Research Article

Journal of PeptideScience

Received: 2 April 2010 Revised: 26 August 2010 Accepted: 26 August 2010 Published online in Wiley Online Library: 26 October 2010

(wileyonlinelibrary.com) DOI 10.1002/psc.1306

A retro-inverso α-melanocyte stimulating hormone analog with MC1R-binding selectivity

Timothy Weeden,[a]*‡ Jim Stefano,[b]‡ Su Duan,[a] Andrea Edling,[c] Lihui Hou,[b] Wei-Lien Chuang,[b] Michael A. Perricone,[c] Clark Pan[b] and John L. Dzuris[a]

α-melanocyte stimulating hormone (α-MSH) is a tridecapeptide fragment of pro-opiomelanocortin (POMC) with broad effects on appetite, skin pigmentation, hormonal regulation, and potential roles in both inflammation and autoimmunity. The use of this peptide as an anti-inflammatory agent is limited by its low selectivity between the melanocortin receptors, susceptibility to proteolytic degradation, and rapid clearance from circulation. A retro-inverso (RI) sequence of α-MSH was characterized for receptor activity and resistance to protease. This peptide demonstrated surprisingly high selectivity for binding the melanocortin receptor 1 (MC1R). However, RI-α-MSH exhibited a diminished binding affinity for MC1R compared to α-MSH. Mapping of the residues critical for agonist activity, receptor binding, and selectivity by alanine scanning, identified the same critical core tetrapeptide required for the native peptide. Modest improvements in affinity were obtained by conservative changes employing non-natural amino acids and substitution of the *C*-terminal sequence with a portion of a MC1R ligand peptide previously identified by phage display. Recombination of these elements yielded a peptide with an identical K_i as α-MSH at MC1R and a lower EC_{50} in Mel-624 melanoma cells. A number of other structural modifications of the RI peptide were found to differ in effect from those reported for the L-form α-MSH, suggesting a significantly altered interaction with the MC1R.

Supporting information may be found in the online version of this article

Keywords: MSH; retro inverso; peptide; analog; autoimmunity; anti-inflammatory

Introduction

Pro-opiomelanocortin (POMC) peptides have been shown to activate adenylate cyclase upon binding specific G-protein coupled receptors (GPCRs) and accumulate intracellular cyclic adenosine monophosphate (cAMP). α-melanocyte stimulating hormone (α-MSH), a tridecapeptide fragment of POMC, has been shown to bind to a specific subset of GPCRs, the melanocortin receptors (MC1R, MC2R, MC3R, MC4R, and MC5R). This family of GPCRs has a broad array of physiological functions and exerts their effects through differential expression in different cell types. Among these receptors, MC1R and MC3R have been identified as the key receptors in the anti-proliferative effects on mononuclear phagocytes and B lymphocytes *in vitro* [1]. Activation of NF-κβ in response to a number of pro-inflammatory stimuli has been shown to be suppressed by α-MSH in a cAMP-dependent manner [2]. NF-κβ has been shown to upregulate pro-inflammatory cytokines, cell surface receptors involved in immune regulation, and synthesis of tissue destructive factors such as nitric oxide [3,4].

Localized immunosuppressive functionality of α-MSH has been demonstrated in the C57BL/6, Balb/C, and Bleomycin (BLM) mouse models with regard to contact hypersensitivity, antigen tolerance, and reduced tissue fibrosis in scleroderma, respectively [5–7]. Even though α-MSH has some effects as an anti-inflammatory agent, its use as a systemic therapeutic agent is not appealing because of undesirable effects on hormonal regulation. Energy homeostasis through MC4R agonism results in appetite suppression, increased lipid metabolism, and weight loss [8,9]. In addition, bioavailable small molecule MC4R antagonists have shown efficacy in erectile dysfunction [10]. Exocrine hormone expression can also be agonized by α-MSH binding to the MC5R [11]. However, a selective MC1R partial agonist with sub-μM affinity was shown to suppress tumor necrosis factor-alpha (TNF-α) production in response to lipopolysaccharide (LPS) *in vivo* [12], generating interest in MC1R as a potential target for anti-inflammatory agents. The potential therapeutic applications of α-MSH have been reviewed [13]. However, a major hindrance for use of the native neuropeptide as a therapeutic is its rapid degradation by proteases *in vivo* [14].

Modifications to α-MSH have been described in the literature for the development of both agonists and antagonists with selectivity for individual melanocortin receptors. Specifically, MC1R binding by α-MSH has been targeted by substituting *N*-terminal aliphatic and aromatic compounds, D-amino acid substitutions within the

* *Correspondence to: Timothy Weeden, Genzyme Corporation, 49 New York Avenue, Framingham, MA 01701, USA.*
E-mail: timothy.weeden@genzyme.com

a *Transplant and Immunology Research, 49 New York Avenue, Framingham, MA 01701, USA*

b *Genzyme Corporation, Department of Therapeutic Protein Research, 1 Mountain Road, Framingham, MA 01701, USA*

c *Immunotherapy Research, 49 New York Avenue, Framingham, MA 01701, USA*

‡ *Authors with equal contribution.*

core tetrapeptide His-Phe-Arg-Trp, and replacement of multiple *N*-terminal residues by a sequence identified through phage display [15–18]. A picture of the α-MSH binding site of MC1R has evolved, including potential polar or dipole interactions, hydrogen bonding, and $\pi-\pi$ interactions with an alkyl aromatic acid [16]. These results also revealed that MC1R, MC3R, and MC4R share an open hydrophobic binding site, but MC1R possesses a unique aromatic π-binding site spaced four carbons from the *N*-terminus of the core tetrapeptide. A full agonist was obtained that showed a high affinity (0.01 nM) and selectivity for MC1R by appending a phenylbutyryl group *N*-terminal to a D-Phe substituted core tetrapeptide [16].

High specificity while retaining sub-nanomolar affinity to the MC1R has been achieved through substitution of the SYSME amino acid sequence with the SSIIS sequence at the *N*-terminus of α-MSH [18]. The effect of this sequence modification representing substitution with neutral or hydrophobic for aromatic or charged amino acids was shown to be dependent on the transmembrane (TM) domains 1–3 and 6–7 of MC1R [19]. Together, these observations suggest significant differences in the binding pockets of the different melanocortin receptors and provide potential means for increasing the selectivity for MC1R.

We prepared a D-amino acid, reverse-sequence peptide of the native α-MSH [retro-inverso (RI)-α-MSH] in an effort to confer protease resistance and biological stability while approximating the structure and activity of the native peptide. Recognition and biological activity of several RI (e.g. retro-enantio) peptides have been described as similar to their parent peptides [20–22]. Although alteration of the backbone (from CO–NH to NH–CO) blocks the susceptibility to protease, reversal of the sequence allows the side chains to approximate their spatial orientation in the native structure [20,23,24]. However, significant structural differences may occur with consequential effects on receptor affinity and activation [25]. RI of two α-MSH analogs, SHU9119 and Melanotan-II has been reported to have a strongly adverse effect on binding and activation of MC3R, MC4R, and MC5R [26], suggesting this may be a common effect at all of the melanocortin receptors (MCRs). We report here the properties and effects of sequence and structural modifications on the receptor selectivity, affinity, and agonist activity of a RI version of α-MSH.

Materials and Methods

Peptide Synthesis

Peptides were synthesized using a Ranin Symphony automated peptide synthesizer using standard Fmoc-chemistry on rink amide resin. Amino acids (EMD Biosciences, San Diego, CA, USA or Anaspec, San Jose, CA, USA) were orthogonally protected with *tert* BOC, *tert*-butyl (tBu), 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl(Pbf), or trityl(Trt) groups. Couplings were performed using an amino acid/HBTU/HOBt/DIEA/resin molar ratio of 6/6/3/12/1. Twenty percent piperidine in N,N-Dimethylformamide (DMF) was used to remove Fmoc from amine terminus during each cycle. *N*-terminal acetylation was performed on resin using acetic anhydride/N-Methylmorpholine/resin in DMF in a molar ratio of 30/8/1. Deprotection/cleavage from resin was performed using a cocktail of 15 ml/0.1 mM resin of 2.5% water/2.5% Triisopropylsilane/5% anisole/90% TFA v/v ratio for 3 h. Supernatant was precipitated in diethyl-ether (−80 °C) and pelleted at 3000 × g for 10 min. Ether was decanted and pellet was washed again. Crude peptide was lyophilized and purified using semi-preparative RP-HPLC column, XBridge C18, 10 × 250 mm, 5 µm particle size (Waters, Milford, MA). Purification was performed at 5 ml/min with a load gradient of 99% buffer-A (water, 0.1% TFA) and 1% buffer-B (Acetonitrile, 0.1% TFA) (gradient slope, 0.3% B/min). Peptide purity >95% was evaluated by analytical RP-HPLC and MALDI-TOF. Fractions were pooled, lyophilized, and redissolved in appropriate assay buffers. Peptides 847int, 880, 881, and 882 were used desalted because of hydrophobicity or material limitations.

Membrane Preparations

Membrane preparations for MC3R, MC4R, and MC5R were obtained from Perkin/Elmer Corp (Waltham, MA, USA). A human MC1R-transfected cell line (ASC0179) was obtained from Atto Biosciences (Rockville, MD, USA) and used for preparation of MC1R membranes. Briefly, cells grown to confluence were lifted using versene and washed with phosphate buffered saline (PBS). The cell pellet was swollen with 1 mM EDTA, 5 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) pH7 with 1 × complete protease inhibitors (Roche) and Dounce homogenized. The lysate was mixed with equal volume 0.5 M sucrose, 1 mM EDTA, 5 mM HEPES pH7 with protease inhibitors and the mixture cleared by centrifugation at 500*g*. The pellet was then rehomogenized and washed with 0.25 M sucrose/HEPES/EDTA. The wash and first supernatants were combined and centrifuged 30 min at 21,000*g*. The pellet was rehomogenized with 1 volume 0.25 M sucrose/HEPES/EDTA and stored at −80 °C before use.

Receptor Binding Assay

Peptides were diluted in binding buffer [25 mM HEPES pH7, 1.5 mM $CaCl_2$, 1 mM $MgSO_4$, 0.1 M NaCl, 1 mM 1,10-*O*-phenanthroline, 0.2% BSA, and 1 × complete protease inhibitor (Roche, Indianapolis IN, USA)]. Diluted peptides (25 µl) were mixed with equal volumes of 0.75 nM [^{125}I]-[Nle^4, D-Phe^7]α-MSH (^{125}I-NDP-MSH) (Perkin-Elmer, Waltham MA, USA) and receptor (1–3 fmol) in binding buffer and incubated 1 h at 37 °C. The reaction mixtures were passed through glass fiber (GFC)/polyvinylidene fluoride (PVDF) filters (Multiscreen HTS/FC, Millipore Corp, Billerica, MA, USA) preblocked with 0.5% polyethyleneimine. The filters were washed with binding buffer without BSA, dried, and counted in scintillation fluid. Nonspecific binding was determined by competition with 3 µM unlabelled NDP-MSH (Anaspec, Freemont, CA). EC_{50} values were determined through Prism software (Irvine, CA, USA) using best-fit value of log EC_{50} with its standard error and 95% class interval (CI). K_d values for [^{125}I]-NDP-MSH for MC3R, MC4R, and MC5R were 0.24 nM, 0.17 nM, and 1.5 nM, respectively which was determined by Perkin/Elmer for the membrane preps. K_d value for [^{125}I]-NDP-MSH for MC1R = 0.085 nM taken from Schioth *et al.* (1995). The Cheng–Prusoff equation in Prism software was used to calculate K_i from the competition curves. $K_i = EC_{50}/[1 + ([L]/K_d)]$ where $[L]$ is the concentration of radioligand and K_d is the equilibrium dissociation constant for the radioligand. Binding assays were performed in triplicate for each experiment. Three separate experiments performed for analogs with K_i values equal to or with better affinity than RI-α-MSH.

Cell Culture

Human melanoma M624 cell line derived from surgically removed metastatic lesions [27] or B16-F10 melanoma cell line from the American Type Culture Collection (ATCC) were grown in Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA) containing 4.5 g/l of D-glucose, 110 mg/l sodium pyruvate,

Journal of PeptideScience

L-glutamine with 1% pen strep. Cells were cultured M624 (1×10^4 cells/well/200 µl) or B16-F10 (5×10^4 cells/well/200 µl) in 96 well (tissue culture grade) plates and incubated at 37 °C, 5% CO_2 and 95% humidity.

Cyclic Adenosine Monophosphate Assay

Intracellular cAMP levels were measured using a direct enzyme immunoassay kit (EIA) (GE Healthcare, Piscataway, NJ). Briefly, M624 human melanoma line (1×10^4 cells/well) or B16-F10 murine melanoma line (5×10^4 cells/well) was pelleted at 1000 g, decanted, and resuspended in 150 µl of 1 mM IBMX inhibitor (Sigma, St. Louis, MO) in serum free media. Cells were incubated at 37 °C for 60 min then stimulated with α-MSH peptide or analogs for 30 min. Forskolin (Sigma, St. Louis, MO) and a scrambled-sequence RI-MSH peptide were used as positive and negative controls, respectively. After incubation, cells were pelleted and resuspended in lysis buffer, and 100 µl of each sample was combined with the specific antiserum, cAMP-peroxidase conjugate, and a colorimetric enzyme substrate according to the manufacturer's instructions. The final reaction was halted with 1.0 M sulfuric acid and optical density was determined using a plate reader at 450 nm. A standard curve was used to assess cAMP concentrations between 12.5 and 3200 fmol/well. Assays were performed in triplicate in at least three independent experiments.

Plasma Stability

Aliquots (30 µl) of normal mouse (C57BL/6N) plasma or sterile PBS combined with 75 µl of 100 µg/ml RI-α-MSH or α-MSH in PBS + 2% DMSO were incubated at 37 °C. Following incubation, 9 µl of bradykinin solution (1 mg/ml in PBS, used as internal standard) and 228 µl of acetonitrile were added. The tubes were vortexed continuously for 1 min and then centrifuged for 5 min at 12,000g. The supernatants were transferred to HPLC vials and frozen at −80 °C until LC/MS/MS analysis.

LC/MS/MS was performed on an Agilent 1100 HPLC coupled to an Applied Biosystems API4000 triple quadrupole mass spectrometer operating in positive electrospray ionization mode. Twenty microlitre of each sample were injected on to a 15 cm × 4.6 mm, 5 µm supelco ascentis C18 column. The chromatographic separation was achieved using a solvent gradient employing the 100% aqueous mobile phase A with 0.5% formic acid and 5 mM ammonium acetate and 95 : 5 acetonitrile/water mobile phase B with 0.5% formic acid and 5 mM ammonium acetate. Following sample injection, the mobile phase was changed from 98% A to 30% A over 1 min. Another gradient to 0% A at 7.5 min was followed by a ballistic gradient to 98% A at 8.5 min. The mobile phase was held at this composition for 1.5 min for column equilibrium. The flow rate was 1 ml/min and the column was maintained at room temperature.

The multiple reaction monitoring (MRM) mode was used for quantitation. Internal standard concentration in the calibration curve is 1316 ng/ml. Calibration curve in the working range from 0 to 6100 ng/ml was assessed with R^2 values >0.99. For MRM, we used doubly charged precursor and singly charged product ion pairs of m/z 833.3 >762.5 for both α-MSH and RI-α-MSH. Each sample was run in duplicate.

Pharmacokinetics

Pharmacokinetic analysis was performed by bolus IV administration (5 mg/kg) of α-MSH or RI-α-MSH to C57 BL mice (n = 4 mice/group). Samples were drawn at seven time points (10, 30, 60, 120, 240, 480, and 1440 min) and centrifuged at 1000 × g for 15 min to remove plasma and stored at −80 °C until processed. Aliquots were thawed, diluted into PBS, spiked with bradykinin internal control and processed as described above (see Section on Plasma Stability). Duplicate runs on LC-MS/MS for each sample gave eight serum concentration values at each time point for each peptide administered.

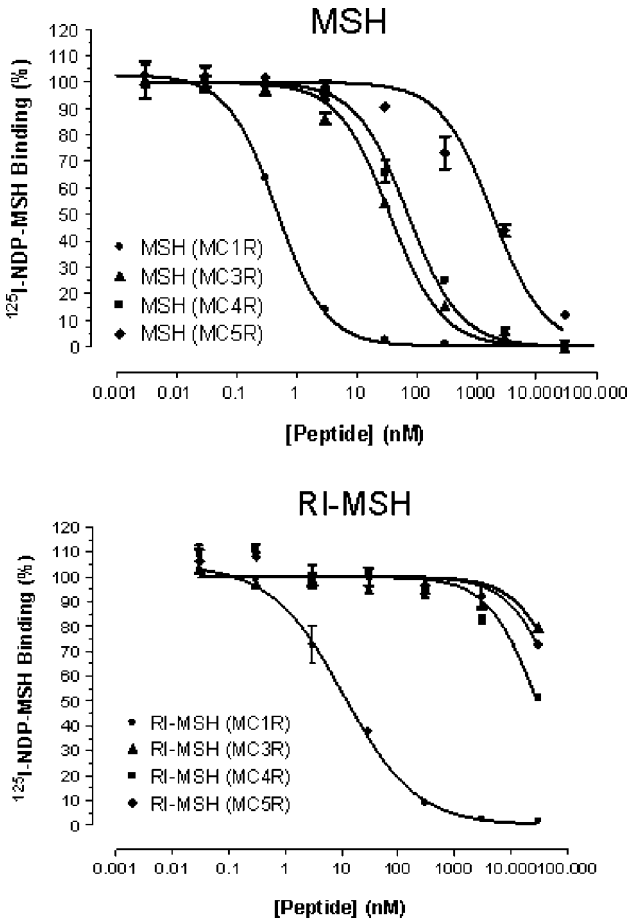

Figure 1. Melanocortin binding of α-MSH and RI-α-MSH. Competitive inhibition of ^{125}I-NDP-MSH to specific melanocortin membrane preps by increasing the concentration of non-isotope labeled peptide. CPM (counts per minute) values were normalized and IC_{50} best-fit values were calculated by Prism software. This is one representative experiment of three independent experiments performed in triplicate.

Results

RI-α-MSH Binding to Melanocortin Receptors *in vitro*

After synthesis and purification of the RI-α-MSH peptide (peptide 720, Table 2), binding at MC1R, MC3R, MC4R, and MC5R was determined by competition assay using membrane preparations from transfected HEK293 cells (See Materials and Methods). As shown in Figure 1, RI-α-MSH was bound by MC1R (K_i = 4.6 nM) although with ~tenfold lower affinity than the parent α-MSH (K_i = 0.41 nM). Unexpectedly, the RI peptide also showed a high selectivity, with binding to MC3R, MC4R, and MC5R >5000-fold weaker than at MC1R. Relative to α-MSH, RI led to a >1000-fold reduction in binding to MC3R and MC4R, and > tenfold

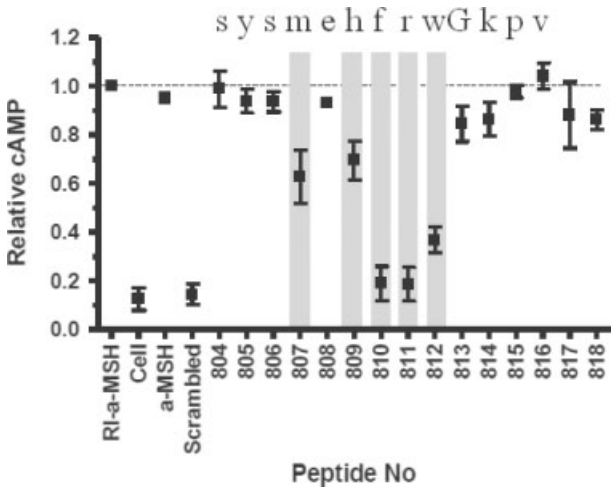

Figure 2. Intracellular cAMP generated in the human melanoma cell line Mel-624 after 30-min stimulation with peptide (10 μg/ml) or forskolin (100 μM). Relative cAMP = measured cAMP(fmol)/cAMP(fmol) of RI-α-MSH. Scrambled: Scrambled sequence of the D-amino acid MSH (Table 1). Values shown are mean of three independent experiments ± standard error of mean (SEM). Shaded grey bars represent critical MC1R binding residues.

Table 1. Alanine substituted peptides designed to measure loss of cAMP as an indicator of essential MC1R binding residues

Number	Sequence	Molecular weight
MSH	Ac-SYSMEHFRWGKPV-amide	1664.9
RI-MSH (720)	Ac-vpkGwrfhemsys-amide	1664.9
804	Ac-vpkGwrfhemsya-amide	1648.9
805	Ac-vpkGwrfhemsas-amide	1572.8
806	Ac-vpkGwrfhemays-amide	1648.9
807	Ac-vpkGwrfheasys-amide	1604.8
808	Ac-vpkGwrfhamsys-amide	1606.8
809	Ac-vpkGwrfaemsys-amide	1598.8
810	Ac-vpkGwrahemsys-amide	1588.8
811	Ac-vpkGwafhemsys-amide	1579.8
812	Ac-vpkGarfhemsys-amide	1549.7
813	Ac-vpkawrfhemsys-amide	1678.9
814	Ac-vpaGwrfhemsys-amide	1607.8
815	Ac-vakGwrfhemsys-amide	1638.8
816	Ac-apkGwrfhemsys-amide	1636.9
817	Ac-aaaGwrfhemsys-amide	1553.7
818	Ac-kkkGwrfhemsys-amide	1725.0
Scrambled	Ac-fsesGvkmprywh-amide	1664.9

reduction in binding to MC5R (K_i > 30 μM, >30 μM, and ~25 μM, respectively). In contrast, α-MSH showed significant binding to the other receptors and only a 50- to 100-fold selectivity for MC1R relative to binding at MC3R and MC4R (Figure 1, Table 2).

Critical Residues for the MC1R Interaction

The reduction in binding at MC1R suggested that RI may have an effect on cAMP induction. The Mel-624 human melanoma cell line (M624) expressing MC1R by both serial analysis of gene expression (SAGE) and fluorescence activated cell sorting (FACS) analysis (data not shown) was used to test cAMP induction by MC1R binding. In contrast to the EC_{50} data, at 10 μM, RI-α-MSH produced an equivalent cAMP response as α-MSH (Figure 2). The residues important for agonist activity were then mapped by following the effects of single alanine substitutions on cAMP induction (Figure 2, Table 1). Significant reductions were observed by substitution at positions 5–8 and 10 (w, r, f, h, and m). However, alanine replacements at other positions in the peptide including the *C*-terminal vpk had no significant effect (Figure 2). Substitution of the *C*-terminal vpk with either ala_3 or lys_3 (peptides 817 or 818) (Table 1) also had at most minor effects. Similar results were obtained in B16-F10 murine melanoma cells (Figure S2, Supporting Information).

The subset of the alanine replacements which showed decreased cAMP induction (peptides 807–812) were then evaluated for binding to MC1R (Table 2). Alanine substitution at each of these positions had a strong effect as previously reported for the L-form α-MSH [18]. The largest reductions in binding (>650-fold) were observed by substitutions of the Trp^5, Arg^6, and Phe^7, while substitutions of His^8 or Met^{10} resulted in over a 250-fold reduction. The rank order of cAMP inhibition at Met^{10}, Glu^9, His^8, and Trp^5 correlated with the K_i for binding at MC1R, suggesting the cAMP response was MC1R-specific. Similar results were obtained in the murine melanoma B16-F10 cell line, except that cAMP was more strongly inhibited by Trp^5 modification (Figure S2).

Amino Acid Analog Substitutions

In an effort to improve the affinity of RI-α-MSH for MC1R, a library with single amino acid substitutions by structurally related and non-natural amino acids for the critical residues identified by alanine substitution was generated. The library was analyzed for MC1R binding (Table 2), and a subset screened for cAMP induction (Figure 3). These results showed that RI-α-MSH tolerates only highly conservative changes. All but three single amino acid substitutions in the analog peptides resulted in reduced or equivalent binding to MC1R compared to RI-α-MSH (Table 2). Two modifications resulted in small but reproducible improvement in binding. The most beneficial substitution was the replacement of the D-Phe with D-cyclohexylalanine (D-Cha) (K_i = 2.2 nM) which retained full agonist activity (>80% of α-MSH). In contrast, the analogous change in native α-MSH had a neutral or slightly inhibitory effect on binding at MC1R (0.51 nM vs 0.41 nM, peptide #892). A twofold increase in MC1R binding affinity was also observed by substitution of the D-Met with D-Buthionine (peptide 878, K_i = 2.3 nM) but without an increase in cAMP. Incorporation of both the D-Cha and D-Buthionine modifications into a single peptide, however, led to only a slight further increase in affinity (890, K_i = 1.9 nM) with no difference in cAMP relative to the RI-α-MSH control. Two analogs, (#858, D-Lys → D-Nle; #868, D-Phe → D-Thi) showed significantly weaker binding but with elevated cAMP, however, they were found to be variable in their response between experiments (Figure 3).

Inversions within the Core Tetrapeptide Element

We used α-MSH modifications known to increase MC1R binding and selectivity to validate our assays as well as compared their effects when applied to RI-α-MSH. A core α-MSH tetrapeptide containing a D-Phe substitution with the sequence (HfRW) has been shown to bind with moderate affinity to both murine [28], (EC_{50} = 20 nM) and human MC1R [16], (EC_{50} = 32 nM). However, we found that a RI version (wrFh, #883) was not bound significantly by the human MC1R (K_i > 100 μM, Table 2). Inversion of the D-Phe

Journal of PeptideScience

Table 2. Melanocortin receptor binding data for RI-α-MSH Analogs

No.	Sequence	Mol. Wt	K_i (nM, except as noted) MC1R	MC3R	MC4R	MC5R
MSH	Ac-SYSMEHFRWGKPV-amide	1664.9	0.41 ± 0.14	23	41	~1500
RI-MSH (720)	Ac-vpkGwrfhemsys-amide	1664.9	4.6 ± 1.3	>30uM	>30uM	~25uM
807	Ac-vpkGwrfheasys-amide	1604.8	1400 ± 190			
808	Ac-vpkGwrfhamsys-amide	1606.8	2.5			
809	Ac-vpkGwrfaemsys-amide	1598.8	1200 ± 270			
810	Ac-vpkGwrahemsys-amide	1588.8	>30uM			
811	Ac-vpkGwafhemsys-amide	1579.8	13 ± 7 uM			
812	Ac-vpkGarfhemsys-amide	1549.7	3 uM			
819	$(Ph(CH_2)_3CO)$-HfRW-amide	789.9	0.07	277	27	~3000
820	Stearyl-HfRW-amide	910.2	1.3	1371	390	860
847	Ac-vpkGwrfh-C_3-Phenyl	1185.5	104 ± 72	660	750	540
847int	Ac-vpkGwrfh	1068.2	150			
857	Ac-vp(D-Orn)Gwrfhemsys-amide	1650.9	3.0			
858	Ac-vp(D-Nle)Gwrfhemsys-amide	1649.9	18			
859	Ac-vpkG(D-Thi(3-Benzo))rfhemsys-amide	1681.9	206			
860	Ac-vpkG(5-Hydroxy-D-Trp)rfhemsys-amide	1680.9	96			
861	Ac-vpkG(5-Methoxy-D-Trp)rfhemsys-amide	1694.9	75			
862-b	Ac-vpkGfrfhemsys-amide	1625.9	128			
863	Ac-vpkGwqfhemsys-amide	1636.8	>100uM			
864	Ac-vpkGwnfhemsys-amide	1622.8	>30uM			
865	Ac-vpkGwhfhemsys-amide	1645.9	2300			
866	Ac-vpkGwr(D-Phg(4-F)hemsys-amide	1667.9	>30uM			
867	Ac-vpkGwr(D-Pal(3))hemsys-amide	1664.9	480			
868	Ac-vpkGwr(D-Thi)hemsys-amide	1670.0	5.4			
869	Ac-vpkGwr(D-Cha)hemsys-amide	1670.9	2.2 ± 0.44	>30uM	>10uM	~25uM
870	Ac-vpkGwrwhemsys-amide	1703.9	~2.5uM			
871	Ac-vpkGwr(D-Phe(4-NO_2)hemsys-amide	1709.9	324			
872	Ac-vpkGwrfremsys-amide	1683.9	13.5			
873	Ac-vpkGwrfwemsys-amide	1714.0	1900			
874	Ac-vpkGwrffemsys-amide	1674.9	>100uM			
875	Ac-vpkGwrfhdmsys-amide	1650.9	400			
877	Ac-vpkGwrfhe(N-alpha-me-D-Met)sys-amide	1678.8	282			
878	Ac-vpkGwrfhe(D-Buthionine)sys-amide	1706.9	2.3 ± 0.8	~40uM	>30uM	~30uM
879	Ac-vpkGwrfheksys-amide	1661.9	832			
880	Ac-vpkGwrFhsiiss-amide	1554.8	1.8 ± 0.5	~18uM	~10uM	>30uM
881	Ac-wrFh-C_3-Phenyl	802.9	>10uM			
882	Ac-wrFh-(1,6-Diaminohexane)stearyl	1085.5	120			
883	Ac-wrFh-amide	685.7	>100uM			
884	Ac-vpkGwrFhemsys-amide	1664.9	72	>30uM	~30uM	>30uM
886	Ac-vpkGwrfhsiiss-amide	1554.8	1.0 ± 0.43	~12uM	4.0 ± 0.6uM	~4.6uM
890	Ac-vpkGwr(D-Cha)he(D-Buthionine)sys-amide	1711.9	1.9 ± 0.01	~15uM	19 ± 2.2uM	~4.5uM
891	Ac-vpkGwr(D-Cha)hsiiss-amide	1560.9	0.43 ± 0.01	~8uM	3.8 ± 1.0uM	~2.5uM
892	Ac-SYSMEH(Cha)RWGKPV-amide	1671.0	0.51			
893	Ac-vpkGWrfhemsys-amide	1664.9	6.5	~28uM	>30uM	~27uM
894	Ac-vpkGwRfhemsys-amide	1664.9	380	>30uM	>30uM	>30uM
895	Ac-vpkGwrfHemsys-amide	1664.9	19	~37uM	~11uM	~37uM
Scrambled	Ac-fsesGvkmprywh-amide	1664.9	*ND	*ND	*ND	*ND

* ND = no detectible binding

K_i values were determined by computer analysis of ^{125}I-NDP-MSH binding competition curves using logEC_{50} on membrane preps from HEK-293 cells expressing MC1, MC3, MC4, or MC5 receptors. K_i values with similar or better affinity compared to RI-α-MSH were evaluated in 3 independent experiments performed in triplicate ± SEM between experiments. K_i values with diminished binding affinity were evaluated in a single experiment in triplicate.

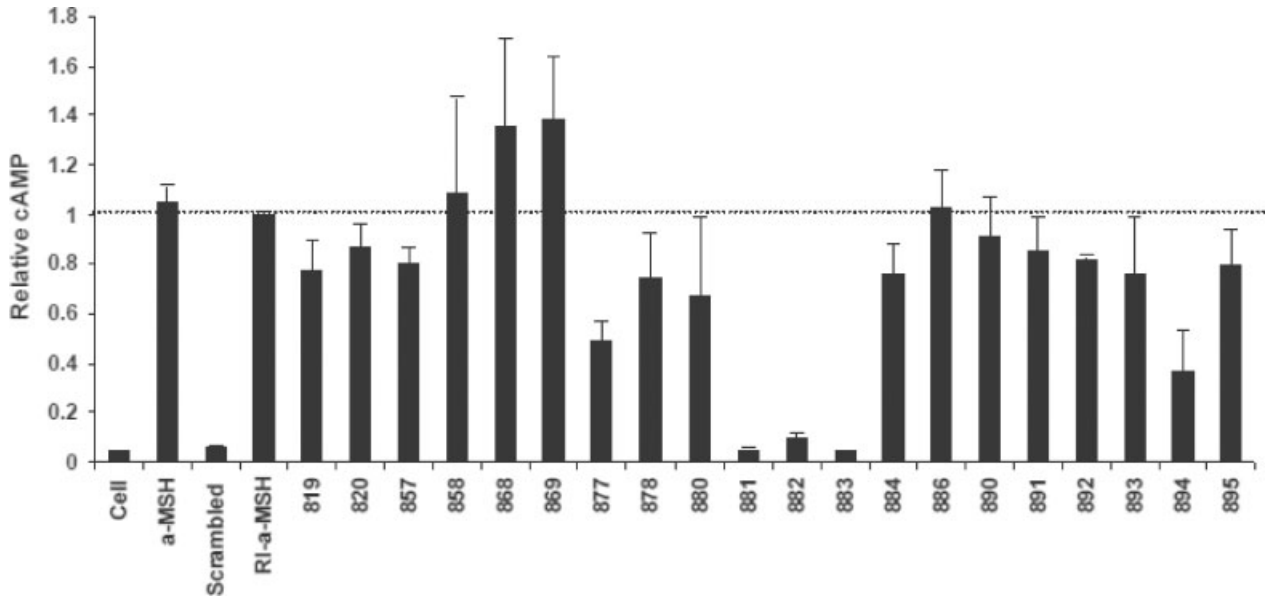

Figure 3. Intracellular cAMP generated in the human melanoma cell line Mel-624 after 30 min stimulation with peptide (10 μM) or forskolin (100 μM). Relative cAMP = measured cAMP(fmol)/cAMP(fmol) of RI-α-MSH. Scrambled: scrambled sequence of the D-amino acid MSH (Table 1). Values shown are mean of at least three independent experiments performed in triplicate ± standard error of mean (SEM).

in full-length RI-α-MSH (#884) also produced a 16-fold loss in affinity for MC1R, whereas inversion of the Phe in [Nle^4]-α-MSH has been reported to produce at least a tenfold increase in activity [29]. Inversion of the residues adjacent to the Phe in RI-α-MSH (Trp^5, Arg^6, His^8, peptides #893, 894, 895) also failed to improve affinity for MC1R.

Extensions off the Histidine and Recombination

Capping of the *N*-terminus of the HfRW core tetrapeptide with aromatic groups or fatty acids has been shown to improve MC1R binding and selectivity, producing low or sub-nanomolar binding affinities for MC1R [15,16]. Moreover, fatty acylation was particularly interesting because a similar modification of both insulin and glucagon-like-peptide (GLP-1) has been shown to produce large improvements in pharmacokinetics [30,31]. We confirmed the effects of both modifications on the α-MSH core tetrapeptide HfRW (#819 and #820). Analogous changes were then applied to the *C*-terminus of the RI version. The terminal histidine of wrFh was capped either with 3-phenyl-1-propylamine or stearic acid through a 1,6-diaminohexane linker. Significant MC1R binding was observed only with the aliphatic extension (#882, Table 2). Although the stimulation was >800-fold (#882 vs #883), binding was still 30-fold weaker than RI-α-MSH. Addition of a phenyl group with 3-carbon spacer to the tetrapeptide elicited only weak binding (>10 μM, #881). Similarly, addition of this phenyl substituent to a truncated version of RI-α-MSH which had the Phe in the opposite entiomeric configuration also produced a negligible increase in binding (#847 : 104 nM vs #847int : 150 n M).

Substitution of the *N*-terminal SYSME tract in α-MSH with the analogous portion of a MC1R binding peptide ligand selected by phage display (MS05) has produced an analog with nM affinity and high selectivity for MC1R [18]. Substitution of the RI version of this sequence (siiss) for the emsys element in RI-α-MSH (#886) produced a fivefold increase in MC1R binding with increases at the other MCRs (Table 2). Substitution of siiss in the weaker-binding L-Phe variant of RI-α-MSH (#884) produced a larger relative response (40-fold, #880) but with a lesser (net 2.5-fold) increase in affinity over RI-α-MSH. Finally, a peptide combining the D-Cha and siiss substitutions (#891) showed identical binding affinity for MC1R as native α-MSH (0.41 vs 0.43 nM) but retained a strong selective preference for MC1R displayed by its parent (Table 2).

Table 3. *In vitro* MC1R binding *vs.* cAMP induction in Mel-624 human melanoma cells

Peptide	Sequence	K_i Factor[1]	EC_{50} Factor[2]
MSH	SYSMEHFRWGKPV	(1.0)	(1.0)
RI-α-MSH	vpkGwrfhemsys	11	6.4
869	vpkGwr(D-Cha)hemsys	5.4	5.0
878	vpkGwrfhe(D-Buthionine)sys	5.6	5.3
880	vpkGwrfhsiiss	4.4	5.3
886	vpkGwrfhsiiss	2.4	2.0
890	vpkGwr(D-Cha)he(D-Buthionine)sys	4.6	0.8
891	vpkGwr(D-Cha)hsiiss	1.0	0.3

[1] K_i with hMC1R relative to α-MSH (0.41 ± 0.14 nM)
[2] EC_{50} with Mel-624 cells relative to α-MSH (44 ± 9 nM)
Cha: Cyclohexylalanine
Factor : [Peptide value/MSH value] for either K_i or EC_{50}
Calculated values are the average of 3 separate experiments performed in triplicate.

EC_{50} values in Mel-624 melanoma cells were also determined for a subset of the RI-α-MSH analogs. In general, the decrease in EC_{50} paralleled the reduction in K_i except for peptide 890 (D-Cha, D-Buthionine) which showed a significantly lower EC_{50} than expected from others in this series (Table 3). The highest affinity variant (#891) showed the lowest EC_{50}, more than threefold lower than α-MSH and 20-fold lower than RI-α-MSH. At saturating concentrations of the peptides, similar cAMP levels were observed as with α-MSH indicating full agonist activity but without superagonistic effects (data not shown).

Journal of PeptideScience

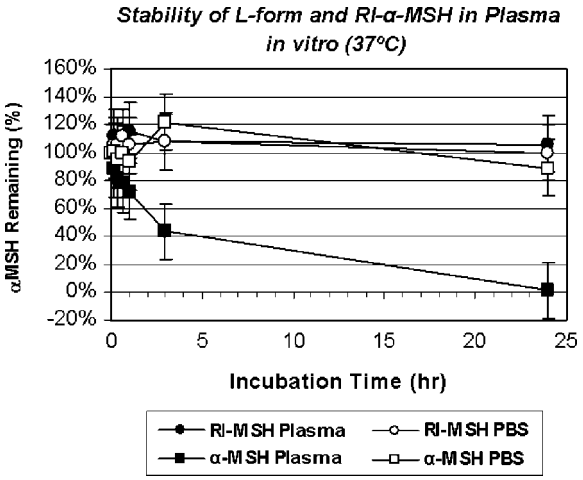

Figure 4. Recovery of α-MSH or RI-α-MSH (#720) as a percentage of the initial concentration spiked into plasma or PBS. Concentration determined with LC-MS/MS in MRM mode (MRM transitions in methods). Values shown are mean of two independent experiments performed in duplicate ± standard error of mean (SEM).

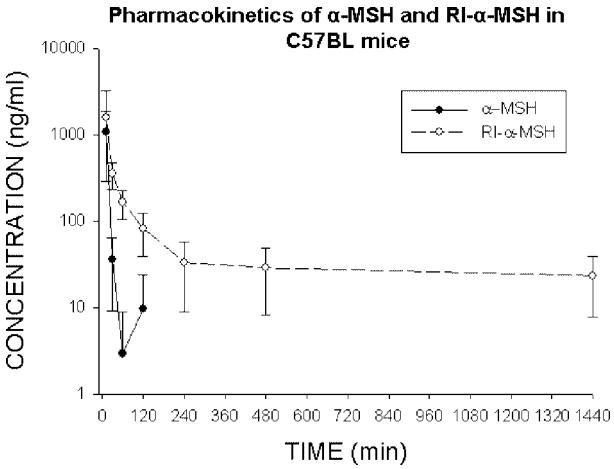

Figure 5. Serum concentration *versus* time plot of α-MSH and RI-α-MSH (#720) after 5 mg/kg IV administration in C57BL mice. Concentration determined with LC-MS/MS in MRM mode (MRM transitions in methods). Data shown as mean ± standard error of mean (SEM) of $n = 4$ mice/group.

Stability in Plasma and Pharmacokinetics

The resistance of RI-α-MSH to degradation in plasma was determined by incubation with pooled plasma from C57 BL mice at 37 °C and followed by LC-MS/MS (See Materials and Methods). While native MSH was completely lost after 24 h incubation, RI-α-MSH was unaffected (#720, Figure 4). Incubation of either α-MSH or RI-α-MSH in PBS had no effect. The pharmacokinetics was determined after bolus administration in C57 BL mice. RI-α-MSH or α-MSH were administered (5 mg/kg IV) and serum peptide levels determined by LC-MS/MS. Within 1 h, over 99% of native α-MSH had cleared from circulation and fell below the limit of detection after 4 h. However, low but detectable levels of RI-α-MSH were observed even after 24 h (Figure 5). Due to the rapid clearance rate, the half-life of native MSH could only be estimated as several minutes in this experiment. In contrast, the terminal half-life of RI-α-MSH was found to be in the range of 0.5–5 days. The residual level of peptide after 24 h (23 ng/ml, 13 nM) was ~threefold above the K_i at MC1R.

Discussion

α-MSH is known to have a role in a number of biological processes including immunomodulatory functions mediated through one or more of the five known receptors for this peptide. A RI version of α-MSH (RI-α-MSH) was examined as a potential MC1R agonist with enhanced proteolytic stability which might be the basis for a therapeutic with broad application in inflammatory and autoimmune disorders. Although RI-α-MSH showed a reduced affinity relative to its parent, it also showed nearly absolute preference for binding MC1R relative to the other melanocortin receptors, an attractive property given their roles in processes potentially leading to undesirable side effects. In initial evaluations, the cAMP level elicited by RI-α-MSH at saturating peptide concentrations in both human and murine melanoma cells was comparable with α-MSH indicating that the RI version retains full agonist activity. Thus, we undertook a detailed analysis and affinity maturation of RI-α-MSH as a potential therapeutic on the basis of its selectivity and an enhanced potency which might be realized as a result of the resistance of D-form peptides to proteolysis. This property was confirmed, and with the unexpected additional benefit of a long terminal half-life in circulation.

A variety of sequence and structural modifications were explored to both improve the affinity and to understand the nature of the receptor interaction and the basis for the high selectivity for MC1R. Most of the key residues were found to be identical in both α-MSH and RI-α-MSH. However, significant differences were observed with Ala and non-natural amino acid substitutions and modifications previously shown to enhance the properties of the native peptide or its analogs. While the effect of some modifications had lesser or negligible affects relative to α-MSH, others had a stronger effects or were unique to RI-α-MSH.

Single Ala substitutions demonstrated that residues within the core tetrapeptide element wrfh and the Met were critical for binding, similar to previous findings for α-MSH [28]. Ala substitution of the Phe, Arg, and Trp had comparable effects on the agonist activity of both RI-α-MSH and α-MSH (Figure 2) [28]. However, significant differences in the magnitude of the effects on binding were observed. While the fold effects were within a factor of three of those seen with α-MSH at the His, Arg, and Trp positions, replacement of the Phe had at least a tenfold stronger effect (>6000-fold) than reported for the native peptide (500-fold) [28]. Moreover, the rank order of the effect on binding RI-α-MSH [Phe(30 μM) > Arg(13 μM) > Trp(3 μM) > His(1.2 μM)] were significantly different than in α-MSH, where the Phe appears to have a lesser role in binding than the Arg or Trp (Arg ~ Trp > Phe > His) [28]. The orientation of the Phe has strikingly different effects on the native and RI peptides. Inversion of the Phe in RI-α-MSH produced a 16-fold decrease in binding (#884, $K_i = 72$ nM), whereas inversion of the Phe in α-MSH has the opposite, stimulatory effect on the agonist activity of [Nle^4]-α-MSH (>tenfold reduction in EC_{50} [29]) and the core HfRW tetrapeptide (380-fold) [32]. These differences suggest the Phe side chain is closer to the preferred orientation in RI-α-MSH for binding MC1R. Substitution of D-Cha for D-Phe (#869) observably improved binding with RI-α-MSH, whereas L-Cha substitution in native α-MSH (#892) had no effect. This suggests that the contact with this more aliphatic side chain may only be achievable with the side chain in the RI orientation, although it is also possible that

the solution conformation of the RI shifts to a form preferred by the receptor as a result of the modification. The effect of a D-Cha substitution in α-MSH has not been tested.

The terminal sequence tract flanking the His contributes significantly to the MCR interaction and shows some properties which distinguish RI-α-MSH from α-MSH. Of the residues in this tract, the Met appears to be the principal contributor to agonistic activity at MC1R (Figure 2). The Met also plays an important role in binding, as demonstrated by the 300-fold drop in affinity upon substitution with Ala, similar to the effect reported with α-MSH (140-fold [28]). However, this residue is tolerant to some structural modification because slightly improved binding was obtained by substitution with the more hydrophobic buthionine (#878, 2.3 nM). However, introduction of an α-methyl group (N-alpha-Me-D-Met #877) strongly interfered with binding (282 nM), suggesting simultaneous contact with the backbone may also be important at this position (Table 2). Deletion of the entire tract including the Met in a truncated RI-α-MSH (#847int) had a lesser effect on binding (30-fold) than Ala replacement of the Met (300-fold), suggesting another residue(s) in the tract may interfere with receptor binding and whose removal partially compensates for the loss of the single residue. A truncated peptide lacking this tract [but containing an ineffective phenyl C_3 appendage (#847)] showed only weak selectivity for binding MC1R (Table 2), suggesting this terminus may also be responsible for much of the discrimination against the other MCRs. Replacement of the corresponding tract SYSME in α-MSH with the sequence SSIIS has been reported to produce a very large increase in selectivity with a small (<twofold) increase in binding at MC1R [18]. However, although replacement of the emsys tract in RI-MSH with siiss (#886) produced a comparable increase in binding (four to fivefold) there was no significant change in receptor selectivity (Table 2). Subsequent inversion of the Phe had a lesser effect on binding with this analog than on RI-α-MSH (#880, 1.8-fold increase in K_i vs #884, 16-fold K_i increase) and again produced no change in selectivity. In contrast, greater than a 300-fold reduction in selectivity occurred in α-MSH with the SSIIS substitution upon inverting the core Phe to the preferred orientation, as a consequence of increases in the affinity for the other MCRs [18]. These data indicate that the selectivity of RI-α-MSH is much less dependent upon the terminal tract and core Phe, suggesting that the source of its selectivity may reside in an intrinsic property such as the structural constraints imposed by the RI backbone. In the case of RI-α-MSH, receptor interaction with the terminal His flanking residues may be largely a relatively nonspecific hydrophobic one that stabilizes the receptor complex. Most dramatic was the ability of a non-peptide aliphatic chain to effectively substitute for the tract (#882, 120 nM) in a RI core peptide with an L-Phe, the unpreferred orientation. The enhancement of binding by such fatty acylation has been demonstrated previously [15], but the effects were over tenfold weaker (80-fold vs >800-fold [11]).

With α-MSH, combination of several TM domain loops in MC1R, TM 1/2/3/6/7, or TM 4/5/6/7 in conjunction with an open hydrophobic binding region near the core tetrapeptide binding pocket have been proposed to be involved in binding α-MSH [33,34]. The strong enhancement in binding of the HfRW core peptide by the addition of an aromatic group to the *N*-terminus through a 5-atom spacer (to the α-carbon) has suggested this receptor region also contains an aromatic group which can establish a $\pi-\pi$ interaction with such a moiety adjacent to the peptide His [16]. We obtained a similar result with the reported optimal compound (phenyl-C_3-HfRW, peptide #819, 70 pM). However, the structurally isologous group on the tetrapeptide wrFh spaced the same distance from the His failed to elicit more than weak binding (#881). Although the core Phe was in the non-preferred orientation in this peptide, the appendage also failed to improve the modest affinity of a larger truncated version of RI-α-MSH with the Phe in the preferred enantiomeric D-configuration (#847, 104 nM vs #847int, 150 nM). The inability of this appendage to stimulate binding indicates that the RI peptide cannot simultaneously contact the residues at the core peptide binding site and the aromatic moiety, although peptide residues neighboring the His or an aliphatic tail appear to be free to interact with the hydrophobic receptor region. It is possible that the peptide *C*-terminus is shifted out of the core tetrapeptide binding site by conformational constraints on the RI backbone in a manner which disallows interaction with the aromatic moiety. However, contact with the His is fully preserved in RI-α-MSH, as shown by the threefold stronger inhibition of binding by Ala substitution than in α-MSH. While the exact interaction of RI-α-MSH with MC1R is not replicated at the other melanocortin receptors, sequence alterations which increased its affinity for MC1R were also accompanied by increases at the other receptors, suggesting some similarities. However, changes which ultimately achieved an identical affinity as α-MSH at MC1R (#891, Table 2) did not result in a significant decline in the selectivity relative to RI-α-MSH. We note that the selectivity of 891 substantially exceeds that of the high affinity phenyl-C_3-HfRW (LK-184, #819) at MC3R and MC4R. It is possible that the features of RI-α-MSH which hinder interaction with the other receptors may be difficult to replicate in such a smaller, minimal-sequence ligand.

Proteolytic susceptibility and rapid clearance are major driving factors that hinder the use of peptides as drugs in the clinic. RI-α-MSH shows resistance to proteolysis expected from the D-form amino acid backbone. Enhanced stability of α-MSH was first reported as a consequence of inversion of the Phe, ultimately yielding a peptide with prolonged bioactivity [29], although with a sacrifice in selectivity. Similar loss of selectivity was observed with the α-MSH containing a SSIIS tract, suggesting a general effect. In contrast, RI-α-MSH displays proteolytic resistance while retaining the ability to discriminate against the other MCRs. The resistance conferred by RI is likely responsible for the longer terminal half-life since there has been no change in molecular weight or primary sequence. Albeit some caution should be taken when interpreting the pharmacokinetic data based on the study size, the RI-α-MSH appears to be consistent with a multi-compartment model, suggesting additional factors such as tissue penetration may play a role. Protease resistance may be a significant contributor, which would likely have significant effects during systemic administration in a number of indications. We note that a protease-resistant α-MSH analog has shown promise in melanoma radiotherapy [35] suggesting the possibility of a broader application for RI-α-MSH and its analogs.

A combination of analog substitutions and rational design has provided peptides with equivalent receptor binding affinity as the native peptide and which retain full agonist activity. The properties of RI-α-MSH and these analogs provide promising tools for further defining the role of MC1R in immunomodulatory processes. Their selectivity may also reduce the risk of side effects associated with the application of melanocortin peptide agonists as therapeutic agents.

Acknowledgements

Special thanks to Partha Manavalan for peptide structure modeling.

Journal of PeptideScience

Supporting information

Supporting information may be found in the online version of this article.

References

1 Cooper A, Robinson SJ, Pickard C, Jackson CL, Friedmann PS, Healy E. Alpha-melanocyte-stimulating hormone suppresses antigen-induced lymphocyte proliferation in humans independently of melanocortin 1 receptor gene status. *J. Immunol.* 2005; **175**: 4806–4813.

2 Manna SK, Aggarwal BB. Alpha-melanocyte-stimulating hormone inhibits the nuclear transcription factor NF-kappa B activation induced by various inflammatory agents. *J. Immunol.* 1998; **161**: 2873–2880.

3 Siebenlist U, Franzoso G, Brown K. Structure regulation and function of NF-kappa B. *Annu. Rev. Cell Biol.* 1994; **10**: 405–455.

4 Grossmann M, Nakamura Y, Grumont R, Gerondakis S. New insights into the roles of ReL/NF-kappa B transcription factors in immune function hemopoiesis and human disease. *Int. J. Biochem. Cell Biol.* 1999; **31**: 1209–1219.

5 Rheins LA, Cotleur AL, Kleier RS, Hoppenjans WB, Saunder DN, Nordlund JJ. Alpha-melanocyte stimulating hormone modulates contact hypersensitivity responsiveness in C57/BL6 mice. *J. Invest. Dermatol.* 1989; **93**: 511–517.

6 Riemann H, Schwarz A, Grabbe S, Aragane Y, Luger TA, Wysocka M, Kubin M, Trinchieri G, Schwarz T. Neutralization of IL-12 in vivo prevents induction of contact hypersensitivity and induces hapten-specific tolerance. *J. Immunol.* 1996; **156**: 1799–1803.

7 Kokot A, Sindrilaru A, Schiller M, Sunderkotter C, Kerkhoff C, Eckes B, Scharffetter-Kochanek K, Luger TA, Bohm M. Alpha-melanocyte-stimulating hormone suppresses bleomycin-induced collagen synthesis and reduces tissue fibrosis in a mouse model of scleroderma: melanocortin peptides as a novel treatment strategy for scleroderma? *Arthritis Rheum.* 2009; **60**: 592–603.

8 Nogueiras R, Wiedmer P, Perez-Tilve D, Veyrat-Durebex C, Keogh JM, Sutton GM, Pfluger PT, Castaneda TR, Neschen S, Hofmann SM, Howles PN, Morgan DA, Benoit SC, Szanto I, Schrott B, Schurmann A, Joost HG, Hammond C, Hui DY, Woods SC, Rahmouni K, Butler AA, Farooqi IS, O'Rahilly S, Rohner-Jeanrenaud F, Tschop MH. The central melanocortin system directly controls peripheral lipid metabolism. *J. Clin. Invest.* 2007; **117**: 3475–3488.

9 Marks DL, Butler AA, Cone RD. Melanocortin pathway: animal models of obesity and disease. *Annu. Endocrinol. (Paris)* 2002; **63**: 121–124.

10 Shadiack AM, Sharma SD, Earle DC, Spana C, Hallam TJ. Melanocortins in the treatment of male and female sexual dysfunction. *Curr. Top. Med. Chem.* 2007; **7**: 1137–1144.

11 Chen W, Kelly MA, Opitz-Araya X, Thomas RE, Low MJ, Cone RD. Exocrine gland dysfunction in MC5-R-deficient mice: evidence for coordinated regulation of exocrine gland function by melanocortin peptides. *Cell* 1997; **91**: 789–798.

12 Ignar DM, Andrews JL, Jansen M, Eilert MM, Pink HM, Lin P, Sherrill RG, Szewczyk JR, Conway JG. Regulation of TNF-alpha secretion by a specific melanocortin-1 receptor peptide agonist. *Peptides* 2003; **24**: 709–716.

13 Getting SJ. Targeting melanocortin receptors as potential novel therapeutics. *Pharmacol. Ther.* 2006; **111**: 1–15.

14 Wallingford N, Perroud B, Gao Q, Coppola A, Gyengesi E, Liu ZW, Gao XB, Diament A, Haus KA, Shariat-Madar Z, Mahdi F, Wardlaw SL, Schmaier AH, Warden CH, Diano S. Prolylcarboxypeptidase regulates food intake by inactivating alpha-MSH in rodents. *J. Clin. Invest.* 2009; **119**: 2291–2303.

15 Todorovic A, Holder JR, Bauzo RM, Scott JW, Kavanagh R, Abdel-Malek Z, Haskell-Luevano C. N-terminal fatty acylated His-dPhe-Arg-Trp-NH(2) tetrapeptides: influence of fatty acid chain length on potency and selectivity at the mouse melanocortin receptors and human melanocytes. *J. Med. Chem.* 2005; **48**: 3328–3336.

16 Koikov LN, Ebetino FH, Solinsky MG, Cross-Doersen D, Knittel JJ. Subnanomolar hMC1R agonists by end-capping of the melanocortin tetrapeptide His-D-Phe-Arg-Trp-NH(2). *Bioorg. Med. Chem. Lett.* 2003; **13**: 2647–2650.

17 Szardenings M, Tornroth S, Mutulis F, Muceniece R, Keinanen K, Kuusinen A, Wikberg JE. Phage display selection on whole cells yields a peptide specific for melanocortin receptor 1. *J. Biol. Chem.* 1997; **272**: 27943–27948.

18 Szardenings M, Muceniece R, Mutule I, Mutulis F, Wikberg JE. New highly specific agonistic peptides for human melanocortin MC(1) receptor. *Peptides* 2000; **21**: 239–243.

19 Muceniece R, Mutule I, Mutulis F, Prusis P, Szardenings M, Wikberg JE. Detection of regions in the MC1 receptor of importance for the selectivity of the MC1 receptor super-selective MS04/MS05 peptides. *Biochim. Biophys. Acta* 2001; **1544**: 278–282.

20 Chorev M, Goodman M. A dozen years of retro-inverso peptidomimetics. *Acc. Chem. Res.* 1993; **26**: 266–273.

21 Verdini AS, Viscomi GC. Synthesis, resolution, and assignment of configuration of potent hypotensive retro-inverso bradykinin potentiating peptide 5a(BPP5a) analogues. *J. Chem. Soc., Perkin Trans. 1* 1985; **14**: 697–701.

22 Goodman M, Coddington J, Mierke DF, Fuller WD. Model for the sweet taste of stereoisomeric retro-inverso and dipeptide amides. *J. Am. Chem. Soc.* 1987; **109**: 4712–4714.

23 Briand JP, Benkirane N, Guichard G, Newman JF, Van Regenmortel MH, Brown F, Muller S. A retro-inverso peptide corresponding to the GH loop of foot-and-mouth disease virus elicits high levels of long-lasting protective neutralizing antibodies. *Proc. Natl. Acad. Sci. USA* 1997; **94**: 12545–12550.

24 Vunnam S, Juvvadi P, Rotondi KS, Merrifield RB. Synthesis and study of normal enantio retro and retroenantio isomers of cecropin A-melittin hybrids their end group effects and selective enzyme inactivation. *J. Pept. Res.* 1998; **51**: 38–44.

25 Freidinger RM, Veber DF. Peptides and their retro enantiomers are topologically nonidentical. *J. Am. Chem. Soc.* 1979; **101**: 6129–6131.

26 Bednarek MA, Silva MV, Arison B, MacNeil T, Kalyani RN, Huang RR, Weinberg DH. Structure-function studies on the cyclic peptide MT-II lactam derivative of alpha-melanotropin. *Peptides* 1999; **20**: 401–409.

27 Marincola FM, Shamamian P, Alexander RB, Gnarra JR, Turetskaya RL, Nedospasov SA, Simonis TB, Taubenberger JK, Yannelli J, Mixon A, Restifo NP, Herlyn M, Rosenberg SA. Loss of HLA haplotype and B locus down-regulation in melanoma cell lines. *J. Immunol.* 1994; **153**: 1225–1237.

28 Sahm UG, Olivier GW, Branch SK, Moss SH, Pouton CW. Synthesis and biological evaluation of alpha-MSH analogues substituted with alanine. *Peptides* 1994; **15**: 1297–1302.

29 Sawyer TK, Sanfilippo PJ, Hruby VJ, Engel MH, Heward CB, Burnett JB, Hadley ME. 4-Norleucine 7-D-phenylalanine-alpha-melanocyte-stimulating hormone: a highly potent alpha-melanotropin with ultralong biological activity. *Proc. Natl. Acad. Sci. USA* 1980; **77**: 5754–5758.

30 Kurtzhals P, Havelund S, Jonassen I, Kiehr B, Larsen UD, Ribel U, Markussen J. Albumin binding of insulins acylated with fatty acids: characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect in vivo. *Biochem. J.* 1995; **312**(Pt 3): 725–731.

31 Knudsen LB, Nielsen PF, Huusfeldt PO, Johansen NL, Madsen K, Pedersen FZ, Thogersen H, Wilken M, Agerso H. Potent derivatives of glucagon-like peptide-1 with pharmacokinetic properties suitable for once daily administration. *J. Med. Chem.* 2000; **43**: 1664–1669.

32 Holder JR, Bauzo RM, Xiang Z, Haskell-Luevano C. Structure-activity relationships of the melanocortin tetrapeptide Ac-His-DPhe-Arg-Trp-NH(2) at the mouse melanocortin receptors: part 2 modifications at the Phe position. *J. Med. Chem.* 2002; **45**: 3073–3081.

33 Haskell-Luevano C, Sawyer TK, Trumpp-Kallmeyer S, Bikker JA, Humblet C, Gantz I, Hruby VJ. Three-dimensional molecular models of the hMC1R melanocortin receptor: complexes with melanotropin peptide agonists. *Drug Des. Discov.* 1996; **14**: 197–211.

34 Schioth HB, Yook P, Muceniece R, Wikberg JE, Szardenings M. Chimeric melanocortin MC1 and MC3 receptors: identification of domains participating in binding of melanocyte-stimulating hormone peptides. *Mol. Pharmacol.* 1998; **54**: 154–161.

35 Miao Y, Hylarides M, Fisher DR, Shelton T, Moore H, Wester DW, Fritzberg AR, Winkelmann CT, Hoffman T, Quinn TP. Melanoma therapy via peptide-targeted {α}-radiation. *Clin. Cancer Res.* 2005; **11**: 5616–5621.

