Structure−Activity Relationships of the Melanocortin Tetrapeptide Ac-His-D-Phe-Arg-Trp-NH_2 at the Mouse Melanocortin Receptors. 4. Modifications at the Trp Position

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The melanocortin pathway is involved in the regulation of several physiological functions including skin pigmentation, steroidogenesis, obesity, energy homeostasis, and exocrine gland function. This melanocortin pathway consists of five known G-protein coupled receptors, endogenous agonists derived from the proopiomelanocortin (POMC) gene transcript, the endogenous antagonists Agouti and the Agouti-related protein (AGRP) and signals through the intracellular cAMP signal transduction pathway. The endogenous melanocortin agonists contain the putative message sequence "His-Phe-Arg-Trp," postulated to be important for melanocortin receptor molecular recognition and stimulation. Herein, we report a tetrapeptide library, based upon the template Ac-His-D-Phe-Arg-Trp-NH_2, consisting of 20 members that have been modified at the Trp^9 position (α-MSH numbering) and pharmacologically characterized for agonist activity at the mouse melanocortin receptors MC1R, MC3R, MC4R, and MC5R. Results from this study yielded compounds that ranged in pharmacological properties from equipotent to a loss of melanocortin receptor activity at up to 100 μM concentrations. Interestingly, modification of the Trp^9 in the tetrapeptide template at the MC1R resulted in only up to a 220-fold potency change, while at the MC4R and MC5R, up to a 9700-fold decrease in potency was observed, suggesting the MC1R is more tolerant of the modifications examined herein. The most notable results of this study include identification that the Trp^9 indole moiety in the tetrapeptide template is important for melanocortin-3 receptor agonist potency, and that this position can be used to design melanocortin ligands possessing receptor selectivity for the peripherally expressed MC1 and MC5 versus the centrally expressed MC3 and MC4 receptors. Specifically, the Ac-His-D-Phe-Arg-Tic-NH_2 and the Ac-His-D-Phe-Arg-Bip-NH_2 tetrapeptides possessed nanomolar MC1R and MC5R potency but micromolar MC3R and MC4R agonist potency. Additionally, these studies identified that substitution of the Trp amino acid with either Nal(2′) or D-Nal(2′) resulted in equipotent melanocortin receptor potency, suggesting that the chemically reactive Trp indole side chain may be replaced with the nonreactive Nal(2′) moiety for the design of nonpeptide melanocortin receptor agonists.

Introduction

The melanocortin receptors belong to the superfamily of seven transmembrane (TM) spanning G-protein coupled receptors (GPCR's) and stimulate the cAMP signal transduction pathway.[1] The endogenous agonist ligands for these melanocortin receptors are derived from 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).[2] All 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.[3−5] The melanocortin receptor family also has two endogenous antagonists, agouti[6] and the agouti-related protein (AGRP),[7,8] which are the only known naturally occurring antagonists of GPCR's 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.[9−11] The most knowledge regarding melanocortin receptor ligands are for the skin melanocortin-1 receptor (MC1R) which are involved in pigmentation and animal coat coloration.[12−14] Additionally, the melanocortin-5 receptor has been deleted from the mouse genome and identified as playing a role in exocrine gland function.[15]

The role of the Trp amino acid at the nine position of α-MSH derivative has not been previously explored at the cloned melanocortin receptors in extensive detail. A deletion of the Trp residue from the Glu-His-D-Phe-Arg-Trp-NH_2 peptide resulted in a ligand that was unable to bind or stimulate the hMC4R.[16] Substitution of the Trp amino acid by Ala or Pro in the cyclic MTII (Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2) agonist template[17,18] resulted in significantly reduced ligand binding affinity and intracellular cAMP stimulation.[19,20] Epimerization at the Trp nine position to the D-configuration in a cyclic melanocortin agonist template did not modify potency at the human MC3-5 receptors.[21] Interestingly, substitution of Trp by Nal(2′) in a cyclic agonist template resulted in equipotent activity at the hMC3R and

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Table 1. 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-D-Phe-Arg-Trp-NH$_2$	3.9	6.9	686.8	686.3, >98
2	Ac-His-D-Phe-Arg-Ala-NH$_2$	0.9	4.0	571.6	571.2, >98
3	Ac-His-D-Phe-Arg-D-Trp-NH$_2$	4.1	6.3	686.8	686.9, >99
4	Ac-His-D-Phe-Arg-His-NH$_2$	2.7	3.9	637.7	637.2, >96
5	Ac-His-D-Phe-Arg-Phe-NH$_2$	3.9	6.6	647.7	647.6, >98
6	Ac-His-D-Phe-Arg-D-Phe-NH$_2$	3.7	6.3	647.7	647.0, >99
7	Ac-His-D-Phe-Arg-Nal(1′)-NH$_2$	5.1	7.1	697.8	698.8, >99
8	Ac-His-D-Phe-Arg-D-Nal(1′)-NH$_2$	5.0	8.1	697.8	696.7, >98
9	Ac-His-D-Phe-Arg-Nal(2′)-NH$_2$	5.2	7.2	697.8	697.9, >98
10	Ac-His-D-Phe-Arg-D-Nal(2′)-NH$_2$	5.0	8.1	697.8	697.3, >98
11	Ac-His-D-Phe-Arg-Tic-NH$_2$	4.3	7.1	659.8	658.9, >98
12	Ac-His-D-Phe-Arg-D-Tic-NH$_2$	4.2	7.0	659.8	659.3, >99
13	Ac-His-D-Phe-Arg-Dip-NH$_2$	5.6	8.9	723.8	723.2, >99
14	Ac-His-D-Phe-Arg-D-Dip-NH$_2$	5.4	8.6	723.8	722.8, >99
15	Ac-His-D-Phe-Arg-Bip-NH$_2$	6.0	9.5	723.8	723.1, >99
16	Ac-His-D-Phe-Arg-D-Bip-NH$_2$	5.9	9.1	723.8	723.8, >98
17	Ac-His-D-Phe-Arg-4Pal-NH$_2$	2.7	3.9	648.7	648.9, >96
18	Ac-His-D-Phe-Arg-3Bal-NH$_2$	5.0	6.5	703.8	703.9, >99
19	Ac-His-D-Phe-Arg-2Thi-NH$_2$	3.7	6.2	653.8	652.8, >99
20	Ac-His-D-Phe-Arg-Atc-NH$_2$ (peak 1)	4.1	7.2	672.8	672.6, >99
21	Ac-His-D-Phe-Arg-Atc-NH$_2$ (peak 2)	4.3	7.6	672.8	672.7, >99

[a] HPLC k' = [(peptide retention time − sovent 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 214λ.

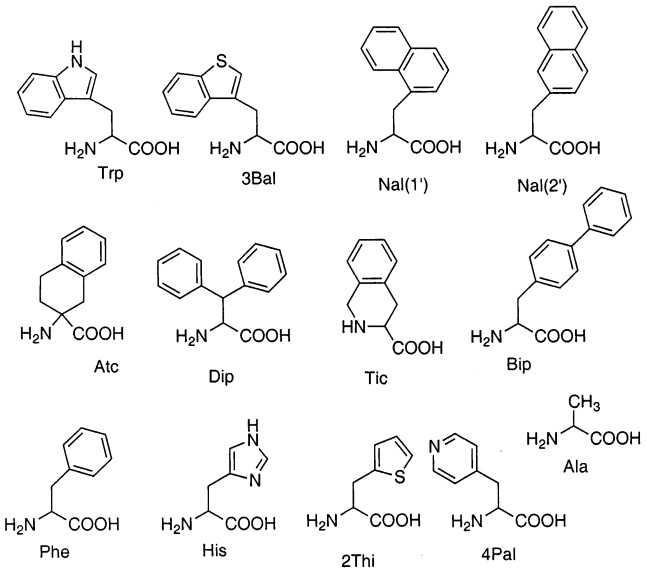

Figure 1. Structures of the amino acids used to replace Trp in the peptide template Ac-His-D-Phe-Arg-Xaa-NH$_2$.

hMC4R, yet was unable to stimulate an agonist response at the hMC5R at up to 5 μM,[22] yet resulted in the conversion of a partial agonist to a MC5R antagonist in a different cyclic peptide template.[23] These limited studies implicate an important role of the Trp amino acid for melanocortin receptor binding and functional activity. Putative ligand−receptor interactions involving the ligand Trp amino acid and the melanocortin-1 and -4 receptors have been postulated,[24,25] but receptor mutagenesis studies[16,26,27] remain inconclusive regarding the specific receptor residues that are interacting with the ligand Trp amino acid. This study was undertaken to examine the role of various aromatic, natural, and unnatural amino acids (Figure 1) in the Trp position of the tetrapeptide Ac-His-D-Phe-Arg-Trp-NH$_2$ for structure−activity-relationships and selectivity properties at the mouse melanocortin receptors. Furthermore, compounds identified from this study resulting in unique receptor pharmacological properties could potentially be utilized to probe melanocortin ligand−receptor interactions at the Trp position to potentially aid in the design of receptor selective small molecules.

Results

Chemical Synthesis and Characterization. The peptides reported herein were synthesized using standard fluorenylmethyloxycarbonyl (Fmoc)[28,29] chemistry and a parallel synthesis strategy on an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY). The peptides were purified to homogeneity using semipreparative reversed-phase high-pressure liquid chromatography (RP-HPLC). The peptides possessed the correct molecular weights, determined by mass spectrometry (Table 1). The purity of these peptides were assessed by analytical RP-HPLC in two diverse solvent systems (Table 1) and one-dimensional ^{1}H NMR (Supporting Information).

Biological Evaluation. Table 2 summarizes the pharmacology at the mouse melanocortin receptors, mMC1R, mMC3R, mMC4R, and mMC5R of the 20 tetrapeptides modified at the Trp nine position (α-MSH numbering) of the tetrapeptide template, Ac-His-D-Phe-Arg-Xaa-NH$_2$ prepared in this study. The amino acids substituted at the Trp^9 position include Ala, D-Trp, His, L/D-Phe, L/D-Nal(1′), L/D-Nal(2′), L/D-Tic, L/D-Dip, L/D-Bip, 4Pal, 3Bal, 2Thi, and racemic Atc. The compounds that did not show agonist activity, >100000 nM EC$_{50}$ values in Table 2, were tested for antagonism at up to 10 μM concentrations, but did not possess antagonistic pharmacological profiles (data not shown). Figure 2 illustrates the amino acid substitutions of Trp and the corresponding effect at the melanocortin receptors.

The tetrapeptide Ac-His-D-Phe-Arg-Trp-NH$_2$ (**1**) is the lead peptide for this study and has been previously reported at the mouse melanocortin receptors[29] to possess 20 nM, 156 nM, 17 nM, and 4 nM agonist

Table 2. Functional Activity of the Trp[9]-Modified Tetrapeptides at the Mouse Melanocortin Receptors[a]

		mMC1R		mMC3R		mMC4R		mMC5R	
peptide	structure	EC_{50} (nM)	fold diff	EC_{50} (nM)	fold diff	EC_{50} (nM)	fold diff	EC_{50} (nM)	fold diff
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-D-Phe-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-D-Phe-Arg-Trp-Lys]-NH_2	0.020 ± 0.003		0.16 ± 0.03		0.087 ± 0.008		0.16 ± 0.03	
1	Ac-His-D-Phe-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-D-Phe-Arg-Ala-NH_2	4400 ± 176	219	>100000		43700 ± 6400	2540	38400 ± 11100	9700
3	Ac-His-D-Phe-Arg-**D**-Trp-NH_2	140 ± 60	7	5400 ± 1100	35	240 ± 30	14	35.3 ± 13	9
4	Ac-His-D-Phe-Arg-His-NH_2	3800 ± 1500	190	>100000		27600 ± 4400	1600	36000 ± 10900	9090
5	Ac-His-D-Phe-Arg-Phe-NH_2	530 ± 240	27	27000 ± 7000	173	7800 ± 2500	453	890 ± 110	224
6	Ac-His-D-Phe-Arg-D-Phe-NH_2	2500 ± 1900	124	15000 ± 2300	96	1000 ± 150	58	470 ± 150	118
7	Ac-His-D-Phe-Arg-Nal(1′)-NH_2	730 ± 320	37	3500 ± 1300	22	260 ± 30	15	33.1 ± 3.9	8
8	Ac-His-D-Phe-Arg-D-Nal(1′)-NH_2	750 ± 220	37	21900 ± 4900	140	5400 ± 1260	314	750 ± 80	189
9	Ac-His-D-Phe-Arg-Nal(2′)-NH_2	17.9 ± 6.0	1	740 ± 160	5	15.8 ± 0.2	1	4.86 ± 1.50	1
10	Ac-His-D-Phe-Arg-D-Nal(2′)-NH_2	127 ± 27	6	1620 ± 630	10	46.2 ± 7.9	3	12.1 ± 3.3	3
11	Ac-His-D-Phe-Arg-Tic-NH_2	43.0 ± 12.5	2	23200 ± 2300	149	8500 ± 930	494	700 ± 99	176
12	Ac-His-D-Phe-Arg-D-Tic-NH_2	1500 ± 210	75	>100000		39400 ± 16500	2290	24800 ± 7300	6263
13	Ac-His-D-Phe-Arg-Dip-NH_2	2300 ± 710	114	53300 ± 12300	342	11600 ± 3600	6,740	7100 ± 1100	1790
14	Ac-His-D-Phe-Arg-D-Dip-NH_2	680 ± 140	34	16000 ± 1300	103	7800 ± 1800	453	7440 ± 1900	1880
15	Ac-His-D-Phe-Arg-Bip-NH_2	51.9 ± 9.9	3	13400 ± 810	86	2700 ± 460	157	100 ± 20	26
16	Ac-His-D-Phe-Arg-D-Bip-NH_2	310 ± 70	15	16200 ± 4800	104	1500 ± 380	87	150 ± 13	38
17	Ac-His-D-Phe-Arg-4Pal-NH_2	2240 ± 770	111	>100000		30000 ± 12300	1740	14500 ± 1400	3660
18	Ac-His-D-Phe-Arg-3Bal-NH_2	79.9 ± 8.7	4	3700 ± 1400	24	143 ± 24	8	47.7 ± 3.8	12
19	Ac-His-D-Phe-Arg-2Thi-NH_2	2780 ± 1130	138	30250 ± 4600	194	8120 ± 1100	472	850 ± 90	215
20-peak 1	Ac-His-D-Phe-Arg-Atc-NH_2	700 ± 250	35	9900 ± 1800	63	3460 ± 1260	201	180 ± 70	46
21-peak 2	Ac-His-D-Phe-Arg-Atc-NH_2	680 ± 190	34	33500 ± 12200	215	8800 ± 1340	512	960 ± 90	243

[a] The indicated errors represent the standard error of the mean determined from at least three independent experiments. >100000 indicates that agonist or antagonist activity was not observed for these compounds at up to 100 μM concentrations.

activity at the mMC1R, mMC3R, mMC4R, and mMC5R, reported herein. Replacement of the Trp indole side chain with the methyl of Ala (**2**) resulted in 200- to 9700-fold decreased activity at the MC1R, MC4R, and MC5R and was unable to stimulate the MC3R at up to 100 μM concentrations. Inversion of stereochemistry from L-Trp to D-Trp (**3**) resulted in 7- to 35-fold decreased MC1R, and MC3-5 R potency, and was 22-fold selective for the MC4R versus the MC3R. Replacement of the indole ring by the imidazole side chain of His (**4**) resulted in a loss of agonist activity at the MC3R at up to 100 μM, and 190- to 9000-fold decreased MC1R, MC4R, and MC5R potency as compared to 1, similarly to the Ala (**2**) substitution. Incorporation of the benzyl moiety in either the Phe (**5**) or D-Phe (**6**) configurations resulted in 27- to 450-fold decreased melanocortin receptor potency, compared to **1**. The Nal(1′) containing tetrapeptide **7**, resulted in a 8- to 37-fold decrease in melanocortin receptor potency, whereas the D-Nal(1′) tetrapeptide **8**, resulted in 37- to 300-fold decreased receptor potency, compared with **1**. Interestingly, the Nal(2′) (**9**) and D-Nal(2′) (**10**) tetrapeptides were equipotent with the lead tetrapeptide **1** (within the inherent 3-fold experimental error) at the MC4R and MC5R and resulted in up to a 10-fold decreased potency at the MC1R and MC3R. Incorporation of a conformationally constrained Tic residue (**11**, Figure 1)[30−32] at the nine position was equipotent with **1** at the MC1R, but possessed 150- to 490-fold decreased potency at the MC3-5Rs. The D-Tic[9] compound **12** resulted in 75-fold decreased potency at the MC1R, 2290- to 6200-fold decreased potency at the MC4R and MC5R, respectively, compared to **1**, and did not possess agonist or antagonist pharmacology at the MC3R. The Dip (**13**) and D-Dip (**14**) analogues resulted in 34- to 6700-fold decreased melanocortin receptor potency, as compared with the Trp amino acid (**1**). The Bip (**15**) containing peptide was equipotent with **1** at the MC1R (within experimental error) and possessed 26- to 150-fold decreased potency at the MC3-5Rs. Tetrapeptide **16**, D-Bip,[9] resulted in 15- to 100-fold decreased potency at the melanocortin receptors, compared with **1**. Incorporation of the unusual amino acid 4Pal (**17**) resulted in similar pharmacology as the Ala (**2**) substitution where a loss of activity was observed at the MC3R and 100- to 3600-fold decreased potency resulted at the MC1R, MC4R, and MC5R, compared with **1**. The 3Bal compound **18**, with the indole N replaced with S (Figure 1), was nearly equipotent with the Trp containing homologue **1** at the MC1R but resulted in 8- to 24-fold decreased MC3-5R potency. The 2Thi[9] moiety (**19**) is a modified His side chain (Figure 1), and resulted in 130- to 470-fold decreased melanocortin receptor potency, compared with **1**. Comparison of the His[9] (**4**) and the 2Thi[9] (**19**) resulted in equipotency at the MC1R and MC4R, agonist activity of **19** at the MC3R, and a 42-fold difference in potency at the MC5R. Finally, incorporation of the racemic Atc[9] amino acid resulted in two peptides that eluted at different retention times (Table 1), and designated peak 1 (**20**) and peak 2 (**21**) based upon the order of their elution from the RP-HPLC column. These Atc containing tetrapeptides **20** and **21** resulted in 34- to 510-fold decreased potency at the melanocortin receptors, compared with **1**.

Discussion

Melanocortin-1 Receptor. The peripheral skin melanocortin receptor, MC1R, is involved in human skin pigmentation[13,33] and animal coat coloration.[14] The lead tetrapeptide **1**, Ac-His-D-Phe-Arg-Trp-NH_2, has been previously reported to possess 25 nM stimulatory activity at the mMC1R,[29] an EC_{50} value of 200 nM in the classical *Rana pipiens* frog skin assay (putative MC1R),[5] and possesses a mMC1R EC_{50} = 20 nM reported herein.

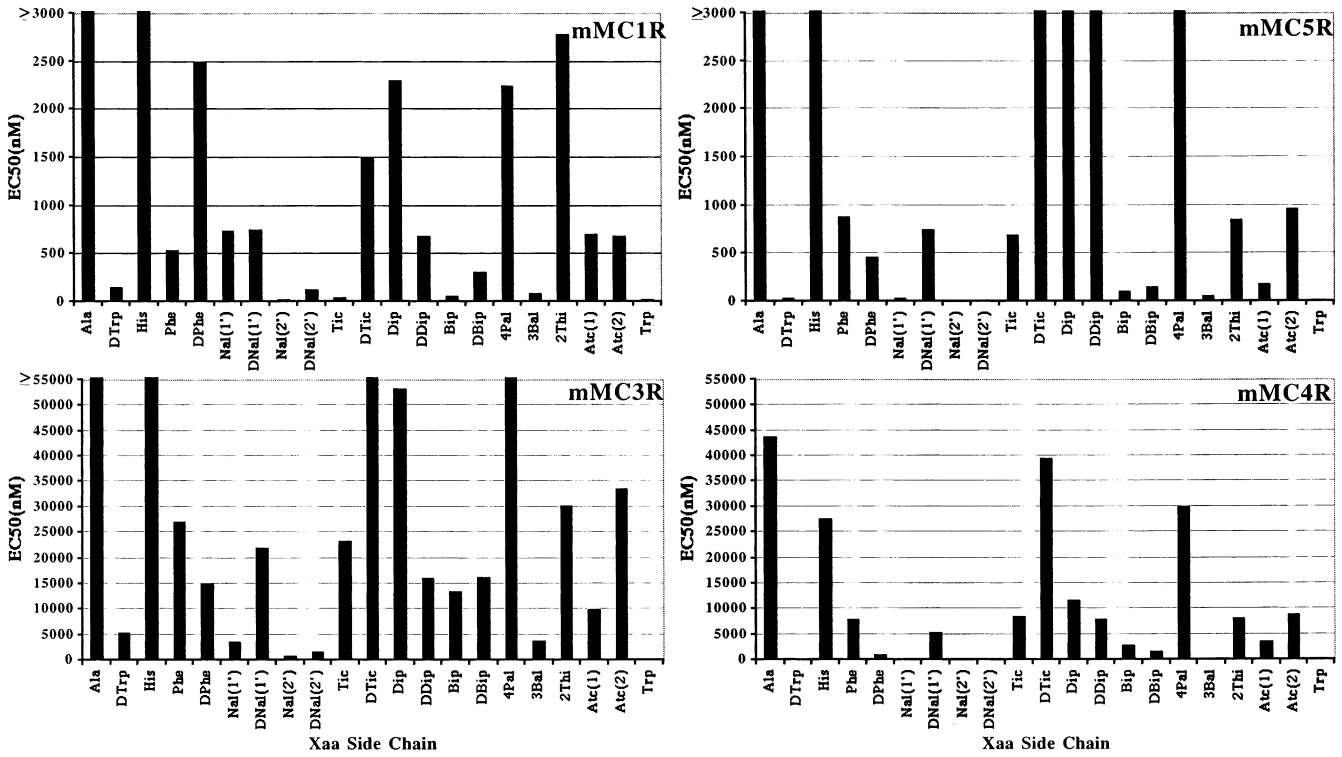

Figure 2. Graphical representation summarizing the effect on melanocortin receptor agonist EC_{50} values of the indicated amino acid substitution of the Trp residue in the tetrapeptide template Ac-His-D-Phe-Arg-Trp-NH_2.

Substitution of the Trp^9 (α-MSH numbering) side chain of **1** with the methyl of Ala (**2**) or the imidazole of His (**4**) resulted in ca. 200-fold decreased MC1R potency, suggesting that putative melanocortin ligand Trp^9 side chain-receptor interactions, versus peptide backbone−receptor interactions, are important at this position for MC1R potency. It has been previously reported that when Trp^9 is replaced with an Ala in the endogenous α-MSH peptide template, a 2000-fold decreased binding was observed in B16 mouse melanoma cells (putative MC1R),[34] consistent with the results presented herein. Substitution of Trp^9 in the tetrapeptide template by D-Trp (**3**), Phe (**5**), Nal(1′) (**7**), D-Nal(1′) (**8**), Nal(2′) (**9**), D-Nal(2′) (**10**), Tic (**11**), D-Dip (**14**), Bip (**15**), D-Bip (**16**), 3Bal (**18**), and Atc (**20, 21**) resulted in nanomolar MC1R potency (Table 2, Figure 2). The Nal(2′) amino acid substitution for Trp in the tetrapeptide Ac-His-D-Phe-Arg-Trp-NH_2 resulted in identical MC1R agonist potency. This observation may be predicted a priori based upon scrutiny of the amino acid side chain structures (Figure 1), and a previous report substituting Nal(2′) at the Trp position in the cyclic MTII and SHU9119 templates.[23] Interestingly, the Tic (**2**) and Bip (**15**) amino acid modifications that possess significantly different biophysical and structural properties compared with the Trp residue (Figure 1) are equipotent with the Trp containing tetrapeptide at the MC1R. While very limited structure−activity relationships of the Trp residue at the MC1R have been reported, previous studies examining the incorporation of the four isomers of β-MeTrp[35−37] at the nine position of the MTII cyclic agonist template resulted in the discovery of the importance of the Trp amino acid for MC1R potency, prolonged biological activity, and receptor dissociation kinetics.[38−40] These previous reports, and the data presented herein, suggest that the importance of the indole side chain at the nine position of melanocortin ligands is important and warrants further experimental probing to determine if this site can be utilized for the design of receptor selective ligands.

Melanocortin-3 Receptor. The MC3R is expressed both peripherally and centrally and appears to be involved in metabolism and energy homeostasis.[9,10,41,42] The lead tetrapeptide **1**, Ac-His-D-Phe-Arg-Trp-NH_2, has been previously reported to possess a 195 nM agonist EC_{50} at the mMC3R,[29] a 1000 nM EC_{50} at the hMC3R,[43] and a 156 nM EC_{50} value reported herein. Substitution of the Trp amino acid side chain at the nine position (α-MSH numbering) of the lead tetrapeptide by Ala, His, D-Tic, and 4Pal (Figure 1) resulted in a loss of MC3R agonist or antagonist activity at up to 100 μM concentrations. Incorporation of the Nal(2′), D-Nal(2′), Nal(1′), and 3Bal resulted in only up to a 24-fold decreased MC3R potency, compared to the Trp indole ring. Previous reports of Trp^9 substitution by Ala^9 and Pro^9 in the cyclic peptide MTII resulted in slight MC3R binding and 2% cAMP stimulation at the hMC3R,[19,20] similar to the results presented herein for the tetrapeptide Ac-His-D-Phe-Arg-Ala-NH_2. When Trp^9 is modified to the D-Trp configuration, a 35-fold decreased MC3R potency is observed. Inversion of chirality of Trp to D-Trp in the cyclic SHU9119 (Ac-Nle-c[Asp-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2) antagonist template resulted in equivalent binding affinity at the hMC3R.[21] Similar to the effects of Nal(2′)^9 incorporation into the tetrapeptide template **9**, substitution of Trp^9 with Nal(2′) in other melanocortin cyclic peptide templates resulted in a equipotent MC3R agonist[22] or antagonist.[23] Generally, modifications at the Trp^9 position in the tetrapeptide template examined herein resulted in micromolar agonist MC3R values, suggesting that the MC3R is less tolerant of substitu-

tions at this position, compared with the MC1, MC4, and MC5 receptors.

Melanocortin-4 Receptor. The central MC4R has been identified as physiologically participating in food consumption[44] and obesity in mice[11] with several polymorphisms of the MC4R observed in obese humans.[45−50] The lead tetrapeptide in this study, Ac-His-D-Phe-Arg-Trp-NH_2 (**1**), was previously reported to possess a 10 nM agonist EC_{50} value at the mMC4R,[29] and 8 nM[16] and 47 nM[43] agonist EC_{50} values at the hMC4R, with a potency at the mMC4R of 17 nM reported herein. Substitution of the Trp^9 indole side chain with the side chain moieties of Ala, His, D-Tic, and 4-Pal resulted in >25 μM EC_{50} values, demonstrating that these modifications are not well tolerated at the MC4R. The substitution of Ala^9 and Pro^9 in the MTII cyclic template also resulted in peptide analogues that did not bind or stimulate the hMC4R.[19,20] Substitution of the Trp^9 residue by Nal(2′) and D-Nal(2′) resulted in equipotent MC4R agonists suggesting that the Nal(2′) side chain may be used to substitute for the chemically reactive Trp indole for the design of nonpeptidic MC4R selective ligands. Previous substitution of the Trp^9 with Nal(2′) in cyclic templates also resulted in equipotent compounds at the MC4R,[22,23] supporting the use of these substitutions in nonpeptide as well as peptide templates developed for MC4R receptor selective ligands. The 3Bal amino acid that contains a sulfur instead of the nitrogen heteroatom in the side chain (Figure 1) resulted in 8-fold decreased MC4R agonist potency. Incorporation of Leu and Phe at the nine position of the NDP-MSH template resulted in 13-fold decreased MC4R potency for the Leu substitution and equipotency for the Phe substitution at the hMC4R, compared to NDP-MSH.[16] Substitution of the Trp^9 by Phe^9 in the tetrapeptide **5**, however, resulted in 450-fold decreased MC4R potency, suggesting that the indole ring interactions with the MC4R may be necessary for potency in melanocortin ligands of decreased molecular weights.

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.[1,15,51] The lead tetrapeptide **1**, Ac-His-D-Phe-Arg-Trp-NH_2 has been previously reported to possess a 3.4 nM agonist EC_{50} at the mMC5R,[29] and a 17% response at a 5 μM concentration at the hMC5R,[43] and possesses a 3.9 nM EC_{50} at the mMC5R reported herein. Incorporation of Ala, His, D-Tic, Dip, D-Dip, and 4Pal at the nine position in the tetrapeptide template Ac-His-D-Phe-Arg-Xaa^9-NH_2 resulted in less potent micromolar EC_{50} values than for Ac-His-D-Phe-Arg-Trp-NH_2 (**1**) at the MC5R. Conversely, substitution of the Trp^9 indole with the side chains of Nal(1′), Nal(2′), D-Nal(2′), and 3Bal resulted in up to only a 12-fold decrease in MC5R agonist potency. Interestingly, substitution of the Trp^9 with Nal(2′) in cyclic peptide templates resulted in a lack of MC5R agonist activity at up to 5 μM,[22] or conversion of a MC5R antagonist into a MC5R partial agonist.[23] At the MC5R, tetrapeptides modified at the indole Trp^9 side chain resulted from equipotent to 9700-fold decreased potency. These data suggest that the MC5R is less tolerant of Trp^9 modifications that appear to be important for potent agonist activity. Furthermore, the MC5 receptor may provide a tool to putatively identify melanocortin ligand−receptor indole interactions using a combination of receptor and ligand complementary mutagenesis experiments.

Stereochemistry L to D Amino Acid Modifications. Epimerization from the natural L-configuration to the D-configuration at the Phe seven position of melanocortin peptides generally results in enhanced agonist or antagonist potency.[12,18,52−54] Using the tetrapeptide template Ac-His-D-Phe-Arg-Trp-NH_2, modification of the stereochemistry at the Trp position did not result in consistent stereochemical preference, other than the D-Trp analogue resulted in 7- to 35-fold decreased melanocortin receptor potency, compared with the lead tetrapeptide. Inversion of chirality of the Trp residue in cyclic melanocortin ligand templates generally resulted in equipotent hMC3-5 receptor agonists.[21] At the MC1R, the stereochemical inversion of the Nal(1′) and Dip amino acids resulted in equipotent compounds. The Phe, Nal(2′), and Bip stereochemical inversions resulted in 3- to 5-fold differences in MC1R potency with the L-configuration being the more potent. The most significant difference in MC1R potency was the Tic to D-Tic modification that resulted in a 38-fold change in potency. At the MC3R, the epimer of the L-amino acids Phe, Nal(2′), Dip, and Bib amino acids resulted in equipotent derivatives, whereas the Nal(1′) analogues resulted in a 6-fold difference in potency. At the MC4R, only the Nal(2′) and Bip residues resulted in equipotency upon stereochemical inversion, with the Phe, Nal(1′), Tic, and Dip residues resulting in 4- to 20-fold changes in potency. At the MC5R, the Phe, Nal(2′), Dip, and Bip amino acids retained equipotency, but the Nal(1′) and Tic L- to D-configurations resulted in 23- to 36-fold potency changes. These data suggest that unlike the general increase in potency observed for D-Phe^7 (α-MSH numbering)-containing compounds, potency of the melanocortin ligand depends on the chirality and particular amino acid substitution at the Trp^9 position in addition to the melanocortin receptor isoform.

Melanocortin Receptor Selectivity. The melanocortin pathway consists of five known receptor isoforms, located in a variety of tissues, and is involved in a variety of physiological functions.[55] The melanocortin receptor knock out mice have provided valuable information attributing which melanocortin receptor to a particular phenotype, but not all the physiological functions attributed to the melanocortin pathway have been linked to a specific melanocortin receptor(s). Therefore, the search for novel melanocortin receptor selective ligands with unique pharmacology is being pursued.[19,21,22,54,56−60] Comparison of the centrally expressed MC3 and MC4 receptors resulted in identification of the D-Nal(1′)^9-containing tetrapeptide that possessed 46-fold MC4R versus MC3R selectivity. The D-Nal(2′)^9 tetrapeptide possessed 35-fold MC4R versus MC3R selectivity. The $3Bal^9$ tetrapeptide possessed 26-fold MC4R versus MC3R selectivity. The most notable results from this Trp^9 substitution study is the identification of the Tic and Bip substitutions that result in nanomolar agonist activity at the peripherally expressed MC1R and MC5R, but possessed micromolar agonist potencies at the centrally expressed MC3 and MC4 receptors (Figure 3). The Tic^9 tetrapeptide is 540- and

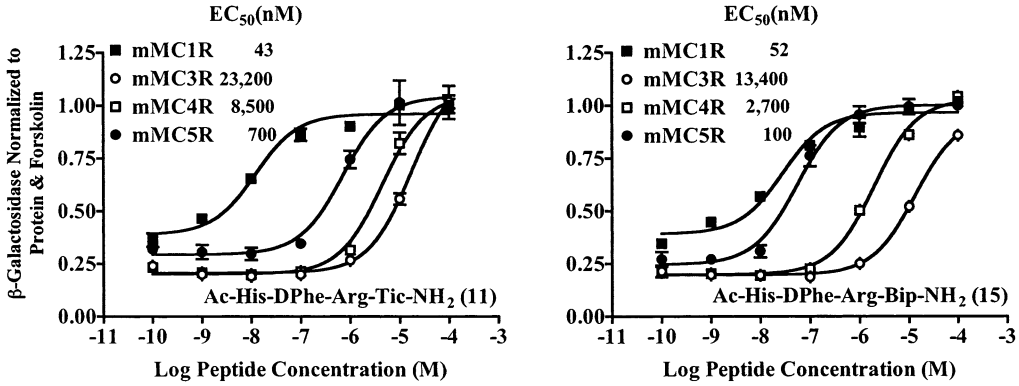

Figure 3. Comparison of the Tic^9 (**11**) and Bip^9 (**15**) containing tetrapeptide EC$_{50}$ values at the mMC1 and mMC3-5 receptors. These tetrapeptides are selective for the MC1R and MC5R versus the MC3 and MC4 receptors.

197-fold selective for the MC1R versus the MC3R and MC4R, respectively, and 12- to 33-fold selective for the MC5R versus the MC4R and MC3R, respectively. The Bip^9 tetrapeptide is 257- and 52-fold selective for the MC1R versus the MC3R and MC4R, respectively, and 130- to 26-fold selective for the MC5R versus the MC3R and MC4R, respectively. These data provide experimental evidence that the Trp^9 position of melanocortin ligands may be further explored for the design of melanocortin ligands that are potent MC1R and MC5R molecules with little or no activity at the central MC3 and MC4 receptors.

Conclusions

This study reports modifications of the Trp^9 side chain indole ring with various aromatic and nonaromatic substitutions in the tetrapeptide template Ac-His-D-Phe-Arg-Trp-NH$_2$. The Trp^9 indole moiety in the tetrapeptide template is important for melanocortin receptor potency, particularly at the MC3R. Substitutions at the Trp nine position by Tic and Bip may used for the design of the peripheral MC1R and MC5R versus the central MC3R and MC4R selective ligands.

Experiemental Section

Peptide synthesis was performed using standard Fmoc methodology[28] on an automated synthesizer (Advanced ChemTech 440MOS, Louisville, KY). The amino acids Fmoc-His(Trt), Fmoc-Arg(Pbf), Fmoc-Trp(Boc), Fmoc-Ala, Fmoc-Phe, Fmoc-D-Phe, Fmoc-3-(1-naphthyl)alanine [Nal(1′)], Fmoc-3-(1-naphthyl)-D-alanine [D-Nal(1′)], Fmoc-3-(2-naphthyl)alanine [Nal(2′)], Fmoc-3-(2-naphthyl)-D-alanine [D-Nal(2′)], Fmoc-3,3-diphenyl-alanine (Dip), and Fmoc-D-3,3-diphenyl-alanine (D-Dip) were purchased from Peptides International (Louisville, KY). Fmoc-D-Trp(Boc) was purchased from Advanced ChemTech (Louisville, KY). Fmoc-β-(3-benzothienyl)alanine (3Bal), Fmoc-3-(2-thienyl)alanine (2-Thi), and Fmoc-3-(4-pyridinyl)alanine (4-Pal) were purchased from Bachem (Torrance, CA). Fmoc-4-phenyl-phenylalanine (Bip), Fmoc-4-phenyl-D-phenylalanine (D-Bip), Fmoc-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) and Fmoc-D-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (D-Tic) were purchased from Synthetech (Albany, OR). Fmoc-(racemic)-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 mmol) 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[61] 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 of 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 0.5M 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 l h 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, 4 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 cleavage cocktail (95% TFA, 2.5% water, 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 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 to 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 × 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 1.

One-Dimensional ^{1}H Nuclear Magnetic Resonance Spectroscopy (NMR, Supporting Information). Peptides were analyzed for purity and structural integrity by nuclear magnetic resonance (NMR). Peptides were dissolved in 600 μL of DMSO-d_6 that contained 0.1% TMS, with an approximate final concentration of 2 mM. ^{1}H NMR spectra were obtained at 27 °C on a Bruker Avance 500 MHz spectrometer in the Advanced Magnetic Resonance Imaging and Spectroscopy facility at the McKnight Brain Institute, University of Florida. One-dimensional ^{1}H data were collected using the decoupler coil of a Bruker 5 mm BBO probe with 128 scans, 26684 total time domain points, a tip angle of 45°, an acquisition time of 2 s, and a delay time of 3 s. The spectral widths were 12 ppm, and TMS was referenced to 0.0 ppm. To correctly determine the integral values of peaks that occasionally occurred in the region around 3.3 ppm, a standard presaturation procedure (Bruker zgf2pr) for H_2O in DMSO was used. Prior to Fourier transformation, the FID was apodized with an exponential line broadening of 0.5 Hz and transformed with minimal zero-filling to 16K data points. The data were processed and analyzed using Bruker XWINNMR and XWINPLOT software.

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 cell/100-mm dish. Melanocortin receptor DNA in the pCDNA$_3$ expression vector (20 μg) were 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 CRE/β-galactosidase reporter gene as previously described.[26,29,62] Briefly, 5000 to 15000 post transfection cells were plated into 96 well Primera plates (Falcon) and incubated overnight. Forty-eight hours post-transfection the cells were stimulated with 100 μL peptide (10^{-4} to 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° 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 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, 200 mg of 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 Bio Rad G250 protein dye:water to the 10 μL of 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.

Data Analysis. EC_{50} values represent the mean of duplicate experiments performed in quadruplet or more independent experiments. EC_{50} value 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 the peptides examined in this study were determined to have approximately equal peptide content as determined by using Beers Law.

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