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Substitution of Arginine with Proline and Proline Derivatives in Melanocyte-Stimulating Hormones Leads to Selectivity for Human Melanocortin 4 Receptor

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Abstract

A new series of melanotropin analogues with His or Arg residues in the core pharmacophores of MTII, SHU9119 and Ac-NDP- γ -MSH-NH₂ replaced by Pro or *trans*-/cis- 4-guanidiny-Pro derivatives were designed and synthesized to introduce selectivity toward the human melanocortin 4 receptor (*h*MC4R). Analogues **1**, **2**, **3**, **6**, **7**, **8** were found to be *h*MC4R selective. Second messenger studies have demonstrated that analogues **1** and **2** are insurmountable inhibitors of MTII agonist activity at the *h*MC4R. Molecular modeling studies suggest that the *h*MC4R selectivity is due to a β -turn shift induced by the Pro ring that makes the global minimum structures of these analogues resemble the NMR solution structure of the *h*ASIP melanocortin receptor binding motif. Substitution of His in MTII also provided functional selectivity for the *h*MC3R or the *h*MC4R. These findings are important for a better understanding of the selectivity mechanism at the *h*MC3R/*h*MC4R, and the development of therapeutic ligands selectively targeting the *h*MC4R.

Introduction

Melanocortin receptors along with their endogenous agonists and antagonists are involved in the modulation of a wide range of important physiological functions¹ that include skin pigmentation,^{2–4} blood pressure and heart rate,⁵ erectile function,^{6,7} feeding behavior and energy homeostasis,^{7–14} aggressive/defensive behavior,¹⁵ and modulation of pain.^{16–18} To date, five human melanocortin receptor subtypes, namely the human melanocortin receptors 1 to 5 (*h*MC1–5R), have been identified and cloned. All five receptor subtypes belong to rhodopsin family of seven-transmembrane G-protein coupled receptors (GPCRs), which intracellularly mediates its effects by activating cAMP-dependent pathways.¹⁹ The *h*MC1R is mainly expressed in the melanocytes and leukocytes, and is involved in skin pigmentation, pain modulation, inflammation and control of immune system.^{18,20–22} The *h*MC2R, also known as the ACTH receptor, is expressed in the adrenal cortex and mediates glucocorticoneogenesis and other effects.^{23,24} The *h*MC3R and *h*MC4R are primarily found in the central nervous system (CNS) with different patterns of distribution.^{25,26} Both of them have been implicated to play roles in feeding regulation and weight control.^{1,27,28} The *h*MC3R also has been suggested to play an important role in mediating some of the anti-inflammatory effects of its endogenous agonist α -MSH.²⁹ Erectile function, anxiety, and stress are also

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Supporting Information Available ¹H NMR spectra of peptide analogues **1–10** in DMSO *d*₆ are available free of charge via the internet at <http://pubs.acs.org>.

primarily mediated through the *hMC4R*.^{6,7,30,31} The *hMC5R* is distributed in a variety of peripheral tissues and in the brain, cortex, and cerebellum. Its effects in the central nervous system have not yet been well studied, but it has been shown to be important in regulating exocrine gland function^{32,33} and coordinating central and peripheral signals for aggression.^{15,34} Of particular interest for therapeutic applications is the *hMC4R*,^{7,35} as there is an extensive body of preclinical and clinical evidence, which suggests that the *hMC4R* is intimately involved in the regulation of food intake and energy expenditure.^{7,8,36} It has been shown that *hMC4R* selective agonists can be used to treat obesity, though there are other side effects to be overcome.³⁷ On the other hand, *hMC4R* selective antagonists are promising candidates for treating anxiety & stress, anorexia and cancer- and HIV-related weight loss.^{31,38} Furthermore, it has been shown that targeting the *hMC4R* may provide an alternative approach to treat sexual dysfunctions in both male and female.^{6,39} Unlike current therapies that target end organ vascular tissue, this new approach is centrally mediated, and thus, will have significant advantages over existing ones for the treatment of a variety of sexual dysfunctions in both sexes.^{6,40} Despite the central importance of the *hMC4R*, rational design of potent selective *hMC4R* antagonists is still challenging. In this paper, we have introduced a rational structure-based design of selective *hMC4R* antagonists.

Design of Novel Ligands

The endogenous antagonists of the *hMCRs* include the agouti signaling protein (*hASIP* or the “agouti protein”) which primarily binds to the *hMC1R* and *hMC4R* and displays weaker affinity towards the *hMC3R*, and the agouti-related protein (*hAGRP*) which is selective for the *hMC3, 4R*.⁴¹ These two antagonists are expressed in different parts of the body⁴² and it has been postulated that they interact with the melanocortin receptors *via* the core pharmacophore RFF in their central Cys-constrained loops (Figure 1).⁴³⁻⁴⁵ The high selectivity of the *hASIP* for the *hMC4R* (46-fold, *hMC4R/hMC3R*) prompted us to compare its structures with potent nonselective *hMC3R/hMC4R* antagonist SHU9119 (Ac-Nle-c[Asp-His-DNal(2')-Arg-Trp-Lys]-NH₂) by using computational modeling methods, and these studies demonstrated considerable similarity. First of all, both of these molecules' binding domains are constrained cyclic peptides with similar ring size. Secondly, they all have guanidinyll and aromatic moieties in their core pharmacophores and all of them have β -turn structures. However, the sequences of their β -turns are somewhat different. The NMR solution structure of the SHU9119 suggests a β -turn encompassed by the Asp-His-DNal(2')-Arg residues,⁴⁶ whereas for the *hASIP*, its NMR solution structure suggests a β -turn at Phe-Phe-Arg-Ser residues.⁴¹ We envisioned that a forced turn spanning between the DNal(2') and Arg residues in SHU9119 may provide short and stable peptide analogues that could mimic the *hASIP* core pharmacophore thus achieving greater selectivity for the *hMC4R*. Since SHU9119 and MTII (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH₂) share considerable sequence and secondary structural similarities,⁴⁶ we also decided to extend similar modifications to MTII.

One possible approach to have such a turn in the globally constrained SHU9119 and MTII is to substitute arginine⁸ with structurally more constrained proline or its derivatives.⁴⁷ Presumably, the two Phe residues in the *hASIP* central loop could be mimicked by the His⁶ and DPhe⁷/DNal(2')⁷ residues in the MTII and SHU9119. In order to keep the guanidinyll groups in MTII or SHU9119 for possibly mimicking the guanidinyll group in the *hASIP* pharmacophore sequence, proline derivatives with guanidinyll moieties on the proline ring are needed. In this regard, Tamaki et al. have developed a methodology for the synthesis of both *trans*- and *cis*-4-guanidinyll-Pro derivatives in their protected forms.⁴⁸ These novel constrained amino acids would also allow us to study the topographic importance of the guanidinyll group when it is highly constrained. Therefore, we designed MTII and SHU9119 analogues **2**, **3**, **7**, **8** in which the Arg⁸ residues in their core sequences were replaced by 4-*trans*- or *cis*-guanidinyll-Pro residues (Figure 2). Additionally, to test how this modification compares with

a potent nonselective linear peptide, Ac-NDP- γ -MSH-NH₂ (Ac-Tyr¹-Val²-Nle³-Gly⁴-His⁵-DPhe⁶-Arg⁷-Trp⁸-Asp⁹-Arg¹⁰-Phe¹¹-Gly¹²-NH₂),⁴⁹ analogues **12**, **13** were designed which contained a *trans*- or *cis*-4-guanidiny-Pro in the key arginine⁷ positions (Figure 2). For comparison, control analogues **1**, **6** and **11** were also synthesized to investigate the importance of the guanidiny groups. Furthermore, previous studies have indicated that the His⁶ residue in the MTII and SHU9119 can be replaced with a Pro residue without causing any selectivity changes at the hMC3R and hMC4R.^{50,51,52} However, replacement of His⁶ residue in the SHU9119 with a variety of proline-like, conformationally constrained, hydrophobic amino acid residues led to the discovery of several hMC3R and hMC4R selective antagonists.⁵³ Therefore, analogues **4**, **5**, **9**, **10** (Figure 2) were prepared by replacing the His⁶ residues in the MTII and SHU9119 with 4-*trans*- or *cis*-guanidiny-Pro derivatives. Such substitution will help in investigating the possible effects of introducing additional positively charged guanidiny groups in position 6.

Peptide Synthesis

A well-known side reaction in Fmoc peptide synthesis of MTII analogues is aspartimide formation (Scheme 1).^{54,55} To avoid this side reaction, the synthesis was designed in such a way that all coupling reactions were performed under neutral conditions using DIC/Cl-HOBt as coupling reagents. In addition, the side chain to side chain cyclization was performed immediately after the attachment of the Fmoc-Asp(Allyl) residue to the growing peptide chain under neutral condition (Scheme 2). The cyclization was then followed by aspartic acid Fmoc removal and the coupling of the next residue, Nle.

Results

The protected *trans*- and *cis*- 4-guanidiny-Pro analogues were synthesized from *trans*-4-hydroxyproline following a facile method developed by Tamaki et al.^{48,56} All peptides were synthesized *via* the Fmoc SPPS strategy outlined above with high yields and purity as determined by HPLC, TLC, NMR and high resolution mass spectrometry (Table 1). To see whether aspartimide derivatives could be formed in basic conditions, we also synthesized Ac-Nle-Asp-His-DPhe-*trans*-4-guanidiny-Pro-Trp-Lys-NH₂, the linear analogue of **2**, using HBTU/HOBt/DIPEA as coupling reagents. The linear peptide was cleaved from the resin after the removal of the side chain Allyl/Alloc protecting groups in the last step. Analytical HPLC and mass spectrometry identified an aspartimide derivative (35%) which has exactly the same mass as the corresponding cyclic peptide **2**. Considering the possible ring opening of the aspartimide derivative that could lead to the formation of linear, epimerized α -peptide or β -peptide that might be difficult to separate from the desired α -peptide by HPLC,^{54,57} the actual percentage of the aspartimide peptide formed could be higher. This result is consistent with the report by Flora et al. regarding the Fmoc synthesis of MTII.⁵⁵ We did not observe the aspartimide derivative during the synthesis of peptide **2** using the strategy shown in Scheme 2.

The binding affinities and biological activities of the peptides **1-13** were determined at the hMC1R, hMC3R, hMC4R and hMC5R, and the results are summarized in Table 2. Considering very high receptor density used for the assays that may give increased apparent drug potency and/or efficacy, and 10-20% errors in the experiments, we define a drug that binds to the hMCRs and triggers a maximal cAMP level that is 20%-80% of MTII triggered maximal cAMP response as a partial agonist, and when 80%-100% level of MT II as a full agonist. Also, we define a drug that binds to the hMCRs and triggers less than 20% maximal cAMP level of MTII as an antagonist.⁵⁸ Control peptides **1**, **6** show high affinity and selectivity towards the hMC1,4Rs, and no binding at the hMC3,5Rs at up to 10 μ M. Peptide **1** is considered a selective antagonist for the hMC4R (IC₅₀=11 nM) with very weak partial agonist activity at the hMC1R

(EC₅₀>1000 nM, 75% max activation). Peptide **6** is a moderate partial agonist selective for the hMC1R (EC₅₀=160 nM, 20-fold, hMC1R/hMC4R). Peptide **11** is a nonselective partial agonist at the hMC1R (EC₅₀=120 nM, 53% max activation) and hMC4R (EC₅₀=210 nM, 46% max activation). Its binding affinity at the hMC4R is 25 and 8-fold lower than peptides **1** and **6**, respectively. Analogue **2** is a potent antagonist selective for the hMC4R (IC₅₀=50 nM, >2000-fold, hMC4R/hMC3R). The presence of a *trans*-4-guanidiny group also introduces weak partial agonist activity at the hMC5R (EC₅₀=2500 nM, 24% max activation). Schild analysis of analogues **1** and **2** based on cAMP accumulation (Figure 3) show that both peptides **1** and **2** are not competitive inhibitors of MTII towards the hMC4R, which suggests that they might be allosteric binders. The binding affinity of peptide **3** is 4-fold weaker than that of the control peptide **1** at the hMC4R. However, the EC₅₀ and maximal cAMP response at the same receptor are increased by 18-fold and 3-fold, respectively, converting peptide **3** to a potent hMC4R selective partial agonist (EC₅₀=11 nM, 36% max activation; 70-fold, hMC4R/hMC1R). Compared to peptide **6**, peptide **7** displays a 5-fold increase in IC₅₀, 29-fold increase in EC₅₀ and 44% decrease in maximal cAMP response at the hMC4R showing moderate partial agonist activity (EC₅₀=200 nM, 31% max activation). It also shows very weak partial agonist activity at the hMC5R (EC₅₀=2400 nM, 59% max activation). Peptide **8** is an antagonist for the hMC4R (IC₅₀=23 nM), with no binding at the hMC3,5Rs and very weak full agonist activity at the hMC1R (EC₅₀=19000 nM, 100% max activation). In the Ac-NDP- γ -MSH-NH₂ series, the additional guanidiny groups in the 4' positions of the proline ring in analogues **12** or **13** produce weak full agonists that are hMC1R selective, but with a 4-5 fold drop in binding affinity and more than 11-fold drop in potency.

For MTII or SHU9119 analogues **4**, **5**, **9**, **10** in which the His⁶ residues in the parent peptides are replaced by the *trans*- or *cis*-4-guanidiny-Pro, the binding at the hMC3,4Rs remains strong and their IC₅₀ values are still in the single-digit nanomolar range. However, there are large changes in ligand potency and/or efficacy at individual receptors. The *trans*-4-guanidiny-Pro⁶ analogue **4** is a potent hMC3R full agonist (EC₅₀=14 nM) and hMC5R partial agonist (EC₅₀=1.6 nM, 68% max activation) with high selectivity against the hMC4R (250-fold, hMC3R/hMC4R) and hMC1R (60-fold, hMC3R/hMC1R). Intriguingly, the isomeric *cis*-4-guanidiny-Pro⁶ analogue **5** displays a reversal of hMC3R/hMC4R selectivity (60-fold, hMC4R/hMC3R), showing potent hMC4R/hMC5R full agonist activity (EC₅₀=41 nM, 21 nM, respectively). Such functional selectivity at the hMC3R and hMC4R is not seen in peptides **9**, **10** that show no or low partial agonist activity. Evidently, the melanocortin receptor 3/4 binding pockets are extremely sensitive to slight modifications of the structure of residue 6 in MTII template.

Peptides **1** and **2** were chosen for further molecular modeling study due to their selective antagonist activity at the hMC4R vs. hMC3R. The modeling experiments were performed using Kolossvary's Monte Carlo Multiple Minima/Low Frequency Mode conformational search method (MCMMLMCS)⁵⁹ with the OPLS_2005 force field⁶⁰ and the GB/SA continuum dielectric water solvent model.⁶¹ It was revealed that the type II β -turn spanning the His⁶-DPhe⁷/DNaI(2')⁷ residues, prominent in the NMR solution structures of MTII or SHU9119,⁴⁶ was no longer possible in Pro⁸-analogue **1** (Figure 4). The 3D structure of analogue **2** displays a shift of the β -turn motif to Asp⁵-His⁶ residues (Figure 5), which is now closer to a type II' turn ($\phi_5 = 75^\circ$, $\psi_5 = -74^\circ$, $\phi_6 = -143^\circ$ and $\psi_6 = 9^\circ$). At the same time, the 3D structure of analogue **2** was found to produce a good fit within the pharmacophore region of the NMR solution structure of the hASIP central loop (Figure 5).⁴¹ The side chains of His⁶ and DPhe⁷ residues in analogue **2** align well with the side chains of the Phe¹¹⁸ and Phe¹¹⁹ residues, respectively, in the putative Arg¹¹⁷-Phe¹¹⁸-Phe¹¹⁹-Arg¹²⁰-Ser¹²¹ pharmacophore sequence of the hASIP, as well as the guanidiny groups of guanidiny-Pro⁸ of analogue **2** and Arg¹²⁰ of the hASIP. Yet, analogue **2** lacks the functionality to mimic the potentially critical Arg¹¹⁷ residue of the hASIP pharmacophore. The unique conformational profiles of the

peptides in this study stemmed from employment of the dihedrally constrained arginine analogues and inferred an opportunity to discover peptide analogues with enhanced melanocortin receptor selectivity.

Discussion

It has been known that it is difficult to separate ligand interactions with the *h*MC3R from those with the *h*MC4R based on rational modifications on MTII or SHU9119. In the present study, Pro residues in the positions of Arg residues in the MTII, SHU9119 and Ac-NDP- γ -MSH-NH₂ have been shown to be able to introduce high binding selectivity for the *h*MC4R vs. *h*MC3R as indicated by the binding data of the three control peptides **1**, **6**, **11**. It should be pointed out that Bednarek et al. reported lower selectivity and weaker binding of peptides **1**, **6** to the *h*MC4R.⁶² These discrepancies may be caused by different assay systems used. Nonetheless, it was shown in both systems that peptide **1** was an antagonist at the *h*MC4R. The altered selectivity/activity of those novel peptides is presumably caused by constrained proline-induced peptide backbone conformational changes as suggested in Figure 4. These changes probably altered the topography of the core pharmacophore elements in a way that favors interaction with the *h*MC1R and/or *h*MC4R. The new 3D positions of the core pharmacophore at least partially resemble that of the *h*ASIP as supported by the molecular modeling results and receptor selectivity displayed by the three control peptides. The much larger affinity drop seen in peptide **11** is likely due to its flexible linear structure, which is easily altered by the presence of a proline residue.

The replacement of Arg with Ala in MTII/SHU9119 has been previously reported to be able to cause at least 100-fold affinity drop at the *h*MC3R, *h*MC4R and *h*MC5R due to loss of direct interactions between the guanidinyll group and the melanocortin receptors.⁶³⁻⁶⁶ However, there is only 1.5-5 fold difference in affinity between peptides **2**, **3**, **7**, **8** and their respective control peptides at the *h*MC4R. In addition, control peptide **1** shows stronger binding (4-5 fold) than its analogues **2**, **3** that have additional guanidinyll groups in position 8. These data suggest that the additional guanidinyll groups in peptides **2**, **3**, **7**, **8** do not contribute to binding to the *h*MC4R. And their presence is not required for excluding binding to the *h*MC3R as indicated by the lack of control peptides binding to the *h*MC3R at up to 10 μ M concentration. The molecular modeling study (Figure 5) shows that the guanidinyll group in peptide **2** is quite far away from the crucial guanidinyll group in Arg¹¹⁷ of the *h*ASIP core pharmacophore. Therefore, the additional guanidinyll groups in novel analogues **2**, **3**, **7**, **8** are probably not mimicking Arg¹¹⁷ of the *h*ASIP.⁴¹ The poor mimicry of the Arg¹¹⁷ guanidinyll group is at least in part due to the sequence difference. To better mimic the core pharmacophore of *h*ASIP central loop, future analogues based on the templates of analogues **1** and **6** will incorporate an Arg residue before the His⁶ residue.

Although the additional guanidinyll groups in positions 8 of peptides **2**, **3**, **7**, **8** do not bind to the *h*MC4R, they do seem to affect ligand potency (up to 29-fold) and efficacy (up to 3-fold) at the *h*MC4R and ligand selectivity over other receptor subtypes depending on the stereochemistry of the guanidinyll groups. Figure 5 shows that the guanidinyll group is in close proximity with the imidazole group of His⁶, which mimics Phe¹¹⁸ of the *h*ASIP. Therefore it is possible that the additional guanidinyll groups can change the 3D positions of the critical His⁶ side chain, which may affect ligand functional activity and selectivity.

In addition to position 8 of these novel MTII/SHU9119 analogues, position 7 also displays SAR that is different from MTII/SHU9119 SAR. In the MTII/SHU9119 binding sites on the *h*MC3R and *h*MC4R, the proper positioning of different type of side chains in position 7 determines ligand functional activity. A phenyl group usually provides agonist activity as in MTII while a 2'-naphthyl group often displays antagonist activity as in SHU9119. Such

observations are not closely followed by the MTII analogues **1**, **2**, **3** and SHU9119 analogues **6**, **7**, **8**. The different SARs in positions 7, 8 further support the hypothesis based on Schild analysis of peptides **1** and **2** that analogues **1**, **2** are non-competitive inhibitor of hMC4R and might be allosteric binders for the hMC4R.

From the cAMP experiments based Schild analyses, it is clear that the maximal stimulation of cAMP level has decreased, and the EC₅₀ values are right shifted along with increasing the concentration of antagonists (Figure 3). Similar observations were reported by other labs for ASIP and AGRP at the hMC4R.^{42,67} Allosteric binding sites are also suggested for other novel hMC4R selective antagonists designed on the basis of MTII.^{68,69}

Another approach to achieve high hMC4R/hMC3R selectivity is via His⁶ substitution in the SHU9119 template. Previously investigations have shown that the side chain of His⁶ in MTII/SHU9119 was not important for binding/activation of hMCRs,^{51,63,66} and that replacement of His⁶ in MTII and SHU9119 with proline does not change ligand biological profiles at the hMC3R and hMC4R.^{53,66} However, Grieco et al. showed that other His⁶ substitutions in the SHU9119 template dramatically changed the selectivity between the hMC3R and hMC4R suggesting slightly different binding pockets for interactions with residues in position 6.⁵³ Furthermore, Cai et al.⁷⁰ suggested that the hMC3R and hMC4R binding pocket could not accommodate a bulky *trans*-3-substituent on the proline ring in position 6 of MTII, whereas the hMC5R binding pocket can tolerate such bulky substituents. While retaining strong binding of peptides **4**, **5**, **9**, **10** at the hMC5R supports such hypothesis, the lack of binding selectivity of these analogues at the hMC3R and hMC4R suggest that the additional positively charged guanidiny groups are not involved in binding to the proposed binding pockets. Grieco et al. also showed that His⁶ substitutions were less likely to affect other key residue 3D conformations, and a small *trans*-4-hydroxyl group in position 6 of Ac-Nle-c[Asp-Hyp-DNal(2')-Arg-Trp-Lys]-NH₂ did not affect binding affinity/selectivity at the hMC3R and hMC4R. Therefore, this lack of influence on binding to the hMC3,4Rs may be due to the relatively small size of the guanidiny groups.⁵³ However, the opposite functional selectivity between peptides **4** (250-fold, hMC3R/hMC4R) and **5** (60-fold, hMC4R/hMC3R) at the hMC3/4R do suggest that the guanidiny groups can affect receptor activation by interacting with the slightly different binding pockets. It has been established that DNal(2')⁷ side chain in SHU9119 is the sole determinant of antagonist activity by interacting with Leu¹³³ in the hMC4R TM3.⁷¹ And Leu¹⁶⁵ in TM3 of the hMC3R is critical for agonist or antagonist selectivity at this receptor.⁷² Both receptor binding pockets are sensitive to positioning of Phe/Nal(2') side chains. It is possible that the phenyl moieties in peptides **4** and **5** can induce receptor functional conformational changes at the hMC3/4R, but such changes are partially or completely blocked by the constrained guanidiny groups depending on their orientations. Other MTII analogues with His⁶ substitution (Ac-Nle-c-[Asp-Aba-DPhe-Arg-Trp-Lys]-NH₂ and c[CO-(CH₂)₂-CO-Nle-DPhe-Arg-Trp-Lys]-NH₂) also have been reported to be able to bind to the hMC3,4Rs without agonist activity, and can be explained by this theory.^{73,74} Peptides **9** and **10** do not show functional selectivity at the hMC3/4R probably because the bulky naphthyl moiety can prevent receptor conformational changes in the first place. The positive charges on the guanidiny groups in these analogues may be important for high functional selectivity because [Hyp(Bzl)⁶]MTII shows lower hMC3R/hMC4R (23-fold) functional selectivity than peptide **4** (250-fold).⁵⁰ Though less likely, we cannot exclude the possibility that the presence of the 4-guanidiny-Pro⁶ can alter the 3D positioning of the DPhe⁷/DNal(2')⁷ side chain in position 7, thus changing ligand potency/efficacy at melanocortin receptors. In any case, this study confirms previous findings by us and others that a constrained side chain in position 6 can influence not only ligand binding affinity but also receptor activation at melanocortin receptors.⁷⁵ These findings are important for understanding the different binding mechanisms of hMC3R and hMC4R, and for the rational design of selective ligands for melanocortin receptors.

Summary

Both cyclic and linear analogues of melanotropins were designed to have a Pro or 4-guanidiny-Pro residues in the His or Arg positions of the melanotropin pharmacophore. The Pro residue was incorporated to investigate the effects of a backbone conformational change on biological activity in those analogues and the guanidiny group on the Pro ring was used to probe possible electrostatic interactions with the melanocortin receptors. These peptides were synthesized successfully *via* a carefully designed solid phase peptide synthesis strategy to avoid the formation of the aspartimide derivatives. Competitive binding and cAMP assays revealed a series of novel ligands selective for the *h*MC1R and/or *h*MC4R. Some of these analogues are high affinity noncompetitive antagonists for the *h*MC4R. The results are consistent with the finding from our molecular modeling studies of analogues **1** and **2**, whose global minimum structures fit well with the NMR solution structure of the endogenous *h*MC1/4R selective antagonist *h*ASIP. The β -turn shift induced by the proline ring was identified to be responsible for this selectivity. The guanidiny group on Pro⁸ is not required for *h*MC4R selectivity. In addition, the 4-guanidiny group on Pro⁶ was shown to be able to affect ligand functional selectivity for the *h*MC3R or *h*MC4R. These findings are useful for the rational design of novel selective ligands targeting melanocortin receptors, especially the *h*MC4R, which is involved in many major diseases.

Experimental Section

Materials

N ^{α} -Fmoc-amino acids and Rink amide MBHA (w/Nle) resin were obtained from Chem-Impex International (Wood Dale, IL). DIC and Cl-HOBt were purchased from Bachem (King of Prussia, PA). Pd(PPh₃)₄ and PhSiH₃ were purchased from Sigma-Aldrich (Milwaukee, WI). HSW syringes (Torviq, Niles, MI) (10 mL) with frits on the bottom were used for all peptide syntheses. Analytical HPLC was performed using a Vydac C₁₈ 218TP104 column (Western Analytical Products, Murrieta, CA) monitored at 230 and 254 nm. Preparative HPLC was done on XTerra® Prep MSC18 Column (10 μ m, 19mm $\times$ 250mm, part#186002259) obtained from Waters (Milford, MA). Analytical TLC was carried out on Anatech Uniplate silica gel TLC plates (250 micron) (Newark, DE). The TLC chromatograms were visualized by UV light and by potassium permanganate solution. ¹H NMR spectra were recorded on Bruker DRX600 or 500 or Varian Inova600 spectrometers with DMSO-*d*₆ as internal standard. Mass spectra were obtained from Mass Spectrometry Facility, Department of Chemistry, University of Arizona.

Peptide Synthesis

All peptides in this study were synthesized manually by Fmoc solid phase methodology using DIC and Cl-HOBt or HOAt as coupling reagents. The following procedures were used for the synthesis of the cyclic peptides. The linear peptides were synthesized likewise. Rink amide MBHA resin (250 mg, 0.7 mmol/g) was placed into a 10 mL HSW polypropylene syringe and swollen in DCM (5 mL) for 30 min. After removal of the Fmoc protecting group (25% piperidine in DMF, 5mL, 5 and 10 min), the resin was washed with DMF (4 $\times$ 5mL) and DCM (4 $\times$ 5mL). Then, Fmoc-Lys(Alloc)-OH, Fmoc-Trp(Boc)-OH, (2*S*,4*R*)-N ^{α} -Fmoc-4-N,N'-di-Boc-guanidiny-Pro-OH or (2*S*,4*S*)-N ^{α} -Fmoc-4-N,N'-di-Boc-guanidiny-Pro-OH or Fmoc-Arg(Pbf)-OH, Fmoc-DPhe-OH or Fmoc-DNal(2')-OH, Fmoc-His(Trt)-OH or (2*S*,4*R*)-N ^{α} -Fmoc-4-N,N'-di-Boc-guanidiny-Pro-OH or (2*S*,4*S*)-N ^{α} -Fmoc-4-N,N'-di-Boc-guanidiny-Pro-OH, Fmoc-Asp(Allyl)-OH were coupled to the resin following the above order. For each coupling, 3 equivalents of amino acids, Cl-HOBt, DIC were used with 10 min preactivation in DMF. All couplings were performed for 1 h and monitored by either the Kaiser Test or the Chloranil Test. In case of a positive test result, the coupling was repeated until a negative test result was observed. Fmoc protecting groups were removed as described above. Once the

Fmoc-Asp(Allyl)-OH was coupled to the peptide chain, the side chain Alloc protecting group of Lys and Allyl protecting group of Asp were removed simultaneously with 0.25 equivalent of $\text{Pd}(\text{PPh}_3)_4$ and 20 equivalents of PhSiH_3 in 5 mL of dry DCM (30 min $\times$ 2). The cyclization was performed with 6 equivalents of DIC and HOAt in DMF for 72 h and was monitored by the Kaiser Test. Upon completion of the cyclization, the resin was treated with 5 mL of 5% sodium diethyldithiocarbamate trihydrate in DMF (10 min) and then washed with DMF (4 $\times$ 5 mL), DCM (4 $\times$ 5 mL). Fmoc-Nle-OH was then coupled to the peptide chain following the procedures described above. Next the N^α -Fmoc was removed and the amino group was acetylated with 10 equivalents of acetic anhydride and 2 equivalents of DIPEA in 5 mL of DCM for 30 min. The resin was washed with DCM (4 $\times$ 5 mL), DCM/diethylether (1:1, 4 $\times$ 5 mL) and dried under vacuum for 4 h. The peptides were cleaved from the resin with a mixture of 95% TFA, 2.5% water and 2.5% TIPS for 3 h. After evaporation of the TFA under vacuum, the peptides were precipitated and washed 3 times with 5 mL of cold diethyl ether for each wash. The liquid was separated from the solid by centrifuge and was decanted. The crude peptides were dried in air and dissolved in acetonitrile and 0.1% TFA in water (2:1) before it was purified by preparative RP-HPLC and eluted with a linear gradient of 15-40% acetonitrile in aqueous 0.1% TFA solution over 35 min with a 15 mL/min flow rate. The fractions containing the desired products were collected, concentrated and lyophilized. The purified peptides were isolated in 40-50% overall yields and were >95% pure as determined by analytical RP-HPLC. The structures of the pure peptides were confirmed by high-resolution mass spectrometry and ^1H NMR in $\text{DMSO}-d_6$.

Binding Assays

Competition binding experiments were performed on whole cells. Stably transfected HEK293 cell lines having the individual *hMCRs*^{70,76-77} were seeded on 96 well plate, 48 h before the assay and grown to 100,000 cells/well. For the assay, the medium was removed and cells were washed twice with a freshly prepared binding buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthroline, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. Cells were then incubated with different concentrations of unlabeled peptides and ^{125}I -labeled [$\text{Nle}^4, \text{DPhe}^7$]- α -MSH (PerkinElmer Life Science, 100,000 cpm/well, 0.1386 nM) for 40 min at 37 °C. The medium was subsequently removed and each well was washed twice with the binding buffer. The cells were lysed by the addition of 250 μL of 0.1 mM NaOH and 250 μL of 1% Triton X-100. The lysed cells were transferred to the 12 $\times$ 75 mm glass tubes and the radioactivity was measured using a Wallac 1470 WIZARD Gamma Counter. Data were analyzed using Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

Adenylate Cyclase Assays

HEK 293 cells stably transfected with individual human melanocortin receptors⁷⁰ were grown to confluence in MEM medium (GIBCO) containing 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96 well plates 48 h before assay and grown to 100,000 cells/well. For the assay, the medium was removed and cells were rinsed with 1 mL of MEM buffer (GIBCO) or with Earle's balanced salt solution (EBSS, GIBCO). An aliquot (0.4 mL) of the Earle's balanced salt solution was placed in each well along with isobutylmethylxanthine (IBMX, 5 μL , 0.5 mM) for 1 min at 37 °C. Next, various concentrations of melanotropins (0.1 mL) were added and the cells were incubated for 3 min at 37 °C. The reaction was stopped by aspirating the buffer and adding ice cold Tris/EDTA buffer to each well (0.15 mL). After dislodging the cells with the help of trypsin, the cells were transferred to polypropylene microcentrifuge tubes, capped, and placed in a boiling water bath for 15 min. The cell lysate was then centrifuged for 2 min (6500 rpm), and 50 μL of the supernatant was aliquoted into a clean Eppendorf tube. The total cAMP content was measured by competitive binding assay according to the assay kit instructions (TRK 432,

Amersham Corp., Arlington Heights, IL). The antagonist properties of analogues **1**, **2** were evaluated by its ability to competitively displace the MTH agonist in a dose dependent manner, at up to 10 μ M.

Data Analysis

IC₅₀ and EC₅₀ values represent the mean of duplicate experiments performed in triplicate. IC₅₀ and EC₅₀ estimates and their associated standard errors were determined by fitting the data using a nonlinear least-squares analysis, with the help of Graphpad Prism 3.1 (Graphpad Software, San Diego, CA).

Computational Procedures

Molecular modeling experiments employed MacroModel version 9.1 equipped with Maestro 7.5 graphical interface (Schrödinger, LLC, New York, NY, 2005) installed on a Linux Red Hat 9.0 system, and were performed as previously described.⁷⁸ Peptide structures were built into extended structures with standard bond lengths and angles, and they were minimized using the OPLS_2005 force field⁶⁰ and the Polak-Ribier conjugate gradient (PRCG).⁷⁹ Optimizations were converged to a gradient RMSD less than 0.05 kJ/Å mol or continued until a limit of 50,000 iterations was reached. Aqueous solution conditions were simulated using the continuum dielectric water solvent model (GB/SA).⁶¹ Extended cut-off distances were defined at 8 Å for Van der Waals, 20 Å for electrostatics and 4 Å for H-bonds.

Conformational profiles of the cyclic peptides were investigated by the hybrid Monte Carlo/Low Frequency Mode (MCMM/LMCS)⁵⁹ procedure as implemented in MacroModel using the energy minimization parameters as described above. MCMM torsional variations and Low Mode parameters were set up automatically within Maestro graphical user interface. A total of 20,000 search steps were performed and the conformations with energy difference of 50 kJ/mol from the global minimum were saved. The superimpositions of peptide structures were performed using the α -carbons of the core sequence His-DPhe/DNal(2')-Xaa-Trp.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Aba, 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one
Ac, acetyl
Ac-NDP- γ -MSH-NH₂, Ac-Tyr-Val-Nle-Gly-His-D-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH₂
AGRP, agouti related protein
Alloc, allyloxycarbonyl
ASIP, agouti signaling protein
Boc, tertbutoxycarbonyl
Bzl, benzyl
cAMP, cyclic adenosine monophosphate
Cl-HOBt, 6-chloro-1-hydroxybenzotriazole
CNS, central nervous system
DCM, dichloromethane
DIC, 1,3-diisopropylcarbodiimide

DIPEA, *N,N*-diisopropylethylamine
 DMF, *N,N*-dimethylformamide
 DMSO, dimethyl sulfoxide
 D-Nal(2'), 3-(2-naphthyl)-D-alanine
 ESI, electrospray ionization
 Et₂O, diethyl ether
 EtOAc, ethyl acetate
 FAB, fast atom bombardment
 Fmoc, 9-fluorenylmethoxycarbonyl
 Fmoc-OSu, *N*-[(9-fluorenylmethoxycarbonyl)oxy]-succinimide
 GB/SA, generalized born/surface area
 GPCR, G-protein coupled receptor
 HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
 HIV, human immunodeficiency virus
 hMC1R, human melanocortin 1 receptor
 hMC2R, human melanocortin 2 receptor
 hMC3R, human melanocortin 3 receptor
 hMC4R, human melanocortin 4 receptor
 hMC5R, human melanocortin 5 receptor
 HOAt, 1-hydroxy-7-aza-benzotriazole
 HOBt, 1-hydroxybenzotriazole
 HPLC, high-pressure liquid chromatography
 HRMS, high resolution mass spectrometry
 Hyp, *trans*-4-hydroxyproline
 IC₅₀, 50% inhibitory concentration
 MBHA, 4-methylbenzhydramine
 α-MSH, alpha melanocyte-stimulating hormone
 Nle, *L*-norleucine
 NMR, nuclear magnetic resonance spectroscopy
 OPLS, optimized potential for liquid simulations
 Pbf, 2,2,4,6,7-pentamethyldihydrobenzofurane-5-sulfonyl
 POMC, proopiomelanocortin
 PRCG, Polak-Ribier conjugate gradient
 RMSD, root mean square deviation
 RP-HPLC, reverse phase high-pressure liquid chromatography
 SAR, structure activity relationship
 SPPS, solid phase peptide synthesis
 TFA, trifluoroacetic acid
 TIPS, triisopropylsilane
 TLC, thin layer chromatography
 TMS, tetramethylsilane
 Trt, trityl

References

1. Cone RD. Studies on the physiological functions of the melanocortin system. *Endocr. Rev* 2006;27:736–749. [PubMed: 17077189]
2. Hadley, ME.; de Lauro Castrucci, AM. Melanotropin mechanisms of action: melanosome movements. In: Hadley, ME., editor. *The Melanotropic Peptides. Mechanisms of Action and Biomedical Applications*. Vol. Vol. III. CRC Press; Boca Raton, FL: 1988. p. 15-25.
3. Cone RD, Lu D, Koppula S, Vage DI, Klungland H, Boston B, Chen W, Orth DN, Pouton C, Kesterson RA. The melanocortin receptors: agonists, antagonists, and the hormonal control of pigmentation. *Recent Prog. Horm. Res* 1996;51:287–318. [PubMed: 8701084]

4. Hadley ME, Hruby VJ, Jiang J, Sharma SD, Fink JL, Haskell-Luevano C, Bentley DL, Al-Obeidi F, Sawyer TK. Melanocortin receptors: Identification and characterization by melanotropic peptide agonists and antagonists. *Pigment Cell Res* 1996;9:213–234. [PubMed: 9014208]
5. (a) Li SJ, Varga K, Archer P, Hruby VJ, Sharma SD, Kesterson RA, Cone RD, Kunos G. Melanocortin antagonists define two distinct pathways of cardiovascular control by α - and γ -melanocyte stimulating hormones. *J. Neurosci* 1996;16:5182–5188. [PubMed: 8756446] (b) Nicholson JR, Peter JC, Lecourt AC, Barde YA, Hofbauer KG. Melanocortin-4 receptor activation stimulates hypothalamic brain-derived neurotrophic factor release to regulate food intake, body temperature and cardiovascular function. *J. Neuroendocrinol* 2007;19:974–982. [PubMed: 18001327]
6. (a) Wessells H, Gralnek D, Dorr R, Hruby VJ, Hadley ME, Levine N. Effect of an α -melanocyte stimulating hormone analogue on penile erection and sexual desire in men with organic erectile dysfunction. *Urol* 2000;56:641–646. [PubMed: 11018622] (b) Wessells H, Fuciarelli K, Hansen J, Hadley ME, Hruby VJ, Dorr R, Levine N. Synthetic melanotropic peptide initiates erections in men with psychogenic erectile dysfunction: Double-Blind placebo controlled crossover study. *J. Urol* 1998;160:389–393. [PubMed: 9679884] (c) Bertolini A, Vergoni W, Gessa GL, Ferrari W. Induction of sexual excitement by action of adrenocorticotrophic hormone in brain. *Nature* 1969;221:667–669. [PubMed: 4304587] (d) Bertolini, A.; Vergoni, W.; Gessa, GL.; Ferrari, W. Erection and Ejaculation: A central effect of ACTH-like peptides in, ammals. In: Sandler, M.; Gessa, GL., editors. *Sexual Behavior: Pharmacology and Biochemistry*. Raven Press; New York: 1975. p. 247-257. (e) Wessells H, Hruby VJ, Hackett J, Han G, Balse-Srinivasan P, Vanderah TW. Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH₂ induces penile erection via brain and spinal melanocortin receptors. *Neuroscience* 2003;118:755–762. [PubMed: 12710982] (f) King SH, Mayorov AV, Balse-Srinivasan P, Hruby VJ, Vanderah T, Wessells H. Melanocortin receptors, melanotropic peptides and penile erection. *Curr. Topics Med. Chem* 2007;7:1098–1106.
7. Wikberg JES, Mutulis F. Targeting melanocortin receptors: an approach to treat weight disorders and sexual dysfunction. *Nat. Rev. Drug Discov* 2008;7:307–323. [PubMed: 18323849]
8. Fan W, Boston BA, Kesterson RA, Hruby VJ, Cone RD. Role of the melanocortineric neurons in feeding and the Agouti obesity syndrome. *Nature* 1997;385:165–168. [PubMed: 8990120]
9. Vergoni AV, Poggioloi R, Bertolini A. Corticotropin inhibits food intake in rats. *Neuropeptides* 1986;7:153–158. [PubMed: 3010169]
10. Vergoni AV, Poggioloi R, Marrama D, Bertolini A. Inhibition of feeding by ACTH-(1-24): Behavioral and pharmacological aspects. *Eur. J. Pharmacol* 1990;179:347–355. [PubMed: 2194823]
11. Ramos EJB, Meguid MM, Campos ACL, Coelho JCU. Neuropeptide Y, α -melanocyte-stimulating hormone, and monoamines in food intake regulation. *Nutrition* 2005;21:269–279. [PubMed: 15723758]
12. Yang YK, Harmon CM. Recent developments in our understanding of melanocortin system in the regulation of food intake. *Obes. Rev* 2003;4:239–248. [PubMed: 14649374]
13. Ellacott KLJ, Cone RD. The central melanocortin system and the integration of short- and long-term regulators of energy homeostasis. *Recent Prog. Horm. Res* 2004;59:395–408. [PubMed: 14749511]
14. Zimanyi IA, Pellemounter MA. The role of melanocortin peptides and receptors in regulation of energy balance. *Curr. Pharm. Des* 2003;9:627–641. [PubMed: 12570796]
15. Morgan C, Thomas RE, Cone RD. Melanocortin-5 receptor deficiency promotes defensive behavior in male mice. *Horm. Behav* 2004;45:58–63. [PubMed: 14733892]
16. Mogil JS, Wilson SG, Chesler EJ, Rankin AL, Nemmani KVS, Lariviere WR, Groce MK, Wallace MR, Kaplan L, Staud R, Ness TJ, Glover TL, Stankova M, Mayorov AV, Hruby VJ, Grisel JE, Fillingim RB. The melanocortin-1 receptor gene mediates female-specific mechanisms of analgesia in mice and humans. *Proc. Natl. Acad. Sci* 2003;100:4867–4872. [PubMed: 12663858]
17. Vrinten DH, Kalkman CJ, Adan RAH, Gispen WH. Neuropathic Pain: A possible role for the melanocortin system? *Eur. J. Pharmacol* 2001;429:61–69. [PubMed: 11698027]
18. Mogil JS, Ritchie J, Smith SB, Strasburg K, Kaplan L, Wallace MR, Romberg RR, Bijl H, Sarton EY, Fillingim RB, Dahan A. Melanocortin-1 receptor gene variants affect pain and μ -opioid analgesia in mice and humans. *J. Med. Genet* 2005;42:583–587. [PubMed: 15994880]
19. Mountjoy, K.; Mountjoy, G. Cloning of the melanocortin receptors. In: Cone, RD., editor. *Melanocortin Receptors*. Humana Press; Totowa, NJ: 2000. p. 209-235.

20. Vaudry H, Eberle AN. The Melanotropic Peptides. *Ann. N.Y. Acad. Sci* 1993;680:1–687.
21. Maaser C, Kannengesser K, Kucharzik T. Role of the Melanocortin System in Inflammation. *Ann. N.Y. Acad. Sci* 2006;1072:123–134. [PubMed: 17057195]
22. Catania A. The melanocortin system in leukocyte biology. *J. Leukoc. Biol* 2007;81:383–392. [PubMed: 17041004]
23. Gispén WH, Isaacson RL. ACTH-induced excessive grooming in the rat. *Pharmacol. Ther* 1981;12:209–246. [PubMed: 6114501]
24. Gantz I, Fong TM. The melanocortin system. *Am. J. Physiol. Endocrinol. Metab* 2003;284:E468–E474. [PubMed: 12556347]
25. Chhajlani B. Distribution of cDNA for melanocortin receptor subtypes in human tissues. *Biochem. Mol. Biol. Int* 1996;38:73–80. [PubMed: 8932521]
26. Cone, RD., editor. *The Melanocortin Receptors*. Humana Press; Totowa, NJ: 2000. p. 1-554.
27. Raffin-Sanson ML, Bertherat J. MC3 and MC4 Receptors: Complementary role in weight control. *Eur. J. Endocrinol* 2001;144:207–208. [PubMed: 11248737]
28. (a) Marks DL, Hruby VJ, Brookhart G, Cone RD. The regulation of food intake by selective stimulation of the type 3 melanocortin receptor (MC3R). *Peptides* 2006;27:259–264. [PubMed: 16274853] (b) Lee M, Kim A, Conwell IM, Hruby VJ, Mayorov A, Cai M, Wardlaw SL. Effects of selective modulation of the central melanocortin-3-receptor on food intake and hypothalamic POMC expression. *Peptides* 2008;29:440–447. [PubMed: 18155809]
29. (a) Getting SJ. Melanocortin peptides and their receptors: new targets for anti-inflammatory therapy. *Trends Pharmacol. Sci* 2002;23:447–449. [PubMed: 12368061] (b) Getting SJ, Christian HC, Flower RJ, Perretti M. Activation of melanocortin type 3 receptor as a molecular mechanism for adrenocorticotrophic hormone efficacy in gouty arthritis. *Arthritis. Rheum* 2002;46:2765–2775. [PubMed: 12384937] (c) Getting SJ, Christian HC, Lam CW, Gavins FNE, Flower RJ, Schiöth HB, Perretti M. Redundancy of a functional melanocortin 1 receptor in the anti-inflammatory actions of melanocortin peptides: studies in the recessive yellow (e/e) mouse suggest an important role for melanocortin 3 receptor. *J. Immunol* 2003;170:3323–3330. [PubMed: 12626592]
30. Martin WJ, McGowan E, Cashen DE, Gantert LT, Drisko JE, Hom GJ, Nargund R, Sebbat I, Howard AD, Van der Ploeg LHT, MacIntyre DE. Activation of melanocortin MC4 receptors increases erectile activity in rats ex copula. *Eur. J. Pharmacol* 2002;454:71–79. [PubMed: 12409007]
31. Chaki S, Okubo T. Melanocortin-4 receptor antagonists for the treatment of depression and anxiety disorders. *Curr. Topics Med. Chem* 2007;7:1145–1151.
32. Van der Kraan M, Adan RA, Entwistle ML, Gispén WH, Burbach JP, Tatro JB. Expression of melanocortin-5 receptor in secretory epithelia supports a functional role in exocrine and endocrine glands. *Endocrinology* 1998;139:2348–2355. [PubMed: 9564844]
33. Chen WB, Kelly MA, Opitz Araya X, Thomas RE, Low MJ, Cone RD. Exocrine gland dysfunction in MC5-R deficient mice: Evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell* 1997;91:789–798. [PubMed: 9413988]
34. Caldwell HK, Lepri JJ. Disruption of the fifth melanocortin receptor alters the urinary excretion of aggression-modifying pheromones in male house mice. *Chem. Senses* 2002;27:91–94. [PubMed: 11751473]
35. Palucki BL, Park MK, Nargund RP, Tang R, MacNeil T, Weinberg DH, Vongs A, Rosenblum CI, Doss GA, Miller RR, Stearns RA, Peng Q, Tamvakopoulos C, Van der Ploeg LHT, Patchetta AA. 2-Piperazinecarboxamides as potent and selective melanocortin subtype-4 receptor agonists. *Bioorg. Med. Chem. Lett* 2005;15:1993–1996. [PubMed: 15808454]
36. (a) Coll AP. Effects of pro-opiomelanocortin (POMC) on food intake and body weight: mechanisms and therapeutic potential? *Clin. Sci* 2007;113:171–82. [PubMed: 17623013] (b) Van der Ploeg LH, Kanatani A, MacNeil D, Ming FT, Strack A, Nargund R, Guan XM. Design and synthesis of (ant)-agonists that alter appetite and adiposity. *Prog. Brain Res* 2006;153:107–118. [PubMed: 16876570]
37. Lam DD, Farooqi IS, Heisler LK. Melanocortin Receptors as Targets in the Treatment of Obesity. *Curr. Topics Med. Chem* 2007;7:1098–1110.
38. (a) Chaki S, Okuyama S. Involvement of melanocortin-4 receptor in anxiety and depression. *Peptides* 2005;26:1952–1964. [PubMed: 15979204] (b) Foster AC, Chen C. Melanocortin-4 receptor antagonists as potential therapeutics in the treatment of cachexia. *Curr. Topics Med. Chem*

- 2007;7:1131–1136. (c) Chen C, Jiang W, Tucci F, Tran JA, Fleck BA, Hoare SR, Joppa M, Markison S, Wen J, Sai Y, Johns M, Madan A, Chen T, Chen CW, Marinkovic D, Arellano M, Saunders J, Foster AC. Discovery of 1-[2-[(1S)-(3-dimethylaminopropionyl)amino-2-methylpropyl]-4-methylphenyl]-4-[(2R)-methyl-3-(4-chlorophenyl)-propionyl]piperazine as an orally active antagonist of the melanocortin-4 receptor for the potential treatment of cachexia. *J. Med. Chem* 2007;50:5249–5252. [PubMed: 17918824]
39. Hadley ME, Dorr RT. Melanocortin peptide therapeutics: historical milestones, clinical studies and commercialization. *Peptides* 2006;27:921–930. [PubMed: 16412534]
 40. Shadiack AM, Sharma SD, Earle DC, Spana C, Hallam TJ. Melanocortins in the treatment of male and female sexual dysfunction. *Curr. Topics Med. Chem* 2007;7:1137–1144.
 41. McNulty JC, Jackson PJ, Thompson DA, Chai B, Gantz J, Barsh GS, Dawson PE, Millhauser GL. Structures of the agouti signaling protein. *J. Mol. Biol* 2005;346:1059–1070. [PubMed: 15701517]
 42. Ollmann MM, Wilson BD, Yang YK, Kerns JA, Chen Y, Gantz I, Barsh GS. Antagonism of central melanocortin receptors in vitro and in vivo by agouti-related protein. *Science* 1997;278:135–138. [PubMed: 9311920]
 43. Yang YK, Fong TM, Dickinson CJ, Mao C, Li JY, Tota MR, Mosley R, Van der Ploeg LHT, Gantz I. Molecular determinants of ligand binding to the human melanocortin-4 receptor. *Biochemistry* 2000;39:14900–14911. [PubMed: 11101306]
 44. Haskell-Luevano C, Cone RD, Monck EK, Wan YP. Structure activity studies of the melanocortin-4 receptor by in vitro mutagenesis: identification of agouti-related protein (AGRP), melanocortin agonist and synthetic peptide antagonist interaction determinants. *Biochemistry* 2001;40:6164–6179. [PubMed: 11352754]
 45. Wilczynski A, Wang XS, Joseph CG, Xiang Z, Bauzo RM, Scott JW, Sorensen NB, Shaw AM, Millard WJ, Richards NG, Haskell-Luevano C. Identification of putative agouti-related protein (87-132)-melanocortin-4 receptor interactions by homology molecular modeling and validation using chimeric peptide ligands. *J. Med. Chem* 2004;47:2194–2207. [PubMed: 15084118]
 46. Ying J, Koeber KE, Gu X, Han G, Trivedi D, Kavarana MJ, Hruby VJ. Solution structures of cyclic melanocortin agonists and antagonists by NMR. *Biopolymers* 2003;7:696–716. [PubMed: 14991679]
 47. (a) Pettit GR, Srirangam JK, Herald DL, Xu J, Boyd MR, Cichacz Z, Kamano Y, Schmidt JM, Erickson KL. Isolation and crystal structure of stylopeptide 1, a new marine porifera cycloheptapeptide. *J. Org. Chem* 1995;60:8257–8261. (b) Imperiali B, Moats RA, Fisher SL, Prins TJ. A conformational study of peptides with the general structure Ac-L-Xaa-Pro-D-Xaa-LXaa-NH₂: spectroscopic evidence for a peptide with significant β -turn character in water and in dimethyl sulfoxide. *J. Am. Chem. Soc* 1992;114:3182–3188.
 48. Tamaki M, Han G, Hruby VJ. Practical and efficient synthesis of orthogonally protected constrained 4-guanidinyprolines. *J. Org. Chem* 2001;66:1038–1042. [PubMed: 11430070]
 49. Cai M, Mayorov AV, Cabello C, Stankova M, Trivedi D, Hruby VJ. Novel 3D pharmacophore of α -MSH/ γ -MSH hybrids leads to selective human MC1R and MC3R analogues. *J. Med. Chem* 2005;48:1839–1848. [PubMed: 15771429]
 50. (a) Grieco P.; Han, G.; Hruby, VJ. New dimensions in the design of potent and receptor selective melanotropin analogues. In: Field, GB.; Tam, JP.; Barany, G., editors. *Peptides for the New Millennium*. Kluwer Academic Publ.; Dordrecht, The Netherlands: 2000. p. 541-542. (b) Grieco P, Cai M, Han G, Trivedi D, Campiglia P, Novellino E, Hruby VJ. Further structure-activity studies of lactam derivatives of MT-II and SHU-9119: Their activity and selectivity at human melanocortin receptors 3, 4, and 5. *Peptides* 2007;28(6):1191–6. [PubMed: 17482720] (c) Grieco P, Han G, Weinberg D, MacNeil T, Van der Ploeg LHT, Hruby VJ. Design and synthesis of highly potent and selective melanotropin analogues of SHU9119 modified at position 6. *Biochem. Biophys. Res. Commun* 2002;292:1075–1080. [PubMed: 11944925]
 51. Prusis P, Muceniece R, Mutule I, Mutulis F, Wikberg JE. Design of new small cyclic melanocortin receptor-binding peptides using molecular modelling: Role of the His residue in the melanocortin peptide core. *Eur. J. Med. Chem* 2001;36:137–146. [PubMed: 11311745]
 52. Bednarek MA, Macneil T, Kalyani RN, Tang R, Van der Ploeg LH, Weinberg DH. Analogs of MTII, lactam derivatives of alpha-melanotropin, modified at the N-terminus, and their selectivity at human

- melanocortin receptors 3, 4, and 5. *Biochem. Biophys. Res. Commun* 1999;261:209–213. [PubMed: 10405347]
53. Grieco P, Lavecchia A, Cai M, Trivedi D, Weinberg D, MacNeil T, Van der Ploeg LHT, Hruby VJ. Structure-activity studies of the melanocortin peptides: Discovery of potent and selective affinity antagonists for the *h*MC3 and *h*MC4 receptors. *J. Med. Chem* 2002;45:5287–5294. [PubMed: 12431055]
54. Lauer JL, Fields CG, Fields GB. Sequence dependence of aspartimide formation during 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis. *Lett. Peptide Sci* 1994;1:197–205.
55. Flora D, Mo H, Mayer JP, Khan MA, Yan LZ. Detection and control of aspartimide formation in the synthesis of cyclic peptides. *Bioorg. Med. Chem. Lett* 2005;15:1065–1068. [PubMed: 15686913]
56. (a) The Mitsunobu reaction gives a mixture of *trans*- and *cis*- 4-bromo-substituted analogues which can be separated by silica gel flash column chromatography; (b) The final purification was done by using only the MeOH/CHCl₃ system described in Tamaki's paper.⁴⁸
57. Wade JD, Mathieu MN, Macris M, Tregear GW. Base induced side reactions in Fmoc-solid phase peptide synthesis: Minimization by use of piperazine as N^α-deprotection reagent. *Lett. Peptide Sci* 2000;7:107–112.
58. Ge H, Olli-Lähdesmäki T, Kallio J, Scheinin M. α 2B-Adrenoceptor levels govern agonist and inverse agonist responses in PC12 cells. *Biochem. Biophys. Res. Commun* 2003;308:12–18. [PubMed: 12890472]
59. Kolossváry I, Guida WC. Low-mode conformational search elucidated. Application to C₃₉H₈₀ and flexible docking of 9-deazaguanine inhibitors to PNP. *J. Comput. Chem* 1999;20:1671.
60. Jorgensen WL, Maxwell DS, Tirado-Rives J. Development and testing of the OPLS all-atom force field on conformational energetics and properties of organic liquids. *J. Am. Chem. Soc* 1996;118:11225–11235.
61. Still WC, Tempczyk A, Hawley RC, Hendrickson T. A General treatment of solvation for molecular mechanics. *J. Am. Chem. Soc* 1990;112:6127–6129.
62. Bednarek MA, MacNeil T, Tang R, Fong TM, Cabello MA, Maroto M, Teran A. Potent and selective agonists of human melanocortin receptor 5: cyclic analogues of α -melanocyte-stimulating hormone. *J. Med. Chem* 2007;50:2520–2526. [PubMed: 17455928]
63. Bednarek MA, Silva MV, Arison B, MacNeil T, Kalyani RN, Huang RR, Weinberg DH. Structure-function studies on the cyclic peptide MT-II, lactam derivative of α -melanotropin. *Peptides* 1999;20:401–409. [PubMed: 10447101]
64. Sahn UG, Olivier GW, Branch SK, Moss SH, Pouton CW. Synthesis and biological evaluation of α -MSH analogues substituted with Alanine. *Peptides* 1994;15:1297–1302. [PubMed: 7854984]
65. Chen M, Cai M, Aprahamian CJ, Georgeson KE, Hruby VJ, Harmon CM, Yang Y. Contribution of the conserved amino acids of the melanocortin-4 receptor in D-[Nle⁴,Phe⁷]- α -melanocyte-stimulating hormone binding and signaling. *J. Biol. Chem* 2007;282:21712–21719. [PubMed: 17545153]
66. Bednarek MA, MacNeil T, Tang R, Fong TM, Angeles Cabello M, Maroto M, Teran A. Potent and selective peptide agonists of α -melanocyte stimulating hormone (α -MSH) action at human melanocortin receptor 5; their synthesis and biological evaluation in vitro. *Chem. Biol. Drug Des* 2007;69:350–355. [PubMed: 17539827]
67. Yang YK, Ollmann MM, Wilson BD, Dickinson C, Yamada T, Barsh GS, Gantz I. Effects of recombinant agouti-signaling protein on melanocortin action. *Mol. Endocrinol* 1997;11:274–280. [PubMed: 9058374]
68. Ying J, Gu X, Cai M, Dedek M, Vagner J, Trivedi D, Hruby VJ. Design, synthesis, and biological evaluation of new cyclic melanotropin peptide analogues selective for the human melanocortin-4 receptor. *J. Med. Chem* 2006;49:6888–6896. [PubMed: 17154518]
69. Cain JP, Mayorov AV, Cai M, Wang H, Tan B, Chandler K, Lee Y, Petrov RR, Trivedi D, Hruby VJ. Design, synthesis, and biological evaluation of a new class of small molecule peptide mimetics targeting the melanocortin receptors. *Bioorg. Med. Chem. Lett* 2006;16:5462–5467. [PubMed: 16931008]

70. Cai M, Cai C, Mayorov AV, Xiong C, Cabello CM, Soloshonok VA, Swift JR, Trivedi D, Hruby VJ. Biological and conformational study of substituted prolines in MT-II template: Steric effects leading to human MC5 receptor selectivity. *J. Pept. Res* 2004;63:116–131. [PubMed: 15009533]
71. Yang Y, Chen M, Lai Y, Gantz I, Georgeson KE, Harmon CM. Molecular determinants of human melanocortin-4 receptor responsible for antagonist SHU9119 selective activity. *J. Biol. Chem* 2002;277:20328–20335. [PubMed: 11912210]
72. Chen M, Georgeson KE, Harmon CM, Haskell-Luevano C, Yang Y. Functional characterization of the modified melanocortin peptides responsible for ligand selectivity at the human melanocortin receptors. *Peptides* 2006;27:2836–2845. [PubMed: 16730390]
73. Ballet S, Mayorov AV, Cai M, Tymecka D, Chandler KB, Palmer ES, Rompaey KV, Misicka A, Tourwé D, Hruby VJ. Novel selective human melanocortin-3 receptor ligands: use of the 4-amino-1,2,4,5-tetrahydro-2-benzazepin-3-one (Aba) scaffold. *Bioorg. Med. Chem. Lett* 2007;17:2492–2498. [PubMed: 17314042]
74. Mayorov AV, Cai M, Palmer ES, Dedek MM, Cain JP, Van Scoy AR, Tan B, Vagner J, Trivedi D, Hruby VJ. Structure-activity relationships of cyclic lactam analogues of alpha-melanocyte-stimulating hormone (alpha-MSH) targeting the human melanocortin-3 receptor. *J. Med. Chem* 2008;51:187–195. [PubMed: 18088090]
75. Cheung AW, Danho W, Swistok J, Qi L, Kurylko G, Rowan K, Yeon M, Franco L, Chu XJ, Chen L, Yagaloff K. Structure-activity relationship of cyclic peptide penta-c[Asp-His(6)-DPhe(7)-Arg(8)-Trp(9)-Lys]-NH(2) at the human melanocortin-1 and -4 receptors: His(6) substitution. *Bioorg. Med. Chem. Lett* 2003;13:1307–1311. [PubMed: 12657270]
76. Haskell-Luevano C, Miwa H, Dickinson C, Hruby VJ, Yamada T, Gantz I. Binding and cAMP studies of melanotropin peptides with the cloned human peripheral melanocortin receptor, *hMC1R*. *Biochem. Biophys. Res. Commun* 1994;204:1137–1142. [PubMed: 7980588]
77. Gantz I, Miwa H, Konda Y, Shimoto Y, Tashiro T, Watson SJ, Delvalle J, Yamada T. Molecular cloning of a novel melanocortin receptor. *J. Biol. Chem* 1993;268:15174–15179. [PubMed: 8392067]
78. Mayorov AV, Cai M, Chandler KB, Petrov RR, Scoy AR, Yu Z, Tanaka DK, Trivedi D, Hruby VJ. Development of cyclic γ -MSH analogues with selective *hMC3R* agonist and *hMC3R/hMC5R* antagonist activities. *J. Med. Chem* 2006;49:1946–1952. [PubMed: 16539382]
79. Grippo L, Lucidi S. A globally convergent version of the Polak-Ribière conjugate gradient method. *Math. Program* 1997;77:375–391.

*h*AGRP(110-117) central loop c[C**RFF**NAFC]
*h*ASIP(116-123) central loop c[C**RFF**RSAC]

Figure 1.

Central Loops of *h*AGRP and *h*ASIP and their core pharmacophore RFF

- 1: Ac-Nle-c[Asp-His-DPhe-**Pro**-Trp-Lys]-NH₂
- 2: Ac-Nle-c[Asp-His-DPhe-**trans-Xaa**-Trp-Lys]-NH₂
- 3: Ac-Nle-c[Asp-His-DPhe-**cis-Xaa**-Trp-Lys]-NH₂
- 4: Ac-Nle-c[Asp-**trans-Xaa**-DPhe-Arg-Trp-Lys]-NH₂
- 5: Ac-Nle-c[Asp-**cis-Xaa**-DPhe-Arg-Trp-Lys]-NH₂
- 6: Ac-Nle-c[Asp-His-DNal(2')-**Pro**-Trp-Lys]-NH₂
- 7: Ac-Nle-c[Asp-His-DNal(2')-**trans-Xaa**-Trp-Lys]-NH₂
- 8: Ac-Nle-c[Asp-His-DNal(2')-**cis-Xaa**-Trp-Lys]-NH₂
- 9: Ac-Nle-c[Asp-**trans-Xaa**-DNal(2')-Arg-Trp-Lys]-NH₂
- 10: Ac-Nle-c[Asp-**cis-Xaa**-DNal(2')-Arg-Trp-Lys]-NH₂
- 11: Ac-Tyr-Val-Nle-Gly-His-DPhe-**Pro**-Trp-Asp-Arg-Phe-Gly-NH₂
- 12: Ac-Tyr-Val-Nle-Gly-His-DPhe-**trans-Xaa**-Trp-Asp-Arg-Phe-Gly-NH₂
- 13: Ac-Tyr-Val-Nle-Gly-His-DPhe-**cis-Xaa**-Trp-Asp-Arg-Phe-Gly-NH₂

Figure 2.
Peptide design with use of proline, or trans/cis-4-guanidinyl-Pro

Figure 3a.

Analysis of **1** inhibition of **MTII** triggered cAMP response by the antagonist peptide **1**. The panel shows the inhibition of the agonist MT-II log dose response curve in the presence of the increasing concentration of **1**.

Figure 3b.

Analysis of the **2** inhibition of MTII triggered cAMP response by the antagonist peptide **2**. The panel shows the inhibition of the agonist MT-II log dose response curve in the presence of increasing concentration of **2**.

Figure 4. Stereoview of the global minimum of analogue **1** obtained by MCMM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulation.

Figure 5. Stereoview of the global minimum structure of analogue **2** (orange) obtained by MCM/LMCS (Monte Carlo Multiple Minima-Low Frequency Mode)-OPLS 2005 simulation, superimposed on the NMR solution structure of the *h*ASIP central loop (blue).

Scheme 1.

Aspartimide formation and the consequence of the side reaction

Scheme 2.
SPPS Strategy used to avoid aspartimide derivative formation

Physicochemical properties of the peptides synthesized

Table 1

no.	HRMS(<i>m/z</i>)		HPLC <i>t_R</i> ^a				TLC <i>R_f</i> ^b	
	calcd	found	1	2	1	2	1	2
1	511.7701 (2H)	511.7725 (ESI)	7.90	11.00	0.67	0.36	0.67	0.36
2	511.7701 (2H)	511.7725 (ESI)	8.09	11.70	0.67	0.36	0.67	0.36
3	483.2538 (2H)	483.2546 (ESI)	10.26	14.81	0.74	0.4	0.74	0.4
4	521.2912 (2H)	521.2907 (ESI)	8.98	13.03	0.68	0.37	0.68	0.37
5	521.2912 (2H)	521.2889 (ESI)	8.94	12.86	0.68	0.37	0.68	0.37
6	536.7779 (2H)	536.7768 (ESI)	10.02	14.59	0.73	0.44	0.73	0.44
7	536.7779 (2H)	536.7756 (ESI)	10.00	14.50	0.73	0.44	0.73	0.44
8	508.2616 (2H)	506.2624 (ESI)	12.28	17.82	0.78	0.51	0.78	0.51
9	546.2990 (2H)	546.3014 (ESI)	10.38	15.12	0.73	0.44	0.73	0.44
10	546.2990 (2H)	546.2995 (ESI)	11.05	16.17	0.73	0.45	0.73	0.45
11	531.2693 (3H)	531.2697 (ESI)	9.70	14.31	0.71	0.33	0.71	0.33
12	531.2693 (3H)	531.2695 (ESI)	9.95	14.70	0.71	0.33	0.71	0.33
13	767.8837 (2H)	767.8868 (ESI)	11.85	17.44	0.74	0.37	0.74	0.37

^a HPLC column, Vydac 218TP104, 250 × 4.6 mm, 10 μm, 300 Å. *t_R* = peptide retention time. System 1: Solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient 10-90% B in 40 min, flow rate 1.0 mL/min at 40 °C. System 2: solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient 10-60% B in 40 min, flow rate 1.0 mL/min at 40 °C.

^b TLC system 1: n-butanol/acetic acid/water/pyridine (4:1:2:1). TLC system 2: n-butanol/acetic acid/water (4:1:1)

Table 2

Binding affinities and cAMP activities of the designed peptides

Peptide ^a	IC ₅₀ nM	<i>h</i> MCIR ^b EC ₅₀ nM	%max	IC ₅₀ nM	<i>h</i> MC3R EC ₅₀ nM	%max	IC ₅₀ nM	<i>h</i> MC4R EC ₅₀ nM	%max	IC ₅₀ nM	<i>h</i> MC5R EC ₅₀ nM	%max
MTII	0.20±0.01	0.30±0.04	100	1.3±0.2	1.9±0.2	100	1.1±0.3	2.9±0.5	100	7.5±0.2	3.3±0.7	100
1	>4800	>1000	75±10	NB	0	0	11±0.9	200±40	13±5	NB	0	0
2	NB	0	0	NB	0	0	50±10	43±8	3.9±0.3	590±90	2500±480	24±3
3	3500±400	760±150	91 ±8	NB	0	0	39±0.2	11±2	36±12	NB	0	0
4	2.7±0.5	820±150	77±17	1.0±0.2	14±2.5	91±7	0.60±0.20	3500±660	64±17	2.5±0.4	1.6±0.2	68±8
5	1.1±0.2	2700±200	100±5	2.8±0.1	2500±500	68±15	5.7±0.5	41±4	89±15	1.1 ±0.4	21±3	100
SHU9119	1.0±0.2	1.5±0.1	100	2.3±0.2	0	0	0.60±0.10	0	0	0.90±0.20	1.2±0.1	97±10
6	600±120	160±30	66±10	NB	0	0	33±3	5700±1000	55±7	NB	0	0
7	>3000	0	0	>10000	620±120	56±7	7.2±1.4	200±10	31±1	22±3	2400±460	59±8
8	400±80	19000±3700	100±5	NB	0	0	23±1	250±30	7.3±1	NB	0	0
9	2.3±0.4	620±110	25±5	2.3±0.1	130±25	28±2	1.5±0.3	200±40	17±2	0.70±0.20	0.30±0.06	18±2
10	1.2±0.2	1700±300	90±18	2.2±0.2	0	0	2.5±0.5	1400±280	6.2±2	2.2±0.4	85±15	52±17
Ac-NDP-γ-MSH-NH ₂	0.50±0.01	1.5±0.1	100	2.0±0.2	2.0±0.2	100	1.2±0.2	1.4±0.1	100	2.4±0.3	1.9±0.2	100
11	190±35	120±20	53±10	NB	0	0	270±30	210±30	46±5	NB	0	0
12	830±150	1400±250	92±14	NB	0	0	NB	0	0	NB	0	0
13	1000±200	>5000	100	NB	0	0	NB	0	0	NB	0	0

^aX_{aa}=4-guainidinyI-Pro.

^bIC₅₀=concentration of peptide at 50% specific binding (N=4). EC₅₀=effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation (N=4). %max, ratio of the highest cAMP level triggered by peptides over the highest cAMP level triggered by MTII. The peptides were tested at a range of concentrations from 10⁻¹⁰ to 10⁻⁵ M. NB: No Binding at 10⁻⁵ M.