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Design of MC1R Selective γ-MSH Analogues with Canonical Amino Acids Leads to Potency and Pigmentation

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Abstract

Melanoma is a lethal form of skin cancer. Skin pigmentation, which is regulated by the melanocortin 1 receptor (MC1R), is an effective protection against melanoma. However, the endogenous MC1R agonists lack selectivity for the MC1R and thus can have side effects. The use of noncanonical amino acids in previous MC1R ligand development raises safety concerns. Here we report the development of the first potent and selective hMC1R agonist with only canonical amino acids. Using γ-MSH as a template, we developed a peptide, [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 (compound **5**), which is 16-fold selective for the hMC1R (EC_{50} = 4.5 nM) versus other melanocortin receptors. Conformational studies revealed a constrained conformation for this linear peptide. Molecular docking demonstrated a hydrophobic binding pocket for the melanocortin 1 receptor. In vivo pigmentation study shows high potency and short duration. [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 is ideal for inducing short-term skin pigmentation without sun for melanoma prevention.

Graphical abstract

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[†]**Author Contributions** Y.Z. and S.M.H. contributed equally. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. M.C. designed the experiments. M.C. performed the conformational study and in vivo study. Y.Z. performed the bioassays. S.M.H., J.R.S., and V.J.H. synthesized the compounds. I.Z. performed the docking studies. Y.Z., M.C., S.M.H., and I.Z. wrote the manuscript. V.J.H. contributed to the writing.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.7b01295. Physiochemical and spectral data (^{1}H NMR, mass spectrometry), HPLC chromatograms, hMC1R binding site prediction, and overall conformation of compound **5** bound to hMC1R (PDF)

Molecular formula strings and some data (CSV)

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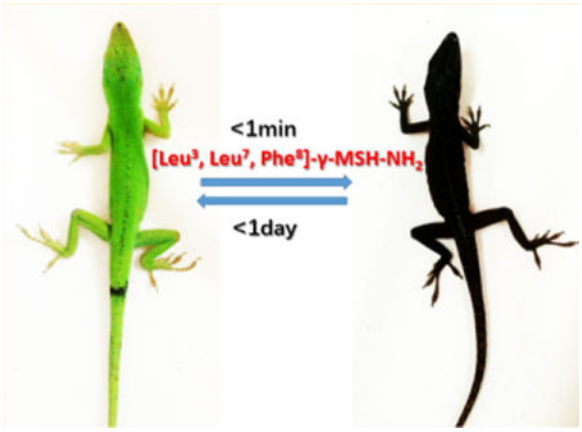
Notes

The authors declare no competing financial interest.

ADDITIONAL NOTE

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.*[40]



INTRODUCTION

Melanoma is the most dangerous form of skin cancer. In 2016, there is an estimated 73 870 new cases of melanoma, with an estimated 10 130 deaths in the U.S. alone.[1] Exposure to UV radiation is the primary risk factor for melanoma.[2] Skin pigmentation, on the other hand, is an effective protection against melanoma. Melanocytes can produce melanin, which is able to dissipate over 99.9% of UV radiation.[3] The strong correlation between skin pigmentation and melanoma risk is also evidenced by the fact that in the U.S., Caucasians have 20–30 times higher chances of getting melanoma than Asians and Blacks.[4] Current efforts seek to prevent UV damage to human skin, which in many cases leads to melanoma and other skin cancers. As a result, inducing skin pigmentation without UV exposure is considered an effective way to prevent UV induced melanoma.

Skin pigmentation is regulated by the melanocortin 1 receptor (MC1R) on melanocytes.[5] Activation of MC1R by melanocyte stimulating hormones (MSH) leads to cyclic adenosine monophosphate (cAMP) production, which eventually leads to transcription of multiple pigment synthesis genes and melanin production.[6–22] The natural MSHs consist of three peptides: α-melanocyte-stimulating hormone (α-MSH), β-melanocyte-stimulating hormone (β-MSH), and γ-melanocyte-stimulating hormone (γ-MSH). All MSHs share the same pharmacophore of His-Phe-Arg-Trp. The MSHs not only activate MC1R but also are able to activate the melanocortin 3 receptor (MC3R), melanocortin 4 receptor (MC4R), and melanocortin 5 receptor (MC5R), which regulate distinct physiological processes such as feeding behavior, energy homeostasis, sexual function, immune responses, and sebaceous gland secretion.[23] Thus, improving selectivity to MC1R is of critical importance for MSHs to trigger skin pigmentation for melanoma prevention without interfering with other physiological functions.

The major challenge is that sunscreen lotion is viewed as both a cosmetic and a drug in the United States and thus must follow regulations from the U.S. Food and Drug Administration (FDA).[24] Since 2014, the α-MSH analogue [Nle^4, D-Phe^7]α-MSH (NDP-α-MSH, brand name Scenesse) has been shown to effectively induce skin pigmentation[25] and has been approved in Europe for treating erythropoietic protoporphyria (EPP). However, the FDA approval for its use in the U.S. is still pending. Two major aspects on NDP-α-MSH can be

improved to pose less health concerns and better fit FDA regulations. One is that NDP-α-MSH is a universal agonist for all melanocortin receptors. The other is that it uses noncanonical amino acids that may not be metabolized the same way as canonical amino acids. Our goal is to develop MC1R selective peptides with canonical amino acids so that it will be more safe and easy degraded into natural building block amino acids in the body.

Design of Selective MC1R γ-MSH Analogues

Our earlier research led to a potent drug NDP-α-MSH (MT-I) or "afamelanotide" for the treatment of congenital erythropoietic porphyria in Europe and skin color disorders in Australia. However, MT-I does not have high selectivity for any one of the melanocortin receptor subtypes, which can lead to unexpected side effects such as headache and nausea.[25] One of our previous studies of a γ-MSH analogue (compound **1**, Table 1) had improved potency and selectivity for the MC1R. Efforts have been made to move compound **1** to be a melanoma prevention agent, but the process of drug development has been very slow due to the un-natural amino acids that are involved.

This motivated us to design a selective MC1R ligand with only canonical amino acids starting from this γ-MSH analogue. We introduced modifications on five different sites of γ-MSH: (1) Introducing the C-terminal amide group to γ-MSH and its analogues was shown to increase their binding affinities to all melanocortin receptor subtypes but MC3R.[26] (2) To keep the similarity and stability of compound **1**, we substitute Nle^3 with Leu^3 since natural γ-MSH has Met^3 which is easily oxidized. (3) His^5 was substituted to proline, which was previously shown to increase potency and selectivity to MC1R.[26] (4) Trp^8 was substituted to phenylalanine, which was also shown to improve MC1R selectivity.[27] (5) Most importantly, our chimeric receptor studies demonstrated that the electrostatic interaction, Arg(L)-Asp(R), between the Arg^8 of the NDP-α-MSH and the Asp122, Asp126 of the hMC4R is of critical importance to achieve binding and receptor activation, as Asp126Asn mutation on the MC4R caused more than a 400-fold increase in the EC_{50} value.[28] Similarly, a key interaction between the Arg^8 of NDP-α-MSH and the Asp154 as well as the Asp158 of the MC3R is necessary, as Asp158Ala mutation on MC3R caused more than 350-fold increase for the EC_{50} value.[29] In contrast, mutation of either Asp117 or Asp121 to alanine on MC1R only had around a 10-fold influence on the IC_{50} and EC_{50} of NDP-α-MSH, suggesting a smaller role of ionic interactions between MC1R and its agonists.[30] Finally, our latest docking studies of hMC1R with selective hMC1R ligand revealed that there is a very hydrophobic binding pocket for the hMC1R. This suggested that increasing hydrophobicity of the hMC1R ligand can improve the selectivity (data in the process of publication). Therefore, our hypothesis is switching the arginine in the γ-MSH analogue to a neutrally charged amino acid, in particular leucine should reduce binding toward the hMC3R and the hMC4R. We envision that enhanced selectivity toward the MC1R can be reached with reduced electrostatic interaction between the Arg(L)-Asp(R) of the γ-MSH analogues and the respective aspartic acids on the MC3R and MC4R receptors. Herein, a series of Leu^3, Pro^5, Leu^7, Phe^8 γ- MSH-NH_2 analogues are designed and synthesized to test these hypotheses (Table 1).

RESULTS

Bioassay Results

Competitive binding assays with [^{125}I]NDP-α-MSH and adenylate cyclase assays were performed with HEK293 cells stably expressing human MC1R (hMC1R), human MC3R (hMC3R), human MC4R (hMC4R), and human MC5R (hMC5R) on the γ-MSH analogues (Table 1, Table 2). γ-MSH (compound **11**) is a universal agonist to melanocortin receptors with 1.8-fold selectivity to hMC1R (EC_{50} = 300 nM). By addition of the C-terminal amide group to γ-MSH, compound **12** loses its selectivity to hMC1R, as the potency on hMC1R (EC_{50} = 70 nM) is in the same range as the potency on hMC4R (EC_{50} = 65 nM). However, the potency to hMC1R is enhanced 3-fold. Compounds **9** and **10** with methionine substituted by leucine both have enhanced potency and selectivity to the hMC1R. Compound **9** has a 10-fold increase in potency (EC_{50}= 31 nM) to the hMC1R compared to γ-MSH with 13-fold selectivity. Compound **10** has better potency and selectivity with EC_{50} of 8 nM on the hMC1R and a selectivity of over 22-fold. The methionine to leucine substitution also caused compounds **9** and **10** to have less binding efficiency and no activation on the hMC5R, which is consistent with the D-amino acid scan showing that Met^3 in γ-MSH is important for hMC5R activation.[31] Thus, we focused our further design using Leu^3 γ-MSH-NH_2 (compound **10**) as a template.

To improve selectivity on the hMC1R, we did modifications on the melanotropin pharmacophore His^5-Phe^6-Arg^7-Trp^8. Peptides were designed with a combination of mutations on His^5, Arg^7, and Trp^8. As expected, compound **2** with the proline substitution had improved potency, but it lost all selectivity as it activates hMC1R (EC_{50} = 4.6 nM) and hMC3R (EC_{50} = 5.0 nM) with almost the same potency. The leucine substitution on compound **4** did affect its binding to hMC3R and hMC4R, as they only showed weak binding and no activity on these receptors. However, the potency to hMC1R (EC_{50} = 604 nM) is also decreased by 76-fold. A double replacement of His^5 to Pro^5 and Arg^7 to Leu^7 (compound **6**) did not show synergistic effects, as compound **6** lost all activities for any melanocortin receptor. The loss of potency is also seen in the triple replacements of His^5 to Pro^5, Arg^7 to Leu^7, and Trp^8 to Phe^8 (compound **7**). The replacements of His^5 to Pro^5 and Trp^6 to Phe^6 (compound **3**) also showed loss of activities at all melanocortin receptors. The purpose of our design was achieved with a double replacement of Arg^7 to Leu^7 and Trp^8 to Phe^8 (compound **5**, [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2). Compound **5** ([Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2) has 1.8-fold enhanced potency for the hMC1R (EC_{50} = 4.5 nM) than the template compound (compound **10**). The selectivity to hMC1R is over 16-fold. Furthermore, compound **5** ([Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2) has only enhanced potency for the hMC1R (EC_{50}= 4.5 nM) compared with the template compound (compound **10**). The selectivity to hMC1R is over 16-fold. Furthermore, compound **5** ([Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2) is only a partial agonist with 54% maximal activation on the hMC4R. The template compound (compound **10**), on the other hand, is a full agonist on the hMC4R. These results suggest that [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 is an ideal peptide with strong potency and selectivity for the hMC1R.

Human Serum Stability—To examine the stability of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$ in comparison to γ-MSH and NDP-MSH, serum stability assays were performed. The endogenous ligand γ-MSH has the shortest half-life of around 5 min. Compared to γ-MSH, compound **5** has an improved serum stability of 17.5 min. The elongated half-life is possibly due to the replacement of methionine on the γ-MSH template. NDP-MSH, which contains D-Phe, has the longest half-life in human serum of around 30 min.

In Vivo Study Results—Pigmentation studies of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$ in the *Anolis carolinensis* show it is a superpotent agonist in vivo. The black color appeared in 1 min after injection (ip, 2 μg/g) (Figure 1b). This is exactly the same response of injection of the same dose of NDP-α-MSH (Figure 1a). In comparison, intraperitoneal injection of the vehicle did not produce any pigmentation effect (data not shown). The green color was able to resume in less than 24 h after [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$ injection but not for the NDP-α-MSH. It took 2 weeks to resume the green color for the NDP-α-MSH (Figure 1a). The much longer half-life of the NDP-α-MSH in vivo is probably due to two un-natural amino acids, Nle and D-Phe, which are not recognized by proteases in vivo, thus resulting in biological stability. However, [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$ is composed of all of natural amino acids. This indicates that [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$ will be ideal to be used in a skin care product to induce skin pigmentation and protect against melanoma during sun exposure. The natural color can be resumed in 1 day with a lower dosage. Furthermore, [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$ is highly selective for hMC1R and therefore avoids possible side effects due to activation of the other hMCRs subtypes.

Conformational Study of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$—The lowest energy conformation search via MacroModel (Schrodinger) revealed a very constrained sphere-like structure for the [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$ (Figure 2a). The major force for the constraint is hydrophobicity due to the presence of dominant hydrophobic amino acids in this peptide. The Ramachandran plot shows that all of the amino acids are in the region of a 3_{10} helix (Figure 2b) except for the Gly ^{4}, Phe^6, and Asp^9. These three amino acids are in the turn region.

Molecular Docking Studies—The initial hMC1R structure applied for this study is from previous published work from the Mosberg lab.[32] First we searched for a potential binding site with Maestro SiteMap and identified the one with the highest druggability score of 1.06 (Figure 3). We then performed a flexible docking simulation with Maestro Glide targeting that pocket.

Molecule docking revealed a very hydrophobic binding pocket for the hMC1R. A docking score of −11.32 kcal/mol was achieved, and key interactions between [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$ and the hMC1R were identified (Figure 4). [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$ showed an antiparallel β-sheet conformation with the modified pharmacophore, His^5-Phe^6-Leu^7-Phe^8, at the turn region (Figure 4, Table 3). His^5 and Phe^6 interact with the transmembrane segment 6 (TM6) of the hMC1R. His^5 is stabilized by π–π stacking interactions with Thr262 and His260 inside the hMC1R. Phe^6 has π–π stacking interactions with both Trp254 and Phe257. Both His260 and Phe257 have been shown by previous mutagenesis studies to be important for melanocortin receptor activation.[28,30] Phe^8 interacts

with Cys125 on the TM3 domain. Other residues on [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 interact with the TM1 and TM3. The relative movement on the TM6 was shown to be critical for G protein coupled receptor activation.[33] Our results indicated that the pharmacophore of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 interacts with TM6 to generate its movement while other residues on [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 anchor to other transmembrane segments on hMC1R. It has to be noted that this peptide maintains all the important interactions as reported from previous studies. The key for its high affinity and selectivity lies in the fact that it introduces new types of interactions. It also has many hydrogen bond interactions, indicative of stronger binding.

We also emphasize that the evaluation of the binding site where our ligand was bound led to an increased druggability score of 1.21, suggesting that the peptide targeted a highly druggable part of the binding site.

Furthermore, we tested compound **1** for comparison purposes, and after having it docked inside the receptor cavity and analyzing its interactions, we note that compound **1** has a slightly higher and therefore worse docking score (−11.08 kcal/ mol) compared to [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2. It also presents less interactions with the receptor, although most of them are hydrogen bonds as shown in Table 4. Surprisingly, only the pharmacophore of compound **1** (His-D-NaI(2′)-Arg-Trp) interacts with hMC1R (Figure 5), whereas in [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 the majority of its amino acids are participating in interactions. In [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 we see more different types of interactions which can partially explain its enhanced selectivity toward the hMC1R.

DISCUSSION AND CONCLUSIONS

Seeking effective treatments of melanoma is very hot in the current era of science and technology. However, prevention of melanoma has been neglected. We are providing a very useful natural AA made peptide here, and it will be more applicable and beneficial for most people. In this study, we first successfully developed a natural aa made peptide, [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2, which is a potent selective hMC1R agonist. The composition of canonical amino acids ensures that the peptide can be easily degraded into natural building block amino acids. The use of only natural amino acids versus unnatural can alleviate most safety concerns. The high selectivity of the [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 for the hMC1R and shorter half-life provide a safer and reduced-side-effect agent. As peptides have been widely used in cosmeceuticals as active ingredients and can be applied transdermally,[34] [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 is suitable as a safe skin care product for the prevention of melanoma skin cancer.

Compared to our previously developed peptide (compound **1**), [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 seems to have less selectivity to hMC1R in terms of EC_{50}. However, compound **1** is also an antagonist at the hMC3R, hMC4R, and hMC5R as suggested by its strong binding affinity to those MCRs. The binding affinity of compound **1** to the hMC1R (IC_{50} = 0.3 nM) is only 12-fold stronger than hMC5R (IC_{50} = 3.5 nM). As hMC5R regulates sebum production in the skin,[35] compound **1** may cause hMC5R antagonism and decreased production of sebum. On the other hand, [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 does not have

detectable binding to hMC5R in our biological assays. Binding of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 to the hMC3R and hMC4R is also weak as evidenced by high IC_{50} and low % binding. More importantly, MC4R is exclusively expressed in the central nervous system.[36] Long linear peptides such as NDP-α-MSH cannot pass through the blood–brain barrier to reach the brain.[37] With similar length and sequence as NDP-α-MSH, compound **5** is unlikely to reach the brain and interfere with MC4R's function. As a result, compound **5** is selective to hMC1R and less likely to interfere with physiological processes regulated by other MCRs.

The serum stability assay showed a half-life of 17.5 min for [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 in human serum. It is worth noting that the skin pigmentation effect lasts longer than the peptide's half-life in serum. Even though the half-life of NDP-α-MSH in human serum is 30 min, the European standard is to have NDP-α-MSH in a controlled-release implant and subcutaneously administrated to EPP patients once every 2 months.[38] It was further confirmed with our in vivo assay that the pigmentation effect would last for less than 24 h. It is worth mentioning that even though pigmentation effect in lizards is mostly regulated by melanosome dispersion rather than melanogenesis, the melanosome dispersion process in coldblooded animals is also regulated by the MC1R/cAMP pathway.[39] [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 is expected to effectively induce short period skin pigmentation in human and thus can be used in skin care products for short-term melanoma prevention.

In our in silico study, we discovered that hMC1R has a very hydrophobic binding pocket. The modified pharmacophore of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2, His-Phe-Leu-Phe, has enhanced hydrophobicity compared to the natural MSHs pharmacophore (His-Phe-Arg-Phe) which contributes to hMC1R selectivity. Computational chemistry combined with chimeric receptors studies verifies our hypothesis that enhanced hydrophobicity of the MSHs pharmacophore can increase the hMC1R selectivity. The newly designed hMC1R selective agonist [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 fits perfectly within the hydrophobic binding pocket of hMC1R and provides new insights for future drug design for the hMC1R.

Molecular docking is a very useful tool in structural biology and computer aided drug design, especially for GPCR based drug design owing to the limited crystal structures available (none of the melanocortin receptors) and the dynamic environment of the membrane protein. The major goal of ligand–receptor docking is to evaluate the feasible binding geometries of a putative ligand with a target protein of known three-dimensional structure. Normally, docking calculations can help obtain an idea about the binding affinity of the ligand. It also provides useful insights regarding the interactions between ligand and receptors and their roles. Here we study the [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 and hMC1R interaction by conjugating the study of biological information from multiple mutagenesis, chimeric receptor studies of the hMC1R, and molecular docking.

Finally, structure-based drug design has become a useful approach for current drug discovery. In our long-term peptide-based drug development, peptide truncation and amino acid scans have been used to discover the major pharmacophore. Conformational constraints were applied to produce numerous stable and selective melanotropins, and the three-dimensional structures of ligands using NMR spectroscopy combined with computational

based drug design have led to several selective compounds. Peptide mimetic studies also led to selectivity and potency. However, few of these strategies led to a drug. In this study, we first used a strategy by applying useful information from multiple mutagenesis and chimeric receptor studies along with computational chemistry to rationally design a hMC1R peptide ligand using canonical amino acids. The economic favorability of canonical amino acids and straightforward synthesis strategy can provide lower cost for mass production. Our strategy represents the rational design to fulfill specific requirements for developing skin care products for short-term skin pigmentation and melanoma prevention.

EXPERIMENTAL SECTION

Synthesis

Nα-Fmoc-amino acids were obtained from Bachem, NovaBiochem, and Advanced ChemTech. The side chain protecting groups were Boc and ^{t}Bu [Fmoc-Asp(^{t}Bu)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Arg(pbf)-OH, Fmoc-His(trt)-OH, Fmoc-Ser(^{t}Bu)-OH, and Fmoc-Tyr(^{t}Bu)-OH]. Fmoc-Rink amide resin was purchased from Polymer Laboratories. Organic solvents and reagents were purchased from Aldrich and used without further purification. All peptides were synthesized by the *N*-Fmoc solid-phase peptide strategy using DIEA and HCTU as the coupling reagents. Rink amide resin (0.65 mmol/g) or 2-chlorotrityl resin (1 mmol/g) was placed into a 5 mL polypropylene syringe with a frit on the bottom and swollen in DCM (2 mL) and DMF (2 mL) for 1 h. The Fmoc protecting group on the Rink linker was removed by 20% piperidine in DMF. After 20 min the solution of piperidine was removed and the resin was washed with DMF (2 mL, 4 times) and DCM (2 mL, 4 times). *N*-Fmoc amino acid (3 equiv) and HCTU (3 equiv) were dissolved in DMF, and then DIEA (3 equiv) was added. The coupling mixture was transferred into the syringe with the resin and shaken for 30 min. Coupling completion was monitored with a Kaiser test. The coupling mixture was removed, and the resin was washed with DMF (2 mL, 4 times) and DCM (2 mL, 4 times). *N*-Fmoc groups were removed with 20% piperidine in DMF in 20 min. Each coupling and deprotection step was repeated until a linear peptide was assembled. The final wash of the resin was done with DMF (2 mL, four times) and DCM (2 mL, four times). The product was cleaved from the resin with a mixture of 95% TFA, 2.5% TIPS, and 2.5% water during 3 h. Side chain protecting groups were removed during the cleavage step as well. The cleaved mixture was evaporated on a rotary evaporator, and the crude peptide was dissolved in H_2O/methanol and purified by HPLC.

The peptide was lyophilized and purified by preparative RP-HPLC on a C18 bonded silica column (Vydac 218TP152022, 250_22 mm, 15–20 μm, 300 Å). A C18 analytical column (YMC-Pack ODS-AM 150_4.6 mm, S-3 μm, 120 Å) was used to analyze the purity, eluted with a linear gradient of acetonitrile (gradient, 2–80% B in A over 30 min, flow rate 0.8 mL/min). System 1: solvent A, 0.1% TFA in water; solvent B, 0.08% TFA in acetonitrile. System 2: solvent A, 1% formic acid in water; solvent B, 1% formic acid in methanol) and aqueous 0.1% TFA (v/v). The major peak of all compounds accounted for ≥95% of the combined total peak area monitored by a UV detector at 254 nM.

Biological Assays

Competitive binding assays with [^{125}I]NDP-MSH and adenylate cyclase assays were performed using previously described protocols.[26,41–43]

Pigmentation Study

Lizards *Anolis carolinensis* were purchased from Carolina online. Peptide samples were dissolved in saline at the concentration of 1 mM. Lizards were anesthetized by diethyl ether before injection. The total amount of peptide was through ip injection with 3 μg/g of each lizard. The strategy follows previous publications.[44–48]

Serum Stability Assay

Compounds **5** and **15** were dissolved at 10 mg/mL as stock solution. An amount of 1 mL of RPMI supplemented with 25% of human serum was allocated into a 1.5 mL tube and warmed up to 37 °C before adding 5 μL of peptide stock solution. An amount of 100 μL of the reaction solution is removed from times 0, 10, 20, 30, and 40 min. An amount of 200 μL of 96% ethanol was added for precipitation of serum proteins. The reaction sample was cooled (4 °C) for 15 min and then spun at 18 000*g* (Eppendorf centrifuge) for 2 min. The reaction supernatant was then analyzed using RP-HPLC on a 5 μm, 25 cm × 0.4 cm Vydac C-18 column. A linear gradient from 100% buffer A to 50–50% of buffer A and buffer B is used over 30 min. The absorbance was detected at 280 nm. Area under the expected peak is expected to be proportional to the amount of remaining peptide. The half-life was estimated by fitting the data into an exponential decay curve.

Molecular Modeling

Molecular modeling experiments employed MacroModel version 10.5 equipped with Maestro 10.5 graphical interface (Schrödinger, LLC, New York, NY, 2016) installed on a Linux Red Hat 11 system and were performed as previously described. Peptide structures were built into extended structures with standard bond lengths and angles, and they were minimized using the OPLS3 force field and the Polak–Ribier conjugate gradient (PRCG). Optimizations were converged to a gradient rmsd of less than 0.05 kJ/Å mol or continued until a limit of 50 000 iterations was reached. Aqueous solution conditions were simulated using the continuum dielectric water solvent model (GB/SA). Extended cutoff distances were defined at 8 Å for van der Waals, 20 Å for electrostatics, and 4 Å for H-bonds.

Conformational profiles of the cyclic peptides were investigated by the hybrid Monte Carlo/low frequency mode (MCMM/LMCS) procedure as implemented in MacroModel using the energy minimization parameters as described above. MCMM torsional variations and low mode parameters were set up automatically within Maestro graphical user interface. A total of 20 000 search steps were performed, and the conformations with energy difference of 50 kJ/mol from the global minimum were saved. Interatomic dihedral angles were measured for each peptide analogue using the Maestro graphical user interface.

Molecular Docking

Molecular docking studies used the Glide programs (version 7.0, Schrodinger, LLC, New York, 2016). To analyze the docking results and execute the protocol, the Maestro user interface (version 10.5, Schrodinger, LLC, New York, 2016) was employed. Docking was performed using the SP (standard precision mode) protocol. This includes the following. (1) Preparation of protein: The protein was subjected to energy minimization using Schrodinger implementation of OPLS3 force field. (2) Preparation of ligand: The ligand was prepared using the LigPrep 3.7 module of the Schrodinger suite using the standard protocol with OPLS3 force field. (3) Active site prediction: We employed Sitemap (version 3.8) to search for potential binding sites. Sitemap applies theoretical methods and predicts the most accurate binding site. Again, we used Sitemap after we had docked our ligand to evaluate the binding site. (4) Grid generation–docking calculation: Glide used a series of hierarchical filters to search for possible locations for the ligand in the active site region of the receptor. For the grid-based ligand docking, the receptor grid generation process was used. A grid box of 30 × 30 × 30 $Å^3$ with a default inner box (10 × 10 × 10 $Å^3$) was centered on the corresponding ligand. The receptor grid was defined as an enclosed box at the centroid of the ligand. Lastly, we performed a flexible docking calculation using the “standard precision” Glide algorithm and after the postdocking minimization we kept the pose with the best docking score.

Preparation of Protein and Ligand

The hMC1R model was obtained from Chai et al.,[32] imported and prepared by a multistep process through the Protein Preparation Wizard of Maestro. The protocol was especially used to obtain the optimized and minimized energy conformation of the protein. First, we assigned bond orders and added hydrogen atoms. Water molecules that did not participate in interactions were removed. Following the above steps of preparation, the protein was subjected to energy minimization using Schrodinger implementation of OPLS3 force field. The structure for compound **5** and compound **1** was built in 3D coordinates and minimized in MacroModel to obtain the lowest energy conformation, and retaining the geometry was then prepared using the LigPrep 3.7 module of the Schrodinger suite using the standard protocol with OPLS3 force field.

Active Site Prediction

We employed Sitemap (version 3.8) to search for potential binding sites. Sitemap applies theoretical methods and predicts the most accurate binding site. The OPLS3 force field generates site points, possible for ligand interaction within the protein. Sitemap searches for positions favorable for a donor, acceptor, and hydrophobic group to be present in the receptor.

We used Sitemap to identify the top ranked potential receptor binding sites, keeping all parameters as default. Five sites with different site scores were obtained as output, and the site with the highest score (SiteScore and Dscore) was selected.

We again used Sitemap after we had docked our ligand to evaluate the binding site based on the druggability score (Dscore). Druggability is a term used in drug discovery to describe a

biological target that is known to bind or is predicted to bind with high affinity to a drug. If that score is higher than 0.75, the target is considered to be druggable.

Molecular Docking Studies

We performed molecular docking studies using the Glide program (version 7.0, Schrödinger, LLC, New York, 2016). To analyze the docking results and execute the protocol, the Maestro user interface (version 10.5, Schrödinger, LLC, New York, 2016) was employed.

We used the set of site points from the proposed binding site to generate a grid box for the docking calculation. The receptor grid was defined as an enclosing box at the centroid of the ligand. Then we performed a flexible docking calculation using the “standard precision” Glide algorithm, and after the postdocking minimization we kept the pose with the best docking score.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS USED

AAA	amino acid analysis
Boc	*tert*-butyloxycarbonyl
Fmoc	fluorenylmethoxycarbonyl
Fmo	fluorenylmethyl
Bzl	benzyl
***t*Bu**	*tert*-butyl
CH_3CN	acetonitrile
DCM	dichloromethane
DIEA	diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DIC	diisopropylcarbodiimide
HOBt	*N*-hydroxybenzotriazole
Nal(2)	naphthylalanine
TFA	trifluoroacetic acid

TIPS	triisopropylsilyl
SPPS	solid-phase peptide synthesis
RP-HPLC	reverse phase high-performance liquid chromatography
hMC1R	human melanocortin 1 receptor
MSH	melanocyte-stimulating hormone

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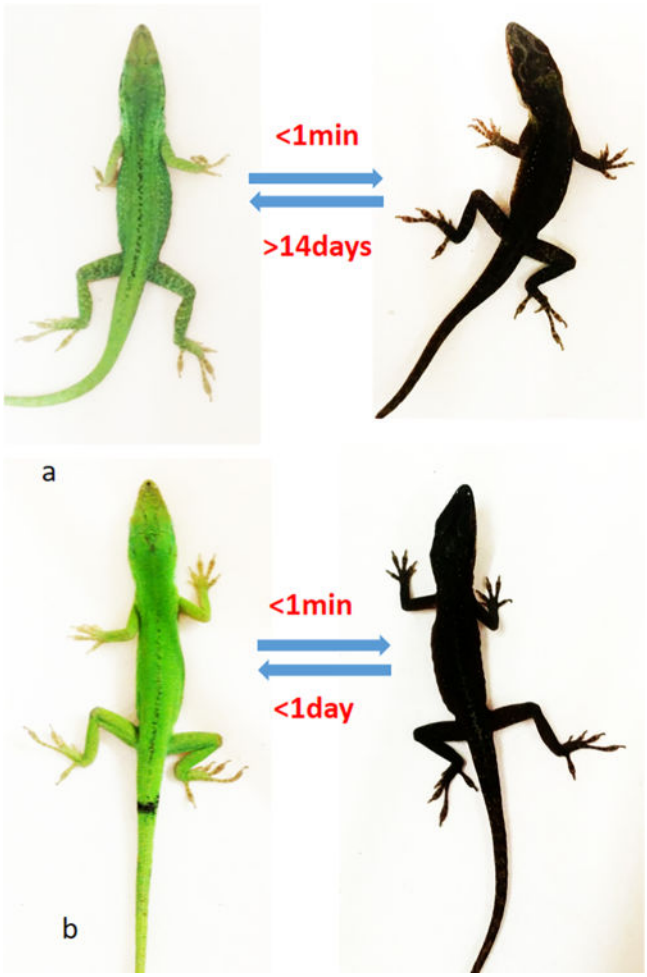

Figure 1.

(a) NDP-α-MSH induced pigmentation on the lizard: (left) before injection; (right) after injection in 1 min. (b) [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 induced pigmentation on the lizard: (left) before injection; (right) after injection in 1 min.

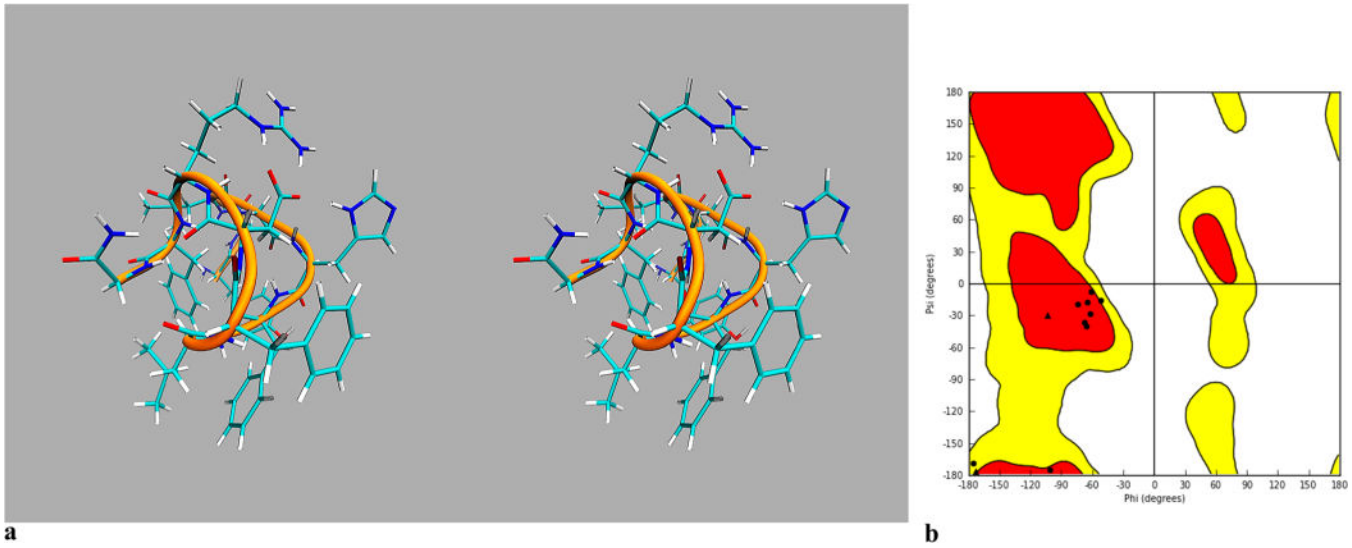

Figure 2.

Conformational studies of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$: (a) stereoview of lowest energy conformation search derived structure of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$; (b) Ramachandran plot of lowest energy conformation search derived structure of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH$_2$, showing that most of the amino acids are in the 3_{10} helix region except for the Gly4, Phe6, and Asp9, which are in the loop area.

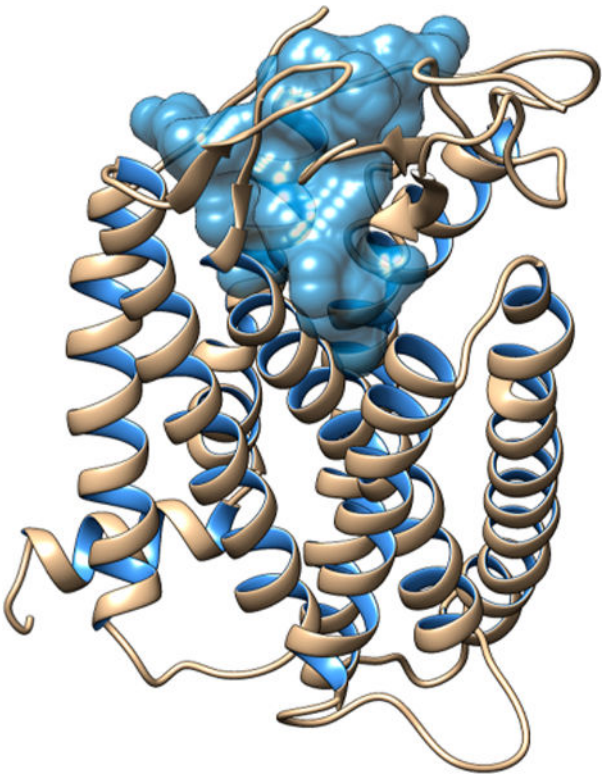

Figure 3.
Binding site of hMC1R for the [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2.

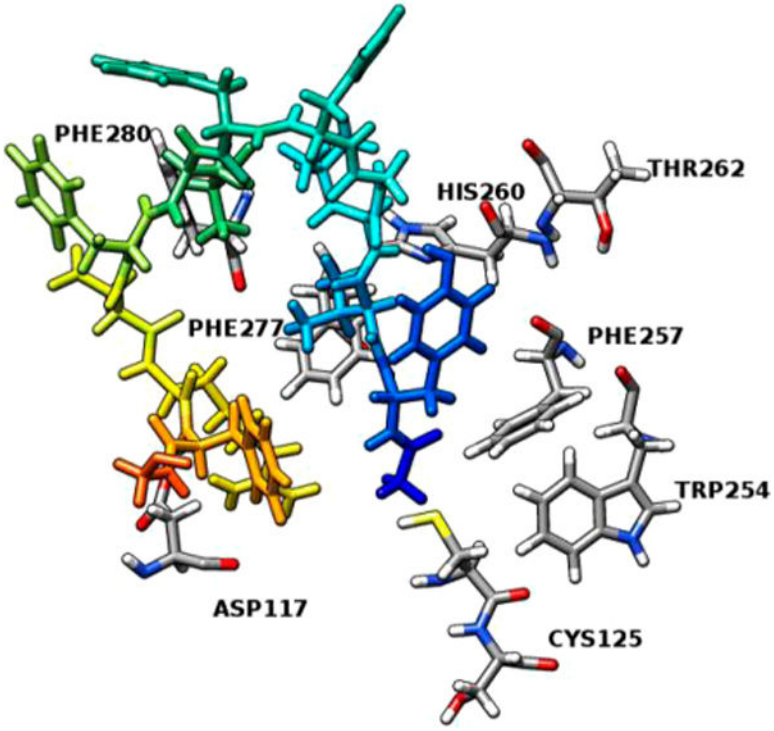

Figure 4.

Molecular interactions between the modified pharmacophore -His-Phe-Leu-Phe- of [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 and the hMC1R.

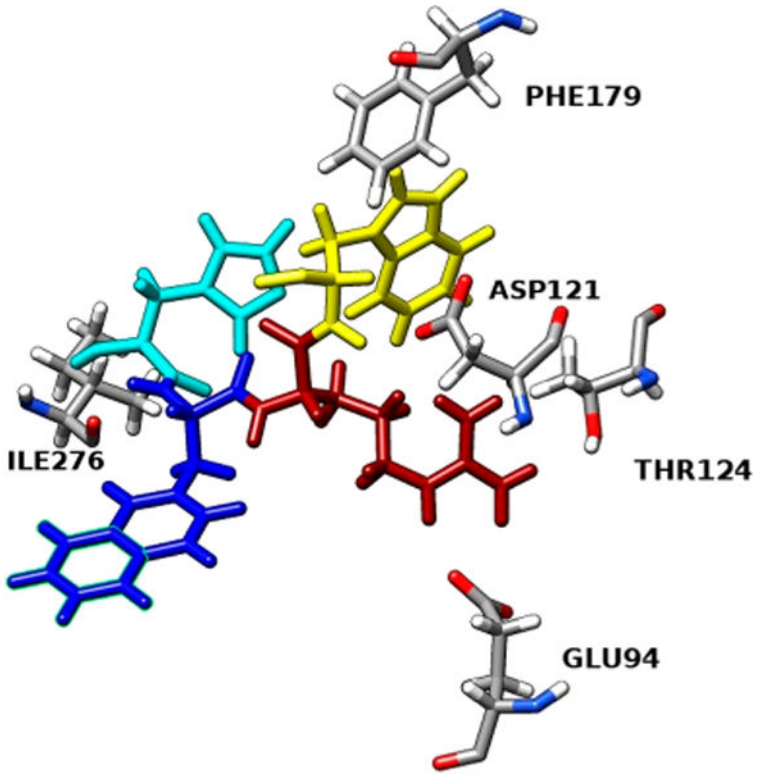

Figure 5.
Molecular interactions between the pharmacophore -His-D-NaI(2′)-Arg-Trp- of compound **1** and the hMC1R.

Table 1

Sequence of γ-MSH Analogues

no.	peptide sequence
1. Nle^3, $DNal(2')^6$, $DTrp^8$-γ-MSH-NH_2	H-Tyr-Val-Nle-Gly-His-D-Nal(2)-Arg-D-Trp-Asp-Arg-Phe-Gly-NH_2
2. Leu^3, Pro^5, γ-MSH-NH_2	H-Tyr-Val-**Leu**-Gly-Pro-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2
3. Leu^3, Pro^5, Phe^8 γ-MSH-NH_2	H-Tyr-Val-**Leu**-Gly-Pro-Phe-Arg-Phe-Asp-Arg-Phe-Gly-NH_2
4. Leu^3, Leu^7, γ-MSH-NH_2	H-Tyr-Val-**Leu**-Gly-His-Phe-**Leu**-Trp-Asp-Arg-Phe-Gly-NH_2
5. Leu^3, Leu^7, Phe^8 γ-MSH-NH_2	H-Tyr-Val-**Leu**-Gly-His-Phe-**Leu**-Phe-Asp-Arg-Phe-Gly-NH_2
6. Leu^3, Pro^5, $Le\ u^7$ γ-MSH-NH_2	H-Tyr-Val-**Leu**-Gly-Pro-Phe-**Leu**-Trp-Asp-Arg-Phe-Gly-NH_2
7. Leu^3, Pro^5, Leu^7, Phe^8 γ-MSH-NH_2	H-Tyr-Val-**Leu**-Gly-Pro-Phe-**Leu**-Phe-Asp-Arg-Phe-Gly-NH_2
8. Pro^5 γ-MSH-NH_2	H-Tyr-Val-Met-Gly-Pro-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2
9. Leu^3, γ-MSH	H-Tyr-Val-**Leu**-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH
10. Leu^3, γ-MSH-NH_2	H-Tyr-Val-**Leu**-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-NH_2
11. γ-MSH	H-Tyr^1-Val^2-Met^3-Gly^4-His^5-Phe^6-Arg^7-$Trp^{8-}Asp^9$-Arg^{10}-Phe^{11}-Gly^{12}-OH
12. γ-MSH-NH_2	H-Tyr^1-Val^2-Met^3-Gly^4-His^5-Phe^6-Arg^7-$Trp^{8-}Asp^9$-Arg^{10}-Phe^{11}-Gly^{12}-NH_2
13. Ac-NDP-γ-MSH-NH_2	Ac-Tyr^1-Val^2-Nle^3-Gly^4-His^5-D-Phe^6-Arg^7-Trp^8-Asp^9-Arg^{10}-Phe^{11}-Gly^{12}-NH_2
14. α-MSH	Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
15. NDP-α-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

Table 2

Competitive Binding Assay and cAMP Assay Results of γ-MSH Analogues[a]

	hMC1R				hMC3R				hMC4R				hMC5R			
no.	**IC_{50}, nM**	**% BE**	**EC_{50}, nM**	**% max effect**	**IC_{50}, nM**	**% BE**	**EC_{50}, nM**	**% max effect**	**IC_{50}, nM**	**% BE**	**EC_{50}, nM**	**% max effect**	**IC_{50}, nM**	**% BE**	**EC_{50}, nM**	**% max effect**
1	0.30 ± 0.02	100	3.0 ± 0.2	70	5.0 ± 0.6	100	300 ± 6	6	6.0 ± 1	100	630 ± 70	18	3.5 ± 0.5	100	NA	6
2	82 ± 7	100	4.6 ± 0.9	100	4.1 ± 0.4	100	5.0 ± 0.4	100	5.0 ± 0.4	100	23 ± 3	90	18 ± 3	100	16 ± 1	82
3	NB	0	>1000	100	18 ± 2	33	NA	0	NB	0	NA	0	NB	0	NA	0
4	137 ± 11	100	604 ± 74	100	17 ± 1	26	NA	0	>1000	47	NA	0	NB	0	NA	0
5	24 ± 3	100	4.5 ± 0.4	100	>1000	45	>1000	50	210 ± 5	58	71 ± 8	54	NB	0	NA	0
6	16 ± 2	29	NA	0	117 ± 24	32	NA	0	NB	0	NA	0	0.42 ± 0.07	32	NA	0
7	1.1 ± 0.1	32	>1000	100	>1000	47	>1000	100	6.7 ± 0.9	36	200 ± 25	100	NB	0	NA	0
8	>1000	52	40 ± 6	100	118 ± 14	73	521 ± 72	100	360 ± 42	62	128 ± 13	78	36 ± 1	47	>1000	100
9	23 ± 2	100	31 ± 6	100	56 ± 3	69	>1000	100	20 ± 1	86	444 ± 69	100	254 ± 41	38	NA	0
10	42 ± 7	100	8 ± 1.8	100	>1000	53	>1000	100	68 ± 11	100	178 ± 23	100	>1000	60	NA	0
11	1000 ± 20	100	300 ± 30	100	71 ± 10	100	700 ± 89	100	760 ± 80	100	710 ± 70	100	2200 ± 200	100	550 ± 60	100
12	1.2 ± 0.2	100	70 ± 10	100	40 ± 10	100	520 ± 50	38	45 ± 5	100	65 ± 12	76	330 ± 57	100	390 ± 25	73
13	0.5 ± 0.01	100	1.5 ± 0.1	100	2.0 ± 0.02	100	2.0 ± 0.2	100	1.2 ± 0.2	100	1.4 ± 0.1	100	2.4 ± 0.3	100	1.9 ± 0.2	100
14	0.4 ± 0.01	100	0.7 ± 0.01	100	30 ± 3.9	100	6.7 ± 1	100	5 ± 1	100	2.1 ± 0.6	100	18 ± 2	100	8.1 ± 1.5	100
15	0.01	100	0.01	100	3.3 ± 0.3	100	0.8 ± 0.1	100	0.4 ± 0.02	100	0.2 ± 0.04	100	2.2 ± 0.5	100	1 ± 0.3	100
[a] IC	is the		peptide at 50%	fic	(N = 4).	% BE	the	of [^{125}	α-MSH		at 10 μM.	means	that 0% of [^{125}		-MSH	

[a] IC50 is the concentration of peptide at 50% specific binding (N = 4). % BE is the percentage of [^{125}I]NDP-α-MSH displacement at 10 μM. NB means that 0% of [^{125}I]NDP-α-MSH displacement was observed at 10 μM. EC_{50} is the effective concentration of peptide that could generate 50% maximal intracellular cAMP accumulation (N = 4). % max effect is the percentage of cAMP produced at a ligand concentration of 10 μM, in relation to MT-II. NA indicates 0% cAMP accumulation observed at 10 μM. The peptides were tested over a range of concentrations from 10^{-10} to 10^{-5} M. Compounds **1** and **11**–**15** were previously published data and are included for comparison.

Table 3

Molecular Interactions between [Leu^3, Leu^7, Phe^8]-γ-MSH-NH_2 and hMC1R

interacting residue on 5	interacting residue on hMC1R	distance, Å	type of interaction
His5 NE2	Thr262	1.14	H-bond
His5	His260	5.12	π–π stacking
Phe6	Trp254	3.68	π–π stacking
Phe6	Phe257	3.16	π–π stacking
Tyr1	Phe277	5.32	π–π stacking
Tyr1	Phe280	3.99	π–π stacking
Leu7 N	Cys125	2.79	H-bond
Asp9 OD1	Ser126	2.62	H-bond
Arg10 NH2	Asp117	1.79	salt bridge
Phe11	Phe45	3.55	π–π stacking

Table 4

Molecular Interactions between Compound 1 and hMC1R

interacting residue on 1	interacting residue on hMC1R	distance, Å	type of interaction
His N	Ile276	2.30	H-bond
Arg NE	Glu94	1.93	H-bond
Arg NH2	Glu94	2.27	H-bond
Arg NH2	Thr124	2.31	H-bond
Arg NH1	Asp121	2.72	H-bond
Trp	Phe179	5.02	π–π stacking
Trp N	Asp121	1.90	H-bond