Development of Melanotropin Antagonists: Investigating Potent and Specific Ligands for New Receptors

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α-Melanotropin (Ac-Ser-Tyr-Ser-Met-Glu^5-His-Phe-Arg-Trp-Gly^{10}-Lys-Pro-Val-NH_2) is one of the first peptide hormones to be isolated and have its structure determined. It was early recognized to have essentially the same N-terminal tridecapeptide sequence as adrenocorticotropic hormone (ACTH) except that the N-terminal was acetylated in the case of α-MSH but not in the case of ACTH, indicating that their biosyntheses were different (Figure 1). Subsequently it was discovered that α-MSH and ACTH were derived from the same gene, currently referred to as proopiomelanocortin (POMC). Its original bioactivity was pigmentation, but it also was recognized that it may have activity in the central nervous system, though the precise nature of these central activities have been controversial. The recent cloning and expression of five melanocortin receptors, with the MC3 and MC4 receptors found primarily in the brain and the MC5 receptor (MC5-R) found throughout the body, has provided new impetus to understand the structure-activity relationships of α-MSH at these receptors. 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 have provided much information on agonist activity related to pigmentary effects, only recently has it been possible to begin to systematically develop potent and selective antagonists.

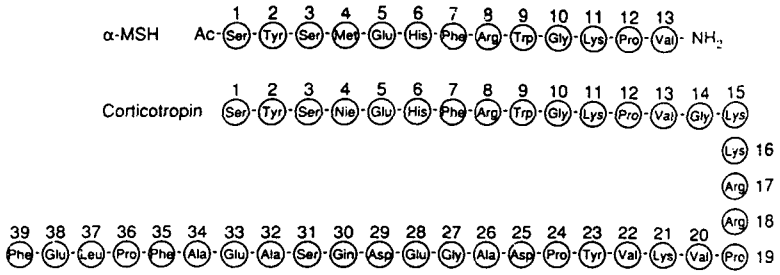

Figure 1. Strucuures of α-MSH and Corticotropin (ACTH)

Classical Structure-Activity Relationships for α-MSH

Extensive structure-activity studies have been made especially with regard to its pigmentary activities by use of the classical frog (*Rana pipiens*) and lizard (*Anolis carolinensis*) skin bioassay. These studies have led to the design of superpotent (up to 60 times that of α-MSH), ultraprolonged-acting (hours in vitro, days in vivo) analogs such as [Nle^4,D-Phe^7] α-MSH. Table 1 lists some of the key findings of these early studies. The key points are that the central tetrapeptide -His-Phe-Arg-Trp- (residue 6 to 9) is sufficient for all activities at the classical melatropin receptors such as the frog skin and lizard skin. Both the N-terminal pentapeptide and C-terminal tetrapeptide are primarily potency modulators. In this regard, the Met^4 residue was particularly important since oxidation of the sulfur led to a large decrease in potency (10-100 times) for the hormone. On the other hand, replacement of Met^4 by the isosteric norleucine (Nle^4) led to an increase in potency. The stereochemistry of amino acids, especially in the critical 6-9 sequence was very important, and in general replacement of the naturally occurring L-amino acids with D-amino acids led to a decrease in potency. A highly significant exception was Phe^7, since substitution of L-Phe^7 by D-Phe^7 led to a large increase in potency for hormone and fragment analogs of the hormone. This led to design of [Nle^4,D-Phe^7] α-MSH, one of the most potent melanotropins known and the subject of numerous investigations. This peptide has many other desirable properties including resistance to enzymatic degradation, high stability in solution or in powder form, no toxicity, excellent biodistribution characteristics, and good solubility in a variety of solvents and solutions. It is currently undergoing clinical trials.

Table 1. Summary of Classical Structure-Activity Relationships for α-MSH.

Ac - Ser - Tyr - Ser - Met - Glu - His - Phe - Arg - Trp - Gly - Lys - Pro - Val - NH_2
1 **5** **10** **13**

1. N-Acetyl and carboxamide terminal groups important for potency
2. Sequence -His-Phe-Arg-Trp- is sufficient for all activities at classical peripheral receptors
3. Residues 1, 2, 3, 10, 11, 12, and 13 primarily modulate potency
4. Met^4 oxidation causes large drop in potency
5. Stereochemistry of central region residues important
6. D-Phe^7-substitution often leads to prolonged residual activity except for α-MSH_{4-10}
7. D-Phe^7-substitution generally makes melanotropins resistant to proteolytic inactivation
8. Prolonged activity not a function of potency or enzymatic stability

Design of Superpotent Cyclic Lactam α-MSH Analog

The discovery of the high potency of the D-Phe^7-containing melanotropins prompted us to consider in detail the conformational and topographical properties that might be responsible for their large potency enhancements. These results indicated that a reverse-turn conformation centered at the Phe^7 position was critical to biological potency since D-amino acids at the i + 1 position are known to stabilize reverse-turn conformations (β-turns). Indeed, application of conformational calculations and model building led to the suggestion that such a conformation might be necessary for the biological activity of melanotropin at the classical peripheral receptor. After examining several different models, we were led to the hypothesis that stabilization of this reverse-turn could be accomplished by a conformational constraint imposed using a backbone-to-side-chain cyclization. This led to the design of a series of cyclic lactam analogs in which cyclization was effected between a carboxylate group on the side chain group of an amino acid residue in position 5 and an amino group on the side chain moiety of the amino acid residue in position 10. This in turn led to the synthesis of the cyclic lactam analog Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}] α-MSH-(4-10)-NH_2 (Figure 2), which was exceptionally potent in the lizard skin (90 times that of α-MSH) and in addition exhibited prolonged biological activity.

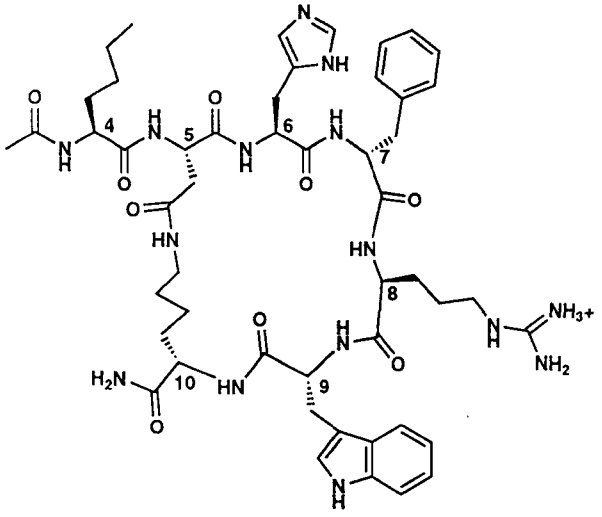

Figure 2. Structure of Cyclic Lactam α-MSH Analog, Ac-Nle^4-c[Asp^5,D-Phe^7,Lys^{10}] α-MSH-(4-10)-NH_2

Design of Highly Potent and Selective α-MSH Antagonists

Using strategies we have previously suggested for peptide hormone and neurotransmitter antagonist development, 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. The analog Ac-Nle^4-c[Asp^5,D-Phe(*p*I)^7,Lys^{10}] α-MSH-(4-10)-NH_2 (3; Table 2) has only minimal agonist activity in the frog skin (*R. pipiens*) assay but, as shown in Figure 3, is a potent inhibitor of the biological response of α-MSH. Evaluation of the dose response displacement curves (Figure 3) showed that 3 was an exceptionally potent antagonist analog (pA_2 = 10.3, Table 2) in this in vitro melanocortin 1 receptor assay. Interestingly, the analog was a potent agonist (EC_{50} = 0.60nM, data not shown) in the lizard (*A. carolinensis*) skin assay, a highly potent agonist at the human MC1-R (EC_{50} = 55 pM, Table 2), and a modestly potent agonist at the mouse MC1-R (EC_{50} = 0.19 nM, Table 2). The cyclic analog 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 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 2). Interestingly, the D-*p*-fluorophenylalanine- and D-*p*-chlorophenylalanine-containing analogue 1 and 2 (Table 2) were both potent agonists at all melanocortin 1 receptors.

Table 2. Agonist and Antagonist Activities of Cyclic Lactam α-MSH Analogs at Various Melanocortin Receptors

Compounds	EC_{50} (nM)		
	frog skin	mMC1-R	hMC1-R
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	0.10 ± 0.35	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	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	pA_2 = 10.3 (Ant.)	0.19 ± 0.13	0.055 ± 0.031
4, Ac-Nle^4-c[Asp^5.D-Nal(2)^7, Lys^{10}]α-MSH(4-10)-NH_2	$pA_2 \geq 10.5$ (Ant.)	0.039 ± 0.029	0.036 ± 0.012

Modification of D-Phenylalanine^7 at the para position or substitution of the phenyl group with a naphthyl group (4, Table 2) had little effect on agonist activity of the compounds at the human MSH receptor (hMC1-R) or mouse MC5 receptor (Table 3). In contrast, replacement of the D-Phe^7 with D-Phe(*p*I)^7 dramatically reduced agonist activity at the MC3 and MC4 receptors. Both compounds are partial agonists with greatly increased EC_{50}s. Interestingly, the D-Phe(*p*Cl)^7-substituted analog 2 was a full agonist and actually was more potent than [Nle^4,D-Phe^7] α-MSH at every receptor

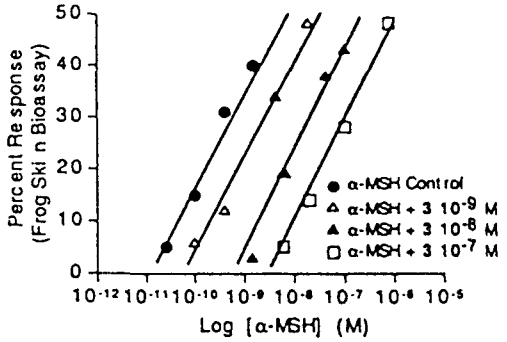

Figure 3. Demonstration that Ac-Nle^4-c[Asp^5,D-Phe(*p*I)^7,Lys^{10}] α-MSH-(4-10)-NH_2 (**3**) is a potent antagonist of α-MSH in the frog skin MC1 receptor assay system. Antagonism of the dose-response curve of α-MSH by 10^{-9}(△), 10^{-8}(▲) and 10^{-7}(□)M antagonist **3** is demonstrated by the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

except the hMC3-R. The D-Phe(*p*F)^7-substituted analog **1** likewise was a very potent full agonist in all assays. The two compounds acting as weak partial agonists at the MC3-R and MC4-R (analogs **3** and **4**) were then examined for antagonist activity at these receptors. As can be seen, the D-Nal(2)^7-substituted cyclic lactam analog **4** is a potent antagonist of the MC4-R and MC4-R, with pA_2 values of 8.3 and 9.3, respectively. 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

Table 3. 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 Analogs at the Different Melanocortin Receptors

Compounds	EC_{50} (pM)			
	hMC1-R	hMC3-R	hMC4-R	mMC5-R
α-MSH	91 ± 69	669 ± 355	210 ± 57	807 ± 125
[Nle^4,D-Phe^7]α-MSH	23 ± 7	132 ± 31	17 ± 18	ND
1	16 ± 3	191 ± 9	19 ± 14	1360 ± 549
2	5 ± 4	63 ± 26	18 ± 14	117 ± 70
3	55 ± 31	1134 ± 197 P. Agonist pA_2 = 8.3	573 ± 357 P. Agonist pA_2 =9.7	684 ± 227 P. Agonist
4	36 ± 12	2813 ± 575 P. Agonist pA_2 = 8.3	no activity Antagonist	434 ± 260 Full Agonist

significant partial agonist activity, stimulating cAMP-dependent β-galactosidase activity to 50% of maximal levels at the hMC4-R and hMC3-R as well.

Investigation to Find Selective Ligands for Melanocortin Receptors

When a bulky aromatic α-amino acid is placed at position 7 of the cyclic 4 to 10 lactam analogs of α-MSH such as **3** and **4** (**Table 3**) potent antagonists at the classical frog skin MC1-R and at the hMC3-R and hMC4-R with some selectivity for the MC4 receptor were obtained. Interestingly **3** and **4** are potent agonists at the hMC1-R. However, a bulky substituent at position 7 is not sufficient for antagonist activity since substitution with L-Nal(2) at position 7, or with D-Nal(2) with Nle^8 (instead of Arg^8) led respectively to agonist **5** which was selective for the hMC4-R (20 to 200 fold), and **6** which was selective for the hMC1-R (100 to 1000 fold) (Table 4). Moreover the D-homophenylalanine analog **7** also was an agonist at all three receptors with selectivity for the hMC1-R and to a lesser extent the hMC4-R. For the cyclic 5 to 11 lactam analogs, the $D\text{-}Phe^7$, $D\text{-}Trp^9$ analog **8** was potent and selective for the hMC1-R (50 to 530 fold), but when the Nle^4 residue was removed as in **9**, the analog became modestly selective for the hMC4-R. Surprisingly the [$D\text{-}Phe^6$]γ-MSH analog **10** was potent at all three receptors, with modest selectivity for the hMC1 and hMC4 receptors. These studies have uncovered important leads for the development of highly selective receptor agonists and antagonists and already have led to insights into new physiological roles for α-MSH.

Table 4. Bioassay Results for α-Melanotropin Agonist Analogs with Selectivity for Melanocortin Receptors

Compounds	EC_{50} (nM)		
	hMC1-R	hMC3-R	hMC4-R
α-MSH	0.09	0.67	0.21
5, Ac-Nle^4-c[Asp^5,Nal(2)^7, Lys^{10}]α-MSH(4-10)-NH_2	2	20	~ 0.1
6, Ac-Nle^4-c[Asp^5,D-Nal(2)^7, Nle^8,Lys^{10}]α-MSH(4-10)-NH_2	0.1	100	~ 10 (P. Ant.)
7, Ac-Nle^4-c[Asp^5,D-Hph^7, Lys^{10}]α-MSH(4-10)-NH_2	~ 0.5	150	~ 0.9
8, Ac-Nle^4-c[Asp^5,D-Phe^7,D-Trp^9,Ala^{10},Lys^{11}]α-MSH(4-11)-NH_2	0.30	160	15
9, Ac-c[Asp^5,D-Phe^7, Ala^{10},Lys^{11}]α-MSH(4-11)-NH_2	13	89	2.9
10, [$D\text{-}Phe^6$]γ-MSH	~ 0.2	2.7	0.29

Conclusion

These exciting and intriguing results provide new insights into structural requirement for melanocortin receptor antagonist at different pigmentary receptors in different species and at different melanocortin receptors in the same species. The results presented here demonstrate that modifications of the phenyl ring of the $D\text{-}Phe^7$

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. 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.