Potent and Selective Agonists of Human Melanocortin Receptor 5: Cyclic Analogues of α-Melanocyte-Stimulating Hormone†

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The physiological role of melanocortin receptor 5 (MC5R) in humans is not clear despite its broad presence in various peripheral sites and in the brain, cortex, and cerebellum. To differentiate between functions of this receptor and those of the other melanocortin receptors (hMC1,3,4R), peptides with improved receptor subtype selectivity are needed. The endogenous ligands, melanocortins, and their various synthetic analogues are not particularly selective for hMC5R. In this study, cyclic peptides derived from MTII, Ac-Nle-cyclo-(Asp-His^6-D-Phe^7-Arg^8-Trp-Lys)-NH_2 (a pan-agonist at the melanocortin receptors) were prepared and tested in binding and functional assays on CHO cells expressing hMC1b,3−5R. The analogues included in their structures sterically constrained hydrophobic amino acids in positions 6 (His) and 8 (Arg), and the D-4,4′-biphenyl residue in position 7 (D-Phe). Several of the new compounds were selective potent agonists at hMC5R. They are exemplified by peptide **29**, Ac-Nle-cyclo(Asp-Oic^6-D-4,4′-Bip^7-Pip^8-Trp-Lys)-NH_2 (Oic = octahydroindole-2-COOH; 4,4′-Bip = 4,4′-biphenylalanine; Pip = pipecolic acid) of IC_{50} = 0.95 nM and EC_{50} = 0.99 nM at hMC5R and selectivity for this receptor with respect to the other melanocortin receptors greater than 5000-fold.

Introduction

Specific membrane proteins called melanocortin receptors (MC1−5R) mediate diverse biological effects of melanocyte-stimulating hormones (α-, β-, and γ-MSH) and adrenocorticopin hormone (ACTH) in mammals.[1−9] Melanocortin receptor 1, detected mainly in the skin and immune cells, plays a role in skin and hair pigmentation and in immune system control.[3−13] Melanocortin receptor 2 (MC2R), found in the adrenal gland and known as the ACTH receptor, is involved in the ACTH-mediated control of steroid production.[3−13] The other two receptors, melanocortin receptors 3 and 4 (MC3R and MC4R), are predominantly expressed in the brain and are involved in the regulation of metabolism and feeding behavior.[3−15] MC4R also plays a role in controlling sexual function, whereas MC3R may be involved the regulation of cardiovascular effects.[3−13]

Melanocortin receptor 5 (MC5R) shows the highest sequence homology to MC4R, but its physiological functions are less understood, despite the MC5R broad presence in various peripheral sites and in the brain, cortex, and cerebellum.[16−26] Effects mediated by MC5R in the central nervous system have not yet been elucidated, although there are indications this receptor might be involved in the control of luteinizing hormone secretion.[27] Effects mediated by MC5R in the peripheral tissues have been explored through rodent pharmacology and genetics.[25−30] In mice, MC5R has been shown to play a role in pheromone and lipid production in the exocrine glands.[25,26] MC5R-deficient mice (MC5R−/−) have displayed defective sebaceous and Harderian glands, which are manifested in decreased content of hair lipids, diminished production of sebum, and reduced content of porphyrins in the Harderian glands.[25−28] The MC5R−/− mice have impaired thermoregulation due to decreased water repulsion, but their appetite and body weight remain normal.[25−28] Lack of MC5R, which regulates the release of a pheromone that suppresses aggression, is also manifested in abnormal behavior of MC5R knockout male mice.[28,29] In humans, MC5R and MC4R genes have been linked with obesity-related phenotypes.[31]

The widespread peripheral distribution of MC5R in mammals—in skin, exocrine glands, adrenal gland, adipocytes, leucocytes, stomach, lung, spleen, skeletal muscle, thymus, bone marrow, testis, leukocytes, lymph nodes, mammary gland, ovary, uterus, liver, fat cells, and others, but not in heart[9,17−20]—suggests this receptor may mediate other actions of melanocortins as well. There are some indications that MC5R might be involved in inflammatory and anxiolytic effects and in the secretion of stress hormones.[4−8,18] It has been suggested that MC5R antagonists could be useful for the treatment of acne in humans and that appropriate MC5R agonists, those which are able to increase glandular secretion, may help to elevate conditions such as dry eyes and mouth.[6,7] Human pharmacological validation is not yet available for these suggestions.

Ac-Ser-Tyr-Ser-Met^4-Glu-His-Phe^7-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (αMSH)

Ac-Ser-Tyr-Ser-Nle^4-Glu-His-D-Phe^7-Arg-Trp-Gly-Lys-Pro-Val-NH_2 (NDP-αMSH)

Ac-Nle^4-cyclo-(Asp^5-His-D-Phe^7-Arg-Trp-Lys^{10})-NH_2 (MTII)

Ac-Nle^4-cyclo-[Asp^5-His-D-Nal(2′)^7-Arg-Trp-Lys^{10}]-NH_2 (SHU9119)

To understand a role of MC5R in various melanocortin-mediated physiological effects, high affinity, specific MC5R ligands are needed. The natural ligands, melanocortins, have

† Dedicated to the memory of Dr. Miklos Bodanszky.

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Table 1. Analogues of Ac-Nle[4]-cyclo(Asp[5]-His[6]-X[7]-Pro[8]-Trp[9]-Lys[10])-NH_2

		binding assay, IC_{50}[a] (nM)				cAMP functional assay, EC_{50}[b] (nM)			
no.	X^7	MC1bR	hMC3R	hMC4R	hMC5R	MC1bR	hMC3R	hMC4R	hMC5R
1	D-Phe	2500 ± 310	990 ± 120	590 ± 190	250 ± 35	1300 ± 35	2%@10	6%@10	360 ± 110
2	D-Nal(2)	880 ± 110	>10 000	4800 ± 1100	50 ± 20	89 ± 16 (75%)	0%@10	2%@10	50 ± 7
3	D-Trp	>5000	>5000	>5000	2700 ± 340	880 ± 520	1%@5	0%@2.5	>5000
4	D-Tyr	>10 000	>10 000	>10 000	>10 000	>2500	2%@5	0%@5	0%@5
5	D-His	>10 000	>10 000	>10 000	>10 000	17%@5	3%@5	2%@5	1%@5
6	D-Tyr(Me)	500 ± 66	>10 000	>10 000	2600 ± 440	50 ± 17 (70%)	7%@10	0%@5	1500 ± 420
7	D-Tyr(Et)	430 ± 44	>10 000	13%@10	340 ± 40	60 ± 18 (70%)	>2500	3%@5	270 ± 29
8	D-Phe(4-NO_2)	>2000	>2000	12%@2	780 ± 220	>500	1%@1	0%@1	770 ± 92
9	D-4-Thz	>5000	>5000	0%@5	>5000	27%@2.5	2%@5	0%@5	5%@5
10	D-2-Thi	>5000	>5000	3%@5	>5000	540 ± 150	3%@5	0%@5	>1500
11	D-Phe(4-Cl)	560 ± 30	>20 000	4500 ± 210	1300 ± 35	63 ± 8.3 (70%)	>5000	9%@10	82 ± 6.5
12	D-Bth	2200 ± 130	>5000	3600 ± 390	360 ± 83	300 ± 55 (40%)	0%@5	0%@2.5	670 ± 70
13	D-4,4-Bip	45 ± 2.5	1500 ± 110	1200 ± 58	4.1 ± 0.95	5.3 ± 1.5 (53%)	1400 ± 240	2%@2.5	2.5 ± 1.8
14	D-4-Dip	2900 ± 130	>10 000	74 ± 7.9 (52%)	1500 ± 300	11%@5	3%@5	0%@5	12%@5
15	D-Tic	>10 000	>10 000	10%@1 0	>10 000	7%@5	1%@5	92%@5	0%@5
16	D-Cha	>10 000	>10 000	>10 000	>10 000	>2500	6%@5	1%@5	14%@5
17	D-dehNal (2)	4500 ± 340	>10 000	>10 000	9400 ± 450	700 ± 140	2%@10	0%@5	9%@10
18	D-Nle	>10 000	>10 000	>10 000	>10 000	11%@5	3%@5	1%@5	3%@5
19	D-Gln	>10 000	>10 000	>10 000	>10 000	12%@5	2%@5	1%@5	4%@5
20	D-Glu	>10 000	>10 000	>10 000	>10 000	0%@5	3%@5	0%@5	1%@5
21	D-Lys	>10 000	>10 000	11%@1 0	>10 000	0%@5	2%@5	99%@5	1%@5
22	L-Phe	980 ± 83	860 ± 86	1600 ± 110	390 ± 76	>1000	5%@10	7%@10	2000 ± 420
23	L-Tyr(Me)	>2000	>2000	5%@2	>2000	16%@1	2%@1	56%@1	2%@1

[a] Concentration of peptide at 50% specific binding, or the percentage of inhibition (relative to [^{125}I]-NDP-α-MSH) observed at a given peptide concentration (micromolar). [b] Concentration of peptide at 50% maximum cAMP accumulation, or the percentage of cAMP accumulation (relative to [^{125}I]-NDP-α-MSH) observed at a given peptide concentration (micromolar)

relatively low affinities for MC5R and are not receptor-subtype-selective. Of those peptides, αMSH displays the highest affinity for MC5R, whereas γMSH is the weakest binder: αMSH > ACTH > βMSH ≫ γMSH.[4−8,13] Synthetic ligands NDP-αMSH, MTII, and SHU9119 are also not selective at the melanocortin receptors.[32−34] NDP-αMSH, a potent pan-agonist at MC1R and MC3−5R,[32] is a linear analogue of αMSH with Nle[a] in place of Met[4] and D-Phe in place of Phe.[7] [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 linear and cyclic peptides.] The other two compounds, MTII and SHU9119, are cyclic derivatives of αMSH, which encompass the 4−10 segment of αMSH. The MTII peptide is a potent pan-agonist at MC1R and MC3−5R,[33] whereas SHU9119 is a high-affinity antagonist at the MC3/4R and a potent agonist at the MC1/5 receptors.[34] Several analogues of αMSH and MTII of enhanced selectivity for hMC5R have been reported, but their selectivity with respect to hMC1R is unknown.[35−37] Recently the tetrapeptide 3,3,3-triphenylpropionyl-His-D-Phe-Arg-Trp-NH_2 with EC_{50} = 140 nM at the mouse MC5R was shown to be about 100-fold selective versus the mouse MC1, -3, and -4 receptors.[38]

Previously,[39−41] we synthesized and evaluated in vitro at human MC3−5R a number of αMSH, MTII, and SHU9119 analogues with the aim of exploring interactions between melanocortins and their receptors. A closer examination of the binding data for one of those compounds, the Pro[8]-MTII peptide Ac-Nle[4]-cyclo-(Asp[5]-His-D-Phe-Pro[8]-Trp-Lys[10])-NH_2, showed it was a rather poor binder to MC3−5R. Yet when compared, its affinity for hMC5R was noticeably higher than that for hMC3R or hMC4R.[40] The peptide displayed moderate agonist potency at hMC5R but was practically inactive at hMC3R and hMC4R even at micromolar concentrations.[40]

In the present study, we synthesized and evaluated in vitro at hMC1b,3−5R an analogue of SHU9119 with the same modification to the lactam ring: Pro in place of Arg in position 8. The new peptide, Ac-Nle[4]-cyclo-[Asp[5]-His-D-Nal(2′)-Pro[8]-Trp-Lys[10]]-NH_2, displayed higher potency at and selectivity for hMC5R than the Pro[8]-MTII peptide. Consequently, additional analogues of Pro[8]-MTII with other amino acids in position 7 were evaluated in this study. Several of the new cyclic peptides were found to be potent and selective hMC5R agonists. Their synthesis and pharmacological evaluation in vitro at human MC1bR and MC3−5R are reported herein.

Results

Analogues of Pro[8]-MTII listed in Tables 1 and 2 were prepared by solid-phase syntheses as previously described (see ref 39 and Experimental Section.) They were evaluated for their binding affinities to the human melanocortin receptors 1b, 3, 4, and 5 in competitive binding assays using the radiolabeled ligand [^{125}I]-NDP-αMSH and for their agonist potency in cAMP assays employing the CHO cells expressing these receptors. The human melanocortin 1b receptor (hMC1bR) possesses pharmacological properties similar to that of its isoform, human melanocortin receptor 1a (hMC1aR).[42] Functional antagonism of peptides discussed in this study was not determined.

Binding and activation data for Pro[8]-MTII, and for its analogues with various amino acids in place of Phe,[7] have been compiled in Table 1. The parent compound, peptide **1**, displayed low affinity for melanocortin receptors 1b, 3, and 4 and was not able to activate these receptors effectively at micromolar peptide concentration. It was, however, a moderate binder to and agonist at hMC5R (IC_{50} = 250 nM, EC_{50} = 360 nM). Interestingly, a similar analogue of SHU9119 with the Pro in position 8, compound **2**, was more potent at and specific for hMC5R (IC_{50} = 50 nM, EC_{50} = 50 nM). At micromolar peptide concentration, Pro[8]-SHU9119 was practically inactive at the human MC3 and -4 receptors but showed moderate potency at hMC1bR (EC_{50} = 89 nM, 75% activation at 2.5 μM peptide concentration). This peptide hence displayed a rather interesting

[a] Abbreviations: 4,4′-Bic, bicyclohexylalanine; 4,4′-Bip, 4,4′-biphenylalanine; Bth, 3-benzothienylalanine; Cha, cyclohexylalanine; Chg, cyclohexylglycine; 3,3-Dip, 3,3-diphenylalanine; dehNal(2), dehydronaphthylalanine; Nal(2′), 2′-naphthylalanine; Oic, octahydroindole-2-COOH; Phg, phenylglycine; Pip, pipecolic acid; Thi, 2-thienylalanine; Thz, 4-thiazoylalanine; Tic, tetrahydroquinoline-3-COOH.

Table 2. Analogues of Ac-Nle[4]-cyclo(Asp[5]-His[6]-D-4,4′-Bip[7]-Arg[8]-Trp[9]-Lys[10])-NH_2

		binding assay[a]							cAMP functional assay[b]			
		IC_{50} (nM)				selectivity			EC_{50} (nM)			
no.	compd	MC1bR	hMC3R	hMC4R	hMC5R	1b/5	3/5	4/5	MC1bR	hMC3R	hMC4R	hMC5R
24	Pip[8]	5.9 ± 1	1400 ± 250	570 ± 45	0.72 ± 0.058	8	1940	790	3.3 ± 0.47 (40%)	370 ± 73 (65%)	41%@2.5	0.37 ± 0.032
25	Pro[6],Pro[8]	2100 ± 310	630 ± 200	1000 ± 160	2.9 ± 1.3	720	217	340	350 ± 23 (70%)	2%@2.5	40%@10	1.6 ± 0.2
26	Oic[6],Pro[8]	7800 ± 750	4400 ± 760	4700 ± 290	10 ± 2.3	780	440	470	>2500	1%@5	97%@5	17 ± 2.4
27	Pro[6],Pip[8]	65 ± 4	270 ± 31	160 ± 6	0.15 ± 0.03	430	1800	1060	28 ± 4.8 (30%)	8%@2	9%@1	0.031 ± 0.006
28	Tic[6],Pip[8]	710 ± 150	3900 ± 1000	720 ± 190	2.7 ± 0.91	260	1440	260	130 ± 21 (38%)	10%@2.5	0%@2.5	0.45 ± 0.065
29	Oic[6],Pip[8] (OBP-MTII)	>5000	>5000	>5000	0.95 ± 0.1	>5000	>5000	>5000	18%@2	5%@2	40%@2	0.99 ± 0.13
30	Val[6],Pip[8]	4900 ± 2200	>10 000	4700 ± 2400	7.3 ± 3.3	670		640	330 ± 64 (33%)	3%@1	77%@1	0.23 ± 0.064
31	Chg[6],Pip[8]	>10 000	>10 000	>10 000	50 ± 22				>500	1%@1	43%@1	1 ± 0.095
32	Phg[6],Pip[8]	>1000	>1000	990 ± 160	2.1 ± 0.33			470	16%@1	0%@1	72%@1	0.29 ± 0.028
33	Oic[6],D-4,4-Bic[7],Pip[8]	1300 ± 290	>1000	25% @2	700 ± 100	1.8			13%@1	>500	39%@1	>500
34	Oic[6],D-pBzlPhe[7],Pip[8]	>1000	>1000	1600 ± 100	24 ± 4.1			66	3%@1	2%@1	93%@1	31 ± 5.8

[a,b] As described for Table 1.

pharmacological profile at the melanocortin receptors studied: it was a selective, dual hMC1b/5R agonist.

In the other analogues of **1** listed in Table 1, various aromatic (compounds **3**−**15**, **22**, **23**) and aliphatic amino acid (both hydrophobic and charged, compounds **16**−**21**) were incorporated in place of Phe[7]. The new peptides were poor ligands for hMC3R and hMC4R: they were not able to form stable complexes with these receptors even at micromolar concentrations. With a few exceptions, peptides **3**−**23** were poor ligands for hMC1bR and hMC5R as well. Only analogues with D-Tyr(Me), D-Tyr(Et), D-Phe(4NO_2), D-Phe(4Cl), or D-Bth in position 7 (compounds **6**−**8**, **11**, and **12**) showed moderate binding affinity and agonist potency at hMC1bR and/or hMC5R. The most interesting compound in Table 1 was undoubtedly peptide **13**, with the D-4,4′-biphenylalanine residue in position 7. This was a highly potent agonist at hMC5R (IC_{50} = 4.1 nM, EC_{50} = 2.5 nM) yet a weak micromolar agonist at hMC3R and hMC4R; it was a partial agonist at hMC1bR (IC_{50} = 45 nM, EC_{50} = 5.3 nM, 53% activation at 2.5 μM).

The novel signal transduction properties of compound **13** were subsequently studied with its analogue in which pipecolic acid (Pip) was incorporated in place of Pro[8], peptide **24** in Table 2. This rather conservative alteration to the side chain in position 8—replacement of the 5-membered ring of proline with the slightly larger, 6-membered ring of pipecolic acid—resulted in peptide **24**, with an enhanced affinity for all studied human melanocortin receptors. The Pip[8] peptide was a full agonist at hMC5R (IC_{50} = 0.72 nM, EC_{50} = 0.37 nM), about 6-fold more potent than the Pro[8] peptide **13** at this receptor. It was not able to activate hMC4R at micromolar concentrations but was a partial agonist at hMC3R and hMC1bR.

In an attempt to improve selectivity of agonists **13** and **24** with respect to hMC1bR, their analogues with Pro or another sterically constrained, primary, or secondary amino acid in position 6 (His) were studied (peptides **25**−**32** in Table 2). The first analogue of **13** with an altered side chain in position 6, the Pro[6],Pro[8] peptide **25**, showed higher human MC1/5 receptor selectivity than the parent compound. The new analogue was about a 700-fold less effective binder to and about a 200-fold less potent agonist at hMC1bR than at hMC5R, but it activated hMC5R as efficiently (EC_{50} = 1.6 nM) as compound **13** (EC_{50} = 2.5 nM).

Incorporation at position 6 of a bulkier analogue of Pro, octahydroindole-2-carboxylic acid (Oic), enhanced the receptor subtype selectivity of compound **13**, but the Oic[6] peptide, analogue **26**, was about 6-fold less potent at hMC5R than the parent peptide **13**. Compound **26** was nearly inactive at the other melanocortin receptors.

Cyclic peptides **27**−**29** were designed to incorporate changes in the structure of **13** that were described above as favorable for hMC5R selectivity. Thus in these analogues amino acid residues in both positions 6 and 8 were replaced: His[6] with Pro, Tic, or Oic and Pro[8] with Pip. The Pro[6],Pip[8] analogue **27** was an exceptionally potent agonist at hMC5R: EC_{50} = 0.03

Table 3. Analogues of Compound **1**: Analytical Data Obtained from RP HPLC and Electrospray Mass Spectra

	RP HPLC retention time (min)			electrospray MS	
no.	G1[a]	G2[a]	purity (%)	mass (theor) [amu]	mass (exptl) [amu]
1	16.7	13.4	>99	965.1	965.7
2	16.9	14.2	>99	1015.2	1015.5
3	16.6	13.8	>98	1004.2	1004.6
4	16.7	14.0	>97	981.1	981.7
5	15.0	12.8	>98	955.0	955.6
6	16.6	13.6	>98	995.2	995.4
7	16.9	13.5	>99	1009.2	1009.9
8	20.1	18.1	>97	1010.1	1010.6
9	17.5	15.6	>97	972.1	972.6
10	20.2	17.9	>98	971.1	971.5
11	20.3	18.0	>97	999.6	999.8
12	20.7	18.2	>99	1021.2	1021.7
13	22.8	19.8	>98	1041.2	1041.9
14	20.1	17.6	>98	1041.2	1041.3
15	18.4	16.0	>98	977.1	977.39
16	18.9	15.9	>99	971.2	971.6
17	19.3	16.7	>98	1025.2	1025.7
18	17.1	15.3	>98	931.1	931.4
19	15.2	12.9	>99	945.0	946.4
20	15.6	13.2	>97	947.0	947.4
21	16.6	14.1	>97	946.1	947.2
22	16.4	14.0	>98	965.1	965.7
23	18.6	16.0	>98	995.1	995.5
24	16.7	14.2	>98	1055.2	1055.6
25	15.1	13.8	>97	1001.2	1002.3
26	16.2	13.9	>99	1055.3	1055.7
27	16.4	14.1	>97	1014.2	1015.2
28	17.6	15.3	>98	1077.3	1077.7
29	17.4	15.1	>99	1069.3	1069.9
30	17.2	15.1	>99	1017.3	1017.7
31	17.8	15.2	>97	1056.3	1056.5
32	17.5	15.2	>97	1051.3	1051.6
33	21.7	19.2	>98	1081.4	1081.6
34	23.0	20.1	>98	1097.3	1098.5

[a] Gradient system 1 (G1) consisted of 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. Gradient system 2 (G2) consisted of 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.

nM, more than 400-fold selective with respect to the other melanocortin receptors. The Tic^6,Pip^8 analogue **28** exhibited about 10-fold lower affinity for and agonist potency at hMC5R than the Pro^6, Pip^8 peptide **27**. The next analogue, the Oic^6,-Pip^8 peptide **29** (IC_{50} = 0.95 nM, EC_{50} = 0.99 nM at hMC5R), was a poor binder to human melanocortin receptors 1b, 3 and 4 (IC_{50} > 5000 nM) and was not able to activate these receptors effectively even at micromolar concentrations. It demonstrated the highest receptor subtype selectivity observed in this study, greater than 5000-fold with respect to all studied melanocortin receptors.

In the next three cyclic peptides **30**−**32**, sterically constrained amino acids were incorporated in position 6 as well. The new substituents, however, were not analogues of Pro but primary amino acids with bulky hydrophobic side chains: valine, cyclohexylglycine, and phenylglycine. Peptides **30**−**32** were nanomolar agonists at hMC5R but of a somewhat lower receptor subtype selectivity than that of peptide **29**.

Among compounds tested in this study, compound **29** emerged as the most selective and potent hMC5R agonist. This peptide and the other selective hMC5R agonists listed in Table 2 share a common structural feature: a rigid aromatic 4,4′-biphenyl side chain in position 7. To explore the role of this moiety in molecular recognition processes, analogues of **29** with a modified 4,4′-biphenyl group, peptides **33** and **34**, were studied. Incorporation of an aliphatic D-4,4′-bicyclohexylalanine in place of its aromatic counterpart, D-4,4′-biphenylalanine in position 7, was detrimental to potency at the melanocortin receptors. The new analogue **33** was more than 500-fold less potent at hMC5R than the parent peptide **29**. Analogue **34**, with a conformationally flexible *p*-benzylphenyl side chain in position 7, the place occupied by the rigid 4,4′-biphenyl group in peptide **29**, was about 30-fold less potent at hMC5R.

Conclusions

Our search for hMC5R-selective agonists originated with the previously disclosed Pro^8-MTII analogue.[40] This compound was a weak activator of hMC5R but was virtually inactive as an agonist at melanocortin receptors 3 and 4. The structure of Pro^8-MTII differs from that of MTII only in position 8, where aliphatic, charged Arg has been replaced with lipophilic, sterically constrained Pro. This single alternation changes the overall charge of MTII and increases steric constraints in its lactam ring. Restriction of conformational freedom in position 8 and/or lack of the hydrophilic side chain of Arg appeared not to be deleterious to molecular recognition at hMC5R but was detrimental to the formation of stable ligand−receptor complexes with hMC3R and hMC4R. Binding and functional data collected in this study for another Pro^8 analogue of MTII, Pro^8-SHU9119,[2] further supported this observation. Similarly, the proline residue in position 8 of SHU9119 severely interfered with binding to hMC3R and hMC4R but was not deleterious to interactions with hMC1bR and hMC5R. In fact, Pro^8-SHU9119 was only about 16-fold less potent at hMC5R (EC_{50} = 50 nM) than SHU9119 (EC_{50} = 3 nM) and was a partial agonist at hMC1bR. It was interesting to notice that Pro^8-SHU9119 was about 7-fold more potent at hMC5R than Pro^8-MTII. Apparently, the aromatic side chain of D-Nal^7 allowed for the formation of more stable complexes with hMC5R than the aromatic side chain of D-Phe in the same position. Unmodified MTII and SHU9119 displayed similar activity patterns at hMC5R, with SHU9119 being about a 10-fold more potent agonist than MTII.

The aforementioned potency and selectivity of Pro^8-SHU9119 at hMC5R prompted us to examine additional analogues of Pro^8-MTII with various aromatic or aliphatic residues in position 7 with the aim of securing hMC5R agonists of even higher potency and selectivity. In a series of compounds, D-Phe^7 of Pro^8-MTII was replaced with more polar aromatic amino acids. These compounds had in position 7 a D-enantiomer of either a natural aromatic amino acid such as Trp, Tyr, or His or an unusual aromatic amino acid such as Tyr(Me), Tyr(Et), Phe-($4NO_2$), 4-Thz, 2-Thi, Phe(4Cl), or Bth. None of these modifications yielded a better hMC5R agonist than Pro^8-SHU9119. In fact, almost all new peptides were practically inactive at hMC5R even at micromolar concentrations. Only analogues with D-Tyr(Et), D-Phe($4NO_2$), D-Phe(4Cl) and D-Bth in position 7 showed weak agonist potency at hMC5R. The Pro^8 peptides with a D-enanthiomer of aliphatic, hydrophobic [Cha, Nle, or dehydro-Nal(2)] or hydrophilic (Gln, Glu, or Lys) residues in place of D-Phe^7, were also not able to form stable complexes with hMC5R. These observations corroborated earlier reports[32−34,39−41] that the side chain of D-Phe^7 or D-Nal(2′)^7 is critical for the efficient interaction of MTII and related peptides with melanocortin receptors. Apparently, a lipophilic aromatic side chain in position 7 is necessary for efficient binding of the melanocortin peptides to a putative binding pocket in the melanocortin receptors, presumably through stacking of the aromatic side chains. The pharmacological properties of yet another Pro^8 cyclic peptide, that with lipophilic, aromatic D-4,4′-biphenylalanine (D-4,4′-Bip) in position 7, supported this conclusion. The D-4,4′-Bip^7,Pro^8 analogue of MTII was more potent at hMC5R (EC_{50} = 2.5 nM) than the analogous D-Nal(2′)^7 and D-Phe^7 peptides (EC_{50} = 50 nM and 350 nM, respectively) and was practically inactive at hMC3R and hMC4R. The agonist potency order at hMC5R for the Pro^8 compounds with different residues in position 7 was therefore D-4,4′-Bip^7,Pro^8-MTII > D-Nal(2′)^7,-Pro^8-MTII > D-Phe^7,Pro^8-MTII. In the peptide with D-4,4′-biphenylalanine, the extended aromatic side chain in position 7, a tandem of two phenyl rings, seems to allow for more effective stacking of the aromatic side chains and stronger interaction with the hydrophobic binding pocket of hMC5R than the single phenyl ring of Phe or fused rings of Nal(2′). In contrast, unfavorable to such interactions was a geminal arrangement of two phenyl rings of diphenylalanine (Dip) in position 7. The D-Dip^7,Pro^8 peptide was devoid of agonist activity at all melanocortin receptors studied even at micromolar concentrations. The sterically hindered side chain of Dip may not properly fit into a putative binding pocket of the receptor and also may destabilize bioactive conformations of the D-Dip^7 cyclic peptide.

Conformations favorable to binding and agonism at the melanocortin receptors were apparently also destabilized when a tetrahydroquinoline-3-carboxylic acid residue (Tic) was incorporated in position 7 of Pro^8-MTII. Additional steric restrictions imposed on the lactam ring by this conformationally constrained, bulky analogue of Pro in position 7 were detrimental to molecular recognition of the D-Tic^7,Pro^8 peptide at the studied melanocortin receptors. Similarly, conformational changes in the lactam ring of Pro^8-MTII introduced by the reversal of chirality in position 7 through the replacement of D-Phe^7 with L-Phe, and also with L-Tyr(Me), were unfavorable to interactions with hMC5R. The all-L-amino acid residue cyclic peptides were poor ligands for this and other melanocortin receptors studied.

Hence, the initial part of this study has shown that Pro in position 8 of MTII or SHU9119 is not deleterious to agonism at hMC1bR and hMC5R but is detrimental to activity at hMC3R and hMC4R. Additionally, a residue in position 7 has been found

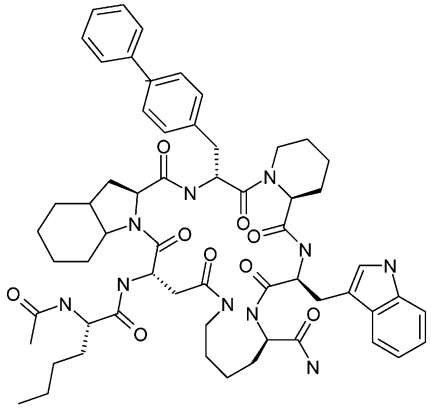
to determine potency of the Pro[8] peptides at hMC5R (and hMC1bR.) The new D-4,4′-Bip[7],Pro[8] analogue of MTII has emerged as the lead compound in the further search for hMC5R agonists of high receptor subtype selectivity.

In the course of a separate study on SHU9119 analogues, those with different secondary amino acid residues in place of Arg[8] (manuscript in preparation), we had learned that incorporation of the pipecolic acid residue (Pip) in position 8 yields a cyclic peptide of higher affinity for the melanocortin receptors than that of the Pro[8] compound. Pipecolic acid, similarly to proline, introduces steric constraints into the lactam ring of MTII/SHU9119, but its larger 6-membered ring may allow for stronger ligand−receptor interactions. The approximately 10-fold higher hMC5R agonist potency of D-4,4′-Bip[7],Pip[8]-MTII than that of its Pro[8] counterpart observed in this study was hence rather expected. The Pip[8] peptide was a subnanomolar agonist at hMC5R, more than 800-fold selective with respect to hMC3R and hMC4R, but still with undesired high binding affinity for hMC1bR.

In the past few years, several research groups have shown[40,43,44] that the side chain of His[6] in peptides derived from αMSH is not critical for efficient interactions with the melanocortin receptors. For example, studied by us and others,[40,43] an analogue of MTII with Pro instead of His in position 6 was a full agonist at hMC1bR, hMC4R, and hMC5R with potency similar to or slightly lower than that of MTII. This Pro[6] peptide also was a partial agonist at hMC3R. In the present study we were interested to evaluate whether a conformationally constrained residue such as Pro in position 6 (His) could affect potency of our new hMC5R agonists at hMC1bR and perhaps improve their receptor 1/5 selectivity. Binding and functional data collected for Pro[6],D-4,4′-Bip[7],Pro[8]-MTII revealed that this peptide retained high affinity for and potency at hMC5R but was more than a 700-fold less effective binder to hMC1bR than the His[6] parent compound. The improved selectivity for hMC5R was similarly displayed by yet another analogue of MTII, that with more sterically hindered octahydroindole-2-carboxylic acid (Oic) in position 6, Oic[6],D-4,4′-Bip[7],Pro[8]-MTII. Additional steric constraints imposed by a secondary amino acid residue such as Pro or Oic in position 6 of our Pro[8] MTII analogues appear to interfere with the formation of stable complexes with hMC1bR but do not disturb molecular recognition at hMC5R.

Cyclic peptides **27**−**29**, designed to include several structural changes to the lactam ring of MTII that are favorable to high agonist potency and selectivity at hMC5R—Pro (or Tic or Oic) in place of His[6], D-4,4′-Bip in place D-Phe[7], and Pip (or Pro) in place of Arg[8]—were highly potent hMC5R agonists. They were unable (or only partially able) to activate other melanocortin receptors studied. The selectivity of the new hMC5R agonists with respect to the human MC receptors 1b, 3, and 4 ranged from 300- to 5000-fold. The Oic[6],D-4,4′-Bip[7],Pip[8]-MTII peptide **29**, abbreviated OBP-MTII ($IC_{50} = 0.95$ nM, $EC_{50} = 0.99$ nM), displayed the highest selectivity for hMC5R observed in this study, greater than 5000-fold with respect to all other hMCRs.

Previously,[45] analogues of MTII with several α,α′-dialkylamino acid residues in position 6 were reported to be potent hMC4R agonists but of substantially lower activity at hMC1R. Incorporation of similar building blocks in position 6 of the linear peptide, His[6]-Phe[7]-Arg[8]-Trp[9]-(Gly[10]), which encompassed the so-called "essential core" of the melanocortins, also resulted in potent hMC4R agonists of low potency at human or mouse MC1R.[46,47] In the present study we speculated that bulky, conformationally constrained amino acid residues in position 6, other than Pro or its analogues, may also yield hMC5R



Ac-Nle-cyclo(Asp-Oic[6]-D-4,4'-Bip[7]-Pip[8]-Trp-Lys)-NH (**29**)

compounds of high selectivity with respect to hMC1bR. Incorporation of valine or cyclohexylglycine or phenylglycine in place of Oic[6] in peptide **29** did not affect molecular recognition at hMC5R and only slightly increased binding affinity of the new hMC5R agonists for human MC 1b, 3, and 4 receptors. The new peptides remained practically inactive at other human melanocortin receptors. The high hMC5R selectivity of these analogues implied that a secondary amino acid residue in position 6, such as Pro or its analogue, is indeed not mandatory for high receptor subtype selectivity of the hMC5R agonists. Similar selectivity could be achieved by incorporation of a primary, aliphatic, or aromatic amino acid residue in position 6.

Separately, the last two compounds in Table 2 emphasized a critical role of the biphenyl group in the molecular recognition of our hMC5R agonists. Replacement of the 4,4′-biphenyl side chain of Oic[6],D-4,4′-Bip[7],Pip[8]-MTII with the aliphatic 4,4′-bicyclohexyl group or the aromatic, more conformationally flexible phenylbenzyl group, not unexpectedly, was unfavorable to agonism at hMC5R. Presumably, unlike the 4,4′-biphenyl group, the new side chains could not fit (stack) properly into a putative binding pocket of hMC5R.

Our study thus yielded several cyclic peptides that are potent and selective agonists at hMC5R. These are analogues of MTII with D-4,4′-Bip in place of D-Phe[7] and with sterically hindered lipophilic amino acids in place of His[6] and Arg[8]. Steric constraints imposed on the lactam ring by the new substituents apparently disturb conformations required for high potency at hMC1b,3,4R but do not affect (or perhaps even favor) the formation of stable complexes with hMC5R. The hMC5R agonists reported here might be useful in evaluation of the physiological role of hMC5R in various peripheral tissues and in the brain. Our continuing studies on conformational properties of these new agonists should provide additional insight into the structural requirements for selectivity at melanocortin receptors.

Experimental Section

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.[39] 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), Table 3.

Competitive Binding Assays. Binding activity of compounds was measured by use of 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 20 000 nM. Reactions were incubated for 1.5 h and then filtered as described previously.[39] Binding data were analyzed with GraphPad curve-fitting software. Active peptides were evaluated in three independent experiments.

cAMP Assays. Agonist activity of all compounds were measured by use of Chinese hamster ovary (CHO) cells expressing the cloned melanocortin receptors (see ref 39 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 *N*-(2-hydroxyethyl)-piperazine-*N*′-ethanesulfonic acid (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 3-isobutyl-1-methylxanthine, 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.

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