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Cyclic lactam hybrid α-MSH/Agouti-related protein (AGRP) analogues with nanomolar range binding affinities at the human melanocortin receptors

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ABSTRACT

A novel hybrid melanocortin pharmacophore was designed based on the topographical similarities between the pharmacophores of Agouti related protein (AGRP) an endogenous melanocortin antagonist, and α-melanocyte-stimulating hormone (α-MSH), an endogenous melanocortin agonist. When employed in two different 23-membered macrocyclic lactam peptide templates, the designed hybrid AGRP/MSH pharmacophore yielded non-competitive ligands with nanomolar range binding affinities. The topography-based pharmacophore hybridization strategy will prove useful in development of unique non-competitive melanocortin receptor modulators.

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Proopiomelanocortin (POMC) is a primordial gene found in virtually all vertebrates. The peptide hormones and neurotransmitters derived from POMC by post-translational processing, including α-, β-, and γ-melanocyte stimulating hormones (MSHs) and adrenocorticotropin (ACTH),[1] (Fig. 1) as well as their targets, the melanocortin receptors (MCRs), are responsible for many physiological functions critical for survival.[2,3] These functions include regulation of feeding behavior and energy homeostasis,[3–5] control of the immune system and inflammation,[1,2] skin pigmentation,[1,2] cardiovascular function,[6] sexual function and procreation,[4,7,8] modulation of aggressive/defensive behavior,[9] thermoregulation,[10] and mediation of pain.[11,12] The multitude of biological functions displayed by the melanocortin receptors and their ligands offer attractive opportunities in addressing a variety of medical conditions including obesity,[4] cachexia,[13] inflammatory disorders,[14,15] sexual dysfunction,[4,16] and even infectious diseases.[17]

Significant efforts have been made in development of more potent and selective melanocortin ligands based on the endogenous agonists α-, β-, and γ-MSH,[18–21] while structure–activity relationship studies on the endogenous antagonists Agouti-signaling (ASIP)[22] and Agouti-related (AGRP)[23] proteins (Fig. 1) have received comparatively less attention.[24] The Agouti (-Arg-Phe-Phe-) tripeptide pharmacophore[25] differs significantly from the MSH (-His-Phe-Arg-Trp-) tetrapeptide pharmacophore,[26] and is a part of the central loop within the inhibitor cystine knot (ICK) motif in both Agouti proteins,[27] which suggests that the SAR trends observed for MSH peptides are unlikely to be manifested in the Agouti protein-derived analogues (Fig. 1). Previous literature reports describe truncation of both ASIP and AGRP sequences resulting in substantial loss of both binding affinities and antagonist/inverse agonist potencies,[28,29] while the Ac-mini-AGRP(87–120, C105A)-NH_2 variant has been reported to be equipotent to the full-length AGRP,[30] which points to possible significance of N- and C-terminal sequences of these proteins in receptor–ligand interactions. When the His-D-Phe-Arg-Trp MSH tetrapeptide pharmacophore was used to replace the Arg-Phe-Phe tripeptide sequence, the resulting cyclic peptide was a relatively potent agonist,[31,32] which can be attributed to the well-known propensity of the His-D-Phe-Arg-Trp tetrapeptide sequence to inducing melanocortin agonist activity in a wide variety of linear and cyclic peptide templates.[19,20,26,33–36] In another instance, replacement of the MSH pharmacophoric D-Phe-Arg-Trp tripeptide sequence with the agouti Arg-Phe-Phe sequence in the linear and cyclic α-MSH templates produced nanomolar range mMC1R agonists, which however registered >300 fold lower than the agonist potency of the super-agonist MT-II control as determined by CRE/β-galactosidase assay.[37] Our recent report[38] described a novel MSH/ASIP hybrid pharmacophore Arg-L/D-Phe-Xaa-L/D-Trp (Xaa = Cys or Glu), which, when incorporated into a monomeric cyclic disulfide template, yielded peptides with hMC3R-selective non-competitive binding affinities. In contrast, in a cyclodimeric lactam template this pharmacophore produced

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α-MSH Ac-Ser-Tyr-Ser-Met-Glu-His-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

hASIP(92-132) SCVATRDSCKPPAPACCDPCASCQCRFFRSACTCRVLNPNC

hAGRP(86-132) RCVRLHESCLGQQVPCCDPCATCYCRFFNAFCYCRKLGTAMNPCSRT

MT-II Ac-Nle-c[Asp-His-*D*-Phe-Arg-Trp-Lys]-NH_2

SHU9119 Ac-Nle-c[Asp-His-*D*-Nal(2')-Arg-Trp-Lys]-NH_2

Figure 1. Sequences of some endogenous and synthetic melanotropin peptides.

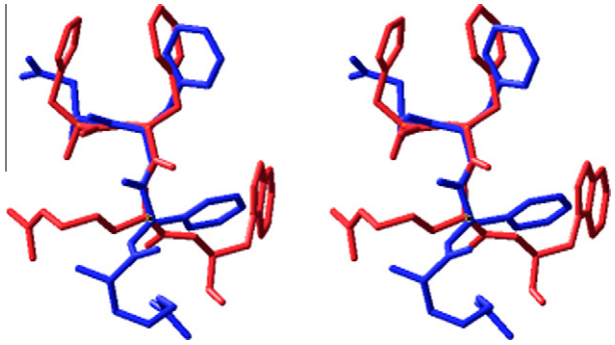

Figure 2. Stereo view of the retro-directed superposition of NMR structures of the pharmacophoric tetrapeptide sequences of endogenous hMC3/4R antagonist AGRP (blue) and of non-selective super agonist MT-II (red) (rmsd = 0.17 Å, C^{α} atoms of His[6], D-Phe[7], and Arg[8] residues of MT-II overlapped with the C^{α} atoms of the Asn[114], Phe[113], and Phe[112] residue of AGRP, respectively). Hydrogens are omitted for clarity.

nanomolar range (25–120 nM) hMC1R-selective full and partial agonists.

Comparison of the NMR structures of AGRP (110–117)[27] and MT-II[39] revealed striking similarities. Both structures feature a β-turn-like motif within the pharmacophore region, which spans over the first two residues in the His-D-Phe-Arg-Trp MSH pharmacophore, and the over the last two residues of the Arg-Phe-Phe-Asn AGRP pharmacophore. Intriguingly, a superposition of these pharmacophoric sequences in a retro-directed fashion (i.e., the C^{α} atoms of His[6], D-Phe[7], and Arg[8] residues of MT-II overlapped with the C^{α} atoms of the Asn[114], Phe[113], and Phe[112] residue of AGRP, respectively) revealed fairly similar overall topographies, matching the loci of the three pharmacophoric elements (two aromatic side chains, and a positively charged Arg side chain) that are believed to be involved in receptor–ligand interactions of both α-MSH analogues and AGRP (Fig. 2). The positions of Trp[9] (MT-II) and Arg[111] (AGRP) showed significant deviation, which was deemed unsubstantial, as much structural flexibility around Trp[9] in MSH analogues is known to be well tolerated by the hMCRs.[40] The hypothesis that the pharmacophores of α-MSH-derived ligands and AGRP are retro-directed in relation to one another suggests that a hybrid pharmacophore can be obtained by (a) replacement of Phe[112] with Trp; (b) D-amino acid substitutions in Phe[113] and Asn[114] positions to stabilize the β-turn motif; (c) Asn[114] position substitutions with other amino acids, in a fashion similar to His[6] position substitutions in α-MSH analogues; specifically, N114Q, N114H and N114R substitutions were planned, since similar modifications were reported previously for MSH-derived templates to affect the potency and receptor selectivity of the resulting peptide analogues (Fig. 3).[41,42] The resulting pharmacophore sequences were incorporated into two different 23-membered macrocyclic lactam templates, which provide global constraints (Table 1).[43–45]

The binding affinities and the agonist activities of the cyclic α-MSH analogues at the hMC1 3, 4 and 5R are summarized in

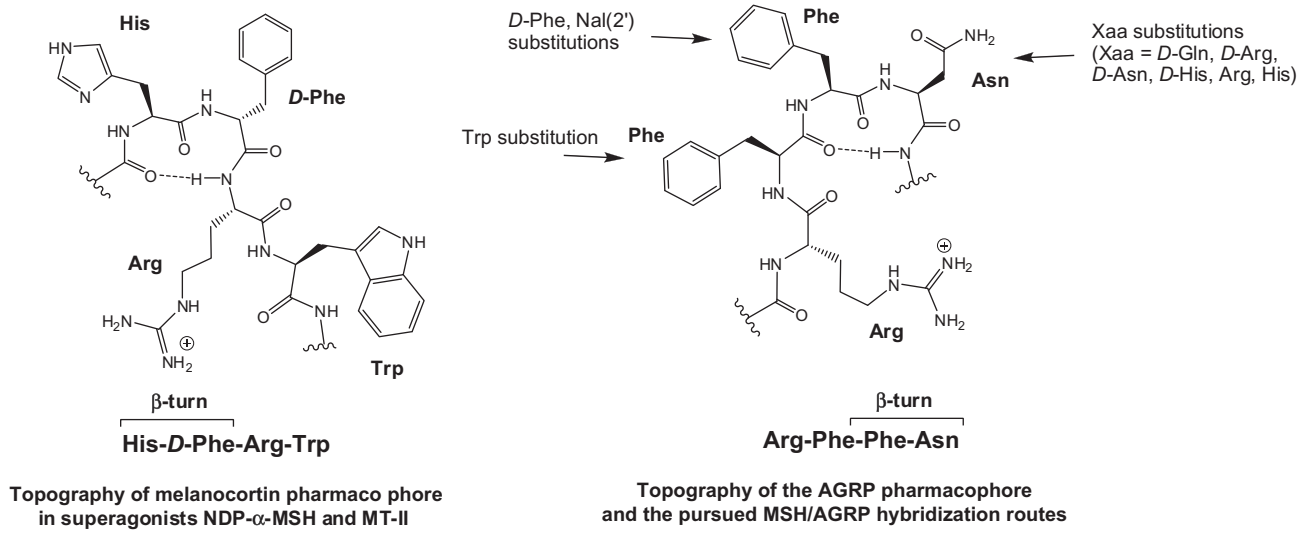

Figure 3. Design of the hybrid α-MSH/Agouti-related protein (AGRP) pharmacophore.

Table 1
Binding affinities and cAMP activities of cyclic α-MSH/AGRP hybrid analogues at hMCRs[a]

No.	Sequence	hMC1R				hMC3R				hMC4R				hMC5R			
		IC_{50} (nM)	% BE	EC_{50} (nM)	Act%	IC_{50} (nM)	% BE	EC_{50} (nM)	Act%	IC_{50} (nM)	% BE	EC_{50} (nM)	Act%	IC_{50} (nM)	% BE	EC_{50} (nM)	Act%
1	Ac-c[Asp-Arg-Trp-Phe-D-Asn-Lys]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
2	Ac-c[Asp-Arg-Trp-Nal-D-Asn-Lys]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
3	Ac-c[Asp-Arg-Trp-Phe-D-Gln-Lys]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
4	Ac-c[Asp-Arg-Trp-Nal-D-Gln-Lys]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
5	Ac-c[Asp-Arg-Trp-Phe-D-Arg-Lys]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
6	c[Arg-Trp-Phe-D-Asn-Ala-Phe-Glu]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
7	c[Arg-Trp-Nal-D-Asn-Ala-Phe-Glu]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
8	c[Arg-Trp-Phe-D-Gln-Ala-Phe-Glu]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
9	c[Arg-Trp-Nal-D-Gln-Ala-Phe-Glu]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
10	c[Arg-Trp-Phe-D-His-Ala-Phe-Glu]-NH_2	>4000	40	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
11	c[Arg-Trp-Phe-D-Arg-Ala-Phe-Glu]-NH_2	>4000	80	NA	0	500 ± 100	30	NA	0	NB	0	NA	0	~1000	30	NA	0
12	c[Arg-Trp-Phe-Asn-Ala-Phe-Glu]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
13	c[Arg-Trp-D-Phe-Asn-Ala-Phe-Glu]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
14	c[Arg-Trp-D-Phe-His-Ala-Phe-Glu]-NH_2	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0	NB	0	NA	0
15	c[Arg-Trp-D-Phe-Arg-Ala-Phe-Glu]-NH_2	>1000	65	26 ± 10	30	400 ± 80	80	NA	0	515 ± 40	40	NA	0	300 ± 30	75	NA	0
MT-II	Ac-Nle-c[Asp-His-D-Phe-Arg-Trp-Lys]-NH_2	2.3 ± 0.9	100	1.02 ± 0.4	100	8.0 ± 1.3	100	5.1 ± 0.3	100	2.3 ± 0.85	100	2.1 ± 0.6	100	4.2 ± 1.3	100	5.7 ± 2.2	100

[a] IC_{50} = concentration of peptide at 50% specific binding ($N = 4$).% BE = maximal% of ^{125}I-NDP-α-MSH displacement observed at 10 μM. EC_{50} = 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. The peptides were tested at a range of concentration from 10^{-10} to 10^{-5} nM.

Table 1.[46–50] Analogues **1**–**5**, employing the Ac-c[Asp-Arg-Trp-Xaa-Yaa-Lys]-NH_2 template (Xaa = Phe, Nal(2′); Yaa = D-Asn, D-Gln, D-Arg) exhibited no binding affinity and no cAMP stimulation at 10 μM at any of the melanocortin receptors which suggested that restricting the hybrid pharmacophore to the first four amino acids of the AGRP pharmacophore sequence Arg^{111}-Phe^{112}-Phe^{113}-Asn^{114}-Ala^{115}-Phe^{116} may be insufficient to induce receptor recognition in the antagonist series.

The second cyclic lactam template c[Arg-Trp-Xaa-Yaa-Ala-Phe-Glu]-NH_2 (Xaa = Phe, Nal(2′), D-Phe; Yaa = Asn, Arg, His, D-Asn, D-Gln, D-Arg, D-His) was constructed to include all six of these amino acids, while maintaining the same macrocycle size. Analogues **6**–**9**, possessing Phe^{113}/Nal(2′)^{113}-D-Asn^{114}/D-Gln^{114} modifications, showed no improvement over the first template and had no binding affinity and no cAMP stimulation at 10 μM. Since binding affinities and agonist potencies of α-MSH-derived peptides generally benefit from polar basic amino acids in the position 6, the Asn^{114} residue in the hybrid peptide template was replaced with D-His and D-Arg to yield analogues **10** and **11**, respectively. Analogue **10** exhibited a weak binding affinity at the hMC1R (IC_{50} > 4 μM) at about 40% binding efficiency (maximal% displacement of [^{125}I]-NDP-α-MSH). The beneficial influence of basic amino acid substitution at position 114 was further confirmed with analogue **11**, which was found to have a nanomolar range binding affinity at the hMC3R (IC_{50} = 500 nM), albeit with only 30% binding efficiency, as well as weaker binding affinities at the hMC1R (IC_{50} >4 μM) and the hMC5R (IC_{50} >1 μM). The all-L Asn^{114}-analogue **12**, D-Phe^{113}, Asn^{114}-analogue **13** and D-Phe^{113}, His^{114}-analogue **14** were devoid of any binding affinity or agonist activity at 10 μM. In contrast, the D-Phe^{113}, Arg^{114}-analogue **15** was determined to be a nanomolar partial agonist at the hMC1R (EC_{50} = 26 nM, 30% maximal cAMP) with a weak binding affinity (IC_{50} >1 μM, 65% binding efficiency), and with nanomolar binding affinity at the hMC3R (IC_{50} = 400 nM, 80% binding efficiency), the hMC4R (IC_{50} = 515 nM, 40% binding efficiency), the hMC5R (IC_{50} = 300 nM, 75% binding efficiency). This result is consistent with our earlier reports, which linked the augmented binding affinity and agonist potency with increased charge[42], although comparison with analogue **11** may suggest that D-amino acid substitution at position 113 may be needed for a suitable secondary structure of the hybrid pharmacophore for favorable receptor–ligand interactions.

In summary, a hybrid AGRP/MSH pharmacophore was designed on the basis of the hypothesis that the pharmacophores of α-MSH-derived ligands and AGRP are retro-directed in relation to one another. The resulting hybrid pharmacophore was explored in two macrocyclic lactam templates. Asn^{114} → Arg^{114} substitutions with a simultaneous D-amino acid substitutions yielded analogues with high nanomolar range binding affinities at the hMC3–5R and nanomolar range partial agonist activity at the hMC1R, such as analogue **15**, c[Arg-Trp-D-Phe-Arg-Ala-Phe-Glu]-NH_2. These new peptide analogues will aid in elucidation of the mechanism of non-competitive hMCR antagonism and GPCR modulation.

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50. *Adenylate cyclase assay*: HEK 293 cells transfected with human melanocortin receptors[47] were grown to confluence in MEM Gibco) containing 10% fetal bovine serum, 100 units/mL penicillin and streptomycin, and 1 mM sodium pyruvate. The cells were seeded on 96-well plates 48 h before assay (50,000 cells/well). For the assay, the cell culture medium was removed and the cells were rinsed with 100 μL MEM buffer (Gibco). An aliquot (100 μL) of the Earle's balanced salt solution with 5 nM isobutylmethylxanthine (IBMX) was placed in each well along for 1 min at 37 °C. Next, aliquots (25 μL) of melanotropin peptides of varying concentrations 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 μL 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 2300 g. A 50 μL aliquot of the supernatant was transferred to another 96-well plate and placed with 50 μL [^{3}H]cAMP and 100 μL protein kinase A (PKA) buffer in an ice bath for 2–3 h. The PKA-buffer consisted of Tris/EDTA-buffer with 60 μg/mL PKA and 0.1% bovine serum albumin by weight. The incubation mixture was filtered through 1.0 lm glass fiber filters in MultiScreen™-FB 96-well plates (Millipore, Billerica, MA). The total [^{3}H]cAMP was measured by a Wallac MicroBeta TriLux 1450 LSC and Luminescence Counter (Perkin–Elmer Life Science, Boston, MA). The cAMP accumulation data for each peptide analogue were determined with the help of a cAMP standard curve generated by the same method as described above. IC_{50} and EC_{50} values represent the mean of two experiments performed in triplicate. IC_{50} and EC_{50} estimates and their associated standard errors were determined by fitting the data using a nonlinear least squares analysis, with the help of GRAPHPAD PRISM 4 (GRAPHPAD SOFTWARE, San Diego, CA).