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Design, synthesis, and evaluation of proline based melanocortin receptor ligands

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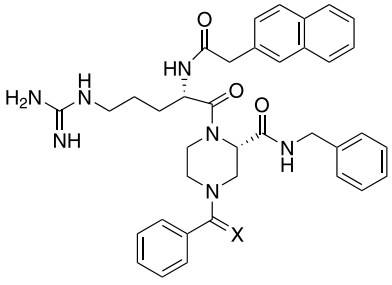
Abstract—A series of proline based melanocortin ligands has been developed on the basis of initial piperazine leads by using a more conformationally rigid scaffold. A number of these novel ligands showed significant binding affinity for MC3 and MC4 receptors.

The melanocortin receptors (MCRs) are a family of five 7-transmembrane G-protein coupled receptors (MC1R–MC5R). These receptors are activated by the peptide ligands: α, β, γ-melanocyte stimulating hormones (MSH), and adrenocorticotropin (ACTH), which are derived from a common precursor protein, proopiomelanocortin (POMC) by post-translational cleavage.[1] The MCRs have been found to mediate a variety of physiological responses that include skin pigmentation, inflammation, steroidogenesis, feeding behavior, sexual function, and exocrine gland secretion. Recently, many research groups have invested intensive effort toward the design of selective, non-peptidic small molecule ligands as potential therapeutic agents for melanocortin-mediated diseases.[2] While the majority of this activity has been focused on developing MC4R agonists as a treatment for obesity or sexual dysfunction, small-molecule MC4R antagonists have also been pursued as possible therapeutics to treat disease associated involuntary weight loss.[2d,3]

The initial leads for our drug discovery effort emerged from screening libraries that were designed to capture key recognition elements from the 'message' sequence, His-Phe-Arg-Trp, present in endogenous melanocortin agonists.[4] A particularly successful library was built on a piperazine-2-carboxamide scaffold and ultimately led to the identification of numerous leads with sub-micromolar binding to the MC4 receptor. Chemotype features that were common to all of these leads included two aromatic side chains attached at the 2,4-positions of the piperazine ring and an acylated Arg residue at the top side chain as exemplified by compounds **1** and **2**. Despite being devoid of functional activity, these leads provided a viable starting point for small molecule MCR ligand design so as to further improve affinity and, more importantly, to achieve melanocortin functional activity (Fig. 1).

Among a variety of potential ways envisioned to modify structures of the initial piperazine lead molecules, we chose first to explore the use of a more rigid five-membered proline ring to replace the 2-carboxylate piperazine motif as the constraining element for two aromatic moieties, while keeping a flexible Arg side chain. The goal was to gain insight into the effect of reducing conformational mobility of two phenyl side chains on



1. X=H2, Ki=376 nM at MC4R; **2.** X=O, Ki=649 nM at MC4R.

Figure 1. Piperazine leads.

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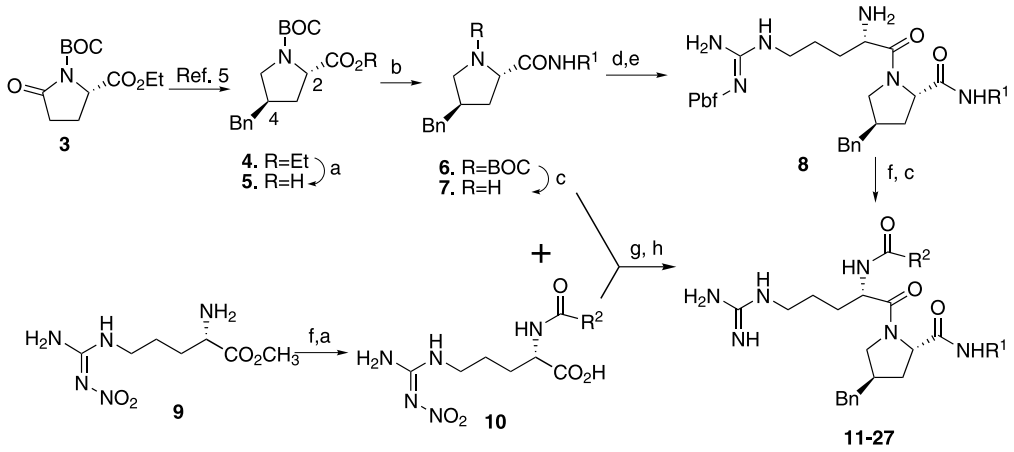


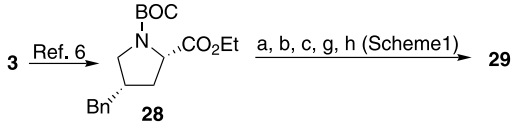
Scheme 1. Synthesis of *trans* proline analogs. Reagents and conditions: (a) LiOH, CH_3OH/H_2O, 18 h, 92%; (b) R^1NH_2, EDCI, HOBt, NMM, DMF, 4 h, 54–79%; (c) TFA/CH_2Cl_2, 3 h, ~100%; (d) Pbf-(Fmoc)-Arg-OH, EDCI, HOBt, NMM, DMF, 4 h, ~90%; (e) piperidine, DMF, 0.5 h, ~91%; (f) R^2CO_2H, EDCI, NMM, HOBt, DMF, 3 h, 61–81%; (g) EDCI, HOBt, NMM, DMF, 5 h, 54–62%; (h) H_2, Pd/$BaSO_4$, HOAc, CH_3OH, 10 h, 30–50%.

biological activity. Herein, we would like to report on synthesis, binding affinity, and functional activity of a class of proline based MCR ligands.

The synthesis of *trans* L-proline analogs was carried out using approaches shown in Scheme 1 and all reactions starting from **4** were performed at room temperature. The (2*S*,4*R*) 4-benzyl proline ester **4** was prepared from **3** by a three-step sequence as reported in the literature.[5] Hydrolysis of the ester followed by the amide formation afforded the proline core structure **6** possessing two aromatic side chains. Cleavage of the Boc group gave rise to **7**, which was then transformed into the final compounds for assay by means of two approaches. Coupling **7** with the pbf-(Fmoc)-Arg-OH followed by treatment of the product with piperidine afforded **8**. Acylation of the amino group of Arg moiety and cleavage of protection groups led to the desired analogs. Alternatively, amine **7** was coupled with an acylated Arg **10**, which was prepared from **9**, followed by hydrogenation to deliver final products in a more convergent fashion.

(2*S*,4*S*)-*cis* L-proline analogs were synthesized from **10** and **28** in a similar convergent manner as described in Scheme 1. *cis* Proline derivative **28** was, in turn, prepared from **3** in four steps[6] (Scheme 2).

All analogs were screened in binding and functional assays against the human MC1R, MC3R, and MC4R. Binding affinity (calculated as IC_{50} and K_i values) was determined by measuring the displacement of a constant concentration of europium labeled NDP-α-MSH[7] with competing unlabeled ligands. The agonist activity of MCR ligands was evaluated at three human hMCR

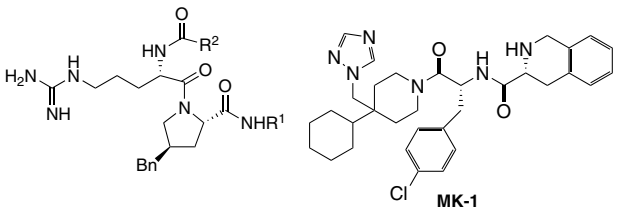


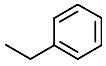
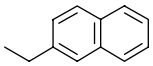
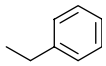
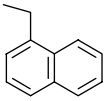
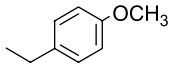
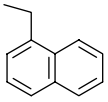
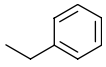
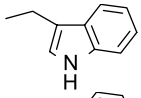
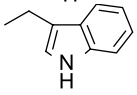
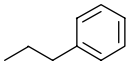
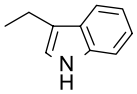
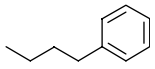
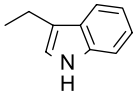
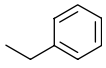
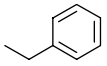
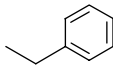
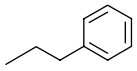
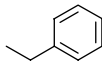
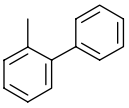
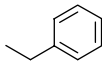
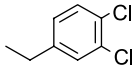
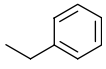
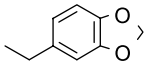
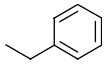
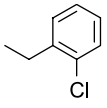
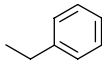
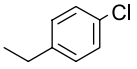
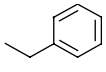
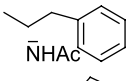
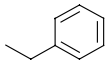
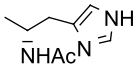
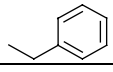
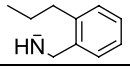
Scheme 2. Synthesis of *cis* proline analogs.

using a cell based assay that is specific for each subtype of MCR (MC1R, MC3R, MC4R). Each subtype of receptor was stably transfected into HEK293 cells. The MCR expressing cells were stably transfected with a reporter system consisting of a cyclic-AMP responsive element (CRE) coupled to a luciferase reporter gene. Responses were compared to the effect of NDP-MSH (MT-I) and expressed as a % of maximum activity of MT-1 (E_{max}). MT-1 is considered to be a full agonist at each of the three MCR subtypes.

Binding affinity at the MC3 and MC4 receptors for *trans* proline ligands are listed in Table 1. The K_i values at the MC1R for all the compounds are >5000 nM. For comparison, a potent and selective 4,4-disubstituted piperidine containing MC4 agonist (**MK-1**) reported by Sebhat and co-workers[2j] was also tested in our assays and the binding data at the MC3 and MC4 receptors are included in Table 1 (K_i for MC1R, 591 nM). The first analog synthesized in this series was compound **11**, which was found to possess K_i values of 139 nM at MC4R and 250 nM at MC3R. Interestingly, it was also one of a few analogs that exhibited weak partial MC1 agonism (EC50, 1684 nM; E_{max}, 51%), although being inactive in MC3 and MC4 functional assays. In fact, none of analogs in this series showed functional activity for MC3 and MC4 receptors. Replacement of 2-naphthylacetic capping group of the Arg residue with 1-naphthylacetyl resulted in a ~5-fold increase in affinity at MC4R (**12**, 29 nM), while maintaining MC3 and MC1 affinity. Although **12** was inactive in functional assays across the three receptors, it represents a significant improvement over the piperazine 2-carboxamide lead compounds in terms of binding affinity. Based on the MC4 binding affinity value and lack of functional MC4 activity for **12**, it was evaluated as an antagonist of the MC4 receptor. Toward this end, MC4-mediated luciferase responses to the MT-1 were determined in the absence and presence of **12** at five different concentrations.[8] Compound **12** caused a rightward-shift of the MT-1 concentration-effect curve with reduction of the E_{max} (Fig. 2), suggesting that it is an insurmountable

Table 1. Binding affinity of *trans* proline analogs



Compound	R^1	R^2	MC4 K_i (nM)[a]	MC3 K_i (nM)[a]
MK-1			9 ± 1	736 ± 49
11			138 ± 47	250 ± 38
12			29 ± 8	258 ± 11
13			47 ± 9	871 ± 242
14			243 ± 91	1110 ± 110
15			1057 ± 296	2422 ± 503
16			667 ± 143	1486 ± 93
17			226 ± 85	527 ± 136
18			1845 ± 85	1360 ± 435
19			8017 ± 2242	4861 ± 616
20			2780 ± 547	10,733 ± 752
21			255 ± 62	900 ± 109
22			333 ± 81	270 ± 55
23			310 ± 76	692 ± 171
24			109 ± 17	66 ± 13
25			31,392 ± 6392	7780 ± 894
26			4756 ± 266	11,250 ± 0
27			10,788 ± 5133	7061 ± 358

[a] The K_i values represent the mean of the at least three experiments ± SEM.

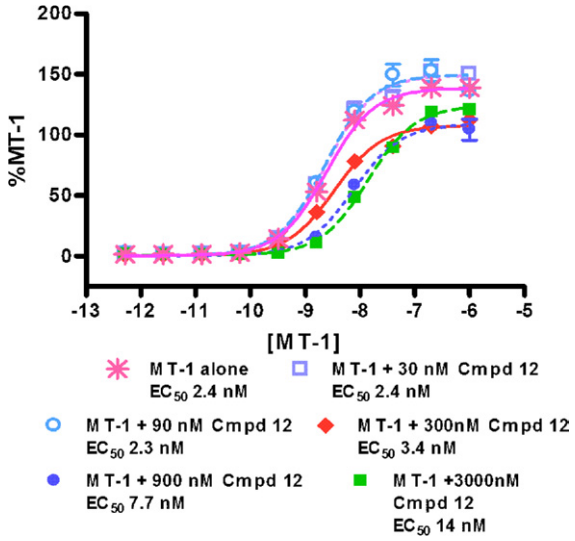


Figure 2. MT-1 dose response curves ± compound **12**: luciferase response in MC4cre Cells.

antagonist at the MC4 receptor. Introduction of a methoxyl group to the *para* position of C-2 amide phenyl ring of **12** afforded an analog, **13** that maintained MC4 binding but was 3-fold less potent at MC3R relative to **12**.

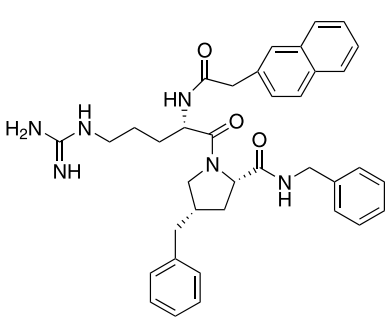
Substitution of the naphthyl group present in **12** for a hydrogen-bonding capable indole ring led to a ~8-fold loss in MC4 affinity and a ~4-fold loss in MC3 affinity (**14**). Additional indole analogs were then prepared to examine whether the affinity could be regained by varying the linkage length for the C-2 phenyl ring. Interestingly, shortening (**15**) or lengthening (**16**) the space between the phenyl ring and the nitrogen atom of C-2 amide moiety by one-carbon resulted in 4-fold and 3-fold loss, respectively, in binding at MC4R and a slight drop in MC3R. However, lengthening the space by two-carbon (**17**) had no effect on MC4 affinity but led to a slight increase at MC3 affinity.

A fused bicyclic naphthyl moiety present in capping group of the Arg residue in **11** appears to be important for significant binding. Replacement of the naphthyl methyl group with benzyl (**18**), 2-phenyl ethyl (**19**), and 2-biphenyl (**20**) significantly reduced binding at MC4R and MC3R in each case. The trend was observed as well when 3,4-dichlorobenzyl (**21**), 3,4-(methylenedioxy)phenyl methyl (**22**), and 2-chlorobenzyl (**23**) were employed. Nevertheless, when 4-chlorobenzyl was used in place of the naphthyl methyl of **11**, the resulting analog (**24**) retained the MC4 affinity but showed 4-fold better affinity at MC3R with a K_i value of 66 nM, the best MC3 affinity achieved in this series.

Use of an amino acid as the capping group of the Arg residue was briefly explored to probe if introducing chirality in conjunction with the hydrogen bonding capacity would promote affinity and functional activity. Three amino acids, Phe (**25**), His (**26**), and Tic (**27**), were examined along this line and all of them significantly decreased affinity at the MC3 and MC4 receptors as compared to the bicyclic naphthylacetyl group.

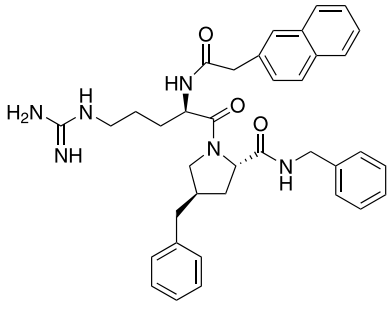
To determine the stereochemistry requirement at the C-4 position of the proline ring, (2*S*,4*S*)-*cis* analog **29**, a stereoisomer of **11**, was synthesized and screened. However, it did not show significant affinity at MC1R, MC3R, and MC4R. Similarly, inverting the chirality of the Arg residue of **11** to *R* configuration (**30**) led to a complete loss of binding as well (Fig. 3).

Fostch et al. recently reported a conceptually similar approach of the design of *cis*-cyclohexane diamine based MC4 agonists from a potent D-Phe-Arg-Trp containing MCR peptide ligand.[9] Their strategy was to use a set of low energy structures derived from NMR data for the peptide ligand to identify ring systems that could position groups in close proximity to the side chains of the D-Phe-Arg-Trp tripeptide. Lack of the functional activity with our proline analogs is likely because spatial orientation of four groups intended to mimic the side chains of the tetrapeptide could not result in bioactive conformations required for activating MCRs under current topographical pattern and conformational restriction mode. Our subsequent efforts toward investigating a variety of approaches of introducing conformational restriction between two adjacent amino acid residues of the tetrapeptide His-D-Phe-Arg-Trp have identified an optimum constraining mode for designing potent MCR peptidomimetic agonists, which will be reported in due course.



29

MC4R, Ki>50,000 nM



30

MC4R, Ki>50,000 nM

Figure 3. Stereochemical effect on affinity.

In summary, a series of proline-based MCR ligands displaying significant binding affinity at the MC3 and MC4 receptors has been prepared and evaluated. The discovery of these compounds originated with lead compounds that emerged from screening libraries built on an analogous piperazine-2-carboxamide scaffold. Key features of these compounds include two conformationally restricted aromatic side chains and a N-capped Arg moiety. Although significant MCR agonism was not achieved with this type of structures, some of these analogs displayed high affinity at MC4R (**12**, 29 nM) and MC3R (**24**, 66 nM). Further, this work has identified a useful scaffold for further MCR ligand designs.

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8. Stably transfected Hek293 cells were seeded into poly-D-lysine coated 96-well plates at a density of 20,000 cells per well. Twenty-four hours later the media was removed and fresh media containing decreasing concentrations of MT-1 agonist and a constant concentration of **12** was added. After 4 h at 37 °C this media was removed and the cells were lysed. Luciferin substrate was injected and read immediately on a 1450 Microbeta Wallac Jet.
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