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Synthesis and Biological Evaluation in Vitro of Selective, High Affinity Peptide Antagonists of Human Melanin-Concentrating Hormone Action at Human Melanin-Concentrating Hormone Receptor 1

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ABSTRACT: Human melanin-concentrating hormone (hMCH) and many of its analogues are potent but nonspecific ligands for human melanin-concentrating hormone receptors 1 and 2 (hMCH-1R and hMCH-2R). To differentiate between the physiological functions of these receptors, selective antagonists are needed. In this study, analogues of Ac-Arg^6-cyclo(S−S)(Cys^7-Met^8-Leu^9-Gly^{10}-Arg^{11}-Val^{12}-Tyr^{13}-Arg^{14}-Pro^{15}-Cys^{16})-NH_2, a high affinity but nonselective agonist at hMCH-1R and hMCH-2R, were prepared and tested in binding and functional assays on cells expressing these receptors. In the new analogues, 5-aminovaleric acid (Ava) was incorporated in place of the Leu^9-Gly^{10} and/or Arg^{14}-Pro^{15} segments of the disulfide ring. Several of these compounds turned out to be high affinity antagonists selective for hMCH-1R. Moreover, even at micromolar concentrations, they were devoid of agonist potency at both hMCH receptors and not effective as hMCH-2R antagonists. For example, peptide **14**, Gva^6- cyclo(S−S)(Cys^7-Met^8-Leu^9-Gly^{10}-Arg^{11}-Val^{12}-Tyr^{13}-Ava^{14,15}−Cys^{16})-NH_2, (Gva = 5-guanidinovaleric acid), was a full competitive hMCH-1R antagonist (IC_{50} = 14 nM, K_B = 0.9 nM) with more than 1000-fold selectivity over hMCH-2R. Examination of various compounds with Ava in positions 9,10 and/or 14,15 revealed that the Leu^9-Gly^{10} and Arg^{14}-Pro^{15} segments of the disulfide ring are the principal structural elements determining hMCH-1R selectivity and ability to act as a hMCH-1R antagonist.

Human melanin-concentrating hormone (hMCH)[1] is a cyclic peptide present in the regions of the mammalian brain (the lateral hypothalamus and the zona incerta) believed to play a role in regulation of energy balance and food intake (*1*−*5*).

Human (rat) MCH

Asp^1-Phe^2-Asp^3-Met^4-Leu^5-Arg^6-Cys^7-Met^8-Leu^9-
Gly^{10}-Arg^{11}-Val^{12}-Tyr^{13}-Arg^{14}-Pro^{15}-Cys^{16}-Trp^{17}-
Gln^{18}-Val^{19}

Several lines of pharmacological and genetic evidence in rodents support the involvement of MCH in feeding behavior (*6*−*12*). Intracerebroventricular injection of MCH promotes feeding in mice and in rats, transgenic mice lacking the MCH gene are lean and hypophalgic and in *ob/ob* mice and rats, MCH mRNA is overexpressed and upregulated in the hypothalamus during fasting. MCH antagonizes the appetite-suppressing effects of αMSH but does not act through the melanocortin receptors (*6*−*12*). Thus, it appears that compounds able to inhibit actions of MCH might be suitable as drugs for the treatment of obesity.

At present, two human receptors that bind MCH are known: hMCH-1R and hMCH-2R (*13*−*23*). They are seven-helix transmembrane-spanning receptors that belong to the family of the G-protein-coupled receptors. Activation of hMCH-1R and hMCH-2R causes an increase in the levels of inositol phosphate turnover and release of calcium (*13*−*23*). Also, activation of hMCH-1R, but not hMCH-2R, leads to reduction of forskolin-elevated cyclic AMP levels. The amino acid sequence of the second receptor (hMCH-2R) is about 38% identical with the amino acid sequence of hMCH-1R (*19*−*23*). The physiological role of hMCH-2R is less well understood than that of hMCH-1R, but the second receptor might also be involved in energy homeostasis since the human MCH-2R gene maps to a region of chromosome 6 implicated in obesity (*21*).

Melanin-concentrating hormone binds to and activates both human receptors equally well (*13*−*23*). To characterize and differentiate the functions of these receptors present in the brain, new, receptor-subtype specific agonists and antagonists are necessary. The synthetic ligands known at present either do not distinguish between the MCH receptor subtypes or have not yet been tested for binding to and activation at hMCH-2R (*24*−*30*).

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[1] Abbreviations: Aoct, 8-aminooctanoic acid; Ava, 5-aminovaleric acid; Gbu, 4-guanidinobutyric acid; Gpr, 3-guanidinopropionic acid; Gva, 5-guanidinovaleric acid (des-amino-arginine); CHO, chinese hamster ovary; hMCH, human melanin-concentrating hormone; hMCH-1R, human melanin-concentrating hormone receptor 1; hMCH-2R, human melanin-concentrating hormone receptor 2.

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Previously, we reported (*30*) synthesis and biological evaluation in vitro of Ac-hMCH(6−16)-NH_2, compound of agonist potency at both receptors similar to that of the full-length hMCH.[2]

Ac-hMCH(6−16)-NH_2

Ac-Arg^6-Cys^7-Met^8-Leu^9-Gly^{10}-Arg^{11}-Val^{12}-Tyr^{13}-Arg^{14}-Pro^{15}-Cys^{16}-NH_2

This smaller than native hMCH agonist encompasses only the cyclic segment and the external to the disulfide ring Arg^6 of hMCH. The side chains of Arg^6, Met^8, Arg^{11}, and Tyr^{13} in this peptide, similarly to the equivalent side chains in hMCH, are critical for the efficient interaction with both receptors. Moreover, reversal of chirality in position 6 (Arg, from L to D) in our new agonist significantly reduced potency at hMCH-2R without affecting activity at hMCH-1R, thus suggesting that modifications of the N-terminus (Ac-Arg^6) could lead to ligands selective for hMCH-1R.

The present study aimed at the synthesis of high-affinity antagonists specific for hMCH-1R. First, several N-methylated analogues of Ac-hMCH(6−16)-NH_2 were evaluated in binding assays and in functional calcium release assays at both hMCH-1R and hMCH-2R. The slightly lower agonist potency of the N-Me-Gly^{10} analogue than that of Ac-hMCH-(6−16)-NH_2 contrasted with the negligible activity of the N-Me-Leu^9 and N-Me-Arg^{14} analogues at both human MCH receptors, suggesting that αNH of Gly^{10} is not necessary, but αNH-s of Leu^9 and Arg^{14} are critical, for molecular recognition. This observation, in conjunction with the previous reports (*25*−*30*) that the side chains of residues in position 9 (Leu), 14 (Arg), and 15 (Pro) are not required for efficient ligand−receptor interactions, led us to examine in a greater detail the role of the Leu^9-Gly^{10} and Arg^{14}-Pro^{15} segments in the formation and stabilization of ligand−receptor complexes. Several analogues of Ac-hMCH(6−16)-NH_2 were designed in which these segments were replaced with *ω*-amino-valeric acid. Additionally, in some of the new compounds L-Arg in position 6 (external to the disulfide ring) was replaced with its D-enantiomer or des-amino-derivative (5-guanidinovaleric acid) with anticipation that their binding and agonist potency at hMCH-2R would be reduced.

Syntheses and biological evaluation in vitro at human MCH receptors 1 and 2 of a family of cyclic peptides which are high-affinity antagonists selective for human MCH receptor 1 is reported here.

EXPERIMENTAL PROCEDURES

Peptide Synthesis, Purification, and Characterization. Elongation of peptide chains on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin, deprotection and cleavage of peptides from a resin were performed as described in detail in ref *30*. The crude peptides were dissolved in 5% acetic acid in water, and the pH of the solutions were adjusted to pH about 8.2 with dilute ammonium hydroxide. The reaction mixtures were stirred vigorously while a 0.05% solution of potassium ferricyanide [$K_3Fe(CN)_6$] in water was added dropwise till the solution remained yellow for about 5 min. After additional 20 min, oxidation was terminated with ca. 1 mL of acetic acid and the reaction mixtures were lyophilized. The lyophilized crude peptides were analyzed by analytical reversed-phase high-pressure liquid chromatography (RP HPLC) on a C18 Vydac column attached to a Waters 600E system with automatic Wisp 712 injector and 991 Photodiode Array detector. A standard gradient system of 0 to 100% buffer B in 30 min was used for analysis: buffer A was 0.1% trifluoroacetic acid in water and buffer B was 0.1% triflouroacetic acid in acetonitrile. HPLC profiles were recorded at 210 and 280 nm. Preparative separations were performed on a Waters Delta Prep 4000 system with a semipreparative C18 RP Waters column. The above-described solvent system of water and acetonitrile, in a gradient of 0 to 70% buffer B in 60 min, was used for separation. The chromatographically homogeneous products (purity > 97%) were analyzed by electrospray mass spectrometry.

MCH-1R and MCH-2R Radioligand Filter Binding Assays. Membrane binding assays were performed on transiently transfected COS-7 cells expressing human MCH-2R from the plasmid vector pCI-neo (Promega, Madison, WI) and a CHO cell line stably expressing human MCH-1R from pcDNA3.1. For transient expression, COS-7 cells were cultured in Dulbecco's modified Eagle medium (Gibco BRL, Rockville, MD) with 10% heat inactivated fetal calf serum. A suspension of 7×10^6 COS-7 cells were transfected with 20 μg of pCI-neo/MCH-2R plasmid by electroporation and cells were harvested after 60−72 h. Membranes were prepared from transient and stable transfectants by hypotonic lysis, frozen in liquid nitrogen, and stored at −80 °C as described in ref *27*. A filter binding assay was developed to measure the specific binding of [^{125}I]-[Phe^{13}Tyr^{19}]-hMCH (*24*). Scintillation proximity assays were carried out using wheat-germ agglutinin-polyvinyltoluene beads (Amersham Corp., Arlington Heights, IL), in 96-well OptiPlates (Packard, Meriden, CT). Each well contained 0.5−10 μg of membrane protein, and 200 μL of binding buffer (50 mM Tris pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, 12% glycerol, 0.1% BSA). Binding buffer contained 50 mM Tris, pH 7.4, 10 mM $MgCl_2$, 2 mM EDTA, and protease inhibitors: 200 μg/mL of Bacitracin (Sigma, St. Louis, MO), 1 μM phosphoramidon (Peninsula Laboratories). Assays were optimized with respect to membrane preparations: for CHO/MCH-1R membranes, 1 μg of membranes/well yielded a 10× specific binding window and for COS MCH-2R membranes, 8 μg of membrane protein yielded a window of about 8×. Specific binding is defined as the difference between total binding and nonspecific binding conducted in the presence of 500 nM unlabeled hMCH. In 96-well dishes, the membranes were combined with peptide at various dilutions and the radioligand [^{125}I]-[Phe^{13}Tyr^{19}]-hMCH at 0.3 nM final concentration and incubated at room temperature for 1 h. The membrane bound counts were collected by filter harvesting through a Filtermate harvester (Packard Instruments, Meridien, CT) washing with binding buffer as described above with added 0.04% Tween detergent, dried, scintillant was added and the plates were read in a TopCount (Packard, Meriden, CT). IC_{50} calculations were performed using Prism 3.0 (GraphPad Software, San Diego, CA). The IC_{50} values were measured in three different experiments.

[2] Throughout this report, the numbering of the amino acid residues in hMCH has been retained for all cyclic analogues of this neuropeptide.

Table 1: Analogues of Ac-hMCH(6-16)-NH_2 with *N*-Methyl-Amino Acids

Ac-Arg[6]-Cys[7]-Met[8]-Leu[9]-Gly[10]-Arg[11]-Val[12]-Tyr[13]-Arg[14]-Pro[15]-Cys[16]-NH_2

		MCH-1R			MCH-2R		
		binding assay[a]	aequorin functional assay[b]		binding assay[a]	aequorin functional assay[b]	
no.	compd	IC_{50} (nM)	EC_{50} (nM)	% activation	IC_{50} (nM)	EC_{50} (nM)	% activation
	hMCH	0.17 ± 0.01	28 ± 3.2	100	2.9 ± 2.1	50 ± 16	100
	hAc-MCH(6-16)-NH_2	0.16 ± 0.03	20 ± 2.5	120	2.7 ± 1	9.1 ± 2.6	94
1	N-Me-Nle[8]	0.34 ± 0.29	33 ± 9.1	110	18 ± 4	86 ± 4	39
2	N-Me-Leu[9]	62 ± 3% at 10	>10000	8.2	45 ± 10% at 10	>10000	14
3	N-Me-Gly[10]	3.3 ± 2.5	310 ± 140	94	37 ± 21	110 ± 15	90
4	N-Me-Arg[11]	0.55 ± 0.11	36 ± 14	110	5 ± 5.5	30 ± 3.5	86
5	N-Me-Arg[14]	330 ± 120	ND[c]	ND[c]	2010 ± 160	>10000	11

[a] Filter binding assays. IC_{50} is a concentration of peptide at 50% specific binding. When peptide is not able to reach 50% specific binding, the percentage of [^{125}I]-[Phe[13], Tyr[19]]-hMCH displayced at 10 mM peptide concentration is reported. [b] Aequorin bioluminescence assay. EC_{50} is the concentration of peptide at 50% maximum calcium accumulation. 100% activation is the bioluminescence value obtained with 10 μM hMCH.[c] ND, not determined.

Aequorin Bioluminescence Functional Assays (30−32). For the functional receptor activation assays, stable cell lines expressing either the MCH-1R or the MCH-2R and the aequorin reporter protein were used. The assays were performed with aid of a Luminoskan RT luminometer (Invitrogen Inc., Gaithersburg, MD) controlled by custom software written for a PC compatible computer. 293AEQ17/MCH-1R (or MCH-2R) cells were cultured for 72 h and the apo-aequorin in the cells was charged for 1 h with coelenterazine (10 μM) under reducing conditions (300 M reduced glutathione) in ECB buffer (140 mM NaCl, 20 mM KCl, 20 mM HEPES−NaOH, pH 7.4, 5 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mg/mL bovine serum albumin). The cells were harvested, washed once in ECB medium, and resuspended to 500 000 cells/mL. A total of 100 μL of cell suspension (corresponding to 5×10^4 cells) was then injected into the test plate containing the hMCH related peptides, and the integrated light emission was recorded over 30 s, in 0.5-s units. A total of 20 μL of lysis buffer (0.1% final Triton X-100 concentration) was then injected and the integrated light emission recorded over 10 s, in 0.5-s units. The "fractional response" values for each well were calculated by taking the ratio of the integrated response to the initial challenge to the total integrated luminescence including the Triton X-100 lysis response. The EC_{50} values were measured in three different experiments.

Functional antagonism of selected peptides was determined similarly to the determination of agonist potency except that the 293AEQ17/MCH-1R or 293AEQ17/MCH-2R cells were preincubated with the hMCH related peptides for 10 min and then the bioluminescence response was initiated by injecting 100 μL of the EC_{50} concentration of agonist (final agonist concentration was one-half of EC_{50} concentration). Functional EC_{50} and IC_{50} values were calculated using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego California). K_B values were determined from IC_{50}s (*33*).

RESULTS

Analogues of hMCH (Tables 1 and 2) were prepared by solid-phase syntheses as described in Experimental Procedures. They were evaluated for their respective binding affinities at the cloned human MCH receptor 1 and 2 in competition binding assays with [^{125}I]-[Phe[13],Tyr[19]]-hMCH as the radiolabeled ligand (*24*). Functional receptor activation was assessed by stimulation of IP_3-coupled mobilization of intracellular calcium in human HEK-293 cells expressing hMCH-1R and hMCH-2R (*30−32*).

Binding and functional data for analogues of Ac-hMCH-(6−16)-NH_2 with N-Me-amino acids are listed in Table 1. At MCH-2R, binding affinities of N-Me-Nle[8] and N-Me-Arg[11] analogues, compounds **1** and **4**, were reduced about 2 to 6-fold, but at hMCH-1R, binding affinities of these peptides were unchanged. Replacement of Leu[9] or Arg[14] with their N-methylated derivatives led to compounds **2** and **5**, respectively, which were virtually inactive at both receptors. In contrast, an analogue **3** with N-Me-Gly in position 10 was only slightly less potent at both hMCH receptors than the parent compound.

Peptides listed in Table 2 were not able to form stable complexes with hMCH-2R even at micromolar peptide concentrations. Their agonist potencies at this receptor were also negligible, thus showing that they are poor ligands for hMCH-2R. However, most of these peptides bound to hMCH-1R with high or moderate affinity but, interestingly, poorly activated this receptor (less than 5% activation at 10 μM concentration). This indicated that they may act as antagonists at hMCH-1R, hence they were tested for their ability to reverse release of intracellular calcium induced by a nonselective agonist, the Ac-hMCH(6−16)-NH_2 peptide, in cells expressing this receptor. Peptides showing binding IC_{50}s < 200 nM and negligible agonist activity at micromolar concentrations (EC_{50} > 10 000 nM) were preincubated with cells expressing the hMCH-1R (293AEQ/MCH-1R) and activity was measured as a bioluminescence response by injecting 100 μL of the EC_{50} concentration of the Ac-hMCH-(6−16)-NH_2 agonist.

In compounds **6**−**32**, the Leu[9]-Gly[10] and/or Arg[14]-Pro[15] segments were replaced with 5-aminovaleric acid (Ava) or its smaller or conformationally constrained analogues. Because 5-aminovaleric acid mimics in length the main chains of these dipeptide units, its incorporation into the Ac-hMCH-(6−16)-NH_2 structure leaves the size of the disulfide ring unchanged (32-membered cycle). However, these analogues lack one or two amide bonds (between Leu[9] and Gly[10] and/or Arg[14] and Pro[15]) and the side chains of amino acids replaced by 5-aminovaleric acid. Substitution of the Leu[9]-Gly[10] segment with Ava led to compound **6** with 20-fold

Table 2: Analogues of Human Ac-hMCH(6-16)-NH_2

Ac-Arg[6]-Cys[7]-Met[8]-Leu[9]-Gly[10]-Arg[11]-Val[12]-Tyr[13]-Arg[14]-Pro[15]-Cys[16]-NH_2

			MCH-1R				MCH-2R		
				aequorin functional assay[b]				aequorin functional assay[b]	
no.	compd	ring size	binding assay[a] IC_{50} (nM)	agonist EC_{50} (nM)	% activation	antagonist K_b (nM)	binding assay[a] IC_{50} (nM)	agonist EC_{50} (nM)	% activation
	hMCH	32	0.17 ± 0.01	28 ± 3.2	100		2.9 ± 2.1	50 ± 16	100
	Ac-hMCH(6-16)-NH_2	32	0.16 ± 0.03	20 ± 2.5	120		2.7 ± 1	9.1 ± 2.6	94
6	Ava[9,10]	32	4 ± 0.5	560 ± 66	56	110 ± 52	300 ± 140	>10000	30
7	D-Arg[6],Ava[9,10]	32	6.8 ± 2.8	1020 ± 200	50	180 ± 110	23 ± 25% at 10	>10000	7
8	Gva[6],Ava[9,10]	32	5 ± 0.7	>1000	16	260 ± 115	48 ± 3% at 10	>10000	31
9	Aoct[8,9,10]	32	4800 ± 780	>10000	0.1	ND[c]	63 ± 25% at 10	>1000	27
10	Ava[14,15]	32	1.8 ± 0.4	430 ± 48	75	61 + 29	50 ± 19% at 10	>10000	30
11	des(Ac-Arg[6]),Ava[14,15]	32	300 ± 54	ND[c]		ND[c]	77 ± 12% at 10	>10000	45
12	desArg[6],Ava[14,15]	32	190 ± 60	>10000	3	940 ± 540	48 ± 9% at 10	>1000	6
13	D-Arg[6],Ava[14,15]	32	11.3 ± 7.9	>1000	28	210 ± 180	65 ± 13% at 10	>10000	18
14	Gva[6],Ava[14,15]	32	14 ± 9.6	>10000	3	0.9 ± 0.2	34 ± 14% at 10	>10000	91
15	Gbu[6],Ava[14,15]	32	7.9 ± 4.1	>10000	6	2.2 ± 0.5	45 ± 24% at 10	>10000	14
16	Gpr[6],Ava[14,15]	32	43 ± 7.4	>10000	6	65 ± 31	43 ± 10% at 10	>10000	35
17	Gva[6],γAbu[14,15]	31	24 ± 16	>10000	3.5	3.3 ± 0.4	48 ± 9.6% at 10	>10000	35
18	Gva[6],βAla[14,15]	30	53 ± 17	>10000	3.6	4.9 ± 0.6	64 ± 19% at 10	>10000	32
19	Gva[6],Gly[14,15]	29	67 ± 47	>10000	3	89 ± 40	43 ± 17% at 10	>10000	6
20	Gva[6],Ava[14,15], OH	32	66 ± 34	>10000	3.8	230 ± 59	38 ± 10% at 10	>10000	38
21	Gva[6],Nle[8],Ava[14,15]	32	4.7 ± 0.7	>10000	3	8.5 ± 0.7	70 ± 14% at 10	>10000	59
22	Gva[6],αAbu[8],Ava[14,15]	32	320 ± 78	>10000	3	313 ± 153	43 ± 13% at 10	>10000	7
23	Gva[6],Ala[8],Ava[14,15]	32	83 ± 50	>10000		28 ± 7.3	33 ± 25% at 10	>10000	45
24	Ala[8],Ava[14,15]	32	2200 ± 79	>10000	0.1	ND[c]	38 ± 10% at 10	>1000	25
25	Gva[6],desLeu[9],Ava[14,15]	29	1350 ± 140	>10000	0.1	ND[c]	20 ± 8% at 10	>10000	26
26	Gva[6],Ala[12],Ava[14,15]	32	25 ± 6.1	>10000	3.4	1.1 ± 0.3	35 ± 13% at 10	>10000	38
27	Gva[6],*cis*Acx[14,15]	32	10 ± 1.6	>10000	3	13.5 ± 4.6	3100 ± 110	>10000	26
28	D-Cys[7],Ava[14,15]	32	340 ± 87	>10000	3	ND[c]	20 ± 14% at 10	>10000	17
29	Gva[6],D-Cys[7],Ava[14,15]	32	120 ± 63	>10000	0.1	410 ± 300	25 ± 17% at 10	>10000	25
30	Ava[9,10], Ava[14,15]	32	65 ± 30	>10000	3	3.6 ± 0.5	49 ± 20% at 10	>10000	16
31	D-Arg[6],Ava[9,10], Ava[14,15]	32	69 ± 33	>10000	2	13 ± 5.2	45 ± 10% at 10	>10000	28
32	Gva[6],Ava[9,10], Ava[14,15]	32	45 ± 35	>10000	3	32 ± 10.5	40 ± 8% at 10	>10000	91

[a] Filter binding assays. IC_{50} is a concentration of peptide at 50% specific binding. When peptide is not able to reach 50% specific binding, the percentage of [^{125}I]-[Phe[13], Tyr[19]]-hMCH displayced at 10 mM peptide concentration is reported. [b] Aequorin bioluminescence assay. EC_{50} is the concentration of peptide at 50% maximum calcium accumulation. 100% activation is the bioluminescence value obtained with 10 μM hMCH. [c] ND, not determined.

lower binding affinity at hMCH-1R than that of the parent compound and with about 30-fold lower agonist potency. However, this partial hMCH-1R agonist was virtually inactive at hMCH-2R even at micromolar concentrations. Subsequent reversal of chirality in position 6 (Arg) or incorporation of des-amino-arginine in this position (acetyl group and the amino group of Arg[6] are omitted) did not affect binding affinity and activity (compounds **7** and **8)**. These peptides, similarly to analogue **6**, were moderately effective as antagonists at hMCH-1R. Peptide **9** with 8-aminooctanoic acid replacing the longer Met[8]-Leu[9]-Gly[10] segment bound poorly to hMCH-1R and hMCH-2R; it lacks the side chain of Met[8] which is critical for ligand−receptor interaction.

In compound **10**, 5-aminovaleric acid was coupled in place of the Arg[14]-Pro[15] segment. At hMCH-1R, the resulted peptide was about a 10-fold weaker binder and 20-fold weaker agonist than Ac-hMCH(6−16)-NH_2. Further, omission of Ac-Arg[6] or Arg[6] from the structure of **10** yielded compounds **11** and **12**, respectively, which showed a substantial loss of binding affinity at this receptor (>100-fold). However, incorporation of D-Arg or des-amino-arginine in position 6 of compound **10** led to high affinity peptides for hMCH-1R (compounds **13** and **14**). At this receptor, they displayed no agonist activity even at micromolar concentrations but were able to act as functional antagonists. In fact, compound **14** was the most effective and hMCH-1R selective competitive antagonist (K_B = 0.9 nM) described in the present study (see also Figure 1).

In compounds **15** and **16**, 5-guanidinovaleric acid of analogue **14** was replaced with lower homologues: 4-guanidinobutyric acid or 3-guanidinopropionic acid. Similarly to peptide **14**, these were high affinity antagonists at hMCH-1R.

Incorporation of 5-aminovaleric acid into the 32-membered ring of Ac-hMCH(6−16)-NH_2 increases significantly conformational freedom of the disulfide ring. To determine the minimal ring size which would allow for high binding affinity and selectivity for hMCH-1R, analogues of **14** were prepared with γ-aminobutyric acid or β-alanine or glycine in place of 5-aminovaleric acid. Compound **17** with γ-aminobutyric acid displayed similar binding affinity for hMCH-1R as peptide **14**, and compounds **18** and **19** with smaller β-alanine and glycine, respectively, were only 4-fold weaker binders.

Unfavorable to antagonism at hMCH-1R was replacement of the C-terminal amide group of compound **14** with a carboxyl group; the acid analogue (compound **20)** was about 250 times less efficient antagonist than the amide peptide.

Substitution of Met[8] in peptide **14** with the stereoisomeric Nle affected only slightly binding and antagonism at hMCH-1R (compound **21**). However, analogues of **14** (or **21**) with

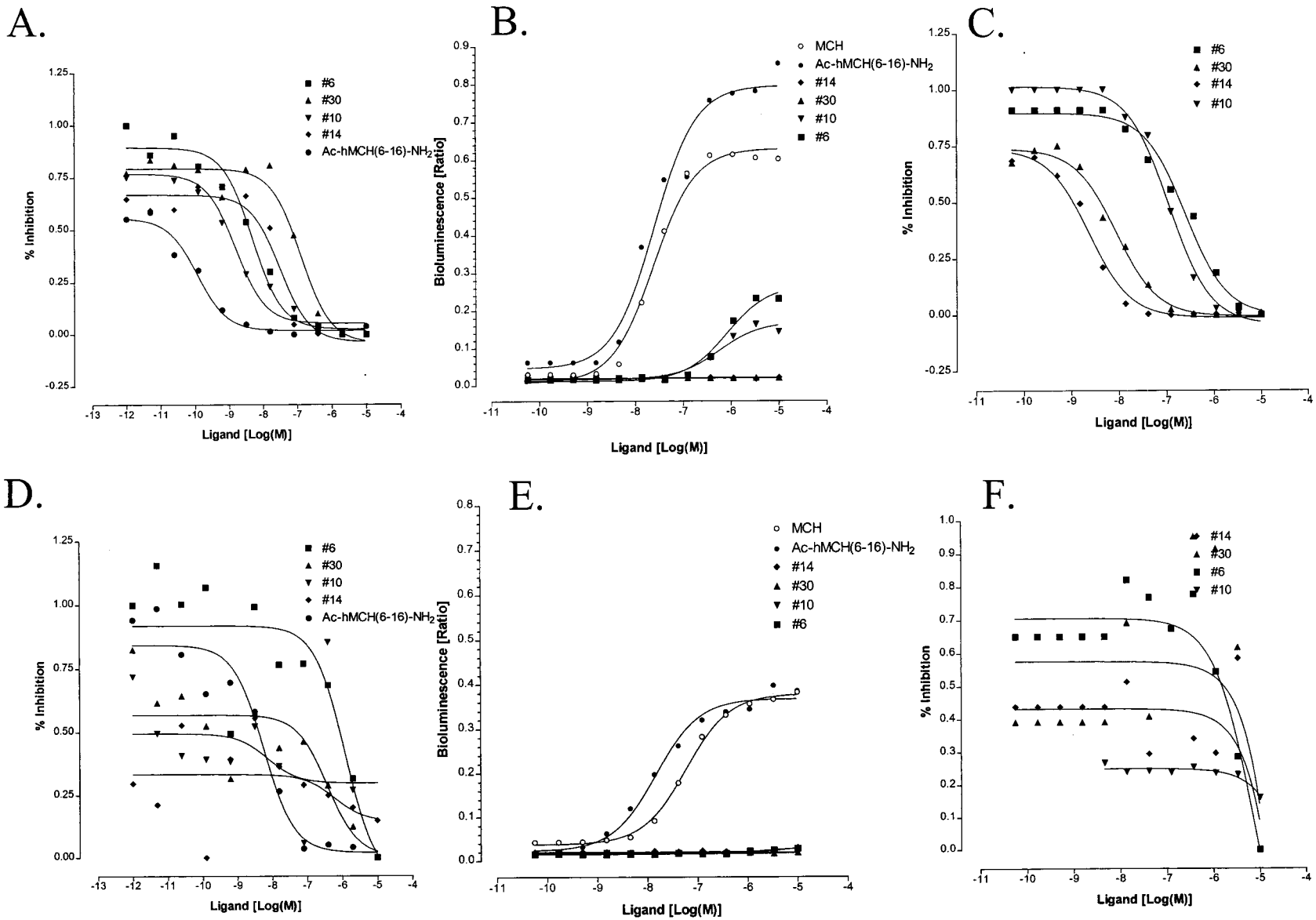


FIGURE 1: Binding and functional agonism and antagonism of selected human MCH peptides. Panels A, B, and C represent binding, functional agonism and antagonism, respectively, of human MCH peptides on the human MCH-1R. Panels C, D, and E represent binding, functional agonism and antagonism, respectively, of the same human MCH peptides on the human MCH-2R.

α-aminobutyric acid or alanine in the same position (amino acid residues with the side chains shorter by 2 and 3 carbons than the side chain of Nle or Met) were more than 10 times weaker binders and 30-fold less effective antagonists at hMCH-1R than the parent compound (peptides **22** and **23**). Additionally, analogue of compound **10** with Ala in place of Met[8] was not able to form stable complexes with hMCH-1R (compound **24)**. This confirmed the critical role of the Met[8] (or Nle[8]) side chain in molecular recognition. As anticipated, replacement of the nonessential for binding Val[12] with Ala in compound **14** yielded peptide **26** of slightly lower affinity for hMCH-1R and of similar antagonistic properties at this receptor.

Omission of Leu[9] from peptide **14** yielded the 29-membered cyclic peptide **25** which was a poor ligand for both MCH receptors. This was in agreement with the previous observation (*30*) that the 32-membered ring is necessary for the efficient interactions of the Ac-hMCH(6−16)-NH_2 analogues with hMCH-1R and hMCH-2R.

Attempts to restrict conformational freedom of the disulfide ring of compound **14** by incorporation of the cyclic conformationally constrained *cis*-4-amino-1-cyclohexanecarboxylic acid in place of a long and flexible chain of Ava led to peptide **27**. This analogue displayed similar to compound **14** binding affinity for hMCH-1R but about 15-fold lower antagonist efficiency.

Analogues of peptides **10** and **14** with D-Cys in position 7 instead of L-Cys were about 180 and 10-fold weaker binders to hMCH-1R than the parent compounds. At the same receptor, they new analogues were also significantly weaker antagonists.

In compounds **30**−**32**, both dipeptide segments of Ac-hMCH(6−16)-NH_2 discussed in this study, Leu[9]-Gly[10] and Arg[14]-Pro[15], were replaced with 5-aminovaleric acid. Binding affinity for hMCH-1R of peptide **30** with two Ava residues in its disulfide ring was about 30-fold lower than those of compounds **6** and **10** with a single Ava residue in their cycles, either in place of Leu[9]-Gly[10] or Arg[14]-Pro[15]. However, this peptide was effective hMCH-1R antagonist (K_B = 3.6 nM), devoid of agonist potency even at micromolar concentrations. Similar moderate binding affinity for hMCH-1R displayed analogues of **30** with D-Arg or des-amino-arginine in position 6 (compounds **31** and **32**, respectively). Binding and functional agonism and antagonism of peptides **6**, **10**, **14**, and **30** at both hMCH receptors is depicted in Figure 1.

DISCUSSION

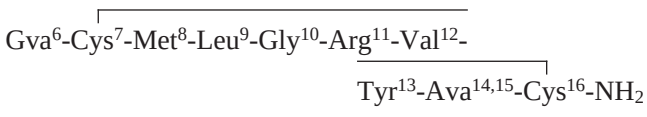
Recently, several antagonists of hMCH action at hMCH-1R were reported in the literature (*25*, *26*, *29*). These were analogues of hMCH and hMCH(6−17) with single or multiple amino acid substitutions. They acted as moderately effective antagonists, $K_B > 0.1$ μM, when tested for their abilities to reverse MCH-induced [^{35}S]GTPγS binding to membranes expressing hMCH-1R, or MCH-induced release of intracellular calcium in cells expressing this receptor (the FLIPR detection system). Because binding and functional data at hMCH-2R are not available, selectivity of these compounds for hMCH-1R is not known.

Our search for hMCH-1R antagonists started from an analogue of hMCH, the Ac-hMCH(6−16)-NH_2 peptide, which is a nonselective agonist of potency similar to that of native hMCH at both human MCH receptors (*30*). This cyclic peptide encompasses the disulfide ring and the external to the ring Arg[6] of hMCH. For the efficient interactions with both receptors, the side chains of Arg[6], Met[8], Arg[11], and Tyr[13] appear to be essential, whereas the side chains of the other residues seem to be unnecessary. Examination, in the present study, of binding and functional data for several N-methylated analogues of this nonselective agonist revealed that αN−Hs of the Leu[9] and Arg[14] residues are critical for molecular recognition at both receptors, but αNH of the Gly residue is not essential. This indicated that potential H-bonds involving αN−H of Leu[9] and Arg[14] may stabilize biologically significant conformations of this peptide and complexes with the MCH receptors, whereas potential H-bonds involving αN−H of Gly[10] appear not to contribute significantly to the interactions. The N-methyl groups may also perturb biologically significant conformations of Ac-hMCH(6−16)-NH_2 leading to weaker receptor−ligand interactions. Taken together, these conclusions prompted us to design analogues of Ac-hMCH(6−16)-NH_2 in which the Leu[9]-Gly[10] or Arg[14]-Pro[15] segment was replaced with 5-aminovaleric acid. In the new compounds, 5-aminovaleric acid mimics in length the main chains of the dipeptide units leaving the size of the disulfide rings unchanged (32-membered cycles). The Ava analogues lack an amide bond between residues 9 and 10, or between 14 and 15, but they retain free N−H in position 9 (N−H of the Ava[9,10] residue) or in position 14 (N−H of the Ava[14,15] residue). The side chains of Leu[9] or Arg[14] and Pro[15] nonessential for molecular recognition are omitted as well. A long and flexible chain of 5-aminovaleric acid in positions 9,10 or 14,15 increases significantly conformational freedom of the disulfide ring. This seems to have only slight effect on binding of the Ava analogues to hMCH-1R but appears to be deleterious to interactions with hMCH-2R, thus suggesting that the topological requirements for the formation of stable complexes with hMCH-2R are more stringent than with hMCH-1R. The Ava[9,10] and Ava[14,15] peptides were inactive at hMCH-2R and displayed moderate agonist potency at hMCH-1R at micromolar concentrations. But, most interestingly, they were able to antagonize release of calcium induced by Ac-hMCH(6−16)-NH_2 (nonselective agonist) in cells expressing hMCH-1R but not hMCH-2R. Thus, these analogues, partial agonists, were our first high affinity ligands of improved selectivity for hMCH-1R. Apparently, the enhanced conformational freedom of the disulfide ring in the Ava peptides affects significantly the biologically active conformations at both human MCH receptors and conformations necessary for antagonism at hMCH-2R but allows for conformations required for antagonism at hMCH-1R. Results obtained from testing, in binding and functional assays, of a compound with two Ava residues in its ring, the Ava[9,10],Ava[14,15] analogue, supported this conclusion. This compound with the highly flexible ring was even more effective as hMCH-1R antagonist (K_B = 3.6 nM) than peptides with one Ava residue in their structures ($K_B > 60$ nM). It was a full competitive antagonist devoid of agonist potency at hMCH-1R even at micromolar concentrations.

Interestingly, the antagonist-receptor interactions were only slightly perturbed when the conformationally constraining *cis*-4-amino-1-hexanecarboxylic acid was incorporated in compound **14** in place of Ava[14,15].

Previously, we noted (*30*) that chirality of Arg in position 6 determines, to some extent, receptor selectivity of the hMCH analogues; the D-Arg^6 analogue of Ac-hMCH(6−16)-NH_2 displayed significantly lower affinity and agonist potency at hMCH-2R than at hMCH-1R. We anticipated therefore that incorporation of D-Arg in position 6 of our Ava peptides might enhance their hMCH-1R selectivity. Evaluation in binding and activation of the D-Arg^6 peptides, which also encompassed one or two Ava residues in their disulfide rings, did not confirm our assumption. Binding affinities and agonist potencies of these peptides were similar to those of the parent Ava compounds. Hence, the Leu^9-Gly^{10} and Arg^{14}-Pro^{15} segments, not Arg^6, appear to be the principal structural features determining hMCH-1R selectivity of our analogues and, also, their ability to act as antagonists at this receptor. Negligible binding affinity and agonist potency at hMCH-2R but high affinity for hMCH-1R of the other Ava analogues compiled in Table 2 corroborated this conclusion. High affinity antagonists selective for hMCH-1R were also the Ava analogues lacking a chiral center in position 6, e.g., compounds with des-amino-arginine (5-guanidinovaleric acid, Gva) in place of Arg. This was in agreement with our previous conclusion (*30*) that for the formation of stable complexes with hMCH-1R, the guanidinyl side chain in position 6 does not need to be in a preferred orientation. One of these peptides, the Gva^6,Ava^{14,15} analogue, compound **14**, in fact, turned out to be the most selective (>1000 times over hMCH-2R) and effective antagonist discussed in this study (K_B = 0.9 nM); it was about 60-fold more effective than the L-Arg^6,Ava^{14,15} peptide. Also, even at micromolar concentrations, the Gva^6,Ava^{14,15} peptide did not possess any agonist activity at hMCH-1R or hMCH-2R.

Compound **14**



*Compound **14**.* The effect of the chain length in position 6 on binding to hMCH-1R of our new antagonist **14** was probed with compounds in which 4-guanidinobutyric acid and 3-guanidinopropionic acid were incorporated in place of 5-guanidinovaleric acid. These analogues with the guanidine group in the side chain in position 6 shortened by one or two carbons were similarly effective binders to hMCH-1R as the Gva^6 compound. Apparently, a shorter distance between the guanidine group and the disulfide ring allows for efficient interaction with the Arg^6 binding pocket on hMCH-1R. However, lack of the guanidine group in the side chain in position 6 was unfavorable to binding and antagonism at hMCH-1R. Analogues of **10** lacking Arg^6 or Ac-Arg^6 bound poorly to hMCH-1R and were rather ineffective as hMCH-1R antagonists. This was in agreement with reports (*26*, *29*, *30*) that the side chain of Arg^6, along with the side chains of Met^8, Arg^{11}, and Tyr^{13}, is critical for the efficient interactions of hMCH and its analogues with hMCH-1R.

To evaluate involvement of the thioether group of Met^8 in interactions of our hMCH-1R selective antagonist **14** with the MCH receptors, an analogue lacking sulfur was made (compound with Nle in position 8). The Nle^8 analogue was about 10-fold less effective as an hMCH-1R antagonist than the parent compound, indicating that the thioether group of Met^8 may contribute, to some extent, to the interactions with the receptor. Shortening of the length of the side chain in position 8 by one carbon at a time through replacement of Nle^8 with its lower homologues, αAbu or Ala or Gly, affected significantly binding and antagonism at hMCH-1R. Also, omission of the side chain of Met^8 from the structures of compounds **6** and **14** through replacement of the Met^8-Leu^9-Gly^{10} segment with 8-aminoactanoic acid or 5-aminovaleric acid resulted in compounds **9** and **38**, respectively, which were not able to form stable complexes with hMCH-1R and hMCH-2R. This emphasized yet again the crucial role of the Met^8 side chain in molecular recognition (*26*, *29*, *30*). Similarly, the nonessential role of the Val^{12} side chain was confirmed (*26*, *29*, *30*) by incorporation of Ala in position 12 of compound **14**; the Ala^{12} analogue was as effective an antagonist as the Val^{12} peptide.

To evaluate the effect of the ring size in compound **14** on binding affinity and hMCH-1R antagonism, the 32-membered disulfide cycle was shortened by one carbon at a time through replacement of Ava^{14,15} with its lower homologues, γ-aminobutyric acid, β-alanine, and glycine. The resulted compounds with the 30- and 31-membered rings were effective as antagonists at MCH-1R, but steric constrain in the 29-membered cyclic peptide affected significantly antagonism at hMCH-1R. Another 29-membered cyclic peptide, an analogue of **14** without Leu^9 in the ring, was also not able to form stable complexes with both MCH receptors. Similarly, a carboxyl group at the C-terminus of compound **14** was detrimental to antagonism at hMCH-1R. This group might be repelled by the acidic side chains of hMCH-1R or might form salt bridges and new H-bonds that destabilize the antagonist−receptor complexes. Moreover, the C-terminal amide group might be necessary for the stabilization of these complexes.

Our previous structure−function studies on Ac-hMCH-(6−16)-NH_2 showed (*30*) that reversal of chirality at position 7 (occupied by ring-forming Cys) results in the D-Cys^7 analogue of a noticeably lower affinity for hMCH-2R than for hMCH-1R. In the present study, we speculated that presence of D-Cys in position 7 may enhance the hMCH-1R selectivity of our antagonists **10** and **14** with a single Ava residue in their rings. The D-Cys^7 analogues of the Ava peptides, however, showed about 180 and 10-fold lower affinities for hMCH-1R than those of the parent peptides. This implied that reversal of chirality in position 7 (Cys) from L to D in the Ava peptides unfavorably changes the ring conformation and/or the orientation of the side chains.

Our study thus yielded several cyclic peptides which are selective high-affinity antagonists for the hMCH-1R. They are analogues of Ac-hMCH(6−16)-NH_2 with 5-aminovaleric acid incorporated in place of the Leu^9-Gly^{10} and/or Arg^{14}-Pro^{15} segments. These dipeptide units were found to be the principal structural elements determining the MCH receptor selectivity of our new analogues and their ability to act as hMCH-1R antagonists.

In summary, we report here a family of high-affinity antagonists for hMCH-1R, compounds which are more than 1000-fold selective with respect to hMCH-2R. These peptides might be useful in the evaluation of the physiological role of hMCH-1R in vivo.

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