **5**, possessed agonist potencies of 32 and 40 nM respectively, most similar to the reported value for Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂. An additional compound was a full agonist at the mMC3R (**4**; EC₅₀ = 260 nM). Three compounds did not possess agonist activity at the mMC3R (**10**, **13**, and **14**) and five ligands partially stimulated the mMC3R at concentrations up to 100 μM (**6**, **7**, **8**, **9**, and **12**). Three peptides displayed partial agonist efficacy (**2**, **3**, and **11**), stimulating cAMP to 85%, 65%, and 70% of maximal NDP-MSH response and EC₅₀ values of 300, 510, and 3000 nM, respectively. The decreased potencies and activities at the mMC3R may indicate that this scaffold is not well situated for this receptor, perhaps due to intrinsic lower potency of AGRP at the mMC3R versus the mMC4R (~10-fold) or by the addition of the Dap amino acid which previously was shown to impart some selectivity for the mMC4R over the mMC3R when the antagonist Arg-Phe-Phe tripeptide sequence was examined in this scaffold.³⁹ Interestingly, when the His-Phe-Arg-Trp is moved around active hexapeptide loop in mini-AGRP, stimulation of the MC3R is reported to be less relative to the MC1R and MC4R, although the lack of dose-response curves do not allow a direct comparison.³⁴

Melanocortin 4 Receptor Pharmacology

The MC4R is expressed in numerous tissues; mice lacking this receptor possess an obese phenotype that is also observed in many mutations of the human form of the MC4R.^{16,20} Chimeric AGRP/NDP-MSH ligands Tyr-c[Cys-His-DPhe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH₂, Tyr-c[Asp-His-DPhe-Arg-Trp-Asn-Ala-Phe-Dap]-Tyr-NH₂, and Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂ have been reported to possess 0.3 nM, 0.2 nM, and 1.4 nM potencies at the mMC4R.^{35–37,53} Two ligands (**1** and **5**) possessed similar 1.4 nM and 1.6 nM potencies, respectively. Unlike the mMC3R, all ligands were able to stimulate the mMC4R, though three compounds (**7**, **13**, and **14**) stimulated a fraction of the maximal NDP-MSH response at 100 μM concentrations and peptides **4** and **10** were partial agonists (70% and 85% maximal NDP-MSH stimulation; EC₅₀ values of 23 and 1,100 nM).

Melanocortin 5 Receptor Pharmacology

The MC5R is expressed ubiquitously in the periphery and has been hypothesized to be involved in exocrine gland function.⁵⁴ The previously reported ligands Tyr-c[Cys-His-DPhe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH₂, Tyr-c[Asp-His-DPhe-Arg-Trp-Asn-Ala-Phe-Dap]-Tyr-NH₂, and Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂ possessed agonist potencies of 2 nM, 0.5 nM, and 0.6 nM at the mMC5R.^{35–37} The two most potent compounds, **1** and **5** possessed similar 0.45 nM and 0.3 nM agonist potencies at the mMC5R. One peptide (**13**) was unable to stimulate the mMC5R at concentrations up to 100 μM, while two ligands (**10** and **14**) partially stimulated the receptor at 100 μM concentrations. The remainder of the synthesized ligands possessed nanomolar to micromolar agonist potencies at this receptor.

Structure-Activity Relationship Trends

Unlike the antagonist pharmacology previously reported which showed a beneficial antagonist potency and selectivity increase with a Dap residue,³⁹ there is not a clear trend for the Asn or Dap residue following the Trp amino acid for increased agonist potency or selectivity. Of the two most potent ligands that had similar activities at the MCRs, one possessed an Asn amino acid (**1**) and one a Dap residue (**5**). These peptides were also different lengths, with a heptapeptide sequence cyclized through a DPro-Pro motif in **1** (His-DPhe-Arg-Trp-Asn-Ala-Phe) and a hexapeptide in **5** (His-DPhe-Arg-Trp-Dap-Ala). The sequential removal of the Phe and then Ala from **1** resulted in decreased potencies at the receptor subtypes, indicating the heptapeptide sequence may be ideal for this sequence. Comparatively, the addition of the Phe residue or subtraction of the Ala from **5** both decreased potency. It appears as if both the Asn and the Dap substitutions are tolerable for an agonist ligand, and the size of the resulting cyclic peptides influences potency. Interestingly, **1** possess the same purported active loop sequence as Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂ and possesses nearly identical pharmacology at all four receptors (EC₅₀ values of 0.35, 32, 1.4, and 0.45 nM for **1** at the mMC1R, mMC3R, mMC4R, and mMC5R versus 0.16, 30, 1.4, and 0.56 nM for Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂),³⁵ though these were measured using different cAMP assays which must be accounted for when comparing these data. Overall, **1** is 26 residues shorter compared to Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂ and possesses similar pharmacology, and may represent a better lead molecule since it is more quickly synthesized, less expensive to make, and does not possess disulfide bonds that could scramble to non-native linkages. The **1** and **5** peptides also possess modest selectivity for the mMC4R over the mMC3R (23- and 25-fold, respectively), more favorable than the 7- and 5-fold reported for the Tyr-c[Cys-His-DPhe-Arg-Trp-Asn-Ala-Phe-Cys]-Tyr-NH₂ and Tyr-c[Asp-His-DPhe-Arg-Trp-Asn-Ala-Phe-Dap]-Tyr-NH₂ AGRP/NDP-MSH chimeric ligands.^{36,37} This selectivity is also better than the melanocortin tetrapeptide scaffold Ac-His-DPhe-Arg-Trp-NH₂, which is 4-fold selective for the mMC4R over the mMC3R (Table 2) and 10-fold less potent at the mMC4R (14 nM) compared to **1** and **5**.⁴⁷ The inherent scaffold selectivity for the MC4R from these compounds may be beneficial for leads in generating MC4R selective probes.

The DPhe-Arg-Trp tripeptide sequence has previously been shown to be the minimal sequence with activity at the MCRs, with the addition of His increasing ligand potency.⁴⁹ This trend was also observed with this set of AGRP/NDP-MSH chimeric ligands, where removal of the His resulted in ligands with similar or decreased agonist potencies. An exception was **11**, which possessed full agonist efficacy at the mMC4R with an EC₅₀ of 100 nM compared to **4** possessing a His residue, which was a partial agonist at the mMC4R (70% maximal NDP-MSH stimulation, EC₅₀ = 23 nM). These two compounds also presented a unique pharmacology at the mMC1R, mMC3R and mMC4R. Peptide **11**, lacking a His residue, was a partial agonist at the mMC1R and mMC3R and a full agonist at the mMC4R. The opposite pattern was observed in **4**, which possessed a His residue and was a full agonist at the mMC1R and mMC3R and was a partial agonist at the mMC4R. The presence of the His residue dictated whether the ligand was a full or partial agonist for this sequence at receptor subtypes.

The control peptide NDP-MSH is a synthetic analogue of α -MSH that is potent at the mMC1R, mMC3R, mMC4R, and mMC5R, and possesses modest selectivity (6 to 26-fold) for the mMC1R over the other receptors.⁴⁵ Select ligands from this study did possess significant selectivity (>100-fold) for one receptor subtype, often due to low potency of this ligand series at the mMC3R. Three compounds were >100-fold selective for the mMC1R over the mMC3R (**6**, **8**, **9**), while the partial agonist **10** was >2500-fold selective for the mMC1R over the mMC3R. Three of these compounds (**8**, **9**, and **10**) were derived from the serial truncation of Phe and Ala residues lacking a His, indicating a possible series effect for selectivity. For the mMC4R over the mMC3R, **9** was >100-fold selective and **8** and **12** were >300-fold selective. Five compounds were selective for the mMC5R over the mMC3R, with **5** and **6** possessing >100-fold selectivity, and **9**, **8** and **12** possessing >1000-fold selectivity for the mMC5R over the mMC3R. One compound (**13**) was a partial agonist at the mMC1R (75% NDP-MSH stimulation, EC₅₀ = 110 nM) and possessed no agonist activity at the mMC3R or mMC5R and partially stimulated the mMC4R at concentrations up to 100 μ M (Figure 1), and possessed >500-fold selectivity for the mMC1R over the other receptor subtypes. No compounds were selective for the mMC3R, and there was no significant selectivity observed between the mMC4R and mMC5R.

The ligands described in this study represent an initial report involving a novel melanocortin agonist macrocyclic scaffold, derived from a potent, small peptide MC4R antagonist.³⁹ Select ligands possessed sub-nanomolar agonist potencies and were >1000-fold selective for MCR subtypes. Extensive studies of the macrocycle cores of the potent, non-selective melanocortin agonist MTII⁵⁵ and potent MC1R/MC5R agonist, MC3R/MC4R antagonist SHU9119⁵⁶ [SHU9119 has a DNaI(2') substitution to replace the DPhe of MTII] have resulted in many ligands with exquisite potency and selectivity. Replacement of the His with a Pro-Pro or Trp-Pro in SHU9119 resulted in nanomolar agonists at the hMC3R with over 100-fold selectivity for the hMC3R over the hMC4R.⁵⁷ Substituting a Pro for His in the MTII scaffold with the addition of the Pro-Val dipeptide sequence at the C-terminal outside the cyclized structure resulted in a sub-nanomolar agonist at the hMC4R and minimal efficacy (12%, EC₅₀ = 29 nM) at the hMC3R.⁵⁸ The Pro to His substitution in MTII alone was previously shown to result in a highly potent agonist at the hMC3R, hMC4R, and hMC5R (EC₅₀ = 5.1, 0.34, 0.93 nM, respectively),⁵⁹ while the same substitution in the SHU9119 scaffold resulted in a highly potent MC5R agonist and potent hMC3R/hMC4R antagonist.⁶⁰ The ability to tune the MTII/SHU9119 scaffold for select MCR activity has been developed over many decades; more investigation of the current scaffold is needed to achieve a similar result.

One advantage the present scaffold may afford over agonists derived from MTII/SHU9119 is that while MTII and SHU9119 are derivatives of the endogenous agonist α -MSH, the DPro-Pro macrocyclic scaffold originates from the active loop of AGRP. While mutagenesis work at the MC4R identified many receptor residues that interact with both α -MSH and AGRP, each ligand also interacted with distinct receptor residues,⁴⁰ suggesting overlapping but unique binding pockets. A chimeric macrocyclic ligand may therefore interact with the receptor using a combination of interactions that cannot be achieved with either parent molecule, resulting in unique molecular signaling. This could potentially limit the off-target

effects of the new scaffold of melanocortin macrocyclic agonists that have previously been reported.^{27,28} The potential for unique ligand-receptor interactions may also influence the cellular signaling pathway stimulated upon ligand binding. In addition to the well-established cAMP pathway for melanocortin signaling, the melanocortin receptors have also been reported to signal through a $G_{i/o}$ pathway,⁶¹ MAPK cascade,⁶² and Kir7.1 ion channel.⁶³ The interactions of chimeric ligands with the melanocortin receptors may produce unique receptor-ligand conformations that have distinct biased signaling profiles, resulting in inimitable pharmacological and biological effects.

Conclusions

The present structure-activity relationships study involved the conversion of potent, AGRP antagonist macrocycles into agonists at the melanocortin receptors. Two compounds, **1** and **5**, were full agonists with EC_{50} values in the nanomolar range at the mMC4R, previously indicated to be involved with energy homeostasis and food intake, and possessed some modest selectivity for the mMC4R over the mMC3R (23- and 25-fold). The most potent peptides in this series possessed equivalent potencies to Ac-mini-(His-DPhe-Arg-Trp)hAGRP-NH₂ at the mMCRs despite possessing 26 fewer residues, and could serve as valuable leads to generate more potent, selective, and/or biased signaling ligands that could probe the different biological pathways associated with melanocortin receptor signaling.

Experimental

Peptide Synthesis

All peptides were synthesized using standard Fmoc chemistry.⁴¹ Amino acids Fmoc-DPro, Fmoc-Phe, Fmoc-Ala, Fmoc-Asn(Trt), Fmoc-Trp(Boc), Fmoc-Arg(Pbf), Fmoc-DPhe, and Fmoc-His(Trt), H-Pro-2-chlorotrityl resin, and coupling reagents 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBt), and benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate (BOP) were purchased from Peptides International (Louisville, KY). Fmoc-Dap(Boc) was purchased from Peptides International and Bachem (Torrance, CA). Dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), dimethylformamide (DMF), trifluoroethanol (TFE), acetic acid, and anhydrous ethyl ether were purchased from Fisher (Fair Lawn, 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 0.10 mmol scale using a H-Pro-2-Chlorotrityl resin (0.76 meq/g) with a manual microwave synthesizer (CEM Discover SPS). Syntheses consisted of two repeated steps: (i) removal of the Fmoc group with 20 % piperidine (1× at rt for 2 min, 1× 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 DIEA (5 eq) in DMF using microwave irradiation (75°C, 5 min, 30W). A lower temperature was utilized for His (50°C) to avoid epimerization. The Arg coupling utilized more Arg (5 eq), HBTU (5 eq), and DIEA (7 eq), and a longer irradiation time (10 min). After completion of the syntheses,

peptides were cleaved with either a 99:1 DCM:TFA solution or 1:1:8 acetic acid:TFE:DCM solution (no difference in cleavage yields was observed). The cleavage solutions were then concentrated and side-chain protected peptides were precipitated using ice-cold ethyl ether. Peptides were cyclized in DCM with BOP (3 eq) and HOBt (3 eq) overnight, 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 hrs, the solution was then concentrated, and peptides precipitated using ice-cold ethyl ether.

Crude peptides were purified by RP-HPLC using a Shimadzu system with a photodiode array detector and a semi-preparative RP-HPLC C18 bonded silica column (Vydac 218TP1010, 1.0×2.5 cm). The peptides were at least 95% pure as assessed by analytical RP-HPLC 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

Peptide ligands were dissolved in DMSO at a stock concentration of 10⁻² M and were characterized pharmacologically using HEK293 cells stably expressing the mouse MC1R, MC3-5R by the cAMP AlphaScreen® assay (PerkinElmer) according to the manufacturer's instructions and as previously described.⁴²⁻⁴⁴

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

Following stimulation, streptavidin donor beads (0.5 µg) and biotinylated-cAMP (0.62 µmol) were added to the wells in a subdued light environment with 10 µL lysis buffer (5 mM HEPES, 0.3% Tween-20, 0.1% BSA, pH = 7.4) and the plates were incubated in the dark at room temperature for an additional 2 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 values represent the mean of duplicate replicates performed in at least three independent experiments. The EC₅₀ 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.). The ligands were assayed as TFA salts and not corrected for peptide context.

Acknowledgments

This work has been supported by NIH Grants R01DK091906 and R01DK097838. Mark D. Ericson is a recipient of an NIH Postdoctoral Fellowship (F32DK108402).

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

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

HBSS

Hanks' Balanced Salt Solution

HEPES

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

IBMX

3-isobutyl-1-methylxanthine

BSA

bovine serum albumin

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Figure 1.

Illustrations of the *in vitro* pharmacology of NDP-MSH, **1**, **5**, **11**, and **13** at the mMC1R, mMC3R, mMC4R, and mMC5R. The cAMP signal was normalized as previously described.

Table 1

Analytical Data for the Peptides Synthesized in this Study.^a

Peptide	Structure	Retention Time (system 1)	Retention Time (system 2)	M+1 (calcd)	M+1 (obs), purity %
1	c[Pro-His-DPhe-Arg-Trp-Asn-Ala-Phe-DPro]	15.5	23.8	1153.6	1153.5 (>99%)
2	c[Pro-His-DPhe-Arg-Trp-Asn-Ala-DPro]	12.7	19.9	1006.5	1006.2 (>95%)
3	c[Pro-His-DPhe-Arg-Trp-Asn-DPro]	12.4	18.9	935.5	935.2 (>96%)
4	c[Pro-His-DPhe-Arg-Trp-Dap-Ala-Phe-DPro]	14.6	23.7	1125.6	1125.4 (>99%)
5	c[Pro-His-DPhe-Arg-Trp-Dap-Ala-DPro]	12.2	18.6	978.5	978.1 (>95%)
6	c[Pro-His-DPhe-Arg-Trp-Dap-DPro]	12.6	18.4	907.5	907.5 (>96%)
7	c[Pro-His-DPhe-Arg-Trp-DPro]	12.5	18.6	821.4	821.2 (>98%)
8	c[Pro-DPhe-Arg-Trp-Asn-Ala-Phe-DPro]	16.7	25.7	1016.5	1016.5 (>99%)
9	c[Pro-DPhe-Arg-Trp-Asn-Ala-DPro]	14.8	22.9	869.4	869.1 (>97%)
10	c[Pro-DPhe-Arg-Trp-Asn-DPro]	13.4	20.9	798.4	798.0 (>95%)
11	c[Pro-DPhe-Arg-Trp-Dap-Ala-Phe-DPro]	15.9	24.4	988.5	988.4 (>99%)
12	c[Pro-DPhe-Arg-Trp-Dap-Ala-DPro]	14.0	21.4	841.4	841.2 (>98%)
13	c[Pro-DPhe-Arg-Trp-Dap-DPro]	12.9	19.6	770.4	770.4 (>96%)
14	c[Pro-DPhe-Arg-Trp-DPro]	15.9	23.9	684.4	684.2 (>96%)

^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 Chimeric NDP-MSH/AGRP β -Hairpin Loop Analogues at the Mouse Melanocortin Receptors.^a

Peptide	Structure	EC ₅₀ (nM)			
		mMC1R	mMC3R	mMC4R	mMC5R
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH ₂	0.015±0.005	0.09±0.02	0.39±0.07	0.11±0.02
MTII ^b	Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH ₂	0.05±0.01	0.15±0.04	0.14±0.02	0.12±0.02
HFRW ^b	Ac-His-DPhe-Arg-Trp-NH ₂	14±3	60±10	14±2	10±3
1	c[Pro-His-DPhe-Arg-Trp-Asn-Ala-Phe-DPro]	0.35±0.05	32±8	1.4±0.4	0.45±0.07
2	c[Pro-His-DPhe-Arg-Trp-Asn-Ala-DPro]	12±2	PA, 85% NDP (300±100)	29±2	2.1±0.2
3	c[Pro-His-DPhe-Arg-Trp-Asn-DPro]	50±20	PA, 65% NDP (510±50)	170±20	16.8±0.8
4	c[Pro-His-DPhe-Arg-Trp-Dap-Ala-Phe-DPro]	24±4	260±90	PA, 70% NDP (23±8)	18±2
5	c[Pro-His-DPhe-Arg-Trp-Dap-Ala-DPro]	1.1±0.4	40±10	1.6±0.2	0.3±0.1
6	c[Pro-His-DPhe-Arg-Trp-Dap-DPro]	460±60	65% @ 100 μ M	950±80	330±30
7	c[Pro-His-DPhe-Arg-Trp-DPro]	900±200	60% @ 100 μ M	65% @ 100 μ M	6,000±2,000
8	c[Pro-DPhe-Arg-Trp-Asn-Ala-Phe-DPro]	400±200	45% @ 100 μ M	130±70	40±10
9	c[Pro-DPhe-Arg-Trp-Asn-DPro]	500±60	55% @ 100 μ M	420±40	70±10
10	c[Pro-DPhe-Arg-Trp-Asn-DPro]	PA, 85% NDP (40±20)	>100,000	PA, 85% NDP (1,100±100)	25% @ 100 μ M
11	c[Pro-DPhe-Arg-Trp-Dap-Ala-Phe-DPro]	PA, 45% NDP (190±90)	PA, 70% NDP (3,000±1,000)	100±40	46±6
12	c[Pro-DPhe-Arg-Trp-Dap-Ala-DPro]	600±200	70% @ 100 μ M	150±10	48±5
13	c[Pro-DPhe-Arg-Trp-Dap-DPro]	PA, 75% NDP (110±20)	>100,000	35% @ 100 μ M	>100,000
14	c[Pro-DPhe-Arg-Trp-DPro]	1,800±500	>100,000	50% @ 100 μ M	75% @ 100 μ M

^aThe indicated errors represent the standard error of the mean determined from at least three independent experiments. >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. PA denotes a partial agonist with the percent maximal NDP stimulation and apparent EC₅₀ value (compounds showing >90% maximal NDP response were considered full agonists).

^bThe values for MTII and HFRW have previously been reported.^{44,47}