Expedited Articles

Cyclic Lactam α-Melanotropin Analogues of Ac-Nle[4]-cyclo[Asp[5],D-Phe[7],Lys[10]] α-Melanocyte-Stimulating Hormone-(4-10)-NH_2 with Bulky Aromatic Amino Acids at Position 7 Show High Antagonist Potency and Selectivity at Specific Melanocortin Receptors

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The cloning of the melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH) receptors (MC1-R and MC2-R, respectively) recently has led to the identification of three additional melanocortin receptors, MC3-R, MC4-R, and MC5-R. The MC2 receptor primarily recognizes only ACTH peptides, but the other four receptors all recognize α-melanocyte-stimulating hormone (α-MSH) and potent α-MSH agonists such as [Nle[4],D-Phe[7]]α-MSH-NH_2 and Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4-10)-NH_2 as well as ACTH. The absence of any known physiological role for these new receptors, expressed both in the brain (MC3-R and MC4-R) and throughout a number of peripheral tissues (MC5-R), has necessitated a search for potent and receptor selective agonists and antagonists. We report here that analogues of the superpotent cyclic agonist analogue Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4-10)-NH_2, in which a bulky aromatic amino acid is substituted in the 7-position, can produce potent and selective antagonists for melanocortin receptors. Thus, the D-*p*-iodophenylalanine[7]-containing analogue Ac-Nle[4]-c[Asp[5],D-Phe(pI)[7],Lys[10]]α-MSH-(4-10)-NH_2 is a potent antagonist ($pA_2 = 10.3$) in the classical frog skin (*Rana pipiens*) assay (MC1-R), as is the D-2′-naphthylalanine[7] (D-Nal(2)[7])-containing analogue Ac-Nle[4]-c[Asp[5],D-Nal(2)[7], Lys[10]]α-MSH-(4-10)-NH_2 ($pA_2 > 10.3$). Interestingly, the D-*p*-chloro- and D-*p*-fluorophenylalanine[7]-containing analogues lacked antagonist activities at all melanotropin receptors, and both exhibited full agonist potency in the frog skin assay. The activity of these analogues also was examined at four mammalian melanocortin receptors. Interestingly, Ac-Nle[4]-c[Asp[5],(D-Nal(2)[7], Lys[10]]α-MSH-(4-10)-NH_2 was found to be a potent antagonist of the MC4-R ($pA_2 = 9.3$) with minimal agonist activity, a less potent antagonist of the MC3-R ($pA_2 = 8.3$) with minimal agonist activity, and a full agonist of the MC1 and MC5 receptors. Surprisingly, Nle[4]-c[Asp[5],D-Phe(pI)[7],Lys[10]]α-MSH was found to be a potent agonist at the cloned human MC1-R ($EC_{50} = 0.055$ nM) and mouse MC1-R ($EC_{50} = 0.19$ nM) but had potent antagonist activities at the human MC4-R ($pA_2 = 9.7$) and human MC3-R ($pA_2 = 8.3$) with significant partial agonist activities ($EC_{50} = 0.57$ and 0.68 nM, respectively) as well. Thus, highly potent and receptor selective antagonist analogues can arise from substitution of the D-Phe[7] residue with a bulky aromatic amino acid. These analogues can be used to help determine the functional roles of these receptors.

Introduction

While pharmacological methods have been traditionally used to define receptor subtypes, receptor-cloning experiments have often led to the discovery of novel receptor subtypes within many receptor families. Following the cloning of the melanocyte-stimulating hormone (MSH)[1] and adrenocorticotropic hormone (ACTH) receptor genes,[2,3] for example, three unique yet related genes were identified that also encoded functional, high-affinity receptors for the melanocortin (MSH/ACTH) peptides[4-13]. Labeled numerically in the order of their discovery, the melanocortin-3, melanocortin-4, and melanocortin-5 receptor genes have been demonstrated to be expressed primarily in the hypothalamus, midbrain, and brain stem (MC3-R and MC4-R) or in a wide distribution of peripheral tissues (MC5-R).

α-Melanotropin (α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2) was among the first peptide hormone to be isolated and to have its structure determined. This hormone plays an important biological role in pigmentation,[14,15] and numerous central nervous system (CNS)-related activities also have been proposed for this hormone.[14-17] Extensive structure-activity relationships have established the central role of α-melanotropin in pigmentation in vertebrates, and super potent, enzymatically stable, super prolonged acting agonist analogues such as [Nle[4],D-

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Table 1. Agonist and Antagonist Activities of Cyclic Melanotropin Analogues at Various Melanocortin 1 Receptors

compound	EC_{50} values (nM)		
	frog skin assay	mMC1-R assay	hMC1-R assay
α-MSH	0.10 ± 0.03	1.3 ± 1.4	0.091 ± 0.070
1, Ac-Nle[4]-c[Asp[5],D-Phe(pF)[7],Lys[10]]α-MSH-(4−10)-NH_2[a] (SHU9128)	0.10 ± 3.5	0.026 ± 0.010	0.016 ± 0.003
2, Ac-Nle[4]-c[Asp[5],D-Phe(pCl)[7],Lys[10]]α-MSH-(4−10)-NH_2 (SHU9203)	2.0 ± 0.8	0.0095 ± 0.0053	0.005 ± 0.004
3, Ac-Nle[4]-c[Asp[5],D-Phe(pI)[7],Lys[10]]α-MSH-(4−10)-NH_2 (SHU8914)	antagonist pA_2 = 10.3	0.19 ± 1.3	0.055 ± 0.031
4, Ac-Nle[4]-c[Asp[5],D-Nal(2)[7],Lys[10]]α-MSH-(4−10)-NH_2 (SHU9119)	antagonist pA_2 ≥ 10.5	0.039 ± 0.029	0.036 ± 0.012

[a] The complete structure of 1 is Ac-Nle[4]-Asp-His-D-Phe(pF)-Arg-Trp-Lys[10]-NH_2. Compounds **2**−**4** are the same except for the amino acid in the 7-position.

Phe[7]]α-MSH[18] and the cyclic lactam analogue Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4−10)-NH_2[19,20] have been developed and widely used in biological studies related to the role of α-melanotropin in pigmentation. The effects of α-MSH on pigmentation are mediated by the MC1-R expressed specifically on the surface of melanocytes. Similarly the MC2-R is involved in the regulation of adrenal steroidogenesis by ACTH. However, given the complexity of expression of the MC3, MC4, and MC5 receptors, it has not been possible to identify any simple correlations between these receptors and the reported biological activities of the melanocortin peptides. Consequently, potent and receptor specific agonists and especially antagonists would be extremely valuable tools for the determination of the physiological roles of the MC3, MC4, and MC5 receptors.

Though the extensive structure−activity relationships mentioned above have provided much information on agonist activity related to pigmentary effects (see, for example, refs 14−17, 21), until recently there have been only a few reports[22−25] on the development of α-MSH antagonists. We report here on the discovery of two highly potent and selective antagonists for certain amphibian MC1 receptors and for the mammalian neural MC3 and MC4 receptors.

Results

Design and Synthesis. The antagonists reported here are based on the super potent and super prolonged acting cyclic lactam-containing agonist Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4−10)-NH_2. Structure−activity and conformation−activity[19,20,26] studies have led us to propose a bioactive conformation for α-MSH at the classical pigment cell receptor.[27,28] These studies indicate that the side chain residues of His[6], Phe[7], Arg[8], and Trp[9] are critical for agonist activity. Using strategies we have previously suggested for peptide hormone and neurotransmitter antagonist development,[29] we have sought ways to disrupt the proposed bioactive conformation necessary for transduction while maintaining strong binding to the inactive form of the receptor. One of these approaches has involved modification of the critically important Phe[7] residue by a variety of bulky aromatic amino acid residues at this position. We report here that substitution of D-2′-naphthylalanine (D-Nal(2)) and D-*p*-iodophenylalanine in position 7 of the potent cyclic agonist analogue Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4−10)-NH_2 produced potent antagonist analogues at certain melanocortin receptors.

Synthesis of the peptide analogues 1−4 (Table 1) was accomplished by solid-phase methods of peptide chemistry similar to those previously reported for the synthesis of Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4−10)-NH_2 using an automatic peptide synthesizer. Briefly, the synthesis was accomplished on a *p*-methylbenzhydrylamine resin using an N^{α}-Boc strategy and via cyclization of the macrocyclic lactam rings on the solid support using (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) reagent in the presence of diisopropylethylamine as described previously.[20] The cyclic, partially protected peptide was deprotected and cleaved from the resin by treatment with HF−anisole for 45 min at 0 °C.[20] The resulting crude peptides were purified by reversed-phase HPLC and characterized by fast atom bombardment mass spectrometry and amino acid analysis. Purity was assessed by analytical RP-HPLC and thin layer chromotography (TLC) in three different solvent systems (see the Experimental Section for details).

Biological Assay Methods. The analogues were assayed for agonist and antagonist activity using the classical frog skin (*Rana pipiens*)[30] and, in a few cases, the lizard skin (*Anolis carolinensis*)[31] bioassays. The analogues also were assayed for agonist and antagonist activities at cloned mammalian melanocortin receptors using a newly developed cAMP-dependent colorimetric β-galactosidase assay[32] (see the Experimental Section for details). This assay uses clonal 293 cell lines expressing human MSH receptor (hMC1-R), human MC3 receptor, human MC4 receptor, and mouse MC5 receptor which were transfected with a β-CRE(β-galactosidase) construct using a $CaPO_4$ method[33] (see the Experimental Section for details). Antagonist pA_2 values in all assays were determined using the method of Schild.[34]

The results for the *R. pipiens*, human, and mouse MSH (MC1) receptors for the four D-Phe[7]-substituted analogues of Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4−10)-NH_2, namely, the D-Phe(pF)[7], D-Phe(pCl)[7], D-Phe(pI)[7], and D-Nal(2)[7] analogues **1**−**4**, respectively, are given in Table 1. The comparative results for the four mammalian melanocortin receptors are given in Table 2.

The analogue Ac-Nle[4]-c[Asp[5],D-Phe(pI)[7],Lys[10]]α-MSH-(4−10) (**3**; Table 1) has only minimal agonist activity in the frog skin (*R. pipiens*) assay but, as shown in Figure 1, is a potent inhibitor of the biological response of α-MSH. Evaluation of the dose−response displacement curves (Figure 1) showed that **3** was an exceptionally potent antagonist analogue (pA_2 = 10.3, Table 1) in this *in vitro* melanocortin 1 receptor assay. Interestingly, the analogue was a potent agonist (EC_{50} = 0.60 nM, data not shown) in the lizard (*A. carolinensis*) skin assay, a highly potent agonist at the human MC1-R (EC_{50} = 55 pM, Table 1), and a modestly potent agonist at the mouse MC1-R (EC_{50} = 0.19 nM, Table 1). The cyclic analogue Ac-Nle[4]-c[Asp[5],D-Nal(2)[7],Lys[10]]α-MSH-(4−10)-NH_2 (**4**) which has the bulky aromatic amino

Table 2. EC_{50} Values (pM) for D-Phe[7]-Substituted Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4–10)-NH_2 Analogues at the Different Melanocrotin Receptors

	EC_{50} values (pM)			
	hMSH1-R	hMC3-R	hMC4-R	mMC5-R
α-MSH	91 ± 69	669 ± 355	210 ± 57	807 ± 125
[Nle[4],D-Phe[7]]α-MSH-NH_2	23 ± 7	132 ± 31	17 ± 18	ND[a]
1, SHU9128 (pF)	16 ± 3	191 ± 9	19 ± 14	1360 ± 549
2, SHU9203 (pCl)	5 ± 4	63 ± 26	18 ± 14	117 ± 70
3, SHU8914 (pI)	55 ± 31	1134 ± 197 partial agonist pA_2 = 8.3	573 ± 357 partial agonist pA_2 = 9.7	684 ± 227 partial agonist
4, SHU9119 (D-Nal(2))	36 ± 12	2813 ± 575 partial agonist pA_2 = 8.3	no activity antagonist pA_2 = 9.3	434 ± 260 full agonist

[a] ND = not determined.

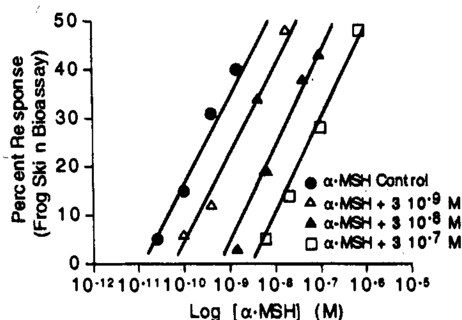

Figure 1. Demonstration that Ac-Nle[4]-c[Asp[5],D-Phe(pI)[7],-Lys[10]]α-MSH-(4–10)-NH_2 (**3**) is a potent antagonist of α-MSH in the frog skin MC1 receptor assay system. Antagonism of the dose–response curve of α-MSH by 10^{-9} (△), 10^{-8} (▲) and 10^{-7} (□) M antagonist **3** is demonstrated by the rightward shift of the dose– response curve of α-MSH.

acid D-Nal(2) in position 7 also exhibited potent antagonist activity in the frog skin MC1-R assay ($pA_2 \geq 10.5$, Table 1) and potent agonist activity at the mouse and human MC1-R receptors (Table 1). Interestingly, the D-*p*-fluorophenylalanine- and D-*p*-chlorophenylalanine-containing analogues **1** and **2** (Table 1) were both potent agonists at all melanocortin 1 receptors.

In the frog skin bioassay, it was noted that the antagonist activity of both **3** and **4** was prolonged in a dose-dependent manner. The antagonist effects of both analogues can be blocked by pretreating the frog skin with the potent prolonged acting agonist [Nle[4],D-Phe[7]]α-MSH. Concentrations of the antagonists at 10^{-6}–10^{-7} M generally produced an irreversible antagonism in that, following removal of analogues from the assay medium, subsequent challenges with α-MSH failed to activate frog MC1 receptors for several hours after removal of the antagonists. The antagonism of frog skin MC1 receptor was specific for α-MSH since the skins would still maximally darken in response to theophylline (a phosphodiesterase inhibitor). These observations may prove of great importance if any of these antagonists were to prove to be of clinical/physiological usefullness.

A newly described cAMP-dependent colorimetric β-galactosidase assay[32] was used to determine the agonist and antagonist activities of the new cyclic lactam derivatives of α-MSH at cloned mammalian melanocortin receptors. This assay utilizes a β-galactosidase reporter gene fused to a cAMP-regulated promoter to detect changes in intracellular cAMP downstream of receptor activation and was useful in these studies since it has been shown that all the melanocortin receptors couple to the effector adenylyl cyclase.[2–13]

Modification of D-phenylalanine[7] at the para position or substitution of the phenyl group with a naphthyl group (compound **4**, Table 1) had little effect on agonist activity of the compounds at the human MSH receptor (hMC1-R) or human MC5 receptor (Figure 2). In contrast, replacement of the D-Phe[7] with D-Phe(pI)[17] dramatically reduced agonist activity at the MC3 and MC4 receptors (Figure 2). Both compounds are partial agonists with greatly increased EC_{50}s (Table 2). Interestingly, the D-Phe(pCl)[7]-substituted analogue **2** was a full agonist and actually was more potent than [Nle[4],D-Phe[7]]α-MSH at every receptor except the hMC3-R. The D-Phe(pF)[7]-substituted analogue **1** likewise was a very potent full agonist in all assays (Table 2, Figure 2).

The two compounds acting as weak partial agonists at the MC3-R and MC4-R (analogues **3** and **4**) were then examined for antagonist activity at these receptors (Figure 3). As can be seen, the D-Nal(2)[7]-substituted cyclic lactam analogue **4** is a potent antagonist of the MC3-R and MC4-R, with pA_2 values of 8.3 and 9.3, respectively (Table 2). Very little agonist activity is seen with these compounds at the hMC4-R. In contrast, the *p*-iodo-substituted compound **3** is also a potent antagonist but retains significant partial agonist activity, stimulating cAMP-dependent β-galactosidase activity to 50% of maximal levels at the hMC4-R and hMC3-R as well (Figure 3).

Competition binding experiments (see the Experimental Section for details) were performed to determine if iodination of the phenylalanine aromatic ring or replacement of the phenyl ring with a naphthyl ring had any effect on the affinity of the cyclic lactam compounds for the MC3 MC4 receptors (Figure 4, Table 3). No significant alteration in IC_{50} values was observed relative to those calculated for [Nle[4],D-Phe[7]]-α-MSH.

Discussion

These exciting and intriguing results provide new insights into antagonist structure–activity relationships for melanocortin receptors and point to different requirements for antagonist activity at different pigmentary receptors in different species and at different melanocortin receptors in the same species. In previous studies from our laboratory, we demonstrated that Ac-[D-Trp[7],D-Phe[10]]α-MSH-(7–10)-NH_2 was a very weak antagonist in both the frog skin and lizard skin melanocortin receptor assays (pA_2 = 4.8 and 5.7, respectively), and other closely related analogues were either weak agonists or weak antagonists but only at one of the two receptors.[23] In another study, the linear 4–10 α-MSH analogue Ac-Nle-Asp-Trp-D-Phe-Nle-Trp-Lys-

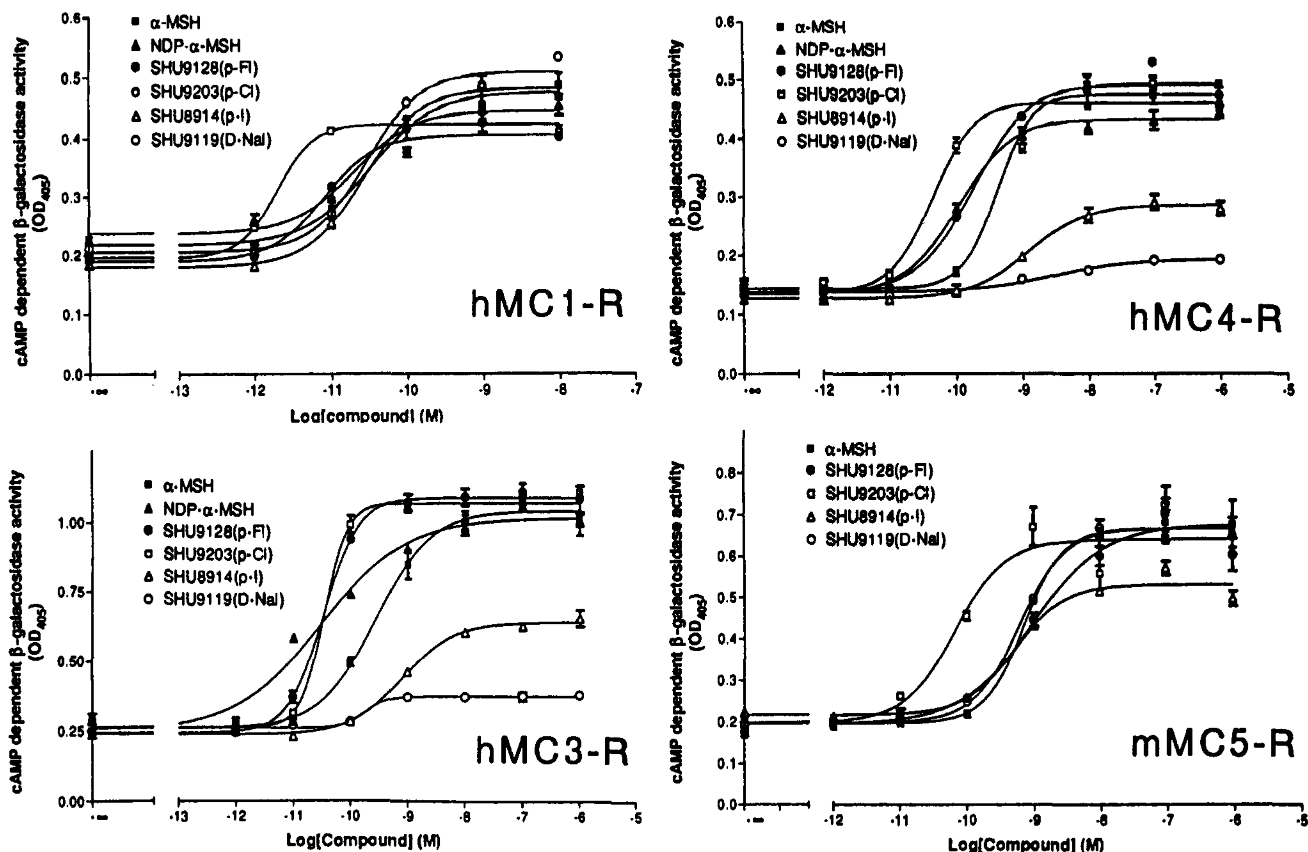

Figure 2. Effect of D-Phe[7] modifications and substitution on the agonist activity of Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4−10)-NH_2. Cell lines expressing the human MC1, human MC3, human MC4, and mouse MC5 receptors were incubated with varying concentrations of each of the analogues shown. Agonist activity was determined as a function of cAMP-dependent β-galactosidase activity. NDP-α-MSH is [Nle[4],D-Phe[7]]α-MSH-NH_2.

NH_2 was found[24] to be an antagonist in the frog skin assay (pA_2 = 8.4) but was active in the lizard skin assay only as an agonist. Interestingly, the corresponding cyclic lactam analogue of the above linear peptide, Ac-Nle-c[Asp[5],Trp[6],D-Phe[7],Nle[8],Lys[10]]α-MSH-(4−10)-NH_2, was a weak agonist in the frog skin assay (EC_{50} = 0.1 μM).[24] Very recently Jayawickreme et al.[25] reported the discovery of other α-MSH antagonists for the *Xenopus laevis* MC1 receptor using a library approach and an assay using cultured *X. laevis* melanophores and cAMP accumulation as the bioassay. Several antagonists with IC_{50} values in the range of 5 μM−11 nM were obtained. Apparently no effort was made to assess partial agonist/antagonist activity in this assay. The nonapeptide H-Met-Pro-D-Phe-Arg-D-Trp-Phe-Lys-Pro-Val-NH_2 was the most potent inhibitor (IC_{50} = 11 nM), which would correspond to a pA_2 value of ca. 8 if the assay conditions represent an equilibrium. At this time it is difficult to make any general statements about what substitutions in α-MSH will lead to antagonist activities of the MSH receptors. Indeed it is extremely intriguing and quite unusual to our knowledge that an analogue of a native hormone such as **3** and **4** would be such a potent antagonist at one physiological receptor in one species (in this case the frog) but a potent agonist in other species (the human and mouse MC1 receptors) (Table 1). However, this should provide a very useful insight for the future. Obtaining the structure of the α-MSH receptor (MC1-R) for the frog and lizard should provide key insights into structural requirements for agonist vs antagonist activity in these receptors and greatly aid in the development of a potent antagonist for the human MC1 receptor.

Equally intriguing are the antagonist activities for the new analogues at the human MC3 and human MC4 receptors. The potent and reasonably selective antagonist potencies for the D-Nal(2)[7] analogue **4** (Figure 3, Table 2) and the partial agonist, yet potent antagonist, properties for the D-Phe(pI)[7] analogue **3** at the hMC4 receptor and the hMC3 receptor, respectively, should prove to be very useful for further design. Though it is not possible with the presently limited group of analogues to provide a model for a particular approach to antagonist design or receptor selectivity, this work suggests that large stereoelectronic steric modifications in the message sequence (His-Phe-Arg-Trp) of a melanotropin can provide potent antagonist analogues for the MC3 and MC4 receptors.

As this manuscript was being written, Adan et al.[35] reported on the antagonist properties for certain linear ACTH-(4−10) analogues using rat MC3, human MC4, and ovine MC5 receptors transfected in 293 HEK cells. The analogues had modifications in the 6−10 positions of ACTH-(4−10). pA_2 values of <6−8.6 were reported, with the analogue H-Met-Glu-His-Phe-Pro-Gly-Pro-OH ([Pro[8,10],Gly[9]]ACTH-(4−10)) being the most potent, with its most potent antagonist activity at the MC4 receptor (pA_2 = 8.6).[35] Interestingly, the L-*p*-iodophenylalanine-containing linear analogue H-Met-Glu-His-Phe(pI)-Arg-Trp-Gly-OH was reported to have good antagonist activity (pA_2 = 7.4−8.4) at all three melanocortin

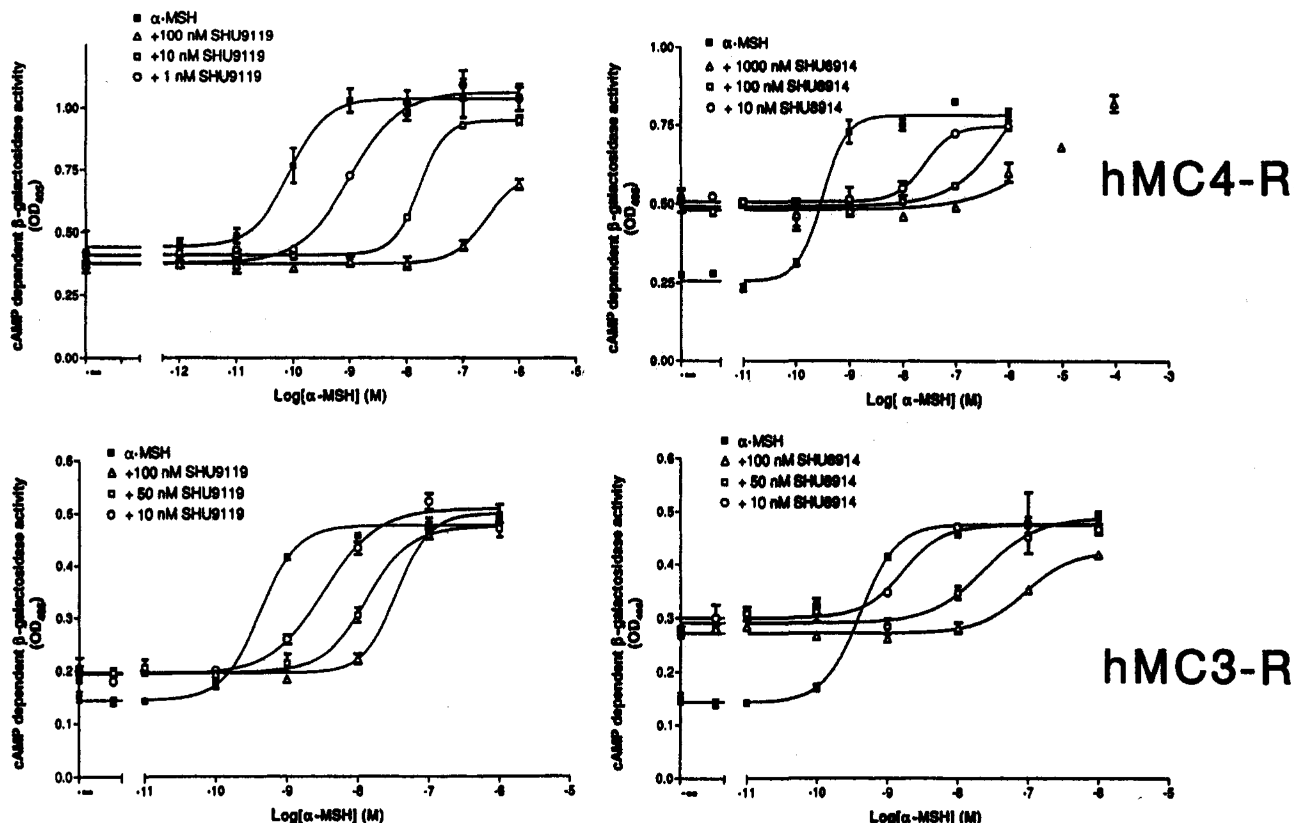

Figure 3. Dose–response curves for antagonism of the MC3 and MC4 receptors by Ac-Nle^4-c[Asp^5,D-Nal(2)^7,Lys^{10}]α-MSH-(4–10)-NH_2 (**4**, SHU9119) and Ac-Nle^4-c[Asp^5,D-Phe(pI)^7,Lys^{10}]α-MSH-(4–10)-NH_2 (**3**, SHU8914). Cell lines expressing the MC3 and MC4 receptors were stimulated with varying concentrations of α-MSH from 0 to 10^{-6} M as indicated on the *x* axis, with or without the analogues indicated. Agonist activity was determined as a function of cAMP-dependent β-galactosidase activity. The α-MSH concentrations given in these studies are respectively 10^{-9}, 10^{-8}, and 10^{-7} molar.

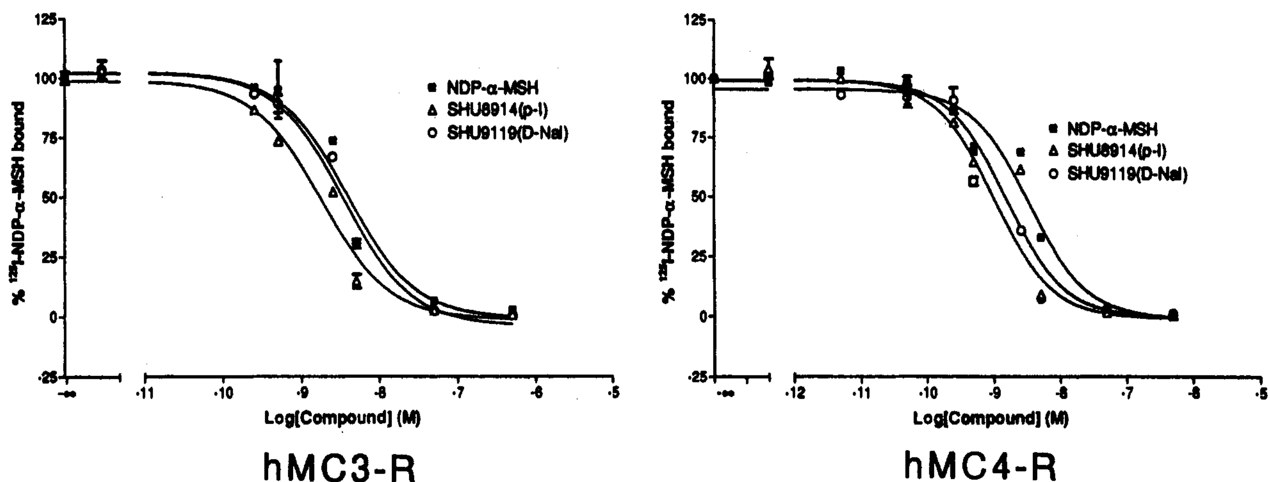

Figure 4. Competition of [^{125}I]Ac-[Nle^4,D-Phe^7]α-MSH binding to the MC3 and MC4 receptors by Ac-Nle^4-c[Asp^5,D-Nal(2)^7,Lys^{10}]α-MSH-(4–10)-NH_2 (SHU9119) and Ac-Nle^4-c[Asp^5,D-Phe(pI)^4,Lys^{10}]α-MSH-(4–10)-NH_2 (SHU8914). Cell lines expressing the MC3 and MC4 receptors were coincubated with [^{125}I]Ac-[Nle^4,D-Phe^7]α-MSH and varying concentrations of the analogues shown. Data indicate specific binding following the subtraction of nonspecific from total counts bound. NDP-α-MSH is [Nle^4,D-Phe^7]α-MSH-NH_2.

receptors investigated. No activities at melanocortin 1 receptors were reported.

In summary, the results presented here demonstrate that modifications of the phenyl ring of the D-Phe^7 residue of a cyclic lactam derivative of α-MSH-(4–10) that retain aromatic character can result in melanocortin receptor antagonists with high potency and specificity. The *in vivo* stability and efficacy of the parent cyclic lactam derivative Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}]α-MSH-(4–10)-NH_2 in the induction of pigmentation[19,20,36] suggest that these antagonists may be very useful for probing the proposed physiological functions of the MC3 and MC4 receptors.[3,10,37] We do not yet understand why the modifications tested antagonize the mammalian MC3 and MC4 receptors while retaining full agonist activity at the mammalian MC1 and MC5 receptors. Nor do we understand why they antagonize the frog MC1-R while having full agonist activity at the mammalian MC1-R. Nonetheless, the results reported here suggest that it should be possible to obtain potent and highly selective ligands for different melanocortin receptor types as well as for the subtypes of the same receptor

Table 3. IC_{50} Values (nM) for Phe[7]-Substituted Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4–10)-NH_2 Analogues

	hMC3R	hMC4R
[Nle[4],D-Phe[7]]α-MSH	3.8	3.6
3, SHU8914 (pI)	1.8	2.5
4, SHU9119 (D-Nal(2))	3.3	1.8

that apparently are found in different species. In addition, analogue **3** can be obtained in its radioiodinated form by a technique reported earlier by us,[38] and this could serve as a useful tool in various biological studies. Though an interesting beginning has been made, much more exploration of structure–activity and conformation–activity relationships is needed to develop predictive insights into the differential requirements for the various melanocortin receptors.

Experimental Section

General Methods for Peptide Synthesis and Purification. All peptides were synthesized by the solid-phase method of peptide synthesis using procedures similar to those used for the synthesis of Ac-Nle[4]-c[Asp[5],D-Phe[7],Lys[10]]α-MSH-(4–10)-NH_2 and related analogues.[20] p-Methylbenzhydrylamine (pMBHA) resin (0.35 mmol/g) was used as the solid support using an N^α-BOC protection strategy and an orthogonal side chain protection stragegy[20,39] that allowed cyclization of the macrocyclic lactam rings on the solid support. N^α-BOC-protected amino acids were purchased from Bachem Inc. (Torrance, CA) or prepared by standard literature procedures. The reactive side chain groups were protected as follows: Lys with Fmoc, Asp with OFm, His with (benzyloxy)methyl (BOM), Arg with tosyl, and Trp with formyl. All reagent and solvents were ACS grade or better and used without further purification except as noted. Following assembly on the solid support as described below, the peptides were cleaved from the resin with anhydrous HF (10 mL/g of resin) in the presence of 10% anisole at 0 °C for 45 min in the usual manner. The HF was removed rapidly by vacuum distillation and the resin filtered off and washed with ether (2 × 25 mL). The crude peptide was dissolved into glacial acetic acid (2 × 30 mL) and 30% aqueous acetic acid (2 × 30 mL), the combined acetic acid and aqueous acetic acid fractions were lyophilized, and the crude peptide was purified by reversed-phase preparative HPLC (RP-HPLC) on a C18-bonded silica gel column (Vaydac 218TP 1010, 1.0 × 25 cm) eluted with a linear acetonitrile gradient (20–40%) with a constant concentration of 0.1% aqueous trifluoroacetic acid. The separation was monitored at 280 nm with a UV absorbance detector. The fractions corresponding to the desired analogue were collected, and the combined fractions were lyophilized to give the final product as a white powder. The purity of the final products was checked by thin layer chromatography in at least three solvent systems and by RP-HPLC in two different gradient systems with UV monitoring at 280 and 223 nm (see Table 4 for details of the solvent systems and gradient systems used). The structures of the pure peptides were confirmed by fast atom bombardment (FAB) mass spectrometry (Table 4) and amino acid analyses which were consistent with the expected values in all cases. Optical rotations were taken (Table 4). Amino acid analyses were performed on a 42017 ABI amino acid analyzer following hydrolysis at 160 °C in propionic acid which destroys tryptophan. The unusual amino acids were not determined by the Biotechnology Facility (University of Arizona) due to its policy and excessive costs. α-MSH was purchased from Bachem.

Ac-[Nle[4],D-Phe[7]]α-MSH. The title compound was synthesized by standard solid-phase methods, purified by methods previously reported, and identical in all respects with the compound previously synthesized.[18]

Ac-Nle[4]-c[Asp[5],D-Phe(pF)[7],Lys[10]]α-MSH-(4–10)-NH_2 (1). The title compound was prepared using methods similar to those previously reported for cyclic lactam analogues of α-MSH-(4–10)[19,20] with cyclization on the solid support. The protected peptide resin to the title compound was prepared

Table 4. Analytical Properties of New Cyclic α-Melanotropin Peptides Used in This Study

compd	$[\alpha]^{22}_{589}$ in 10% HOAc (deg)	TLC R_f[a] values A	B	C	HPLC[b] K′	FAB-MS,[g] M + H found (calcd)
1	−27.6	0.82	0.04	0.69	5.71[c]	1042.5
	(c 0.023)				6.71[d]	(1042.1)
2	−41.8	0.80	0.05	0.69	4.42[c]	1059.9
	(c 0.038)				3.11[e]	(1058.6)
3	−45.4	0.84	0.02	0.68	6.83[c]	1151
	(c 0.032)				8.14[f]	(1150.1)
4	−65.2	0.79	0.01	0.65	7.28[c]	1075
	(c 0.018)				9.59[f]	(1075.3)

[a] Solvent systems: (A) 1-butanol/HOAc/pyridine/H_2O (5:1:5:4), (B) EtOAc/pyridine/(HOAc/H_2O (5:5:1:3), (C) 1-butanol/HOAc/H_2O (4:1:5). [b] Analytical HPLC performed on a C18 column (Vydac 218TP 104) using a gradient of acetonitrile in 0.1% aqueous TFA in 30 min at 1.5 mL/min. The following gradients were used. [c] 20–40% acetonitrile in 30 min. [d] 20–30% acetonitrile in 30 min. [e] 25–35% acetonitrile in 30 min. [f] 10–40% acetonitrile in 30 min. [g] Fast atom bombardment mass spectrometry.

from 1.5 g of pMBHA resin (0.35 mmol of NH_2/g of resin) by first coupling N^α-BOC-Lys(N^ϵ-FMOC) to the resin. The following amino acids were then added to the growing peptide chain by stepwise addition of N^α-BOC-Trp(N^i-For), N^α-BOC-Arg(N^G-Tos), N^α-BOC-D-Phe(pF), N^α-BOC-His(N^π-BOM), N^α-BOC-Asp(β-OFm), and N^α-BOC-Nle using standard solid phase methods. Each coupling reaction was achieved using a 3-fold excess of DIC and a 2.4-fold excess of HOBt after coupling the last amino acid. The N^ϵ-FMOC and β-OFm protecting groups were removed by treating the N^α-BOC-protected peptide resin with 20% piperidine in NMP for 30 min. The peptide resin was washed with DMF (3 × 40 mL), dichloromethane (DCM) (3 × 40 mL), and 10% diisopropylethylamine (DIEA) (3 × 40 mL) and then suspended in 15 mL of NMP and mixed with 6-fold excess of BOP reagent in the present of an 8-fold excess of DIEA for 2 h. The coupling was repeated twice if needed until the resin gave a negative ninhydrin test.[40] Then the N^α-BOC protecting group was removed in the usual manner with 50% TFA in DCM. The amino group was neutralized with 10% DIEA in DCM and acetylated with 25% acetic anhydride in DCM for 20 min. The peptide resin was cleaved in 15 mL of anhydrous HF as outlined above. The HF–anisole and dithioethane were removed rapidly by vacuum distillation at 4 °C. The residue was washed with ethyl ether (2 × 25 mL) and the cleaved peptide dissolved in acetic acid (2 × 25 mL) and 30% aqueous acetic acid (2 × 30 mL). The pooled acetic acid and aqueous acid phase fractions were lypophilized to give a white powder that was purified by preparative HPLC on a C18-bonded silica gel column (Vydac 218TP 1010, 1.0 × 25 cm) eluted with a linear gradient of acetonitrile (20–40%) in aqueous 0.1% trifluoroacetic acid (v/v). The purification was monitored at 280 nm, and the fractions corresponding to the major peak were collected, combined, and lyophilized to give the title compound **1** as a pure (>98%) white powder. Analytical data are given in Table 4.

Ac-Nle[4]-c[Asp[5],D-Phe(pCl)[7],Lys[10]]α-MSH-(4–10)-NH_2 (2). The title compound was prepared in a manner very similar to that reported above for analogue **1** except that N^α-BOC-D-Phe(pCl) was added to the growing peptide chain in the appropriate sequence. The compound was purified as before for **1** to give **2** as a white powder. Analytical data are given in Table 4.

Ac-Nle[4]-c[Asp[5],D-Phe(pI)[7],Lys[10]]α-MSH-(4–10)-NH_2 (3). The title compound was prepared in a manner very similar to that reported above for analogue **1** except that N^α-BOC-D-Phe(pI) was added to the growing peptide chain in the appropriate sequence. The peptide product was purified as before for **1** to give **3** as a white powder. Analytical data are given in Table 4.

Ac-Nle[4]-c[Asp,D-Nal(2)[7],Lys[10]]α-MSH-(4–10)-NH_2 (4). The title compound was prepared by methods very similar to those reported above for analogue **1** except that N^α-BOC-D-Nal(2) was added to the growing peptide chain in the appropriate

sequence. The peptide product was purified as reported above for 1 to give **4** as a white powder. Analytical data are given in Table 4.

General Biological Methods. All products for cell culture were purchased from Gibco-BRL (Grand Island, NY). $CaCl_2$, NaCl, and Na_2HPO_4 were purchased from Mallinckrodt Chemical (Paris, KN). Triton X-100, *o*-nitrophenyl-β-D-galactopyranoside (ONPG), isobutylmethylxanthine, and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO). G250 protein reagent concentrate and 2-mercaptoethanol were purchased from Bio-Rad (Hercules, CA). β-Galactosidase activity was measured and normalized by protein concentration in each well of a 96-well plate. The 96-well plate spectrophotometer used to monitor β-galactosidase activity at 405 nM and protein concentration at 595 nM was from Molecular Devices (Sunnyvale, CA).

Bioassays. Bioassays using frog (*R. pipiens*) and lizard (*A. carolinensis*) skins were performed by published procedures.[41-43] In these bioassays, skins become dark as a result of melanin granule dispersion within melanocytes in response to a melanotropin. This darkening response can be conveniently monitored by photoreflectance methods. The potency of each peptide was determined from log dose–response curves comparing the melanotropic activity of the analogues with that of the native hormone, α-MSH. Competitive antagonist activity was measured by constructing a dose–response curve of α-MSH in the presence of fixed amounts of the antagonist. A rightward shift in the log dose–response curve corresponds to antagonism. The inhibition potency (pA_2 values) was determined by the method of Schild.[34]

β-Galactosidase Activity Assay. Clonal 293 cell lines expressing the human MSH receptor, human MC3 receptor, human MC4 receptor, and mouse MC5 receptor were transfected with a pCRE/β-galactosidase construct using a $CaPO_4$ method;[33] 4 μg of pCRE/β-gal DNA was used for transfection of a 10 cm dish of cells. After 15–24 h posttransfection, cells were split into 96-well plates with 20 000–30 000 cells/well and incubated at 37 °C in a 5% CO_2 incubator until 48 h posttransfection. Cells were then stimulated with different α-MSH analogues diluted in stimulation medium (Dulbecco's modified Eagle's medium containing 0.1 mg/mL bovine serum albumin and 0.1 mM isobutylmethylxanthine) for 6 h at 37 °C in a 5% CO_2 incubator. Agonist activity was measured by stimulating cells with varying concentrations of α-MSH, Ac-[Nle^4,D-Phe^7]α-MSH, and cyclic analogues **1**–**4** (Table 1). Antagonist activity was measured by stimulating MC3 and MC4 receptor cell lines with varying concentrations of α-MSH or α-MSH plus varying concentrations of the compounds SHU8914 (pI) or SHU9119 (D-Nal(2)). After stimulation, cells were lysed in 50 μL of lysis buffer (250 mM Tris-HC1, pH 8.0, 0.1% Triton X-100), frozen, thawed, and then assayed for β-galactosidase activity as described.[32] Data represent means and standard deviations from triplicate data points, and curves were fitted by linear regression using GraphPad Prism software. Antagonist pA_2 values were determined using the method of Schild.[34]

Competition Binding Assay. Cell lines expressing the hMC3-R or hMC4-R were plated at 1×10^6 cells/well in 24-well plates. Prewarmed PBS–BSA solution (1 mg/mL BSA in PBS solution) containing 3.1×10^{-10} [^{125}I][Nle^4,D-Phe^7]α-MSH (100 000 cpm) and different concentrations of Ac-[Nle^4,D-Phe^7]α-MSH-NH_2, Ac-Nle^4-c[Asp^5,D-Nal(2)^7, Lys^{10}]α-MSH-(4–10)-NH_2, or Ac-Nle^4-c[Asp^5,D-Phe(pI)^7, Lys^{10}]α-MSH-(4–10)-NH_2 was added to each well. Cells were incubated for 30 min at 37 °C in a 5% CO_2 incubator, washed twice with PBS–BSA at 37 °C, lysed with 500 μL of 0.5 N NaOH, and counted with a γ-counter. Nonspecific binding, determined as the amount of radioactivity bound at 5×10^{-6} M cold [Nle^4,D-Phe^7]α-MSH, was 3–5% of the total counts bound. Data represent means and standard deviations from duplicate data points and were analyzed using GraphPad Prism software.

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