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Biological and Conformational Examination of Stereochemical Modifications Using the Template Melanotropin Peptide, Ac-Nle-c[Asp-His-Phe-Arg-Trp-Ala-Lys]-NH_2, on Human Melanocortin Receptors

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Examination of conformationally constrained melanotropin peptides (Ac-Nle[4]-c[Asp[5]-His-Phe[7]-Arg-Trp[9]-Ala-Lys]-NH_2) on four human melanotropin receptors (hMC1R, hMC3R, hMC4R, and hMC5R) resulted in identifying the importance of ligand stereochemistry at positions 5, 7, and 9 for agonist binding affinity and receptor selectivity. A trend in ligand structure−activity relationships emerged for these peptides, with the hMC1R and hMC4R possessing similar tendencies, as did the hMC3R and hMC5R. α-MSH (Ac-Ser-Tyr-Ser-Met[4]-Glu-His-Phe[7]-Arg-Trp-Gly-Lys-Pro-Val-NH_2), NDP-MSH (Ac-Ser-Tyr-Ser-Nle[4]-Glu-His-D-Phe[7]-Arg-Trp-Gly-Lys-Pro-Val-NH_2), and MTII (Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]-α-MSH(4−10)-NH_2) were also examined at each of these melanocortin receptors. Interestingly, the linear NDP-MSH possessed greater binding affinity for the hMC3R and hMC5R than did the cyclic analogue MTII. The peptide Ac-Nle-c[Asp-His-Phe-Arg-D-Trp[9]-Ala-Lys]-NH_2 demonstrated the greatest differentiation in binding affinity between the hMC1R and hMC4R (78-fold). Analogue Ac-Nle-c[Asp-His-Phe[7]-Arg-Trp-Ala-Lys]-NH_2 resulted in micromolar binding affinity (or greater) at the hMC3R and hMC5R, demonstrating the importance of D-Phe[7] for ligand binding potency at these receptors. Ac-c[Asp-His-Phe-Arg-Trp-Ala-Lys]-NH_2 resulted in loss of binding affinity at the hMC5R, implicating the importance of Nle[4] (or a hydrophobic residue in this position) for binding to this receptor. Ac-Nle-c[D-Asp[5]-His-Phe-Arg-Trp-Ala-Lys]-NH_2 was unable to competitively displace [^{125}I]NDP-MSH binding at micromolar concentrations on the hMC3R and hMC5R, suggesting the importance of chirality of Asp[5] either for ligand−receptor interactions or for orientation of the side chain lactam bridge and the structural integrity of the peptide conformation. Energy calculations performed for these peptides resulted in the identification of a low-energy ligand conformer family that is common to all the ligands. The differences in ligand binding affinities observed in this study are postulated to be a result of different ligand−receptor complexed interactions and not solely to the ligand structure.

Introduction

The melanotropin peptides make up a family of hormones which are derived by posttranslational modification of the pro-opiomelanocortin (POMC) prohormone.[1] This family is composed of α-, β-, and γ-melanocyte stimulating hormones (MSH) and adrenocorticotropin (ACTH). These hormones, and the melanocortin receptors, have been implicated in a multitude of physiological effects[2,3] including obesity,[4,5] cognitive affects related to learning, memory, and attention,[1,6] analgesic effects,[7] immunolomodulatory effects,[8] erectogenic activity,[9] follicular melanogenesis,[10] cardiovascular regulation,[11] and tanning.[12] Within the past decade, five human melanocortin receptors (hMC1R−hMC5R) have been cloned and characterized.[13−17] These receptors were characterized as belonging to the superfamily of seven transmembrane-spanning receptors which are coupled to G-proteins. All of these melanocortin receptors possess unique tissue distributions and recognize all the melanotropin peptides, with the exception of hMC2R, which is only activated by ACTH.[13] With the cloning and stable expression of these human melanocortin receptors, a new challenge of identifying ligands which are selective agonists or antagonists, and understanding the conformational, structural, topographical, and substructural features which lead to these properties, has yet to be revealed. Other than the commercially available melanotropin peptides, a limited number of synthetic analogue structure−activity studies on the human melanocortin receptors have been reported. These include modifications of γ-MSH examined on the hMC3R and hMC4R,[18] characterization of antagonists,[19−21] and topographically modified cyclic peptides on hMC1R.[22]

Identification of the minimal amino acid sequence of α-melanotropin required for the generation of a minimal physiological response in the frog[23] and lizard[24] was Ac-His-Phe-Arg-Trp-NH_2, which is also considered the "message sequence". This peptide was 15000−300000-fold less potent than α-MSH in the frog and lizard skin, respectively, and did not possess prolonged biological activity in either assay. Inversion of chirality of Phe

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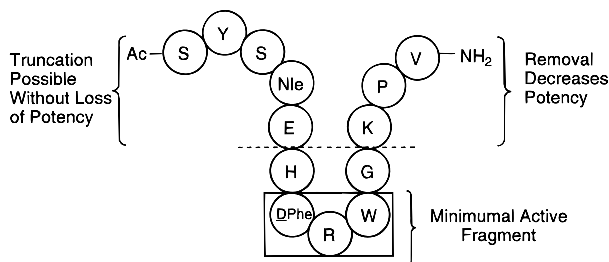

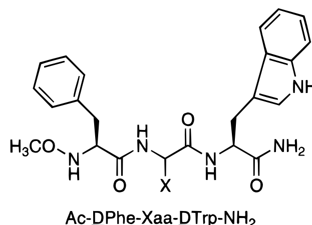

Figure 1. (A) Amino acid sequence of NDP-MSH summarizing the importance of residues evaluated on the classical frog and lizard skin bioassays. (B) Proposed side chain pharmacophores of D-Phe[7] and D-Trp[9] in the peptide template which resulted in a micromolar EC_{50} in the frog skin bioassay.

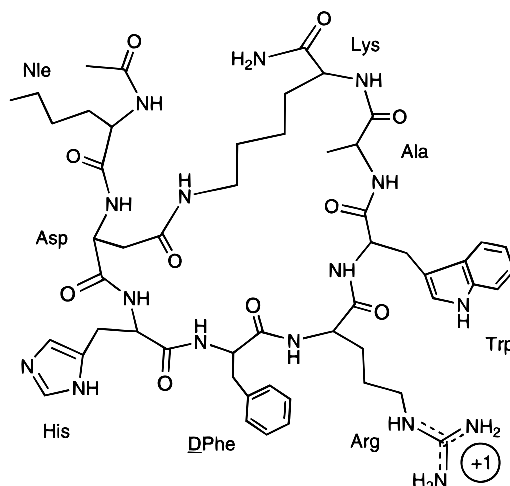

Figure 2. Structural depiction of Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Ala-Lys]-NH_2 (analogue **I**), which differs from MTII by the insertion of Ala[10] and increased cyclization size to a 26-membered ring instead of the 23-membered ring of MTII.

at position 7 (Ac-His-D-Phe-Arg-Trp-NH_2) resulted in only 10−1,500-fold decreased potency, compared to α-MSH, in the lizard and frog skin assays, respectively.[25] Examination of the tripeptide Ac-D-Phe-Arg-Trp-NH_2 resulted in 1000−100000-fold decreased potency in the lizard and frog skin assays, respectively, as compared to α-MSH.[25] Further stereochemical and substructural modifications of the tri- and tetrapeptides resulted in the identification of the frog skin melanotropin side chain pharmacophore model. This model was based on the Ac-D-Phe-Xaa-D-Trp-NH_2 template where Xaa can be a variety of amino acids, but increased potency resulted if Xaa = Arg (Figure 1).[26] In summary, all of these studies illustrate the importance of the stereochemical and resulting substructural conformations of the Phe and Trp residues in the message sequence, His-Phe-Arg-Trp.

Linear peptides are highly flexible and make determination of biologically relevant conformations required for receptor recognition and binding, signal transduction, and receptor dissociation difficult. One approach to determine biologically relevant conformations has been the utilization of side chain to side chain cyclic constraints.[27,28] Several endeavors to "lock" in the bioactive conformations of α-melanotropin have been reported previously for the frog and lizard skin melanocortin receptors. These approaches have sought to identify the bioactive conformations of the peptide backbone and topographical orientation of the amino acid side chains in the melanotropin active site region His-Phe-Arg-Trp. These side chain monocyclic analogues include Ac-c[Cys[4],Cys[10]]-α-MSH-NH_2,[29] Ac-c[Cys[4], D-Phe[7], Cys[10]]α-MSH(4−11)-NH_2,[30] Ac-[Nle[4]-c(D-Orn[5],-Xaa[7],Glu[8])]α-MSH(4−11)-NH_2 where Xaa is L- or D-Phe,[31] Ac-[Nle[4]-c(Yaa[5], D-Phe[7],Lys[10])]α-MSH(4−10)-NH_2 where Yaa is Asp or Glu,[32,33] Ac-[Nle[4]-c(Asp[5],D-Phe[7],-Zaa[10],Lys[11])]α-MSH(4−11)-NH_2 where Zaa is Ala, Aib, or Sar,[34] the N- to C-terminal monocyclic analogues c[Gly-His-Xaa-Arg-Trp-Gly] where Xaa is L- or D-Phe,[35] and bicyclic analogues based on the templates Ac-c[Cys[4]-c(Xaa[5],D-Phe[7],Cys[10])-Lys[11]]-NH_2 and Ac-c[Cys[4]-c(Xaa[5],D-Phe[7],Lys[10]]-Cys[11])-NH_2, where Xaa is Glu or Asp.[36] The cyclic disulfide Ac-c[Cys[4],Cys[10]]-α-MSH-NH_2, and cyclic lactam Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH(4−10)-NH_2 analogues were shown to be superpotent, prolonged acting analogues whereas the other analogues generally were less potent than the native hormone.

The peptides examined in this study were originally designed to study the "bioactive" backbone conformations on the classical frog (*Rana pipiens*) skin melanocortin receptor,[34,37] and have been characterized here on the human melanocortin receptors which recognize all the endogenous melanotropin peptides (hMC1R and hMC3R−hMC5R). NDP-MSH, Ac-Ser-Tyr-Ser-Nle[4]-Glu-His-D-Phe[7]-Arg-Trp-Gly-Lys-Pro-Val-NH2,[38,39] differs from α-MSH by having Met[4] substituted with the stable nonoxidizable Nle[4], and the stereoisomer D-Phe[7] instead of L-Phe[7]. This linear peptide is the superpotent and prolonged acting peptide which has been used to characterize all of the cloned melanocortin receptors to date. Upon iodination at the Tyr residue at position 2, NDP-MSH has become the standard radiolabeled peptide for competitive binding studies. A cyclic peptide, Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2 (MTII),[33] has been identified as a lead peptide for a multitude of structure−function studies on the frog and lizard skin melanocortin receptors and is also a superpotent and prolonged acting peptide on the human receptors.[40] Insertion of Ala between Trp and Lys increases the lactam bridge from 23-membered to a more flexible 26-membered ring system (Figure 2). This modification of a lactam bridge between positions 5 and 11 has previously been proposed to be important for a biologically active peptide conformation.[41] Further substructural and topographical modifications by inverting the chirality at positions 5, 7, and 9 were hypothesized to affect the substructural integrity of the peptide conformation and, therefore, receptor selectivity. The goal of this study was to identify topographical and/or substructural peptide templates which may be initial leads for the design of further receptor selective compounds and for probing ligand−receptor interactions at the human melanocortin receptors.

Results

The syntheses of the melanotropin analogues discussed herein were accomplished by solid-phase synthetic methods.[34,37] These analogues were purified by reversed-phase high-pressure liquid chromatography (RP-HPLC). The properties and purity of these peptides were assessed by fast atom bombardment mass spectrometry (FAB-MS), RP-HPLC, thin-layer chromatography (TLC) in three solvent systems, amino acid analysis, and optical rotation (see the Experimental Section).

Biological Evaluation. Table 1 summarizes the binding IC_{50} values and the second messenger cAMP EC_{50} values of these cyclic melanotropin peptides on the human melanocortin receptors which recognize all the endogenous melanotropin peptides (hMC1R, hMC3R, hMC4R, and hMC5R). In analyzing ligand−receptor interactions (ability of the peptide to competitively displace binding of [^{125}I]NDP-MSH), α-MSH, the endogenous melanotropin peptide, was less potent than MTII on all the melanocortin receptors and possessed greatest affinity for the hMC1R, with 10- to 6-fold decreased potency for the hMC3R and the hMC4R, respectively. Interestingly, there was a 93-fold decrease in binding affinity of α-MSH for the hMC5R compared with the hMC1R. NDP-MSH possessed nanomolar affinity on all the melanocortin receptors examined. This linear peptide possessed decreased potency compared with cyclic MTII, at the hMC1R and hMC4R. At the hMC3R and hMC5R, however, NDP-MSH possessed 7−50-fold greater binding affinity than MTII, respectively. MTII possessed sub-nanomolar affinities at the hMC1R and hMC4R, 8 nM binding affinity at the hMC3R, and 174-fold decreased binding affinity at the hMC5R (43.6 nM) compared to the hMC1R (0.25 nM). Analogue **I** differs from MTII by the insertion of an Ala between the Trp and Lys amino acids. Surprisingly, only a maximum of 4-fold decreased binding affinity (hMC3R) resulted from this structural modification, as compared to MTII. Deletion of Nle[4] (analogue **II**) resulted in a 5.5-fold decreased binding affinity at the hMC1R, a 4-fold decreased binding affinity at the hMC3R, a 2-fold decreased binding affinity at the hMC4R, and a loss of ability to competitively displace [^{125}I]NDP-MSH at the hMC5R, as compared with analogue **I**. When the D-Phe[7] was changed to L-Phe[7] (analogue **III**), up to a 100-fold ligand selectivity was observed between the hMC1R and the hMC3R, while only a 30-fold difference in selectivity between the hMC3R and hMC4R resulted. Interestingly, however, analogue **III** lacked the ability to competitively displace [^{125}I]NDP-MSH at the hMC5R. Analogue **IV**, which contains D-Trp at position 9, resulted in 2.6-fold decreased binding affinity at the hMC1R, 5.4-fold decreased binding affinity at the hMC3R, 80-fold decreased binding affinity at the hMC4R, and 5.6-fold decreased binding affinity at the hMC5R, as compared with the corresponding L-Trp analogue (**I**). When the Asp residue comprising a component of the lactam bridge was inverted to the D configuration (analogue **V**), a remarkable loss in ability to competitively displace [^{125}I]NDP-MSH binding resulted at both the hMC3R and hMC5R. Analogue **V** possessed 754-fold decreased binding affinity at the hMC1R and 102-fold decreased binding affinity at the hMC4R compared to analogue **I**,

Table 1. Binding and Intracellular cAMP Accumulation of These Melanotropin Peptides on the Human Melanocortin Receptors

peptide	structure	hMC1R binding IC_{50} (nM)[a]	hMC1R cAMP EC_{50} (nM)[b]	hMC3R binding IC_{50} (nM)	hMC3R cAMP EC_{50} (nM)	hMC4R binding IC_{50} (nM)	hMC4R cAMP EC_{50} (nM)	hMC5R binding IC_{50} (nM)	hMC5R cAMP EC_{50} (nM)
α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	5.97 ± 0.33	ND	50.40 ± 10.1	ND	38.70 ± 1.44	ND	557 ± 198	ND
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2	0.51 ± 0.11	0.19 ± 0.04	1.17 ± 0.14	4.10 ± 0.62	1.16 ± 0.09	0.72 ± 0.04	0.86 ± 0.04	0.58 ± 0.17
MTII	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2	0.25 ± 0.03	0.19 ± 0.099	8.56 ± 2.28	0.24 ± 0.05	0.72 ± 0.02	0.04 ± 0.008	43.6 ± 19.7	1.48 ± 0.29
I	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Ala-Lys]-NH_2	0.35 ± 0.05	0.098 ± 0.003	25.5 ± 4.74	27.6 ± 1.51	0.89 ± 0.11	2.52 ± 0.98	24.8 ± 1.58	6.01 ± 0.85
II	Ac-c[Asp-His-D-Phe-Arg-Trp-Ala-Lys]-NH_2	1.94 ± 0.36	13.4 ± 3.35	102 ± 14.6	88.5 ± 24.0	2.03 ± 0.41	2.92 ± 0.64	>1000*	
III	Ac-Nle-c[Asp-His-Phe-Arg-Trp-Ala-Lys]-NH_2	22.4 ± 1.37	2.61 ± 0.19	2400 ± 939	136.4 ± 29.4	77.8 ± 3.16	6.08 ± 0.35	>1000*	
IV	Ac-Nle-c[Asp-His-D-Phe-Arg-D-Trp-Ala-Lys]-NH_2	0.91 ± 0.01	0.30 ± 0.13	137 ± 68	161.4 ± 43.3	71.5 ± 47.7	14.6 ± 0.77	141 ± 32.3	269 ± 61.7
V	Ac-Nle-c[D-Asp-His-D-Phe-Arg-Trp-Ala-Lys]-NH_2	264 ± 77.4	34.2 ± 6.5	>1000*		91.0 ± 7.14	3.38 ± 0.15	>1000*	

[a] IC_{50} = Concentration of peptide at 50% specific binding (N = 4−6). The peptides were tested at a range of concentrations (10^{-6} M−10^{-12} M). An asterisk (*) >1000 identifies peptides that were unable to competitively displace [^{125}I]NDP-MSH binding at concentrations up to 1 μM. [b] EC_{50} = Effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation (N = 4).

Table 2. Effect of Modifications in Various Analogues on Their Binding Potencies Toward Different Melanocortin Receptors

modification type[a]	receptor type			
	hMC1R	hMC3R	hMC4R	hMC5R
presence of Ala[10] (**I**/MTII)	1.40	2.98	1.24	0.57
presence of Nle[4] (**I**/**II**)	0.18	0.25	0.44	<0.024
configuration of Phe[7] (**I**/**III**)	0.016	0.011	0.011	<0.024
configuration of Trp[9] (**I**/**IV**)	0.38	0.19	0.012	0.18
configuration of Asp[5] (**I**/**V**)	0.001	<0.025	0.01	<0.024

[a] In parentheses are comparisons of the ratios of IC_{50} value of analogue **I** divided by the IC_{50} value of the analogue indicated. Values indicated in boldface italic highlight important peptide modifications.

which contains L-Asp. All these peptides were agonists as determined by a functional cAMP assay (Table 1).

Energy Calculations. Low-energy conformers for NDP-MSH, MTII, and analogues **I**−**V** were either found by molecular modeling methods described in the Experimental Section or obtained from our earlier calculations (α-MSH itself was not taken into consideration).[34,37] To proceed with geometrical comparisons, we had to determine which compounds should be regarded as "potent" as opposed to "nonpotent," for each receptor separately and which functional groups are especially important for binding toward a particular receptor. Similar quantitative criterion for both issues were utilized. For example, if binding affinity of a given compound, according to IC_{50} values (Table 1), is at least 2 orders of magnitude lower than for the compound with the highest binding affinity for a particular receptor subtype, the given compound is defined as "nonpotent." If a particular modification of analogue **I** results in increasing/decreasing potency greater than 2 orders of magnitude, this modification is regarded as "important" (see Table 2, where values equal to or less than 0.01 represent the important modifications and are shown in boldface italics). According to Table 2, "nonimportant" functional groups (residues) were represented in geometrical comparison by their C^{α} atoms, and "important" ones by C^{α} and C^{β} atoms.

It was decided that for all receptors a "standard" compound for geometrical comparison (the compound to compare with) would be NDP-MSH, since it possesses uniform nanomolar binding affinities at all the melanocortin receptors (Table 1), and its radiolabeled analogue [^{125}I] NDP-MSH was employed in binding studies. Additionally, since presence of the Ala[10] residue is not important for binding to all receptors (see Table 2), only C^{α} atoms of the Asp[5] to Trp[9] fragments were selected as obligatory atomic centers for geometrical comparison of these peptides at all the human melanocortin receptors. Geometrical comparison have been performed for each particular receptor starting with the following considerations: (A) *for the hMC1R*, potent analogues are NDP-MSH, MTII, **I**, **II**, and **IV** with the $C^{\alpha,\beta}$Asp, C^{α}His, $C^{\alpha,\beta}$Phe, C^{α}Arg, and C^{α}Trp atomic centers used for comparison; (B) *for the hMC3R*, potent analogues are NDP-MSH, MTII, **I**, and **II**, with the $C^{\alpha,\beta}$Asp, C^{α}His, $C^{\alpha,\beta}$Phe, C^{α}Arg, and C^{α}Trp atomic centers used for comparison; (C) *for the hMC4R*, potent analogues are NDP-MSH, MTII, **I**, **II**, and **IV**, with the $C^{\alpha,\beta}$Asp, C^{α}His, $C^{\alpha,\beta}$Phe, C^{α}Arg, and $C^{\alpha,\beta}$Trp atomic centers used for comparison; and (D) *for the hMC5R*, potent analogues are NDP-MSH, MTII, and **I**, with the C^{α}Nle, $C^{\alpha,\beta}$Asp, C^{α}His, $C^{\alpha,\beta}$Phe, C^{α}Arg, and C^{α}Trp atomic centers used for comparison.

Despite having performed geometrical comparisons for each receptor individually, with different sets of potent compounds and atomic centers being employed, the general geometrical shape of peptide backbone which resulted for the ligands appeared to be nearly identical for all the melanocortin receptors examined in this study. The same geometrical type of spatial arrangement of side chain groups in the fragment Nle-Trp emerged as a common low-energy conformation type for all compounds potent at all receptors. In terms of dihedral angle values, three main subtypes of backbone conformations for NDP-MSH, two for MTII, one for analogue **I**, one for analogue **II** (these two are identical), and two for analogue **IV** were distinguished. All of these conformers are described in Table 3 (note that Table 3 lists only the lowest-energy conformers belonging to each receptor subtype). Interestingly, the peptide conformations binding to each melanocortin receptor subtype differ from one another only by slight rotations of the planes of amide bonds between some residues.

Discussion

These studies examine the effect of stereochemical and substructural modifications of cyclic melanotropin peptides on the human melanocortin receptor subtypes hMC1R, hMC3R, hMC4R, and hMC5R for receptor selectivity and agonism. Surprisingly, inclusion of the Ala residue between the Trp and Lys of MTII (analogue **I,** Figure 2) did not significantly result in decreased binding affinity. Previous studies examining ring size of monocyclic melanotropin peptides for superpotency identified a 23-membered ring for side chain to side chain cyclizations to be optimal in the lizard skin bioassay.[29,30,33] For the human melanocortin receptors, the increased flexibility of a 26-membered ring was found not to significantly alter ligand binding, suggesting that the 23-membered ring structure of MTII may not be optimal for these receptors. Additionally, the linear NDP-MSH analogue is more potent than cyclic MTII at the hMC3R and hMC5R. These data suggest that, for future design strategies for selective melanocortin ligands, evaluation of cyclization ring size for the hMC1R and the hMC4R may be appropriate, while modifications using a linear template may be appropriate for the hMC3R and the hMC5R.

Examination of Table 1 reveals an emerging trend in SAR for these cyclic peptides. Comparative ligand binding IC_{50} values identify similar SAR profiles between the hMC1R and hMC4R and in a like manner, between the hMC3R and hMC5R. This observation, as indicated above, has potential for the design of ligands that can differentiate between these two different subclasses of melanocortin receptors. This SAR grouping has also been reported for the antagonism of the hMC1R and hMC4R by the endogenous agouti protein.[4] When examining the human melanocortin receptor homology and amino acid sequences, only about a 60% receptor homology is present when considering functionally similar amino acids comparing the hMC1R with the hMC4R, and the hMC3R with the hMC5R, with even less homology if identical residues are considered.[42,43] Tissue distribution using poly(A)$^{+}$ Northern Blot analysis identified the hMC1R mRNA expression in the melanocytes,[13,14] hMC2R mRNA expression in adrenal tissue,[13] hMC3R mRNA expression in the brain and peripheral tissues,[16,44] hMC4R mRNA expression

Table 3. Backbone Conformations of Asp-Trp Fragment Common for All Potent Compounds[a]

peptide	Asp/Glu φ	Asp/Glu ψ	His φ	His ψ	DPhe φ	DPhe ψ	Arg φ	Arg ψ	L/D-Trp φ	L/D-Trp ψ
NDP−MSH	−72	−22	−63	103	128	−21	−104	−52	63	74
	−84	−19	−66	110	114	−9	−116	−62	57	66
	−84	−19	−62	113	78	17	−154	37	−67	−56
MTII	−101	18	−56	121	85	11	−144	122	58	48
	−75	80	−130	130	82	−61	−78	89	−82	−20
I	−75	−33	−58	130	101	−39	−74	−40	−112	146
II	−	−33	−58	130	101	−39	−74	−40	−112	146
IV	−76	89	−137	120	74	−71	−56	147	111	−157
	−75	−5	−50	138	89	−57	−65	129	74	29

[a] The structures listed are only the lowest-energy conformers belonging to each subset.

in the brain,[17] and mMC5R mRNA expression in the brain, skeletal muscle, and peripheral tissues.[45] Other studies using experimental methods such as PCR techniques report mRNA expression of these receptors in other tissues.[46] These types of biological and physiological information should be kept in mind when evaluating ligand−receptor structure−activity relationships and receptor selectivity. For example, both the hMC3R and hMC4R have been located in the brain, with extensive mapping identifying regions which express both subtypes, and regions of the brain where each subtype is expressed individually.[42,47] Therefore, the observation that similar SAR profiles exist between the hMC1R and hMC4R, or the hMC3R and hMC5R, may actually be beneficial when the purpose of identifying an agonist or antagonist targeted for a particular receptor subtype (*in vitro*) in certain tissues (i.e., hMC3R and hMC4R in the brain) is to identify physiological effects associated with a particular receptor subtype. Thus, analogue **V**, which possesses 91 nM binding affinity at the hMC4R and is unable to competitively displace radiolabel at micromolar concentrations at the hMC3R (Table 1) may provide a tool to differentiate physiological activities of these two melanocortin receptors.

In evaluating the cyclic peptides examined in this study for receptor selectivity, it appears that the Nle residue is important for ligand binding at the hMC5R. This loss in ability to competitively displace [^{125}I]NDP-MSH binding at micromolar concentrations of analogue **II** would implicate complementary hMC5R hydrophobic interactions as important. Modification of the chirality of the lactam bridge in analogue **V**, (D-Asp^5) resulted in 1056-fold decreased potency at the hMC1R and a 126-fold decreased potency at the hMC4R, as compared to MTII, while losing ability to competitively displace [^{125}I]-NDP-MSH at both the hMC3R and hMC5R at 1 μM concentration. This particular modification (c[D-Asp^5,-Lys^{11}]) may be a structural template which can be optimized to differentiate receptor selectivity between the hMC1R and hMC4R.

Analogue **IV** (Ac-Nle-c[Asp-His-D-Phe-Arg-D-Trp-Lys]-NH_2) possesses the greatest hMC1R selectivity compared with the hMC4R, albeit it is only a 79-fold difference. This analogue possesses sub-nanomolar potency for the hMC1R (0.91 nM) with approximately a 150-fold selectivity compared with both the hMC3R and hMC5R and only 3-fold decreased potency compared with analogue **I** at the hMC1R. Comparison of the primary amino acid sequences of the hMC1R and hMC4R reveals transmembrane region 4 to diverge significantly between these two subtypes (Table 4). Only 38% identical amino acid homology is found, and

Table 4. Alignment of Transmembrane Domain Four of the Human Melanocortin Receptors Examined

Melanocortin Receptor Subtype	Residue Number Initiating the Transmembrane Domain 4	Amino Acid Sequence of Transmembrane Domain 4[a]	
hMC1R	159	PRARQAVAAIWVA	SVV**FST**LFIAY
hMC4R	164	KRVGIIISCIWAA	CTV**SGI**LFIIY
hMC3R	196	RKALTLIVAIWVC	CGV**CGV**VFIVY
hMC5R	157	RRSGAIIAGIWAF	CTG**CGI**VFILY
Residues Conserved in the G-Protein Coupled Receptor Family		----------W--	-----------

[a] The transmembrane domains were defined according to the Baldwin allignment.[69] The 11 amino acids enclosed in the box indicate residue which may be involved in ligand−receptor interactions and are located in the "top" portions of the transmembrane binding pocket. Amino acids in bold are highlighted due to the dramatic differences in side chain functional moieties of the hMC1R compared with the other residues for the subtypes indicated in identical positions.

58% functionally conserved residue homology is present in this transmembrane domain.

Extensive homology molecular modeling implicated Phe175 of the hMC1R to be located in the putative binding pocket, and specifically proposed to interact with the Trp^9 residue of the ligand.[43,48] Specifically, this Phe175 residue is proposed to be involved in a network of hydrophobic/aromatic interactions involving up to seven aromatic residues, including the ligand D-Phe^7 and Trp^9. In the absence of this residue, we proposed that this continuity would be disrupted and result in decreased ligand binding affinity of cyclic peptides containing D-Trp^9. Interestingly, this Phe residue is only present in the human MC1R and is replaced with a Ser or Thr residue in the mouse,[13] bovine,[49] fox,[50] chicken,[51] and panther (R. D. Cone, personal communication). This is of particular note since the mouse and human melanocortin-1 receptors possess different pharmacological profiles in response to the melanocortin peptides (20-fold for α-MSH).[52] The Phe^{175} residue is a likely candidate for the differences observed between these two receptors. Additionally, this aromatic residue has been substituted by other functional moieties in the hMC3R, hMC4R, and hMC5R subtypes (Table 4). In analogue **IV**, the Trp residue has been inverted to the D configuration and shows a 78-fold selectivity for the hMC1R over hMC4R, whereas the L-Trp stereochemical configuration containing peptide (**I**) only possesses approximately 3-fold selectivity, which is within experimental error. Precedent for stereochemical specificity of the melanocortin ligand with the hMC1R has been illustrated previously by site-directed mutagenesis of Asp117 (TM3) and His260 (TM 6) to Ala's. This study demonstrated that α-MSH (which contains L-Phe^7,

Table 5. Binding Affinities of Two Sets of Melanotropin Peptides Containing L- to D-Trp Modifications Examined on the Endogenous hMC1R and the Mutated hMC1R Phe175Ala

peptide	structure	binding IC_{50} (nM)[a]	
		hMC1R	hMC1R F175A
MTII	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2	0.25 ± 0.03	0.12 ± 0.01
VI	Ac-Nle-c[Asp-His-D-Phe-Arg-D-Trp-Lys]-NH_2	0.40 ± 0.17[b]	0.69 ± 0.04
I	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Ala-Lys]-NH_2	0.35 ± 0.05	0.34 ± 0.01
IV	Ac-Nle-c[Asp-His-D-Phe-Arg-D-Trp-Ala-Lys]-NH_2	0.91 ± 0.01	0.59 ± 0.05

[a] IC_{50} = concentration of peptide at 50% specific binding ($N = 3-6$). The peptides were tested at a range of concentrations ($10^{-6}-10^{-12}$ M). [b] Previously reported value.[22]

Table 1) binding affinity was significantly affected by these two mutations (up to 267-fold), as compared with the wild-type receptor, whereas NDP-MSH (which contains D-Phe[7], Table 1) did not possess a difference in binding affinities at these mutated receptors.[53] These observations above allowed us to generate the hypothesis that the receptor Phe[175] of hMC1R may be specifically interacting with the ligand D-Trp[9] residue. To test this hypothesis, the ligand should distinguish differential binding affinities between wild type hMC1R and the hMC1R Phe[175]Ala (F175A) point mutation, with the latter modification resulting in a loss of affinity mutation (ca. 70-fold) and resembling the binding affinity observed at the hMC4R. Table 5 summarizes the ligand binding data on the hMC1R F175A mutant on ligands containing L-Trp (MTII and **I**) and D-Trp (**VI** and **IV**). Analogue **VI** has been synthesized and reported on the hMC1R previously.[22] Although the above hypothesis of ligand (analogue **IV**, D-Trp)–receptor (Phe[175]) appears to be incorrect because no significant differences in binding were observed between the wild-type hMC1R and F175A hMC1R, precedent has been identified for stereochemical specificity in the melanocortin hMC1R subtype.[53] This process of examining ligand–receptor complementary interactions is not unique and has been performed successfully in other studies.[54–56]

Energy Calculations. Differences observed in binding potencies of analogues at a single receptor type may be a combination or result of multiple physiological and thermodynamic factors. The "two-state or ternary complex model"[57–59] proposes that the role of the ligand is to stabilize a particular receptor conformation that is "active (coupled to a G-protein)" for an agonist, or "inactive (not coupled to a G-protein)" for an antagonist. The classical concerted mechanism, which involves ligand binding to the receptor, with the ligand playing a subsequent role in this "receptor–ligand" complex which then undergoes a conformational change and becomes "activated" by coupling to a G-protein. Although examples supporting both mechanisms are in the literature, controversy remains, and the actual mechanism(s) may involve a combination of both proposals for "receptor activation."[60] Theory in designing conformationally constrained ligands includes the hypothesis that by providing the "correct" ligand constraint, the overall energy necessary for the ligand to obtain the "bioactive" conformation for receptor binding is decreased, and hence, overall ligand binding affinity is increased. Determination of these possible ligand backbone structure(s) for agonism and receptor selectivity was the main goal for the conformational studies performed in this study. The rational approach involving computational procedures is to employ energy calculations and determine sets of low-energy conformers (or "families") for each compound, which "bind" to a receptor. Considerations include potent binding affinities for a particular receptor subtype, and comparisons of spatial arrangements including functionally important side chain groups present for low-energy conformational families of the compound for comparisons with the other analogues in the study (i.e., see Nikiforovich[61]). Conformers with similar spatial arrangements of the functionally important groups for all highly potent compounds can then be used as models for "receptor-bound" conformation.

The approach outlined above requires two main assumptions. First, it is postulated that all compounds with high binding affinities interact with the same site of the same receptor, and presumably in the same binding mode. Second, the same functional groups are considered to be important for binding for all those compounds. In this study, the latter requirement means that compounds listed in Table 1 are involved in binding only through their 4–11 fragments, which is a reasonable assumption when keeping in mind the data of extensive earlier structure–activity studies for synthetic α-melanotropin derivatives.[2,62,63] Additionally, there is no direct evidence that compounds listed in Table 1 interact with the same receptor in the same binding mode, or even at the same receptor site. On the other hand, the finding of a common low-energy ligand "receptor-bound" conformation at each human melanocortin receptor examined in Table 1 may support this latter assumption.

Resulting calculations suggest that the three-dimensional (3D) model of the "receptor-bound" conformer for all potent α-MSH related peptides listed in Table 1 can be described by similar spatial structure of the Nle-His-D-Phe-Arg-L/D-Trp fragment at the melanocortin receptors examined. Suggested differences in binding of different ligands to different receptor subtypes may be attributed not only to differences in the "receptor-bound" conformers required by each particular receptor subtype but to some specific elements within the ligand–receptor interactions. These interactions may depend on configurations of particular residues in the ligand. The configuration of residues Asp and Phe are necessary elements for binding affinities at all melanocortin receptor subtypes examined. However, only the hMC4R requires L-configuration of the Trp residue, and only the hMC5R requires the presence of the Nle residue for potent binding affinity. The suggested 3D model of analogue **I** bound to the melanocortin receptor complex is depicted in Figure 3. One can speculate that the stereoview depicted in Figure 3 is oriented in a manner that a viewer is "at the receptor side", since the lactam bridges in all cyclic analogues are under the plane of the picture in this particular orientation. "The foreground" of this model consists of the Arg and Nle residues, and the aromatic side chains of the His, Phe, and Trp residues are placed "in the background". It

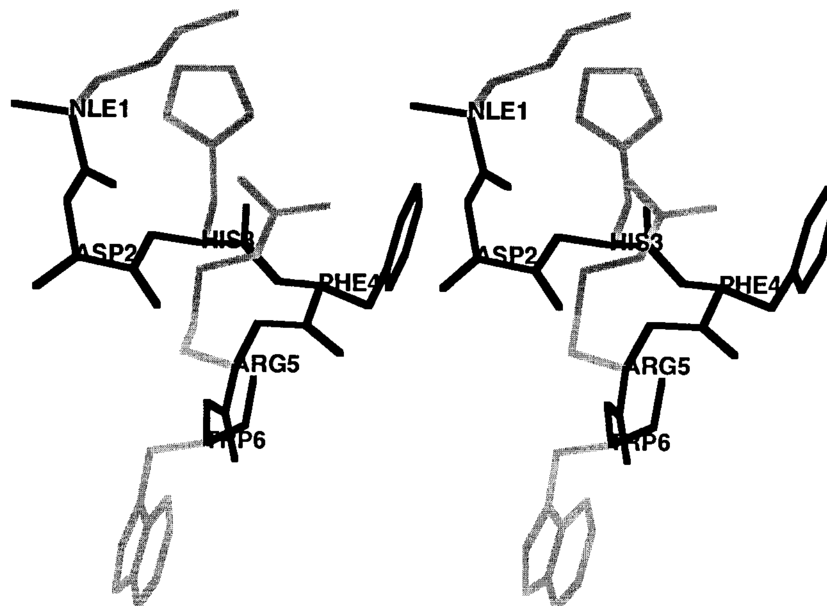

Figure 3. Stereoview of the proposed 3D model of Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Ala-Lys]-NH_2 (analogue **I**), interacting within the melanocortin receptor. For simplicity, only the residues from Nle through Trp are indicated. The residue side chains of Nle, His, Arg, and Trp whose configurations can vary for different receptors, are shown in gray lines.

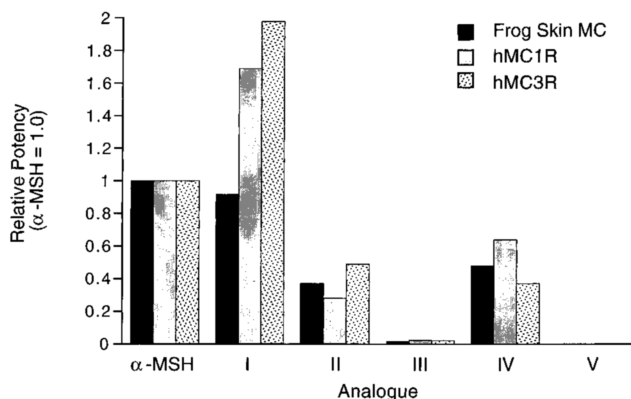

Figure 4. Comparison of the relative potencies of the analogues examined in this study, as compared with α-MSH, on the frog skin melanocortin receptor (MCR), human MC1R, and human MC3R. Correlations exist between these three MC receptors which support the suggested ligand–receptor models of the frog skin MC receptor may extend to the human MC receptors as well.

might suggest that the primary "anchor" group of these ligands are the Arg side chain that is involved in forming the ligand–receptor complex, whereas, the aromatic groups become involved in this process at the later steps.

Interestingly, the suggested 3D ligand–receptor complexed model is identical to what was proposed earlier for the "receptor-bound" conformer of the central tetrapeptide of α-MSH (His-Phe-Arg-Trp) interacting with the frog skin melanocortin receptor.[37] Figure 4 depicts comparison of relative potencies for several compounds obtained on the frog skin[34] and in this study of binding affinities for the melanocortin receptors hMC1R and hMC3R. Correlations among all three melanocortin receptors are observed (Figure 4), which is consistent with the mentioned similarity of suggested ligand–receptor models of the frog skin melanocortin receptor. However, in the "frog skin" model, that the primary ligand interactions with the receptor(s) would be predominately through aromatic side chains, whereas the Arg residue would be turned away from the receptor.[34,37] Thus, the present study confirms the 3D model for melanotropin (His-Phe-Arg-Trp) "receptor-bound" structure as to its conformation, while refining the possible orientation of the peptide ligand toward the human melanocortin receptors.

The conformational analysis performed in this study allows for the following hypotheses to be confirmed or generated. First, it is reasonable to predict that different α-MSH related peptides (containing the melanotropin pharmacophores) would bind to different human melanocortin receptor subtypes in similar receptor-bound conformation(s), which are described in this study. The differences in binding affinities toward different receptor subtypes could be attributed to different elements in the suggested 3D model of the ligand–receptor complex, and not to the ligand model in general. One can expect that these differences in binding affinities may also be related to differences (both in sequences and in conformations) of the receptors themselves, as alluded to previously. Second, the suggested 3D model for ligand binding toward human melanocortin receptors is identical to the ligand models proposed for binding to frog skin melanocortin receptors. Correlation between the data obtained on the frog skin, hMC1R, and hMC3R were observed. Finally, results of conformational studies performed in this study suggest not only the 3D model for ligand binding but also its possible orientation toward the receptor. These hypotheses may be helpful for future studies on 3D modeling of melanocortin receptors and their complexes with α-MSH-related ligands.

Experimental Section

Materials. TLC was done on Merck silica gel 60 F_{254} plates using the following solvent systems: (A) 1-butanol/acetic acid/pyridine/water (5:5:1:4); (B) ethyl acetate/pyridine/acetic acid/water (5:5:1:3); (C) upper phase of 1-butanol/acetic acid/water (4:1:1). The peptides were detected on the TLC plates using iodine vapor. Final peptide purification was achieved using a semipreparative RP-HPLC C_{18}-bonded silica column (Vydac 218TP1010, 1.0 × 25 cm). The peptides were eluted with a

Table 6. Physicochemical Properties of the Melanotropin Analogues

peptide	optical rotation $[\alpha]_{598}^{23}$ = (10% aq HOAc)	R_f, TLC solvents[a] A	B	C	HPLC K' [b]	FAB-MS obs (M + 1)	calcd (M + 1)
I	−45.2 (c = 0.044)	0.79	0.07	0.70	3.34[1] 3.95[2]	1096.5	1096.3
II	−40.0 (c = 0.029)	0.79	0.08	0.71	5.97[5] 3.34[4]	982.5	983.1
III	−43.8 (c = 0.041)	0.78	0.07	0.69	8.41[5] 3.19[4]	1096.5	1096.3
IV	−46.1 (c = 0.048)	0.73	0.06	0.65	6.39[1]	1095.6	1096.3
V	−45.3 (c = 0.024)	0.77	0.06	0.68	1.24[2]	1096.5	1096.3

[a] R_f values on thin-layer chromatograms of silica gel were observed in the following solvent systems: (A) 1-butanol/AcOH/pyridine/H_2O (5:5:1:4); (B) EtOAc/pyridine/AcOH/H_2O (5:5:1:3); (C) upper phase of 1-butanol/AcOH/H_2O (4:1:1). [b] HPLC K' = [(peptide retention time − solvent retention time)/solvent retention time] was performed on a C-18 column (VYDAC 218TP104) using a gradient of CH_3CN in 0.1% aqueous TFA in 30 min with a flow rate of 1.5 mL/min. The following gradients were used: [1]20−30%, [2]20−40%, [3]15−25%, [4]15−30%, [5]10−30%, and [6]10−25%.

linear acetonitrile gradient (10−50%) over 30 min at a flow rate of 5.0 mL/min, with a constant concentration of TFA (0.1% v/v). The linear gradient was generated with a Perkin-Elmer 410 LC Bio pump system. The separations were monitored at 280 nm and integrated with a Perkin-Elmer LC-235 diode array detector. Amino acid analyses were performed at the University of Arizona Biotechnology Core Facility. The system used was an Applied Biosystems Model 420A amino acid analyzer with automatic hydrolysis (Vapor Phase at 160 °C for 1 h 40 min using 6 N HCl) and precolumn phenylthiocabamyl-amino acid (PTC-AA) analysis. No corrections are made for amino acid decomposition. FAB-MS analyses were performed at the University of Arizona Core Facility. The instrument was custom made in Breman, Germany, and consists of a LIQUID SIMS4 Sectors AMD mass spectrometer. The experimental conditions consisted of a glycerol matrix-scan of 200−2000 Da in the positive ion mode. Optical rotation values were measured on an Autopol III at 589 nm in 10% acetic acid. The pMBHA resin (0.37 mmol of NH_2/g) was purchased from Peptides International Inc. (Louisville, KY 40224). N^{α}-*tert*-Butyloxycarbonyl (Boc)-protected amino acids and amino acid derivatives were purchased from Bachem (Torrance, CA). All purchased amino acids were of the L configuration except for specified. *Tert*-Butyloxycarbonyl (Boc) was used for N^{α} protection, and the reactive side chains of the amino acids were protected as follows: Lys, with fluorenylmethyloxycarbonyl (Fmoc); Asp, with fluorenylmethyl ester (OFm); His, with (benzyloxy)methyl (Bom); Arg, with tosyl (Tos); and Trp, with formyl (For). All reagents and solvents were ACS grade or better and were used without further purification. The purity of the finished peptides were checked by TLC in three solvents and analytical RP-HPLC at 280 and 220 nm in all cases were greater than 95% pure as determined by these methods. The structures of the pure peptides were confirmed by fast atom bombardment (FAB) mass spectrometry (Table 6).

General Procedure for the Synthesis and Cleavage of Melanotropin Ac-Nle-c[Asp-His-Phe-Arg-Trp-Ala-Lys]-NH_2 Peptides. The peptides were synthesized using a manual synthesizer adapted from previously published methods.[33] Approximately 0.5 mmol of *p*-methylbenzhydrylamine (pMBHA) resin (0.37 mmol of NH_2/g of resin, or 0.24 mmol of NH_2/g of resin) was neutralized with 10% diisopropylethylamine (DIEA) in dichloromethane (DCM) (2 × 50 mL), followed by a DCM wash (4 × 50 mL). The amino acid N^{α}-Boc-Lys (N^{ϵ}-Fmoc) (1.2 excess) was coupled to the resin for 2 h in *N*-methylpyrrolidinone (NMP) using (benzotriazolyloxy)-tris(dimethylamino)phosphonium (BOP) (1.4-fold excess) and DIEA (1.6-fold excess). The N^{α}-Boc protecting group was removed by washing the resin 2 min in 50% trifluoroacetic acid (TFA), 2% anisole in DCM (1 × 50 mL) followed by a 20 min 50% TFA, and 2% anisole in DCM wash (1 × 50 mL). The resin was then washed with DCM (3 × 50 mL), neutralized with 10% DIEA in DCM (2 × 50 mL), and washed with DCM (4 × 50 mL). N^{α}-Boc-Trp(N^{in}-For) or was coupled using identical coupling conditions as described above. The sequential coupling of the amino acids (4-fold excess) N^{α}-Boc-Arg (N^{g}-Tos), N^{α}-Boc-Phe, and N^{α}-Boc-His (N^{π}-Bom), using DIC (6.5 mmol) and HOBT (6.5 mmol) as coupling reagents for 2 h, and N^{α}-Boc removal conditions described above, added to the growing peptide chain. Then N^{α}-Boc-Asp (β-OFm) (1.2 excess) was coupled to the peptide resin for 2 h in NMP using BOP (1.4-fold excess) and DIEA (1.6-fold excess) and N^{α} deprotected as described above. At this stage, the N^{ϵ}-Fmoc and β-OFm protecting groups of lysine and aspartic acid, respectively, were removed by the addition of 20% piperidine/NMP (1 × 50 mL) and mixed for 20 min. The resin was washed with DCM (7 × 50 mL), followed by cyclization of the free acid side chain of Asp to the free amine side chain of Lys by the addition of BOP (5-fold excess) and DIEA (6-fold excess) in NMP for 2 h. This process was repeated until a negative Kaiser test resulted. Upon complete formation of the lactam cycle, N^{α}-Boc-Nle (4-fold excess) was coupled to the growing peptide chain using DIC/HOBT coupling conditions. After removal of the N^{α}-Boc protecting group, N-terminal acetylation was carried out by the addition of 2 mL of acetic anhydride and 1 mL pyridine for 20 min. The resin was washed with DCM (6 × 50 mL) and dried *in vacuo* to yield approximately 2.0 g of peptide resin. Approximately half of the peptide-resin was added to an equivalent amount of 10% anisole followed by the addition of approximately 10 mL of anhydrous HF. The mixture was stirred at 0 °C for 60 min followed by the removal of the scavengers and HF under high vacuum. To ensure complete removal of the scavengers and nonpeptide material, anhydrous ethyl ether (5 × 30 mL) was added to each vessel and the mixture filtered using a course glass frit. The crude peptide was dissolved in glacial acetic acid (4 × 50 mL) and lyophilized to give 100 to 200 mg of crude peptide. A portion of the crude peptide was purified by preparative RP-HPLC and yielded 50 to 60% of pure peptide. The analytical properties for each peptide are given in Tables 6 and 7.

Table 7. Amino Acid Analysis of the Melanotropin Peptides[a]

peptide	Nle	Asp	His	Phe	Arg	Ala	Lys
I	1.1	0.95	0.92	1.0	1.02	1.01	0.91
II		0.90	0.98	1.0	0.97	0.95	0.94
III	1.0	0.91	0.96	1.03	1.05	1.1	0.93
IV	1.0	0.90	0.91	1.1	1.05	1.08	0.91
V	0.97	0.94	0.97	1.0	0.94	1.1	0.89

[a] Trp was not determined.

Binding Assays. The coding region of the human melanocortin receptors cloned from a human genomic EMBL3 phage library (Clontech, Palo Alto, CA) was placed into the eukaryotic expression vector, CMVneo, and stably transfected into L-cells as previously described.[16,17,22] Transfected cells (0.6 × 10^6 cells/well) were grown to confluence in 12-well (2.4 × 1.7 cm) tissue culture plates. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 4.5 g/100 mL glucose, 10% fetal calf serum, 100 units/mL penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/mL Geneticin. For the assays, this medium was removed and cells were washed twice with a freshly prepared binding buffer consisting of minimum essential medium with Earle's salt (MEM, GIBCO), 25 mM HEPES (pH 7.4), 1% bovine serum albumin, and 200 mg/L bacitracin. A 450 μL sample of the

peptide concentration being tested was added to the well, with the concentrations ranging between 10^{-12} and 10^{-6} M. Next, 50 μL solution of [^{125}I]NDP-MSH, (100000 cpm/well) was added to each well and the cells were incubated at 37 °C for 1 h. The medium was subsequently removed, and each well was washed twice with assay buffer. The cells were lysed by the addition of 0.5 mL of 0.1 M NaOH and 0.5 mL of 1%Triton X-100. The mixture was left to lyse the cells for 10 min, and the contents of each well were transferred to labeled 16 × 150-mm glass tubes and quantified in a γ-counter. [^{125}I]NDP-MSH was prepared and purified by methods described previously.[64]

cAMP Assays. A commercially available cAMP assay kit (TRK 432, Amersham Corp.) was employed. L-cells transfected with the human melanocortin receptors were grown to confluence in 12-well (2.4 × 1.7 cm) tissue culture plates. The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 4.5 g/100 mL glucose, 10% fetal calf serum, 100 units/mL penicillin and streptomycin, 1 mM sodium pyruvate, and 1 mg/mL Geneticin. For the assays, the medium was removed and cells were washed twice with Earle's balanced salt solution containing 10 mM HEPES (pH 7.4), 1 mM glutamine, 26.5 mM sodium bicarbonate, and 100 mg/mL bovine serum albumin. An aliquot (0.5 mL) of Earle's balanced salt solution (EBSS, GIBCO) was placed into each well along with 5 μL of 2 × 10^{-2} M isobutylmethylxanthine. Varying concentrations of melanotropins were added, and the cells were incubated for 1 h at 37 °C. Ice-cold 100% ethanol (0.5 mL/well) was added to stop the reaction. The incubation medium and scrapped cells were transferred to 16 × 150-mm glass tubes and then placed at 4 °C for at least 30 min. The precipitate was then centrifuged for 10 min at 1900*g*, and the supernatant was dried under a nitrogen stream and resuspended in 50 mM Tris, 2 mM EDTA (pH 7.5). The cAMP content was measured by competitive binding assay according to the assay instructions.

Data Analysis. IC_{50} and EC_{50} values represent the mean of duplicate experiments performed in triplicate. IC_{50} estimates, EC_{50} estimates, and their associated standard errors were determined by fitting the data using nonlinear least-squares analysis.[65] The results are not corrected for peptide content.

Energy Calculations. Energy calculations were performed only for analogues **I**, **III**, **IV**, and **V**. Sets of low-energy conformers for NDP-MSH, MTII, and **II** were borrowed from our earlier studies.[34,37] Energy calculations were performed using the ECEPP potential field[66,67] with the dielectric constant value of 2.0 as a standard value. Dihedral angles were the only variables in the process of energy minimization, since rigid valence geometry with the planar peptide bonds was assumed. Aliphatic and aromatic hydrogens were generally included in united atomic centers of CH_n type. Calculation protocol consists of adding the Nle residue to sets of low-energy conformers of the corresponding cycles (Ac-c[L/D-Asp-His-L/D-Phe-Arg-L/D-Trp-Ala-Lys]-NH_2, see Nikiforovich et al.,[37] in different backbone conformations ($\varphi = -140°$, $\psi = 140°$; $\varphi = -75°$, $\psi = 80°$; and $\varphi = -60°$, $\psi = -60°$). In total there were 56 low-energy backbone conformers of the cyclic moiety of analogue **I**, 35 of analogue **III**, 88 for analogue **IV**, and 9 for analogue **V**, none of the conformers possessing cis peptide bonds). The dihedral angle values of side chain groups (χ_1's) and of the terminal groups of the backbone were optimized before energy minimization to achieve their most favorable spatial arrangements according to an algorithm described previously.[68] Subsequently, energy minimization involved all dihedral angles. Finally, 22 low-energy backbone conformations were found for analogue **I**, 41 for analogue **III**, 165 for analogue **IV**, and 12 for analogue **V**, with the criterion for selecting low-energy conformers being $\Delta E = E_i - E_{min} \leq 10$ kcal/mol. Geometrical comparison of low-energy conformers was assessed by the best fit for the same atomic centers, which was calculated for a pair of conformations A and B as follows

$$\text{rms} = (1/N)\sum_{i=1}^{N}[(x^A{}_i - x^B{}_i)^2 + (y^A{}_i - y^B{}_i)^2 + (z^A{}_i - z^B{}_i)^2]^{1/2}$$

where N is the number of the atomic pairs chosen for superposition and x, y, and z are their atomic Cartesian coordinates. Two conformers were regarded as similar when the corresponding rms value, and all distances between overlapping atomic centers were less than 1.0 Å.

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