Design of Novel Chimeric Melanotropin−Deltorphin Analogues. Discovery of the First Potent Human Melanocortin 1 Receptor Antagonist

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A number of novel α-melanotropin (α-MSH) analogues have been designed, synthesized, and assayed for bioactivity at the melanocortin-1 (MC1) receptor from *Xenopus* frog skin, and selected potent analogues were examined at recombinant human MC1, MC3, and MC4 receptors expressed in human embryonic kidney (HEK) cells. These ligands were designed from Deltorphin-II, by a new hybrid approach, which incorporates the hydrophobic tail and the address sequence of Deltorphin-II (Glu-Val-Val-Gly-NH_2) and key pharmacophore elements of melanotropins. Some of the ligands designed, *c*[Xxx-Yyy-Zzz-Arg-Trp-Glu]-Val-Val-Gly-NH_2 {XXX = nothing, Gly, β-Ala, γ-Abu, 6-Ahx; YYY = His, His(3-Bom), (*S*)-cyclopentylglycine (Cpg); ZZZ = Phe, D-Phe; D-Nal(2′)}, show high potency at melanocortin receptors. One ligand, GXH-32B-*c*[β-Ala-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2, the most potent of the chimeric analogues tested, displayed agonist activity at each of the MC receptor subtypes analyzed, with an EC_{50} of 2 nM at the amphibian MC1 receptor. In contrast, GXH-38B-*c*[Gly-Cpg-D-Nal-(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (Cpg = cyclopentyl glycine) was an antagonist with a IC_{50} of 43 nM at the amphibian receptor, and among the human subtypes tested, was the most potent at the MC1 receptor subtype where it also acted as an antagonist (K_i = 53 nM), which is the first potent antagonist discovered for the human MC1 receptor. These results provide strong evidence supporting our hypothesis that ligand scaffolds for different G-protein coupled receptors (GPCRs) can be used to design ligands for other GPCRs and to design more potent ligands to treat diseases associated with the human MC1 receptor.

Introduction

α-Melanocyte stimulating hormone, α-melanotropin (α-MSH)[1], a tridecapeptide (Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), and one of the products generated from the proopiomelanocortin gene, is generated in the brain, pituitary, epidermis, and elsewhere by posttranslational modification of proopiomelanocortin (POMC). α-MSH was discovered primarily for its function in pigmentation,[2−4] exerted at the melanocortin-1 (MC1) receptor, decades ago.[4−6] (The nonprefaced "melanocortin receptor" of earlier years is generally synonymous to the MC1 receptor as currently defined.) Recent research has revealed that this peptide and its analogues have several other profound biological activities,[1,7−22] including improvement of learning and memory, cardiovascular functions, reproduction, treatment for erectile dysfunction, and effects on feeding behaviors which are believed to contribute to the obesity syndrome. These findings have prompted us to seek an understanding of how these ligands interact with their corresponding receptors and to correlate structure−activity relationships.

Since its amino acid sequence was first characterized by Lerner and co-workers in the late 1950s, many analogues of α-MSH have been designed and tested for biological activity.[23,24] However, rational approaches to the design of α-MSH analogues were not successful until the development of MT-I[25] (Ac-Ser-Tyr-Ser-Nle[4]-Glu-His-D-Phe[7]-Arg-Trp-Gly-Lys-Pro-Val-NH_2; [Nle,[4] D-Phe[7]]α-MSH). MT-I showed greater metabolic stability and improved potency compared with α-MSH, and since its development, numerous attempts have been made to rigidify its structure by addition of conformational and topographical constraints. Such efforts to obtain shorter, more rigid and potent analogues of α-MSH were well rewarded with the discovery of Ac-Nle[4]-*c*[Asp[5],D-Phe[7], Lys[10]]α-MSH(4−10)-NH_2 (MT-II) in the late 1980s.[26,27]

Significant advances in the design of selective and potent melanocortin (MC) receptor ligands have been made,[e.g.25,28−44] but there still is much room for improvement, and a number of important questions have yet to be addressed adequately. For example, highly selective agonists or antagonists for the human melanocortin 1 (*h*MC1) receptor have yet to be developed, even though the MC1 receptor was the first of the MC receptors to be identified. While antagonists do exist that are relatively selective for the MC3 and MC4 receptors, there is still room for enhancement. For the MC5 receptor, both selectivity and potency need considerable improvement. Second, the biophysical properties of ligands need to be tuned to enable them to access the physiological compartments where their target receptors reside. Currently, most melanotropic ligands cannot penetrate the epidermal layer of mammalian skin to

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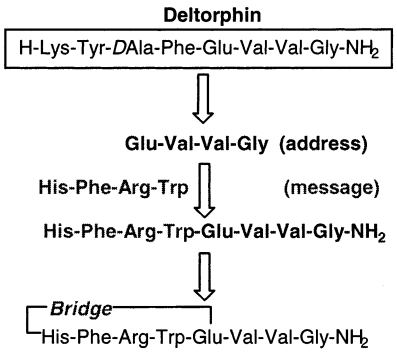
interact with MC1 receptors located there, or effectively penetrate the blood−brain barrier (BBB)[45−47] where they have access to central nervous system MC receptors that are involved in regulation of erectile function, temperature homeostasis, feeding behavior, among a host of other functions.[1,48] Better penetration of the BBB permits lower concentrations of drugs to be used to achieve equivalent pharmacological effects, and lower dosages in turn, help reduce the number of significant side effects. Third, almost all of the MSH analogues designed so far are based on α-MSH itself.[42] The chance to discover more potent and selective ligands through this traditional approach may be getting more difficult. Thus, we have searched for alternative starting points to introduce new templates for structural diversity. By developing new templates from which to design new sets of selective and potent MC receptor ligands, we also hope to gain insight into how MC ligands interact with their target receptors and what the chemical and structural basis for this interaction could be.

In humans and many other organisms, opioid peptides are encoded by a set of related but distinct gene families, including the Orphanin FQ (Nociceptin) Prohormone, Prepro-Enkephalin A, Proopiomelanocortin (POMC), and the Prepro-Enkephalin B gene that gives rise to the dynorphin, neo-endorphin, and other peptides. In addition to α-MSH, POMC also encodes *β*-endorphin,[49] a prominent opioid peptide that contains the methionine-enkephalin sequence at its amino terminus. Met-enkephalin is also derived from the Prepro-Enkephalin A gene.[50] Yet despite the obvious genetic relationships that exists between parental POMC, melanocortin, and opioid peptides, little is known about their possible direct interactions;[50] though considerable evidence exists that they and their derivatives have interactions through unknown mechanisms.[16,51−59]

More than two decades ago, it was reported that enkephalin could desensitize frog skin melanophores to α-MSH.[60] Later, it was shown that the Met-enkephalin analogue, [D-Met^2, Pro^5]enkephalinamide (DMPEA), could stimulate α-MSH secretion by acting directly through opiate receptors at the level of the neurointermediate lobe.[54] Using dual microinjections into the nucleus tractus solitarius of anaesthetized rats, it was found that α-MSH may play a role in directly antagonizing the central cardiovascular effects of endogenous opioid peptides.[53] Additionally, functional antagonism between opioids and melanocortins has been noted in behavioral and a number of other related biological processes.[61] For example, melanocortin peptides facilitate yawning, while opioid peptides, on the other hand, appear to prevent the stretching−yawning syndrome induced by MSH peptides.[62] Furthermore, it has been shown that both melanotropins and *β*-endorphin peptides can exert potent effects on early postnatal growth in rats. Ironically, des-Ac-α-MSH displayed greater activity in promoting body trophism, than α-MSH itself, despite the fact that α-MSH displays similar, if not greater, potency in stimulating MC receptors. In contrast to MSH, high doses of *β*-endorphin exert inhibitory effects on body growth and organ trophism.[55]

Intriguing though these reports may be, they do not provide direct evidence of interaction between melanotropin peptides and opioid (OP) receptors or, vice versa, between opioid peptides and MC receptors. Although there remains little solid evidence that direct interactions of this sort occur in vivo, evidence has been building to suggest they can and do occur in vitro. This evidence has prompted our research into how analogues from these two distinct neuroendocrine systems come to interact with receptors of the opposite family.With this in mind, we decided to construct hybrid structures of α-MSH and analogues of the *δ*-opioid receptor selective Deltorphin II (Delt-II), itself devoid of MC receptor activity at the *Xenopus* frog skin. Our strategy (Scheme 1) to make novel α-MSH analogues involves taking a hybrid approach by combining the cores for α-MSH and constrained analogues of Deltorphin II (Delt-II). At this point, we decided to put the address sequence Glu-Val-Val-Gly at the C-terminus, and the bioactive message sequence of α-MSH, His-Phe-Arg-Trp,[63,64] at the N-terminus, with C-terminal amidation of Gly. We believed that this would be more logical than the other way around, because α-MSH has a hydrophobic C-terminus, Lys-Pro-Val-NH_2. Hence, H-His-Phe-Arg-Trp-Glu-Val-Val-Gly-NH_2 became our initial targeted model, though linear molecules were never the ultimate goal in our studies for two reasons. First, linear compounds tend to have very flexible conformations, which make it hard to study the conformation−activity relationships. On the other hand, flexible conformations often do not have good selectivity binding closely related receptors, such as opioid receptors and melanocortin receptors. Consequently, constrained analogues became our priority in the design of new ligands. One way to create constraint is through cyclization, which has four possibilities: side chain to side chain (backbone to backbone in its many variations), side chain to head (N-terminus), side chain to tail (C-terminus), and head to tail.

Scheme 1. Design of Novel Chimeric Melanotropin Analogues



The research in this project was focused on the side chain to head group since the side chain of Glu has an acidic functional group (COOH) in its side chain while the N-terminus is a free amino group (NH_2). Cyclization of side chain to head group can be achieved easily by simple lactam formation between the two functional groups (Scheme 1).

Results

Peptide Synthesis. Solid-phase peptide synthesis (SPPS) was used in the synthesis all the peptide analogues designed. None of the syntheses was optimized for yields. For lactam-bridged peptides, the

Table 1. High Throughput Screening of Chimeric α-MSH Analogues at *Xenopus* Frog MC1 Receptor

c[**ZZZ**-**XXX**-**YYY**-Arg-Trp-Glu]-Val-Val-Gly-NH_2

peptide nos.	peptide designation nos.	peptide sequence	EC_{50} (nM)	E_{max} (arbitrary units)	IC_{50} nM[a] (0.50 nM α-MSH)
1	GXH-15	*c*[His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	>10000	NA	8200 (SE 1900)
2	GXH-17	*c*[His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	825 (SE 80)	125 (SE 1)	455 (SE 28)
3	GXH-21	*c*[His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	60 (SE 20)	200 (SE 1)	none
4	GXH-22	*c*[Pro-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	>10000	NA	1500 (SE 900)
5	GXH-07	*c*[Gly-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	5200 (SE 1500)	308 (SE 98)	none
6	GXH-11	*c*[Gly-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	510 (SE 134)	257 (SE 12)	none
7	GXH-12	*c*[Gly-Pro-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	>10000	NA	~10000
8	GXH-13	*c*[Gly-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	60 (SE 10)	317 (SE 10)	none
9	GXH-14	*c*[Gly-Pro-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	>10000	NA	637 (SE 81)
10	GXH-25B	*c*[γ-Abu-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2700 (SE 200)	274 (SE 14)	none
11	GXH-26	*c*[6-Ahx-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	>10000	NA	none
12	GXH-28B	*c*[β-Ala-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	31 (SE 22)	208 (SE 13)	none
13	GXH-29B	*c*[γ-Abu-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	313 (SE 181)	218 (SE 17)	none
14	GXH-30B	*c*[6-Ahx-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	23 (SE 19)	245 (SE 18)	none
15	GXH-32B	*c*[β-Ala-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2 (SD 0.3)	263 (SE 24)	none
16	GXH-33B	*c*[γ-Abu-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	23 (SE 11)	318 (SE 15)	none
17	GXH-35B	*c*[Gly-His(3-Bom)-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	>10000	NA	440 (SE 48)
18	GXH-36B	*c*[Gly-Cpg-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	>10000	NA	953 (SE 31)
19	GXH-38B	*c*[Gly-Cpg-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	>1000	NA	43 (SE 5)

[a] IC_{50} is the competitive binding in the presence of 500 pM of α-MSH.

cyclizations were carried out on the solid support before the peptides were cleaved from resin[65] (See Experimental Section for details).

***Xenopus laevis* Frog MC1 Receptor Assays.** The design of melanotropin analogues from Deltorphin-II (Scheme 1) was based on the following considerations. Initially it was decided to make the lactam bridge as simple as possible. Since the acid group from the side chain of glutamic acid can form an amide bond with the amino group of the N-terminal, this amide bond became the first lactam bridge of choice. On the basis of previous studies, His-Phe-Arg-Trp is an essential sequence for α-MSH analogues to possess agonist biological activity,[63,64] and thus it was incorporated into our designed analogues. However, the analogue GXH-15 (**1**, XXX = His, YYY = Phe) displayed almost no agonist activity in the *Xenopus* frog skin assay, but was a very weak antagonist with an IC_{50} of 8.2 μM (Table 1). Replacement of Phe by D-Phe led to *c*[His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**2**, GXH-17), which was an agonist with an EC_{50} of 0.8 μM, with partial antagonist activity (IC_{50} of 0.45 μM vs α-MSH). Further modifying position 7 by D-Nal(2′) gave *c*[His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**3**, GXH-21), a more potent agonist analogue with an EC_{50} around 60 nM and no antagonist activity in this assay. In addition, replacement of His^6 with Pro led to *c*[Pro-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**4**, GXH-22), which gave no agonist activity, but instead was an antagonist with an IC_{50} of about 1.5 μM.

In the next step of our design, expansion of the ring size became one strategy because ligands **1** to **4** are all 17-membered ring compounds. Compared with MT-II[27] which has a 23-membered ring, the analogues designed above might be too constrained for the core sequence to attain the proper conformation for maximum binding to the receptor. Hence, a Gly was inserted into the lactam bridge. Consequently, all of the compounds in this series possess 20-membered rings. *c*[Gly-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**5**, GXH-07, Table 1) derived from **1**, showed improved agonist activity with an EC_{50} of 5 μM, while the antagonist activity of **1** was completely lost. *c*[Gly-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**6**, GXH-11) derived from **2**, showed almost the same agonist activity as GXH-17 (**2**, Table 1). However, unlike **2** (GXH-17), GXH-11 had no antagonist activity. Replacement of His^6 of **6** (GXH-11) with a Pro gave *c*[Gly-Pro-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**7**, GXH-12), which did not have any agonist activity. Instead, it showed very weak antagonist activity with an IC_{50} of around 10 μM. Interestingly, replacing the D-Phe of **6** (GXH-11) with a D-Nal(2′) led to *c*[Gly-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**8**, GXH-13) which showed much improved agonist activity (EC_{50} of 60 nM) with no antagonist activity. Further, replacing His^6 of **8** (GXH-13) with a Pro led to *c*[Gly-Pro-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**9**, GXH-14), which loses essentially all agonist activity and becomes a weak antagonist with an IC_{50} of 640 nM (Table 1).

To further evaluate the effect of ring size, we expanded the ring by replacing Gly with other non-α-amino acids by inserting CH_2 units between the amino and the acidic group of Gly. Hence amino acid derivatives (commercially available β-alanine (β-Ala), γ-aminobutyric acid (γ-Abu), and 6-aminohexanoic acid (6-Ahx)) with different lengths of CH_2 units were used to replace Gly. As can be seen from Table 1, *c*[γ-Abu-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**10**, GXH-25B), in which γ-Abu replaced the Gly in **5**, showed slightly improved agonist activity. Further expanding the ring by incorporating γ-Ahx instead of Gly gave *c*[6-Ahx-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**11**, GXH-26) which did not have any agonist or antagonist activity. When Gly was replaced by a β-Ala and Phe by D-Phe to give *c*[β-Ala-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**12**, GXH-28B), the analogue became a potent agonist with an EC_{50} around 30 nM. Further expansion of the ring by incorporating γ-Abu, which has one more CH_2 unit than β-Ala, gave *c*[γ-Abu-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**13**, GXH-29B) which lost 10-fold in potency compared with **12** (GXH-28B). However, when 6-Ahx replaced γ-Abu of GXH-29B, the derivative (**14**, GXH-

Table 2. Bioassay Results of Selected Analogues at Human MC Receptors

peptide nos.	peptide designation nos.	peptide sequence	EC_{50} or IC_{50}[a] (nM) MC1	MC3	MC4
15	GXH-32B	*c*[*β*-Ala-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	75 (SE 82)	3.8 (SE 2.1)	77 (SE 64)
19	GXH-38B	*c*[Gly-Cpg-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	12 (SE 28)	44 (SE 31)	1300 (SE 1200)

[a] EC_{50} for GXH-32B; IC_{50} for GXH-38B.

30B) became more potent than GXH-29B and was as potent as **12** (GXH-28B).

The D-Nal(2′)[7] derivative *c*[Gly-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**8**, GXH-13), in which Gly was replaced by *β*-Ala, gave *c*[*β*-Ala-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**15**, GXH-32B) which is an agonist that is 30-fold more potent than the parent molecule **8** (GXH-13). Further replacement of *β*-Ala with a *γ*-Abu gave *c*[*γ*-Abu-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**16**, GXH-33B), which lost 10-fold of potency, compared with **15**. On the basis of the above results, it can be concluded that the *β*-Ala derivatives, all of which have a 21-membered ring, showed the highest potency among all the derivatives tested. Larger or smaller ring size compounds have weaker bioactivities.

Early studies on the function of imidazole ring of His[6] had revealed that hydrogen bonding from the imidazole group might play a role in the activity of α-MSH and its analogues. For example, Ac-Nle-*c*[Asp-His(1′-Me)-D-Nal(2′)-Arg-Trp-Lys]-NH_2 was a *m*MC5 receptor selective antagonist.[36] Recently, replacement of the His[6] in SHU9119 with a Pro led to Ac-Nle-*c*[Asp-Pro-D-Nal(2′)-Arg-Trp-Lys]-NH_2, which was found to be a potent and selective MC5 receptor agonist.[66] These results demonstrated that position 6 in α-MSH analogues plays a significant role in both receptor selectivities and biological activities. In our studies reported here, we found that incorporation of proline into analogues **6** and **8** to give **7** (GXH-13) and **9** (GXH-14), respectively, gave analogues that are weak antagonists in the *Xenopus* frog skin MC1 receptor assay (Table 1).

When a highly bulky His derivative (3-Bom)His was incorporated into **6** (GXH-11) to give *c*[Gly-His(3-Bom)-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**17**, GXH-35B, Table 1), a weak ($IC_{50} \sim 1$ *μ*M) antagonist with no agonist activity was obtained. This can be compared to its parent compound *c*[Gly-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**6**, GXH-11) which was a pure agonist ($EC_{50} = 510$ nM).

Interestingly, replacement of His[6] in *c*[Gly-His-D-Nal-(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**8**, GXH-13) with cyclopentylglycine (Cpg) led to *c*[Gly-Cpg-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**19**, GXH-38B, Table 1) which lost agonist activity in the *Xenopus* frog skin assay and became a potent antagonist (IC_{50} of 43 nM), making it the most potent antagonist among all the chimeric analogues.

Human MC Receptors Assays. Two of the most potent analogues, one an agonist (**15**, $EC_{50} = 2$ nM), and one an antagonist (**19**, $IC_{50} = 43$ nM) at *Xenopus Laevis* frog MC1 receptor assays, were selected for human MC receptors assays (Table 2). Analogue **15** displayed higher potency at the human MC3 receptor ($EC_{50} = 3.8$ nM) than at other human MC receptors ($EC_{50} = 75$ nM and 77 nM for human MC1 and MC3 receptors, respectively). Analogue **19** (GHX-38B) was an antagonist at all human melanocortin receptors. It was most potent at MC1 receptor antagonist with an $IC_{50} = 12$ nM. It also displayed potent antagonistic activity at the human MC3 receptor ($IC_{50} = 44$ nM), while it is a very weak antagonist ($IC_{50} = 1300$ nM) at the human MC4R. A Schild plot[67] (Figure 1) shows that the compound is an excellent competitive antagonist against α-MSH at human MC1 receptor ($K_i = 53$ nM).

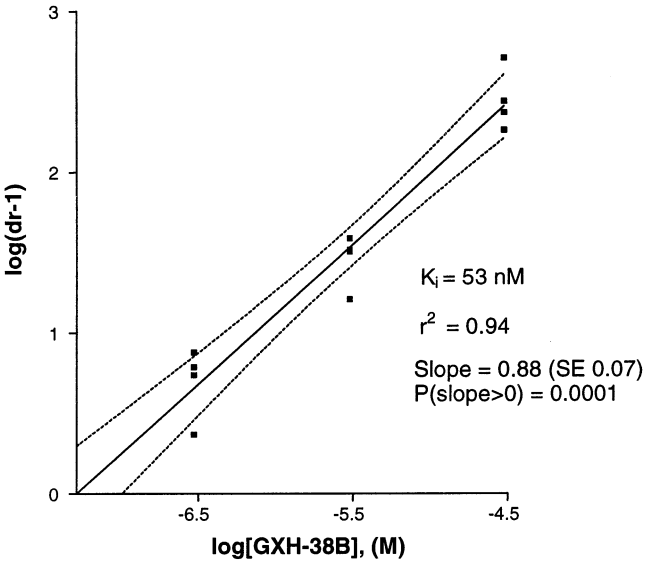


Figure 1. Schild plot for **19** (GXH-38B).

Discussion

Prior to this report, it has been shown that some opioid analogues can interact with the MC1 receptor found in *Xenopus* dermal melanophores.[56,58] Among these analogues were two *μ*-opioid receptor selective antagonists CTAP (H-D-Phe-*c*[Cys-Tyr-*D*Trp-Arg-Thr-Pen]-Thr-NH_2) and CTOP (H-D-Phe-*c*[Cys-Tyr-*D*Trp-Orn-Thr-Pen]-Thr-NH_2).[68,69] Both compounds are similar in structure, both are powerful *μ*-opioid (OP3) receptor antagonists, and both show significant binding affinity toward MC receptors found in *Xenopus* frog skin. However, CTAP and CTOP have completely opposite activities; CTAP is a weak antagonist ($IC_{50} = 3$ *μ*M) with no significant agonist activity, while CTOP is a relatively potent agonist ($EC_{50} = 70$ nM) with no antagonist activity.[56] Other well-known *μ*-opioid receptor ligands, such as DAMGO, a *μ* agonist, had no significant activity, suggesting no simple correlation between opioid activity and binding of the MC1 receptor.

Among the *δ*- (OP1) and *κ*- (OP2) opioid receptor ligands tested for interactions with MC receptors, the Dynorphin A (DynA) series analogues showed interesting results.[58] Full length sequences of DynA(1−17)NH_2 and DynA(1−13)NH_2 were found to possess significant ability to interact with *Xenopus* and human MC receptors, as did similar sequences where the Tyr residue at position 1 had been removed—a residue critical for DynA analogues to have potent *κ*-opioid receptor activity. In

addition, DynA(1−8) and shorter sequences appeared to have virtually no binding at all, including Leu-Enkephalin (i.e., DynA(1−5)). A number of other DynA analogues of different fragments than mentioned above, also were found to have no significant binding activity (A. Burritt, J. M. Quillan and V. J. Hruby, unpublished results), while Dynorphin B was a weak antagonist with an IC_{50} in the micromolar range. Potent δ opioid receptor selective ligands such as, β-endorphin, orphanin-PQ, DTLET, DADLE, DSLET, and most notably DPDPE, a highly selective δ-opioid receptor ligand, did not appear to bind at all to the *Xenopus* frog skin MC1 receptor.

On the basis of these published results, it is difficult to deduce correlations of amino acid sequences that can be used to explain the interactions observed. Hence, much more information about structural correlates involved in receptor interaction is necessary to fully understand how some opioids come to bind to MC receptors.

***Xenopus* Frog MC1 Receptor Bioassay. Ring Size of Chimeric α-MSH Analogues.** Previous studies have revealed that the ring size in cyclic α-MSH analogues has a significant impact on the potency of these analogues.[70−72] For example, Ac-Nle-c[Asp-His-DPhe-Arg-Trp-Lys]α-MSH(4−10)-NH_2 (MT-II) has a 23-membered ring structure with a lactam bridge between Asp^5 and Lys^{10}. When, Lys^{10} (with 4 CH_2 repeating units) was replaced by 2,3-diaminopropoinic acid (Dpr, with only 1 CH_2 repeating unit), the resulting analogue Ac-Nle^4-*c*[Asp^5, D-Phe^7,Dpr^{10}]α-MSH(4−10)-NH_2 has a 20-membered ring, and this new and more restricted analogue lost about 80-fold potency in the *Rana pipiens* frog skin bioassay and also was 15 times less potent in the lizard skin bioassay.[70]

These findings led us to investigate how ring size and orientation of the lactam bridge would affect the bioactivity of the α-MSH analogues at other MCRs, and thus we decided to use the *Xenopus* frog skin MC1 receptor as an initial screen. The chimeric α-MSH ligand *c*[His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**1**, GXH-15, Table 1) has a 17-membered ring as compared with MT-II (which has a 23-membered ring). Compound **1** exhibited no detectable bioactivity (EC_{50} < 1 μM) in the *Xenopus* frog skin assay. When the ring size of GXH-15 was expanded to 20 members by inserting a Gly in front of His^6, the resulting ligand *c*[Gly-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**5**, GXH-07, Table 1) showed weak agonist potency (EC_{50} = 5.2 μM) in the *Xenopus* frog skin assay. This improvement encouraged us to further expand the ring size by two CH_2 units with the insertion of a γ-Abu into GXH-12 (**7**) instead of Gly at the same position. The analogue obtained, *c*[γ-Abu-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**10**, GXH-25B), displayed a similar potency (EC_{50} = 2.7 μM, Table 1) as **5**. Further expansion of ring size by two CH_2 units by substituting γ-Abu with 6-Ahx led to a 24-membered analogue *c*[6-Ahx-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**11**, GXH-26), which lost essentially all bioactivity. Thus when the ring size was 17 to 22, the potency of the corresponding ligands increased, while when the ring size was further expanded to 24, the resulting analogue, **11** had no bioactivity.

Interestingly, *c*[His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**11**, GXH-26, EC_{50} = 0.8 μM), which has a 17-membered ring, and *c*[Gly-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**6**, GXH-11, EC_{50} = 0.5 μM), which has a 20-membered ring are essentially equipotent. Replacing Gly with β-Ala led to *c*[β-Ala-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**12**, GXH-28B) which has a 21-membered ring, led to a dramatic improvement in potency (EC_{50} = 31 nM). Further expansion of the ring size to 22 by replacing β-Ala with γ-Abu gave *c*[γ-Abu-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**13**, GXH-29B) resulted in a 10-fold loss of potency (EC_{50} = 0.3 μM) compared with **12**. On the other hand, when a 6-Ahx residue was inserted to give *c*[6-Ahx-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**14**, GXH-30B). The analogue showed a 10-fold increase in potency (EC_{50} = 23 nM), very similar to **12** (GXH-28B).

The overall behavior in the potency for this series of compounds is likely caused by conformational changes in these ligands since no other functional groups (just the number of CH_2 units) were introduced in **6** (GXH-11), **12** (GXH-28B), **13** (GXH-29B), and **14** (GXH-30B). Interestingly, when D-Nal(2′) was used in position 7 with a 17-membered ring, the analogue *c*[His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**3**, GXH-21) had subnanomolar agonist potency (EC_{50} = 60 nM, Table 1). Insertion of a Gly led to an equipotent ligand, *c*[Gly-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**8**, GXH-13, EC_{50} = 60 nM, Table 1). Additional insertion of one CH_2 unit by replacing Gly with β-Ala led to *c*[β-Ala-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**15**, GXH-32B), which had an EC_{50} of 2 nM. The increase in potency, about 30-fold, from a 20-membered ring to a 21-membered ring was also seen between **6** (GXH-11) and **12** (GXH-28B) where the Gly in **6** was replaced by β-Ala in **12** (GXH-28B). The only difference between these two pairs (**6**, **12** and **8**, **15**) of ligands is D-Phe (**6**, **12**) versus D-Nal(2′) in position 7 (**8**, **15**). A further increase of CH_2 units by 1 led to *c*[γ-Abu-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**16**, GXH-33B) which has a 22-membered ring and was about 10-fold less potent than **15** (Table 1).

In conclusion, the ring size of these chimeric ligands affects the bioactivities of the resulting analogues significantly regardless of the amino acids used at position 7 (either D-Phe or D-Nal(2′)). It appears that analogues with different ring sizes have different conformations in which functional side chain groups of residues 6 to 9 are arranged differently in 3D space.

Position 7 in the Chimeric Analogues. Position 7 of α-MSH has been well studied in the past,[23−25,35,36,72−75] as this position is one of the most critical residues in the core sequence His-Phe-Arg-Trp. Earlier, it was demonstrated that the change from Phe to D-Phe led to more potent, stable, and prolonged acting α-MSH ligands.[23−25,72−75] These improvements were due to an enhancement of a β-turn structure in the core sequence, and the D configuration of phenylalanine made the analogue much less susceptible to enzymatic degradation. Later, the introduction of D-Nal(2′) led to the discovery of the first generation of potent melanocortin antagonists, such as SHU-9119.[35] In the present study, we investigated how changes in these residues would affect the bioassay results.

The 17-membered ring α-MSH derivative, *c*[His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**1**, GXH-15) with a Phe^7, had no detectable bioactivity (EC_{50} > 10 μM) in the *Xenopus* frog skin assay. When Phe was replaced by D-Phe in *c*[His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**2**, GXH-17), the analogue had weak potency with an EC_{50} around 1 μM. Further replacement of $D\text{-}Phe^7$ with a D-Nal(2′) led to *c*[His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**3**, GXH-21) which had increased potency (EC_{50} = 60 nM). Hence, for the 17-membered cyclic chimeric analogues, the potencies increase stepwise when the residue at position 7 changes from Phe to D-Phe, and to D-Nal(2′).

The 20-membered ring structured derivatives with a Gly inserted at the N-terminus to give *c*[Gly-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**5**, GXH-07, Phe at position 7) had a weak potency with an EC_{50} around 5 μM. Replacement of Phe^7 with D-Phe led to *c*[Gly-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**6**, GXH-11), which showed a much better potency in the *Xenopus* frog skin assay (EC_{50} = 0.5 μM). Replacement of $D\text{-}Phe^7$ by D-Nal(2′) led to *c*[Gly-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**8**, GXH-13), which showed a further 9-fold increase in potency (EC_{50} = 60 nM). Thus for the 20-membered ring cyclic chimeric analogues, the potencies also increase stepwise when the residue at position 7 changes from Phe to D-Phe, and to D-Nal(2′). Hence, the pattern of the change in bioactivities for 20-membered ring analogues is similar to that of 17-membered ring counterparts. Interestingly, the insertion of Gly did not change the ligands' bioactivities to any significant extent (see above).

The 22-membered ring structured analogues with a γ-Abu at the N-terminus, *c*[γ-Abu-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**10**, GXH-25B), showed a weak potency (EC_{50} = 2.7 μM), and when Phe was replaced by D-Phe, the activity of *c*[γ-Abu-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**13**, GXH-29B) improved about 9-fold (EC_{50} = 0.30 μM). Replacing D-Phe with a D-Nal(2′) led to *c*[γ-Abu-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**16**, GXH-33B), whose potency further improved more than 14-fold to an EC_{50} of 23 nM. These bioassay results for the 22-membered ring structured chimeric analogues together with the earlier discussed results from 17- and 20-membered ring structured analogues demonstrate that ring size generally is not a critical factor for improving the bioactivity for these analogues. For example, for analogues with a D-Nal(2′) at position 7, **3** (17-membered ring, EC_{50} = 60 nM), **8** (20-membered ring with a Gly insertion, EC_{50} = 60 nM), and **16** (22-membered ring with a γ-Abu insertion, EC_{50} = 23 nM) all have similar potencies.

All these examples clearly showed that the change of phenylalanine's configuration in position 7 from (*S*) to (*R*) led to improved bioactivities of around 10-fold regardless of the ring size (17, 20, and 22). Further replacement of D-Phe with a D-Nal(2′) at position 7 led to another 10-fold increase in bioactivities in all three analogues with different ring sizes presented above. These examples also demonstrate that the ring sizes (17, 20, and 22) do not have any significant impact on the potencies for these specific ligands. However, this does not apply to the analogues with a 21-membered ring as discussed in the previous section.

Position 6 in the Chimeric Deltorphin-II Analogues. Among all the residues of the core sequence of α-MSH, His-Phe-Arg-Trp, His at position 6 is one that is not well studied for its structure−activity relationships in full of α-MSH analogues. Recently, it was found that modification of imidazole ring could lead to *m*MC5 receptor selective antagonists, such as substitution with methyl group at position 1 of imidazole ring,[33] which led to first generation *m*MC5 receptor antagonist, Ac-Nle-*c*[Asp-His(1-Me)-D-Nal(2′)-Arg-Trp-Lys]-NH_2 (WY012), with a modest pA_2 of 7.1.[36] However, similar modifications at position 3′ of the imidazole ring with a methyl group substitution did not change the activity profile in terms of either selectivities or potency. For example, Ac-Nle-*c*[Asp-His(3-Me)-D-Nal(2′)-Arg-Trp-Lys]-NH_2 (WY013) was a potent agonist at the *m*MC5 receptor (EC_{50} = 3.7 nM). Interestingly, replacing His^5 with Trp, the later of which has a bulky indole aromatic side chain, led to a partial agonist.[36] All these results demonstrated that certain substitution at position 6, if properly placed, can lead to changes in the bioactivity profile for the designed ligands.

Most recently, following this hypothesis, it was realized that incorporation of proline in position 6 might help β-turn formation. The α-MSH derivative Ac-Nle-*c*[Asp-Pro-D-Nal(2′)-Arg-Trp-Lys]-NH_2 (PG-901) was a highly selective and potent MC5 receptor agonist.[66] In the current study, we have investigated how modifications at position 6 affect the bioactivity of the new analogues.

For the 17-membered ring *c*[His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**3**, EC_{50} = 60 nM), replacement of His^6 with a Pro led to *c*[Pro-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**4**, GXH-22) which was inactive (Table 1), with weak antagonist activity (IC_{50} = 1.5 μM), changing the bioactivity profile of the 17-membered ring ligand from agonist to antagonist. For the 20-membered ring structure *c*[Gly-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**6**, GXH-11, EC_{50} = 0.5 μM), a similar replacement at position 6 with a Pro led to *c*[Gly-Pro-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**7**, GXH-12), which also was a weak antagonist (IC_{50} = 10 μM) in the presence of 500 pM of α-MSH. In addition, when Pro replaced His^6 in *c*[Gly-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**8**, EC_{50} = 60 nM), the resulting compound *c*[Gly-Pro-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**9**, GXH-14), lost all agonist activity and exhibited modest antagonist activity (IC_{50} = 0.6 μM) in the presence of 500 pM of α-MSH.

Thus, in these analogues, replacement of His^6 with a Pro always led to a loss of agonist activity, and the appearance of some antagonist activity against 500 pM α-MSH. Substitution of the side chain in His^6 was used to further examine the importance of His^6 in this series of analogues. Substitution of 3′ position in the side chain of His^6 with a Bom group (benzyloxymethyl) led to *c*[Gly-His(3-Bom)-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**17**, GXH-35B), which lost agonist activity, and showed only very weak antagonist activity in the *Xenopus* skin assay (IC_{50} = 0.5 μM) in the presence of 500 pM of α-MSH.

In addition to the changes made above for His^6, a further modification of this residue was investigated using (*S*)-cyclopentylglycine (Cpg). Cpg has a side chain bearing a cyclopentyl ring which has a similar size as

the imidazole ring, but the cyclopentyl ring in Cpg is saturated while the imidazole ring is hetero aromatic, cyclopentyl group in Cpg is neutral while the imidazole group is a Lewis acid or Lewis base, and the cyclopentyl group in Cpg is not a hydrogen bond donor or acceptor while the imidazole group is a strong hydrogen-bond donor and acceptor, and finally Cpg is much more constrained in χ-space compared with His in that the former lacks of a free-rotating CH_2 group in its β-position, while the cyclopentyl group directly connects with the α-carbon. We envisioned that these changes would lead to Cpg-containing analogues which would have different properties than the His-containing counterparts.

c[Gly-Cpg-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**18**, GXH-36B), where Cpg replaced His^6 in **6** was inactive (Table 1), but showed very weak antagonist activity ($IC_{50} = \sim 1\ \mu M$), half of that for **2**, in the presence of 500 pM of α-MSH. *c*[Gly-Cpg-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**19**, GXH-38B), where Cpg replaced His^6 in **5** which has a D-Nal(2′) at position 7 instead of D-Phe in **6** (GXH-11), lost more than 15-fold in agonist activity ($EC_{50} \geq 1\ \mu M$). However, it displayed good competitive inhibition activity ($IC_{50} = 43$ nM) against 500 pM of α-MSH. Thus **19** is the most potent antagonist among all the chimeric analogues tested.

Human MC Receptors Bioassay. To determine how well these new chimeric analogues could bind to human MC receptors, we chose the most potent analogue **15** ($EC_{50} = 2$ nM, Table 1) in the *Xenopus* frog MC receptor assays for human MC receptors assay. The results (Table 2) are very interesting as this ligand binds to the *h*MC1 and *h*MC4 receptors with almost equal potency, $EC_{50} = 75$ and 77 nM for *h*MC1 and *h*MC4 receptors, respectively. However, it binds at *h*MC3R with an EC_{50} of 3.8 nM, almost the same potency at frog MC1 receptor assay. Thus **15** is selective (about 20-fold) for the *h*MC3 receptor vs the *h*MC1 and *h*MC4 receptors. This opens a new lead structure for the design of potent and selective *h*MC3 receptor ligands.

Prior to our research, there has been no potent human MC1 receptor antagonist reported. Our primary focus in the current research using this chimeric strategy is trying to fill the gap of finding a potent human MC1 receptor antagonist. After screening the designed ligands at *Xenopus* frog assay, we decided to select ligand **19** (GXH-38B), the most potent antagonist with an EC_{50} of 43 nM, for human MC receptor assays. Ligand **19** displayed some potency at the *h*MC4 receptor with an EC_{50} of 1300 nM. However, it displayed subnanomolar range EC_{50}s at *h*MC1R (12 nM) and *h*MC3R (44 nM). Subsequent Schild analysis (Figure 1) revealed that this ligand has a subnamolar K_i value of 53 nM.

In comparing the structures of the agonist **15** (GXH-32B) and the antagonist **19** (GXH-38B), the difference lies in the N-terminal residues with β-Ala-His for GXH-32B and Gly-Cpg for GXH-38B. His is flexible, hydrophilic, and also has the aromatic imidazole ring which is a Lewis acid and a Lewis base and can act as a hydrogen-bond doner or acceptor, while Cpg is very constrained in its side chain, very hydrophobic without any hydrogen-bonding character, and aliphatic without any aromaticity. This difference plays a significant role in binding at human MC receptors as well as at the *Xenopus* frog MC receptor. Further research is underway to find more potent and selective *h*MC1 receptor antagonists.

Conclusions. The hydrophobic tail of Deltorphin-II (Glu-Val-Val-Gly-NH_2) and the core sequence of α-MSH, His-D-Phe-Arg-Trp, were incorporated into chimeric analogues with the general sequence *c*[Xxx-Yyy-Zzz-Arg-Trp-Glu]-Val-Val-Gly-NH_2 {Xxx = nothing, Gly, β-Ala, γ-Abu, 6-Ahx; Yyy = His, His(3-Bom), (*S*)-cyclopentylglycine (Cpg); Zzz = Phe, D-Phe; D-Nal(2′)}. All these ligands were bioassayed at the *Xenopus* frog MC1 receptor using a fast high-throughput screening method. The agonist activities for this series ranged from no activity to nM (EC_{50}) activity. For example, *c*[Gly-Cpg-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**18**) displayed no binding at *Xenopus* frog MC1 receptor, whereas *c*[β-Ala-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (GXH-32B, **15**) exhibited potent agonist bioactivity with an EC_{50} of 2 nM. By modification of His^6 with substitutions of the side chain imidazole ring, or replacement His^6 with a proline or Cpg, we obtained weak antagonists in the *Xenopus* frog MC1 receptor assay. The Cpg-containing analogue *c*[Gly-Cpg-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2 (**19**) was the most potent antagonist ($IC_{50} = 43$ nM) among these analogues. This ligand is also the first potent *h*MC1 receptor antagonist ($EC_{50} = 12$ nM, $K_i = 53$ nM) discovered.

These results provide strong additional evidence for our hypothesis that ligand scaffolds for different G-protein-coupled receptors (GPCRs) can be used to design ligands for other GPCRs. These results will help direct us to design more selective and potent ligands at the different human melanocortin receptors.

Experimental Section

Materials. All peptides designed in this investigation were prepared by solid-phase synthesis methods with N^{α}-Fmoc/*tert*-butyl chemistry. N^{α}-Fmoc protected amino acids and Rink-amide resin were purchased from Advanced ChemTech (Louisville, KY), Bachem Science (King of Prussia, PA), American Peptide Company (Sunnyvale, CA) and Chiral Technologies (Great Britain). Other chemicals and solvents were purchased from the following sources: trifluoroacetic acid (TFA; Halocarbon Products, NJ); *N*,*N*-diisopropylethylamine (DIEA), anisole, 1,2-ethanedithiol, dimethyl sulfide, piperidine, acetic anhydride, tetrakis(triphenylphosphine)palladium(0), and phenylsilane (Aldrich, Milwaukee, WI); dichloromethane (DCM); *N*,*N*-dimethylforamide (DMF; Fischer Scientific, Pittsburgh, PA); 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt; Quantum Biotechnologies, Montreal, Canada)); HPLC-quality acetonitrile (Fischer Scientific, Pittsburgh, PA). All amino acids were of the L-configuration unless otherwise stated. The purity of the peptides was checked by analytical reverse-phase high-pressure liquid chromatography (HPLC) using VYDAC 218 TBP-16 column (4.6 × 250 mm) at 230, 254, and 280 nm (Table 3), and the structures of the purified peptides were characterized by electrospray mass spectrometry (Finnigan LCQ Ion Trap Mass Spectrometer; Table 3). The purification of the peptides was achieved using a Ranin HPXL HPLC instrument with UV-D detector for preparative high-pressure liquid chromatography on a C_{18}-bonded silica columns (VYDAC, 10 × 250 mm, 300 Å, semipreparative columns, Cat. No. 218TP1010). The peptides were eluted with a linear acetonitrile in 0.1% aqueous TFA gradient at a flow rate of 10.0 mL/min. The separations were monitored at 230 and 280 nm. The analytical data is summarized in Table 3.

Peptide Synthesis. General Protocol. All peptides were synthesized in a stepwise fashion via the solid-phase method, using 1 or 2% cross-linked Rink amide resin (0.4−0.7 meq/g,

Table 3. Analytical Data for α-MSH Analogues[a]

peptide nos.	peptide designation nos.	peptide sequence	HPLC k'	MF	MS $[M+H]^+$ calcd	MS $[M+H]^+$ found	HR-MS $[M+H]^+$ calcd	HR-MS $[M+H]^+$ found
1	GXH-15	*c*[His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.27	$C_{49}H_{67}N_{15}O_9$	1011.15	1010.5	1010.5246	1010.5543
2	GXH-17	*c*[His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	1.94	$C_{49}H_{67}N_{15}O_9$	1011.15	1010.5	1010.5246	1010.5240
3	GXH-21	*c*[His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.44	$C_{53}H_{69}N_{15}O_9$	1060.21	1060.5	1060.5403	1010.5394
4	GXH-22	*c*[Pro-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	3.38	$C_{52}H_{69}N_{13}O_9$	1021.18	1020.5	1020.5341	1020.5328
5	GXH-07	*C*[Gly-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.33	$C_{51}H_{70}N_{16}O_{10}$	1068.20	1067.5	1067.5461	1067.5453
6	GXH-11	*c*[Gly-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.07	$C_{51}H_{70}N_{16}O_{10}$	1068.20	1067.5	1067.5461	1067.5455
7	GXH-12	*c*[Gly-Pro-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	3.16	$C_{50}H_{70}N_{14}O_{10}$	1028.18	1027.6	1027.5399	1027.5388
8	GXH-13	*c*[Gly-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.55	$C_{55}H_{72}N_{16}O_{10}$	1118.26	1117.6	1117.5617	1117.5602
9	GXH-14	*c*[Gly-Pro-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	3.78	$C_{54}H_{72}N_{14}O_{10}$	1078.23	1077.6	1077.5556	1077.5543
10	GXH-25B	*c*[γ-Abu-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.40	$C_{53}H_{74}N_{16}O_{10}$	1096.26	1095.6	1095.5852	1095.5836
11	GXH-26	*c*[6-Ahx-His-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.55	$C_{54}H_{76}N_{16}O_{10}$	1124.31	1123.9	1123.6165	1123.6149
12	GXH-28B	*c*[β-Ala-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.33	$C_{52}H_{72}N_{16}O_{10}$	1082.23	1081.9	1081.5696	1081.5685
13	GXH-29B	*c*[γ-Abu-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.12	$C_{53}H_{74}N_{16}O_{10}$	1096.26	1095.9	1095.5852	1095.5862
14	GXH-30B	*c*[6-Ahx-His-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.27	$C_{54}H_{76}N_{16}O_{10}$	1124.31	1123.9	1123.6165	1123.6149
15	GXH-32B	*c*[β-Ala-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.81	$C_{56}H_{74}N_{16}O_{10}$	1132.29	1131.8	1131.5852	1131.5836
16	GXH-33B	*c*[γ-Abu-His-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	2.59	$C_{57}H_{76}N_{16}O_{10}$	1146.32	1145.9	1145.6009	1145.5996
17	GXH-35B	*c*[Gly-His(3-Bom)-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	3.11	$C_{59}H_{78}N_{16}O_{11}$	1188.55	1187.9	1187.6114	1187.6134
18	GXH-36B	*c*[Gly-Cpg-D-Phe-Arg-Trp-Glu]-Val-Val-Gly-NH_2	3.85	$C_{52}H_{74}N_{14}O_{10}$	1056.23	1055.8	1055.5790	1055.5782
19	GXH-38B	*c*[Gly-Cpg-D-Nal(2′)-Arg-Trp-Glu]-Val-Val-Gly-NH_2	4.33	$C_{56}H_{76}N_{14}O_{10}$	1106.29	1105.8	1105.5947	1105.5942

[a] Purity of each peptide was >98% based on RP-HPLC.

Advance ChemTech, Louisville, KY). The protected amino acids incorporated in positions other than the amino terminus were purchased from commercial sources (Bachem California, Torrance, CA; Advanced ChemTech, Louisville, KY; Peptech-Corp, Cambridge, MA; Bachem Science, King of Prussia, PA; Chiral Technology, Great Britain). The side chains' protecting groups[75] were Arg(N^{ϵ}-Pbg), His(N^{im}-Trt), Trp(N^{im}-Boc), Glu-(allyl). Each N^{α}-Fmoc-protected amino acid was coupled to this resin using a 3-fold excess of the protected amino acids. The coupling reagents were *N*-hydroxybenzotrizaole (HOBt) and HBTU, and the coupling was monitored by the Kaiser test.[77] Each natural amino acid was coupled in this fashion, while nonnatural amino acids were coupled using a 1.5−2-fold excess of the protected amino acid, with HBTU and HOBt (1 equiv to amino acid used) and diisopropylethylamine (DIPEA, DIEA) as the coupling reagents. The detailed protocol for peptide synthesis and side chain lactam bridge cyclization using N^{α}-Fmoc chemistry used standard methods in our laboratory.[65] The N^{α}-Fmoc protecting group in the final Fmoc-peptide resin was removed using standard procedures. A mixture of TFA, triethylsilane (TES), and water (v/v/v = 87/7/6) was used (3 h at room temperature) to cleave the peptide from the peptide-resin and remove all of the side chain protecting groups. The crude peptide mixture was filtered from the solid support, which was washed with acetic acid (2 ×). Anhydrous ether was added to the resulting solution mixture. The peptide precipitated as a white solid. After centrifugation, the solution was decanted and discarded, and the solid was thoroughly washed with anhydrous ether (2 ×). The resulting crude peptide was dried, and purified by HPLC using a preparative C-18 column (reverse phase), with a gradient, 90−10% B in 40 min [component A: acetonitrile (Biograde, Fisher, Pittsburgh, PA); component B: deionized water containing 0.1% (v/v) trifluoroacetic acid (TFA)]. The absorbance of the eluents was detected by a Rainin UVD detector, monitored at 230 and 280 nm. The fractions collected were checked for purity by analytical reverse HPLC using a Hewlett-Packard HPLC 1090II with a diode array detector monitored at various wavelengths, typically at 230, 254, and 280 nm. The pure fractions were pooled and lyophilized. Yields varied from 5% to 50%. Neither syntheses nor separations were optimized.

Peptides synthesized were characterized (Table 3) by RP-HPLC, low and high-resolution mass spectroscopy, and some by NMR spectroscopy (vide infra). The peptides all were demonstrated to be pure (purity > 98%) as determined by RP-HPLC chromatograms. The low- and high-resolution mass spectral results all agreed with the calculated values. For peptides checked by NMR, all amino acids in the designed peptide analogues were present in the corresponding TOCSY spectra (see Supporting Information).

***Xenopus* Frog Skin Bioassays.** Test compounds were assayed for melanotropic activity using a cultured embryonic *Xenopus* melanophores essentially as described in Quillan and Sadee.[56,58] Briefly, melanophore cells, grown until confluent in Falcon 96-well culture plates, were treated with increasing concentrations of test compound 1 pM to 100 μM in the presence or absence of melatonin. (Compounds were tested over the range of concentrations from 10^{-9} M to 10^{-4} M. Only compounds exhibiting activity at concentrations below 10^{-6} M were selected for further testing using concentrations below 10^{-9} M.) Agonist activity was monitored by measuring transmittance at 485 nm from cells that were aggregated by application of melatonin (10 nM) 90 min prior to addition of test compound, and antagonist activity was monitored from cell layers that had dispersed pigment in the absence of melatonin[56] at the same wavelength. (Cells from both the melatonin treated and nontreated groups were exposed to ordinary room light for at least 90 min prior to addition of test compounds which disperses pigment in the nonmelatonin treated group.) Melanotropic selectivity was confirmed using known MC agoinists and antagonists and performing Schild regression analysis.

Human MC Receptor Bioassays. Test compounds displaying melanotropic activity in the *Xenopus* melanophore bioassay were then selected for further testing using recombinant human melanocortin receptors MC1, MC3, and MC4, stably expressed in human embryonic kidney (HEK-293). MC receptor activity was monitored in HEK cells using calcium measurements essentially as described by Lin et al.[78] Briefly, HEK-293 cells were maintained in modified Eagle's medium (DMEM/H-16/F-12) with 10% fetal calf serum (FBS), 100 U/mL penicillin "G" and 100 μg/mL streptomycin (P/S; Gemini) and loaded with Oregon Green 488 BAPTA-1/AM (Molecular Probes). Fluorescence was measured from 96-well plates using a FLUOstar fluorometer (BMG LabTechnologies, Durham, NC), in the presence or absence of test ligand (as indicated), upon injection of agonist into wells containing 150 μL of cells (∼200000 cell/mL). Agonist test ligands were also examined for their ability to directly evoke calcium currents by injecting them directly onto nonpretreated cells. Fluorescence intensities generated with test compounds were compared to cells injected with buffer alone.

Gain was calibrated at 80% of resting baseline intensity and instrument linearity, precision and cross-talk were calibrated over the range of measured fluorescence intensities using the salt form of Oregon Green 488 BAPTA-1 dissolved in Krebs-

HEPES buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 11.7 mM D-Glucose, 1.3 mM $CaCl_2$, 10 mM HEPES, pH 7.4). Intracellular calcium concentration ($[Ca^{2+}]_i$) was also calibrated by measuring fluorescence intensities before and after permeabilizing cells with 1% Triton X-100 to release all the dye (F_{max}) and subsequently chelating with 10 mM EGTA (F_{min}). Calcium concentrations were calculated using the following equation: $[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F_{min})$.

Concentration−response curves and standard errors were analyzed by fitting experimental observations to a logistic equation, defined by DeLean et al.,[79] using SigmaPlot curve fitting software (SPSS Inc., Chicago, IL).

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Supporting Information Available: ^{1}H NMR spectral assignments and TOCSY spectra of selected peptides (**1**, **3**, **5**, **8**, **15**, **19**). This material is available free of charge via the Internet at http://pubs.acs.org.

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