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Design of a New Peptidomimetic Agonist for the Melanocortin Receptors Based on the Solution Structure of the Peptide Ligand, Ac-Nle-cyclo[Asp-Pro-DPhe-Arg-Trp-Lys]-NH_2

Christopher Fotsch,* Duncan M. Smith, Jeffrey A. Adams, Janet Cheetham, Michael Croghan, Elizabeth M. Doherty, Clarence Hale, Mark A. Jarosinski, Michael G. Kelly,† Mark H. Norman, Nuria A. Tamayo, Ning Xi and James W. Baumgartner

Departments of Small Molecule Drug Discovery and Metabolic Disorders, Amgen Inc., One Amgen Center Drive, Thousand Oaks, CA 91320, USA

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Abstract—The solution structure of a potent melanocortin receptor agonist, Ac-Nle-cyclo[Asp-Pro-DPhe-Arg-Trp-Lys]-NH_2 (**1**) was calculated using distance restraints determined from ^{1}H NMR spectroscopy. Eight of the lowest energy conformations from this study were used to identify non-peptide cores that mimic the spatial arrangement of the critical tripeptide region, DPhe-Arg-Trp, found in **1**. From these studies, compound **2a**, containing the *cis*-cyclohexyl core, was identified as a functional agonist of the melanocortin-4 receptor (MC4R) with an IC_{50} and EC_{50} below 10 nM. Compound **2a** also showed 36- and 7-fold selectivity over MC3R and MC1R, respectively, in the binding assays. Subtle changes in cyclohexane stereochemistry and removal of functional groups led to analogues with lower affinity for the MC receptors.

The melanocortin receptors (MCRs)[1,2] are a class of G-protein coupled receptors that are involved in inflammation,[3] skin pigmentation,[4] steroidogenesis,[5] fat metabolism,[6] sexual function,[7,8] feeding behavior,[9,10] and exocrine gland secretion.[11] Their function is regulated by peptide agonists (e.g., α-, β-, γ- melanocyte stimulating hormones (MSH), and adrenocorticotropic hormone (ACTH)) and antagonists (e.g., agouti and agouti-related protein). The MCRs are attractive targets for drug discovery,[12,13] but the use of their endogenous ligands as therapeutic agents is not practical. In general, peptides are metabolically unstable, poorly absorbed, and are costly to prepare. In this report we describe our initial efforts to design and synthesize non-peptide agonists for the melanocortins. Our approach was to determine the structure of a peptide ligand, and use that structure to design non-peptide agonists for the receptor.[14] Since our group was interested in developing new therapies for obesity, we focused on agonists for MC4R because of its role in feeding behavior.[9,10]

Endogenous agonists for the MCRs all contain the tetrapeptide sequence, His-Phe-Arg-Trp, which has been labeled the 'message sequence', that is, the minimal peptide sequence necessary for activating the receptor.[15] Further studies with MCR agonists have shown that histidine can be replaced with alanine without much loss in activity,[16] and phenylalanine when replaced with its D-stereoisomer has better activity at the MCRs.[17] Therefore, we focused on mimicking the tripeptide, DPhe-Arg-Trp. This tripeptide is only weakly active at the MCRs,[18] but the cyclic peptide, Ac-Nle-cyclo[Asp-Pro-DPhe-Arg-Trp-Lys]-NH_2 (**1**), reported by Bednarek and co-workers,[19] has low nM affinity at MC3, 4, and 5R. Apparently, in the cyclic peptide (**1**), DPhe-Arg-Trp is positioned in a way that better complements the MCRs. To understand the bioactive conformation of the DPhe-Arg-Trp, we set out to determine the solution structure of peptide **1**. After we determined the structure of **1**, we would prepare compounds that could position groups in close proximity to side-chains found on the DPhe-Arg-Trp.

*Corresponding author. Tel.: +1-805-447-7746; fax: +1-805-480-1337; e-mail: cfotsch@amgen.com
†Current address: Renovis, 270 Littlefield Avenue, South San Fransico, CA 94080, USA.

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We used 600MHz ^{1}H NMR spectroscopy[20] to determine the solution structure of peptide **1**. The structure was calculated using eighty-two distance restraints between hydrogen atoms at least four bonds apart. One dihedral angle (Lys χ^1 fixed at 180 ± 30^o), and one hydrogen bond (Lys αNH to Asp C=O, obtained from NH temperature coefficients) were also included in the calculations. Through this analysis, an ensemble of 35 low energy structures were calculated, and eight of the lowest energy structures were used for modeling (see Fig. 1). Peptide **1** did not exist in any classical turn structure, but we saw a significant number of short-range nuclear Overhauser effects that suggested conformational preferences for the peptide backbone. These findings are in contrast to the NMR structure of a closely related analogue Ac-Nle-cyclo[Asp-His-DPhe-Arg-Trp-Lys]-NH_2, MT-II, reported by Bednarek and co-workers.[16] They concluded that the peptide backbone did not have any preferred conformation. The structure of peptide **1** was determined at 5 °C in aqueous buffer rather than in DMSO-d_6 or CD_3OH at 25 °C used for the NMR of MT-II, which could account for the differing observations.

With the structure of **1** in hand, various ring systems (carbocyclic and heterocyclic) appended with groups that mimicked the side-chains on DPhe-Arg-Trp were overlapped with our NMR structure. Those compounds that showed the best overlap were synthesized. Two examples, cyclohexanes **2a** and **2b** (see Fig. 2), contain a tryptamine group at the 4-position intended to mimic tryptophan, a butyl guanidine on the 1-position mimicking arginine, and D-phenylalanine at the 1-position. Since the backbone of peptide **1** has no charge, acyl groups were added to D-phenylalanine and to the β-nitrogen on the tryptamine to keep the compound neutral at those positions. Compound **2a** can adopt one of two chair conformations that have either H(a) or H(b) in the equatorial position. Isomer **2b** (*trans*) exists as one chair conformation with H(a) and H(b) both in the axial orientation. All three conformations of **2** overlapped with the tripeptide region of **1** equally well.

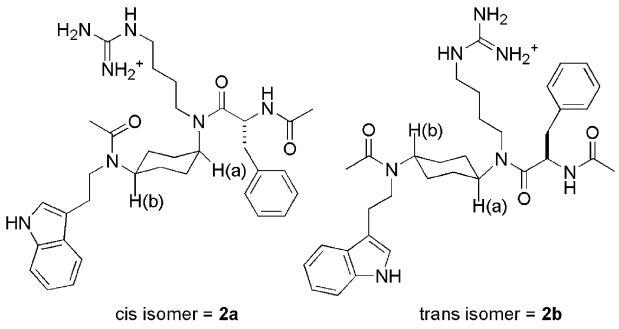


Figure 2.

Compound **2** was synthesized (see Scheme 1) by first appending tryptamine onto 1,4-cyclohexanedione ethylene ketal (**3**) through a reductive amination. Intermediate **4** was then acylated and deprotected to give ketone **5**. A second reductive amination was performed

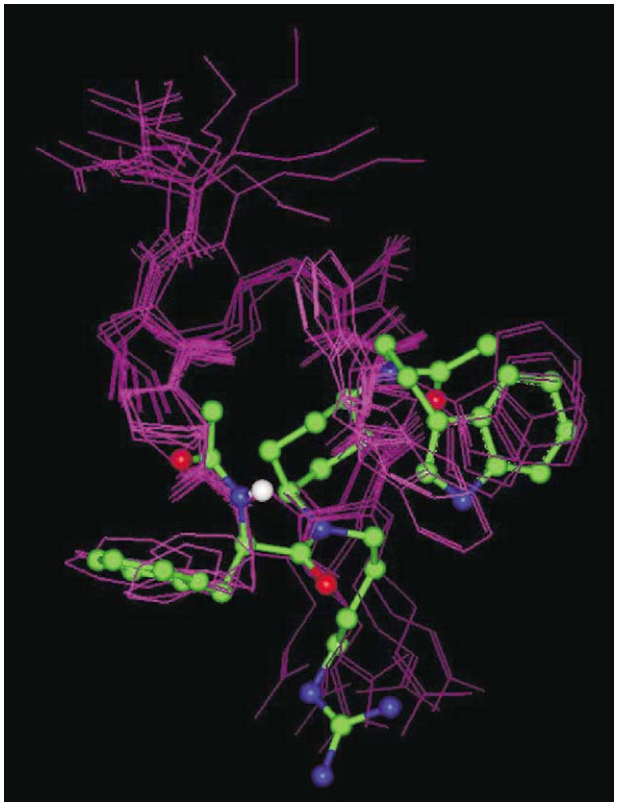
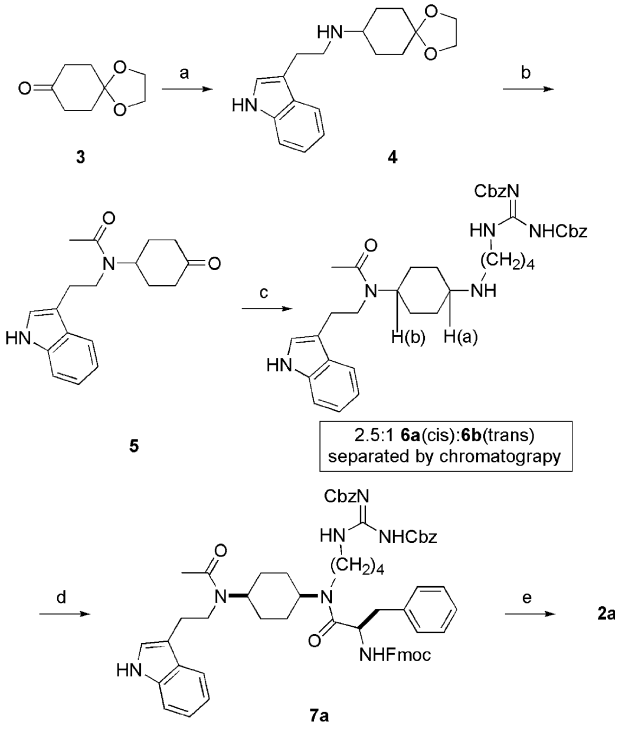
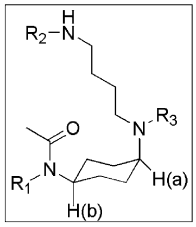


Figure 1. Ensemble of eight lowest energy conformations of (**1**) (in purple); compound **2a** is overlaid (carbon atoms are in green, hydrogen atoms are removed for clarity).



Scheme 1. Synthesis of Compound **2a**: (a) tryptamine, $NaHB(OAc)_3$, DCE; (b) (i) Ac_2O, Et_3N; (ii) aq AcOH, (65% from **3**); (c) $H_2N(CH_2)_4NHC(=NCbz)NHCbz$, $NaHB(OAc)_3$, DCE (89%); (d) FmocDPheCl, BSA, CH_2Cl_2 (66%); (e) (i) TAEA, CH_2Cl_2; (ii) Ac_2O, Et_3N, CH_2Cl_2 (46%, 2 steps); (iii) H_2, Pd-C, CH_3OH; AcOH (50%).



	R_1	R_2	R_3
8	2-(Indol-3-yl)-ethyl-	$H_2N(HN=)C$-	Ac-
9	H_3C-	$H_2N(HN=)C$-	AcDPhe-
10	2-(Indol-3-yl)-ethyl-	H-	AcDPhe-
11	2-(Indol-3-yl)-ethyl-	$H_2N(HN=)C$-	AcLPhe-

Scheme 2. Analogues of Compound **2a**.

Table 1. Biological activity of analogues of compound **2**

Compd	mMC1R, IC_{50}, nM[a]	hMC3R, IC_{50}, nM[a]	hMC4R, IC_{50}, nM[a]	hMC4R, EC_{50}, nM[b]
1	nd	23 (±6)[c]	3.0 (±2)	0.17 (±0.1)[c]
2a	51 (±20)[d]	280 (±80)[e]	7.7 (±2)[f]	4 (±3) (93%)[g]
2b	>10,000	>10,000	3500 (±500)	680 (±90) (90%)[g]
8	>10,000	>10,000	>10,000	nd
9	9000	>10,000	>10,000	nd
10	>10,000	>10,000	9250 (±750)	nd
11	nd	nd	500 (±90)	nd

[a]^{125}I-NDP-α-MSH binding to the melanocortin receptors, SEM of two IC_{50}s determined over six dilutions except where noted.
[b]Intracellular levels of cAMP in cells expressing melanocortin receptors, SEM of two EC_{50}s determined over six dilutions.
[c]Data from ref 19.
[d]$n=4$.
[e]$n=3$.
[f]$n=8$.
[g]Percentage of maximal response with respect to α-MSH.

with the bis-benzylcarbamate (Cbz) protected (4-aminobutyl)guanidine[21] to provide a mixture of *cis* and *trans* isomers **6a** and **6b** that were separable by flash chromatography. Isomers **6a** and **6b** were stereochemically assigned by examining the coupling constants of the H(a)-signal. The aliphatic region in the ^{1}H NMR contained many overlapping signals, so to simplify the spectrum, H(b) (δ 3.6 ppm, CD_3OD) was irradiated in a TOCSEY experiment to enhance the peaks on the cyclohexane ring. The H(a) peak (δ = 2.9 ppm, CD_3OD) on the *cis*-isomer **6a** was a multiplet, but after irradiating the H(b) peak, a $J \approx 4$ Hz was observed for H(a), which is an indication of either an equatorial/equatorial or equatorial/axial coupling. Doing the same for the *trans*-isomer **6b**, a $J \approx 10$ Hz was measured for the H(a) peak (δ = 2.4 ppm, CD_3OD) which correlates to an axial/axial coupling. Each isomer was then independently carried through the remainder of the synthesis (the *cis*-isomer is shown for the remaining part of the synthesis in Scheme 1). The secondary amine on **6** proved to be a difficult coupling partner for Fmoc-DPhe-OH. After extensive experimentation with standard coupling reagents, we found that by pre-treating **6** with *N,O*-bis(trimethylsilyl)acetamide (BSA)[22] and then adding the acid chloride of Fmoc-DPhe-OH, we were able to obtain **7** in adequate yields (66% for **7a**). The Fmoc protecting group was removed with *tris*-(2-aminoethyl)amine (TAEA) using the protocol described by Carpino and co-workers,[23] the α-amino group was acetylated, and the Cbz protecting groups were then removed by hydrogenolysis to provide the desired product **2**. Analogous synthetic methods were used to prepare compounds **8–11**.

Compounds were tested at the mouse MC1R, human MC3R, and human MC4R. Table 1 summarizes the results of a ligand (NDP-MSH) displacement assay.[24] For comparison the activity of peptide **1** is included in Table 1. The *cis*-isomer **2a** had sub-micromolar IC_{50}s at all of the MCRs, and was the most potent at MC4R with an $IC_{50} = 7.7$ nM. Compound **2a** was also 36- and 7-fold selective over MC3R and MC1R, respectively. The IC_{50}s for the *trans* isomer **2b** were >10,000 nM for MC1R and MC3R and ca. 3000 nM at MC4R. The functional activities of **2a** and **2b**, measured by cAMP production of HEK 293 cells transfected with recombinant human MC4R,[25] were 4 and 680 nM, respectively. The large difference in activity between the *cis* and *trans* isomers of **2** cannot be explained by their overlap with the structure of **1**. There seems to be enough flexibility in the side chains of DPhe-Arg-Trp that either isomer of **2** could be positioned to overlap with the functionality found on the key tripeptide. Compound **2a** has one group appended in the axial orientation, so the indole group and the phenylalanine/guanidine groups are closer together than in the trans isomer **2b**. Apparently, the closer arrangement of these groups on **2a** better approximates the active conformation of **1**.

Analogues of **2a** were also prepared to determine the significance of each functional group (compounds **8–11**, see Scheme 2). Compounds **8–10** were weakly active ($IC_{50} = 9000$ to >10,000 nM) at each of the receptor sub-types. The replacement of either the D-phenylalanine group with acetyl (**8**) or the replacement of the tryptamine group with a methyl (**9**) was detrimental to activity. Bednarek and co-workers reported[16] similar results in their work with the potent melanocortin peptide agonist MTII (Ac-Nle-cyclo[Asp-His-DPhe-Arg-Trp-Lys]-NH_2). In their studies they also showed that removing the amino acid side chains on D-phenylalanine or tryptophan, by substituting with alanine, was deleterious to activity at the MCRs. In compound **10**, the amino group replacement for the guanidine also adversely affected activity even though both groups are positively charged at physiological pH. Compound **2a** may be more potent than **10** since the guanidine group is able to form additional hydrogen bonding interactions with the receptor. Finally, the LPhe analogue, compound **11**, was 65-fold less potent than **2a**, which is similar to the trend observed for peptide ligands.[17]

By using a set of low energy structures calculated from NMR data collected for the potent MCR peptide ligand (**1**), we were able to design molecules that overlapped with the tripeptide region of DPhe-Arg-Trp. Among those compounds were those that contained the cyclohexane core. When substituted with D-phenylalanine, guanidine, and indole we were able to identify compound **2a**, which had an IC_{50} <10 nM at MC4R, and showed selectivity versus MC3R and MC1R. Compound **2a** was also a low nM functional agonist of MC4R. The overall strategy for identifying a peptidomimetic agonist from the structure of a peptide ligand was successful. Compound **2a** still has liabilities that may prevent it from being well absorbed in vivo (e.g.,

presence of polar guanidine group), but it could be used as a lead to discover compounds that are more 'drug-like'.

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20. The sample was dissolved in 90% H_2O/10% D_2O, and the pH was adjusted to 5.0 with dilute HCl or NaOH. The spectra were acquired at 5 °C, and the resonances were assigned using TOCSEY and ROESY spectra. Intranuclear distances were obtained from NOESY data, and structures were calculated using XPLOR.
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24. **Ligand Binding Assay**. Binding assays were performed in 96-well U-bottom plates. Membranes (200 μg tissue) were incubated at 30 °C for 2 h in assay buffer with various compounds in the presence of 0.2 nM ^{125}I-NDP-α-MSH (AmershamPharmacia Biotech, Piscataway, NJ) in 100 μL total volume. Non-specific binding was assessed in the presence of 1 μM cold NDP–MSH. The reaction was terminated by rapid filtration through Unfilter-96 GF/C glass fiber filter plates (FilterMate 196 Harvester, Packard Instrument Company, Meriden, CT) followed by three washes with 300 μL of ice-cold water. Bound radioactivity was determined using a TopCount microplate scintillation and luminescence counter (Packard Instrument Company, Meriden, CT). Nonlinear regression analyses of test compound concentration curves were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).
25. **Functional (cAMP) Assay:** Human embryonic kidney cells (HEK 293) expressing the melanocortin 4 receptor were seeded into 96-well fibronectin coated plates to a density of 75,000 cells/well. Plates were used for assay ca. 48 h after seeding. Assay was initiated by removing media from cells and replacing with 90 μL of assay buffer (DMEM + 100 μM 3-Isobutyl-1-Methylxanthine, IBMX), followed by addition of 10 μL of the test peptide ranging in concentration from 0.1 nM to 10 μM. Cells were then incubated for 30 min at 37 °C. Removal of assay media and addition of 100 μL Tropix Lysis Buffer terminated the reaction. Production of cAMP was measured using Tropix cAMP-Screen 96-well assay (Applied Biosystems, Foster City, CA). The kit used was a chemiluminescent ELISA system designed for quantitation of cAMP from mammalian cell extracts. Chemiluminescent activity was measured using a Labsystems Luminoscan (Labsystems, Inc., Franklin, MA). Nonlinear regression analyses of test compound concentration curves were performed using GraphPad Prism.