Structure−Activity Relationships of the Melanocortin Tetrapeptide Ac-His-DPhe-Arg-Trp-NH_2 at the Mouse Melanocortin Receptors: Part 2 Modifications at the Phe Position

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The melanocortin pathway is an important participant in skin pigmentation, steroidogenesis, obesity, energy homeostasis and exocrine gland function. The centrally located melanocortin-3 and melanocortin-4 receptors (MC3R, MC4R) are involved in the metabolic and food intake aspects of energy homeostasis and are stimulated by melanocortin agonists such as α-melanocyte stimulation hormone (α-MSH). The melanocortin agonists contain the putative message sequence "His-Phe-Arg-Trp," and it has been well-documented that inversion of chirality of the Phe to DPhe results in a dramatic increase in melanocortin receptor potency. Herein, we report a tetrapeptide library, based upon the template Ac-His-DPhe-Arg-Trp-NH_2, consisting of 26 members that have been modified at the DPhe^7 position (α-MSH numbering) and pharmacologically characterized for agonist and antagonist activity at the mouse melanocortin receptors MC1R, MC3R, MC4R, and MC5R. The most notable results of this study include the identification of the tetrapeptide Ac-His-(pI)DPhe-Arg-Trp-NH_2 that is a full nanomolar agonist at the mMC1 and mMC5 receptors, a mMC3R partial agonist with potent antagonist activity (pA_2 = 7.25, K_i = 56 nM) and, but unexpectedly, is a potent agonist at the mMC4R (EC_{50} = 25 nM). This ligand possesses novel melanocortin receptor pharmacology, as compared to previously reported peptides, and is potentially useful for in vivo studies to differentiate MC3R vs MC4R physiological roles in animal models, such as primates, where "knockout" animals are not viable options. The DNal(2′) substitution for DPhe resulted in a mMC3R partial agonist with antagonist activity (pA_2 = 6.5, K_i = 295 nM) and a mMC4R (pA_2 = 7.8, K_i = 17 nM) antagonist possessing 60- and 425-fold decreased potency, respectively, as compared with SHU9119 at these receptors. Examination of this DNal(2′)-containing tetrapeptide at the F254S and F259S mutant mMC4Rs resulted in agonist activity of this mMC4R tetrapeptide antagonist, similar to that observed for the SHU9119 peptide, supporting our previously proposed hypothesis that the Phe 254 and 259 transmembrane six receptor residues are important for differentiating melanocortin sequence-based MC4R antagonists vs the agouti-related protein (AGRP) sequence-based antagonists.

Introduction

The melanocortin receptors belong to the superfamily of seven transmembrane (TM)-spanning G-protein-coupled receptors (GPCRs) and stimulate the adenosine cyclic 3′,5′-phosphate (cAMP) signal transduction pathway.[1] The endogenous agonist ligands for these melanocortin receptors are derived by posttranslational cleavage of the pro-opiomelanocortin (POMC) gene transcript, which upon differential processing results in the generation of the α-, β-, and γ-melanocyte-stimulating hormones (MSH) and adrenocorticotropin (ACTH). All of these melanocortin peptide agonists contain a core His-Phe-Arg-Trp tetrapeptide sequence that has been attributed to the ligand selectivity and stimulation of the melanocortin receptors.[2−4] The melanocortin receptor family also has two endogenous antagonists, agouti[5] and the agouti-related protein (AGRP),[6,7] which are the only known naturally occurring antagonists of GPCRs discovered to date. The centrally located melanocortin-3 and -4 receptors (MC3R, MC4R) have been identified in knockout mice to be involved in feeding behavior, obesity, metabolism, and energy homeostasis.[8−10] The peripheral melanocortin-1 receptor (MC1R) is expressed in the skin and is involved in skin pigmentation and animal coat coloration.[11−13] The melanocortin-5 receptor (MC5R) is expressed in a variety of peripheral tissues and has been deleted from the mouse genome and identified as playing a role in exocrine gland function.[14]

The Phe side chain at the seven position (α-MSH numbering) has been modified and resulted in the discovery of antagonists for the central MC3 and MC4 receptors.[15] The DNal(2′) and para iodine (pI)DPhe substitutions of the Phe amino acid in the MTII cyclic peptide template (Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2) resulted in the MC3R and MC4R partial agonists and antagonists SHU9119 and SHU8914, respectively.[15] The (pI)DPhe substitution of the DPhe amino acid in the NDP-MSH linear peptide template (Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) resulted in the SHU9005 partial agonist and antagonist activity at the MC3R and MC4R.[16] The MTII (agonist) and SHU9119 (antagonist) ligands were further utilized in rodent feeding studies to demonstrate that the central melanocortin pathway could modify feeding behavior by increasing food intake upon antagonist administration

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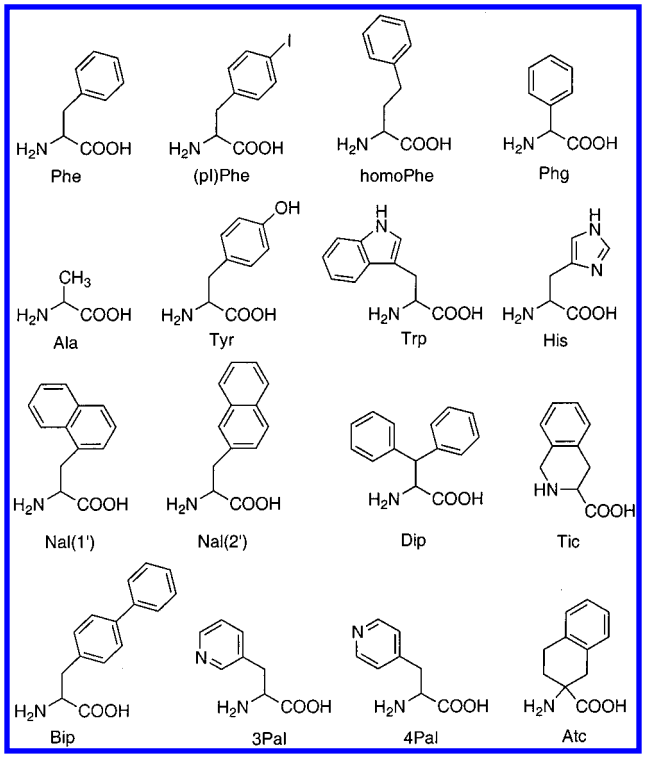

Figure 1. Structures of the amino acids used to replace DPhe in the peptide template Ac-His-Xaa-Arg-Trp-NH_2.

and decreasing food intake upon agonist administration into the brain.[17] Subsequent studies substituting the DNal(2′) amino acid into various melanocortin agonist peptide templates at the Phe position resulted in MC3 and/or MC4 receptor antagonists.[18−23] Computer GPCR homology molecular modeling of the MC1R[24] and receptor mutagenesis studies of the peripheral MC1R[25] and the central MC4R[16,26] resulted in the identification of the melanocortin ligand DPhe residue as putatively interacting with a hydrophobic receptor pocket consisting of several aromatic receptor side chains.[27] The study presented herein utilizes the tetrapeptide template Ac-His-Xaa-Arg-Trp-NH_2, where Xaa is substituted with natural, unnatural, and aromatic amino acids (Figure 1) to examine the melanocortin ligand side chain properties important for melanocortin receptor selectivity, potency, structure−activity relationship (SAR) trends, and differentiation of agonist vs antagonist activities at the mouse MC1, MC3, MC4, and MC5 receptors.

Results

Table 1 summarizes the pharmacology at the mouse melanocortin receptors mMC1R, mMC3R, mMC4R, and mMC5R of the 26 tetrapeptides modified at the Phe seven position (α-MSH numbering) of the tetrapeptide template Ac-His-Xaa-Arg-Trp-NH_2 prepared in this study. The compounds that did not show agonist activity, >100 000 nM EC_{50} values in Table 1, were tested for antagonism at up to 10 μM concentrations but did not possess antagonistic pharmacological profiles (data not shown).

Tetrapeptide **1**, Ac-His-DPhe-Arg-Trp-NH_2, is the lead peptide for this study and has been previously reported at the mouse melanocortin receptors[28] to possess 20, 156, 17, and 4 nM agonist activity at the mMC1R, mMC3R, mMC4R, and mMC5R, reported herein. The (pI)DPhe^7-containing tetrapeptide **3** maintained equipotency at the mMC1R, mMC4R, and mMC5R (within the inherent 3-fold experimental error) with DPhe^7 (**1**) but was a partial agonist possessing potent antagonist activity (pA_2 = 7.25, K_i = 56 nM) at the mMC3R (Figure 2). The homoPhe^7 tetrapeptide **4** resulted in 1650- and 2980-fold decreased potencies at the mMC1R and mMC5R, respectively, as compared with **1**, but was not able to stimulate the mMC3 and mMC4 receptors at up to 100 μM concentrations. The homoDPhe^7 tetrapeptide **5** resulted in only 310-, 645-, and 700-fold decreased potency at the mMC1R, mMC4R, and mMC5R, respectively, as compared with **1** and similarly to the homoPhe-containing tetrapeptide **4** was unable to stimulate the mMC3R at up to 100 μM concentrations. The Phg^7 tetrapeptide **10**, which contains one less CH_2 in the side chain length than Phe, resulted in 731-, 282-, 2360-, and 1010-fold decreased potency as compared with the Phe^7 tetrapeptide **1** at the mMC1R, mMC3R, mMC4R, and mMC5R, respectively. Tetrapeptide **11** (DPhg^7) resulted in 841-fold decreased mMC1R potency as compared with **1** and possessed only slight agonist activity (at 100 μM) at the mMC5R and was unable to stimulate either the mMC3 or the mMC4 receptors. The Nal(1′)^7-containing tetrapeptide **12** possessed 756-fold decreased potency at the mMC1R and 2830-fold decreased mMC5R potency, as compared with DPhe^7 (**1**), and did not stimulate the mMC3R or mMC4R. However, the DNal(1′)^7 tetrapeptide **13** resulted in slight and nearly equal 13−26-fold decreased potency, as compared with **1**, at the mMC1−5 receptors. Tetrapeptide **14**, containing Nal(2′) at the Phe position, resulted in an equipotent decreased potency at the mMC1R (1100-fold) and mMC5R (1870-fold) as compared with **1** and was also unable to stimulate the mMC3 and mMC4 receptors. The tetrapeptide Ac-His-DNal(2′)-Arg-Trp-NH_2 (**15**) resulted in 8- and 9-fold decreased agonist potency at the mMC1R and mMC5R, respectively, as compared with **1** and was a partial agonist with antagonist activity at the mMC3R (pA_2 = 6.5, K_i = 295 nM) and a mMC4R antagonist (pA_2 = 7.78, K_i = 17 nM); see figure in the Supporting Information. The Tic^7-containing tetrapeptide **18** resulted in 2670-fold decreased mMC1R potency and 5380-fold decreased mMC5R potency, as compared with **1**, and was unable to stimulate the mMC3 and mMC4 receptors. Tetrapeptide **19** (DTic^7) possessed 2030-fold decreased mMC1R potency as compared with the DPhe^7 tetrapeptide **1** and did not stimulate the mMC3−5 receptors at up to 100 μM concentrations. Tetrapeptide **20**, containing the Bip amino acid in the Phe position, resulted in a 527-fold decreased mMC1R potency and 1390-fold decreased mMC5R potency, as compared with **1**, and possessed slight agonist activity at the mMC4R (100 μM) but was unable to stimulate the mMC3R. Interestingly, DBip^7 (**21**) was equipotent with **1** at the mMC1R, mMC4R, and mMC5R (within experimental error) but was 13-fold less potent at the mMC3R. Incorporation of the racemic Atc amino acid into the tetrapeptide template resulted in the separation of two separate peaks by reversed-phase high-performance liquid chromatography (RP-HPLC); they are designated as peptides **25** and **26**. Tetrapeptide **25**, the first Atc-containing peptide eluted by RP-HPLC, resulted in equal 63-fold decreased potency at both the

Table 1. Functional Activity of the DPhe[7]-Modified Tetrapeptides at the Mouse Melanocortin Receptors

		mMC1R		mMC3R		mMC4R		mMC5R	
peptide	structure	EC_{50} (nM)	fold difference	EC_{50} (nM)	fold difference	EC_{50} (nM)	fold difference	EC_{50} (nM)	fold difference
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.55 ± 0.09		0.79 ± 0.14		5.37 ± 0.62		0.44 ± 0.09	
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-DPhe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.038 ± 0.012		0.098 ± 0.013		0.21 ± 0.03		0.071 ± 0.012	
MTII	Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]-NH_2	0.020 ± 0.003		0.16 ± 0.03		0.087 ± 0.008		0.16 ± 0.03	
α-MSH (7−9)	Ac-His-Phe-Arg-Trp-NH_2	7690 ± 3590*		4370 ± 535*		2,110 ± 243*		103 ± 28*	
1	Ac-His-**DPhe**-Arg-Trp-NH_2	20.1 ± 0.57	1.0	156 ± 9.2	1.0	17.2 ± 2.78	1.0	3.96 ± 0.94	1.0
2	Ac-His-**Ala**-Arg-Trp-NH_2	30 000 ± 6100	1490	>100 000		>100 000		>100 000	
3	Ac-His-**(pI)DPhe**-Arg-Trp-NH_2	60.4 ± 13.4	3	partial agonist $pA_2 = 7.25 \pm 0.18$	antagonist	25.0 ± 9.78	1	1.60 ± 0.35	−2
4	Ac-His-**homoPhe**-Arg-Trp-NH_2	33 100 ± 8300	1650	>100 000		>100 000		11 800 ± 4700	2980
5	Ac-His-**homoDPhe**-Arg-Trp-NH_2	6250 ± 2000	310	>100 000		11 100 ± 2000	645	2800 ± 780	700
6	Ac-His-**Tyr**-Arg-Trp-NH_2	61 400 ± 17 100	3050	>100 000		>100 000		32 700 ± 9400	8260
7	Ac-His-**DTyr**-Arg-Trp-NH_2	3200 ± 1300	159	34 000 ± 5000	218	2400 ± 690	140	631 ± 97	159
8	Ac-His-**Trp**-Arg-Trp-NH_2	33 400 ± 10 700	1660	>100 000		34 300 ± 7900	1990	18 400 ± 6800	4650
9	Ac-His-**DTrp**-Arg-Trp-NH_2	25 100 ± 15 000	1250	>100 000		>100 000		6950 ± 1780	1760
10	Ac-His-**Phg**-Arg-Trp-NH_2	14 700 ± 2000	731	44 000 ± 13 700	282	40 500 ± 9300	2360	4000 ± 1300	1010
11	Ac-His-**DPhg**-Arg-Trp-NH_2	16 900 ± 3200	841	>100 000		>100 000		slight agonist at 100 μM	
12	Ac-His-**Nal(1′)**-Arg-Trp-NH_2	15 200 ± 3100	756	>100 000		>100 000		11 200 ± 2300	2830
13	Ac-His-**DNal(1′)**-Arg-Trp-NH_2	356 ± 64	18	4100 ± 1000	26	303 ± 65	18	51.4 ± 4.07	13
14	Ac-His-**Nal(2′)**-Arg-Trp-NH_2	22 000 ± 5600	1100	>100 000		>100 000		7400 ± 1800	1870
15	Ac-His-**DNal(2′)**-Arg-Trp-NH_2	167 ± 41	8	partial agonist $pA_2 = 6.53 \pm 0.09$	antagonist	$pA_2 = 7.78 \pm 0.18$	antagonist	34.7 ± 6.75	9
16	Ac-His-**Dip**-Arg-Trp-NH_2	19 000 ± 7100	945	>100 000		>100 000		>100 000	
17	Ac-His-**DDip**-Arg-Trp-NH_2	1500 ± 280	75	>100 000		5000 ± 750	291	4300 ± 695	1090
18	Ac-His-**Tic**-Arg-Trp-NH_2	53 600 ± 13 600	2670	>100 000		>100 000		21 300 ± 9700	5380
19	Ac-His-**DTic**-Arg-Trp-NH_2	40 800 ± 19 900	2030	>100 000		>100 000		>100 000	
20	Ac-His-**Bip**-Arg-Trp-NH_2	10 600 ± 3700	527	>100 000		slight agonist at 100 μM		5500 ± 2300	1390
21	Ac-His-**DBip**-Arg-Trp-NH_2	28.4 ± 11.4	1	2050 ± 734	13	68.4 ± 15.8	4	11.6 ± 3.92	3
22	Ac-His-**His**-Arg-Trp-NH_2	41 700 ± 19 200	2080	>100 000		>100 000		41 200 ± 14 400	10 400
23	Ac-His-**3Pal**-Arg-Trp-NH_2	22 900 ± 1200	1140	>100 000		>100 000		36 100 ± 9300	9100
24	Ac-His-**4Pal**-Arg-Trp-NH_2	36 700 ± 10 100	1830	>100 000		24 400 ± 2000	1420	9700 ± 1500	2450
25 (peak 1)	Ac-His-**Atc**-Arg-Trp-NH_2	2350 ± 930	117	9900 ± 2900	63	1780 ± 163	103	263 ± 81	66
26 (peak 2)	Ac-His-**Atc**-Arg-Trp-NH_2	5800 ± 2070	289	7800 ± 1300	50	7130 ± 1400	415	2070 ± 657	523

[a] The indicated errors represent the standard error of the mean determined from at least four independent experiments. Slight agonist denotes that some stimulatory response was observed but not enough to determine an EC_{50} value. Compounds possessing >100 000 EC_{50} values were not found to possess antagonist properties at up to 10 μM concentrations. *These α-MSH (7−9) peptide values have been previously reported[28] and are included herein for reference purposes.

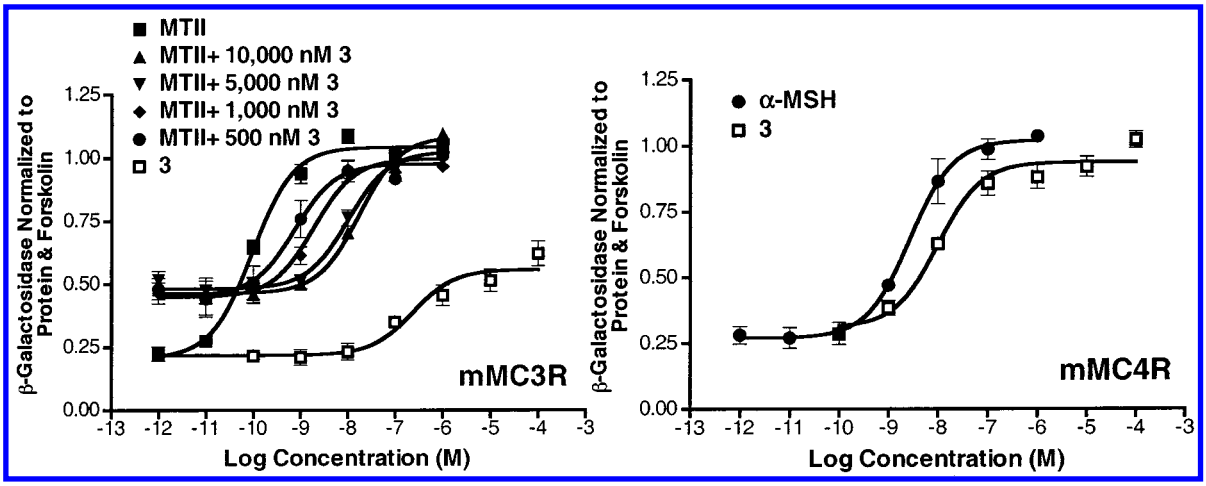

Figure 2. Illustration of the tetrapeptide **3** possessing partial agonist and antagonist pharmacology at the mouse MC3R and agonist pharmacology at the mouse MC4R. The endogenous agonist for the melanocortin receptors, α-MSH, is only 5-fold more potent at the mMC4R than tetrapeptide **3**, Ac-His-(pI)DPhe-Arg-Trp-NH_2.

mMC3R and the mMC5R and equal 103-fold decreased potency at the mMC1R and mMC4R, as compared with **1**. Interestingly, tetrapeptide **26** containing the second Atc peptide eluted resulted in 50-fold decreased mMC3R potency, 289-fold decreased mMC1R potency, 415-fold decreased mMC4R potency, and 523-fold decreased mMC5R potency, as compared with the DPhe[7]-containing tetrapeptide **1**.

Discussion

Mouse Melanocortin-1 Receptor. The peripheral skin melanocortin receptor, MC1R, is involved in human skin pigmentation[12,29] and animal coat coloration.[13] The lead tetrapeptide **1**, Ac-His-DPhe-Arg-Trp-NH_2, has been previously reported to possess 25 nM stimulatory activity at the mMC1R,[28] an EC_{50} value of 200 nM in the classical *Rana pipiens* frog skin assay (putative MC1R),[4] and possesses a mMC1R EC_{50} = 20 nM reported herein. Interestingly, all of the substitutions at the DPhe position of the tetrapeptide **1** were tolerated by the mMC1R and EC_{50} values and full agonism resulted (Table 1), unlike the pharmacology of these compounds at the mMC3R and mMC4R. Upon substitution of the Phe side chain with Ala, a 1500-fold decreased potency as compared with **1** is observed at the mMC1R. Substitution at the Phe position of these tetrapeptides with homoPhe (**4**), Tyr (**6**), Trp (**8**), DTrp- (**9**), Nal(2′) (**14**), Tic (**18**), DTic (**19**), His (**22**), 3Pal (**23**), and 4Pal (**24**) all resulted in approximately the same fold decreased potency as the Ala-containing tetrapeptide **2**, as compared with the lead tetrapeptide **1**. The most potent Phe substitution in the tetrapeptide template is the DBip amino acid (Figure 1) in tetrapeptide **21**, which resulted in equipotent mMC1R activity, as compared with the lead tetrapeptide **1**. Tetrapeptides possessing nanomolar mMC1R agonist activity include the (pI)DPhe (**3**), DNal(2′) (**15**), and DNal(1′) (**13**) amino acid substitutions at the Phe position of tetrapeptide **1**. Interestingly, SHU8914 (pIDPhe[7]) was a potent (pA_2 = 10.3) antagonist in the frog skin bioassay (putative MC1R) but was a potent agonist at both the mouse and the human MC1 receptors.[15]

Receptor mutagenesis and GPCR homology molecular modeling studies of the MC1R identified a putative hydrophobic receptor pocket consisting of multiple Phe receptor residues proposed to interact with the melanocortin ligand Phe amino acid.[24,25,27] The tetrapeptides reported herein that possessed nanomolar agonist pharmacology (**1**, **3**, **14**, **15**, and **21**) consisted of a D-configured amino acid derivative of the benzyl side chain. These data are consistent with the receptor mutagenesis and modeling studies, suggesting that an aromatic network of interactions is formed between the melanocortin agonist DPhe amino acid and multiple Phe residues of the MC1 receptor.

Mouse Melanocortin-3 Receptor. The MC3R is expressed both peripherally and centrally and appears to be involved in metabolism and energy homeostasis.[8,9,30,31] The lead tetrapeptide **1** has been previously reported to possess a 195 nM agonist EC_{50} at the mMC3R,[28] a 1000 nM EC_{50} at the hMC3R,[32] and a 156 nM EC_{50} value reported herein. Substitution of the Phe amino acid side chain at the seven position (α-MSH numbering) of the lead tetrapeptide by Ala, homoPhe, DhomoPhe, Tyr, Trp, DTrp, DPhg, Nal(1′), Nal(2′), Dip, DDip, Tic, DTic, Bip, His, 3Pal, and 4Pal (Figure 1) resulted in loss of agonist activity at up to 100 μM concentrations (Table 1). Replacement of DPhe with Ala at the seven position of the cyclic MTII template resulted in only 19% total binding at 10 μM and 2% agonist stimulation at 20 μM concentrations at the human MC3R.[33] Incorporation of the pI moiety onto the DPhe amino acid in tetrapeptide **3** resulted in a partial mMC3R agonist with potent antagonist activity (pA_2 = 7.25), which corresponds to a K_i value of 56 nM (Figure 2). In previous studies with the (pI)DPhe substitution in either the cyclic MTII template (SHU8914, hMC3R pA_2 = 8.3, K_i = 5 nM)[15] or the linear NDP-MSH template (SHU9005, mMC3R pA_2 ca. 9.0; Haskell-Luevano, C.; Hruby, V. J.; Cone, R. D. Unpublished results.), partial agonism was observed in addition to MC3R antagonistic pharmacology. Similarly to the previously reported peptides containing (pI)DPhe at the seven position, when the (pI)DPhe amino acid is inserted into the tetrapeptide template **3**, a compound results that possesses partial agonist activity at the mMC3R (Figure 2). The tetrapeptide Ac-His-DNal(2′)-Arg-Trp-NH_2 (**15**) is also a mMC3R antagonist (pA_2 = 6.53, K_i = 295 nM) that possesses slight agonist activity, less than **3**, at greater than 1 μM concentrations

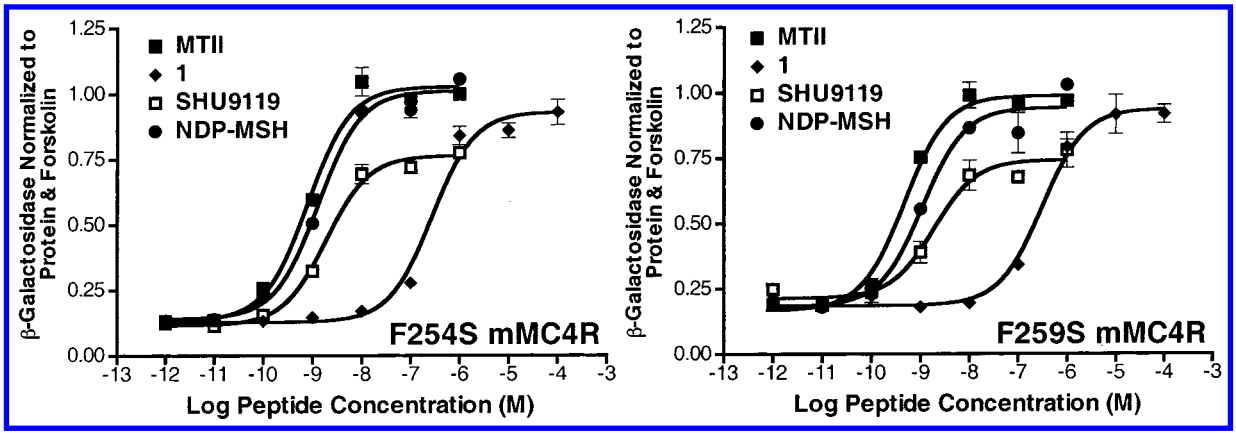

Figure 3. Comparison of the mMC4R agonist peptides MTII, NDP-MSH, and **1** (Ac-His-DPhe-Arg-Trp-NH_2) and the mMC4R antagonist SHU9119 at the mutant F254S and F259S mMC4 receptors. The SHU9119 peptide possesses partial agonist activity at these mutant receptors vs possessing competitive antagonist pharmacology at the wild-type mMC4R.[15]

(Supporting Information). This DNal(2′) substitution for the Phe side chain in the cyclic MTII template resulted in the identification of the first MC3R antagonist, albeit with partial agonist activity.[15] Subsequently, this DNal(2′) substitution in several other cyclic melanocortin templates also resulted in antagonists of the MC3 receptor.[18−22] Furthermore, a study of a modified molecule based upon the His-DNal(2′)-Arg-Trp peptide motif, RO27-4680, resulted in a MC3R antagonist.[23] The most potent agonist tetrapeptides substituted at the Phe position reported herein include the DBip[7] (**21**)- and the DNal(1′)[7] (**13**)-containing molecules that are 13- and 26-fold less potent than the DPhe tetrapeptide **1** at the mMC3R.

Mouse Melanocortin-4 Receptor. The central MC4R has been identified as physiologically participating in food consumption[17] and obesity in mice[10] with several polymorphisms of the MC4R observed in obese humans.[34−39] The lead tetrapeptide in this study, **1**, was previously reported to possess a 10 nM agonist EC_{50} value at the mMC4R,[28] 8[26] and 47 nM[32] agonist EC_{50} values at the hMC4R, and a potency at the mMC4R of 17 nM reported herein. Similarly to the mMC3R, substitution of the Phe benzyl side chain with a methyl group of Ala (**2**) resulted in a loss of a determinable EC_{50} value at the mMC4R. Interestingly, when the DPhe of the linear 13 amino acid NDP-MSH peptide was replaced with a DAla, only ca. 1200-fold decreased binding and agonist potency was reported at the human MC4R.[26] In a separate paper, replacement of DPhe with Ala at the seven position of the cyclic MTII template resulted in only 13% total binding at 10 μM and 32% agonist stimulation at 20 μM concentrations at the human MC4R.[33] Substitution of the Phe amino acid with homoPhe (**4**), Tyr (**6**), DTrp (**9**), DPhg (**11**), Nal(1′) (**12**), Nal(2′) (**14**), Dip (**16**), Tic (**18**), DTic (**19**), His (**22**), and 3Pal (**23**) resulted in a loss of agonist activity at up to 100 μM concentrations. Tetrapeptide **20** containing the Bip amino acid instead of DPhe (**1**) possessed slight agonist activity at up to 100 μM concentrations at the mMC4R but not enough agonist response to determine an EC_{50} value. Substitutions of the DPhe (**1**) residue in the tetrapeptide template resulting in nanomolar mMC4R agonist potency include the (pI)DPhe (**3**), DBip (**21**), and DNal(1′) (**13**) amino acids. These later results suggest that substitution of the DPhe residue in melanocortin small molecules by either the (pI)DPhe or the DBip amino acids may result in nonpeptide compounds possessing similar MC4R agonist potency as homologous compounds containing the DPhe moiety. Surprisingly, tetrapeptide **3** containing the (pI)DPhe[7] amino acid resulted in a potent mMC4R agonist (EC_{50} = 25 nM) that is only 5-fold less potent than α-MSH (Table 1), instead of an antagonist pharmacology like at the mMC3R (Figure 2). Previous studies incorporating a pI moiety onto the DPhe at the seven position (α-MSH numbering) in both the seven amino acid MTII cyclic template, SHU8914,[15] and the linear 13 amino acid NDP-MSH template, SHU9005,[16] resulted in peptides possessing partial agonism and potent antagonism ($pA_2 = 9.7$, $K_i = 0.2$ nM) at the human and mouse MC4 receptors. Because the tetrapeptide (**3**) containing the (pI)DPhe[7] residue is a potent MC4R agonist at the mouse receptor, we further examined this peptide using the human MC4R clone and also observed full agonist activity with a similar nanomolar EC_{50} potency (data not shown). Thus, the agonist pharmacology for compound **3** does not appear to be species specific (human vs mouse). It is therefore interesting to speculate that differences in antagonist activity of the larger (pI)-DPhe[7]-containing peptides vs agonist activity of the tetrapeptide reported herein may be attributed to the additional amino acids at the N and C terminus that may be modifying the secondary structure and/or topography of how these ligands may be interacting with the MC4 receptor putative binding pocket. As predicted, tetrapeptide **15**, containing DNal(2′),[7] resulted in a mMC4R antagonist ($pA_2 = 7.8$, $K_i = 17$ nM, Supporting Information). In the linear tetrapeptide template used in this study, a 427-fold decreased potency results, as compared with the more potent cyclic SHU9119 mMC4R antagonist Ac-Nle-c[Asp-His-DNal(2′)-Arg-Trp-Lys]-NH_2.[15,16]

Receptor mutagenesis studies of the mouse MC4R identified mutations in the putative TM6 domain as converting the SHU9119 and SHU9005 MC4R antagonists into agonists.[16] Interestingly, these two mutant receptors, F254S mMC4R and F259S mMC4R, did not result in converting the endogenous antagonist hAGRP-(83−132) into a ligand possessing agonist activity as the SHU9119 and SHU9005 ligands were, nor did the hAGRP(109−118) decapeptide result in any agonist

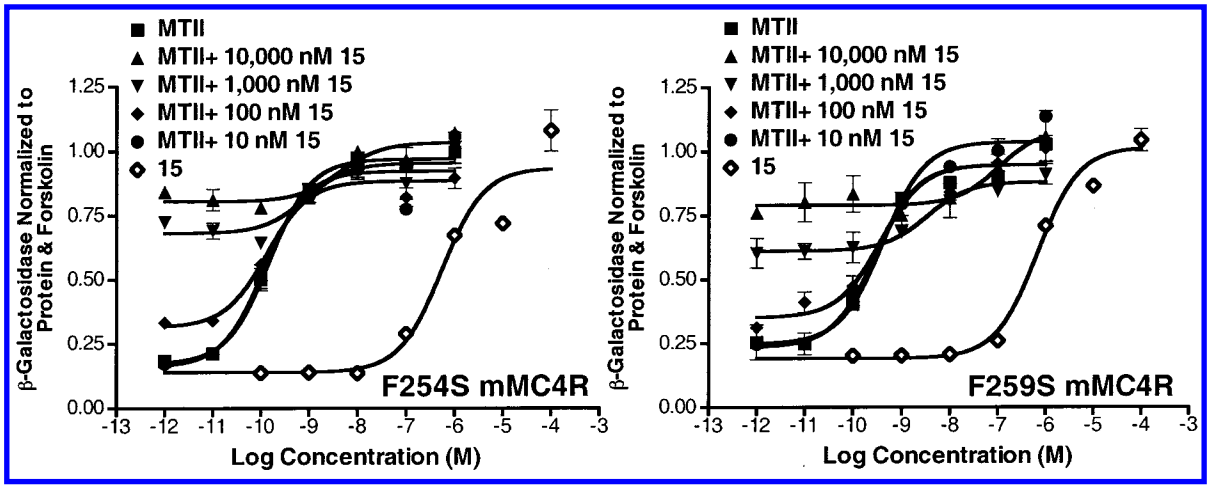

Figure 4. Illustration of the tetrapeptide **15**, Ac-His-DNal(2′)-Arg-Trp-NH_2, pharmacology at the mutant F254S and F259S mouse MC4 receptors. Similarly to the SHU9119 peptide, this DNal(2′)[7]-containing tetrapeptide results in agonist pharmacology at these two mutant mMC4 receptors whereas at the wild-type mMC4R (Supporting Information), competitive antagonist pharmacology results.

activity at these mutant receptors. Figure 3 shows the agonist pharmacology of the MTII, NDP-MSH, and tetrapeptide **1** presented herein and the partial agonist activity of the SHU9119 MC4R antagonist at these F254S and F259S mutant mMC4 receptors. We have previously identified these two mutant mMC4 receptors as being able to differentiate melanocortin sequence-based antagonists (i.e., SHU9119 and SHU9005) vs the endogenous antagonist AGRP(83−132) ligand pharmacology. To further test this hypothesis, we tested the tetrapeptide **15**, Ac-His-DNal(2′)-Arg-Trp-NH_2, at these F254S and F259S mutant mMC4 receptors to determine if these receptors would possess pharmacology similar to the melanocortin-based antagonist SHU9119. Figure 4 summarizes the pharmacology of the DNal(2′)-containing tetrapeptide **15** at the F254S and F259S mMC4Rs, resulting in agonist activity of the compound **15** that is a competitive antagonist at the wild-type mMC4R (Supporting Information). These data support our previous hypothesis that the mMC4R Phe 254 and 259 residues in the putative TM6 domain interact with the melanocortin DNal(2′) amino acid, which is substituted for the DPhe amino acid in the agonists.[16]

Mouse Melanocortin-5 Receptor. The peripheral MC5R is expressed in a variety of tissues and has been implicated as physiologically participating in the role of exocrine gland function.[1,14,40] The lead tetrapeptide **1** has been previously reported to possess a 3.4 nM agonist EC_{50} at the mMC5R[28] and a 17% response at a 5 μM concentration at the hMC5R[32] and possesses a 3.9 nM EC_{50} at the mMC5R reported herein. Similarly to the mMC3 and mMC4 receptors, tetrapeptide **2** containing Ala instead of the DPhe amino acid at the seven position (α-MSH numbering) results in a loss of determinable EC_{50} value at up to 100 μM concentrations at the mMC5R. Tetrapeptide **19**, containing the DTic[7], was the only compound in addition to **2** that lost agonist activity at up to 100 μM concentrations, although **11** (DPhg) had only slight agonist activity at up to 100 μM concentrations. It has been previously reported that replacement of DPhe with Ala at the seven position of the cyclic MTII template resulted in only 14% total binding at 10 μM and 5% agonist stimulation at 10 μM concentrations at the human MC5R.[33] Tetrapeptides possessing nanomolar mMC5R agonist potencies include the (pI)DPhe (**3**), DTyr (**7**), DNal(1′) (**13**), DNal(2′) (**15**), DBip (**21**), and Atc (peak 1, **25**) amino acids. These results suggest that modification of the DPhe[7] position by the (pI)DPhe, DBip, and possibly the DNal(2′) amino acids in small nonpeptidic molecules may possess similar mMC5R agonist potency at homologous compounds containing the DPhe[7] residue. Substitution of the DPhe with the DNal(2′) amino acid in a variety of melanocortin peptide templates resulted in mMC5R agonist activities ranging from nanomolar to micromolar, depending upon the template.[18−22] Amino acid substitutions of the DPhe in this tetrapeptide template that resulted in significantly decreased mMC5R agonist potencies include the homoPhe, Tyr, Trp, DPhg, Nal-(1′), Tic, His, and 3Pal amino acid side chains that appear to be less-tolerated in this position at this melanocortin receptor.

Conclusions

The study presented herein has resulted in the identification of a tetrapeptide, Ac-His-(pI)DPhe-Arg-Trp-NH_2, that possesses novel melanocortin receptor pharmacology in that it is a mMC3R partial agonist with antagonist acitivity ($pA_2 = 7.25$, $K_i = 56$ nM) but, unexpectedly, possess 25 nM mMC4R agonist activity. This molecule is potentially a physiologically useful compound for differentiating the MC3R vs MC4R in vivo activities in wild-type, nongenetically modified rodents and even primates where knocking out a particular receptor to study physiology is more difficult and costly. Furthermore, the tetrapeptide **15** Ac-His-DNal(2′)-Arg-Trp-NH_2, possessing the DNal(2′) amino acid that converts the MTII agonist into the SHU9119 antagonist, is a mMC3R partial agonist with agonist activity ($pA_2 = 6.5$, $K_i = 295$ nM) and a mMC4R antagonist ($pA_2 = 7.8$, $K_i = 17$ nM) and is only 60-fold less potent at the mMC3R and 425-fold less potent at the mMC4R, as compared with SHU9119. Thus, tetrapeptide **15** provides a linear template for the design of small modified peptidic[23] or nonpeptidic MC4R antagonists. After tetrapeptide **15** was examined at the mutant F254S and F259S mMC4 receptors, this tetrapeptide that possessed antagonist activity at the wild-type mMC4R is converted

Table 2. Analytical Data for the Peptides Synthesized in This Study[a]

peptide	structure	HPLC k′ (system 1)	HPLC k′ (system 2)	M + 1 (calcd)	mass spectral analysis (M + 1)	purity (%)
1	Ac-His-DPhe-Arg-Trp-NH_2	3.9	6.9	686.8	686.3	>98
2	Ac-His-**Ala**-Arg-Trp-NH_2	3.1	4.9	610.7	610.3	>98
3	Ac-His-**(pI)DPhe**-Arg-Trp-NH_2	5.0	8.3	812.7	812.0	>98
4	Ac-His-**homoPhe**-Arg-Trp-NH_2	5.3	8.4	700.8	700.3	>97
5	Ac-His-**homoDPhe**-Arg-Trp-NH_2	4.6	7.6	700.8	700.0	>96
6	Ac-His-**Tyr**-Arg-Trp-NH_2	3.8	6.2	702.8	702.2	>99
7	Ac-His-**DTyr**-Arg-Trp-NH_2	3.6	5.7	702.8	702.1	>99
8	Ac-His-**Trp**-Arg-Trp-NH_2	4.9	7.7	725.8	725.3	>99
9	Ac-His-**DTrp**-Arg-Trp-NH_2	4.2	6.9	725.8	725.3	>96
10	Ac-His-**Phg**-Arg-Trp-NH_2	4.3	6.9	672.8	672.3	>97
11	Ac-His-**DPhg**-Arg-Trp-NH_2	3.8	5.8	672.8	672.4	>98
12	Ac-His-**Nal(1′)**-Arg-Trp-NH_2	5.6	9.5	736.8	737.2	>98
13	Ac-His-**DNal(1′)**-Arg-Trp-NH_2	4.8	8.1	736.8	736.0	>97
14	Ac-His-**Nal(2′)**-Arg-Trp-NH_2	5.6	9.4	736.8	736.4	>98
15	Ac-His-**DNal(2′)**-Arg-Trp-NH_2	5.0	8.3	736.8	736.3	>98
16	Ac-His-**Dip**-Arg-Trp-NH_2	5.7	9.9	762.9	762.3	>98
17	Ac-His-**DDip**-Arg-Trp-NH_2	4.8	8.1	762.9	762.2	>98
18	Ac-His-**Tic**-Arg-Trp-NH_2	4.5	7.4	698.8	698.1	>96
19	Ac-His-**DTic**-Arg-Trp-NH_2	4.1	6.8	698.8	698.1	>98
20	Ac-His-**Bip**-Arg-Trp-NH_2	6.2	10.2	762.9	761.8	>98
21	Ac-His-**DBip**-Arg-Trp-NH_2	5.6	9.3	762.9	762.1	>98
22	Ac-His-**His**-Arg-Trp-NH_2	3.1	4.7	676.7	676.3	>99
23	Ac-His-**3Pal**-Arg-Trp-NH_2	3.1	4.9	687.8	687.3	>98
24	Ac-His-**4Pal**-Arg-Trp-NH_2	3.1	4.9	687.8	687.2	>98
25	Ac-His-**Atc**-Arg-Trp-NH_2	4.7	7.9	712.8	712.3	>98
26	Ac-His-**Atc**-Arg-Trp-NH_2	4.8	8.2	712.8	712.3	>96

[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.

to an agonist, resulting in similar pharmacology at these mutant mMC4 receptors observed of SHU9119.[16] These latter data further support our hypothesis that the DNal(2′) substitution of DPhe in the melanocortin peptide templates is putatively interacting with the mMC4R Phe 254 and 259 receptor residues.

Experiemental

Peptide synthesis was performed using standard Fmoc methodology[41] on an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY). The amino acids Fmoc-Tyr(tBu), Fmoc-His(Trt), Fmoc-DPhe, Fmoc-Trp(Boc), Fmoc-Ala, Fmoc-Phe, Fmoc-phenylglycine (Phg), Fmoc-D-phenylglycine (DPhg), Fmoc-3-(1-naphthyl)alanine [Nal(1′)], Fmoc-3-(1-naphthyl)-D-alanine [DNal(1′)], Fmoc-3-(2-naphthyl)alanine [Nal(2′)], Fmoc-3-(2-naphthyl)-D-alanine [DNal(2′)], Fmoc-3,3-diphenyl-alanine (Dip), and Fmoc-D-3,3-diphenyl-alanine (DDip) were purchased from Peptides International (Louisville, KY). Fmoc-DTrp(Boc) was purchased from Advanced ChemTech. Fmoc-D-homo-phenylalanine (hDPhe), Fmoc-homo-phenylalanine (hPhe), Fmoc-3-(3-pyridinyl)alanine (3-Pal), and Fmoc-3-(4-pyridinyl)alanine (4-Pal) were purchased from Bachem (Torrance, CA). Fmoc-4-phenyl-phenylalanine (Bip), Fmoc-4-phenyl-D-phenylalanine (DBip), Fmoc-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), Fmoc-D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (DTic), and Fmoc-4-iodo-D-phenylalanine (*p*-I-DPhe) were purchased from Synthetech (Albany, OR). Fmoc-amino-tetrahydro-2-naphthyl carboxylic acid (Atc) was purchased from Pharma Core (High Point, NC). The coupling reagents 2-(1-H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) were purchased from Peptides International. Glacial acetic acid (HOAc), dichloromethane (DCM), methanol (MeOH), acetonitrile (ACN), and anhydrous ethyl ether were purchased from Fisher (Fair Lawn, NJ). *N*,*N*-Dimethylformamide (DMF) was purchased from Burdick and Jackson (McGaw Park, IL). Trifluoroacetic acid (TFA), 1,3-diisopropylcarbodiimide (DIC), pyridine, piperidine, and acetic anhydride were purchased from Sigma (St. Louis, MO). *N*,*N*-Diisopropylethylamine (DIEA) and triisopropylsilane (Tis) were purchased from Aldrich (Milwaukee, WI). All reagents and chemicals were ACS grade or better and were used without further purification.

The peptides were assembled on rink-amide-MBHA resin (0.44 mequiv/g substitution), purchased from Peptides International. The synthesis was performed using a 40 well Teflon reaction block with a course Teflon frit. Approximately 100 mg of resin (0.044 mmole) was added to each reaction block well. The resin was allowed to swell for 2 h in DMF and deprotected using 25% piperidine in DMF for 5 min followed by a 20 min 25% piperidine incubation at 450 rpms. A positive Kaiser[42] test resulted indicating free amine groups on the resin. The growing peptide chain was added to the amide-resin using the general amino acid cycle as follows: 500 μL DMF is added to each reaction well to "wet the frit", 3-fold excess amino acid starting from the C terminus is added (275 μL of 0.5 M amino acid solution containing 0.5 M HOBt in DMF) followed by the addition of 275 μL of 0.5 M DIC in DMF, and the reaction well volume is brought up to 3 mL using DMF. The coupling reaction is mixed for 1 hr at 450 rpms, followed by emptying of the reaction block by positive nitrogen gas pressure. A second coupling reaction is performed by the addition of 500 μL of DMF to each reaction vessel, followed by the addition of 275 μL of the respective amino acid (3-fold excess), 275 μL of 0.5 M HBTU, and 225 μL of 1 M DIEA. The reaction well volume is brought up to 3 mL with DMF and mixed at 450 rpm for 1 h. After the second coupling cycle, the reaction block is emptied and the Nα-Fmoc-protected peptide-resin is washed with DMF (4.5 mL, four times). Nα-Fmoc deprotection is performed by the addition of 4 mL of 25% piperidine in DMF and mixed for 5 min at 450 rpms followed by a 20 min deprotection at 450 rpms. The reaction well is washed with DMF (4.5 mL, four times), and the next coupling cycle is performed as described above. Following Nα-Fmoc deprotection of the final amino acid, acetylation of the Nα-amine was performed by addition of 2 mL of acetic anhydride, 1 mL of pyridine, and 1 mL of DMF to the reaction block wells and mixed for 30 min at 450 rpms. The acetylated peptide-resin was washed with DCM (4 mL, five times) and dried thoroughly prior to cleavage from the resin. Deprotection of the amino acid side chains and cleavage of the acetylated-

peptide from the resin was performed by incubating the peptide-resin with 3 mL of cleavage cocktail (95% TFA, 2.5% water, and 2.5% Tis) for 3 h at 450 rpms. The cleavage product was emptied from the reaction block into a cleavage block containing 7 mL collection vials under positive nitrogen gas pressure. The resin was washed with 1.5 mL of cleavage cocktail for 5 min and 450 rpms and added to the previous cleavage solution. The peptides were transferred to preweighed 50 mL conical tubes and precipitated with cold (4 °C) anhydrous ethyl ether (up to 50 mL). The flocculent peptide was pelleted by centrifugation (Sorval Super T21 high-speed centrifuge using the swinging bucket rotor) at 4000 rpm for 5 min, the ether was decanted off, and the peptide was washed one time with cold anhydrous ethyl ether and again pelleted. The crude peptide was dried in vacuo for 48 h. The crude peptide yields ranged from 60 to 90% of the theoretical yields. A 15−30 mg sample of crude peptide was purified by RP-HPLC using a Shimadzu chromatography system with a photodiode array detector and a semipreparative RP-HPLC C18 bonded silica column (Vydac 218TP1010, 1.0 cm × 25 cm) and lyophilized. The purified peptides were at least >95% pure as determined by analytical RP-HPLC and had the correct molecular mass (University of Florida protein core facility), Table 2.

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

Functional Bioassay. HEK-293 cells stably expressing the melanocortin receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene as previously described.[16,28,43] Briefly, 5000−15 000 post transfection cells were plated into 96 well Primera plates (Falcon) and incubated overnight. Forty-eight hours posttransfection, the cells were stimulated with 100 μL of peptide (10^{-4}−10^{-12} M) or forskolin (10^{-4} M) control in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media 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. To the cell lysate plates, 40 μL of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, and 200 mg 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 was determined by adding 200 μL of 1:5 dilution BioRad 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). Data points were normalized both to the relative protein content and nonreceptor-dependent forskolin stimulation. The antagonistic properties of these compounds were evaluated by the ability of these ligands to competitively displace the MTII agonist (Bachem) in a dose-dependent manner, at up to 10 μM concentrations.[16] The pA_2 ($K_i = -\log pA_2$) values were generated using the Schild analysis method.[44]

Data Analysis. EC_{50} and pA_2 values represent the mean of duplicate experiments performed in quadruplet or more independent experiments. EC_{50} and pA_2 estimates, and their associated standard errors, were determined by fitting the data to a nonlinear least-squares analysis using the PRISM program (v3.0, GraphPad Inc.). The results are not corrected for peptide content, although all of the peptides examined in this study were determined to have approximately equal peptide content as determined by using Beers Law.

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Supporting Information Available: Illustration of antagonist pharmacology at the mMC3 and mMC4 receptors. This material is available free of charge via the Internet at http://pubs.acs.org.

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