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Effects of Macrocycle Size and Rigidity on Melanocortin Receptor-1 and -5 Selectivity in Cyclic Lactam α-Melanocyte-Stimulating Hormone Analogs

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Abstract

The effects of the linker arm rigidity and size on melanocortin receptor selectivity were explored in a series of compounds using cyclic lactam α-melanocyte-stimulating hormone template. A variety of dicarboxylic acid linkers introduced between the α-amino group of His^6 and the ε-amino group of Lys^{10} lead to high-affinity, selective human melanocortin receptor-1 and -5 (hMC1R and hMC5R) antagonists. The incorporation of hydrophilic functions into the linker arm was found to be unfavorable for both binding potency and receptor selectivity. Analogs 8 and 9 containing highly conformationally constrained hydrophobic linkers (*m*- and *p*-phthalic acids) were found to be selective nanomolar range hMC1R antagonists (IC_{50} = 7 and 4 nM, respectively), whereas the employment of a small conformationally constrained linker (maleic acid) resulted in a high-affinity (IC_{50} = 19 nM) and selective hMC5R antagonist (analog 12). These newly developed melanotropins will serve as critical biochemical tools for elucidating the full spectrum of functions performed by the physiologically important melanocortin-1 and -5 receptors.

Keywords

α-melanocyte-stimulating hormone; antagonist; human melanocortin-1 receptor; human melanocortin-5 receptor; macrocyclic; melanocortin; peptide

Abbreviations

All, allyl; Alloc, allyloxycarbonyl; Boc, *tert*-butyloxycarbonyl; Fmoc, fluorenylmethoxycarbonyl; CH_3CN, acetonitrile; DCM, dichloromethane; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; DIC, diisopropyl carbodiimide; HBTU, 2-(1H-benzotriazole-1-yl)-1, 1, 3, 3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; hMCR, human melanocortin receptor; MSH, melanocyte-stimulating hormone; Nal(2′), 2′-naphthylalanine; Pbf, 2, 2, 4, 6, 7-pentamethyldihydrobenzofuran-5-sulfonyl; PyBOP, benzotriazol-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate; TFA, trifluoroacetic acid; Trt, trityl; SPPS, solid-phase peptide synthesis; RP-HPLC, reverse-phase high-performance liquid chromatography; hMC1R, human melanocortin-1 receptor; α-MSH, Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2; NDP-α-MSH, Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

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The natural melanocortin agonists, α-, β-, γ-melanocyte-stimulating hormones (MSHs), and adrenocorticotropin have been receiving great attention in the recent years because of their involvement in a large number of multifaceted biological actions, including skin pigmentation (1–3), control of the immune system (1–4), erectile function (5–9), blood pressure and heart rate (10), control of feeding behavior and energy homeostasis (11–17), modulation of aggressive/defensive behavior (18), and mediation of pain (19). These endogenous neuropeptides are ligands for the five known subtypes of human melanocortin receptors, which are expressed in various tissues, including skin [human melanocortin-1 receptor (hMC1R)] (1–4), adrenal cortex (hMC2R) (20,21), and throughout the central nervous system (hMC3R, hMC4R, and hMC5R) (22).

Previous reports from our laboratories (23–28) had focused on finding potent and selective agonists and antagonists for the hMC3R and hMC4R, which have been implicated to play complementary roles in weight control (11–17). Recently, Kavarana *et al.* (24,25) and Bednarek *et al.* (29) have reported that replacing the Ac-Nle^4-Asp^5 part of the non-selective superagonist MT-II (Ac-Nle^4-c[Asp^5, D-Phe^7, Lys^{10}]α-MSH(4–10)-NH_2) (30) with a variety of dicarboxylic acid linkers yielded several potent and selective hMC3R and hMC4R agonists and antagonists. Extending these studies, a series of novel cyclic α-MSH analogs possessing a variety of flexible aliphatic and constrained aromatic and heterocyclic dicarboxylic acid linkers (c[CO-R-CO-His-D-Phe/D-Nal(2′)-Arg-Trp-Lys]-NH_2) have been designed and synthesized by solid-phase methods to further investigate the effects of macrocycle size and flexibility on melanocortin receptor selectivity.

Peptide Design

MT-II, a superpotent but non-selective human melanocortin receptor agonist (30), along with potent non-selective hMC3R/hMC4R antagonist SHU-9119 (Ac-Nle^4-c[Asp^5, D-Nal(2′)^7, Lys^{10}]α-MSH(4–10)-NH_2) (31) provided an excellent template for design of the more selective melanocortin ligands. The MT-II template in this study was modified to include the following variety of dicarboxylic acid linkers between the α-amino group of histidine and the ε-amino group of lysine: constrained aromatic acids (isophthalic and terephthalic acids), constrained heterocyclic acids (2,6-pyridinedicarboxylic acid and 2,3-pyrazinedicarboxylic acid), constrained unsaturated acid (maleic acid), and flexible aliphatic acids (pimelic and adipic acids). In addition, the effects of linker hydrophilicity were investigated on the analogs containing glutamic acid as the linker (Table 1).

Methods and Materials

N^{α}-fluorenylmethoxycarbonyl (Fmoc)-amino acids, peptide coupling reagents and Rink amide AM resin were obtained from Novabiochem (San Diego, CA, USA), except N^{α}-Fmoc-Glu(O-All)-OH and N^{α}-Fmoc-Lys(Alloc)-OH, which were purchased from Neo-MPS (San Diego, CA, USA). The following side-chain protecting groups were used: Glu(O$^{\gamma}$-All), Trp(N^{in}-Boc), Arg(N^{ε}-Pbf), His(N^{im}-Trt), and Lys(N^{ε}-Alloc). ACS grade organic solvents were purchased from VWR Scientific (West Chester, PA, USA), and other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA) and used as commercially available. The polypropylene reaction vessels (syringes with frits) (32) were purchased from Torviq (Niles, MI, USA). The purity of the peptides was checked by analytical reverse-phase high-performance liquid chromatography (RP-HPLC) using a Vydac C_{18} 218TP104 column (Western Analytical Products, Murrieta, CA, USA) monitored at 230 and 254 nm, and by thin-layer chromatography (TLC), which was performed using three different solvent systems. Analytical TLC was carried out on 0.25-mm glass-backed silica gel 60 F_{254} plates (EM Science 5715, VWR Scientific). The TLC chromatograms were visualized by ultraviolet light, and by dipping in potassium permanganate solution followed by heating (hot plate).

Peptide Synthesis

All peptides in this study were synthesized manually by the N^{α}-Fmoc solid-phase methodology (25,26). Rink amide AM resin [4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin, 4.0 g, 0.637 mmol/g] was placed into a 50-mL polypropylene syringe with the frit on the bottom and swollen in *N*,*N*-dimethylformamide (DMF; 20 mL) for 1 h. The Fmoc protecting group on the Rink linker was removed by 25% piperidine in DMF (1 × 5 and 1 × 15 min). The resin was washed with DMF (6 × 15 mL), and the first N^{α}-Fmoc amino acid was coupled using preactivated ester [3 equiv. of N^{α}-Fmoc amino acid, 3 equiv. of *N*-hydroxybenzotriazole (HOBt), and 3 equiv. of 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)] in DMF solution containing 6 equiv. of diisopropylethylamine (DIPEA). The coupling mixture was transferred into the syringe with the resin and shaken for 60 min. The resin was washed with DMF (3 × 15 mL) and thrice with dichloromethane (DCM) (3 × 15 mL), and the unreacted amino groups were capped using acetic anhydride (2 mL) and pyridine (2 mL) in DCM (15 mL) for 30 min, and the resin was once again washed with DCM (6 × 15 mL). The next four amino acids, Trp, Arg, D-Phe or D-Nal(2′), and His were consecutively coupled using the procedure described earlier, using the Kaiser ninhydrin test (33) to check the extent of coupling. In the case of a positive Kaiser test, the coupling was repeated until a negative Kaiser test was obtained. The resulting batch of the resin-bound protected pentapeptide N^{α}-Fmoc-His-D-Phe/D-Nal(2′)-Arg-Trp-Lys was carefully washed with DMF (5 × 15 mL), DCM (5 × 15 mL), methanol (5 × 15 mL), and diethyl ether (5 × 15 mL), and dried under reduced pressure (16 h). The dry resin was split in eight equal portions, which were placed in separate 10-mL fritted polypropylene syringes, and swollen with DMF as described earlier. The same coupling procedure was followed to append the dicarboxylic acid linkers. Pyrazinedicarboxylic and maleic acids were converted into their corresponding monoallyl esters prior to appending to the peptides to minimize competing formation of cyclic imides, by stirring the corresponding anhydrides with allyl alcohol (1 equiv.) in DCM in the presence of a catalytic (5 mol%) amount of DIPEA until homogeneous solutions were obtained. The resulting solutions were concentrated under reduced pressure and used for peptide synthesis without any further purification. The other dicarboxylic acids were used as commercially available. The orthogonal allylic protection for the side-chain of Lys^{11} and the linker (if applicable) was removed with 0.1 equiv. $Pd(PPh_3)_4$/20 equiv. $PhSiH_3$ in DCM (2 × 30 min) prior to the peptide cyclization (34). The deprotected resin-bound peptide was washed with DCM (6 × 5 mL) and DMF (3 × 5 mL). The peptide cyclizations were accomplished with 6 equiv. benzotriazol-1-yloxy-tris-pyrrolidinophosphonium hexafluorophosphate (PyBOP) (35), 6 equiv. HOBt, and 6 equiv. DIPEA in DMF (36 h), and were monitored by Kaiser ninhydrin test (33). The PyBOP treatment was repeated until a negative Kaiser test was obtained. This procedure was found to be vastly superior to the previously described cyclization methodology that employed HBTU/HOBt/DIPEA (25,26), as it was not accompanied with competing guanylation of the primary amine function of Lys^{10}, commonly associated with acyluronium reagents, such as HBTU (36). The new procedure also resulted in improved overall yield of the target peptides. Upon completion of cyclization, the resin was treated with 5% solution of sodium diethyldithiocarbamate trihydrate in DMF (20 min) to remove any remaining traces of the Pd catalyst, then washed with DMF (5 × 15 mL), DCM (3 × 15 mL), methanol (5 × 15 mL), and diethyl ether (5 × 15 mL), and dried under reduced pressure (16 h). The cyclized peptides were cleaved off the solid support with 82.5% v/v trifluoroacetic acid (TFA), 5% water, 5% thioanisol, 2.5% 1,2-ethanedithiol, and 5% phenol (5 mL, 3 h), and the crude peptides were precipitated out by the addition of a chilled 3:1 mixture of diethyl ether and petroleum ether (50 mL) to give white precipitates. The resulting peptide suspensions were centrifuged for 10 min at 6500 r.p.m., (300 g) and the liquid was decanted. The crude peptides were washed with diethyl ether (4 × 50 mL), and after the final centrifugation, the peptides were dried under vacuum (2 h). The resulting white residues were dissolved in 2 M acetic acid, and the insoluble impurities were removed by passing the solutions through Gelman Laboratory

Acrodisc 13-mm syringe filters with 0.45-μM polytetrafluoroethylene (PTFE) membranes (Pall Corporation, East Hills, NY, USA). The clear filtrates were lyophilized, the obtained white powders (50–80 mg) were redissolved in glacial acetic acid (1 mL), the resulting solutions were diluted with water (4 mL) to a peptide concentration of about 1 mg/mL, and passed through a Sephadex G-15 column (520 × 30 mm) using 1 M aqueous acetic acid as the eluent. Fractions containing the target peptides, as determined by TLC, were combined and lyophilized. Final purification was accomplished by preparative RP-HPLC on a C_{18}-bonded silica column (Vydac 218TP152022, 250 × 22 mm, 15–20 μM, 300 Å) using a Shimadzu SCL-10A HPLC system (Shimadzu Scientific Instruments, Pleasanton, CA, USA). The peptides were eluted with a linear gradient of 20–80% acetonitrile in 0.1% aqueous TFA solution over 50 min with 10-mL/min flow rate. The purified peptides were isolated in 25–30% overall yield. The structures of the pure peptides were confirmed by high-resolution electrospray ionization (ESI) mass spectrometry using an IonSpec Fourier-transform mass spectrometer with a HiRes ESI source.

Biological Activity Assays

Receptor binding assay

Competition binding experiments were carried out as described earlier (37) using whole HEK-293 (Human embryonic kidney) cells stably expressing hMC1R, hMC3R, hMC4R, and hMC5R. Briefly, HEK-293 cells transfected with human melanocortin receptors (38–40) were seeded on 96-well plates 48 h before assay (100 000 cells/well). For the assay, the cell culture medium was aspirated and cells were washed twice with a freshly prepared binding buffer containing 100% minimum essential medium (MEM) with Earle's salt (Gibco Invitrogen, Carlsbad, CA, USA), 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1 mM 1,10-phenanthrolone, 0.5 mg/L leupeptin, 200 mg/L bacitracin. Next, cells were incubated with different concentrations of unlabeled peptide and labeled [^{125}I]-[Nle^{4}, D-Phe^{7}]-α-MSH (Perkin-Elmer Life Science, Wellesley, MA, USA, 100 000 c.p.m./well, 0.1386 nM) for 40 min at 37 °C. The assay medium was subsequently removed and each well was washed twice with the binding buffer. The cells were then lysed by the addition of 250 μL of 0.1 M NaOH and 250 μL of 1% Triton-X-100. The lysed cells were transferred to 12 × 75 mm glass tubes and the radioactivity was measured by a Wallac 1470 WIZARD gamma-counter (Perkin-Elmer).

Adenylate cyclase assay

HEK-293 cells transfected with human melanocortin receptors (38) were grown to confluence in MEM medium (Gibco Life Technologies) containing 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96-well plates 48 h before assay (100 000 cells/well). For the assay, the cell culture medium was removed and the cells were rinsed with 1 mL of MEM buffer (Gibco Life Technologies) or with Earle's balanced salt solution (EBSS; Gibco Invitrogen). An aliquot (0.4 mL) of the EBSS was placed in each well along with 5 μL of 0.5 mM isobutylmethylxanthine (IBMX) for 1 min at 37 °C. Next, aliquots of melanotropin peptide solutions with varying concentrations (0.1 mL) were added, and the cells were incubated for 3 min at 37 °C. The reaction was stopped by aspirating the assay buffer and adding 0.15-mL ice-cold Tris/ethylenediaminetetraacetic acid buffer to each well. After dislodging the cells with the help of trypsin, the cells were transferred to polypropylene micro-centrifuge tubes and placed in a boiling water bath for 15 min. The cell lysate was then centrifuged for 2 min at 14,000 g (max 16000 g), and 50 μL of the supernatant was aliquoted into an Eppendorf tube. The total cAMP content was measured by competitive binding assay according to the TRK 432 assay kit instructions (Amersham Corp., Piscataway, NJ, USA). IC_{50} and EC_{50} values represent the mean of two experiments performed in triplicate. IC_{50} and EC_{50} estimates and their associated standard errors were

determined by fitting the data using a non-linear least-squares analysis, with the help of GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). The maximal cAMP produced at 10 μM concentration of each ligand was compared with the amount of cAMP produced at 10 μM concentration of the standard agonist MT-II, and is expressed in per cent (as % max effect) in Table 2.

Results and Discussion

Analog **1** was found to be a very weak antagonist at the hMC1R and the hMC3R, entirely inactive at the hMC4R, and a weak partial agonist at the hMC5R (maximum stimulation 44%). Replacement of the isophthalic acid linker with terephthalic acid (analog **2**) resulted in no change in the biological profile of the cyclic peptide. Similar results were obtained for the peptides with 2,6-pyridinedicarboxylic acid (analog **3**) and 2,3-pyrazinedicarboxylic acid linkers (analog **4**). This suggests that the macrocycle expansion and introduction of aromatic heterocyclic linkers into the MT-II template do not produce favorable peptide topography for melanocortin activity. The introduction of the small constrained maleic acid linker yielded analog **5**, which exhibited weak antagonist binding at the hMC1R and nanomolar range partial agonist activities at the hMC3R (IC_{50} = 110 nM), hMC4R (IC_{50} = 60 nM), and hMC5R (IC_{50} = 69 nM), with negligible receptor selectivity. This result contrasts sharply with our earlier findings that short aliphatic linkers, such as succinic acid, placed into the MT-II template produced potent hMC4R selective agonist VJH-085 (c[CO-$(CH_2)_2$-CO-His-D-Phe-Arg-Trp-Lys]-NH_2) (25). It seems plausible that the differences in the biological activities of these peptides stem from the unfavorable dihedral constraint introduced by the maleic acid linker, which is locked in the *cis*-configuration, whereas the nuclear magnetic resonance structure of VJH-085 reported by our laboratories (41) indicates the opposite *anti*-conformation of the succinic acid linker. Analogs **6** and **7** were obtained by further expansion of the lactam macrocycle with flexible adipic acid and pimelic acid linkers, respectively. Analog **6** demonstrated nanomolar range partial agonist activities at all four receptor subtypes, while analog **7** displayed no interaction with the hMC1R, full agonist activity at the hMC3R and modest partial agonist activities at the hMC4R and hMC5R. Overall, these aliphatic linkers showed little tendency to produce selective ligands, which may be due to higher flexibility of the corresponding macrocycles.

Analogs **8–14** were produced by replacing D-Phe in position 7 of peptides **1–7** with D-Nal(2′) and the biological activities of these two subsets were compared. Analogs **8** and **9** exhibited very similar biological profiles, where both peptides were found to be antagonists at the hMC1R with high binding affinity (IC_{50} = 7 and 4 nM, respectively), weak partial agonists at the hMC3R, weak antagonists at the hMC4R and weak partial agonists at the hMC5R. These compounds show a good selectivity for the hMC1R (up to 30-fold versus the hMC3R, 25-fold versus the hMC4R, and up to 170-fold versus the hMC5R), which is a particularly important finding as reports of potent hMC1R antagonists are scarce in the literature (42–45). The *meta*- and *para*-stereochemistry of the phthalic acid linker clearly has very little effect on the biological activities of these two compounds. Interestingly, the *ortho*-isomeric peptide (c[CO-*o*-C_6H_4-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2), reported earlier from our laboratories (25), showed similar biological properties at the hMC3–5R, albeit with a somewhat higher binding potencies, which was likely due to the smaller size of the lactam macrocycle. In contrast, replacement of the phthalic acid linker with more polar heterocyclic linkers produced peptides (analogs **10** and **11**) with drastically different biological profiles. Thus, analog **10** exhibited weak antagonist affinities at the hMC1–4R, and a weak partial agonist activity at the hMC5R, while analog **11** was found to be a weak partial agonist at the hMC1R (25% cAMP stimulation), a weak antagonist at the hMC3R, a partial agonist at the hMC4R (36% cAMP stimulation) and a weak agonist at the hMC5R. Analog **12**, with maleic acid linker, showed nanomolar range binding affinities at all four receptor subtypes, and partial agonist activities at the hMC1–4R

(28%, 15%, and 11% maximum cAMP stimulation, respectively). At the same time, analog **12** was found to be a high-affinity hMC5R antagonist (IC_{50} = 19 nM). It is interesting to compare the biological activities of this peptide with those of MK-7 (c[CO-*o*-C_6H_4-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2) (25). While maleic acid linker offers the same type of dihedral constraint for the peptide chain as *o*-phthalic acid linker used in MK-7, it lacks the aromaticity and the steric bulk of the latter. This structural difference may account for the observed conversion of the hMC5R agonist MK-7 to antagonist (analog **12**). Furthermore, analogs **13** and **14**, featuring longer aliphatic linkers, were determined to be weak antagonists at the hMC1R, partial agonists at the hMC3R (23% and 13% maximum cAMP stimulation, respectively) and hMC4R (20% and 19% maximum cAMP stimulation, respectively), and antagonists at the hMC5R, while exhibiting high binding affinities to this receptor sub-type (IC_{50} = 10 and 44 nM, respectively). Interestingly, the increase in the size of the flexible lactam macrocycle results in loss of binding affinities of the respective peptides (analogs **13** and **14**) toward the hMC1R (IC_{50} = 2.8 and 1.0 μM, respectively), while favoring binding to the other receptor subtypes (hMC3–5R). This is in contrast to the observed binding affinities of the peptides containing highly conformationally constrained linkers (analogs **8** and **9**) to the hMC1R (IC_{50} = 7 and 4 nM, respectively). This finding offers evidence for the effects of the macrocycle rigidity on the hMC1R receptor selectivity.

The analogs **15** and **16** were derived from the hMC3R selective antagonist MK-9 (c[CO-$(CH_2)_3$-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2) (25), by introducing a hydrophilic function to the linker via replacement of the glutaric acid linker with L-glutamic acid and *N*-acetyl-L-glutamic acid. Analog **15** was found to be a weak partial agonist at the hMC1R, whereas analog **16** showed a very weak agonist activity at this receptor. Similar to MK-9, analog **15** demonstrated moderate hMC3R binding selectivity, but in contrast to MK-9, it remained a potent partial agonist at this receptor (EC_{50} = 18 nM, 15% maximum stimulation). Analog **15** also showed weak partial agonist activities at the hMC4R (EC_{50} > 1.0 μM, 28% maximum stimulation) and the hMC5R (EC_{50} = 62 nM, 34% maximum stimulation). Acetylation of the N-terminus (analog **16**) resulted in weakened partial agonist activity at the hMC3R (EC_{50} = 374 nM, 75% maximum stimulation), but improved binding affinity and the partial agonist activity at the hMC4R (EC_{50} = 36 nM, 23% maximum stimulation), and produced a potent hMC5R agonist (EC_{50} = 32 nM).

Conclusions

In summary, introduction of a hydrophilic function into the linker arm was found to be unfavorable in terms of both potency and receptor selectivity. On the contrary, increasing the overall rigidity of the cyclic D-Nal(2′)^7-α-MSH scaffold by introducing highly conformationally constrained hydrophobic linkers (*m*- and *p*-phthalic acids) between the α-amino group of His^6 and the ε-amino group of Lys^{10} leads to nanomolar range selective hMC1R antagonists (analogs **8** and **9**), which suggests the important role of hydrophobic aromatic interactions between the linker arm and the hMC1R binding pocket for hMC1R selectivity. Conversely, more polar aromatic heterocycles used as linker arms (analogs **10** and **11**) weakened the peptide binding potencies towards the hMC1R. These findings represent a significant advancement toward development of more potent and highly selective hMC1R antagonists, unprecedented in the current literature. In addition, replacement of *o*-phthalic acid linker in an hMC5R agonist c[CO-*o*-C_6H_4-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2 with a small conformationally constrained maleic acid linker resulted in a high-affinity and selective hMC5R antagonist (analog **12**), which exemplifies an interesting and potentially useful new way of conversion of an hMC5R agonist into an antagonist. These newly developed melanotropin peptides may find application as biochemical and pharmacological tools for determining the complex physiological roles played by the hMC1R and the hMC5R.

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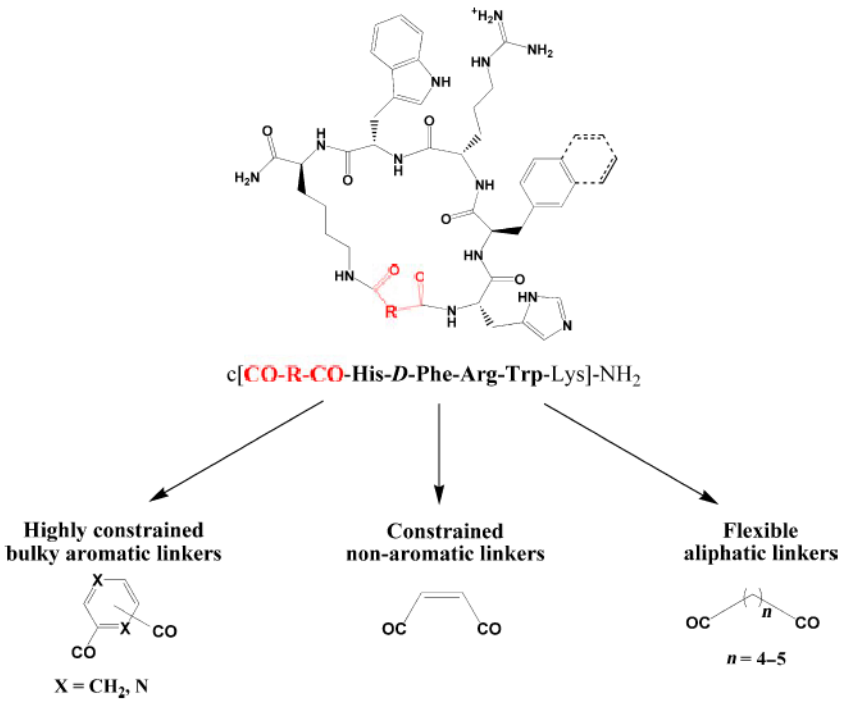

Figure 1.
Design of the α-MSH-derived cyclic lactam scaffold.

Table 1

Sequences and the physicochemical properties of the cyclic α-MSH analogs

No.	Sequence	*m/z* (*M* + 1)		HPLC retention time (min)[a]	TLC R_f[b]		
		Calculated	Observed (ESI)		1	2	3
1	c[CO-*m*-C_6H_4-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	902.4426	902.4440	14.12	0.56	0.10	0.68
2	c[CO-*p*-C_6H_4-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	902.4426	902.4518	15.26	0.53	0.04	0.61
3	c[CO-2,6-pyridine-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	903.4378	903.4237	14.37	0.64	0.11	0.67
4	c[CO-2,3-pyrazine-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	904.4331	904.4195	14.38	0.58	0.04	0.60
5	c[CO-*cis*-CH=CH-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	852.4269	852.4312	12.87	0.63	0.05	0.66
6	c[CO-$(CH_2)_4$-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	882.4739	882.4685	12.39	0.61	0.05	0.63
7	c[CO-$(CH_2)_5$-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	896.4895	896.4908	12.46	0.65	0.06	0.65
8	c[CO-*m*-C_6H_4-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	952.4582	952.4563	16.50	0.71	0.11	0.70
9	c[CO-*p*-C_6H_4-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	952.4582	952.4618	16.98	0.62	0.06	0.69
10	c[CO-2,6-pyridine-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	953.4535	953.4491	16.52	0.71	0.14	0.69
11	c[CO-2,3-pyrazine-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	954.4487	954.4502	15.21	0.41	0.07	0.69
12	c[CO-*cis*-CH=CH-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	902.4426	902.4474	14.63	0.66	0.06	0.69
13	c[CO-$(CH_2)_4$-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	932.4895	932.4916	14.42	0.64	0.05	0.66
14	c[CO-$(CH_2)_5$-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	946.5052	946.4997	14.23	0.69	0.11	0.68
15	H-c[Glu-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	933.4848	933.4884	13.33	0.55	0.03	0.59
16	Ac-c[Glu-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	975.4952	975.4921	15.51	0.63	0.06	0.67

α-MSH, α-melanocyte-stimulating hormone; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; ESI, electrospray ionization.

[a] HPLC column: Vydac 218TP104, 250 × 4.6 mm, 10 μm, 300 Å; HPLC solvent A, 0.1% trifluoroacetic acid in water; solvent B, acetonitrile; gradient: 10–90% B in A over 40 min, flow rate 1.0 mL/min.

[b] TLC system 1: ethyl acetate/acetic acid/water/pyridine (5:1:3:5); TLC system 2: *n*-propanol/acetic acid/water (10:1:1); TLC system 3: *n*-butanol/acetic acid/water/pyridine (5:1:4:5).

Table 2

Binding affinities and cAMP activities of cyclic α-MSH analogs at hMCRs

		hMC1R			hMC3R			hMC4R			hMC5R		
No.	**Code**	**IC_{50} (nM)**	**EC_{50} (nM)**	**% max effect**	**IC_{50} (nM)**	**EC_{50} (nM)**	**% max effect**	**IC_{50} (nM)**	**EC_{50} (nM)**	**% max effect**	**IC_{50} (nM)**	**EC_{50} (nM)**	**% max effect**
1	c[CO-*m*-C_6H_4-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	>1000	>1000	0	550 ± 70	>1000	0	>10 000	>10 000	0	260 ± 30	>1000	44
2	c[CO-*p*-C_6H_4-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	>1000	11 ± 2	4	390 ± 40	>1000	0	>1000	>1000	0	>2000	600 ± 60	17
3	c[CO-2,6-pyridine-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	>1000	>1000	8	420 ± 60	560 ± 60	4	>3500	>5000	0	>1500	>1000	13
4	c[CO-2,3-pyrazine-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	>10 000	670 ± 89	10	4000	>10 000	0	>1000	>1000	0	>10 000	>3000	61
5	c[CO-*cis*-CH=CH-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	330 ± 30	>1100	0	110 ± 10	94 ± 10	69	60 ± 6	110 ± 11	80	69 ± 7	55 ± 6	39
6	c[CO-$(CH_2)_4$-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	140 ± 15	38 ± 4	70	72 ± 8	3 ± 0.3	87	110 ± 11	60 ± 7	90	85 ± 8	6 ± 0.6	46
7	c[CO-$(CH_2)_5$-CO-His-D-Phe-Arg-Trp-Lys]-NH_2	1000	>1000	0	140 ± 15	80 ± 8	100	160 ± 14	250 ± 30	80	150 ± 16	200 ± 23	67
8	c[CO-*m*-C_6H_4-CO-His-D-Nal (2′)-Arg-Trp-Lys]-NH_2	7.0 ± 1	>10 000	0	160 ± 23	>1000	17	140 ± 15	>10 000	0	550 ± 60	110 ± 10	63
9	c[CO-*p*-C_6H_4-CO-His-D-Nal (2′)-Arg-Trp-Lys]-NH_2	4.0 ± 0.8	>10 000	0	120 ± 13	>1000	25	110 ± 11	>5000	0	670 ± 70	210 ± 21	55
10	c[CO-2,6-pyridine-CO-His-D-Nal (2′)-Arg-Trp-Lys]-NH_2	100 ± 10	>1000	0	110 ± 20	>10 000	0	540 ± 60	>10 000	0	>1000	610 ± 70	64

No.	Code	hMC1R			hMC3R			hMC4R			hMC5R		
		IC_{50} (nM)	EC_{50} (nM)	% max effect	IC_{50} (nM)	EC_{50} (nM)	% max effect	IC_{50} (nM)	EC_{50} (nM)	% max effect	IC_{50} (nM)	EC_{50} (nM)	% max effect
11	c[CO-2,3-pyrazine-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	100 ± 11	400 ± 50	25	280 ± 20	>1000	0	420 ± 45	99	36	660 ± 60	>1000	93
12	c[CO-*cis*-CH=CH-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	250 ± 22	96 ± 10	28	56 ± 6	310 ± 40	15	7 ± 8	>1000	11	19 ± 2	>10 000	0
13	c[CO-$(CH_2)_4$-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	>2800	>3000	0	15 ± 2	300 ± 30	23	16 ± 2	>1000	20	10 ± 1.2	59 ± 6	5
14	c[CO-$(CH_2)_5$-CO-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	1000	930 ± 88	0	64 ± 8	940 ± 11	13	60 ± 8	120 ± 13	19	44 ± 5	11 ± 2	4
15	H-c[Glu-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	>1000	42 ± 6	56	27 ± 3	18 ± 2	15	900 ± 100	>1000	28	470 ± 50	62 ± 4	34
16	Ac-c[Glu-His-D-Nal(2′)-Arg-Trp-Lys]-NH_2	500 ± 100	400 ± 122	95	42 ± 4	370 ± 40	75	42 ± 4	36 ± 4	23	77 ± 7	32 ± 3	198
17	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2	0.2 ± 0.01	0.3 ± 0.04	100	1.25 ± 0.2	1.85 ± 0.2	100	1.07 ± 0.3	2.87 ± 0.52	100	7.47 ± 0.23	3.3 ± 0.7	100

hMC1R, -3R, -4R, and -5R, human melanocortin receptor-1, -3, -4, and -5; IC_{50}, concentration of peptide at 50% specific binding (n = 4); EC_{50}, effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation (n = 4); % max effect, % of cAMP produced at 10 μM ligand concentration, in relation to MT-II. The peptides were tested at a range of concentration from 10^{-10} to 10^{-5} nM.