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An Unusual Conformation of MSH Analogues Leads to a Selective Human Melanocortin 1 Receptor Antagonist for Targeting Melanoma Cells

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Abstract

γ -MSH (γ -melanocyte-stimulating hormone: H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH), with its exquisite specificity and potency, has recently created much excitement as a drug lead. However, this peptide like most peptides susceptible to proteolysis in vivo which potentially decreases its beneficial activities. In our continued effort to design a proteolytically stable with specific receptor binding ligand, we have engineered peptides by cyclizing γ -MSH using a thioether bridge. A number of novel cyclic truncated γ -MSH analogues were designed and synthesized, in which a thioether bridge was incorporated between a cysteine side chain and an N-terminal bromoacyl group. One of these peptides, *cyclo*-[(CH₂)₃CO-Gly¹-His²-D-Phe³-Arg⁴-D-Trp⁵-Cys(S-)⁶]-Asp⁷-Arg⁸-Phe⁹-Gly¹⁰-NH₂, demonstrated potent antagonist activity and receptor selectivity for the human melanocortin 1 receptor (hMC1R) (IC₅₀ = 17 nM). This novel peptide is the most selective antagonist for the human hMC1R to date. Further pharmacological studies have shown that this peptide can specifically target melanoma cells. The NMR analysis of this peptide in a membrane-like environment revealed a new turn structure, specific to the hMC1R antagonist, at the C terminal, wherein the side chain and backbone conformation of D-Trp⁵ and Phe⁹ of the peptide are contributors to the hMC1R selectivity. Cyclization strategies represent an approach for stabilizing bioactive peptides while keeping their full potencies and should boost applications of peptide-based drugs in human medicine.

Keywords

α -MSH; γ -MSH; melanocortin receptors; melanotropin antagonist; hMC1R; steric constraints; selective ligands; melanoma

Introduction

The melanocortin system is involved in numerous physiological functions and may have great potential for the novel drug discovery.¹⁻¹⁰ The human melanocortin receptor 1 (hMC1R), mainly present in the periphery for example, in the skin of mammals, is involved in control of the relative amounts of pheomelanin and photoprotective eumelanin.¹¹⁻¹³ Appropriate selective hMC1R ligands may offer protection against damaging and mutagenic

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SUPPORTING INFORMATION AVAILABLE: High resolution mass spectra of peptide analogues and extensive 2D NMR of peptide **6** which provide data needed for conformational analysis are provided. The material is available free of charge via the internet at <http://pubs.acs.org/>.

effects of UV radiation, or be useful in the diagnosis and treatment of certain types of skin cancer, such as melanoma. The hMC1R is also present on the surface of various immune cells, macrophages, fibroblasts, monocytes, mast cells and neutrophils and is a potential target for the development of agents for the treatment of acute and chronic inflammatory diseases, neurodegenerative diseases and systemic host reactions.^{5, 11-14} In the CNS the MC1 receptor is present only on neurons in the periaqueductal grey matter of the midbrain where it is thought to have a role in pain control.¹⁵⁻¹⁷ Despite the multiple physiological functions and the great potential for medical application, there are only a few reports of selective antagonists for the hMC1R available in the literature. Thus design of selective hMC1R agonists and antagonists warrants special interest in the melanocortin research. Therefore, in our continued effort to produce selective melanocortin ligands, we devoted a great deal of time to obtain ligands with selective antagonist activity for the hMC1R and to develop the pharmacological properties that are suitable for medical applications of these compounds.

Structure based drug design is one of the major strategies in drug discovery.¹⁸⁻²¹ In peptide-based drug development, truncations and amino acid scans have been used to find the relevant pharmacophore.^{19,22-25} The same method is important for the rational design of melanocortin receptors ligands. Conformational constraints and β -turn mimetics were applied to develop stable and selective melanotropins; the 3D structure of ligands (NMR) combined with computational aided drug design helped in obtaining several selective compounds.^{26,27} Recently, *N*-methylation of the amide bonds has been introduced into our novel drug design strategy to obtain the most selective agonist of the hMC1R.²⁸ In this study we have designed a selective antagonist of the hMC1R by using a peptide fragment conformation from agouti signaling protein (ASIP)²⁹ as a template combined with the pharmacophore of γ -MSH. ASIP is an endogenous antagonist of the hMC1R. The key feature of the design is a cyclic constraint via a thioether bridge as shown in Scheme 1. Utilizing the thioether bond constraints, γ -MSH leads to improved receptor (subtype) selectivity with enhanced pharmacological and chemical-physical properties that include metabolic stability, efficacy, lipophilicity, potency and bioavailability. Further, the newly discovered cyclic hMC1R peptide antagonist also was examined for its conformational properties and tested on melanoma cells to evaluate its potential for *in vivo* applications.

Design

The thioether bond has been previously used as a stable disulfide replacement in bioactive cyclic peptides to increase the metabolic stability of peptides.³⁰⁻³² The thioether linkage has also been used to prepare conformationally constrained peptides that are analogues of normally linear peptides.^{18,30,32-36} Cyclic peptides with thioether bonds have been found in nature, especially in a family of antibiotics and lantibiotics³⁷⁻³⁹.

Previously, cyclic thioether peptides have been used as more stable alternatives to disulfide bridges. Thioethers are usually prepared by alkylation of thiols. Thiols are powerful nucleophiles and they react with electrophiles in an S_N2 manner to produce thioethers. Nucleophilic substitutions with thiols do not require the use of strong bases and generally proceeds at room temperature. In favorable cases, thiol alkylation can even be performed at room temperature in an acidic environment.

We have considered the synthesis of macrocyclic thioethers to be a good way for global conformational constraint of the natural ligand γ -MSH, since γ -MSH analogues tend to be more selective than α -MSH from our previous studies²⁰. The new ligands were designed to contain an array of rings with sizes from 21, 22, 23 atoms to form a spectrum of cyclic peptides from less flexible to more flexible analogues. Intramolecular thioether bond

formation was proposed to be a good synthetic method for peptide cyclization and a convenient approach for probing peptide conformations. Published data suggest that a 23-membered ring (e.g. MTII, SHU9119)⁴¹⁻⁴³ is an optimal size for melanotropin ligand binding to the melanocortin receptors. So far, the majority of published work is based on α -MSH cyclic analogues with either lactam or disulfide bridges. The application of thioethers for cyclization of γ -MSH derivatives is a novel structural modification for melanotropin ligands. In order to form a thioether we need to introduce a thiol group into a peptide sequence and a choice for the thiol source was a cysteine residue. The next issue was where to incorporate a cysteine residue in γ -MSH and for that we examined two general ways. We can either replace an amino acid with cysteine or we can insert a cysteine between two amino acids in the natural sequence. Finally, we need to consider a suitable site for an electrophilic moiety. A very simple solution was to apply a bromoacetyl group at the amino terminus of the peptide. We then have two very reactive species, which can form intramolecular thioether bridged structures (Figure 1). The synthetic procedure is shown in Scheme 1. In this approach we used bromoacetic as well as 3-bromopropionic acid and 4-bromobutyric acid so as to obtain cyclic structures of different ring size. (Table 1)

Experimental Section

N^α-Fmoc-amino acids were obtained from Bachem (Torrance, CA), Nova Biochem (Gibbstown, NJ) and AdvancedChemTech (Louisville, KY). Fmoc Rink amide resin was purchased from Polymer Laboratories (Santa Clara, CA). Organic solvents and reagents were purchased from Aldrich and used without further purification. All cyclic peptides were synthesized according to a general protocol outline below.

Rink amide resin (100 mg, 0.065 mmol/g) was placed into a 5 mL polypropylene syringe with the frit on the bottom and swollen in DCM (2mL) for 30 min and in DMF (2 mL) for 30 min. Fmoc protecting group on the Rink linker was removed by 50 % piperidine in DMF. After 20 min the solution of piperidine was removed and the resin was washed with DMF (2 mL, 10 times). Fmoc amino acid (3 equiv., 0.195 mmol) and HOBt (3 equiv., 0.195 mmol) were dissolved in 700 μ L of DMF and then DIC (3 equiv., 0.195 mmol) was added. The coupling mixture was transferred into the syringe with the resin and shaken for 1-3 hours. Coupling completion was monitored with bromophenol blue and ninhydrin tests^{40,58}. Coupling mixture was removed and the resin was washed with DMF (2 mL, 5 times). The Fmoc group was cleaved with 50% piperidine in DMF. Each coupling and deprotection step was repeated until a linear peptide was assembled. Bromoacetic acid (3 equiv. 0.195 mmol) was dissolved in 700 μ L of DMF and DIC (3 equiv., 0.195 mmol) was added. The acylation reaction was usually complete after 20 min. The reaction mixture was removed and resin was washed with DMF (2 mL, 5 times) and DCM (2 mL, 5 times). The monomethoxytrityl group was cleaved by treatment with 2% TFA / 5% TIPS / DCM (2 mL, 5 times, 5 min each treatment). The resin was washed with DCM (2 mL, 10 times) and DMF (2 mL, 5 times). Peptide cyclization was performed in a solution of 5 % DIEA in DMF during 10-12 hours at room temperature. The final wash of the resin was done with DMF (2 mL, 5 times) and DCM (2 mL, 5 times). The product was cleaved from the resin with a mixture of 95% TFA, 2.5 % TIPS and 2.5% water in 1.5 hours. Side chain protecting groups were removed during the cleavage step. The cleavage mixture was evaporated on a rotary evaporator. A crude cyclic peptide was dissolved in acetic acid and purified by HPLC.

TLC was performed using two solvent systems. System I was composed of n-butanol-water-acetic acid-pyridine (4 : 2 : 1 : 2) and system II was n-butanol-water-acetic acid (4 : 1 : 2). The R_f values are listed in the Table 2.

For HPLC samples were dissolved in 10-20% of methanol in water (1 mg/mL) and 5 μ L of that solution was injected onto the analytical HPLC column (YMC-Pack ODS-AM 150 $\times$ 4.6 mm, 3 μ m, 120 $\text{\AA}$). Two buffer systems were used for peptide purity assessment. Solvent system I consists of buffers (A) 0.1% TFA in H₂O and (B) 0.08% TFA in ACN. Solvent system II consists of buffers (A) 20 mM Na₂HPO₄ in H₂O and (B) 20 % ACN in H₂O. The retention times (RT) of cyclic peptides are listed in the Table 2. Peptide purification was achieved using preparative HPLC. A crude product was dissolved in 50% of acetic acid in water (10 mg/mL), filtrated through a minidisk filter and 1 mL of clear solution was loaded onto the semi-preparative column (YMC-Pack ODS-AM, 100 $\times$ 10 mm, 5 μ m, 120 $\text{\AA}$). The solvent system for HPLC purification consists of buffers (A) 0.1% TFA in H₂O and (B) 0.08% TFA in ACN. The solvent gradient was usually 2 % (A) to 40 % (B) over 15 minutes with the flow rate of 10 mL/min. Collected fractions of the purified products were lyophilized.

We used high-resolution mass spectrometry for evaluation of molecular formulas of our novel macrocyclic peptide ligands (Table 2). Both ESI and MALDI were used to generate protonated peptides (Peptides **1-6**), respectively. ESI experiments were carried out on an IonSpec 4.7 T Fourier ion cyclotron resonance (ICR) instrument with peak matching technique by using peptide reference standards. MALDI measurements have been carried out on a Bruker Reflex III MALDI-TOF instrument.

Biological Activity Assays

Receptor Binding Assays

Competition binding experiments were carried out using both of cloned cell line and melanoma cells (A375, ATCC). HEK293 cells stably expressing the human MC1, MC3, MC4, and MC5 receptors were described before^{44,45}. HEK293 cells transfected with hMCRs^{44,45} were seeded on 96-well plates 48 hours before assay (50,000 cells/well). For the assay, the cell culture medium was aspirated and the cells were washed once with a freshly prepared MEM buffer containing 100% minimum essential medium with Earle's salt (MEM, GIBCO), and 25 mM sodium bicarbonate. Next, the cells were incubated for 40 min at 37°C with different concentrations of unlabeled peptide and labeled [¹²⁵I]-[Nle⁴,D-Phe⁷]- α -MSH(Perkin-Elmer Life Science, 20,000 cpm/well, 33.06 pM) diluted in a 125 μ L of freshly prepared binding buffer containing 100% MEM, 25 mM HEPES (pH 7.4), 0.2% bovine serum albumin, 1mM 1,10-phenanthroline, 0.5 mg/L leupeptin, 200 mg/L bacitracin. The assay medium was subsequently removed, the cells were washed once with basic medium, and then lysed by the addition of 10 μ L of 0.1M NaOH and 10 μ L of 1% Triton X-100. The radioactivity of lysed cells was measured by a Wallac MicroBeta TriLux 1450 LSC and Luminescence Counter (PerkinElmer Life Science, Boston, MA).

Adenylate Cyclase Assays

Both melanoma as well as cloned cells were grown to confluence in Dulbecco's Modified Eagle's Medium (DMEM) and MEM medium (GIBCO) respectively 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 hours before assay (50,000 cells/well). For the assay, the cell culture medium was removed and the cells were rinsed with 100 μ L of MEM buffer (GIBCO). An aliquot (100 μ L) of the Earle's balanced salt solution with 5 nM isobutylmethylxanthine (IBMX) was placed in each well along for 1 min at 37°. Next, aliquots (25 μ L) of melanotropin peptides of varying concentration were added, and the cells were incubated for 3 min at 37°C. The reaction was stopped by aspirating the assay buffer and adding 60 μ L ice-cold Tris/EDTA buffer to each well, then placing the plates in a boiling water bath for 7 min. The cell lysates were then centrifuged for 10 min at 2,300 $\times$ g.

A 50 μ L aliquot of the supernatant was transferred to another 96-well plate and placed with 50 μ L [3 H]cAMP and 100 μ L protein kinase A (PKA) buffer in an ice bath for 2-3 hours. The PKA buffer consisted of Tris/EDTA buffer with 60 μ g/mL PKA and 0.1% bovine serum albumin by weight. The incubation mixture was filtered through 1.0 μ m glass fiber filters in MultiScreenTM-FB 96-well plates (Millipore, Billerica, MA). The total [3 H]cAMP was measured by a Wallac MicroBeta TriLux 1450 LSC and Luminescence Counter (PerkinElmer Life Science, Boston, MA). The cAMP accumulation data for each peptide analogue was determined with the help of a cAMP standard curve generated by the same method as described above. IC₅₀ and EC₅₀ values represent the mean of two experiments performed in triplicate. IC₅₀ and EC₅₀ values and their associated standard errors were determined for binding and cAMP assays by fitting the data using a nonlinear least squares analysis, with the help of GraphPad Prism 4 (GraphPad Software, San Diego, CA). The maximal cAMP produced at 10 μ M concentration of each ligand was compared to the amount of cAMP produced at 10 μ M concentration of the standard agonist MT-II, and is expressed in percent (as % max effect) in Table 3. The pA₂ is 8.1.

Data Analysis

IC₅₀ and EC₅₀ values represent the mean of two experiments performed in triplicate. IC₅₀ and EC₅₀ estimates and their associated standard errors were determined by fitting the data using a nonlinear least squares analysis, with the help of GraphPad Prism 4 (GraphPad Software, San Diego, CA).

NMR spectroscopy methods

All NMR spectra were recorded on a Bruker DRX600 600MHz spectrometer at 298°K. Peptide concentration for the NMR experiments is 4.7 mM. The micelle samples were prepared by dissolving the peptide and 50 equivalents of perdeuterated SDS in 0.6 ml of acetate buffer (10 mM) containing 10% D₂O. The pH of the each sample was adjusted to 5.5 by using DCl or NaOD as necessary. Deuterated 3-(Trimethylsilyl)propionic acid (TSP) was added as internal standard for referencing. Two-dimensional nuclear Overhauser enhancement (NOESY) and total correlation spectra (TOCSY) were acquired using standard pulse sequences and processed using XWINNMR (Bruker Inc) and FELIX2000 (Accelrys Inc, San Diego, CA). Mixing time for TOCSY spectra were 60 ms, and for NOESY spectra was 300 ms. All experiments were 750 increments in t₁, 16 or 32 scans each, 1.5 s relaxation delay, size 2 or 4K and the spectral processing was with shifted sine bell window multiplications in both dimensions. The water suppression was achieved by using the WATERGATE pulse sequence. Coupling constants ($^3J_{\alpha\text{H-NH}}$) were measured from DQF-COSY spectrum.

Structure calculation methods

Distance constraints for the structure calculation were obtained from integral volumes of the NOESY peaks. The NOE integral volumes were classified into strong, medium and weak with 3.0, 4.0 and 5.0 Å as upper bound distance. Molecular dynamics simulation was done with the INSIGHT/DISCOVER package (Accelrys Inc, San Diego, CA) with consistent valency force field (CVFF). All the calculations were done *in vacuo*. A distance dependent dielectric constant (2.5r where r is the distance in Å) was used. All peptide bonds were constrained to trans conformation by a 100kcal mol⁻¹ energetic penalty. Distance restraints with a force constant of 25 kcal mol⁻¹ were applied in the form of a flat-bottom potential well with a common lower bound of 1.8 Å and an upper bound of 3.0, 4.0 and 5.0 Å, respectively, in accordance with observed weak, moderate or strong NOE intensities. Only the distance restraints from inter-residue NOEs were included in the calculation. Dihedral angle restraints based on C $^{\alpha}$ H chemical shift index (CSI) were imposed on the residues

displaying negative deviation. Thus for a CSI of >-0.10 ppm, the ϕ and ψ restraints were in the range -90° to -30° and -60° to 0° , respectively while for a CSI of -0.10 ppm, the corresponding ranges were -150° to -30° for ϕ and -90° to 150° for ψ . For D-chiral amino acids the opposite dihedral angle values were used.

Results and Discussions

Bioassay Results

It has been shown that D-phenylalanine is a critical amino acid in the tetrapeptide (Pharmacophore) sequence of γ -MSH to enhance potency, whereas a D-tryptophan improves selectivity⁴¹⁻⁴⁷. We have prepared three peptides with the same sequence containing D-Phe, but with different ring sizes (peptides **1**, **3**, **5**) and three other peptides which have D-Phe and D-Trp (Peptides **2**, **4** and **6**) (Table 1) in their sequences with systematic differences in the size of their rings. The binding affinities and the in vitro cAMP accumulation data for these analogues are summarized in Table 3. Analogue **1** with a 21 member ring was found to be a very weak antagonist ($IC_{50} = 2000$ nM) at the hMC1R, a full agonist at the hMC3R ($EC_{50} = 190$ nM), inactive at the hMC4R, and a partial agonist at the hMC5R ($EC_{50} = 86$ nM, 31% activation). Similar observations were made for analogue **2** which also has a 21 membered ring size. This analogue exhibited weak antagonist activity ($IC_{50} = 1800$ nM) at the hMC1R and was inactive at the hMC3R, hMC4R and hMC5R. It is interesting to note that peptide **2** which has a D-Trp residue, though not potent at the hMC1R, is nonetheless very selective. For analogues **3** and **4**, the ring size was increased by one carbon atom to 22 and this improves binding affinity. Analogue **3** had partial antagonist activity towards the hMC3R ($IC_{50} = 76$ nM, 52% activation). Also, this analogue was found to have no binding to the hMC4R and to be a full but weak agonist at the hMC5R ($EC_{50} = 2500$ nM). Analogue **4** in which Trp was replaced with D-Trp had much improved binding affinity for the hMC1R ($IC_{50} = 30$ nM) with no cAMP accumulation activity (antagonist). This analogue was found to be a very weak partial agonist ($EC_{50} = 1100$ nM, 50% activation) at the hMC3R, modest antagonist activity at the hMC5R, and totally inactive towards the hMC4R. Analogue **5** was very similar to analogue **4** with antagonist activity at the hMC1R ($IC_{50} = 29$ nM) but with partial agonist activity at the hMC3R ($EC_{50} = 100$ nM). This analogue was inactive at the hMC4R but exhibited full agonist activity towards the hMC5R (91% activation). Analogue **6** (Table 3) was the most interesting analogue in this series. It demonstrated potent antagonist activity for the hMC1R ($IC_{50} = 17$ nM). This analogue was inactive at the hMC4R and was a very weak partial agonist at the hMC3R ($EC_{50} = 3900$ nM, 75% activation) and weak partial agonist at the hMC5R ($EC_{50} = 1300$, 65% activation). It is interesting to note that the hMC1R selectivity is very much dependent on the conformational constraint. Thus, cyclic analogues of γ -MSH obtained by incorporating a thioether bridge between a cysteine side chain and an N-terminal bromoacyl group were found to be very critical in forming the right conformation constraint for selectivity and in this study the 23 membered ring was most favorable for hMC1R selectivity (peptide **6**).

NMR studies

To explore the conformation underlying the selective antagonist activity of the hMC1R, we have analyzed the structure of peptide **6** using NMR conformational studies. The spin-system of each amino acid present in the peptide was identified using TOCSY spectra. This information was used with NOESY spectra to determine the sequential assignment of the peptide. The finger-print regions of the TOCSY and NOESY spectra are shown in Figure 2. The $d_{\alpha N}(i, i+1)$ and $d_{\beta N}(i, i+1)$ were helpful in unambiguous sequential assignment of the peptide. The chemical shift values along with $^3J_{\alpha NH}$ and temperature coefficient values are given in Table 4. The observed NOEs are summarized in Figure 3. The peptide displayed

sequential strong $d_{\alpha N}(i, i+1)$ and $d_{\beta N}(i, i+1)$ NOEs throughout the sequence along with relatively weaker $d_{NN}(i, i+1)$ NOEs. Observation of strong consecutive $d_{\alpha N}(i, i+1)$ and relatively weaker $d_{NN}(i, i+1)$ NOEs suggests that peptide undergoes rapid interconversion between extended and folded conformations.⁴⁸⁻⁴⁹ Absence of helix diagnostic $d_{\alpha N}(i, i+3)$ and $d_{\alpha N}(i, i+4)$ NOEs and the observation of four $d_{\alpha N}(i, i+2)$ NOEs and one $d_{NN}(i, i+2)$ NOE suggest multiple turns or a 3_{10} -type helical region in the middle segment of the peptide.

The hydrogen bond information of the peptide can be obtained from the temperature coefficient ($d\delta/dT$) values of the amide protons.⁵⁰ The temperature coefficient values were obtained by recording TOCSY spectrum at different temperatures with 5 °C increments starting from 20 °C because the 1D proton NMR spectrum showed lot of overcrowding in the amide proton region. The observation of diminished temperature coefficient values (i.e. less than -4 ppb/K) for amide protons of His⁵, Trp⁸, Arg¹¹ and Gly¹² indicate that those amide protons are involved in hydrogen bonding or are shielded from the solvent, whereas the other amide protons are exposed to solvent.

It is possible to obtain qualitative structural information from $C^{\alpha}H$ chemical shift values as they are sensitive to the secondary structure of the peptide.⁵¹ It is well established that consecutive negative deviations of $C^{\alpha}H$ chemical shift value from random coil values indicate a helical fold and the positive deviation indicates a β -sheet structure. The chemical deviation plot (aka CSI plot) is shown in Figure 4. Negative deviation was observed for all the residues with interruptions at Trp⁸ and Phe¹¹ suggesting that the residues at the N-terminal possibly having conformational preferences in the helical domain. The positive deviation at Trp⁸ and Phe¹¹ implies a turn type fold around those residues.

Structure calculations

In order to obtain the three-dimensional structure of peptide **6**, a simulated annealing molecular dynamics calculation was carried out by applying distance as well as dihedral angle constraints as described in the methods section. The stereochemical quality of the final energy minimized structures from the calculation was checked by the distribution of backbone dihedral angles phi and psi in the Ramachandran plot⁵² as shown in Figure 5. The distribution range is very narrow for all the amino acids except Asp⁷ and Phe⁹. This clearly suggests that all the residues except Asp⁷ and Phe⁹ are in a rigid conformation. Two distinct populations, namely extended and helical, are experienced by the Asp⁷ and Phe⁹. Interestingly, the dihedral space for the D-Trp⁵ falls in the negative quadrant of the Ramachandran plot which is unexpected for a D-chiral amino acid. The same result was obtained even after the calculation was repeated with strong chiral constraints in place. This may be due to the global constraint introduced by the cyclization.

The lowest energy structure is shown in Figure 6. Consecutive type III β -turns are observed for the middle segment -Arg⁴-DTrp⁵-Cys⁶-Asp⁷-Arg⁸- as indicated by the consecutive $d_{\alpha N}(i, i+2)$ NOEs in this region. As the 3_{10} -type helical dihedral angle values are similar to single type III β -turn dihedral values ($\phi=-60$, $\psi=-30$), this region containing consecutive turns can be described as a 3_{10} helical region. Both termini are flexible in nature despite the cyclization. Previous NMR analysis of shorter analogs related to α -MSH suggested a β -turn like structure at -His⁶-D-Phe⁷-Arg⁸-Trp⁹ but the exact type and position of the β -turn depended on the nature of amino acid substitution in the core pharmacophore region.⁵³ The main difference between the peptide discussed here and those peptides are the C-terminal extension by four additional residues. The C-terminal extension was favorable for the formation of consecutive β -turns, i.e. the short 3_{10} helical type fold. This conformation implies that the C-terminal of the peptide could be a new binding site. The analysis of the backbone dihedral angle values is given in Table 5. Overlay of the five lowest energy

structures from the calculation is shown in Figure 7. The backbone atoms aligned very well, but the side-chains of all the amino acids except His² are flexible. This observation parallels our earlier studies in which the His residue in cyclized peptides can be replaced with different constrained amino acids without interfering with the binding.⁵⁴ The stereo view of the potential new binding site of peptide **6** is shown in Figure 8.

To further explore the structural features of the hMC1R antagonist selectivity of peptide **6**, we have systematically compared the backbone and the side chain conformations of peptide **6** with a recently reported selective hMC1R agonist²⁸ as well as the selective endogenous hMC1R antagonist Agouti Signal Protein: ASIP (PDB: 2KZA)⁵⁵ in detail. The compared dihedral angles are given in Table 6. It is interesting to note that the backbone dihedral angles of the lowest energy conformation of peptide **6** for -D-Trp⁵-Cys⁶-Asp⁷-Arg⁸-Phe⁹-Gly¹⁰- matched the NMR derived pharmacophore of ASIP (117-122) -Arg¹¹⁷-Phe¹¹⁸-Phe¹¹⁹-Arg¹²⁰-Ser¹²¹-Ala¹²²- (Table 6) except for the pair of Asp⁴⁷/Arg¹²⁰. These dihedral angle data demonstrate that the backbone conformation of ASIP (117-122) and the C-terminus of the Peptide **6** are similar. This observation imply that there is a new binding site of the peptide **6** in the region of -D-Trp⁵-Cys⁶-Asp⁷-Arg⁸-Phe⁹-Gly¹⁰- instead of -His-D-Phe-Arg-Trp-. This result is consistent with our earlier work⁴⁷ where a new binding site was observed based on the SAR study of γ -MSH. Comparing the backbone dihedral angles of hMC1R agonist (NMe)MTII²⁸ and peptide **6**, one can see that the major difference is at D-Trp⁵(Peptide **6**) vs Trp⁸ (NMeMTII). Both the Phi and Psi angles of D-Trp⁵/Trp⁸ have a difference of 90-180 deg. Therefore, we conclude that the conformation of D-Trp⁵/Trp⁸ in MSHs is critical for selective agonist/antagonist activity at the hMC1R. Comparison of the Ramachandran plot of Peptide **6**, NMe-MTII and ASIP (117-122) (Figure 9) shows the critical conformation properties of the constrained peptide **6**. The D-Trp configuration falls in the negative quadrant of the Ramachandran plot which is unusual for a D-chiral amino acid because of the topographic constraint, but that is not true in the case of NMe-MTII. The Ramachandran plot of ASIP has dihedral angles on both sides of the L- and D-like configuration, even though there is no D amino acid in this natural protein. The D-like configuration of Phe in the ASIP protein is confined by the disulfide bond and the topographic constraint via the whole protein. Thus, the topographic constraint of ASIP (117-122) directs Phe to play its role for the hMC1R antagonist selectivity. This SAR study is an example of conformational based ligand design that may be generally useful in peptide drug design.

Biological Activity at the melanoma cancer cells

To further investigate the potential application of peptide **6** towards melanoma cells, we did both binding as well as cAMP assays using the A375 melanoma cell lines (ATCC). Table 7 shows that the peptide **6** binds to the melanoma cell receptor in the nano-molar range. It is interesting to note that peptide **6** and SHU9119 act as antagonists towards the melanoma cells. On the contrary, MTII kept its agonist activity, but the cAMP efficacy (EC₅₀) is reduced by two orders of magnitude compared to the cloned hMC1R cells. This may be due to the lower density of the hMC1R in the cancer cell line. Peptide **6** is an excellent probe to target specifically melanoma cells for skin cancer diagnosis and treatment in the future. This observation consistence with the latest report of ASIP that is lack of natural antagonist of hMC1R (ASIP) will have risk of melanoma, ASIP pigmentation variants are associated with cutaneous melanoma and basal cell carcinoma⁵⁷. The new result can also explain why the conformation of peptide **6** for -D-Trp⁵-Cys⁶-Asp⁷-Arg⁸-Phe⁹-Gly¹⁰- matched very well with the NMR derived pharmacophore of ASIP(117-122) -Arg¹¹⁷-Phe¹¹⁸-Phe¹¹⁹-Arg¹²⁰-Ser¹²¹-Ala¹²²-.

In summary, structure-activity relationships (SAR) of cyclic γ -MSH analogues were examined to evaluate the multiple factors that contribute to melanocortin 1 receptor selectivity, and are illustrated by the Figures 2-9 and Tables 2-6. Figures 8 and 9 illustrate the new binding site of gamma MSH, and the *D*-Trp⁵ configuration which contributes to selective antagonist activities. Table 3 compares the binding affinities of the *D*-Trp⁵ analogues having different constraints and extension of the C termini, and thus it establishes a trend towards hMC1R selectivity. Finally, the absence of Nle in the gamma-MSH cyclized analogues leads to weak binding compared to the cyclized peptide with Nle (e.g. MTII) and this is consistent with our earlier findings.⁴⁷ Also, NMR analysis reveals a new binding site which is closely related to that of the endogenous antagonist ASIP (117-122) fragment. Table 7 illustrates the nano-molar binding affinity of this peptide towards melanoma cells (A375) which may be very useful for therapeutics.

Conclusions

SAR and conformational studies of novel cyclized γ -MSH analogues have identified a highly selective hMC1R antagonist (analogue **6**). NMR studies of this constrained peptide reveals a new binding site, -*D*-Trp⁵-Cys⁶-Asp⁷-Arg⁸-Phe⁹-Gly¹⁰- of peptide **6**, which is different from the more general -His-D-Phe-Arg-Trp- pharmacophore of melanocortin receptor agonists. The backbone conformation of peptide **6** matches well with the endogenous antatagonist conformation of the hMC1R selective ASIP (117-122) fragment. In this new binding site, *D*-Trp⁵ and Phe⁹ of Peptide **6** plays a critical role for hMC1R antagonist activity and the residue *D*-Trp⁵ is also crucial for binding to the 6th transmembrane domain (6TM) of hMC1R⁵⁶. The newly developed selective melanotropin peptides will facilitate the elucidation of the physiological roles of the hMC1R in pigmentation and immune response related disorders. Finally, the nano-molar binding affinity of this peptide towards melanoma cells indicate that it can be used as a molecular probe for the diagnosis and treatment of skin cancer. Furthermore, the backbone conformation of Peptide **6** can be used for future melanoma inhibitor discovery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* 1972, 247, 977-983. The following additional abbreviations are used:

AAA	amino acid analysis
Boc	<i>tert</i> -butyloxycarbonyl
Fmoc	fluorenylmethoxycarbonyl
Fmo	fluorenylmethyl
Bzl	benzyl

<i>t</i>Bu	<i>tert</i> -butyl
CH₃CN	acetonitrile
DCM	dichloromethane
DIEA	diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
DIC	diisopropyl carbodiimide
HOBt	N-hydroxybenzotriazole
BOP reagent	(benzotriazolyl)oxy tris-(dimethylamino)-phosphonium hexafluorophosphate
Nal(2')-	2'-naphthylalanine
TFA	trifluoroacetic acid
TIPS	triisopropylsilyl
SPPS	solid-phase peptide synthesis
RP-HPLC	reversed 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
γ-MSH	H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH
ASIP(117-122)	Agouti Signal Protein fragment

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Figure 1.
General scheme for peptide cyclization using sulfide bridge where $n=1, 2$ & 3 and $m=1,2$

Figure 2.

a. Finger-print region TOCSY (left panel) and NOESY (right panel) of peptide peptide **6**.

b. Amide (NH-NH) region of NOESY spectrum showing sequential $d_{NN}(i, i+1)$ NOEs of peptide peptide **6**.

Figure 3.

Summary of observed NOEs and temperature coefficient values measured for peptide peptide **6** in SDS micelles at 25 °C and pH 5.5. Lower case letters denotes *D* chiral amino acids. The thickness of the lines is approximately proportional to the intensity of the NOE cross-peaks.

Figure 4.
Chemical shift deviation of C α H chemical shift values from random coil values.

Figure 5. Phi (Φ) and Psi (Ψ) dihedral angle distribution (i.e. Ramachandran Plot) of each amino acid in peptide **6** for the resultant hundred structures from the simulated annealing molecular dynamics calculations.

Figure 6. Stereo-view representation of lowest energy structure from the simulated annealing molecular dynamics calculations of peptide **6**. The hydrogen atoms are not shown for clarity. The ribbon diagram shows the secondary structure of the peptide.

Figure 7. Stereo-view of overlay of five lowest energy structures resulting from the simulated annealing molecular dynamics calculations of peptide **6**. The hydrogen atoms are removed for clarity. The ribbon diagram shows the secondary structure of the peptide.

Figure 8.
Stereo-view of the lowest energy structure showing the new binding site of the peptide **6** with the broken circle.

Figure 9. Ramachandran Plot of lowest energy conformation of the selective antagonist of hMC1R (peptide **6**) and hMC1R selective agonist N(Me)MTII, and ASIP (117-122)

Scheme 1.

Synthesis of a cyclic analogue of γ -MSH Reagents: (i) assembly of linear peptide by Fmoc strategy; (ii) 20% piperidine in DMF; (iii), 4-bromo butyric acid, DIC, DMF (iv) 2% TFA/ 5% Et₃SiH/CH₂Cl₂; (v) 5% DIEA/DMF; (vi) 95% TFA/5% H₂O

Table 1Novel designed cyclic γ -MSH analogues

LIGAND	SEQUENCE	RING SIZE
Peptide 1	c(CH ₂ -CO-Gly-His-D-Phe-Arg-Trp-Cys)-Asp-Arg-Phe-Gly-NH ₂	21
Peptide 2	c(CH ₂ -CO-Gly-His-D-Phe-Arg-D-Trp-Cys)-Asp-Arg-Phe-Gly-NH ₂	21
Peptide 3	c(C ₂ H ₄ -CO-Gly-His-D-Phe-Arg-Trp-Cys)-Asp-Arg-Phe-Gly-NH ₂	22
Peptide 4	c(C ₂ H ₄ -CO-Gly-His-D-Phe-Arg-D-Trp-Cys)-Asp-Arg-Phe-Gly-NH ₂	22
Peptide 5	c(C ₃ H ₆ -CO-Gly-His-D-Phe-Arg-Trp-Cys)-Asp-Arg-Phe-Gly-NH ₂	23
Peptide 6	c(C ₃ H ₆ -CO-Gly-His-D-Phe-Arg-D-Trp-Cys)-Asp-Arg-Phe-Gly-NH ₂	23

Table 2Analytical characterization of cyclic peptides **1-6**

Peptide No.	Molecular Formula	m/z (M+H ⁺)		HPLC retention time, min ^[A]		TLC R _f ^[B]	
		Calculated	Observed (ESI)	System I ^a	System II ^b	System I ^c R _f	System I ^d R _f
1	<i>C₆₀H₇₈N₂₀O₁₃S</i>	1319.58562	1319.5855	13.71	19.97	0.52	0.17
2	<i>C₆₀H₇₈N₂₀O₁₃S</i>	1319.58562	440.5299 ^a	13.14	18.59	0.51	0.15
3	<i>C₆₁H₈₀N₂₀O₁₃S</i>	1333.60127	1333.6080	13.45	18.25	0.48	0.13
4	<i>C₆₁H₈₀N₂₀O₁₃S</i>	1333.60127	1333.5981	13.09	18.43	0.54	0.20
5	<i>C₆₂H₈₂N₂₀O₁₃S</i>	1347.61692	1347.6152	13.86	18.93	0.49	0.13
6	<i>C₆₂H₈₂N₂₀O₁₃S</i>	1347.61692	1347.6187	13.47	18.73	0.50	0.13

MS: the theoretical and experimental masses for peptides 1-6 obtained by FT-MS analysis. ^a[M+3H]³⁺ HPLC: gradient: 10-90% B in A over 40 min, flow rate 1.0 mL/min;

^aSystem I: Buffer A: 0.1% TFA in H₂O, Buffer B: 0.08% TFA in ACN (pH 3);

^bSystem II: Buffer A: 20mM Na₂HPO₄ in H₂O, Buffer B: 20% H₂O in ACN (pH 6.3) TLC:

^cSystem I: n-butanol / water / acetic acid / pyridine (4:2:1:2) pH 6;

^dSystem II: n-butanol / water / acetic acid (4:1:2) pH 2.5

Table 3
Binding and cAMP assays of novel cyclized peptides towards human melanocortin receptors

Code	hMC1R			hMC3R			hMC4R			hMC5R		
	IC ₅₀ (nM)	EC ₅₀ (nM)	Act%	IC ₅₀ (nM)	EC ₅₀ (nM)	Act%	IC ₅₀ (nM)	EC ₅₀ (nM)	Act%	IC ₅₀ (nM)	EC ₅₀ (nM)	Act%
1	2000	>1000	/	1630±300	195±23	140	N.B.	800±90	37	510±60	86±10	31
2	1800	N.A.	/	NB	>1000	30	N.B.	N.A.	/	N.B.	>1000	0
3	330±60	N.A.	10	76±10	170±30	52	N.B.	N.A.	/	160±20	2500	100
4	30±4	N.A.	/	17000	1100±120	50	N.B.	N.A.	NA	115±10	1000	0
5	29±3	N.A.	/	8500	100±14	50	N.B.	N.A.	/	210±30	873±100	91
6	17±2	N.A.	/	3900	59±6	75	N.B.	N.A.	/	1000±110	1300	65
Nle ³ γ-MSH	60±5	80±7	100	62±8	1000±100	100	45±5	70±7	100	640±100	82±8	100
Nle ³ γ-MSH-NH ₂	--	--	--	45±7	1.6±0.2	100	64±7	34±4	105	200±23	99±10	114
γ-MSH	1000±20	300±30	100	71±10	700±89	100	760±80	710±70	100	2200±200	550±60	100

IC₅₀ = concentration of peptide at 50% specific binding (N=4). NB = 0% of [¹²⁵I]-NDP-α-MSH displacement observed at 10 μM. EC₅₀ = Effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation (N=4). Act% = % of cAMP produced at 10 μM ligand concentration, in relation to MT-II. NA = 0% cAMP accumulation observed at 10 μM. The peptides were tested at a range of concentration from 10⁻¹⁰ to 10⁻⁵ M; NB = no binding observed at 10⁻⁵ M.

Table 4

Chemical shift values (ppm) of peptide **6** as measured in SDS micelles at 6.11 mM concentration, pH=5.5 and at 25 °C.

Residue	NH	C ^α H	C ^β H	Δδ ppm	dδ/dT (-ppb/°K)
Gly (1)	8.088	3.782		-0.228	5.40
His (2)	7.946	4.637		-0.133	2.82
Phe (3)	8.235	4.551	2.922 3.097	-0.069	4.90
Arg (4)	7.734	4.118		-0.222	4.94
Trp (5)	8.034	4.729	3.243 3.455	0.059	3.88
Cys (6)	8.120	4.348	2.847 2.972	-0.232	4.26
Asp (7)	8.398	4.606	2.979 2.860	-0.024	5.02
Arg (8)	7.894	4.057		-0.283	3.08
Phe (9)	7.862	4.675	2.976 3.291	0.055	4.64
Gly (10)	8.039	3.866 3.949		-0.103	3.54

Table 5

Phi and Psi angle distribution of peptide **6** from simulated annealing molecular dynamics study. The standard deviation from the average value is given in parenthesis and deviations greater than 30 are highlighted by bold.

Amino acid	All hundred structures		Lowest energy structure	
	Phi (ϕ)	Psi (ψ)	Phi (ϕ)	Psi(ψ)
Gly 1	-72 (7)	-64(3)	-75	-67
His 2	-83(7)	-68(2)	-91	-69
<i>D</i> Phe 3	34(21)	92(2)	34	90
Arg 4	-47(8)	-60(3)	-55	-65
<i>D</i> Trp 5	-26(3)	-60(3)	-28	-61
Cys 6	-42(7)	-50(4)	-49	-46
Asp 7	-87(33)	-45(65)	-70	-59
Arg 8	-66(9)	-43(9)	-68	-33
Phe 9	-107(21)	70(52)	-116	82
Gly 10	-88(3)	-62(1)	-82	-62

Table 6

Comparison of the pharmacophore of the NMR structures of (NMe)-MTII, Peptide 6 and ASIP (117-122): Backbone torsion angles (deg) for the NMR structures of the peptide backbone conformation

Compound	Nle ³		Asp ⁴ /Gly ¹		His ⁵ /His ²		DPhe ⁶ /DPhe ⁴		Arg ⁷ /Arg ¹¹⁷		Trp ⁸ /DTrp ⁵ /Phe ¹¹⁸		Lys ⁹ /Cys ⁶ /Phe ¹¹⁹		Asp ⁷ /Arg ¹²⁰		Arg ⁸ /Ser ¹²¹		Phe ⁹ /Ala ¹²²	
	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ	φ	ψ
(NMe)MTII	-101	109.1	71.4	143.7	-97.8	78.4	96.1	-125.9	-135.2	80.4	-119.8	62.6	-113.7	0.4						
Peptide 6			-75.3	-67.3	-90.9	-69.5	34.1	90.1	-54.8	-64.8	-28.4	-61.1	-45.8	-45.8	-70.3	-59.5	-68.4	-33.5	-115.6	82.0
ASIP ¹¹⁷⁻¹²²									-115.4	-171.7	-41.4	-56.5	-48.8	-45.9	63.5	18.4	-118.2	-24.5	-119.5	64.6

Note: Number labeled for α-MSH analogues shown as red N(Me)MTII (Ac-Nle³-c[(NMe)Asp⁴-N(Me)His⁵-DPhe⁶-N(Me)Arg⁷-N(Me)Trp⁸-Lys⁹]-NH₂; Peptide 6 (cyclo-[(CH₂)₃CO-Gly¹-His²-D-Phe³-Arg⁴-D-Trp⁵-Cys(S-⁶)-Asp⁷-Arg⁸-Phe⁹-Gly¹⁰-NH₂) shown as blue; ASIP loop -Arg¹¹⁷-Phe¹¹⁸-Phe¹¹⁹-Arg¹²⁰-Ser¹²¹-Ala¹²² shown as green.

Table 7Binding and cAMP activities of Peptide **6** at human melanoma cells (A375)

Compound	Structure	IC ₅₀	Binding Efficiency	EC _{50t}	Act%
Peptide 6	c[(CH ₂) ₃ -Gly-His-D-Phe-Arg-D-Trp-Cys(S-)]- Asp-Arg-Phe-Gly-NH ₂	92 ± 65	98 ± 41	22 ± 2.5	NA
MT-II	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH ₂	6.7 ± 2.9	100 ± 10	110 ± 0.6	100
SHU9119	Ac-Nle-c[Asp-His-D-Nal(2')-Arg-Trp-Lys]-NH ₂	100 ± 96	80 ± 34	3.3 ± 2.6	NA

IC₅₀ = concentration of peptide at 50% specific binding (*N*=4). Binding Efficiency = maximal % of [¹²⁵I]-NDP-α-MSH displacement observed at 10 μM. EC₅₀ = Effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation (*N*=4). Act% = % of cAMP produced at 10 μM ligand concentration, in relation to MT-II. NA = 0% cAMP accumulation observed at 10 μM. The peptides were tested at a range of concentration from 10⁻¹⁰ to 10⁻⁵ M.