Biochemical and Biophysical Research Communications **272,** 23–28 (2000)
doi:10.1006/bbrc.2000.2589, available online at http://www.idealibrary.com on IDEAL®

Analogs of Lactam Derivatives of α-Melanotropin with Basic and Acidic Residues

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Received March 24, 2000

A role of the aromatic and of the basic residues of the potent agonist (MTII) and antagonist (SHU9119) at the human melanocortin receptors 4 in the formation and stabilization of ligand-receptor complexes was examined. Analogs of MTII and SHU9119 with glutamic acid replacing one amino acid at a time were synthesized and tested for their ability to bind to and activate human melanocortin receptors 3, 4, and 5. Replacement of Phe (Nal) or Trp with Glu resulted in analogs of MTII and SHU9119 which were practically inactive at the receptors studied. The rather large (and unexpected) tolerance toward the presence of Glu in the position of His or Arg of MTII and SHU9119 clearly suggested that in the ligand receptor complexes these basic residues are not in contact with the receptors but probably face the extracellular environment. This identified the aromatic residues of MTII and SHU9119 as the primary structural features determining interactions of the agonist/antagonist with hMCR3-5.

Key Words: **melanotropin; melanocortin receptor; glutamic acid-scan; lysine-scan; binding affinity; cAMP accumulation assay.**

Various peptide hormones, neurotransmitters, neuropeptides, inflamatory mediators, and other bioactive molecules interact with membrane proteins called G-protein coupled-receptors (GPCRs). This family of receptors is characterized by a common structural motif of seven membrane-spanning helices connected by regions (loops) outside the lipid membrane (1, 2). Binding of an appropriate ligand to the extracellular face of a G-protein coupled-receptor activates the receptor-associated intacellular G-proteins. This activates various signaling pathways in the cells, and prompts the specific physiological events (1, 2). To be effectively recognized by the G-protein coupled-receptors, ligands need to possess distinct structural features. For example, the amino group and the β-hydroxy group of catecholamines seem to be necessary for efficient binding to the adrenergic receptors (1, 2). For antagonism at the same receptors, additionally, an aromatic ring is required.

Similarly, the short peptide motif of α-melanotropin, His^6-Phe^7-Arg^8-Trp^9, also present in the other melanotropins, βMSH, γMSH, and ACTH, has been recognized (3, 4) as necessary for efficient binding of these peptide hormones to the melanocortin receptors (members of the G-protein coupled-receptor family) and for activation of the receptors. The His^6-Phe^7-Arg^8-Trp^9 motif, frequently referred to as the "active core," consists of two basic hydrophilic amino acids (His and Arg) and two bulky aromatic amino acids (Phe and Trp). This suggests that both hydrophobic aromatic interactions and hydrophilic interactions could play a defining role in the recognition of the peptide ligands by the melanocortin receptors.

Throughout the years, numerous analogs of melanotropins (melanocortin peptides) were prepared in an attempt to enhance their biological activity and receptor subtype selectivity (at present, five subtype of human melanocortin receptors are known, hMCR1-5). Not surprisingly, the sequence His^6-Phe^7-Arg^8-Trp^9 was present in many of them (3–14). A particularly valuable synthetic ligand is the lactam derivative of αMSH, a potent but not selective agonist at the human melanocortin receptor 3, 4, and 5, Ac-Nle^4-cyclo(5β->10ϵ)(Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10})-amide, called MTII (7). Another lactam derivative of αMSH, Ac-Nle^4-cyclo(5β->10ϵ)(Asp^5-His^6-D-Nal(2′)^7-Arg^8-Trp^9-Lys^{10})-amide, called SHU9119 (8), is a potent an-

Throughout this report, the numbering of the amino acids 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}-NH$_2$, has been retained for all cyclic and linear analogs of this hormone.

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0006-291X/00 $35.00

tagonist at the human melanocortin 3 and 4 receptors but an agonist at the human melanocortin receptor 5.

The hypothetical three-dimensional models of the complexes of the melanocortin receptor 1 with the melanocortin peptides, α-MSH, NDP-αMSH, and MTII, were described by several groups (9–13). In the construction of these models, bacteriorhodopsin served as template for MCR1. The computer-assisted molecular-dynamic simulation and energy-minimization studies on these complexes (12, 15), combined with the side-directed mutagenesis of MCR1 (13), allowed elucidation of several amino acid residues in this receptor presumably involved in the binding to the "active core" of the melanotropins. The Phe residues 175, 179, 195, 196, 257, 258, and 280, and Tyr residues 182 and 183, located in the transmembrane portion of MCR1, were postulated to interact with Phe^7 and Trp^9 of the melanocortin peptides through stacking of the aromatic rings. The hydrophilic basic Arg^8 residue of the His^6-Phe^7-Arg^8-Trp^9 motif was proposed to interact with several negatively charged residues of MCR1 (Glu^{94}, Asp^{117}, and Asp^{121}). Thus, from the two putative binding pockets on MCR1, one seems to be predominantly hydrophobic (lined up with the side chains of the aromatic amino acids listed above), and the other predominantly hydrophilic (lined up with the side chains of the acidic amino acids, also listed above). Additionally, it was suggested (13, 14) that ionic interactions might play a dominant role in the formation and stabilization of the MCR1 complexes with αMSH, NDP-αMSH, and MTII. Because of the high sequence-similarity among melanocortin receptors, it was reasonable to speculate that these ionic forces might also determine interactions of the same agonists with the other melanocortin receptors, notably the melanocortin receptors 3, 4, and 5, present in the central nervous system.

Our previous structure-function studies on MTII indicated (16), however, that the hydrophilic residues at positions 6 (His) and 8 (Arg) were less essential for the activation of hMCR3-5 than the hydrophobic aromatic residues 7 (Phe) and 9 (Trp) of MTII. Therefore, in the present studies we reexamined the role of the basic residues in the molecular recognition of MTII at the melanocortin receptors 3, 4, and 5. Analogs* of MTII with glutamic acid and lysine were prepared and evaluated in binding assays and also in cAMP accumulation assays at hMC3-5R, because activation of these receptors leads to the elevation of cAMP levels. It was anticipated that incorporation of an acidic or a basic residue into the MTII structure would allow formation of new hydrogen-bonds and salt-bridges between this ligand and the receptors studied, and that the affinities between the new analogs and hMCR3-5 would be different from those between the parent compound and the same receptors. We expected that these studies will provide insight into the effect of ionic interactions on binding to and activation at the receptors mentioned. The rather large tolerance toward charge reversal at position 6 (His → Glu) and at position 8 (Arg → Glu) of MTII reveled that the basic side chains of His^6 and Arg^8 are not the principal structural determinants in the interactions of this agonist with the human melanocortin receptors 3, 4, and 5.

Similarly, the basic amino acids, His^6 and Arg^8 of α-MSH, NDP-αMSH, the "active-core" peptide (Ac-His^6-D-Phe^7-Arg^8-Trp^9-amide), and the SHU9119 antagonist were substituted with glutamic acid to probe the ionic interactions between each of these ligands and the human melanocortin 3, 4, and 5 receptors.

αMSH

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

NDP-αMSH

Ac-Ser^1-Tyr^2-Ser^3-Nle^4-Glu^5-His^6-D-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^{12}-Val^{13}-amide

MTII

Ac-Nle^4-Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10}-amide

"active-site" peptide

Ac-His^6-D-Phe^7-Arg^8-Trp^9-amide

MATERIALS AND METHODS

Peptide synthesis and purification. Building of peptide chains on p-methylbenzhydrylamine resin, formation of the lactam ring on a resin, deprotection, and cleavage of peptides from a resin, and purification of the crude products by high-pressure liquid chromatography were performed as previously described in detail (16, 17). The chromatographically homogenous compounds were analyzed by amino acid analysis and electrospray mass spectrometry.

Competitive binding assays. Binding activity of compounds was measured using membranes from Chinese hamster ovary (CHO) cells expressing the cloned melanocortin receptors. Binding reactions contained membranes, 200 pM [^{125}I]-NDP-α-MSH (New England Nuclear Corp.), and increasing concentrations of unlabelled test compounds. Reactions were incubated for 1.5 h and then filtered as described previously (16). Binding data were analyzed using GraphPad curve-fitting software. Active peptides were evaluated in 3 independent experiments.

cAMP assays. Agonist activity of compounds was measured using Chinese hamster ovary (CHO) cells expressing the cloned melanocortin receptors. Compounds together with 0.6 mM IBMX were incubated at room temperature with dissociated cells for 40 min, lysed with dilute HCl or by incubation at 100°C for 4 min to terminate the assay as described previously (16). cAMP was quantitated by Amersham (Arlington Heights, IL) RPA556, cAMP SPA screening assay or by New England Nuclear (Boston, MA) SMP-001J Flashplate cAMP assay. All active peptides were characterized in 3 independent experiments. Tables 1, 2, and 3 indicate the mean ± SEM (standard error of the mean).

For assay of functional antagonism, the alpha-MSH dose response curves were performed in the absence and presence of different doses

TABLE 1

Analogs of MT-II Substituted with Glutamic Acid or Lysine

Ac-Nle[4]-cyclo(5β → 10ϵ)(Asp[5]-His[6]-D-Phe[7]-Arg[8]-Trp[9]-Lys[10])-[11]amide

	Compound:	Binding assay, IC_{50}(nM)*			cAMP assay, EC_{50}(nM)**		
		hMCR3	hMCR4	hMCR5	hMCR3	hMCR4	hMCR5
#	MT-II	1.6 ± 0.09	0.07 ± 0.02	0.89 ± 0.01	0.75 ± 0.21	0.19 ± 0.04	2.8 ± 0.47
1	His[6] → Glu	785 ± 28	13 ± 2	187 ± 14	950 ± 431	16 ± 6	141 ± 5
2	D-Phe[7] → D-Glu	>10000	>10000	>10000	7% @ 10	2992 ± 64	2511 ± 483
3	Arg[8] → Glu	2066 ± 250	58 ± 5.5	1070 ± 55	65 ± 10	7 ± 3	69 ± 13
4	Trp[9] → Glu	>10000	>10000	>10000	2% @ 10	1% @ 10	23% @ 10
5	His[6], Arg[8] → Glu, Glu	7000 ± 3000	10000	4000 ± 1000	4500 ± 530	1000 ± 44	4400 ± 570
6	Nle[4], His[6] → Glu, Glu	8% @ 4	19% @ 4	69% @ 4	6% @ 5	1500 ± 180	6% @ 5
7	Nle[4], Arg[8] → Glu, Glu	8% @ 1	1% @ 1	60% @ 4	9% @ 5	4700 ± 330	3% @ 5
8	Glu[11] inserted	14 ± 4	1.8 ± 0.2	3 ± 0.3	2.4 ± 0.28	1.6 ± 0.25	3.1 ± 0.82
9	His[6] → Lys	37 ± 6	0.7 ± 0.2	4 ± 0.5	3.0 ± 0.7	0.10 ± 0.03	2.0 ± 0.9
10	D-Phe[7] → D-Lys	0.6% @ 5	10% @ 5	24% @ 5	2% @ 10	1% @ 10	0% @ 10
11	Arg[8] → Lys	190 ± 26	2 ± 0.4	26 ± 1.2	92 ± 47	2.0 ± 0.52	22 ± 7
12	Trp[9] → Lys	4100 ± 1500	880 ± 49	841 ± 240	3% @ 10	12% @ 10	1770 ± 1034
13	Lys[11] inserted	7 ± 3	1.3 ± 0.3	3 ± 0.8	1.2 ± 0.21	0.54 ± 0.04	3.4 ± 0.36

* Concentration of peptide at 50% specific binding. When peptide was not able to reach 50% specific binding, the percentage of ^{125}I-NDP-αMSH displaced at a given peptide concentration (μM) was reported.

** Concentration of peptide at 50% maximum cAMP accumulation or, the percentage of cAMP accumulation (relative to αMSH) observed at a given peptide concentration (μM).

of the selected antagonist. The antagonists were preincubated for 10 min with the receptor expressing cells prior to the addition of alpha-MSH and the assay was finished as described above. In Table 4, the K_B values from Schild analysis are the average from two experiments.

RESULTS AND DISCUSSION

Pharmacological and genetic evidence suggests (18, 19) that melanocortin receptor 4 is involved in the regulation of energy balance and body weight. Thus, for the design of the new MCR4 ligands (compounds which might be useful in the treatment of eating disorders), it is important to understand the forces which govern binding of the known agonist (MTII) and antagonist (SHU9119) to this receptor.

Analogs of MTII substituted with glutamic acid or lysine were synthesized and tested for binding to and for activation of hMCR4 (Table 1). The same compounds were also evaluated for their recognition by the additional brain melanocortin receptors 3 and 5. For the competitive binding assays, [^{125}I]NDP-αMSH was used as radioligand; for the cAMP accumulation assays, the CHO cells expressing these receptors were utilized (see Materials and Methods).

Replacement of the aromatic residues at position 7 (Phe) and 9 (Trp) of MTII with an acidic residue (Glu) or a basic residue (Lys) yielded compounds **2**, **4**, **10**, and **12** inactive at hMCR3-5 at micromolar concentrations (Table 1). Previously, also analogs of MTII with the aliphatic residues, Ala or Pro, in the same positions were reported to be devoid of agonist potency at the same melanocortin receptors (16, 17). Together, these observations suggest that Phe[7] and Trp[9] of MTII are critical for recognition of this agonist at hMCR3-5 and support the model of interactions between peptide ligands and melanocortin receptors described in the literature (9–13). In the previously discussed model, the aromatic side chains (Phe[7] and Trp[9]) of the melanocortin peptides (αMSH, NDP-αMSH) bind to a putative hydrophobic binding pocket in MCR1, presumably through stacking of aromatic side chains. In addition, the aromatic residues of MTII might be required for stabilization of bioactive conformations of this agonist.

Charge reversal at position 6 or 8 of MTII, that is replacement of His[6] or Arg[8] with Glu, led to compounds with neutral overall surface charges (compounds **1** and **3** in Table 1). Both [Glu[6]]MTII and [Glu[8]]MTII were only 10 to 100-fold less potent than the parent compound in the cAMP accumulation assays at hMCR3-5. Apparently, the γ-carboxyl group of Glu in position 6 or 8 was not completely deleterious to the recognition of MTII at hMCR3-5; perhaps, because in the ligand-receptor complexes, the side chain of Glu points away from the receptors. In view of the suggested interactions between Arg[8] of the melanocortin peptides and the acidic residues of the proposed ionic binding pocket on MCR1 (9–13), this result was rather unanticipated. Our present study unequivocally shows that neither the side chain of His[6] nor that of Arg[8] is essential for efficient interactions of MTII with MCR4. Arginine 8 appears to play a significantly smaller role in the for-

TABLE 2

Analogs of αMSH Substituted with Glutamic Acid

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

#	Compound	Binding assay, IC_{50}(nM)*			cAMP assay, EC_{50}(nM)**		
		hMCR3	hMCR4	hMCR5	hMCR3	hMCR4	hMCR5
	αMSH	19 ± 2	19 ± 2	120 ± 19	0.73 ± 0.07	1.6 ± 0.18	19 ± 2.4
14	[Glu[6]]αMSH	20% @ 5	29% @ 5	5% @ 5	1% @ 2.5	8% @ 2.5	11% @ 2.5
15	[Glu[8]]αMSH	4% @ 2	7% @ 2	1% @ 2	3000 ± 1500	4100 ± 590	14% @ 5
	NDP-αMSH	3 ± 1	3.7 ± 1	1 ± 0	0.12 ± 0.01	0.14 ± 0.02	0.33 ± 0.2
16	[Glu[6]]NDP-αMSH	93 ± 27	57 ± 5	19 ± 2	47 ± 7.4	66 ± 11	11 ± 1.3
17	[Glu[8]]NDP-αMSH	158 ± 27	67 ± 5	135 ± 22	11 ± 2.1	13 ± 1.0	20 ± 6.2
	Ac-His[6]-D-Phe[7]-Arg[8]-Trp[9]-amide	5400 ± 33	100 ± 24	30% @ 20	1000 ± 320	47 ± 7.8	17% @ 5
18	$His^6 \rightarrow Glu$	4% @ 5	12% @ 5	5% @ 5	2% @ 5	11% @ 5	12% @ 5
19	$Arg^8 \rightarrow Glu$	52% @ 5	27% @ 5	5% @ 5	5% @ 5	4300 ± 790	3% @ 5

Note. *, ** as for Table 1.

mation and stabilization of complexes between MTII and MCR4 than it might have been assumed.

In contrast, substitution of His^6 or Arg^8 of αMSH with Glu resulted in compounds **14** and **15,** which were practically devoid of agonist potency at hMCR3-5 (Table 2). Sahm and coworkers reported (20) that replacement of the same residues with the neutral alanine also yields analogs of αMSH with the binding affinities and biological activities significantly reduced. The Ala analogs of αMSH were tested on cultured B16 murine melanoma cells expressing mouse melanocortin receptor 1. Unlike in the conformationally constrained cyclic MTII, the basic residues (His^6 and Arg^8) of the linear and thus more flexible αMSH seem to be necessary for efficient interactions between the hormone and the melanocortin receptors 3, 4, and 5, and perhaps also, for the stabilization of biologically active conformations of αMSH.

However, analogs of NDP-αMSH with His^6 or Arg^8 substituted with Glu retained significant binding affinities and agonist potencies at hMCR3-5, albeit 100- to 600-fold lower than those found for the parent compound (peptides **16** and **17** in Table 2). For [Glu^6]-NDP-αMSH, a similarly modest reduction of binding affinities was observed previously by Schioth and coworkers (21). Acidic residues in position 6 or 8 of NDP-αMSH weaken but do not destroy the interaction between the agonist and MCR4; whereas, acidic residues in the same positions of αMSH preclude formation of stable αMSH-melanocortin receptor complexes. The less essential role of His^6 and Arg^8 of NDP-αMSH in the recognition of this potent agonist at MCR3-5 was in contrast with the above discussed critical role of the same residues of αMSH in binding to and activation at hMCR3-5. The enhanced activity of NDP-αMSH (and many of its analogs) relative to αMSH has usually been attributed, among other factors, to the presence of one D-amino acid (Phe^7) in the peptide sequence. D-Phenylalanine in position 7 presumably facilitates a bent orientation of the peptide chain and stabilizes conformations favorable to interactions with the receptor.

Interestingly, analogs of the moderately active short peptide Ac-His^6-D-Phe^7-Arg^8-Trp^9-amide (a segment of NDP-αMSH, encompassing the "active core" of melanotropins) with His^6 or Arg^8 replaced by Glu poorly activated the here studied melanocortin receptors, even at micromolar concentrations (see compounds **18** and **19** in Table 2). In the absence of other points of attachments, that is of the N-terminal and C-terminal segments of NDP-αMSH external to the "active core," the two basic residues of this short peptide might become critical to binding to the here discussed receptors.

The analog of MTII with two acidic residues, one in position 6 ($His \rightarrow Glu$) and another in position 8 ($Arg \rightarrow Glu$), was practically inactive at hMCR3-5 at micromolar concentrations (compound **5** in Table 1). Simultaneous replacement of the two basic residues of MTII by two acidic residues, like in [Glu^6,Glu^8]MTII, changes the overall surface charge of this ligand from positive to negative. This change is apparently unfavorable for the formation of the ligand-receptor complexes. The γ-carboxyl groups of [Glu^6,Glu^8]MTII might be repelled by the acidic residues of the putative ionic binding pockets of hMC3-5R, or they might form new salt-bridges and H-bonds that destabilize the agonist-receptor complexes.

Similarly, the analogs **6** and **7** with the two negatively charged residues, in position 4 ($Nle \rightarrow Glu$) and 6 ($His \rightarrow Glu$), or in position 4 ($Nle \rightarrow Glu$) and 8 ($Arg \rightarrow Glu$) respectively, were inactive even at micromolar concentrations at hMCR3-5. The overall surface charge of these compounds was also negative.

Previously, we reported (17) that the polar residues at position 4 (Glu or Lys) replacing Nle only minimally affected recognition of MTII at hMCR4. Thus, [Glu^4]MTII was 10-fold less potent at hMCR4 than MTII, but about 200 and 70-fold less active at hMCR5 and hMCR3. Additionally, [Lys^4]MTII was as potent as

TABLE 3

Analogs of SHU9119 Substituted with Glutamic Acid

	Ac-Nle^4-cyclo(5$\beta \rightarrow$ 10ϵ)(Asp^5-His^6-D-Nal(2′)^7-Arg^8-Trp^9-Lys^{10})-amide						
	Compound:	Binding assay, IC_{50}(nM)*			cAMP assay, EC_{50}(nM)**		
		hMCR3	hMCR4	hMCR5	hMCR3	hMCR4	hMCR5
#	SHU9119	0.09 ± 0.02	0.04 ± 0.01	0.05 ± 0.02	10% @ 1	4% @ 1	0.090 ± 0.01
20	Nle^4 → Glu	130 ± 18	3.8 ± 0.2	120 ± 14	2% @ 5	5% @ 5	47 ± 9
21	His^6 → Glu	12 ± 0.9	0.7 ± 0.2	1.2 ± 0.2	0% @ 5	2% @ 5	1.8 ± 0.87
22	Arg^8 → Glu	88 ± 12	3.6 ± 0.3	2.5 ± 0.3	5% @ 5	3% @ 5	1.8 ± 0.37
23	Trp^9 → Glu	0% @ 5	11% @ 5	38% @ 5	3% @ 5	2% @ 5	26% @ 5

Note. *, ** as for Table 1.

MTII in binding and functional assays at hMCR4 (see reference 17 for other substitutions at position 4 of MTII). Apparently, Ac-Nle^4 of MTII is not required for potency and selectivity of MTII at human melanocortin receptor 4, probably because this part of MTII is not in direct contact with hMCR4. Similarly, the N-terminal section of NDP-αMSH was reported (21) not to interact with hMCR4.

Insertion of a charged residue, Glu or Lys, at the C-terminus of MTII, external to the lactam ring, only slightly affected interactions of the new peptides with hMCR3-5 (compounds **8** and **13** in Table 1).

Ac-Nle^4-Asp^5-His^6-D-Phe^7-Arg^8-Trp^9-Lys^{10}-

X^{11}-amide 8: X = Glu; 13: X = Lys

At the here studied receptors, these "extended" analogs of MTII displayed agonist activities similar to those of the parent compound, implying that the C-terminus of MTII is also not in contact with hMCR3-5 but probably faces the extracellular environment.

A fairly conservative substitution of His^6 with Lys in MTII resulted in peptide **9**, as a potent agonist at hMCR4 as MTII. The conservative replacement of Arg^8 with Lys led to compound **11**, which is only slightly less active than MTII at hMCR4. A subtle decrease of binding affinities was also observed by Schioth and coworkers (21) when they replaced Arg^8 with Lys in NDP-αMSH.

Analogs of SHU9119 substituted with glutamic acid also were synthesized and tested for binding to and for activation at hMCR3-5 (Table 3). The amino acid sequence of SHU9119 differs from MTII only at position 7 where phenylalanine has been replaced with 2-naphthylalanine (8).

SHU9119

Ac-Nle^4-Asp^5-His^6-D-Nal^7-Arg^8-Trp^9-Lys^{10}-amide

Substitution of the aromatic residue (Trp) at position 9 with Glu yielded [Glu^9]SHU9119, which was inactive both in binding and in functional assays at hMCR3-5 (compound **23** in Table 3). Similarly to the interactions of MTII with hMCR3-5, the aromatic side chain at position 9 of SHU9119 seems to be required for efficient binding to the melanocortin receptors studied and for antagonistic potency at hMCR3 and hMCR4.

Interestingly, replacement of the hydrophobic residue norleucine at position 4 or the basic residues at position 6 (His) or 8 (Arg) with Glu resulted in analogs of SHU9119 (compounds **20**, **21**, and **22** in Table 3), which retained substantial antagonist potencies at hMCR3 and hMCR4 albeit 2 logs lower than those of the parent compound. Functional antagonism of peptides **20**, **21**, and **22** is summarized in Table 4. Moreover, the Glu analogs were also 5 to 100-fold less potent as agonists at the human melanocortin receptor 5.

Our studies demonstrate, therefore, that the aromatic residues of MTII, Phe^7 and Trp^9, are the primary structural features determining interactions of this agonist with the melanocortin receptor 3, 4, and 5. The hydrophobic interaction of Phe^7 and Trp^9 with the aromatic residues of the receptors (of the putative hydrophobic binding pockets) seem to be a significant force in the formation and stabilization of ligand-receptor complexes. In contrast, the basic residues in MTII, His^6

TABLE 4

Functional Antagonism of hMCR3 and hMCR4 by Selected SHU9119 Analogs

	Ac-Nle^4-cyclo(5$\beta \rightarrow$ 10ϵ)(Asp^5-His^6-D-Nal(2′)^7-Arg^8-Trp^9-Lys^{10})-amide		
	Compound:	K_B (nM)	
		hMCR3	hMCR4
#	SHU9119	2.15 ± 0.05	0.39 ± 0.085
20	Nle^4 → Glu	530 ± 14	28 ± 4.0
21	His^6 → Glu	41 ± 14	2.1 ± 0.95
22	Arg^8 → Glu	170 ± 59	9.0 ± 0.40

and Arg^8 appear to play only a negligeable role in molecular recognition at the studied melanocortin receptors. This suggest that in the ligand-receptor complexes these residues are not in direct contact with the receptors but are probably exposed to the extracellular environment. Our conclusions are not in agreement with the previously postulated (3–14) interactions between Arg^8 of NDP-αMSH and the negatively charged residues of the melanocortin receptors (of the putative ionic binding pockets). The N-terminal and C-terminal sections of MTII, external to the lactam ring, also seem not to participate in the formation of the ligand-receptor complexes, perhaps, because they similarly point away from the receptors. Moreover, hydrophobic interactions appear to dominate in the binding of the SHU9119 antagonist to hMCR3-5.

In summary, Glu-substituted analogs of MTII and SHU9119 were used to develop a better recognition of some meaningful structural features of these peptide ligands that promote strong and specific interactions with the human melanocortin receptors 3, 4, and 5. In conjunction with mutagenesis studies on the melanocortin receptors, the new compounds reported here, might be used for the elucidation and confirmation of specific interactions stabilizing these ligand-receptor complexes. The insight gained in this study should be helpful in the design of new agonists and antagonists for hMCR3-5.

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