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Short Segment of Human Melanin-Concentrating Hormone That Is Sufficient for Full Activation of Human Melanin-Concentrating Hormone Receptors 1 and 2

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ABSTRACT: Human melanin-concentrating hormone (hMCH) is a potent but nonselective agonist at human melanin-concentrating hormone receptors 1 and 2 (hMCH-1R and hMCH-2R, respectively). To determine the structural features of this neuropeptide which are necessary for efficient binding to and activation of the receptors, Ala-substituted, open-chain, and truncated analogues were synthesized and tested in the binding assays in CHO cells expressing hMCH-1R and hMCH-2R, and in functional assays measuring the level of intracellular calcium mobilization in human HEK-293 cells expressing these receptors. A compound consisting merely of the cyclic core of hMCH with the Arg attached to the N-terminus of the disulfide ring was found to activate both hMCH-1R and hMCH-2R about as effectively as full-length hMCH. Thus, the sequence Arg-cyclo(S−S)(Cys-Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-Cys) appears to constitute the "active core" that is necessary for agonist potency at hMCH-1R and hMCH-2R. A potent and ∼4-fold more selective agonist at hMCH-1R than at hMCH-2R is also reported.

Human melanin-concentrating hormone (hMCH)[1] is a 19-amino acid cyclic peptide present in the lateral hypothalamus and the zona incerta (*1*−*5*).

$$\mathrm{Asp^{1}\text{-}Phe^{2}\text{-}Asp^{3}\text{-}Met^{4}\text{-}Leu^{5}\text{-}Arg^{6}\text{-}}$$

$$\mathrm{Cys^{7}\text{-}Met^{8}\text{-}Leu^{9}\text{-}Gly^{10}\text{-}Arg^{11}\text{-}Val^{12}\text{-}Tyr^{13}\text{-}Arg^{14}\text{-}Pro^{15}\text{-}Cys^{16}\text{-}}$$

$$\mathrm{Trp^{17}\text{-}Gln^{18}\text{-}Val^{19}}$$

This neuropeptide appears to be involved in numerous biological functions, including regulation of the hypothalamic−pituitary−adrenal axis, energy balance, and feeding behavior (*6*−*12*). Unlike its extensively studied vertebrate counterpart (*13*−*16*), human MCH has not been detected in normal skin melanocytes and is not involved in skin color changes (*15*−*17*). Recently, considerable interest has focused on a role of MCH in regulation of feeding behavior, because a rapidly growing body of pharmacological and genetic evidence in rodents suggests that compounds able to mimic or inhibit actions of MCH might be useful in the treatment of eating disorders (*6*−*12*). It was reported that, among others, rat MCH (with an amino acid sequence identical to the sequence of human MCH) is able to stimulate food intake when injected intracerebroventricularly, the metabolic rate of mice lacking MCH increases, in the hypothalamus of genetically obese (ob/ob) and fasting mice the level of MCH messenger RNA is elevated, and also MCH antagonizes the anorexic effect of αMSH acting through a receptor distinct from the melanocortin 4 receptor (*6*−*12*). In humans, a receptor specific for MCH has been detected mainly in the hypothalamus, and recognized as a member of the family of G-protein-coupled receptors (*17*−*22*). Activation of this receptor leads to reduction of forskolin-elevated cyclic AMP levels and to mobilization of intracellular Ca (*17*−*22*). Recently, a second receptor specific for human MCH has been identified and named hMCH-2R (*23*−*25*). The amino acid sequence of MCH-2R is ∼38% identical with the amino acid sequence of the above-mentioned first MCH receptor (MCH-1R). In mammalian cells, binding of human MCH to MCH-2R causes an increase in the level of inositol phosphate turnover and release of calcium, but it does not cause reduction of forskolin-stimulated cAMP production (*23*−*25*). The MCH-2R messenger RNA appears to be expressed in only several specific regions of the brain, interestingly, in those which are also implicated in the regulation of body weight (*23*). This suggests that the second mammalian MCH receptor might be involved in the control of feeding behavior as well.

Human MCH is a high-affinity, but nonselective, natural ligand for both MCH-1R and MCH-2R (*23*−*25*). To better understand and differentiate the physiological functions of these receptors, both present in brain, new and receptor-subtype selective agonists and antagonists are needed.

The study presented here was designed to determine structural features of human MCH that are crucial for interactions of this peptide hormone with hMCH-1R and hMCH-2R. First, the amino acid side chains of hMCH critical for molecular recognition were identified through evaluation of the Ala-substituted analogues in binding and functional

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[1] Abbreviations: hMCH, human melanin-concentrating hormone; hMCH-1R, human melanin-concentrating hormone receptor 1; hMCH-2R, human melanin-concentrating hormone receptor 2; CHO, chinese hamster ovary.

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assays of the cloned human MCH-1R and MCH-2R. Subsequently, several truncated analogues of hMCH were tested to determine the smallest peptide(s) which can still efficiently bind to and activate MCH-1R and MCH-2R. At both receptors, a compound encompassing Arg (in position 6) and the cyclic segment of hMCH turned out to be equipotent to full-length hMCH.[2]

compound **19**

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

Omission of one amino acid at a time from the cyclic segment of this new agonist led to inactive compounds. Also, the importance of the side chain topology and the peptide backbone for the interaction of the same agonist with hMCH-1R and MCH-2R was investigated through examination of its analogues with chirality of one amino acid at a time reversed, and through testing its enantio, retro, and retro-enantio derivatives.

EXPERIMENTAL PROCEDURES

Peptide Synthesis, Purification, and Characterization. Elongation of peptide chains on 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (0.65 mequiv/g of substitution) was performed with a 431A ABI peptide synthesizer. Manufacturer-supplied protocols were applied for coupling of the hydroxybenzotriazole esters of amino acids in *N*-methylpyrrolidone (NMP). The fluorenylmethyloxycarbonyl (Fmoc) group was used for the semipermanent protection of α-amino groups, whereas the side chains were protected with *tert*-butyl for aspartic acid and tyrosine, 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for arginine, and trityl for cysteine. Peptides were cleaved from the resin with TFA containing 5% anisole. After 2 h at room temperature, the peptidyl resin was filtered and washed with TFA and the combined filtrates were evaporated to dryness in vacuo. The residue was triturated with ether, and the precipitate which formed was filtered, washed with ether, and dried. The crude peptides were dissolved in 5% acetic acid in water, and the pHs of the solutions were adjusted to ~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 ~5 min. After an 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 reverse-phase high-pressure liquid chromatography (RP-HPLC) on a C18 Vydac column attached to a Waters 600E system with an automatic Wisp 712 injector and a model 991 photodiode array detector. A standard gradient system of 0 to 100% buffer B over the course of 30 min was used for analysis. Buffer A was 0.1% trifluoroacetic acid in water, and buffer B was 0.1% trifluoroacetic 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 over the course of 60 min, was used for separation. The chromatographically homogeneous products (>97% pure) were analyzed by electrospray mass spectrometry.

MCH-1R and MCH-2R SPA Radioligand 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), on a Chinese hamster ovary (CHO) cell line stably expressing MCH-2R from the plasmid vector pEF1/V5-HisB (Invitrogen, Carlsbad, CA) or 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's medium (Gibco BRL, Rockville, MD) with 10% heat-inactivated fetal calf serum. A suspension of 7×10^6 COS-7 cells was transfected with 20 μg of the pCI-neo/MCH-2R plasmid by electroporation (*26*), 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 scintillation proximity assay (SPA) was developed to measure the level of specific binding of [^{125}I][Phe^{13}Tyr^{19}]hMCH. SPAs were carried out using wheat germ agglutinin−polyvinyltoluene beads (Amersham Corp., Arlington Heights, IL) in 96-well OptiPlates (Packard, Meriden, CT). Each well contained 0.25 mg of SPA beads, 1−10 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, and 0.1% BSA]. Binding buffer contained 50 mM Tris (pH 7.4), 8 mM $MgCl_2$, 12% glycerol, 0.1% BSA (Sigma, St. Louis, MO), and the following protease inhibitors: 4 μg/mL leupeptin (Sigma), 40 μg/mL bacitracin (Sigma), 5 μg/mL aprotinin (Roche Molecular Biochemicals, Indianapolis, IN), 0.05 M AEBSF (Roche Molecular Biochemicals), and 5 mM phosphoramidon (Boeringer Mannheim). Assays were optimized with respect to membrane preparations. For CHO MCH-1R membranes, 1 μg of membranes per well yielded a >6-fold specific binding window, and for COS or CHO MCH-2R membranes, 8 μg of membrane protein yielded a window of ~3-fold. Specific binding is defined as the difference between total binding and nonspecific binding conducted in the presence of 500 nM unlabeled hMCH. Beads were coated with membranes for 20 min and dispensed to the 96 wells; various concentrations of test compounds in DMSO were added (final DMSO concentration of 1−2%), and then 25 nCi of [^{125}I][Phe^{13}Tyr^{19}]hMCH (~2000 Ci/mmol; NEN Life Sciences, Boston, MA) was added to the wells. After equilibration at room temperature for 3 h, the plates were read in a TopCount apparatus (Packard). IC_{50} calculations were performed using Prism 3.0 (GraphPad Software, San Diego, CA). The IC_{50} values were measured in three different experiments. Panels A and C of Figure 1 depict binding of hMCH, the shortest active peptide (compound **19**) and the short receptor selective peptide (compound **46**) at hMCH-1R and hMCH-2R, respectively.

Aequorin Bioluminescence Functional Assays (28, 29). The aequorin bioluminescence assay is a reliable test for identifying G-protein-coupled receptors which couple through the

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

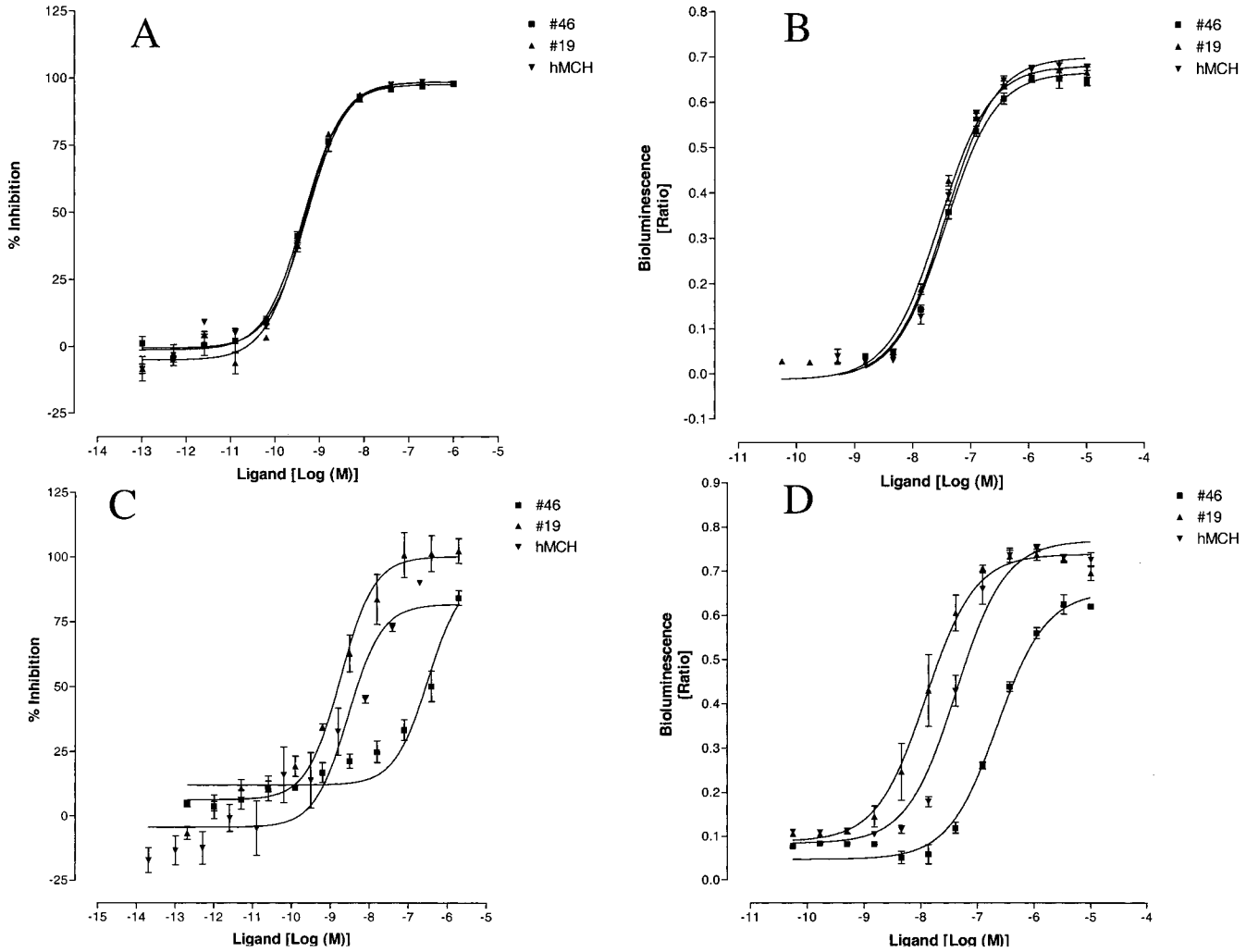

FIGURE 1: Binding and functional activities of selected MCH peptides. Panels A and B depict the binding and function of MCH peptides on human MCH-1R, respectively. Panels C and D depict the binding and function of MCH peptides on human MCH-2R, respectively.

G-protein subunit family consisting of G_q and G_{11} which leads to the activation of phospholipase C, mobilization of intracellular calcium, and activation of protein kinase C. Stable cell lines expressing either MCH-1R or MCH-2R and the aequorin reporter protein were used. The assay was performed using a Luminoskan RT luminometer (Labsystems Inc., Gaithersburg, MD) controlled by custom software written for a Macintosh PowerPC 6100. 293AEQ17/MCH-1R (or MCH-2R) cells were cultured for 72 h, and the apoaequorin 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 $CaCl_2$, and 0.1 mg/mL bovine serum albumin]. The cells were harvested, washed once in ECB medium, and resuspended to a density of 500 000 cells/mL. One hundred microliters of cell suspension (corresponding to 5×10^4 cells) was then injected into the test plate containing the ghrelin peptides, and the integrated light emission was recorded over 30 s, in 0.5 s units. Twenty microliters 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 functional EC_{50} values were measured in three separate assays. Panels B and D of Figure 1 depict the functional activity of hMCH, the shortest active peptide (compound **19**) and the short receptor selective peptide (compound **46**) at hMCH-1R and hMCH-2R, respectively. None of the analogues of hMCH listed in Tables 1−6 was tested for antagonist activity at hMCH-1R or hMCH-2R.

RESULTS

Analogues of hMCH (Tables 1−6) were prepared by solid-phase syntheses as described in Experimental Procedures. They were evaluated for their respective binding affinities for cloned human MCH receptor 1 and 2 in the competitive binding assays with [^{125}I][Phe^{13},Tyr^{19}]hMCH as the radiolabeled ligand (*30*) and, also, for their ability to stimulate IP_3-coupled mobilization of intracellular calcium in human HEK-293 cells expressing hMCH-1R and hMCH-2R (*28*, *29*). The complementary use of assays which involve the direct assessment of ligand binding and functional receptor activation coupled to intracellular signaling provides a thorough pharmacological profile of MCH peptides at MCH-1R and MCH-2R.

Binding and functional data for analogues of hMCH substituted with Ala are listed in Table 1 (compounds **1**−**16**).

Table 1: Alanine Scan of Human Melanin-Concentrating Hormone (hMCH)

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]

		MCH-1R			MCH-2R		
		binding assay[a]	functional assay[b]		binding assay[a]	functional assay[b]	
	compound	IC_{50} (nM)	EC_{50} (nM)	% activation	IC_{50} (nM)	EC_{50} (nM)	% activation
	hMCH	0.37 ± 0.27	35 ± 16	100	1.1 ± 0.7	30 ± 15	100
1	Asp^1 → Ala	1.5 ± 0.74	74 ± 2.3	98	1.8 ± 0.3	30 ± 10	83
2	Phe^2 → Ala	0.28 ± 0.15	30 ± 4.6	99	2.8 ± 0.3	14 ± 2.5	113
3	Asp^3 → Ala	0.59 ± 0.19	44 ± 4.3	98	2.4 ± 0.7	25 ± 12	111
4	Met^4 → Ala	0.51 ± 0.05	32 ± 6.3	99	2.2 ± 0.1	21 ± 11	116
5	Leu^5 → Ala	0.4 ± 0.09	42 ± 6.5	97	4.2 ± 1.1	17 ± 0.8	110
6	Arg^6 → Ala	4.0 ± 1.6	230 ± 38	72	120 ± 42	970 ± 530	85
7	Met^8 → Ala	59 ± 28	910 ± 27	42	99 ± 32	50 ± 17	100
8	Leu^9 → Ala	3.7 ± 3.6	64 ± 8.8	96	6.9 ± 1.4	49 ± 11	90
9	Arg^{11} → Ala	160 ± 20	>10000	39	290 ± 73	>10000	29
10	Val^{12} → Ala	2.1 ± 1.3	99 ± 17	93	15 ± 6.1	54 ± 10	108
11	Tyr^{13} → Ala	220 ± 55	1300 ± 54	40	730 ± 110	690 ± 750	54
12	Arg^{14} → Ala	1.3 ± 0.58	78 ± 15	97	33 ± 15	190 ± 20	81
13	Pro^{15} → Ala	1.4 ± 0.52	130 ± 38	98	24 ± 3.7	110 ± 22	114
14	Trp^{17} → Ala	0.15 ± 0.08	17 ± 9.3	104	3.5 ± 1.3	54 ± 10	95
15	Gln^{18} → Ala	0.8 ± 0.11	57 ± 9.6	97	6.3 ± 2.2	61 ± 14	111
16	Val^{19} → Ala	0.4 ± 0.07	34 ± 6.3	98	3.5 ± 1.8	34 ± 8.1	112

[a] SPA binding assay. IC_{50} is a peptide concentration at 50% specific binding. When peptide is not able to reach 50% specific binding, the percentage of [^{125}I][Phe^{13},Tyr^{19}]hMCH displaced at 2 μM peptide is reported. [b] Aequorin bioluminescence assay. EC_{50} is the concentration of peptide at 50% maximum calcium accumulation. One hundred percent activation is the bioluminescence value obtained with 10 μM hMCH.

Table 2: Truncated Analogues of hMCH

No.	Compound	MCH-1R Binding Assay[a] IC_{50} (nM)	Functional Assay[b] EC_{50} (nM)	% act.	MCH-2R Binding Assay[a] IC_{50} (nM)	Functional Assay[b] EC_{50} (nM)	% act.
	1 3 5 * 9 11 13 15 * 17 19						
hMCH	Asp Phe Asp Met Leu Arg **Cys** Met Leu Gly Arg Val Tyr Arg Pro **Cys** Trp Gln Val	0.37 ± 0.27	35 ± 16	100	1.1 ± 0.7	30 ± 15	100
17	Ac **Cys** Met Leu Gly Arg Val Tyr Arg Pro **Cys** Trp Gln Val	31 ± 38	1200 ± 150	41	53 % ± 4.3	280 ± 760	43
18	Asp Phe Asp Met Leu Arg **Cys** Met Leu Gly Arg Val Tyr Arg Pro **Cys** NH_2	0.24 ± 0.14	24 ± 8.1	99	2.4 ± 0.3	27 ± 12	109
19	Ac Arg **Cys** Met Leu Gly Arg Val Tyr Arg Pro **Cys** NH_2	0.83 ± 0.99	27 ± 7.6	97	1.6 ± 0.4	5.2 ± 3.8	105
20	Ac Arg **Cys** Met Leu Gly Arg Val Phe Arg Pro **Cys** Tyr NH_2	0.32 ± 0.25	50 ± 12	94	0.15 ± 0.1	8.1 ± 8.1	106
21	Ac **Cys** Met Leu Gly Arg Val Tyr Arg Pro **Cys** NH_2	3.5 ± 2.5	270 ± 31	69	38 % ± 9.2	1500 ± 56	84
22	Ac **Cys** Met Leu Gly Arg Val Tyr Arg **Cys** NH_2	72% ± 5.7	> 10000	1	15 % ± 7.3	> 10000	13
23	Ac **Cys** Leu Gly Arg Val Tyr Arg **Cys** NH_2	600 ± 37	> 10000	1	24 % ± 7.8	> 10000	34
24	Ac **Cys** Arg Val Tyr Arg **Cys** NH_2	>1000	> 10000	4	50 % ± 3.3	> 10000	15
25	Ac **Cys** Met Leu Gly Arg Val Tyr **Cys** NH_2	70% ± 6.8	> 10000	1	22 % ± 5.9	> 10000	13
26	Ac **Cys** Gly Arg Val Tyr **Cys** NH_2	700 ± 150	> 10000	2	18 % ± 9.4	> 10000	36

[a] As for Table 1. [b] As for Table 1. *Site of peptide cyclization (S−S).

Replacement of Arg^6, Met^8, Arg^{11}, or Tyr^{13} with Ala led to compounds that were 10−1000 times less potent at both receptors. Also, at hMCH-2R, binding affinities of compounds with Ala in position 9 (Leu), 12 (Val), 14 (Arg), or 9 (Pro) were reduced 10−60-fold, but at hMCH-1R, the same compounds were as potent agonists as the parent ligand. Substitution in the other positions with Ala led to peptides which interacted with both receptors as efficiently as hMCH.

To find the smallest segment(s) of hMCH which can still efficiently activate hMCH-1R and hMCH-2R, truncated analogues of hMCH were prepared (Table 2). Omission of the N-terminal six-amino acid segment, external to the disulfide ring, resulted in compound **17**, a slightly weaker agonist at both receptors, whereas omission of the external C-terminal residues (Trp^{17}, Gln^{18}, and Val^{19}) yielded compound **18** which is as potent as hMCH. Peptide **21** lacking both the N-terminal and C-terminal segments mentioned above, thus consisting of only the disulfide ring of hMCH, bound 10-fold less efficiently to hMCH-1R, and was a weak activator of both receptors. Addition of Arg to the N-terminus of the disulfide ring resulted in peptide **19** with enhanced binding and agonist activity. This new agonist was as potent as the native hMCH at hMCH-1R and hMCH-2R.

In compound **27**, the cysteine residues in both positions 7 and 16 were replaced with Ala. The open chain analogue of hMCH, and several of its fragments, were inactive at both

Table 3: Linear Analogues of hMCH

No.	Compound	MCH-1R Binding Assay [a] IC_{50} (nM)	MCH-1R Functional Assay [b] EC_{50} (nM)	MCH-1R Functional Assay [b] % act.	MCH-2R Binding Assay [a] IC_{50} (nM)	MCH-2R Functional Assay [b] EC_{50} (nM)	MCH-2R Functional Assay [b] % act.
	1 3 5 9 11 13 15 17 19						
hMCH	Asp Phe Asp Met Leu Arg **Cys** Met Leu Gly Arg Val Tyr Arg Pro **Cys** Trp Gln Val	0.37 ± 0.27	35 ± 16	100	1.1 ± 0.7	30 ± 15	100
27	Asp Phe Asp Met Leu Arg **Ala** Met Leu Gly Arg Val Tyr Arg Pro **Ala** Trp Gln Val	90 % ± 1.4	> 10000	3	30 % ± 12	>10000	36
28	Asp Phe Asp Met Leu Arg **Ala** Met Leu Gly NH_2	35% ± 12.1	> 10000	3	28 % ± 1.2	>10000	17
29	Asp Phe Asp Met Leu Arg **Pro** Met Leu Gly NH_2	77 % ± 0.88	> 10000	3	24 % ± 4	>10000	28
30	Ac Gly Arg Val Tyr Arg Pro **Ala** Trp Gln Val	44 %± 1.9	> 10000	3	13 % ± 1.2	>10000	15
31	Ac Gly Arg Val Tyr Arg Pro **Pro** Trp Gln Val	79 % ± 5.2	> 10000	3	18 % ± 9	>10000	16
32	Ac Arg **Ala** Met Leu Gly Arg Val Tyr Arg Pro NH_2	88% ± 0.6	> 10000	3	33 % ± 3.3	>10000	46
33	Ac Met Leu Gly Arg Val Tyr Arg Pro NH_2	>1000	> 10000	3	10 % ± 3.3	>10000	14
34	Ac Gly Arg Val Tyr Arg NH_2	>1000	> 10000	3	7.7 % ± 3	>10000	11
35	Ac Arg **Ala** Met Leu Gly Arg Val Tyr NH_2	>1000	> 10000	3	1.3 % ± 1.3	>10000	14
36	Ac Met Leu Gly Arg Val Tyr NH_2	>1000	> 10000	3	8 % ± 7.5	>10000	13

[a] As for Table 1. [b] As for Table 1.

Table 4: Analogues of Compound **19** with One Amino Acid Residue Omitted

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 binding assay [a]	MCH-1R functional assay [b]		MCH-2R binding assay [a]	MCH-2R functional assay [b]	
	compound	IC_{50} (nM)	EC_{50} (nM)	% activation	IC_{50} (nM)	EC_{50} (nM)	% activation
	hMCH	0.37 ± 0.27	35 ± 16	100	1.1 ± 0.7	30 ± 15	100
19		0.83 ± 0.99	27 ± 7.6	97	1.6 ± 0.4	5.2 ± 3.8	105
37	ΔAc	0.1 ± 0.03	46 ± 12	96	10 ± 1.5	7.1 ± 6.4	109
38	ΔMet[8]	22% ± 6.2	>10000	5	10% ± 1.9	>10000	16
39	ΔLeu[9]	22% ± 7.9	>10000	5	21% ± 9.2	450 ± 310	65
40	ΔGly[10]	53% ± 10.5	>10000	5	30% ± 11	>10000	13
41	ΔArg[11]	41% ± 9.7	>10000	5	39% ± 3.9	>10000	14
42	ΔVal[12]	41% ± 13	>10000	5	920 ± 210	460 ± 270	42
43	ΔTyr[13]	55% ± 0.14	>10000	5	27% ± 4.1	>10000	17
44	ΔArg[14]	25% ± 4.1	>10000	5	3.8% ± 1.3	>10000	14
45	ΔPro[15]	250 ± 69	>10000	5	26% ± 7.1	>10000	18

[a] As for Table 1. [b] As for Table 1.

Table 5: D-Amino Acid Scan of Compound **19**

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 binding assay [a]	MCH-1R functional assay [b]		MCH-2R binding assay [a]	MCH-2R functional assay [b]	
	compound	IC_{50} (nM)	EC_{50} (nM)	% activation	IC_{50} (nM)	EC_{50} (nM)	% activation
	hMCH	0.37 ± 0.27	35 ± 16	100	1.1 ± 0.7	30 ± 15	100
19		0.83 ± 0.99	27 ± 7.6	97	1.6 ± 0.4	5.2 ± 3.8	105
46	D-Arg[6]	0.25 ± 0.22	36 ± 3.2	97	650 ± 110	140 ± 89	87
47	D-Cys[7]	4.6 ± 2.1	910 ± 260	67	590 ± 76	750 ± 11	81
48	D-Met[8]	50%	>10000	5	14% ± 1.7	>10000	11
49	D-Leu[9]	1520	>10000	5	26% ± 8	>10000	9
50	D-Arg[11]	50%	>10000	5	33% ± 8.8	2080 ± 470	30
51	D-Val[12]	381	>10000	5	1500 ± 120	>10000	4
52	D-Tyr[13]	>2000	>10000	5	16% ± 11	>10000	4
53	D-Arg[14]	300 ± 68	>10000	5	44% ± 1.9	>10000	5
54	D-Pro[15]	410 ± 150	>10000	5	46% ± 17	>10000	4
55	D-Cys[16]	0.45 ± 0.23	160 ± 52	86	1.7 ± 0.4	4.8 ± 4.7	91

[a] As for Table 1. [b] As for Table 1.

receptors at micromolar concentrations (peptides **28**−**36**, Table 3).

The disulfide ring of our potent new agonist, compound **19**, consists of 10 amino acid residues. Analogues of this peptide were prepared in which one amino acid at a time was omitted (peptides **38**−**45**, Table 4). None of the compounds with nine amino acid residues in the ring was active at hMCH-1R or hMCH-2R, not even at micromolar concentrations.

Of the derivatives of compound **19** in which chirality of one amino acid was reversed (from L to D), only those with a D-amino acid in position 6 (Arg), 7 (Cys), or 16 (Cys)

Table 6: Enantio, Retro, and Retro-Enantio Analogues of Compound **19**

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]	functional assay[b]		binding assay[a]	functional assay[b]	
	compound	IC_{50} (nM)	EC_{50} (nM)	% activation	IC_{50} (nM)	EC_{50} (nM)	% activation
	hMCH	0.37 ± 0.27	35 ± 16	100	1.1 ± 0.7	30 ± 15	100
19		0.83 ± 0.99	27 ± 7.6	97	1.6 ± 0.4	5.2 ± 3.8	105
57	enantio	>1000	>10000	3	29% ± 1.7	>10000	16
58	retro	>2000	>10000	3	32% ± 1.5	>10000	4
59	retro-enantio	>1000	>10000	3	24% ± 1.9	>10000	7

[a] As for Table 1. [b] As for Table 1.

retained significant binding affinities and biological activities at hMCH-1R and hMCH-2R (analogues **46**, **47**, and **55**, Table 5). Thus, peptide **46** with D-Arg in place of L-Arg^6 displayed binding potency at hMCH-1R similar to that of the parent compound **19**, but bound more than 1000-fold less efficiently to hMCH-2R. Conversely, peptide **55** with D-Cys in position 16 retained binding potency at hMCH-2R but was an ~5-fold less effective activator of hMCH-1R. Analogue **47** with D-Cys in position 7 bound to hMCH-1R about as efficiently as compound **19** but was a more than 30-fold less effective agonist at both hMCH-1R and hMCH-2R. Compounds with a D-amino acid in the other positions (8, 9, and 11−15) were practically inactive at the studied receptors at micromolar concentrations (peptides **48**−**54**, Table 5).

The enantio, retro, and retro-inverso analogues of peptide **19** were not active at the receptors that were studied (compounds **56**−**58**, Table 6).

DISCUSSION

In the last two decades, numerous analogues of salmon melanin-concentrating hormone (sMCH) have been prepared to elucidate the structural features needed for its biological activity (*31*−*34*).

Asp^1-Thr^2-Met^3-Arg^4-

Cys^5-Met^6-Val^7-Gly^8-Arg^9-Val^{10}-Tyr^{11}-Arg^{12}-Pro^{13}-Cys^{14}-Trp^{15}-Glu^{16}-Val^{17}

Changes included amino acid substitutions, reversal of amino acid chiralities, omission of a single amino acid or several amino acids, and opening of the disulfide ring (*31*−*34*). Eventually, it was demonstrated that the ring structure and the Arg^9 and Tyr^{11} residues are crucial for the salmon MCH-induced aggregation of the melanosomes in teleost fish melanophores, and the dispersion of melanosomes in frog and lizard melanophores (*31*−*34*). Because of the high degree of amino acid sequence homology between salmon and human MCH (only six residues are different), it was reasonable to expect that similar structural elements are crucial for biological activity of human MCH. Our systematic evaluation of the Ala-substituted, open chain, and truncated analogues of hMCH, in binding and activation of hMCH-1R, vindicated these assumptions. For instance, the importance of the ring structure for agonistic activity at hMCH-1R was demonstrated by the inability of the open chain analogue of hMCH, and several of its fragments, to activate the receptor even at micromolar peptide concentrations. Similarly, at the same concentrations, these derivatives were unable to bind to and recognize the second hMCH receptor, thus showing that the ring structure of hMCH is necessary for agonistic activity at hMCH-2R as well. Moreover, analogues of hMCH with Ala instead of Arg^{11} or Tyr^{13} (the residues corresponding to Arg^9 and Tyr^{11} in salmon MCH) were practically inactive at hMCH-1R; this confirmed the importance of the side chains of these residues for molecular recognition. Similarly, MacDonald et al. observed (*35*) in the case of [Ala^{11}]hMCH a sharp decrease in binding affinity and agonist potency at hMCH-1R. In the study presented here, the side chains of Ala^{11} and Tyr^{13} also turned out to be crucial for the formation of stable complexes with the second human MCH receptor. Additionally, our Ala scan of hMCH revealed that the side chains of Arg^6 and Met^8 contribute moderately to molecular recognition at both hMCH-1R and hMCH-2R. In contrast, unchanged binding affinities and agonist activities of the other Ala-substituted analogues, relative to the activities of the parent compound, indicated that the side chains of residues Asp^1, Phe^2, Asp^3, Met^4, Leu^5, Leu^9, Val^{12}, Arg^{14}, Pro^{15}, Trp^{17}, Gln^{18}, and Val^{19} are not essential for interactions. Thus, at both hMCH receptors, the side chains of Arg^6, Met^8, Arg^{11}, and Tyr^{13}, and the disulfide ring, appear to be the crucial structural elements in molecular recognition.

The conclusions derived from the Ala scan were further confirmed in this study by results obtained from testing, in binding and activation, of several truncated analogues of hMCH. Hence, a compound lacking the last three C-terminal residues (Trp^{17}, Gln^{18}, and Val^{19}) was as effective an agonist at both receptors as full-length hMCH. In contrast, an analogue without the first six N-terminal residues (Asp^1, Phe^2, Asp^3, Met^4, Leu^5, and Arg^6), thus lacking the essential Arg^6, was a weaker binder and activator. Also, a compound encompassing only the cyclic segment of hMCH, with the crucial Met^8, Arg^{11}, and Tyr^{13} residues, but without the external Arg^6, was similarly less effective. This once again underlined the significant role of Arg^6 in the formation of the stable ligand−receptor complexes. As might have been anticipated, an analogue of the last peptide with all four crucial residues present in its structure, e.g., with its cyclic core (Met^8, Arg^{11}, and Tyr^{13}) and with Arg attached to the N-terminus of the disulfide ring, displayed enhanced agonist activity. In fact, the agonist potency of this compound was

equal to the potency of native hMCH at both receptors.[3]

The above-described potent new agonist consists of 11 amino acid residues of which 10 form the 32-member disulfide ring. Attempts to diminish the conformational mobility of this ring (in compound **19**, and also in **21**) through the omission of one or more residues resulted in inactive peptides at both receptors. Apparently, these changes alter the biologically active conformation of the ring.

The effect of conformational changes on biological activity of our potent new agonist, compound **19**, was also probed with analogues in which the chiralities of amino acids in the cyclic core were reversed (from L to D). The incorporation of one D-residue into a peptide ring frequently facilitates the formation of reversed turns and alters the orientation of the side chains. Interestingly, reversal of chirality in position 7 or 16 (occupied by the ring-forming Cys residues), or in position 6 (Arg, external to the disulfide ring), affected only moderately, or not at all, the potency of the agonist. Yet, of the two cysteine residues, the one in position 7 appeared to be more sensitive to conformational changes, presumably, because it is next to two residues that are essential for activation (Arg^6 and Met^8). The conformational alternations in position 7 might disturb the orientations of the side chains of the neighboring Arg^6 and Met^8 residues which are favorable for receptor interactions; this seems to be supported by the significantly reduced potency (20−100-fold) of the D-Cys^7 analogue. Reversal of chirality in position 16 (Cys) had only a slight effect on the biological potency of the new agonist, suggesting that the orientation of the side chain of Tyr^{13}, located two residues away, was less affected. D-Residues in any other positions were deleterious to agonism, probably because they unfavorably change the conformation of the peptide ring, and also affect the orientation of the side chains of the residues in the cyclic core.

Reversal of chirality of Arg^6 did not diminish agonist activity at hMCH-1R but, interestingly, caused a significant decrease in the binding and agonist potency at hMCH-2R. Hence, this conformational change resulted in a potent agonist at hMCH-1R, and in improved selectivity over hMCH-2R. It seems that for the formation of stable complexes with hMCH-2R, the side chain of Arg^6 needs to be in a preferred orientation, whereas for maximum activity at hMCH-1R, the conformational requirements are less rigid. This suggests differences between the Arg^6 binding pockets of hMCH-1R and hMCH-2R.

Structural requirements for the interaction of agonist **19** with hMCH-1R and hMCH-2R were further investigated with enantio, retro, and retro-enantio analogues. The lack of binding and functional activities for the enantio analogue, with the chiralities of all amino acid residues reversed [Ac-D-Arg^6-cyclo(S−S)(D-Cys^7-D-Met^8-D-Leu^9-Gly^{10}-D-Arg^{11}-D-Val^{12}-D-Tyr^{13}-D-Arg^{14}-D-Pro^{15}-D-Cys^{16})-NH_2], the retro analogue, with the sequence of amino acids reversed but the chiralities of the amino acids unchanged [Ac-cyclo(S−S)-(Cys^{16}-Pro^{15}-Arg^{14}-Tyr^{13}-Val^{12}-Arg^{11}-Gly^{10}-Leu^9-Met^8-Cys^7)-Arg^6-NH_2], and the retro-enantio analogue, with both the directions of the amide bonds and the chiralities of the amino acids reversed [Ac-cyclo(S−S)(D-Cys^{16}-D-Pro^{15}-D-Arg^{14}-D-Tyr^{13}-D-Val^{12}-D-Arg^{11}-Gly^{10}-D-Leu^9-D-Met^8-D-Cys^7)-D-Arg^6-NH_2], implied that both the side chain topology and direction of peptide backbone are important for molecular recognition at both receptors.

Our study has thus revealed that like in salmon MCH, the cyclic segment of hMCH with Arg^{11} and Tyr^{13} constitutes the "essential core" required for efficient binding to and activation of hMCH-1R. We found that for full activity both the Arg^6 external to the disulfide ring and internal Met^8 are necessary as well. Furthermore, we determined in this study that the same principal structural features (ring structure and Arg^6, Met^8, Arg^{11}, and Tyr^{13}) are needed for molecular recognition at hMCH-2R.

In summary, we report here the syntheses of several potent agonists at hMCH-1R and hMCH-2R, one of them with improved selectivity with respect to hMCH-2R. These new peptides (smaller than hMCH) should be useful for evaluation of the physiological roles of both hMCH receptors. Together with the other analogues reported in this study, they should also be helpful in localization and characterization of the hMCH binding domains on hMCH-1R and hMCH-2R, and in the design of potent and selective agonists and antagonists for both receptors.

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REFERENCES

1. Presse, F., Nahon, J.-L., Fischer, W. H., and Vale, W. (1990) *Mol. Endocrinol. 4*, 632−637.
2. Mouri, T., Takahashi, K., Kawauchi, H., Sone, M., Totsune, K., Murakami, O., Itoi, K., Ohneda, M., Sasano, H., and Sasano, N. (1993) *Peptides 14*, 643−646.
3. Nahon, J.-L. (1994) *Crit. Rev. Neurobiol. 8*, 221−262.
4. Burgaud, J.-L., Poosti, R., Fehrentz, J.-A., Martinez, J., and Nahon, J.-L. (1997) *Biochem. Biophys. Res. Commun. 241*, 622−629.
5. Sone, M., Takahashi, K., Murakami, O., Totsune, K., Arihara, Z., Satoh, F., Sasano, H., Ito, H., and Mouri, T. (2000) *Peptides 21*, 245−250.
6. Gonzalez, M. I., Vaziri, S., and Wilson, C. A. (1996) *Peptides 17*, 171−177.
7. Qu, D., Ludwig, D. S., Gammeltoft, S., Piper, M., Pelleymounter, M. A., Cullen, M. J., Mathes, W. F., Przypek, R., Kanarek, R., and Maratos-Flier, E. (1996) *Nature 380*, 243−247.
8. Rossi, M., Choi, S. J., O'Shea, D., Miyoshi, T., Ghatei, M. A., and Bloom, S. R. (1997) *Endocrinology 138*, 351−355.
9. Gonzalez, M. I., Kalia, V., Hole, D. R., and Wilson, C. A. (1997) *Peptides 18*, 387−392.
10. Shimada, M., Tritos, N. A., Lowell, B. B., Flier, J. S., and Maratos-Flier, E. (1998) *Nature 396*, 670−674.
11. Ludwig, D. S., Mountjoy, K. G., Tatro, J. B., Gillette, J. A., Frederich, R. C., Flier, J. S., and Maratos-Flier, E. (1998) *Am. J. Physiol. 274*, E627−E633.
12. Hwa, J. J., Witten, M., and Ghibaudi, L. (2001) *Obes. Res. 9*, 69.
13. Kawauchi, H., Kawazoe, I., Tsubokawa, M., Kishida, M., and Baker, B. I. (1983) *Nature 305*, 321−323.

[3] In the course of preparation of the manuscript, we have learned that Audinot et al. [(2001) *J. Biol. Chem. 276*, 13554−13562] reported that the dodecapeptide MCH_{6-17} [Arg-cyclo(S−S)(Met-Leu-Gly-Arg-Val-Tyr-Arg-Pro-Cys)-Trp] is the smallest peptide with full agonist activity at hMCH-1R. This compound however is longer by one amino acid residue (Trp) than our short peptide **19** which is equipotent to full-length hMCH at both hMCH-1R and hMCH-2R.

14. Oshima, N., Kasukawa, H., Fujii, R., Wilkes, B. C., Hruby, V. J., and Hadley, M. E. (1986) *Gen. Comput. Endocr. 64*, 381−388.
15. Baker, B. I. (1991) *Int. Rev. Cytol. 126*, 1−47.
16. Baker, B. I. (1993) *Ann. N.Y. Acad. Sci. 680*, 279−289.
17. Bachner, D., Kreienkamp, H., Weise, C., Buck, F., and Richter, D. (1999) *FEBS Lett. 457*, 522−524.
18. Chambers, J., Ames, R. S., Bergsma, D., Muir, A., Fitzgerald, L. R., Hervieu, G., Dytko, G. M., Foley, J. J., Martin, J., Liu, W. S., et al. (1999) *Nature 400*, 261−265.
19. Lembo, P. M., Grazzini, E., Cao, J., Hubatsch, D. A., Pelletier, M., Hoffert, C., St-Onge, S., Pou, C., Labrecque, J., Groblewski, T., et al. (1999) *Nat. Cell Biol. 1*, 267−271.
20. Saito, Y., Nothacker, H. P., Wang, Z., Lin, S. H., Leslie, F., and Civelli, O. (1999) *Nature 400*, 265−269.
21. Saito, Y., Nothacker, H., and Civelli, O. (2000) *Trends Endocrinol. Metab. 11*, 299−303.
22. Hawes, B. E., Kil, E., Green, B., O'Neill, K., Fried, S., and Graziano, M. P. (2000) *Endocrinology 141*, 4524−4532.
23. Mori, M., Harada, M., Terao, Y., Sugo, T., Watanabe, T., Shimomura, Y., Abe, M., Shintani, Y., Onda, H., Nishimura, O., and Fujino, M. (2001*) Biochem. Biophys. Res. Commun. 283*, 1013−1018.
24. Hill, J., Duckworth, M., Murdock, P., Rennie, G., Sabido-David, C., Ames, R. S., Szekeres, P., Wilson, S., Bergsma, D. J., Gloger, I. S., Levy, D. S., Chambers, J. K., and Muir, A. I. (2001) *J. Biol. Chem.* (in press).
25. Sailer, A. W., Sano, H., Zeng, Z., McDonald, T. P., Pan, J., Pong, S.-S., Feighner, S. D., Tan, C. P., Fukami, T., Iwaasa, H., Hreniuk, D. L., Morin, N. R., Sadowski, S. J., Nossoughi, R., Ito, M., Ito, M., Bansal, A., Ky, B., Figueroa, D. J., Jiang, Q., Austin, C. P., MacNeil, D. J., Ishihara, A., Ihara, M., Kanatani, A., Van der Ploeg, L. H. T., Howard, A. D., and Liu, Q. (2001) *Proc. Natl. Acad. Sci. U.S.A.* (in press).
26. Strader, C. D., Sigal, I. S., Register, R. B., Candelore, M. R., Rands, E., and Dixon, R. A. F. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 4384−4388.
27. MacNeil, D. J., Occi, J. L., Hey, P. J., Strader, C. D., and Graziano, M. P. (1994) *Biochem. Biophys. Res. Commun. 198*, 328−334.
28. Ungrin, M. D., Singh, L. M., Stocco, R., Sas, D. E., and Abramovitz, M. (1999) *Anal. Biochem. 272*, 34−42.
29. Button, D., and Brownstein, M. (1993) *Cell Calcium 14*, 663−671.
30. Drozdz, R., and Eberle, A. N. (1995) *J. Pept. Sci. 1*, 58−65.
31. Kawazoe, I., Kawauchi, H., Hirano, T., and Naito, N. (1987) *Int. J. Pept. Protein Res. 29*, 714−721.
32. Lebl, M., Hruby, V. J., Castrucci, A. M. L., Visconti, M. A., and Hadley, M. E. (1988) *J. Med. Chem. 31*, 949−954.
33. Castrucci, A. M. L., Lebl, M., Hruby, V. J., Matsunaga, T. O., and Hadley, M. E. (1989) *Life Sci. 45*, 1141−1148.
34. Baker, B. I., Kinsman, R. G., Moss, C. A., White, P. D., Paul, P. K. C., Brown, D. W., Campbell, M. M., and Osguthorpe, A. J. (1990) *Peptides 11*, 1103−1108.
35. MacDonald, D., Murgolo, N., Zhang, R., Durkin, J. P., Yao, X., Strader, C. D., and Graziano, M. P. (2000) *Mol. Pharmacol. 58*, 217−225.

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