Selective, High Affinity Peptide Antagonists of α-Melanotropin Action at Human Melanocortin Receptor 4: Their Synthesis and Biological Evaluation in Vitro†

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Peptide Ac-Nle^4-cyclo(5β→10ϵ)(Asp^5-His^6-D-(2′)Nal^7-Arg^8-Trp^9-Lys^{10})-NH$_2$, compound **1**, a cyclic derivative of α-melanotropin, is a nonselective high affinity antagonist at human melanocortin receptors 3 and 4, and an agonist at melanocortin receptors 1 and 5. To differentiate between the physiological functions of these receptors, antagonists with improved receptor selectivity are needed. In this study, analogues of compound **1** without Ac-Nle^4 or His^6 and/or the amino group of Asp^5 were prepared and tested in binding assays and in functional assays on CHO cells expressing hMC3-5R. Several of these peptides were to be selective, high affinity hMC-4R antagonists. The most interesting was compound **10**, named MBP10, cyclo(6β→10ϵ)-(succinyl^6-D-(2′)Nal^7-Arg^8-Trp^9-Lys^{10})-NH$_2$, an antagonist (IC$_{50}$ = 0.5 nM) with 125-fold selectivity over hMC-3R (and of >300-fold selectivity over MC-1RB). This compound had no agonist activity at hMC-3R or hMC-4R and only weak agonist activity at hMC-5R. Examination of the sequences of these new peptides revealed that the D-(2′)Nal^7-Arg^8-Trp^9 segment of peptide **1** forms the "essential core" required for high affinity and high selectivity of analogues of peptide **1** at hMC-4R, but the "extended core", His^6-D-(2′)Nal^7-Arg^8-Trp^9, is necessary for the maximum affinity for hMC-3R and hMC-5R.

Introduction

Melanocortin peptides or melanotropins, α-MSH, β-MSH, γ-MSH, and ACTH, are involved in many physiological functions in vertebrates, mammals, and humans.[1−2] They regulate skin pigmentation and steroid production, modulate immune responses and learning processes, influence energy balance, growth, and regeneration of nerves, and affect the cardiovascular system.[1−5] Five human receptors are known which interact with melanotropins, hMC-1R to hMC-5R.[5−8] The receptors are seven-helix transmembrane-spanning receptors and belong to the superfamily of G protein-coupled receptors; their activation leads to elevation of cAMP.[6−9] The melanocortin receptors 1, 3, 4, and 5 recognize α-MSH, β-MSH, and γ-MSH, while melanocortin receptor 2 recognizes only ACTH.[6] Considerable attention has recently focused on melanocortin receptors 3 and 4 that are widely expressed in the central nervous system, and also on melanocortin receptor 5, found in the brain and in various peripheral tissues.[7−9] The physiological roles of hMC-3R and hMC-5R are not well defined. However, recent studies of receptor deficient mice suggest an involvement of MC-3R in the regulation of energy homeostasis[10,11] and of MC-5R in the control of lipid and pheromone production in exocrine glands.[12]

A large body of pharmacological and genetic evidence in rodents suggests that also hMC-4R is involved in regulation of the energy balance and body weight.[3,4,13−17] The role of MC-4R in the regulation of food intake and body weight is supported by results obtained from agonist/antagonist administration in rats and from murine genetics. Intraventricular administration of the agonist Ac-Nle^4-cyclo(5β→10ϵ)(Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10})-NH$_2$, called MTII, reduced food intake, and conversely, the antagonist Ac-Nle^4-cyclo(5β→10ϵ)(Asp^5-His^6-D-(2′)Nal^7-Arg^8-Trp^9-Lys^{10})-NH$_2$, known in the literature as SHU9119, compound **1**, increased food intake and body weight.[3,4,13−17] Mice genetically deficient in the melanocortin receptor 4 develop obesity.[17] It could be anticipated therefore that compounds active at MC-4R might be useful in the treatment of eating disorders. Melanocortin receptor 4 appears to play a role in other physiological functions as well, namely in controlling grooming behavior, erection, and blood pressure.[8,9] The natural hormones, melanotropins, however, have relatively low affinity for hMC3-5R and are not particularly selective. To differentiate the physiological role of melanocortin receptor 4 from that of other melanocortin receptors in the brain, in particular from MC-3R, high affinity and selective antagonists are necessary. The synthetic ligands available at present do not distinguish between the melanocortin receptors. A frequently used research tool is Ac-Nle^4-cyclo(5β→10ϵ)(Asp^5-His^6-D-(2′)-Nal^7-Arg^8-Trp^9-Lys^{10})-NH$_2$, compound **1**, a high affinity antagonist at melanocortin receptors 3 and 4 and an agonist at melanocortin receptors 1 and 5, see Table 1 and Chart 1.[18] It is a cyclic derivative of α-MSH with

† Throughout this report, the numbering of the amino acid residues in α-MSH, Ac-Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-amide, has been retained for all cyclic peptides.

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Table 1. Analogues of Compound **1**: Binding Affinities to the Human Melanocortin Receptors 3, 4, and 5, and Signal Transduction Efficacies in cAMP Assay in the CHO Cells Expressing Human Melanocortin Receptor 5

	cyclic peptide[a]						size of lactam ring	binding assay[b] K_i (nM)			selectivity ratio		cAMP[c] EC_{50} (nM)
no.	4 5 6	7	8	9	10			hMC-3R	hMC-4R	hMC-5R	3/4	5/4	hMC-5R
1	Ac Nle Asp* His	D-(2′)Nal	Arg	Trp	Lys*	NH_2	23	0.23 ± 0.02	0.06 ± 0.01	0.09 ± 0.02	3.8	1.5	0.12 ± 0.01
2	Ac NleβAsp* His	D-(2′)Nal	Arg	Trp	Lys*	NH_2	23	29 ± 7.3	0.85 ± 0.11	28 ± 1.5	34	33	19 ± 10
3	Ac Asp* His	D-(2′)Nal	Arg	Trp	Lys*	NH_2	23	0.96 ± 0.35	0.096 ± 0.045	4.3 ± 1.5	10	45	4.2 ± 2.1
4	Asp* His	D-(2′)Nal	Arg	Trp	Lys*	NH_2	23	14 ± 4.5	1.6 ± 0.7	18 ± 4.9	8.8	11	18 ± 8
5	Ac Nle Asp*	D-(2′)Nal	Arg	Trp	Lys*	NH_2	20	20 ± 4.7	0.28 ± 0.055	20 ± 1.7	71	71	87 ± 17
6	Asp*	D-(2′)Nal	Arg	Trp	Lys*	NH_2	20	420 ± 130	4 ± 1	24 ± 3.9	110	6	200 ± 60
7	Ac Asp*	D-(2′)Nal	Arg	Trp	Lys*	NH_2	20	190 ± 10	4.3 ± 0.52	720 ± 160	44	170	1000 ± 23
8	*COCH2CH2CO His	D-(2′)Nal	Arg	Trp	Lys*	NH_2	23	47 ± 6	0.88 ± 0.14	150 ± 26	53	170	170 ± 26
9	*COCH2CH2CO His	D-(2′)Nal	Arg	Trp	Dpa*[d]	NH_2	20	29 ± 7.8	0.26 ± 0.069	65 ± 9.9	110	250	210 ± 88
10	*COCH2CH2CO	D-(2′)Nal	Arg	Trp	Lys*	NH_2	20	150 ± 12	0.5 ± 0.064	540 ± 200	300	1100	530 ± 190
11	*COCH2CH2CO	D-(2′)Nal	Arg	Trp	Orn*	NH_2	19	56 ± 22	0.37 ± 0.19	130 ± 70	150	350	300 ± 200
12	*COCH2CH2CO	D-(2′)Nal	Arg	Trp	Dab*[d]	NH_2	18	3000 ± 800	58 ± 11	2700 ± 580	52	47	13% @ 5
13	*COCH2CH2CO	D-(2′)Nal	Arg	Trp	Dpa*[d]	NH_2	17	1500 ± 58	110 ± 9.5	4800 ± 1500	14	44	>5000
14	*COCH2CH2CO	D-(2′)Nal	Arg	Trp	Apc*[d]	NH_2	17	3000 ± 770	600 ± 160	2500 ± 650	5	4.2	3% @ 5
15	*His	D-(2′)Nal	Arg	Trp	Glu*	NH_2	17	2900 ± 1500	420 ± 67	2400 ± 880	6.9	5.7	>5000
16		*D-(2′)Nal	Arg	Trp	Glu*	NH_2	14	5900 ± 1500	960 ± 120	5000 ± 1300	6.1	5.2	1% @ 10
17[e]	*COCH(CH2)CHCO	*D-(2′)Nal	Arg	Trp	Lys*	NH_2	20	120 ± 13	0.58 ± 0.06	140 ± 28	210	240	240 ± 90
18[e]	*COCH(CH2)CHCO	*D-(2′)Nal	Arg	Trp	Lys*	NH_2	20	240 ± 21	1.5 ± 0.2	400 ± 170	160	270	220 ± 100
19	*COCH2CH2CO	(2′)Nal	D-Arg	Trp	Lys*	NH_2	20	5900 ± 1500	3700 ± 930	7000 ± 3300	1.6	1.9	5% @ 5
20	*COCH2CH2CO	(2′)Nal	Arg	D-Trp	Lys*	NH_2	20	5900 ± 1500	3800 ± 520	4900 ± 1200	1.6	1.3	7% @ 5
21	*COCH2CH2CO	(2′)Nal	Arg	Trp	Lys*	NH_2	20	5900 ± 1500	1300 ± 220	4900 ± 1200	4.5	3.8	0% @ 5
22	Ac Nle Asp* His	(2′)Nal	Arg	Trp	Lys*	NH_2	23	140 ± 12	20 ± 3.5	26 ± 1.8	7	1.3	28 ± 10
23	HS014	see Chart 2 for sequence						25 ± 4.1	1.5 ± 0.3	16 ± 3.1	17	11	42 ± 15
24	HS024	see Chart 2 for sequence						2.6 ± 0.64	0.57 ± 0.2	0.39 ± 0.11	4.6	0.68	0.22 ± 0.05
25	HS028	see Chart 2 for sequence						20 ± 6.3	2.2 ± 0.6	22 ± 5.1	9.1	10	73 ± 21

[a] Cyclization sites denoted with asterisk. [b] Concentration of peptide at 50% specific binding. [c] Concentration of peptide at 50% maximum cAMP accumulation or the percentage of cAMP accumulation (relative to α-MSH) observed at a given peptide concentration (μM). [d] Apc: 4-amino-pyrrolidine-2-carboxylic acid, Dba: 2,4-diamino-butyric acid, Dpa: 2,3-diamino-propionic acid. [e] Two cis isomers tested separately.

Chart 1

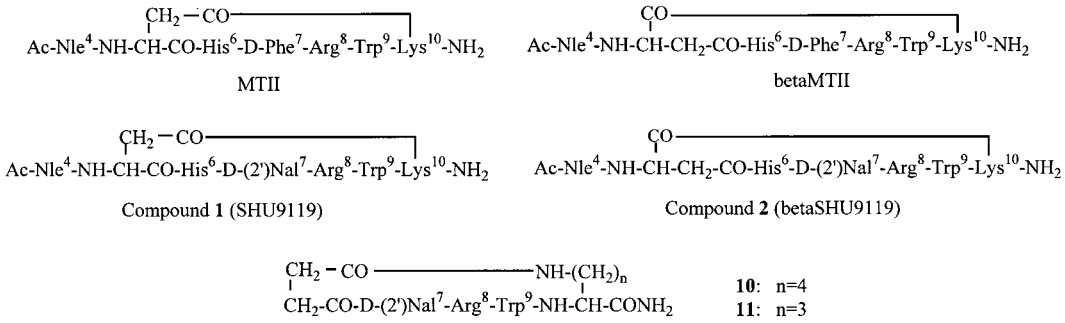
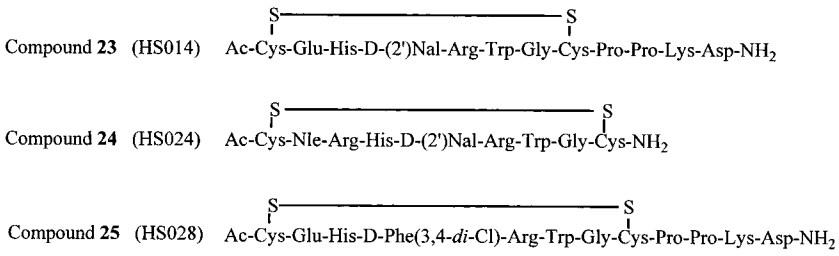


Chart 2



the 6−9 fragment of the hormone forming the lactam ring, and with D-β-(2-naphthyl)-alanine, D-(2′)Nal, substituted for Phe[7]. Compound **1** has been extensively studied in vitro and in vivo; injection of this peptide stimulates food intake in rats.[3,4,15,18] A similar lactam derivative, Ac-Nle[4]-cyclo(5β→10ϵ)(Asp[5]-His[6]-D-Phe[7]-Arg[8]-Trp[9]-Lys[10])-NH_2, is a potent but nonselective agonist at hMC3-5R, see Chart 1.[15]

Schioth and co-workers described[14,19,20] several peptides which were cyclized via disulfides bridges. These compounds antagonized the α-MSH responses at MC-3R and MC-4R. The sequences of the most selective MC-4R antagonists, compounds **23**, **24**, and **25**, are shown in Chart 2. In addition to high affinity and selectivity for MC-4R over MC-3R, it is reported that peptides **23** and **25** are partial agonists at melanocortin receptors 1 and 5, whereas at the same receptors, peptide **24** is an antagonist. In rats these antagonists increase food intake and body weight.[14,19,20]

Previously, we noted[21] that an analogue of Ac-Nle[4]-cyclo(5β→10ϵ)(Asp[5]-His[6]-D-Phe[7]-Arg[8]-Trp[9]-Lys[10])-NH_2, with aspartic acid at position 5 coupled to histidine through its β-carboxyl group displayed 10-fold lower affinity for hMC-4R than the parent compound. How-

Table 2. Analogues of Compound **1**: Analytical Data Obtained from RP HPLC and Electrospray Mass Spectra

	RP HPLC[a]				
	retention time (min)		purity	mass (th),	mass, $(M+H)^{2+}$ (exp),
no.	G1	G2	(%)	[amu]	[amu]
1	15.2	13	>99	1074.2	537.8
2	15.4	13.2	>96	1074.2	537.9
3	14	10.7	>99	961.1	481.3
4	12	9.8	>96	918.1	460.3
5	16.8	15.1	>97	937.1	469.3
6	13	10.3	>98	780.9	391.7
7	13.5	10.7	>99	823.9	412.7
8	13.9	10.9	>97	904.1	452.8
9	12.8	9.9	>98	861.2	431.8
10	14.8	11.2	>99	766.9	384.2
11	14	10.8	>98	752.9	377.3
12	14.5	11.3	>97	738.9	370.3
13	14.1	11.1	>99	724.8	363.2
14	13.8	11	>98	750.8	376.4
15	12.7	10.4	>97	804.9	403.3
16	13.9	10.9	>99	667.8	334.8
17[b]	15	12	>98	778.9	390.3
18[b]	16.1	12.8	>98	778.9	390.4
19	14.1	11.5	>97	766.9	384.2
20	14.5	11.8	>97	766.9	384.4
21	14.2	11.5	>98	766.9	384.3
22	15.2	13.1	>99	1074.2	537.8

[a] G1: 10−100% buffer B in 30 min; buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in acetonitrile. G2: 0−100% buffer B in 30 min; buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in methanol. [b] Two cis isomers.

ever, this β-peptide (named βMTII) was from 20 to 200 times more selective at hMC-4R with respect to hMC-3R and hMC-5R.

In the present study we synthesized and tested, in binding assays and in functional assays for cAMP accumulation at human melanocortin receptors 3, 4, and 5, a β analogue of peptide **1** cyclized as described above for β MTII (Chart 1). The resulting compound **2** (named βSHU9119) was found to be an antagonist about 30 times more selective for hMC-4R over hMC-3R.

Further, structure−function studies revealed that the N-terminal segment of peptide **1** (Ac-Nle,[4] external to the lactam ring), and also histidine in position 6 in the lactam, only minimally affected the binding of this antagonist to hMC-4R. However, the same structural features appeared to be mandatory for the efficient interaction of compound **1** with the other melanocortin receptors in the brain (hMC-3R and hMC-5R).

These observations led us to design new analogues of peptide **1** with smaller lactam rings and with modified N-termini. We report here syntheses and biological evaluation in vitro at human melanocortin receptors 3, 4, and 5 of several cyclic peptides which are selective, high affinity antagonists of hMC-4R.

Results

Analogues of compound **1**, Ac-Nle[4]-cyclo(5β→10ϵ)-(Asp[5]-His[6]-D-(2′)Nal[7]-Arg[8]-Trp[9]-Lys[10])-NH_2, listed in Table 1, were prepared by solid phase syntheses, as previously described (ref 21, see also the Experimental Section and Table 2). They were evaluated for their binding affinities to the human melanocortin receptors 3, 4, and 5 in the competitive binding assays using the radiolabeled ligand [^{125}I]-NDP-α-MSH and for their agonist potency in cAMP assays employing the CHO cells expressing those receptors.[21] At 5 μM concentration, the new analogues, compounds **1**−**22** of Table 1, yielded less than 5% activation at human melanocortin receptors 3 and 4, indicating they have negligible agonist activity. Only at hMC-5R was significant agonist activity observed. Binding data at hMC3-5R and functional data at hMC-5R are compiled in Table 1.

The β analogue of compound **1**, with aspartic acid in position 5 coupled to histidine through β-carboxyl group, compound **2**, displayed about 10-fold lower affinity for hMC-4R than the parent compound. This compound, however, was more selective at the same receptor: about 30-fold with respect to hMC-3R and hMC-5R (Table 1).

Subsequently, the role of the Ac-Nle[4] segment, external to the lactam ring, in interactions of peptide **1** with hMC3-5R was evaluated by testing compound **3** and **4**, analogues without Nle or Ac-Nle in position 4, respectively. These peptides had slightly less affinity than compound **1** at hMC-4R, and had 4 to 300 times less affinity at hMC-3R and hMC-5R.

In the past few years, there were several suggestions in the literature[20,22] that His in position 6 is not essential for efficient binding to hMC-4R of various linear and cyclic peptides derived from α-MSH. In the present study, omission of histidine from the lactam ring of peptide **1** resulted in peptide **5** with 4-fold lower affinity for hMC-4R than that of the parent compound, and more than 100-fold lower affinity for the other brain melanocortin receptors (hMC-3R and hMC-5R). Subsequent deletion of Ac-Nle[4] or Nle[4] from peptide **5** yielded compounds **6** and **7**, respectively, of more than 10-fold lower binding activity at the examined receptors than that of compound **5**. In the absence of histidine in position 6, the signal transduction efficacies at hMC-5R of peptides with 20-membered lactam rings, compounds **5**, **6**, and **7**, were substantially reduced (700-fold or more) in comparison to the agonist activity of peptide **1**.

In several of the cyclic peptides listed in Table 1, the Ac-Nle[4]-Asp[5] segment of compound **1** was eliminated and succinic acid (des-amino-aspartic acid) was used, instead of Asp, to form the lactam ring, see Table 1. Thus, compound **8**, with succinic acid in position 5 closing the 23-membered lactam ring, showed 15 times lower binding affinity for hMC-4R than peptide **1** and more than 200-fold lower binding affinity for both hMC-3R and hMC-5R. Replacement of lysine in position 10 of compound **8** with 2,3-diamino-propionic acid and subsequent lactam closure between the carboxyl group of succinic acid and β-amino group of 2,3-diamino-propionic acid yielded compound **9**. This 20-membered cyclic peptide bound to hMC3-5R as efficiently as compound **8** (23-membered lactam) but activated hMC-5R only with EC_{50} = 210 nM.

The 20-membered cyclic peptide **10**, without His[6] in the lactam ring, was designed to mimic compound **9** (the above-described high affinity and hMC-4R selective antagonist) in term of its ring size. This was accomplished by reinstating Lys in position 10 and closing the lactam bridge between the ϵ-amino group of Lys and the carboxyl group of succinic acid in position 6, Chart 1. Analogue **10** had 10-fold less affinity at hMC-4R than peptide **1**, but it was more selective than compound **9** with respect to the other brain melanocortin receptors, 300 times for hMC-3R and 1100 times for hMC-5R.

Subsequently, smaller cyclic peptides were prepared to determine the minimal size of the lactam ring in compounds which would still maintain high affinity and selectivity for hMC-4R. Hence, substitution of Lys^{10} of compound **10** with ornithine yielded the 19-membered lactam, compound **11**, with similar binding affinities at hMC3-5R and similar signal transduction efficacy at hMC-5R as those of compound **10** (20−membered). Replacement of Lys^{10} of compound **10** with ω-amino-α-amino acids with shorter side chains, 2,4-diamino-butyric acid and 2,3-diamino-propionic acid, further reduced the size of the lactam ring to 18- and 17-membered compounds **12** and **13**, respectively. These peptides, however, displayed 1000 times and 2000 times lower affinity in the binding assays at hMC-4R than peptide **1**. Also, the binding affinity of 17-membered cyclic peptide **14**, with conformationally constrained 4-amino-pyrrolidine-2-carboxylic acid in the lactam ring instead of more flexible 2,3-diamino-propionic acid, was substantially lower at hMC-4R than that of peptide **1**.

A similar 3 log drop in binding affinity at hMC-4R was observed for yet another 17-membered cyclic peptide, compound **15**, in which the lactam ring was closed between α-amino group of His^6 and γ-carboxyl group of Glu^{10} (in place of Lys^{10}). Subsequent omission of His^6 from compound **15** led to cyclic peptide **16** (14-membered ring) with even lower binding affinity for hMC-4R than that of compound **15**. Peptides **13**, **14**, and **15** with the 17-membered lactam rings and peptide **16** with the 14-membered lactam ring bound poorly to hMC-3R and hMC-5R and also elicited less than 5% of maximal cAMP accumulation at micromolar peptide concentrations at hMC-5R.

In an attempt to decrease conformational mobility of the 20-membered ring, a less flexible analogue of succinic acid was incorporated in peptide **10**. Replacement of succinic acid with 1,2-cyclopropane-dicarboxylic acid yielded, however, two 20-membered cyclic peptides, compounds **17** and **18**. These two cis isomers eluted as separate peaks from C18 RP HPLC column. Their individual biological profiles at hMC3-5R were slightly different from each other but similar to those of compound **10** at the same receptors.

Of the compounds tested in this study, peptide **10** emerged as one of the most selective and high affinity antagonists at hMC-4R. In contrast, its analogue with L-(2′)Nal substituted for D-$(2')Nal^7$ (all L-amino acids peptide), compound **21**, was virtually inactive, even at micromolar peptide concentrations, in the binding assays and cAMP assays at hMC3-5R. Interestingly, the similar analogue of peptide **1**, with L-(2′)Nal in place of D-$(2')Nal,^7$ compound **22**, displayed significant binding affinities and biological activities at hMC3-5R, albeit more than 300 times lower than those of peptide **1**. Moreover, the all L-amino acids peptide **1**, compound **22**, was a weak partial agonist for hMC-3R and hMC-4R (19% and 13% activation were observed at 5 μM peptide concentration).

The two analogues of compound **10** with L-(2′)Nal in position 7, but with a D-amino acid either in position 8 (D-Arg, **19**) or in position 9 (D-Trp, **20**) were also inactive at hMC3-5R at micromolar peptides concentration.

Peptides **23**, **24**, and **25** (see Chart 2 for structures), obtained from commercial sources, were also tested in our membrane binding assays and agonist assays of hMC3-5R (Table 1). These antagonists displayed high affinity for hMC-4R and 5- to 20-fold selectivity for hMC-4R over hMC-3R. In contrast to report[19] of the antagonist activity of compound **24** at hMC-5R, we found that this peptide was a full agonist at hMC-5R and also a full agonist of similar potency at the rat MC-5R stably expressed in CHO cells. Differences in expression conferred by stable expression in CHO cells versus expression from transiently transfected COS-1 cells[19] as well as differences in receptor density may contribute to these different observations.

Functional antagonism of the five compounds with highest affinity and selectivity for hMC-4R defined by the receptor binding assays (Table 1) was measured by Schild analysis. The ability of compounds **5**, **9**, **10**, **11**, and **17** and also of peptide **1** to shift the α-MSH dose response curve was measured in both hMC-3R and hMC-4R containing cell lines. Figure 1 shows antagonism of α-MSH stimulated cAMP accumulation at both hMC-3R and hMC-4R by compound **10**. These results suggest that the compound is a competitive inhibitor. Table 3 summarizes Schild analyses for the above-listed six peptides. This assay, like the receptor binding assays of Table 1, shows that peptides **5**, **9**−**11**, and **17** are 20- to 125-fold selective hMC-4R antagonists. Compounds **10** and **11** are the hMC-4R antagonists of highest affinity and selectivity.

Peptides listed in Table 3 were also examined in binding and functional assays at hMC-R1B, a melanocortin 1 receptor isoform with pharmacological properties similar to that of hMC-1RA.[23] Compounds **5**, **10**, **11**, and **17** displayed negligible binding at hMC-1RB with IC_{50}s greater than 3000 nM. They also failed to activate hMC-1RB in stable CHO transfected cells; less than 10% activation, relative to αMSH, was observed with a 10 μM dose of compound. Analogue **9** possessed slightly higher activity at MC-1RB: $EC_{50} = 300 \pm 200$ nM.

Discussion and Conclusions

In the past few years, several analogues of Ac-Nle^4-cyclo(5β→10ϵ)(Asp^5-His^6-D-$(2')Nal^7$-Arg^8-Trp^9-Lys^{10})-NH_2, compound **1**, a high affinity antagonist at hMC-3R and hMC-4R, were reported in the literature.[18,21,24] These compounds were prepared in order to improve the selectivity of peptide **1** at melanocortin receptors. The chiralities of several amino acid residues were changed and some of the amino acids replaced by new constituents.[18,21,24] However, most of the modifications of compound **1** led to peptides with similar selectivities for hMC-4R as that of the parent compounds, or to compounds with substantially lower affinities for melanocortin receptors, or to agonists at these receptors.[18,21,24] D-β-(2-Naphthyl)-alanine in position 7 of peptide **1**, and position 7 of other derivatives of α-MSH, appeared to be important for high affinity and selectivity of antagonists at hMC-4R.[18,25]

It was interesting, therefore, that in our first analogue of compound **1** with improved selectivity for hMC-4R over hMC-3R, the sequence, chiralities, and ring size of the parent compound were left unchanged. This new analogue, compound **2**, differed from peptide **1** only at position 5 where Asp has been coupled to His^6 through its β-carboxyl group (Chart 1). Hence, aspartic acid was

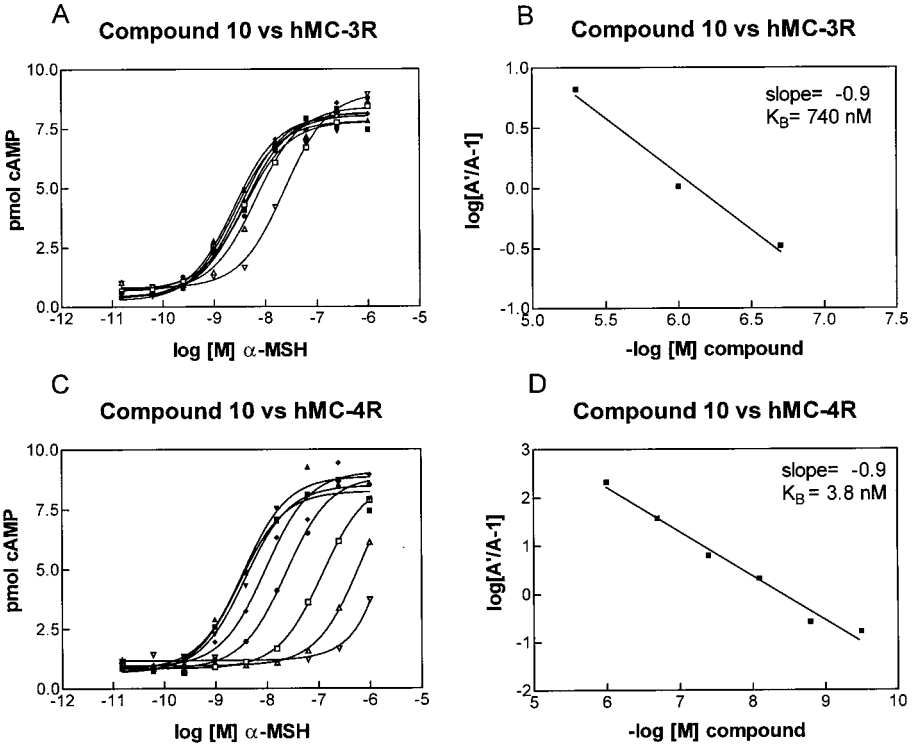


Figure 1. Inhibition of α-MSH activation of hMC-3R and hMC-4R by antagonist peptides. CHO cells expressing a melanocortin receptor were stimulated with α-MSH, and cAMP was measured as described in the Experimental Section. Panels A and C show activation of hMC-3R and hMC-4R, respectively, in the presence of 0, 0.32, 1.6, 8, 40, 200, 1000, and 5000 nM test compound. Schild analyses of the dose response curves are in panels B and D. On the *Y* axis, A is the EC_{50} of α-MSH in the absence of competing compound and A′ is the EC_{50} of α-MSH in the presence of compound.

Table 3. Functional Antagonism of hMC-3R and hMC-4R by Selected Peptides[a]

no.	called	hMC-3R KB (nM)	hMC-4R KB (nM)	selectivity ratio 3/4
1	SHU9119	2.15 ± 0.05	0.39 ± 0.085	5.5
5	MBP5	264 ± 114	11 ± 3.5	24
9	MBP9	360 ± 10	7 ± 2.3	51
10	MBP10	775 ± 35	6.2 ± 2.4	125
11	MBP11	245 ± 5	3.1 ± 0.95	79
17	MBP17	455 ± 95	11 ± 0.5	41

[a] K_B values from Schild Analyses were the average of two experiments such as those shown in Figure 1.

incorporated in the backbone as *β*-amino acid, NH_2-CH-(COOH)-CH_2-COOH. The noticeably lower affinity of compound **2** for hMC-3R and hMC-5R than those of peptide **1** seemed to stem from the presence of the *β*-amide bond in position 5, see Chart 1. Substituents at the carbon atom next to the carboxyl in the *β*Asp-His amide bond appeared to be not necessary for the efficient binding of peptide **1** to hMC-4R, but were required for interaction with hMC-3R and with hMC-5R. Binding and functional data on the truncated analogues of compound **1**, lacking Nle^4 or Ac-Nle^4 at their N-termini, corroborated these observations. Moreover, subsequent omission of the amino group of Asp^5 from the lactam, which is incorporation of des-amino-aspartic acid (succinic acid) in place of aspartic acid, yielded a 23-membered lactam, compound **8**, a high affinity and even more selective hMC-4R antagonist. The improved hMC-4R selectivity of this new peptide, with respect to hMC-3R, could be attributed to the absence of the hydrophobic moiety Ac-Nle at position 4, and/or to the greater conformational freedom of the succinic acid containing 23-membered lactam. However, subsequent reduction of the ring size by three atoms through the replacement of lysine with 2,3-diamino-propionic acid led to a 20-membered ring, peptide **9**, with binding affinity and selectivity for hMC-4R similar to those of 23-membered cyclopeptide, compound **8**. Apparently, decreased conformational mobility in this 20-membered cyclic peptide did not affect interactions with hMC-4R. The biologically active conformations of the 20-membered lactam and the 23-membered lactam might be similar at the receptor sites. Thus, selectivity of peptide **1** for hMC3-5R appears to be determined mainly by its N-terminal section (Ac-Nle^4 and the amino group of Asp^5). In the absence of these structural features, binding affinity of analogues of compound **1** at hMC-4R and their ability to act as antagonists at this receptor were only minimally decreased. However, selectivity of these compounds with respect to hMC-3R was noticeably improved. Previously, we reported[26] a similarly negligible effect of Ac-Nle^4 segment on binding and agonist potency of Ac-Nle^4-cyclo(5*β*→10*ϵ*)(Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10})-NH_2 at hMC-4R. The Ac-Nle^4 segment and the amino group of Asp^5 in the analogues of peptide **1**, in contrast, were both required for maximum activity at hMC-3R and hMC-5R, perhaps because they significantly contribute to hydrophobic and H-bond interactions with these receptors.

Several research groups recently indicated[20−22,24] that His^6 in peptides derived from α-MSH might not be involved in interactions with melanocortin receptor 4. In this study, we noted that replacement of His^6 with Ala in peptide **1** did not affect binding and activity at hMC3-5R (data not included). The lack of involvement

of the histidine residue in interaction of compound **1** with hMC-4R was further confirmed by omission of His^6 from the ring of this antagonist. The 20-membered lactam, compound **5**, had slightly lower affinity for hMC-4R than compound **1**, but was about 20 times more selective than the parent compound with respect to hMC-3R. Moreover, the EC_{50} of agonist activation by this new peptide at hMC-5R was drastically lower than that of peptide **1**. The D-(2′)Nal^7-Arg^8-Trp^9 segment of compound **1** (and of its analogues) seems to constitute the "essential core" required for binding to and for antagonism at hMC-4R. However, the "extended core", His^6-D-(2′)Nal^7-Arg^8-Trp^9, is favored for maximum antagonist activity at hMC-3R and for agonist activity at hMC-5R. The high affinity and selectivity of compound **5** for hMC-4R contrasted with our previous observations concerning an analogue of Ac-Nle^4-cyclo(5β→10ϵ)(Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10})-NH_2 without His; this deshistidine peptide was a very weak ligand of hMC3-5R, even at micromolar peptide concentrations.[21] The similarity between the binding and activity profiles for analogues of compound **5** lacking Nle^4 or Ac-Nle^4 and that of the parent compound supported our earlier conclusions that the presence of Ac-Nle^4 is not required for the high affinity and antagonism of peptide **1** (and its analogues) at hMC-4R. The cyclic peptide designed to accommodate these observations (compound **10**, analogue of peptide **1** without His^6, Ac-Nle^4 and the amino group of Asp^5, that is with succinic acid replacing Asp^5) was shown to be a high affinity hMC-4R antagonist, 125 times more selective with respect to hMC-3R (compound **10** in Table 3).

The effect of the ring size on biological activity of compound **10** was also evaluated in further studies. The lactam bridge of antagonist **10** was shortened by one atom at a time through replacement of Lys^{10} with its lower homologues . The similarly high affinity and selectivity at hMC-4R of the 19-membered cyclic peptide **11** (Table 3) and those of the 20-membered antagonist, compound **10**, implied that the two compounds interact with here discussed receptors in a similar manner. However, further reduction of the size of the lactam ring led to compounds of much lower binding affinities for hMC-4R. The 18- and 17-membered cyclic peptides were practically inactive at hMC3-5R at micromolar peptide concentrations. The enhanced steric constraints in these smaller lactams could severely affect conformations required for binding and activity at the brain melanocortin receptors. The antagonist-receptor interactions were even more perturbed when the conformationally stabilized 4-amino-pyrrolidine-2-carboxylic acid was incorporated (in place of Lys^{10} in compound **10**) into the 17-membered ring. The new analogue was inactive at hMC3-5R even at micromolar peptide concentrations. Similarly, the 17-membered cyclic peptide with the lactam bridge closed by an amide bond between the amine group of His^6 and the γ-carboxyl group of Glu^{10} was unable to form sufficiently stable complexes with hMC3-5R (presumably again because of conformational constraints in the lactam ring). Thus, from the compounds studied, only the 19-membered and 20-membered cyclic peptides appear to possess the lactam ring sizes which provide for high affinity and selectivity of these antagonists at hMC-4R. Incorporation of a conformationally constrained analogue of succinic acid (1,2-cyclopropane-dicarboxylic acid) into the 20-membered lactam of compound **10** only slightly affected affinity and selectivity for hMC3-5R.

The effect of conformational changes on biological activity of our highly selective antagonist **10** was also studied with analogues in which chiralities of amino acids in the "essential core" were reversed. A single D-amino acid in the ring of cyclic peptides frequently defines their preferred conformations, for instance, through facilitation of the formation of the reversed turns. It has been previously shown[16,27] that reversal of chirality at position 7 in peptides derived from α-MSH greatly enhances their biological activity and resistance to proteolytic enzymes. Thus, it was not too surprising that replacement of D-(2′)Nal with L-(2′)Nal in position 7 of compound **10** yielded an analogue that was practically inactive at hMC3-5R even at micromolar peptide concentrations. The additional peptides with a single D-amino acid at position 8 (Arg) or 9 (Trp) also were not active at the receptors studied. Apparently, D-amino acid in the last two peptides was not in a favorable position to allow biologically active conformations of the lactams. However, reversal of chirality in position 7 of peptide **1**, D-(2′)Nal to L-(2′)Nal, was not deleterious to biological activity at hMC3-5R. Presumably, the larger and hence more flexible 23-membered ring of this analogue of peptide **1** (all L-amino acids) was still able to assume conformations favorable for interaction with these receptors.

Our studies thus yielded several 20-membered (and 19-membered) cyclic peptides which are selective, high affinity antagonists for hMC-4R. These peptides failed to activate hMC-1RB, hMC-3R, and hMC-4R and are weak agonists of hMC-5R. The new antagonists are derivatives of compound **1** with its characteristic "essential core", D-Nal^7-Arg^8-Trp^9, in their lactam rings. In these peptides, the N-terminal section of compound **1** (consisting of Ac-Nle^4, His^6, and the amino group of Asp^5) was found not to be necessary for high affinity at hMC-4R. However, the same structural features are required for high affinity and biological activity at the other melanocortin receptors in the brain (hMC-3R and hMC-5R).

In summary, we report here a family of high affinity antagonists at human melanocortin receptor 4, compounds with improved selectivity with respect to melanocortin receptors 1B, 3, and 5. These peptides might be useful in the evaluation of the physiological role of melanocortin receptor 4 found in the brain. Additional studies on conformational properties of these new antagonists should provide further insight into the structural requirements for selectivity at melanocortin receptors.

Experimental Section

Materials. Boc-protected amino acids were obtained from Bachem (King of Prussia, PA), Fmoc-4-amino-1-Boc-pyrrolidine-2-carboxylic acid from Neosystem Laboratoire (Strasburg, France), *p*-methoxybenzhydrylamine resin, 0.18 mequiv/g substitution, from Peptide International (Louisville, KY), and succinic anhydride and 1,2-cyclopropane-dicarboxylic anhydride from Aldrich. Peptides **23**, **24**, and **25** were obtained from Neosystem Laboratoire (Strasburg, France). Peptides **23** and **24** were also purchased from Phoenix Pharmaceutical, Inc. (Mountain View, CA) and Bachem (Torrance, CA).

Peptide Synthesis, Purification, and Characterization. Elongation of peptidyl chains on *p*-methylbenzhydrylamine resin (431A ABI peptide synthesizer), formation of the lactam ring on a resin, deprotection and cleavage of peptides from a resin with HF, and purification of the crude products by high-pressure liquid chromatography were performed as previously described in detail.[21] For several compounds, the peptidyl resin was transferred into a vessel, agitated with 6-fold excess of succinic anhydride and 6-fold excess of diisopropylethylamine in *N*-methylpyrrolidone until a negative Kaiser test was observed, and then thoroughly washed with *N*-methylpyrrolidone and methanol. Subsequent removal of Fmoc group, cyclization, deprotection, and cleavage of peptides from a resin, and purification of the crude products were performed as previously reported.[21] A standard gradient system of 10−100% buffer B in 30 min (G1) was used for analysis; buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in acetonitrile. The second gradient system used for analysis was 0−100% buffer B in 30 min (G2); buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% trifluoroacetic acid in methanol. The chromatographically homogeneous compounds were analyzed by electrospray mass spectrometry (Hewlett-Packard series 1100 MSD spectrometer); correct mass and the RP HPLC analytical data are shown in Table 2.

Competitive Binding Assays. Binding activity of compounds was measured using membranes from Chinese hamster ovary (CHO) cells expressing the cloned melanocortin receptors. Binding reactions contained membranes, 200 pM [^{125}I]-NDP-α-MSH (New England Nuclear Corp.), and increasing concentrations of unlabeled test compounds from 0.05 to 20000 nM. Reactions were incubated for 1.5 h and then filtered as described previously.[21] Binding data were analyzed using GraphPad curve-fitting software. Active peptides were evaluated in three independent experiments.

cAMP Assays. Agonist activity of all compounds were measured using Chinese hamster ovary (CHO) cells expressing the cloned melanocortin receptors (see ref 21 for details). Cells were detached from tissue culture flasks, collected by 5 min centrifugation, and resuspended in Earle's Balanced Salt solution (Life Technologies, Gaithersburg, MD) with addition of 10 mM HEPES pH 7.5, 5 mM $MgCl_2$, 1 mM glutamine, and 1 mg/mL bovine serum albumin. Compounds from 0.003 to 5000 nM concentration together with 0.6 mM IBMX were incubated at room temperature with dissociated cells for 40 min and lysed with 0.1 M HCl to terminate the assay. cAMP was quantitated by Perkin-Elmer Life Sciences (NEN) (Boston, MA) SMP-001J Flashplate cAMP assay. Activation by compounds was compared to the maximum response to α-MSH. Active peptides were evaluated in three independent experiments, and data were analyzed with GraphPad prism curve-fitting software.

For assay of functional antagonism, the α-MSH dose response curves were performed in the absence and presence of different doses of the selected antagonist. The antagonists were preincubated with the receptor expressing cells for 10 min. prior to the addition of α-MSH, and the assay was finished as described above.

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