Development of Cyclic γ-MSH Analogues with Selective hMC3R Agonist and hMC3R/hMC5R Antagonist Activities

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A series of cyclic lactam analogues of γ-MSH (H-Tyr[1]-Val[2]-Met[3]-Gly[4]-His[5]-Phe[6]-Arg[7]-Trp[8]-Asp[9]-Arg[10]-Phe[11]-Gly[12]-OH) with a bulky hydrophobic residue in the direct proximity to the pharmacophore (Xaa-D-Phe/D-Nal(2′)-Arg-Trp) were designed and synthesized by solid-phase methods. A variety of amino acids with a broad range of hydrophobic/hydrophilic properties was introduced in position 5 to further explore their complementary role in receptor selectivity. Biological evaluation of these peptides revealed several analogues with potent hMC3R agonist and hMC3R/hMC5R antagonist activities, and good receptor selectivity. Analogue **4,** c[Nle-Arg-D-Phe-Arg-Trp-Glu]-NH_2, was found to be a very potent and selective hMC3R agonist (EC_{50} = 1.2 nM, 112% act). In addition, analogue **13**, c[Nle-Val-D-Nal(2′)-Arg-Trp-Glu]-NH_2, was identified as an hMC3R/hMC5R antagonist with the best selectivity against the hMC4R in this series (pA_2(hMC3R) = 8.4; pA_2(hMC5R) = 8.7). These results indicate the significance of steric factors in melanocortin receptor selectivity and suggest that introduction of bulky residues in the direct proximity to the melanocortin pharmacophore is an effective approach to design of novel hMC3R and hMC5R selective ligands.

Introduction

The natural melanotropin peptides include α-, β-, and γ-melanocyte-stimulating hormones (MSH) and adrenocorticotropin (ACTH). All of these hormones are derived by posttranslational processing of the pro-opiomelanocortin (POMC) gene transcript,[1] and each of them possess a central "core" sequence His-Phe-Arg-Trp, which is essential for their agonist biological activity.[2,3] It has been discovered that the melanocortin receptors (hMCR) and their ligands control a surprisingly large number of multifaceted biological actions including skin pigmentation,[4−6] erectile function,[7−11] blood pressure and heart rate,[12] control of feeding behavior and energy homeostasis,[13−19] modulation of aggressive/defensive behavior,[20] and mediation of pain.[21] To date, five melanocortin receptor subtypes with different patterns of tissue expression in the brain and in the periphery have been cloned and characterized.[22] The hMC1R is mainly expressed in melanocytes and leukocytes and has been implicated to play a role in skin pigmentation and inflammatory responses and control of the immune system,[5,6,23,24] whereas the hMC2R is expressed in the adrenal cortex and mediates steroidogenesis and other effects.[25,26] Of particular interest for therapeutical applications are the hMC3R and the hMC4R, which have been implicated to play complementary roles in weight control.[13−19] Extensive studies have been carried out toward highly selective and potent agonists at the hMC4R as potential therapeutics for treating obesity, while selective hMC4R antagonists have been targeted as promising drug candidates for treating anorexia and cancer- and HIV-related weight loss.[5,6,27,28] The hMC3R has recently been suggested to be physiologically involved in the control of energy partitioning and body weight,[29] although the broad scope of the physiological functions of this receptor has not been fully investigated. Therefore, selective ligands at this receptor will be important tools in exploring the entire array of biological functions of the hMC3R, and in conjunction with an hMC4R agonist, they might provide a novel approach to the treatment of obesity, anorexia, weight loss, and related disorders. Finally, the hMC5R, found in a variety of peripheral tissues, plays a role in regulating exocrine gland function[30] and coordinating central and peripheral signals for aggression.[20]

Among the natural melanocyte-stimulating hormones, α-MSH and β-MSH show little selectivity for any specific receptor subtype, in contrast to γ-MSH, which exhibits significant hMC3R selectivity.[31,32] Our laboratories have previously reported several linear γ-MSH analogues with substantially improved potency and hMC3R selectivity, out of which D-Trp[8]-γ-MSH[31] and Ac-NDP-γ-MSH-NH_2[32] were the most promising leads. Bednarek et al.[33] have reported several 21-membered or a larger cyclic lactam analogues of α-MSH and γ-MSH, which were found to be potent and hMC4R-selective agonists. At the same time, Kavarana et al.[34] have found that enhancing the hydrophobic properties of the cyclic α-MSH analogues combined with the increased ring size resulted in improved hMC3R selectivity. Furthermore, Grieco et al.[35] have shown that the certain dihedrally constrained amino acid substitutions at the position 6 of Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH(4−10)-NH_2 (MT-II) led to potent and highly hMC3R and hMC4R selective antagonists. Cai et al.[36] have shown that steric interactions in χ-space of β-modified proline-6 analogues of MT-II resulted in enhanced hMC5R selectivity. In addition, various substitutions (e.g. Gln, Asn, Lys, Arg, Ala, Phe, etc.) at the position 6 of α-MSH have been reported to produce analogues with improved hMC4R selectivity.[37,38]

On the basis of these observations, we have designed a series of novel cyclic γ-MSH analogues with the following general sequence: c[Nle-Xaa-D-Phe/D-Nal(2′)-Arg-Trp-Glu]-NH_2, which features the amide bond between the ε-carboxyl group of the C-terminal glutamic acid amide residue and the α-amino group of the N-terminal norleucine residue as the global constrain. We introduced a bulky hydrophobic residue (Nle[4]) in the close proximity to the pharmacophore (Xaa-D-Phe/D-Nal(2′)-Arg-Trp) to investigate the impact of steric hindrance on receptor

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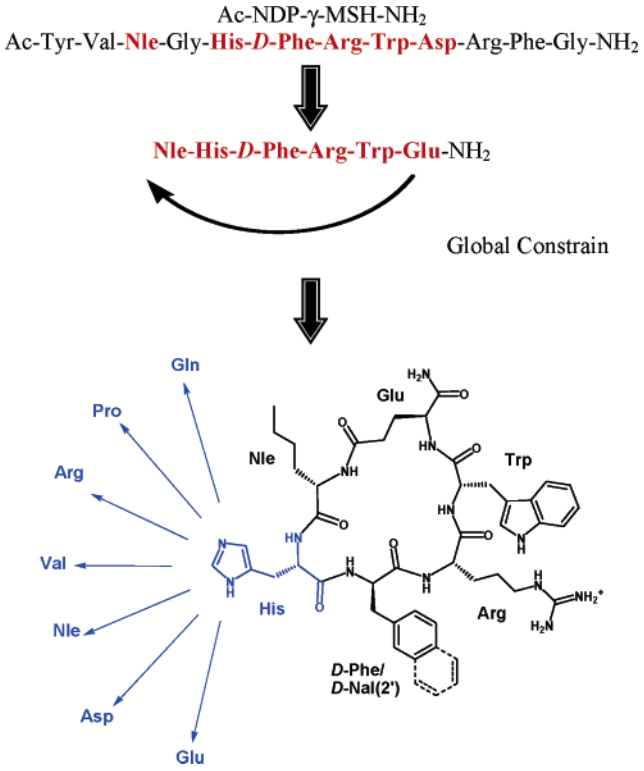

Figure 1. Design of the novel γ-MSH-derived cyclic lactam scaffold.

selectivity. Also, a variety of amino acids with a broad range of hydrophobic/hydrophilic properties was placed in the position 5 to further explore their complementary role in receptor selectivity.

Molecular Modeling and Peptide Design. In our efforts to obtain highly potent and selective melanotropin peptides, we have designed and synthesized a series of novel cyclic γ-MSH analogues (Figure 1). The peptide sequences of the cyclic γ-MSH analogues and their physicochemical properties are listed in Table 1. In the design process we performed computational experiments using hybrid Monte Carlo/low-frequency mode (MCMM/LMCS)[39] simulations with the OPLS-AA force field[40] and the GB/SA continuum dielectric water solvent model[41] implemented in the Macromodel 8.1 software package (Schrödinger, Portland, OR) to identify the three-dimensional structures of the melanotropin peptides that would resemble the secondary structures of the nonselective super agonist MT-II and the hMC3R/hMC4R antagonist SHU9119 (Figures 2 and 3), which were earlier reported by our laboratories.[42] The detailed computational procedures are described in the Experimental Section. As evident from the Figures 2 and 3, the secondary structures of the peptides in this series feature a rigid β-loop, which fits well with the NMR structures of MT-II and SHU9119 and varies little with position 5 substitutions (Table 2). The rigidity of this β-loop scaffold in addition to smaller macrocycle (20-membered ring) as compared to MT-II (23-membered ring) may have contributed to the increased receptor selectivity observed in this series (vide infra). In addition, Figures 2 and 3 also display hydrophobic interactions between the side chains of Nle^4 and Arg^7, which may have led to partial steric hindrance of the binding space of Arg^7, resulting in further increase in receptor selectivity that would favor the receptor subtypes with larger binding pocket (Figure 4). Negatively charged amino acid (Asp^5 and Glu^5) substitutions at position 5 induced intramolecular salt bridge interactions between the side chains of Xaa^5 and Arg^7 (Figure 5), which were expected to limit the involvement of Arg^7 in the receptor−ligand interactions, thus affecting the biological activities of the corresponding analogues. This design approach allowed detailed and systematic evaluation of effects of steric, hydrophobic, and electrostatic factors on ligand−receptor interaction and selectivity in human melanocortin receptors.

Results and Discussion

Table 3 summarizes the binding affinities and the in vitro biological activities of the peptides under study. In the D-Phe^6 series, analogue **1** (c[Nle^4-His^5-D-Phe^6-Arg^7-Trp^8-Glu^9]-NH$_2$) was found to be inactive at the hMC1R, a full agonist at the hMC3R (EC_{50} = 37 nM), a weak partial agonist at the hMC4R (EC_{50} = 2 μM, 48% activation), and an antagonist at the hMC5R. When His^5 was replaced by Pro^5 (analogue **2**), there was a considerable loss in binding affinities as well as bioefficacy for all the receptor subtypes, which may be due to the increased rigidity of the 20-membered macrocycle as compared to the 23-membered MT-II-type cyclic lactams, where such substitutions had been found to increase potency and

Table 1. Sequences and the Physicochemical Properties of the Cyclic γ-MSH Analogues

		m/z (M + 1)		HPLC *k'*[a]		TLC R_f[b]	
no.	sequence	calcd	obsd (FAB)	1	2	1	2
1	c[Nle-His-D-Phe-Arg-Trp-Glu]-NH$_2$	868.4582	868.4582	2.68	4.92	0.64	0.41
2	c[Nle-Pro-D-Phe-Arg-Trp-Glu]-NH$_2$	828.4521	828.4547	3.95	6.44	0.68	0.51
3	c[Nle-Gln-D-Phe-Arg-Trp-Glu]-NH$_2$	859.4579	859.4543	3.32	5.51	0.68	0.56
4	c[Nle-Arg-D-Phe-Arg-Trp-Glu]-NH$_2$	887.5004	887.4988	2.85	5.03	0.64	0.42
5	c[Nle-Val-D-Phe-Arg-Trp-Glu]-NH$_2$	830.4677	830.4656	4.38	7.27	0.69	0.58
6	c[Nle-Nle-D-Phe-Arg-Trp-Glu]-NH$_2$	844.4834	844.4819	4.72	7.38	0.70	0.61
7	c[Nle-Asp-D-Phe-Arg-Trp-Glu]-NH$_2$	918.4739	918.4761	3.69	5.52	0.61	0.45
8	c[Nle-Glu-D-Phe-Arg-Trp-Glu]-NH$_2$	878.4677	878.4672	4.60	7.05	0.69	0.61
9	c[Nle-His-D-Nal(2′)-Arg-Trp-Glu]-NH$_2$	909.4735	909.4760	3.69	6.19	0.66	0.56
10	c[Nle-Pro-D-Nal(2′)-Arg-Trp-Glu]-NH$_2$	937.5161	937.5166	3.43	6.10	0.61	0.45
11	c[Nle-Gln-D-Nal(2′)-Arg-Trp-Glu]-NH$_2$	880.4834	880.4847	4.95	7.79	0.70	0.63
12	c[Nle-Arg-D-Nal(2′)-Arg-Trp-Glu]-NH$_2$	894.4990	894.5003	5.26	8.07	0.72	0.66
13	c[Nle-Val-D-Nal(2′)-Arg-Trp-Glu]-NH$_2$	846.4262	846.4254	3.54	5.74	0.68	0.58
14	c[Nle-Nle-D-Nal(2′)-Arg-Trp-Glu]-NH$_2$	896.4419	896.4428	4.12	6.53	0.70	0.66
15	c[Nle-Asp-D-Nal(2′)-Arg-Trp-Glu]-NH$_2$	860.4419	860.4429	3.40	5.48	0.68	0.57
16	c[Nle-Glu-D-Nal(2′)-Arg-Trp-Glu]-NH$_2$	910.4575	910.4565	4.03	6.51	0.69	0.65
17 MT-II	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH$_2$						

[a] HPLC column: Vydac 218TP104, 250 × 4.6 mm, 10 μm, 300 Å. HPLC *k'* = [(peptide retention time − solvent retention time)/solvent retention time]. System 1: solvent A, 0.1% TFA in water; solvent B, acetonitrile; gradient 10−90% B in A over 30 min, flow rate 1.0 mL/min. System 2: solvent A, 0.1% TFA in water; solvent B, methanol; gradient 10−90% B in A over 30 min, flow rate 1.0 mL/min. [b] TLC system 1: *n*-butanol/acetic acid/water/pyridine (4:1:2:1). TLC system 2: *n*-butanol/acetic acid/water (4:1:1).

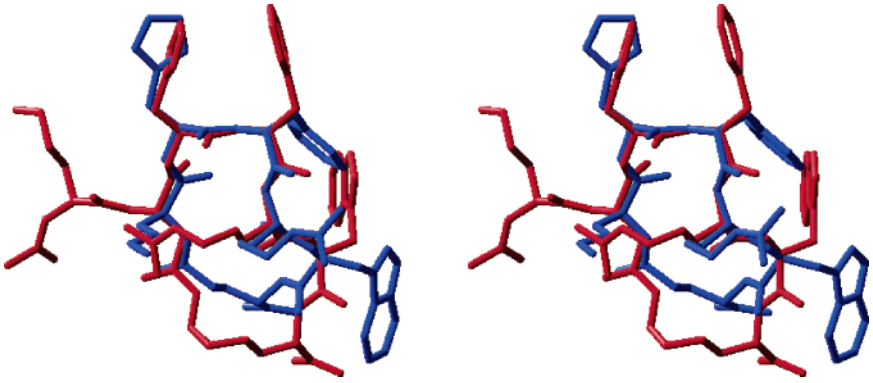

Figure 2. Stereoview of the superimposed global minimum of analogue **1**, c[Nle-His-D-Phe-Arg-Trp-Glu]-NH_2, obtained by MCMM/LMCS (Monte Carlo multiple minima-low-frequency mode)-OPLS-AA simulation with the NMR structure of nonselective superagonist MT-II, Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2 (rmsd = 0.21 Å). Hydrogens are omitted for clarity.

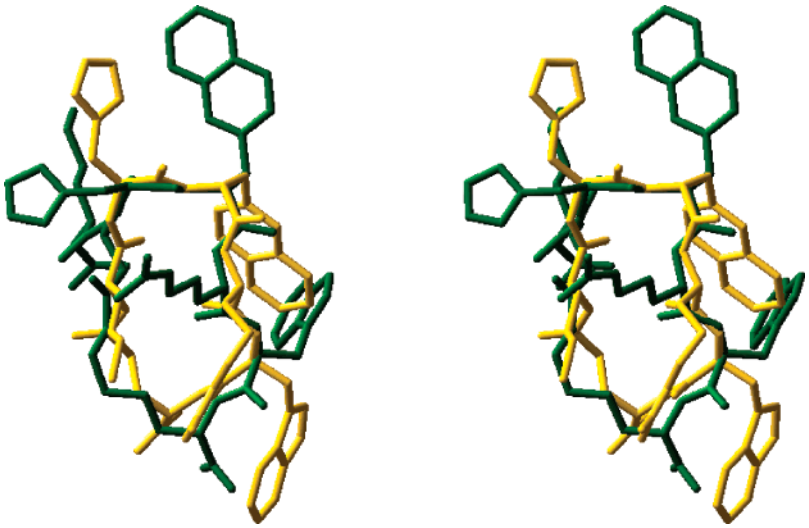

Figure 3. Stereoview of the superimposed global minimum of analogue **9**, c[Nle-His-D-Nal(2′)-Arg-Trp-Glu]-NH_2, obtained by MCMM/LMCS (Monte Carlo multiple minima-low-frequency mode)-OPLS-AA simulation with the NMR structure of hMC3R/hMC4R antagonist SHU9119, Ac-Nle-c[Asp-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2 (rmsd = 0.44 Å). Hydrogens are omitted for clarity.

Table 2. Backbone Torsion Angles (deg) for the Global Minima of Selected Cyclic γ-MSH Analogues Based on MCMM/LMCS-OPLS-AA Calculations Compared with the NMR Structure of MT-II (17)[42]

	Nle/Asp^4		Xaa^5		D-Phe/D-Nal(2)^6		Arg^7		Trp^8		Lys/Glu^9	
no.	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ	Φ	Ψ
1	−153	−159	−69	98	79	29	−134	135	−71	120	−128	44
2	−139	152	−73	140	72	23	−167	−163	−107	158	−78	−37
3	−128	−159	−82	138	84	-66	−85	78	−60	125	−90	−36
4	−164	171	61	22	64	25	−143	−149	−63	145	−108	64
9	−124	−177	−77	79	91	36	−144	152	−62	140	−165	167
13	−130	−170	−74	82	67	35	−153	162	−66	142	−90	−23
17	−85	113	−108	109	84	0	−122	90	−77	108	−101	103

selectivity.[35] Analogue **3** exhibited weak binding affinity and partial agonist activity at the hMC1R, weak partial agonist activity at the hMC3R, no binding affinity and no agonist activity at the hMC4R, and a weak antagonist activity for the hMC5R. Analogue **4**, with an Arg^5 substitution, demonstrated very potent hMC3R agonist activity (EC_{50} = 1.2 nM) and a 150-fold selectivity against the hMC4R (EC_{50} = 174 nM, 63% activation). This increase in potency is likely to be caused by enhanced electrostatic interaction between the ligand and the receptor. It is interesting to note that this compound exhibits only modest binding potency toward the hMC3R, which could be due to the influence of the steric hindrance of Nle^4 on the peptide binding affinity. A similar trend, albeit to a lesser extent, was observed for analogues **1** and **5**. Analogue **4** also showed a very weak binding affinity for the hMC1R and a moderate

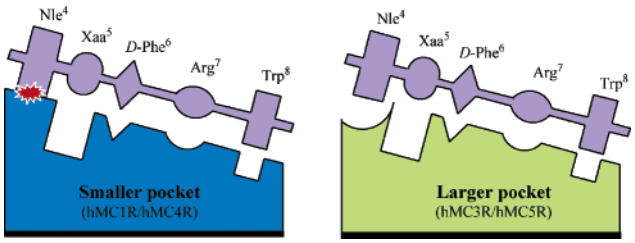

Figure 4. Influence of steric interference from Nle,[4] placed in close proximity to the pharmacophore, on the melanocortin receptor selectivity.

antagonist activity at the hMC5R. Hydrophobic substitutions at position 5 (Val^5, analogue **5**, and Nle^5, analogue **6**) resulted in no affinity for the hMC1R and weak binding affinity and

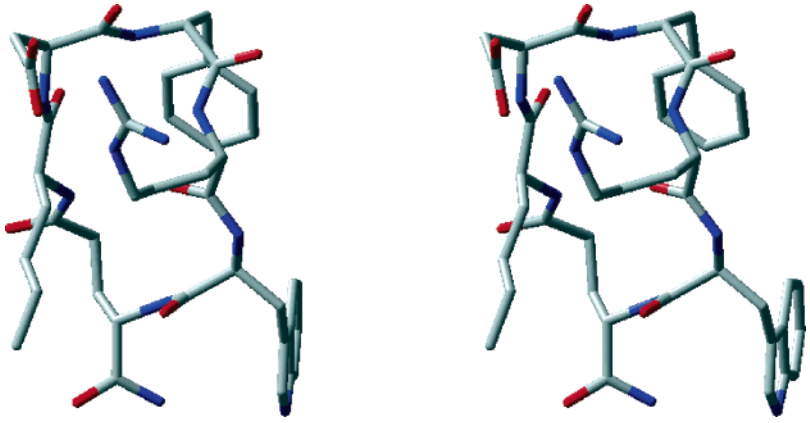

Figure 5. Stereoview of the global minimum of analogue **7**, c[Nle-Asp-D-Phe-Arg-Trp-Glu]-NH_2, obtained by MCMM/LMCS (Monte Carlo multiple minima-low-frequency mode)-OPLS-AA simulation. Hydrogens are omitted for clarity.

Table 3. Binding Affinities and CAMP Activities of Cyclic g-MSH Analogues at HMCRsa

	hMC1R			hMC3R			hMC4R			hMC5R		
no.	IC_{50}, nM	EC_{50},nM	% max effect	IC_{50}, nM	EC_{50}, nM	% max effect	IC_{50}, nM	EC_{50}, nM	% max effect	IC_{50}, nM	EC_{50}, nM	% max effect
1	>10 000	>10 000	0	560 ± 50	37 ± 3	147	170 ± 20	2 000 ± 145	48	100 ± 3	>10 000	0
2	>10 000	>10 000	0	2 700 ± 300	>10 000	0	2 700 ± 15 0	>10 000	0	400 ± 4	850 ± 100	10
3	1 000	1 000	20	270 ± 30	1 700 ± 100	60	>10 000	>10 000	0	500 ± 100	300 ± 30	8
4	1 400 ± 100	>10 000	0	140 ± 10	1.2 ± 0.2	112	270 ± 26	174 ± 18	63	68 ± 2	>10 000	0
5	>10 000	>10 000	0	600 ± 60	27 ± 4	108	2 500 ± 100	3 000 ± 90	25	110 ± 6	180 ± 2	75
6	>10 000	>10 000	0	250 ± 20	300 ± 30	27	600 ± 20 0	>1 000	50	70 ± 4	>10 000	0
7	>10 000	>10 000	0	>10 0 00	>10 000	0	150 ± 14	>10 000	0	>10 000	>10 000	0
8	>10 000	>10 000	0	1 200 ± 79	4 300	54	>10 000	>10 000	0	3060±1 00	>10 000	0
9	>10 000	>10 000	0	12 ± 3	>10 000	0	100 ±20	>10 000	0	2.3 ± 0.0 1	>10 000	0
10	>10 000	>10 000	0	13 ± 0.4	>10 000	0	1 000 ± 30	>10 000	0	10 ± 1	>10 000	0
11	1 000	>1 000	0	1.4 ± 0.4	>10 000	0	65 ± 10	130 ± 10	50	1.4 ± 0.3	>10 000	0
12	1 500	>10 000	0	2.6 ± 0.1	>10 000	0	77 ± 10	530 ± 80	10	2.3 ± 0.1	>10 000	0
13	4 000	>10 000	0	1.7 ± 0.1	>10 000	0	180 ± 20	16 ± 0 .3	13	2.2 ± 0.5	>10 000	0
14	3 000	>1 000	0	6 ± 0.1	>10 000	0	16 ± 1	340 ± 30	31	3.8 ± 0.8	200 ± 2	10
15	>10 000	>10 000	0	820 ± 40	>10 000	0	170 ± 12	>10 000	0	220 ± 11	>10 000	0
16	>10 000	>10 000	0	140 ± 19	2 800 ± 120	7	34 ± 3	>10 000	0	44 ± 4	>10 000	0
17	0.20 ± 0.01	0.30 ± 0.04	100	1.3 ± 0.2	1.9 ± 0.2	100	1.1 ± 0.3	2.9 ± 0.52	10 0	7.5 ± 0.2	3.3 ± 0.7	100

[a] IC_{50} = concentration of peptide at 50% specific binding ($N = 4$). EC_{50} = effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation ($N = 4$). The peptides were tested at a range of concentration from 10^{-10} to 10^{-5} M.

partial agonist activity for the other receptor subtypes, with the exception of full agonist activity at the hMC3R for analogue **5** and a modest antagonist activity at the hMC5R for analogue **6**. Negatively charged amino acid substitutions at position 5 (Asp^5, analogue **7,** and Glu^5, analogue **8**) led to a drastic drop in ligand−receptor interactions for all receptor subtypes, which can be attributed to unfavorable electrostatic interactions. Another possible explanation for this loss in binding affinities is the intramolecular salt bridge interactions between the side chains of Arg^7 and Asp^5/Glu^5 (Figure 5), which would limit the involvement of Arg^7 in receptor−ligand interactions. Similar findings were earlier reported by Bednarek et al.,[33,43] where replacement of His residue with Glu led to a significant decline in binding affinity.

In the D-Nal(2′)^6 series, analogue **9** (c[Nle^4-His^5-D-Nal(2′)^6-Arg^7-Trp^8-Glu^9]-NH_2) exhibited a very potent antagonist activity at the hMC3R and the hMC5R (IC_{50} = 12 and 2.3 nM, respectively), a weak antagonist activity at the hMC4R (IC_{50} = 100 nM), and a complete loss of binding affinity for the hMC1R. Next, His^5 was replaced with Pro^5 (analogue **10**), Gln^5 (analogue **11**), Arg^5 (analogue **12**), Val^5 (analogue **13**), and Nle^5 (analogue **14**). Interestingly, binding affinities of these compounds for the hMC3R and the hMC5R were found to be in the nanomolar range (1.4−13 nM), similar to analogue **9**. Furthermore, analogues **10**−**14** also showed antagonistic activity at these two receptor subtypes. At the same time, these analogues exhibited no significant interaction with the hMC1R and weak partial agonist activities at the hMC4R, except for analogue **10**, which was determined to be a weak hMC4R antagonist. The most selective hMC3R (2400-fold vs hMC1R and 100-fold vs hMC4R) and hMC5R (1800-fold vs hMC1R and 80-fold vs hMC4R) antagonist was found to be analogue **13** (pA_2(hMC3R) = 8.4; pA_2(hMC5R) = 8.7). The higher degree of receptor−ligand interactions observed in the D-Nal(2′)^6 series for the hMC3R (IC_{50} = 1.4−13 nM) and the hMC5R (IC_{50} = 1.4−10 nM) could be due to increased hydrophobicity of these melanotropin peptides and enhanced aromatic receptor−ligand interactions. It is particularly interesting to note that replacing His^5 with a variety of hydrophobic and hydrophilic amino acids has little impact on the hMC3R/hMC5R antagonist activities of the compounds in this series. This clearly indicates that His^5 in cyclic analogues of α-MSH/γ-MSH only has structural importance and most likely is not directly involved in the receptor−ligand interactions critical for agonist or antagonist activities. The significant finding in this study was the pronounced effect of steric hindrance on hMC3R and hMC5R selectivity, which led to the discovery of the novel cyclic peptide antagonist scaffold with good hMC3R/hMC5R selectivity vs the hMC1R (500−2400-fold) and the hMC4R (up to 100-fold) in analogues **9**−**14**. The observation that binding potencies of analogues **9**−**14** at the hMC5R were not significantly affected by the steric bulk of the residues Nle^4 and Xaa^5 used in this study was in

agreement with our earlier findings that the hMC5R has a low sensitivity for steric factors, probably due to a larger size of the receptor pocket.[36] Figure 4 illustrates the effects of steric hindrance on the melanocortin receptor selectivity. In addition, an important consequence of steric hindrance from Nle^4 in the D-Nal(2′)^6 series is the loss of agonist activity at the hMC5R, which is generally observed in a wide variety of D-Nal(2′)^7 analogues of α-MSH and D-Nal(2′)^6 analogues of γ-MSH.[33,35,43,44] This observation suggests that steric effects can be used as valuable tool in converting agonist properties of melanotropin peptides at the hMC5R to antagonist activities at this receptor. Finally, Asp^5 and Glu^5 substitutions (analogues **15** and **16**, respectively) resulted in considerable (20−100-fold) decline in binding affinities to the hMC3R (IC_{50} = 820 and 135 nM) and the hMC5R (IC_{50} = 220 and 44 nM), while retaining the similar binding affinities to the hMC4R (IC_{50} = 168 and 34 nM). Overall, this type of position 5 substitution seems to be unfavorable for melanocortin receptor binding. Consistent with the rest of the peptides in this study, analogues **15** and **16** were also found to be inactive at the hMC1R.

Summary

Computer-aided design of γ-MSH analogues has yielded a novel series of cyclic peptides that feature a rigid β-loop, which is very similar to the secondary structures of the nonselective superagonist MT-II and the hMC3R/hMC4R antagonist SHU9119. Modeling experiments have also suggested that introduction of a bulky hydrophobic residue in the direct proximity to the melanocortin pharmacophore would result in some steric hindrance of the Arg^7 side chain that could potentially affect receptor selectivity. This hypothesis has led to discovery of selective hMC3R agonists and potent hMC3R/hMC5R antagonists with good selectivity against the hMC1R and the hMC4R. Specifically, analogue **4,** c[Nle-Arg-D-Phe-Arg-Trp-Glu]-NH_2, was found to be a very potent and selective hMC3R agonist (EC_{50} = 1.2 nM, 112% act) and analogue **13**, c[Nle-Val-D-Nal(2′)-Arg-Trp-Glu]-NH_2, was identified as an hMC3R/hMC5R antagonist with the best selectivity against the hMC4R in this series (pA_2(hMC3R) = 8.4; pA_2(hMC5R) = 8.7). The unique scaffold described in this study exemplifies the utility of steric effects as the means of discriminating among the melanocortin receptor subtypes. In addition, replacement of His^5 with a variety of hydrophobic and hydrophilic amino acids produced several potent hMC3R agonists and hMC3R/hMC5R antagonists, which is evidence for the structural but not a functional role of His^5 in α-MSH/γ-MSH analogues. Finally, the selective hMC3R agonist **4** and the hMC3R/hMC5R antagonist **13** described in this study will be useful in elucidating the physiological roles of the hMC3R and the hMC4R, particularly in feeding behavior, obesity, and related disorders.

Experimental Section

Materials. Nα-Fmoc-amino acids and Rink amide AM (w/Nle) resin were obtained from Chem-Impex International (Wood Dale, IL), except Nα-Fmoc-Glu(OAll)-OH, which was purchased from NeoMPS (San Diego, CA), and Cl-HOBt was acquired from Bachem (King of Prussia, PA). The following side chain protecting groups were used: Asp(O^δ-*t*Bu); Glu(O^ε-*t*Bu); Glu(O^ε-Allyl); Trp(N^{in}-Boc); Arg(N^ε-Pbf); His(N^{im}-Trt); Gln(N^ε-Trt). ACS grade organic solvents were purchased from VWR Scientific (West Chester, PA), and other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and used as commercially available. The polypropylene reaction vessels (syringes with frits)[45] were purchased from Torviq (Niles, MI). The purity of the peptides was checked by analytical reverse-phase HPLC using a Vydac C_{18} 218TP104 column (Western Analytical Products, Murrieta, CA) monitored at 230 and 254 nm and by thin-layer chromatography (TLC), both performed using two different solvent systems. Analytical thin-layer chromatography (TLC) was carried out on 0.25 mm glass-backed silica gel 60 F_{254} plates (EM Science 5715, VWR Scientific). The TLC chromatograms were visualized by UV light and by dipping in potassium permanganate solution followed by heating (hot plate).

Peptide Synthesis. All peptides in this study were synthesized manually by the Nα-Fmoc solid-phase methodology using DIC and Cl-HOBt as the coupling reagents.[46,47] Rink amide AM (w/Nle) resin (4.0 g, 0.637 mmol/g) was placed into a 50 mL polypropylene syringe with the frit on the bottom and swollen in DMF (20 mL) for 1 h. The Fmoc protecting group on the Rink linker was removed by 25% piperidine in DMF (1 × 5 min and 1 × 15 min). The resin was washed with DMF (3 × 15 mL) and then with DCM (3 × 15 mL). Nα-Fmoc-Glu(OAll)-OH (3 equiv) and Cl-HOBt (3 equiv) were dissolved in 10 mL of DMF, and then DIC (3 equiv) was added. The coupling mixture was transferred into the syringe with the resin and shaken for 30 min. The resin was washed three times with DMF (15 mL) and three times with DCM (15 mL), the unreacted amino groups were capped using acetic anhydride (2 mL) and pyridine (2 mL) in DCM (15 mL) for 30 min, and the resin was once again washed with DCM (6 × 15 mL). The next three amino acids, Trp, Arg, and D-Phe or D-Nal(2′), were consecutively coupled using the procedure described above, using the Kaiser ninhydrin test to check the extent of coupling. In case of a positive Kaiser test, the coupling was repeated until a negative Kaiser test was obtained. The resulting batch of the resin-bound protected tetrapeptide Fmoc-D-Phe/D-Nal(2′)-Arg-Trp-Glu was carefully washed with DMF (5 × 15 mL), DCM (5 × 15 mL), methanol (5 × 15 mL), and diethyl ether (5 × 15 mL) and dried under reduced pressure (16 h). The dry resin was split in 8 equal portions, which were placed in separate 10 mL fritted polypropylene syringes and swollen with DMF as described above. The same coupling procedure was followed to complete the desired peptide sequences, except chloranil test was used to monitor coupling to proline instead of Kaiser test. The orthogonal allyl ester protection for the side chain of Glu^{10} was removed with 0.1 equiv of $Pd(PPh_3)_4$/20 equiv of $PhSiH_3$ in DCM (2 × 30 min) prior to the peptide cyclization.[48] The deprotected resin-bound peptide was washed with 10% DIPEA solution in DCM (1 × 5 mL × 10 min), DCM (6 × 5 mL), and THF (1 × 5 mL). The peptide cyclizations were found to proceed in facile manner with 6 equiv of DIC and 6 equiv of Cl-HOBt in THF (72 h) and were monitored by Kaiser ninhydrin test. Upon completion of cyclization the resin was treated with 5% solution of sodium diethyldithiocarbamate trihydrate in DMF (20 min) and then washed with DMF (5 × 15 mL), DCM (3 × 15 mL), methanol (5 × 15 mL), and diethyl ether (5 × 15 mL) and dried under reduced pressure (16 h). The cyclized peptides were cleaved off the solid support with 82.5% v/v TFA, 5% water, 5% thioanisol, 2.5% 1,2-ethanedithiol, and 5% phenol (5 mL, 3 h), and the crude peptides were precipitated out by the addition of a chilled 3:1 mixture of diethyl ether and petroleum ether (50 mL) to give white precipitates. The resulting peptide suspensions were centrifuged for 10 min at 6500 rpm, and the liquid was decanted. The crude peptides were washed with diethyl ether (4 × 50 mL), and after the final centrifugation, the peptides were dried under vacuum (2 h). The resulting white residues were dissolved in 2 M acetic acid, and the insoluble impurities were removed by passing the solutions through syringe filters (Gelman Laboratory, Acrodisc 13 mm syringe filter with 0.45 μM PTFE membrane). The clear filtrates were lyophilized, the obtained white powders were dissolved in glacial acetic acid (1 mL), and the resulting solutions were diluted with water (4 mL) and passed through a Sephadex G-15 column. Fractions containing the target peptides, as determined by TLC, were combined and lyophilized. Final purification was accomplished by preparative RP-HPLC on a C_{18}-bonded silica column (Vydac 218TP152022, 250 × 22 mm, 15−20 μm, 300 Å) eluted with a linear gradient of 20−80% acetonitrile in 0.1% aqueous TFA solution over 50 min with 10 mL/min flow rate. The purified

peptides were isolated in 30−35% overall yield and were >95% pure as determined by analytical RP-HPLC. The structures of the pure peptides were confirmed by high-resolution fast atom bombardment (FAB) mass spectrometry and ^{1}H NMR in DMSO-d_6/acetonitrile-d_3.

Biological Activity Assays. Receptor Binding Assay. Competition binding experiments were carried out using whole HEK293 cells stably expressing human MC1, MC3, MC4, and MC5 receptors. HEK293 cells transfected with hMCRs[36,49,50] were seeded on 96-well plates 48 h before assay (100 000 cells/well). For the assay, the cell culture medium was aspirated and cells were washed twice with a freshly prepared binding buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, and 200 mg/L bacitracin. Next, cells were incubated with different concentrations of unlabeled peptide and labeled [^{125}I]-[Nle,^{4}D-Phe^7]-α-MSH (Perkin-Elmer Life Science, 100 000 cpm/well, 0.1386 nM) for 40 min at 37 °C. The assay medium was subsequently removed, and each well was washed twice with the binding buffer. The cells were then lysed by the addition of 250 μL of 0.1 M NaOH and 250 μL of 1% Triton X-100. The lysed cells were transferred to 12 × 75 mm glass tubes, and the radioactivity was measured by a Wallac 1470 WIZARD Gamma Counter.

Adenylate Cyclase Assay. HEK 293 cells transfected with human melanocortin receptors[36] were grown to confluence in MEM medium (GIBCO) containing 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96-well plates 48 h before assay (100 000 cells/ well). For the assay, the cell culture medium was removed and the cells were rinsed with 1 mL of MEM buffer (GIBCO) or with Earle's balanced salt solution (EBSS, GIBCO). An aliquot (0.4 mL) of the Earle's balanced salt solution was placed in each well along with 5 μL 0.5 mM isobutylmethylxanthine (IBMX) for 1 min at 37 °C. Next, varying concentration aliquots of melanotropin peptides (0.1 mL) were added, and the cells were incubated for 3 min at 37 °C. The reaction was stopped by aspirating the assay buffer and adding 0.15 mL ice-cold Tris/EDTA buffer to each well. After dislodging the cells with the help of trypsin, the cells were transferred to polypropylene microcentrifuge tubes and placed in a boiling water bath for 15 min. The cell lysate was then centrifuged for 2 min at 6500 rpm, and 50 μL of the supernatant was aliquoted into an Eppendorf tube. The total cAMP content was measured by competitive binding assay according to the TRK 432 assay kit instructions (Amersham Corp., Piscataway, NJ). The antagonist properties of the lead compound were evaluated by its ability to competitively displace the MT-II agonist in a dose-dependent manner, at up to 10 μM. The pA_2 values were obtained using the Schild analysis method.[51]

Data Analysis. IC_{50} and EC_{50} values represent the mean of two experiments performed in triplicate. IC_{50} and EC_{50} estimates and their associated standard errors were determined by fitting the data using a nonlinear least-squares analysis, with the help of GraphPad Prism 4 (GraphPad Software, San Diego, CA).

Computational Procedures. Molecular modeling experiments employed Macromodel 8.1 equipped with Maestro 5.1 graphical interface (Schrödinger, Portland, OR) installed on a Linux Red Hat 9.0 system. Peptide structures were built into extended structures with standard bond lengths and angles, and they were minimized using the OPLS-AA force field[40] and the Polak−Ribier conjugate gradient (PRCG). Optimizations were converged to a gradient RMSD less that 0.05 kJ/Å mol or continued until a limit of 50 000 iterations was reached. Aqueous solution conditions were simulated using the continuum dielectric water solvent model (GB/SA).[41] Extended cutoff distances were defined at 8 Å for van der Waals, 20 Å for electrostatics, and 4 Å for H-bonds.

Conformational profiles of the cyclic peptides were investigated by the hybrid Monte Carlo/low-frequency mode (MCMM/LMCS)[39] procedure as implemented in Macromodel using the energy minimization parameters as described above. MCMM torsional variations and low mode parameters were set up automatically within Maestro graphical user interface. A total of 10 000 search steps were performed, and the conformations with energy difference of 50 kJ/mol from the global minimum were saved. Interatomic dihedral angles were measured for each peptide analogue using the Maestro graphical user interface, and they are described in Table 2. The superimpositions of peptide structures were performed using the α-carbons of the core sequence Xaa-D-Phe/D-Nal(2′)-Arg-Trp.

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Supporting Information Available: ^{1}H NMR spectra of peptide analogues **1**−**16** in DMSO-d_6/acetonitrile-d_3, a listing of chemical shifts and coupling constants, and results from biological activity assays. This material is available free of charge via the Internet at http://pubs.acs.org.

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