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Potent and selective MC-4 receptor agonists based on a novel disulfide scaffold☆

Liang Z. Yan,* David Flora, Patrick Edwards, David L. Smiley, Paul J. Emmerson, Hansen M. Hsiung, Robert Gadski, JeAnne Hertel, Mark L. Heiman, Saba Husain, Thomas P. O'Brien, Steven D. Kahl, Lianshan Zhang, Richard D. DiMarchi and John P. Mayer*

Lilly Research Laboratories, A Division of Eli Lilly & Co., Lilly Corporate Center, Indianapolis, IN 46285, USA

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Abstract—Extensive structure–activity relationship studies utilizing a β-MSH-derived cyclic nonapeptide, Ac-Tyr-Arg-[Cys-Glu-His-D-Phe-Arg-Trp-Cys]-NH_2 (**3**), led to identification of a series of novel MC-4R selective disulfide-constrained hexapeptide analogs including Ac-[hCys-His-D-Phe-Arg-Trp-Cys]-NH_2 (**12**). The structural modifications associated with profound influence on MC-4R potency and selectivity were ring size, ring conformation, and the aromatic substitution of the D-Phe7. These cyclic peptide analogs provide novel and enhanced reagents for use in the elucidation of melanocortin-4 receptor-related physiology, and may additionally find application in the treatment of obesity and related metabolic disorders.

The melanocortin family of peptides includes the pro-opiomelanocortin derived α, β, and γ melanocyte stimulating hormone (MSH) isoforms and adrenocorticotropic hormone (ACTH). These peptides share a conserved His-Phe-Arg-Trp (HFRW) pharmacophore or 'message' sequence[1] and biological activity that is mediated through activation of a family of five G protein-coupled receptors, MC-1R–5R.[2–5] In contrast to the MC-1, 3, 4, and 5 receptors which are activated by peptides containing the common HFRW message sequence, the MC-2 receptor can only be activated by interaction with its unique ligand, ACTH.[6] Signalling through the melanocortin receptors is reported to mediate diverse physiological functions including pigmentation (MC-1R), inflammation (MC-1R and -3R), steroidogenesis (MC-2R), energy homeostasis (MC-3R and -4R), food intake (MC-4R), sexual function (MC-4R), and exocrine gland secretion (MC-5R).[1,7] Further characterization for unique biological function inherent to each receptor is dependent upon higher quality receptor ligands. The non-pharmacologic genetic disruption of the MC-4 receptor in mice has been shown to result in hyperphagia and obesity.[8] Furthermore, mutations in the human MC-4R have been associated with the most common form of monogenic obesity.[9] Validating the biological function of the MC-4R pharmacologically has become the subject of intense investigation.

Hruby and co-workers have reported the discovery of several long-acting, enzymatically stable, non-selective MC receptor superagonists. These include peptide-based superagonists NDP-α -MSH (**1**),[10] a series of lactam-based cyclic analogs which includes the agonist MT-II (**2**),[11] and the MC-3/4 antagonist SHU-9119.[12] The development and availability of these peptides, particularly MT-II and SHU-9119, enabled the initial elucidation of the central role melanocortins serve in body weight regulation and energy homeostasis. These observations have focused attention on the identification of peptides with improved MC-receptor subtype selectivity as a possible means to improved pharmacological treatment of obesity.[13–22]

All peptides reported here were prepared by using standard Fmoc solid-phase chemistry, utilizing either Rink- or Wang-type polystyrene resin. Final products were

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☆ The sequence nomenclature used throughout this report is based on α-MSH (1–13): Ac-Ser^1-Tyr^2-Ser^3-Met^4-Glu^5-His^6-Phe^7-Arg^8-Trp^9-Gly^{10}-Lys^{11}-Pro^2-Val^{13}-amide.

* Corresponding authors. Tel.: +1 317 433 7283; fax: +1 317 276 1177 (L.Z.Y.); tel.: +1 317 277 9152; fax: +1 317 276 1177 (J.P.M.); e-mail addresses: lyan@lilly.com; j.mayer@lilly.com

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purified on reverse-phase HPLC and characterized by analytical HPLC and mass spectral analysis.[23] Our efforts in this field, based on the β-MSH isoform, have produced a number of potent MC-4R selective ligands[17] including analog **3** (Fig. 1). This peptide possesses subnanomolar potency at MC-4R, and respective selectivities at the MC-5R and MC-1R of approximately 600- and 20-fold. The pharmacologic utility of analog **3** is limited by its low solubility at physiological pH, and to some extent its less than optimal selectivity to MC-1R. In view of these shortcomings, we initiated SAR studies to enhance MC-4R, selectivity, and to improve physicochemical properties, most notably aqueous solubility.

We first examined the role of Glu5, the single amino acid within the disulfide-constrained region of the molecule which is not part of the His-Phe-Arg-Trp tetrapeptide

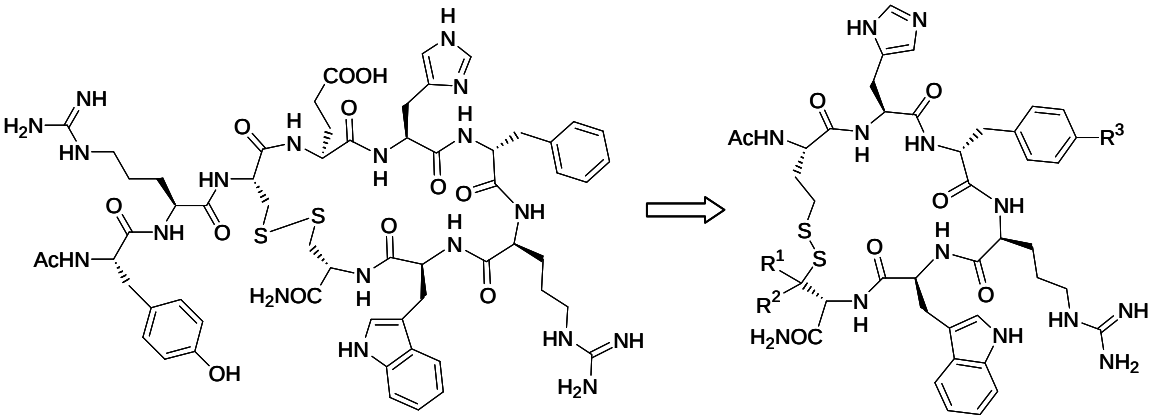

Figure 1. Summary of structure–activity relationship studies.

Table 1. In vitro binding and functional assay data

No.	Structure[a]	Binding affinity K_i (nM)[b]				MC-4R functional activity	
		MC-1R	MC-3R	MC-4R	MC-5R	EC_{50} (nM)[c]	Rel. eff (%)[d]
1	NDP-α-MSH	0.10 ± 0.00	0.23 ± 0.01	0.31 ± 0.01	0.17 ± 0.01	0.36 ± 0.01	100
2	Ac-Nle[**DH**d**FRWK**]-NH_2[e]	0.61 ± 0.07	15.80 ± 1.00	0.68 ± 0.07	8.42 ± 0.94	0.16 ± 0.03	94.7 ± 2.6
3	Ac-**YR**[**CEH**d**FRWC**]-NH_2	17.80 ± 0.90	56.40 ± 2.20	0.77 ± 0.03	>500	0.27 ± 0.02	95.6 ± 1.3
4	Ac-**YR**[**CFH**d**FRWC**]-NH_2	4.06 ± 1.10	28.00 ± 5.70	0.53 ± 0.07	79.10 ± 7.20	0.05 ± 0.00	92.1 ± 2.2
5	Ac-**YR**[**CRH**d**FRWC**]-NH_2	0.08 ± 0.01	29.70 ± 6.10	0.24 ± 0.07	49.40 ± 6.10	0.04 ± 0.01	94.0 ± 3.4
6	Ac-**YR**[**CAH**d**FRWC**]-NH_2	1.62 ± 0.05	34.90 ± 2.70	0.41 ± 0.15	>500	0.04 ± 0.01	120.0 ± 3.8
7	Ac-**YR**[**CGH**d**FRWC**]-NH_2	7.40 ± 2.30	122.0 ± 32.90	1.10 ± 0.31	>500	0.10 ± 0.01	83.4 ± 0.6
8	Ac-**YR**[h**CEH**d**FRWC**]-NH_2	235.0 ± 31.8	>500	25.10 ± 3.70	>500	3.75 ± 0.34	95.8 ± 2.1
9	Ac-**YR**[h**CH**d**FRW**h**C**]-NH_2	10.50 ± 3.30	85.00 ± 26.00	2.10 ± 0.57	186.0 ± 28.20	0.05 ± 0.01	108.0 ± 1.8
10	Ac-**YR**[h**CH**d**FRWC**]-NH_2	2.96 ± 0.74	9.04 ± 2.12	0.38 ± 0.03	14.30 ± 2.40	0.05 ± 0.01	103.0 ± 4.6
11	Ac-**R**[h**CH**d**FRWC**]-NH_2	4.02 ± 0.81	1.90 ± 0.19	0.08 ± 0.01	4.42 ± 0.78	0.05 ± 0.01	108.0 ± 5.2
12	Ac-[h**CH**d**FRWC**]-NH_2	13.80 ± 3.02	10.30 ± 0.43	0.27 ± 0.06	92.30 ± 26.70	0.10 ± 0.01	103.0 ± 4.0
13	Ac-[h**CH**d**FRW**Pen]-NH_2	194.0 ± 31.0	77.90 ± 6.60	1.14 ± 0.21	402.0 ± 74.00	0.68 ± 0.08	93.5 ± 5.0
14	Ac-[h**CH**(*p*F-d**F**)**RWC**]-NH_2	20.40 ± 2.10	7.98 ± 0.46	0.28 ± 0.07	37.20 ± 3.90	0.16 ± 0.01	102.0 ± 3.6
15	Ac-[h**CH**(*p*Cl-d**F**)**RWC**]-NH_2	25.60 ± 1.80	1.86 ± 0.50	0.20 ± 0.02	7.66 ± 0.13	0.23 ± 0.04	56.4 ± 2.7
16	Ac-[h**CH**(*p*Cl-d**F**)**RW**Pen]-NH_2	56.10 ± 7.90	1.29 ± 0.30	0.05 ± 0.02	11.40 ± 2.50	0.60 ± 0.25	66.4 ± 6.4
17	Ac-[**CH**d**FRW**h**C**]-NH_2	>500	360.0 ± 37.00	9.36 ± 0.37	>500	0.55 ± 0.00	89.7 ± 3.5
18	Ac-[**CH**(*p*F-d**F**)**RW**hC]-NH_2	245.0 ± 19.3	128.0 ± 10.00	2.51 ± 0.16	>500	0.42 ± 0.08	96.2 ± 3.1
19	Ac-**YR**[**CEH**d**FRWC**]-OH	112.0 ± 31.0	411.0 ± 54.00	8.78 ± 2.28	>500	1.53 ± 0.07	95.0 ± 2.6
20	Ac-[h**CH**d**FRWC**]-OH	115.0 ± 3.00	115.0 ± 6.00	2.68 ± 0.53	>500	1.20 ± 0.40	87.4 ± 3.4
21	Ac-Nle[h**CH**d**FRWC**]-NH_2	0.06 ± 0.02	0.36 ± 0.05	0.05 ± 0.01	0.27 ± 0.02	0.10 ± 0.02	95.3 ± 6.4

[a] For details of peptide synthesis and characterization, see Ref. 23 and Supplementary material.
[b] K_i values (n > or = 2) ± SEM were determined by a radioligand binding assay using I^{125}-NDP-α-MSH. See Ref. 22 for detailed procedures.
[c] EC_{50} values (n > or = 2) were determined by the concentration of peptide at 50% maximum cAMP release. See Ref. 22 for detailed procedures.
[d] Relative efficacy was defined as the maximum peptide-induced cAMP release relative to the maximum cAMP release induced by NDP-α-MSH (defined as 100%).
[e] Amino acids were abbreviated using one-letter system for brevity, and highlighted in bold. Specifically, **Y**: Tyr; **R**: Arg; **C**: Cys; h**C**: homoCys; **E**: Glu; **H**: His; d**F**: D-Phe; *p*F-d**F**: para-fluoro-D-Phe; *p*Cl-d**F**: *para*-chloro-D-Phe; **W**: Trp; Nle: norleucine; Pen: penicillamine.

'message' sequence. Replacement of Glu5 with a number of amino acids (analogs **4**–**7**, see Table 1) failed improve biological performance, and suggested that this acidic residue was not critical to MC-4R ligand–receptor interaction. This also raised the possibility that this amino acid could be deleted and that potency could be maintained, provided appropriate adjustment was made to the ring size. To systematically test this possibility, we evaluated a number of des-Glu5 analogs where one or both cysteines were replaced with homocysteine in order to expand the disulfide loop by one or two methylene units. Expansion of the 23-membered macrocycle of analog **3** to 24 by substitution of Cys4 with homocysteine (hCys) gave analog **8** of decreased MC-4R potency and selectivity to the MC-1R and MC-5R subtypes. Substitution of both Cys4 and Cys10 with homocysteine, with deletion of Glu5, gave the 22-membered analog **9**, which exhibited improved MC-4R potency compared to analog **3**, but no improvement in selectivity. Deletion of a single methylene group from **9** by replacement of hCys10 with cysteine gave the 21-membered cyclic analog **10**, which further improved MC-4R potency but not selectivity. Unexpectedly, deletion of the N-terminal tyrosine afforded the heptapeptide **11**, which demonstrated a nearly 10-fold enhanced MC-4R potency compared to the lead peptide **3**, and improved selectivity with respect to MC-1R and MC-5R. Further deletion of the exocyclic arginine gave the hexapeptide **12**, which further improved selectivity with only slightly diminished MC-4R potency compared to **11**. At this point, our concerted SAR strategy involving deletion of Glu5, N-terminal truncation and fine-tuning of ring size resulted in a 33% reduction in molecular size of the initial nonapeptide lead **3** (MW 1338) to hexapeptide **12** (MW 904) (Fig. 1). The in vitro profile was improved with respect to lead analog **3** in potency and MC-4R versus MC-1R selectivity. While the absolute affinity for MC-5R increased, a sizable 340-fold selectivity margin relative to the MC-4R was still inherent. Our second stated objective was also accomplished where the solubility of analog **12** improved to approximately 10 mg/mL at neutral pH in saline from 2.5 mg/mL of **3** (as the acetate salt).

Two additional modifications were found to exert a profound influence on potency, or selectivity, and at times both parameters. The geminal dimethyl analog of cysteine, penicillamine, was observed to consistently improve MC-4R subtype selectivity. For example, substitution of Cys10 in **12** with penicillamine (analog **13**) enhanced MC-4R selectivity at MC-1R to 170-fold with only a slight loss in binding affinity. We also examined the influence of aromatic substitution at the D-Phe7 residue. Previous studies found that halogenation of the D-Phe side chain increased potency at the MC-4R.[12,17] While the modification of D-Phe7 in analog **12** with a para-fluoro or- chloro substituent (analogs **14** and **15**) had little overall effect, it resulted in enhanced selectivity when introduced in combination with the penicillamine modification. Analog **16** demonstrated excellent MC-4R binding and significantly improved selectivity versus the MC-1 and MC-5 receptors (approximately 1200- and 250-fold, respectively). However, it exhibited a reduced efficacy relative to NDP-α-MSH (66%), suggesting that this peptide functions as a partial agonist at MC-4R.

An important part of our strategy was the use of cysteine/homocysteine substitution to subtly modify ring size. Interestingly, while the [hCys4, Cys10], and [Cys4, hCys10] based scaffolds share the same ring size, the latter scaffold was consistently associated with lower MC-4R potency: **12** versus **17**, and **14** versus **18**, suggesting that the orientation of the disulfide within the macrocycle is important.

The C-terminal unsubstituted amides were found to possess optimal potency and selectivity. They were found to be consistently more potent than the corresponding C-terminal acids, but of comparable selectivity. This trend, exemplified by analog pairs **3** versus **19** and **12** versus **20**, was general and consistent across each of the melanocortin receptor subtypes, where approximately a 10-fold difference between acids and amides was noted. This observation stands in contrast to changes in the N-terminal region of the peptide series which was found to serve a critical role in MC-receptor selectivity noted previously. An additional example, analog **21**, obtained by addition of norleucine (Nle) to the N-terminus of **12**, exhibited a significant loss in selectivity among the receptor subtypes by gaining both MC-1R and MC-5R binding affinity (230- and 340-fold, respectively). Interestingly, this disulfide containing analog **21** bears structural similarity to the lactam MT-II (**2**), which also exhibits limited selectivity among the four receptor subtypes (MC-1R, MC-3R, MC-4R, and MC-5R).

To summarize, minimization of the lead peptide (**3**) through systematic truncation, optimization of ring size, and linkage in the macrocycle, as well as aromatic substitution of D-Phe7 yielded improved biologic and pharmaceutic properties. The systematic evaluation of cysteine, homocysteine, and penicillamine containing disulfide homologs was shown to be an effective, synthetically straightforward strategy in the development of soluble, potent, and selective peptide agonists at MC-4R. The degree of constraint induced by the size of the macrocycle, introduction of the geminal dimethyl substitution, and even the orientation of the disulfide were found to exert a significant influence on the in vitro properties of these peptides. These peptides provide alternative and improved tools for the study of MC-4 receptor pharmacology.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.06.081.

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