Stereochemical Studies of the Monocyclic Agouti-Related Protein (103−122) Arg-Phe-Phe Residues: Conversion of a Melanocortin-4 Receptor Antagonist into an Agonist and Results in the Discovery of a Potent and Selective Melanocortin-1 Agonist

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The agouti-related protein (AGRP) is an endogenous antagonist of the centrally expressed melanocortin receptors. The melanocortin-4 receptor (MC4R) is involved in energy homeostasis, food intake, sexual function, and obesity. The endogenous hAGRP protein is 132 amino acids in length, possesses five disulfide bridges at the C-terminus of the molecule, and is expressed in the hypothalamus of the brain. We have previously reported that a monocyclic hAGRP-(103−122) peptide is an antagonist at the melanocortin receptors expressed in the brain. Stereochemical inversion from the endogenous L- to D-isomers of single or multiple amino acid modifications in this monocyclic truncated hAGRP sequence resulted in molecules that are converted from melanocortin receptor antagonists into melanocortin receptor agonists. The Asp-Pro-Ala-Ala-Thr-Ala-Tyr-cyclo[Cys-Arg-DPhe-DPhe-Asn-Ala-Phe-Cys]-Tyr-Ala-Arg-Lys-Leu peptide resulted in a 60 nM melanocortin-1 receptor agonist that is 100-fold selective versus the mMC4R, 1000-fold selective versus the mMC3R, and ca. 180-fold selective versus the mMC5R. In attempts to identify putative ligand−receptor interactions that may be participating in the agonist induced stimulation of the MC4R, selected ligands were docked into a homology molecular model of the mMC4R. These modeling studies have putatively identified hAGRP ligand DArg111-mMC4RAsn115 (TM3) and the hAGRP DPhe113-mMC4RPhe176 (TM4) interactions as important for agonist activity.

Introduction

Agouti-related protein (AGRP) (Figure 1) is one of two known endogenous antagonists of G-protein coupled receptors (GPCRs) identified to date, and when it was ectopically expressed in transgenic mice, it resulted in an obese phenotype.[1−3] AGRP antagonizes the melanocortin-3 (MC3R) and -4 (MC4R) receptors expressed in the brain[2,4,5] and is involved in the regulation of hypothalamic based energy homeostasis.[6−9] hAGRP(87−132) possess a C-terminal domain that contains five disulfide bridges[2,10] and has been identified as possessing antagonistic properties at the melanocortin receptors equipotent to the full length peptide.[2,4] Truncation and fragment studies of hAGRP have resulted in the identification that the hAGRP(109−118) decapeptide Tyr-c[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr is the minimal sequence that results in antagonism of the MC4R,[11,12] and elongation studies of this monocyclic decapeptide have identified tetradecapeptides that can additionally antagonize the MC3R.[13] These latter studies resulted in the identification of a monocyclic hAGRP-(103−122) sequence Asp-Pro-Ala-Ala-Thr-Ala-Tyr-cyclo-[Cys-Arg-Phe-Phe-Asn-Ala-Phe-Cys]-Tyr-Ala-Arg-Lys-Leu that resulted in high nM antagonism of the MC3 and MC4 receptors and full agonist at the melanocortin-1 receptor (MC1R, expressed in the skin[14]). Multiple structure−activity relationship (SAR) studies of AGRP resulted in the identification of the hAGRP(111−113) Arg-Phe-Phe amino acids as critical for the ability of AGRP to recognize and antagonize the melanocortin receptors.[5,11,15−19] Classical structure−activity relationship (SAR) studies of the melanocortin receptor agonists resulted in the identification that stereochemical modifications at "key" positions results in increased ligand potency and enzymatic stability. Specifically, modification of the endogenous agonist α-melanocyte stimulating hormone (α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe[7]-Arg-Trp-Gly-Lys-Pro-NH_2) at the Phe seven position resulted in the discovery of the potent and enzymatic resistant NDP-MSH (Ac-Ser-Tyr-Ser-Nle[4]-Glu-His-DPhe[7]-Arg-Trp-Gly-Lys-Pro-NH_2) ligand that has been used extensively to characterize the cloned melanocortin receptors, both in vitro and in vivo.[20−22] Modification of the DPhe position in the MTII (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2)[23,24] peptide template by incorporation of DNal(2′), but not DNal(1′), resulted in the potent MC3 and MC4 receptor antagonist, SHU9119.[25,26] Additionally, using chimeric peptide templates incorporating hAGRP Arg-Phe-Phe residues into the NDP-MSH and MTII templates or, conversely, incorporating the melanocortin agonist His/DPhe-Arg-Trp amino acids into the hAGRP(109−118) templates with stereochemical conversion of L- to D-amino acids resulted in enhanced receptor potency and selectivity.[17,18] The

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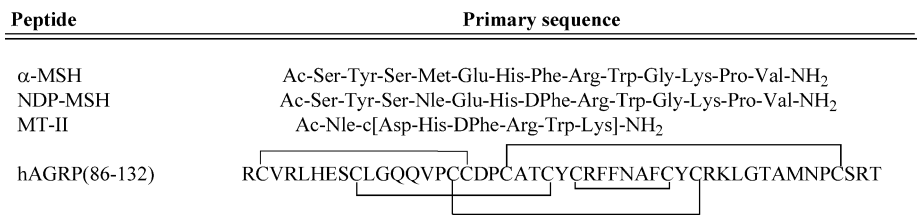

Figure 1. Primary amino acid sequence of the melanocortin agonists and antagonist used as controls.

studies presented herein were originally designed to perform a D-amino acid scan of the hAGRP(111−113) Arg-Phe-Phe residues in the monocyclic hAGRP(103−122) template to increase antagonist potency at the MC3 and MC4 receptors. Excitingly, however, the conversion from an antagonist ligand to an agonist ligand resulted from this hAGRP Arg-Phe-Phe D-scan.

Results

Melanocortin Receptor Pharmacology. The peptides reported herein were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc) chemistry.[27,28] The peptides were purified to homogeneity (>95% pure) using semipreparative reversed-phase high-pressure liquid chromatography (RP-HPLC) and possessed the correct molecular weights, as determined by mass spectrometry. Table 1 summarizes the pharmacological results of control and the monocyclic hAGRP(103−122) peptides prepared in this study at the mouse melanocortin receptor MC1R, MC3−5R isoforms.

The lead peptide **1** (Table 1), monocyclic hAGRP(103−122) pharmacology is consistent, within experimental error, with the previously reported values at the mouse melanocortin receptors.[13] Figure 2 illustrates the ability of **1** to competitively displace the melanocortin agonist MTII at the mMC4R using the classical Schild analysis to determine competitive antagonism.[29] Stereochemical inversion from the L-configuration to the D-configuration of the hAGRP(11−113) Arg-Phe-Phe residues in the lead peptide was performed singly and in combinations. Analogue **2** (DArg111) resulted in a 6-fold less potent mMC1R agonist, became a mMC5R agonist, was only able to stimulate the mMC3R to 70% maximal stimulation at up to 100 μM concentrations, and possessed μM mMC4R full agonist pharmacology (Figure 2), as compared to **1**. Analogue **3** (DPhe112) resulted in 10-fold increased mMC1R agonist potency, was only able to stimulate the mMC3R to 50% and the mMC4R to 75% maximal stimulation at up to 100 μM concentrations, and resulted in 13-fold increased mMC5R potency, compared with **1**. Peptide **4** (DPhe113) resulted in 8-fold increased mMC1R and mMC5R potency as compared with **1**, and was a full μM agonist at both the mMC3R and mMC4R (Figure 2).

Peptides containing dual combinations of stereochemically modified hAGRP(111−113) Arg-Phe-Phe residues were synthesized to determine if a synergistic increase in melanocortin receptor potency would result. Peptide **5** (DArg-DPhe-Phe) resulted in a full μM agonist at all the mMC1 and mMC3−5 receptors. Peptide **6** (DArg-Phe-DPhe) resulted in full agonists at the mMC1, mMC4, and mMC5 receptors but was only able to stimulate the mMC3R to 70% maximal stimulation at 100 μM concentrations (was not an antagonist). Analogue **7** (Arg-DPhe-DPhe) possessed a 15-fold increased mMC1R agonist potency, as compared with peptide **1**, and was a full μM agonist at the mMC3−5Rs. Interestingly, peptide **7** resulted in a mMC1R selective agonist that was approximately 1000-fold selective for the mMC1R versus the mMC3R, 100-fold mMC1R versus mMC4R selective, and 180-fold selective for the mMC1R versus the mMC5R. Finally, analogue **8** with the all-D-configuration (DArg-DPhe-DPhe) resulted in a full μM agonist at all the melanocortin receptors utilized in this study.

Mouse Melanocortin-4 Receptor Homology Molecular Modeling. We have previously generated a three-dimensional model of the mouse MC4R where we "docked" the NMR structure of hAGRP(87−132) provided by Millhauser et al.[30−32] and tested the hypothesis that the hAGRP(111−113) amino acids putatively interact with the mMC4R similarly to the endogenous melanocortin agonist Phe-Arg-Trp residues.[18] Furthermore, we utilized this model and the NMR structure of a bicyclic hAGRP derivative, to determine putative ligand−receptor interactions that might account for differences in mMC4R potency comparing the hAGRP-(87−132) and the bicyclic hAGRP NMR derivative "docked" structures.[33] Herein, we have utilized this mMC4R homology molecular model in attempts to identify putative monocyclic hAGRP−mMC4R interactions that might account for the conversion of the monocyclic hAGRP(103−122) from a mMC4R antagonist into an agonist. Figure 3A illustrates a theoretical model of analogue **1** [monocyclic hAGRP(103−122)] docked into our mMC4R homology molecular model.[18,33] Figure 3B illustrates the monocyclic hAGRP(103−122) Arg111 ligand residue putatively interacting with the mMC4R Glu92 (TM2), Asp114 (TM3), and Asp118 (TM3) amino acids previously identified to be important for melanocortin receptor molecular recognition and stimulation.[5,19,34,35] Figure 3C illustrates the monocyclic hAGRP(103−122) Phe112-Phe113 interactions in the mMC4R Phe176 (TM4), Phe193 (TM5), Phe253 (TM6), and Phe 254 (TM6) putative hydrophobic receptor binding pocket.[5] Figure 3D,E illustrates changes in the monocyclic hAGRP(103−122) derivative **2** (DArg111, panel D) and **4** (DPhe113, panel E) interactions with the putative mMC4R ligand binding pocket. Interestingly, a strong interaction is observed between the hAGRP DArg111 amino acid of **2** and the mMC4R Asn115 (TM3) which is not seen with the Arg111 containing analogue **1** (Figure 3B,D). The hAGRP Phe112 and DPhe113 of **4** are observed to be interacting with the same aromatic-hydrophobic pocket as **1**; however, the DPhe113 of **4** appears to be putatively interacting more physically favorably[36] with the mMC4R Phe176 (TM4), as compared to Phe113 of analogue **1** (Figure 3C,E).

Table 1. Functional Activity of Monocyclic hAGRP(103−122) Peptide Analogues at the Mouse Melanocortin Receptors[a]

ID	peptide sequence	agonist EC_{50} (nM)				antagonist pA_2	
		mMC1R	mMC3R	mMC4R	mMC5R	mMC3R	mMC4R
MTII	Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2	0.021 ± 0.10	0.17 ± 0.038	0.040 ± 0.0047	0.18 ± 0.12		
α-MSH	Ac-SYSMEHFRWGKPV-NH_2	0.28 ± 0.38	0.52 ± 0.060	1.89 ± 0.24	1.12 ± 0.36		
NDP-MSH	Ac-SYS-Nle-EH-DPhe-RWGKPV-NH_2	0.023 ± 0.083	0.085 ± 0.021	0.13 ± 0.20	0.062 ± 0.014		
hAGRP	C-terminal fragment (87−132)	>100			>100	8.90 ± 0.20	9.39 ± 0.10
Ac-Mini-hAGRP-NH_2	Ac-hAGRP(87−120, Cys105Ala)-NH_2	>100			>100	8.08 ± 0.1	8.46 ± 0.06
1	DPAATAYc[C-**Arg-Phe-Phe**-NAFC]YARKL	960 ± 240			>100	6.15 ± 0.15	6.92 ± 0.26
2	DPAATAYc[C-**DArg^{111}-Phe-Phe**-NAFC]YARKL	6330 ± 3880	70%@100μM	37700 ± 8560	18300 ± 5350		
3	DPAATAYc[C-**Arg-DPhe^{112}-Phe**-NAFC]YARKL	93 ± 36	50%@100μM	75%@100μM	1410 ± 280	6.14 ± 0.92	6.56 ± 1.10
4	DPAATAYc[C-**Arg-Phe-DPhe^{113}**-NAFC]YARKL	120 ± 45	13700 ± 1310	3700 ± 1140	2220 ± 920		
5	DPAATAYc[C-**DArg^{111}-DPhe^{112}-Phe**-NAFC]YARKL	1750 ± 570	5510 ± 60	8300 ± 1970	4600 ± 1700		
6	DPAATAYc[C-**DArg^{111}-Phe-DPhe^{113}**-NAFC]YARKL	5270 ± 990	70%@100μM	8330 ± 840	19800 ± 9060		
7	DPAATAYc[C-**Arg-DPhe^{112}-DPhe^{113}**-NAFC]YARKL	64 ± 17	63300 ± 13700	6240 ± 1000	11400 ± 2430		
8	DPAATAYc[C-**DArg^{111}-DPhe^{112}-DPhe^{113}**-NAFC]YARKL	3750 ± 95	22600 ± 7490	21500 ± 3190	13200 ± 4230		

[a] Errors are the standard error of the mean from at least three independent experiments. The pA_2 antagonist value was determined by Schild analysis ($pA_2 = -\log K_i$). >100 indicates that no agonist or antagonist melanocortin receptor pharmacology was observed at the highest concentrations examined.

Discussion

Identification of "key" amino acid residue(s) of a ligand that are necessary for receptor recognition and function is the objective of structure−activity relationship studies. It has been postulated that hAGRP(111−113) Arg-Phe-Phe amino acids are critical for high affinity binding and activity of hAGRP at the melanocortin receptors.[11,16−18] AGRP is an antagonist at the MC3 and MC4 receptors[2] and appears to possess inverse agonist activity at the MC4R.[37−39] Monocyclic hAGRP (103−122) containing 20 amino acid residues and one disulfide bridge has been previously identified as an antagonist of the mMC3R and mMC4 receptors, and agonist at the mMC1R, and null activity at the mMC5R.[13] However, analogue **1** is not significantly selective for either receptor (Table 1). This stereochemical inversion study reported herein was aimed at increasing the monocyclic hAGRP(103−122) potency at the MC3 and MC4 receptors using a strategy involving epimerization of the key hAGRP Arg-Phe-Phe (111−113) residues that had been successful for melanocortin agonist based SAR studies.[20]

We designed a series of hAGRP (103−122) analogues that have one disulfide bridge between Cys residues at positions 110 and 117 (hAGRP numbering), giving rise to a 26-membered ring. Our design strategy included (1) replacing Cys at positions 105, 108, and 119 with Ala to eliminate any possibility of bridge formation and (2) changing the stereochemistry at position 111−113 individually as well as two or more simultaneously. Replacing hAGRP(111−113) Arg-Phe-Phe residues with alanine resulted in reduction of binding affinity at both the hMC3R and hMC4R,[11] indicating that the Arg-Phe-Phe residues are necessary for binding to these receptors. Replacing the hAGRP(111−113) Arg-Phe-Phe with the respective D-enantiomer has not been published to date, and it may render the ligand into a unique conformation(s) promoting differences in melanocortin receptor potency and selectivity.[40] Substitution of hAGRP Arg111 and/or Phe113 with their D-enantiomeric homologue in the monocyclic hAGRP(103−122) series resulted in a complete loss of MC3R and MC4R antagonist pharmacology, and the analogues resulted in ligands that were able to fully or partially stimulate the melanocortin receptors used herein (Table 1). Comparison of compounds **1**−**4** allows us to hypothesize that both the monocyclic hAGRP(103−122) Arg111 and Phe113 residues synergistically are required for mMC3R and mMC4R antagonist pharmacology. The hypothesis of synergy between melanocortin receptor ligand Arg and Phe residues has been previously suggested for the differentiation of the MTII (agonist, Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2[23,24]) and SHU9119 (antagonist, Ac-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH_2[25]) mMC4R pharmacology.[5] Biophysical analysis, using NMR and computer assisted molecule modeling, of a series of compounds based upon the MTII and SHU9119 peptides has been performed,[41] suggesting that cation−π interactions[42−44] may be a predominate chemical force contributing to how the ligand interacts with the mMC4R.

Melanocortin Receptor Selectivity. The melanocortin pathway has been implicated in a variety of physiological processes including pigmentation,[45] energy homeostasis,[2,6−8,46,47] sexual function,[48] inflammation,[49]

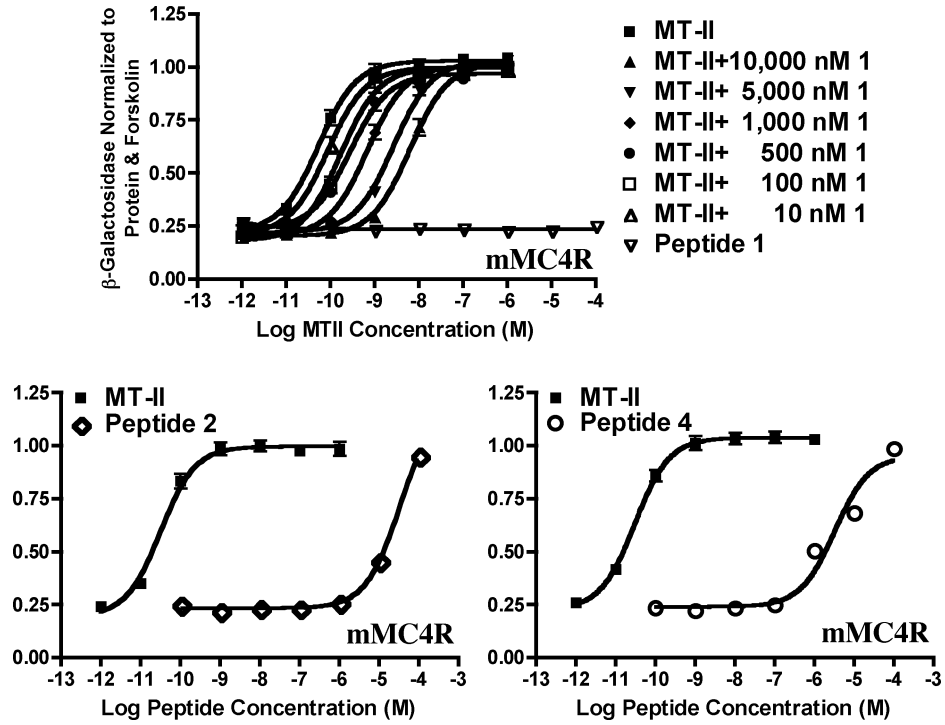

Figure 2. Pharmacology of peptides **1**, **2**, and **4** at the mMC4R. (A) Illustrative competitive antagonist curve of peptide **1** using MTII as the agonist. (B, C) Plots illustrating that peptides **2** and **4**, respectively, are mMC4R full agonists.

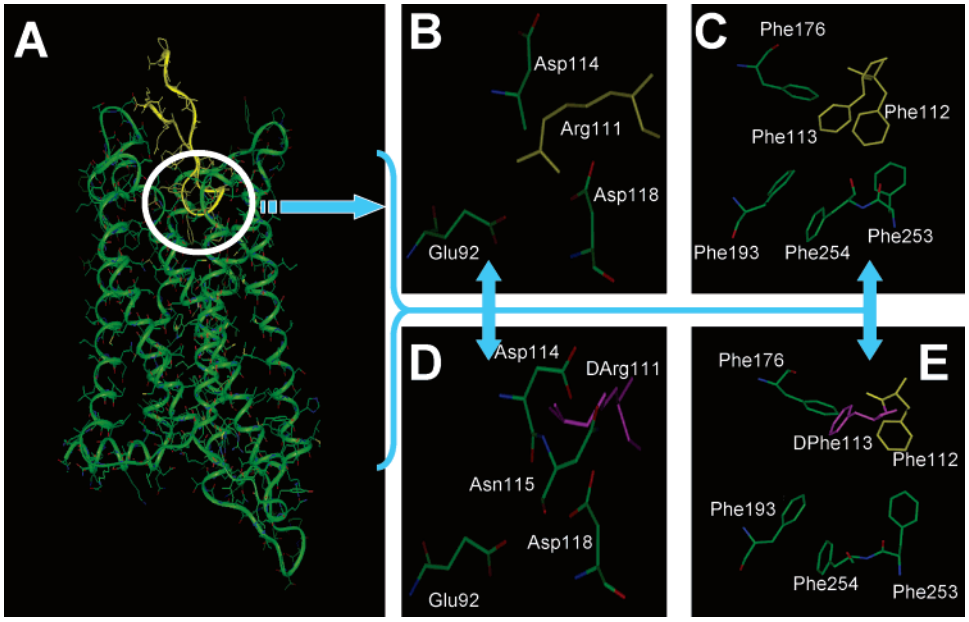

Figure 3. Model of the monocyclic hAGRP(103−122)−mMC4R complex. (A) Ribbon diagram of the monocyclic hAGRP(103−122)−mMC4R model, visualized using CAChe V5.0. The hAGRP(103−122) ligand in yellow and TM helical domain (1−7) of mMC4R in green. (B) The putative hydrophilic binding pocket for hAGRP(103−122) in mMC4R. The side chains of the important residues involved in the postulated electrostatic interactions between the ligand and receptor are highlighted and labeled (L-amino acid of ligand in yellow and D-amino acid in purple). (C) Illustration of the hAGRP(112−113) Phe-Phe amino acid and stereoisomer interactions with the mMC4R residues located in the putative hydrophobic−aromatic binding domain. The side chains of the important residues involved in the hydrophobic−aromatic interactions are highlighted and labeled (L-amino acid of ligand in yellow and D-amino acid in purple). (D) Illustrates the putative interaction of the DArg111 residue (purple) of peptide **2** with the mMC4R Asn115 residue not observed for peptide **1** in panel B. (E) Illustrates the different putative interaction of DPhe113 of peptide **4** (purple), with the mMC4R Phe176 residue not observed for peptide **1** in panel C.

and others, that have been linked to a particular melanocortin receptor isoform. However, central administration of melanocortin peptides has been reported to have thermoregulatory,[50] pressor,[51−53] antipyretic,[54] behavioral,[55,56] and neuroendocrine effects [57] in which the particular melanocortin receptor isoform to which these functions can be attributed remains elusive. Thus, discovery and design of melanocortin receptor selective ligands for each of the receptor isoforms is an important and continuing objective of many academic and industrial research endeavors. Herein, we have discovered peptide **7** which is a 64 nM MC1R agonist that is highly

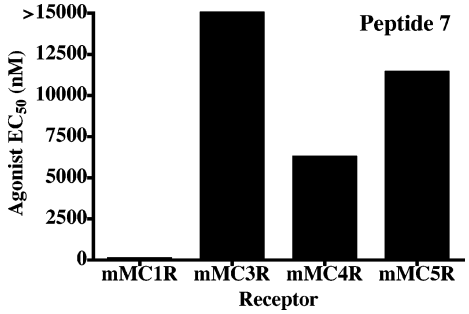

Figure 4. Melanocortin receptor selectivity summary of peptide **7**. The agonist EC_{50} (nM) values are summarized at the mouse melancortin receptors examined in this study. These data illustrate the >90-fold mMC1R selectivity of peptide **7**, versus the other receptors.

selective for this melanocortin receptor isoform (Figure 4). Peptide **7** is 990-fold MC1R versus mMC3R selective, 98-fold MC1R versus mMC4R selective, and 180-fold MC1R versus mMC5R selective. This is an interesting and unexpected discovery, as normally the hAGRP peptide does not bind to or function at the MC1R,[2] and the agouti homologue of AGRP is a MC1R antagonist.[46]

Putative Monocyclic hAGRP(103−122)-mMC4R Interactions. We have identified modifications of the monocyclic hAGRP(103−122) antagonist that convert it from a melanocortin receptor antagonist into an agonist, specifically at the mMC3R and mMC4R isoforms. Modifications of either the endogenous antagonist proteins agouti or AGRP that result in the conversion from an antagonist to agonist have only been reported to date for the "Mini-hAGRP" template,[18] originally reported by Millhauser et al.[32] Thus, while the stereochemical inversion modifications of DArg111 (peptide **2**) and DPhe113 (peptide **4**) resulted in weak μM MC3R and MC4R agonists, these findings are nonetheless seminal in regard to AGRP based rational drug design strategies, putatively understanding melanocortin based ligand−receptor interactions, and GPCR stimulation by one of two known naturally occurring antagonists.

Our laboratory has previously reported the generation and validation of a three-dimensional homology molecular model of the mouse MC4R.[18] This mMC4R model has also been used to examine putative ligand−receptor interactions of a twenty-two amino acid bicyclic derivative of hAGRP and in comparison with hAGRP(87−132) in attempts to identify specific mMC4R residues that might account for the observed pharmacological differences.[33] Herein, we attempt to further utilize this AGRP−mMC4R molecular complex to identify mMC4R amino acids that might account for differences between the monocyclic hAGRP(103−122) ligands and antagonist versus agonist pharmacology. Generally, GPCR homology receptor modeling is based upon the 2.8 Å resolution rhodopsin GPCR structure template,[58] and is inherently built upon many poor assumptions and low resolution starting structures. Unfortunately to date, a high resolution structure of a peptide hormone GPCR is not available for receptor homology modeling studies primarily due to the fact that the cellular lipid bilayer is essential, in most cases, for proper GPCR function, and is not readily amenable to the formation of crystal structures rigorous enough for X-ray crystallographic studies. Thus, utilization of GPCR homology molecular modeling studies is a current medicinal chemistry technique to study putative ligand−receptor interactions in attempts of explaining ligand SAR.[59] X-ray crystallographers have successfully used a "homology" based modeling approach for decades to help solve new structures from similar enzyme classes and identify putative enzyme−substrate interactions that result in the mutagenesis of both the enzyme and substrate experimental design in attempts to discover better drugs. One of the major caveats to performing GPCR homology modeling studies is that putative ligand−receptor interactions need to be verified with experimental data to validate and support the further refinement of GPCR "complexed" models. For the MC4R, several in vitro receptor mutagenesis studies have been reported by different groups.[5,19,60−64] These studies included not only construction of chimeric receptors (two different receptors with different ligand pharmacological properties) but also site-specific receptor residue mutations with Ala's as well as other amino acids at identical positions. Additionally, our laboratory has generated chimeric agonist ligands that took the critical antagonist hAGRP(111−113) Arg-Phe-Phe amino acids and put them into melanocortin based agonist templates (linear NDP-MSH and cyclic MTII) and conversely, using a modified hAGRP(109−118) decapeptide template, substituted the hAGRP Arg-Phe-Phe residues with the melanocortin agonist key residues "His-Phe/DPhe-Arg-Trp," resulting in agonists.[17,18] This latter approach was also applied to the potent MC4R antagonist mini-AGRP template consisting of four disulfide bridges with the mini-His-DPhe-Arg-Trp-hAGRP peptide resulting a potent melanocortin receptor agonist.[18]

Figure 3 summarizes the results of our modeling studies. The "docked" structure of the lead monocyclic hAGRP(103−122) peptide **1** was generated from our previous study that "docked" the NMR structure of hAGRP(87−132) into the mMC4R (see Experimental Section below for details).[18] The positively charged Arg111 of peptides **1** and **4**, and the DArg111 of **2**, was observed as putatively interacting with negatively charged mMC4R residues in TM2 and TM3. These putative electrostatic interactions between Arg111 and the mMC4 receptor E92 (TM2), D114 (TM3), and D118 (TM3) appears to be important for agonist and antagonist binding to this isoform. These result are consistent with MC4R mutagenesis data[5,19] which resulted in reduced binding affinity of hAGRP(87−132) and showed no antagonist activity when point mutations were generated at the mMC4R residues Glu92Lys and Asp118Lys.[5] C-Terminal mutation of the AGRP Arg111 residue resulted in 130-fold decreased binding affinity observed at the hMC4R,[11] implicating the importance of this hAGRP residue for molecular recognition at the MC4R. Interestingly, for peptide **2** (DArg111, mMC4R agonist) an additional putative interaction of DArg111 with the mMC4R Asn115 (TM3) was observed (Figure 3D). The mMC4R residues Asp114 (TM3) and Asn115 (TM3) appear to "lock" the DArg side chain of peptide **2**, and the mMC4R Asp118 (TM3) putative interaction with peptides **1** and **4** is distant from the DArg peptide **2** side chain. This mMC4R Asn115 residue has not been previously examined in MC4R mutagenesis studies,[5,19] but was observed as possessing putative contacts in a bicyclic hAGRP study.[33] Thus, this putative DArg111−

Table 2. Analytical Data of the Monocyclic AGRP Peptides Synthesized[a]

peptide	sequence	HPLC k' system 1	HPLC k' system 2	% purity	*m/z* M, calcd	*m/z* M + 1, expt
1	DPAATAYc[C-**Arg**-**Phe**-**Phe**-NAFC]YARKL	6.5	12.6	>99	2326.7	2327.3
2	DPAATAYc[C-**DArg^{111}**-**Phe**-**Phe**-NAFC]YARKL	6.6	11.2	>96	2326.7	2328.0
3	DPAATAYc[C-**Arg**-**DPhe^{112}**-**Phe**-NAFC]YARKL	6.5	11.2	>99	2326.7	2327.6
4	DPAATAYc[C-**Arg**-**Phe**-**DPhe^{113}**-NAFC]YARKL	6.6	11.5	>97	2326.7	2326.9
5	DPAATAYc[C-**DArg^{111}**-**DPhe^{112}**-**Phe**-NAFC]YARKL	6.3	11.0	>99	2326.7	2326.6
6	DPAATAYc[C-**DArg^{111}**-**Phe**-**DPhe^{113}**-NAFC]YARKL	5.9	10.9	>98	2326.7	2328.1
7	DPAATAYc[C-**Arg**-**DPhe^{112}**-**DPhe^{113}**-NAFC]YARKL	5.9	10.5	>99	2326.7	2326.1
8	DPAATAYc[C-**DArg^{111}**-**DPhe^{112}**-**DPhe^{113}**-NAFC]YARKL	6.0	12.0	>98	2326.7	2327.0

[a] HPLC k' = [(peptide retention time − solvent retention time)/(solvent retention time)] in solvent system 1 (10% acetonitrile in 0.1% trifluoroacetic acid/water and a gradient to 90% acetonitrile over 35 min) or 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 λ.

mMC4R Asn115 interaction may explain the conversion of the monocyclic hAGRP(103−122) antagonist into a mMC4R agonist. This hypothesis remains to be experimentally verified, however.

Consistent with our previous studies and experimental approach,[18,33] comparison of the monocyclic hAGRP-(103−122) Phe113 (**1** and **2**) and DPhe113 (**4**) residues were identified to putatively interact in a mMC4R hydrophobic binding pocket consisting of Phe176 (TM4), Phe193 (TM5), Phe253 (TM6), and Phe254 (TM6). In vitro mutagenesis studies of mMC4R indicated that these residues appear to be important for molecular recognition and functional antagonism of AGRP(87−132) and the decapeptide AGRP(109−118)[5] containing the Arg-Phe-Phe(111−113) residues. The importance of the AGRP Phe113 residue for MC4R binding has been previously demonstrated. Mutation of this AGRP Phe113 residue resulted in decreased binding affinity at the MC4R.[11] The Phe176 of mMC4R has been demonstrated to be important for functional antagonism of AGRP(87−132) and AGRP(109−118). Mutations of the mMC4R Phe176 resulted in a loss of functional activity observed for both of these ligands.[5] In peptide **4** (DPhe113, mMC4R agonist), the homology modeling studies resulted in the identification of altered putative monocyclic hAGRP(103−122) DPhe113−mMC4R Phe176 interactions (Figure 3E), as compared with peptide **1**. As discussed previously, this mMC4R Phe176 amino acid was identified as important for the antagonist function of the hAGRP(87−132) compound, and may potentially explain why peptide **4** becomes an agonist upon epimerization of the ligand Phe113 residue. This hypothesis remains to be further studied and experimentally verified. Nonetheless, these studies have provided specific putative ligand−receptor interaction hypothesis that can be further probed by additional ligand design and receptor mutagenesis studies.

Experimental Section

Peptide Synthesis. Peptide synthesis was performed using standard 9-fluorenylmethoxycarbonyl (Fmoc) methodology[27,28] in a manual reaction vessel or by automation on an Advanced ChemTech 440MOS automated synthesizer (Louisville, KY). The amino acids Fmoc-Lys(Boc), Fmoc-Arg(Pbf), Fmoc-DArg-(Pbf), Fmoc-Ala, Fmoc-Tyr(tBu), Fmoc-Cys(Acm), Fmoc-Phe, Fmoc-DPhe, Fmoc-Asn(Trt), Fmoc-Thr(tBu), Fmoc-Pro, Fmoc-Asp(OtBu), and Fmoc-Val were purchased from Peptides International (Louisville, KY). The peptides were assembled on 9-fluorenylmethoxycarbonyl-L-leucine-*p*-alkoxybenzyl alcohol (Fmoc-Leu Wang) resin (0.73 meq/g substitution), purchased from Peptides International (Louisville, KY). All reagents were ACS grade or better and were used without further purification. The Fmoc protecting groups were removed using 20% piperidine (Sigma Aldrich) in *N*,*N*-dimethylformamide (DMF), and amino acid coupling (3-fold excess) was accomplished using 2-(1*H*−benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, 3-fold excess), 1-hydroxybenzotriazole anhydrous (HOBt, 3-fold excess), and diisopropylethylamine (DIEA, 5.1-fold excess) manually. On the automated synthesizer the synthesis was performed using a 16 well Teflon reaction block with a course frit. Approximately 205 mg of resin (0.15 mmol) was added to each reaction block well. The resin was allowed to swell for 2 h in methylene chloride (DCM) and deprotected using 25% piperidine in DMF twice for 5 min and then for 20 min at 450 rpm. A positive ninhydrin test result indicates free amine groups on the resin.[65] The growing peptide chain was added to the Wang resin using the following general amino acid cycle: 500 μL of DMF is added to each reaction well to wet the frit, 3-fold excess amino acid starting from the C-terminus is added (900 μL of 0.5 M amino acid solution containing 0.5 M HOBt in DMF) followed by the addition of 900 μL of 0.5 M diisopropylcarbodiimide (DIC) in DMF, and the reaction well volume is brought up to 6 mL using DMF. The coupling reaction is mixed for 1 h at 450 rpm, followed by emptying of the reaction block by positive nitrogen gas pressure. A second coupling reaction is performed by addition of 500 μL of DMF to each reaction vessel, followed by addition of 900 μL of 0.5 M of the respective amino acid, 900 μL of 0.5 M HBTU, and 765 μL of 1 M DIEA; the reaction well volume is brought up to 6 mL with DMF and mixed at 450 rpm for 1 h. After the second coupling cycle, the reaction block is emptied and the Fmoc protected resin is washed with DMF (6 mL 4 times). Fmoc deprotection is performed by addition of 6 mL of 25% piperidine in DMF and for 5 min at 450 rpm followed by a 20 min deprotection at 450 rpm. The reaction well is washed with 6 mL of DMF 4 times, and the next coupling cycle is performed as above. Completion of amino acid coupling and Fmoc deprotection were monitored using the ninhydrin test.[65] Final peptide cleavage from the resin and amino acid side chain protecting group removal were performed using 5 mL of (82.5:5:5:5:2.5) trifluoroacetic acid (TFA), phenol, water, thioanisole, and 1,2-ethanedithiol or (88:5:5:2) TFA, phenol, water, and triethylsilane for 2−3 h. Peptide disulfide cyclization was performed by either of two methods, A or B below. The cleavage product was emptied from the reaction block into a cleavage block containing 15 mL collection vials under nitrogen gas pressure. The resin was washed with 3 mL of cleavage cocktail for 5 min at 450 rpm and emptied into the previous cleavage solution. The crude peptides was transferred to preweighed 50 mL conical tubes and precipitated with cold (4 °C) anhydrous ethyl ether (up to 50 mL). The crude peptides were centrifuged (Sorval Super T21 high-speed centrifuge using the swing bucket rotor) at 4000 rpm for 5 min and 4 °C. The ether was decanted off, and the peptide was washed one more time with cold anhydrous ethyl ether and pelleted as before. The crude peptides were dried in vacuo for 48 h. The crude peptide yields ranged from 75% to 95% of the theoretical yields based on resin loading. A 30 to 40 mg

sample of crude peptide was purified by reversed-phase high performance liquid chromatography (RP-HPLC) using a Shimadzu chromatography system with a photodiode array detector. Final peptide purification was achieved using a semi-preparative RP-HPLC C_{18} bonded silica column (Vydac 218TP1010, 1.0 × 25 cm). The purified peptides were >96% pure as determined by analytical RP-HPLC in two diverse solvent systems and had the correct molecular mass (University of Florida protein core facility), Table 2.

Method A: Disulfide Bridge Formation in Solution.[66] The linear peptides were cleaved from the resin using (82.5:5:5:5:2.5) TFA:phenol: water:thioanisole:1,2-ethanedithiol for 3 h at room temperature (RT). The crude linear peptides, the bis(Acm) intermediates, were dissolved in glacial acetic acid−water (HOAc−H_2O; 4:1), and 10 equiv of iodine dissolved in methanol was added. Cyclization was monitored by RP-HPLC and mass spectroscopy. The reaction was mixed in the dark at RT for 90 min, quenched by diluting with water (twice the amount of the total volume used for cyclization), and extracted with carbon tetrachloride (CCl_4; 4 × 15 mL) to remove excess iodine. The aqueous phase was then lyophilized to give the crude cyclic peptide.

Method B: Disulfide Bridge Formation on Resin.[66] In a manual reaction vessel, the synthesized linear peptide attach to the resin was washed with DMF (4 × 10 mL). Iodine (15 equiv) dissolved in DMF was added to the resin and mixed in the dark by bubbling with nitrogen gas for 2 h at RT. The peptide resin was washed with DMF, dichloromethane (DCM), and 1,2-dichloroethane (10 × 2 min, 10 mL) followed by DCM (5 × 2 min, 10 mL). Cleavage of the cyclic peptides from the resin was achieved with (88:5:5:2) TFA:phenol:water: triethylsilane for 2 h at RT.

Cell Culture and Transfection. Briefly, HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM), 10% pen-strep, with 10% fetal calf serum (FCS) and seeded 1 day prior to transfection at 1−2 × 10^6 cells/100 mm dish. Mouse melanocortin receptor cDNA (20 μg) in the $pCDNA_3$ expression vector was transfected by using the calcium phosphate method. Stable receptor populations were generated by using G418 sulfate selection (1 mg/mL) for subsequent bioassay analysis.[5]

β-Galactosidase Functional Bioassay. HEK-293 cells stably expressing the melanocortin receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene as previously described.[67] Briefly, 5000−15000 posttransfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. Forty-eight hours posttransfection, the cells were stimulated with peptide (10^{-4} to 10^{-12} M) or forskolin control (10^{-4} M) in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine, IBMX) for 6 h. The assay medium was aspirated, and 50 μL of lysis buffer (250 mM Tris·HCl, pH 8.0, and 0.1% Triton X-100) was added. The plates were stored at −80 °C overnight. The plates containing the cell lysates were thawed the following day. Aliquots of 10 μL were taken from each well and transferred to another 96-well plate for relative protein determination. Phosphate-buffered saline (PBS) with 0.5% BSA (40 μL) was added to each well of the cell lysate plates. Subsequently, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCL_2$, 10 mM KCl, 5 mM β-mercaptoethanol, 2 mg/mL ONPG) was added to each well, and the plates were incubated at 37 °C. The sample absorbance, OD_{405}, was measured using a 96-well plate reader (Molecular Devices). The relative protein value was determined by adding 200 μL of 1:5 dilution Bio-Rad G250 protein dye: water to the 10-μL cell lysate sample taken previously, and the OD_{595} was measured on a 96-well plate reader (Molecular Devices). Forskolin treatment (10^{-4} M) in six wells of each 96-well plate was used as controls for transfection efficiency of the CRE-β-gal reporter gene. Data points were normalized to both the relative protein content and nonreceptor-dependent forskolin stimulation. Data analysis and EC_{50} values were determined by using nonlinear regression analysis with the PRISM program (v3.0, Graph Pad Inc.). The antagonistic properties were determined by the ability of these ligands to competitively displace the agonist MTII in a dose-dependent manner. The pA_2 values were generated using the Schild analysis method,[29] as previously reported.[12,25] The pA_2 values were then used to calculate the corresponding K_i values (K_i = −log pA_2). The EC_{50} and pA_2 values represent the mean of duplicate wells examined in at least three independent experiments, with the standard errors of the mean presented.

Homology Molecular Modeling Computational Methods. The homology model of the hAGRP(87−132)−melanocortin-4 receptor complex[18] was imported into the Workspace interface of the BioMedCAChe 5.05 software package (CAChe Group, Fujitsu, Portland, OR). The model of the 20-residue peptide **1** complexed with receptor was made directly from the hAGRP(87−132)−mMC4R complex model. Residues 87−102 and 122−132 of hAGRP(87−132) were deleted, and the terminals were capped with charged group. Cys105, Cys108, and Cys119 were replaced with Ala residues, and the whole sequence was renumbered. Complex models of the docked peptides **2** and **4** were created by inverting Arg111 or Phe113 of **1** to the respective d-configuration, and the χ angles were adjusted to favorable orientations as well as to fit to the corresponding binding pockets.

Limited conformation searches of bound monocyclic peptides were carried out within the CONFLEX module to locate the global minimum.[68] CONFLEX generated a sequence of low-energy conformers of any shape systematically and exhaustively, incorporating downstream, reservoir-filling, corner flap, edge flip, stepwise rotation, and precheck. All conformations with Boltzmann populations larger than 1% were subjected to local perturbations and optimized by augmented MM3. All the final structures were analyzed by automatic labeling of hydrogen bonds and bumps in the 3D Structure Window. The calculations were performed on a Dell desktop PC with 1.70 GHz Pentium 4 processor.

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