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A predictive pharmacophore model of human melanocortin-4 receptor as derived from the solution structures of cyclic peptides

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Abstract—Using nuclear magnetic resonance (NMR) spectroscopy, we have determined the solution structures for a series of potent agonists for the human melanocortin-4 receptor (hMC4R), based on the cyclic peptide MT-II [Ac-Nle-cyclo-(Asp-Lys) (Asp-His-(D)Phe-Arg-Trp-Lys)-NH_2]. Members of this series were designed to improve selectivity for MC4R versus the other melanocortin receptors, and to reduce the flexibility of the side chains. The most selective and rigid analog [penta-cyclo(D-K)-Asp-Apc-(D)Phe-Arg-(2*S*,3*S*)-β-methylTrp-Lys-NH_2] was found to be a full agonist of hMC4R with an EC_{50} of 11 nM against hMC4R, and to exhibit 65-fold selectivity against hMC1R. This compound represents the most constrained hMC4R peptide agonist described to date. A β-turn structure was conserved among all of the cyclic peptides studied. The rigidity of the analogs allowed an exceptionally well-defined pharmacophore model to be derived. This model was used to perform a virtual screen using a library of 1000 drug-like compounds, to which a small set of known potent ligands had been intentionally added. The utility of the model was validated by its ability to identify the known ligands from among this large library.

1. Introduction

The melanocortin (MC) peptide family, comprising α-, β-, and γ-melanocyte stimulating hormone (MSH) and adreno-corticotropic hormone (ACTH), is a group of neuropeptides derived from the pro-opiomelanocortin gene product (POMC). The MSH peptides have been implicated in numerous biological functions, including regulation of skin pigmentation, regulation of steroid production, modulation of the immune response, thermoregulation, obesity, and sexual function.[1–4] The physiological actions of these peptides are mediated through five G-protein-coupled receptor (GPCR) subtypes, designated MC1-5R. MC4R, expressed predominantly in the brain, was initially recognized as a promising obesity drug target.[5] Much evidence supports the concept that MC4R is involved in the modulation of food intake.[6–8] MC4R knockout mice display an obesity phenotype that includes maturity onset obesity, hyperglycemia, and hyperinsulinemia.[6] A nonselective cyclic MC4R peptide agonist (MT-II, Fig. 1) inhibits food intake when given icv in mice,[7] while a selective antagonist of MC4R increases food intake in rats.[8] Therefore, much effort has been invested toward developing a selective, orally delivered small molecule MC4R agonist as a possible treatment for obesity.[9–16] So far, most of the successes in the area have been observed with peptide agonists of MC4R.[9–12] While relatively few small molecule agonists have been reported, most of them lack adequate selectivity.[13–17] Therefore, our investigation was aimed at how to make the best use of structural information derived from potent and selective peptides toward the discovery of small molecule agonists. Structural information on the ligand is particularly important in the case of GPCR targets, since structural information for the protein transmembrane receptor is difficult to obtain. Homology models of such a receptor could be derived from the structure of rhodopsin, but would suffer from inaccuracy due to its low sequence similarity to other GPCR's, and from the fact that rhodopsin possesses an unusual binding site where the 'ligand', retinal, is covalently bound.[18,19]

One way to bridge the gap between peptides and small molecules is through the use of a pharmacophore

Keywords: MC4R; Pharmacophore model; NMR; Cyclic peptides.
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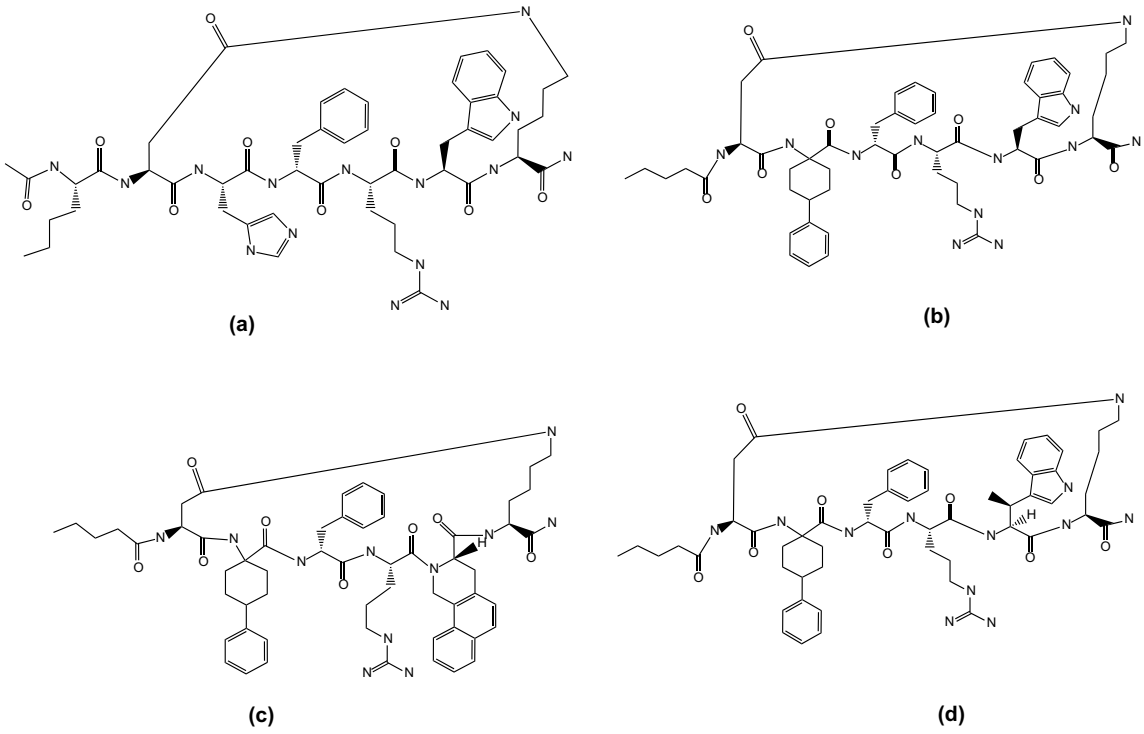

Figure 1. Schematic representation of MT-II (a), peptide **2** (b), peptide **3** (c), and peptide **4** (d).

model.[20,21] A pharmacophore model identifies the minimal chemical features necessary for activity, and defines their relative positions, sometimes also including descriptors for shape and excluded volumes. A good pharmacophore model should be helpful for guiding both optimization of small molecule ligands and for performing virtual screening.[22]

Peptides offer a platform for exploring a large range of structural diversity. A structure–activity relationship (SAR) can be built quickly by replacing side chains with different amino acids (e.g., Ala walk) or varying the chirality of the α-carbons (e.g., D walk). The peptide approach is suitable in the area of GPCR drug discovery, since the natural ligands of many GPCRs are peptides. This approach can also be valuable in reducing risk for GPCR drug discovery processes—proof of concept can be achieved using peptides before the initiation of a small molecule drug discovery project. However, the high flexibility of peptides limits their application in deriving pharmacophore models, where chemical features need to be localized as accurately as possible. Cyclization is a common strategy to rigidify the backbone conformation of a peptide,[23] while introduction of rigid non-natural amino acids and β-carbon methylation can rigidify the side chains to defined conformations.[24] All of these strategies were used in the present study in attempts to achieve an extremely rigid template for pharmacophore modeling.

2. Results

In our efforts to discover potent, selective, and rigid peptide agonists, extensive structure–activity studies were conducted in our laboratories using the previously described cyclic peptide MT-II[12] as the template (Fig. 1). Four sequential residues [His-(D)Phe-Arg-Trp] in MT-II have been identified as key residues in determining the binding affinity and efficacy toward the melanocortin receptors.[24] By systematically replacing each of the four key residues with natural and non-natural amino acids, it was discovered that the side chains of the aromatic residues (D)Phe and Trp are critical for the binding of MT-II analog, while the basic hydrophilic residues His and Arg are less important for binding and agonist activity.[25] The parent peptide MT-II is very potent against hMC4R, but it is nonselective (Table 1). We

Table 1. MT-II and its analogs

No	Compound	MC-4, nM	MC-1, nM
1	Ac-Nle-cyclo(D-K)-Asp-*His*-(D)Phe-Arg-Trp-Lys-NH_2 (MT-II)	0.6	0.6
2	Penta-cyclo(D-K)-Asp-*Apc*-(D)Phe-Arg-Trp-Lys-NH_2	9	654
3	Penta-cyclo(D-K)-Asp-Apc-(D)Phe-Arg-*Tap*-Lys-NH_2	713	3813
4	Penta-cyclo(D-K)-Asp-Apc-(D)Phe-Arg-(2*R*,3*S*)-β-*methylTrp*-Lys-NH_2	49.6 (PA)	1296
5	Penta-cyclo(D-K)-Asp-Apc-(D)Phe-Arg-(2*S*,3*S*)-β-*methylTrp*-Lys-NH_2	11	700

PA = partial agonist.

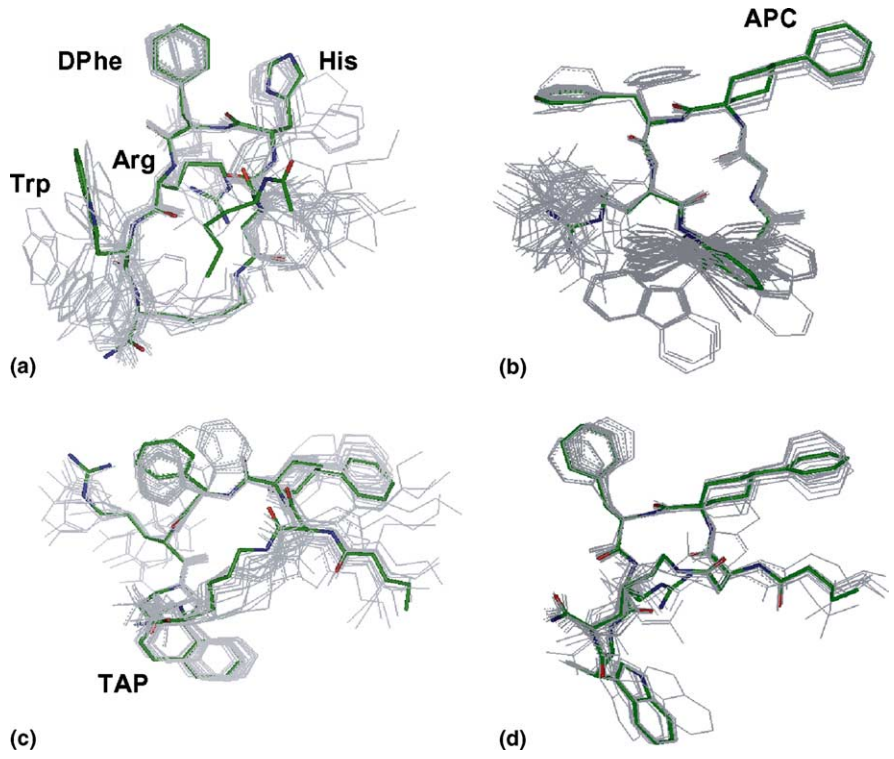

Figure 2. Superposition of low-energy members of the ensemble of structures determined by NMR, for: MT-II (a), peptide **2** (b), peptide **3** (c), and peptide **4** (d). The lowest energy conformations are color coded.

determined its structure in solution using NMR and used the range of conformations compatible with the NOE-derived distance constraints as a measure of flexibility (Fig. 2a). The backbone segment composed of His-(D)Phe-Arg-Trp formed a well-defined β-turn structure, as had been observed in NMR studies of linear analogs of α-MSH,[26] while the side chains appeared to be flexible and their positions were not well defined. Replacement of His with various amino acids, including the novel and conformationally constrained amino acids 2-aminotetraline-2-carboxylic acid (ATC), 1-amino-4-phenylcyclohexane-carboxylic acid (APC), and 4-aminophenylpiperidine-4-carboxylic acid (APPC) (Fig. 2) resulted in peptides, which are highly selective for MC4R.[24,25] For peptide **2**, in which His was substituted with APC, NOEs verified that the phenyl ring and the N-terminus of APC are in the *cis*-configuration (Fig. 3). While the side chain of His was found to be scattered in MT-II, the APC moiety was found to be exceptionally rigid and locked into a single conformation (Fig. 2b). The APC residue apparently confers selectivity by providing a nonadjustable protrusion, which is compatible only with the MC-4 receptor binding site and not with other family members.[27] In this peptide (peptide **2**), the side-chain conformations of (D)Phe were split into two distinct families, but the side-chain positions of Arg and Trp remained diverse (Fig. 2b).

Further NMR structural studies showed that TAP (1,2,3,4-tetrahydro-benzo-isoquinoline-3-carboxylic acid) replacement at the Trp position (peptide **3**) seemed to restrict the side chain, but to an improper conformation, resulting in reduced potency and efficacy. Methyl substitutions on the β-carbon were also found to rigidify the position of the Trp side chain, and allow hMC4R agonist activity to be maintained, with the (2*S*,3*S*) configuration (peptide **5**) being the most favorable (Table 1). While NMR structure determination showed that incorporation of the (2*R*,3*S*)-β-methyl-Trp (peptide **4**) resulted in single side-chain conformations for APC, (D)Phe, and even Nle, the flexibility of the side chain of Arg was not reduced (Fig. 2d). Its counterpart, the (2*S*,3*S*)-β-methyl-Trp (peptide **5**), was not only more active, but resulted in the most rigid peptide yet

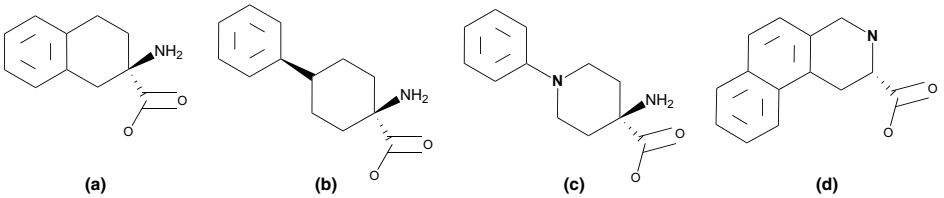

Figure 3. The structures of (a) ATC, (b) APC, (c) APPC, and (d) TAP.

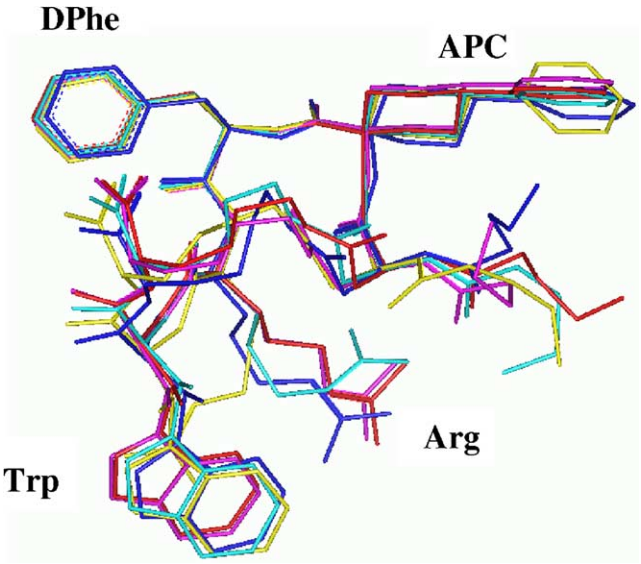

Figure 4. Superposition of five low-energy NMR structures of peptide **5**.

obtained as indicated by NMR. The NMR structure of this peptide, penta-cyclo(D-K)-Asp-APC-(D)Phe-Arg-(2*S*,3*S*)-β-*methylTrp*-Lys-NH_2 (peptide **5**) is shown in Figure 4. In this peptide, the side chains of APC, (D)Phe, and Trp are all locked into specific conformations, and the guanidine of Arg is stacked with the indole ring of Trp, apparently due to an energetically favorable packing.[28]

A 4-point pharmacophore model was generated from the solution structure of peptide **5**. The model was based on the three hydrophobic sites of APC, (D)Phe, and Trp, and a positive-charge center defined by the Arg side chain. To validate the model, a virtual library was constructed, composed of 990 compounds from the Advanced Chemical Directory (ACDv.2000-1)[29] selected and identified as 'drug-like' by Bissantz et al.[30] This library was seeded with 10 compounds possessing known MCR binding activities, as shown in Table 2. The pharmacophore model was used to perform a virtual screen of the 1000-compound library. Each of the compounds was enumerated to 250 conformations by using Catalyst.[31] From the screening process, a total of eight hits was identified. Seven out of these eight were the most potent members of the seeding set of small

Table 2. Binding affinity of 10 reported small molecule MC4R agonists used in virtual screening

Compd	IC_{50} (nM)	Hit/non-hit	Ref.
6	200	H	14
7	238	H	13
8	1.2	H	13
9	0.3	H	13
10	80	H	16
11	7040	N	15
12	13,050	N	15
13	4000	N	17
14	1.2	H	13
15	2400	H	14

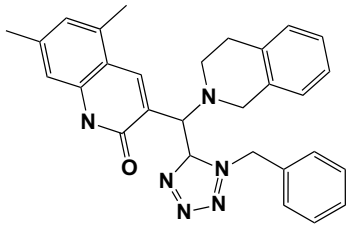

Figure 5. Structure of ASI32768.

molecule agonists that had been added to the library. This verified the ability of the pharmacophore model to successfully identify MC4R ligands from among a large set of diverse drug-like small molecules. One of the eight hits was an ACD compound ASI32768 (Fig. 5). The binding activity of this compound to MC4R is not known. However, ASI32768 might be a false positive, since the nitrogen in the 1,2,3,4-tetrahydro-3-isoquinoline, which was assigned as protonated with SLN (Sybyl Line Notation),[32] is probably not very basic, with a calculated pK_a of 6.45.[33] Of the seven known agonists that were identified as hits, compound **14** (Fig. 6), mapped onto the pharmacophore model at a low-energy conformation with the highest fit scores. Figure 7 shows the best mapping of compound **14** to the 4-point pharmacophore model, where the mapping score was 3.9 out of the perfect score of 4.0, and the conformation energy was zero (the lowest possible energy defined by Catalyst).[31] Its analogs, compound **7**, **8**, and **9**, mapped onto the 4-point pharmacophore with comparable quality, where the scores were better than 3.6, and energies were lower than 5.0 kcal/mol. All of these compounds are potent binders to MC4R. The three least-active compounds, **11**, **12**, and **13**, did not map well to the 4-point pharmacophore model, showing that the model can successfully segregate compounds into high- and low-potency classes.

In Figure 8, the superposition of the solution structure of peptide **5** and the zero-energy conformation of compound **14** is illustrated. A comparable approach, resulting in a different orientation, was reported by Sebhat et al.,[13] although his study utilized the more flexible MT-II peptide (peptide **1**), for which a low-energy conformation was arbitrarily selected. As can been seen from the present overlay (Fig. 8), chloro substitution on the phenyl ring of compound **14** helps the fragment to reach the side chain of (D)Phe, cyclohexane attached to piperidine maps into the APC site, and the 1,2,3,4-tetrahydro-3-isoquinoline hits both the Trp and Arg sites. The contribution of the triazole is not obvious. One possible role of this moiety is to improve the pharmacokinetic or physicochemical properties of the molecule, although another possibility is that the triazole helps to lock in the proper binding conformation. The mapping of compound **7** did not make obvious the basis of its 200-fold lower binding affinity, compared to its closely related analogs, compounds **8**, **9**, and **14**. This represents one of the limitations of pharmacophore-based methods—pharmacophores capture only the essential features determining the activity of a compound, but they do not include the subtle features

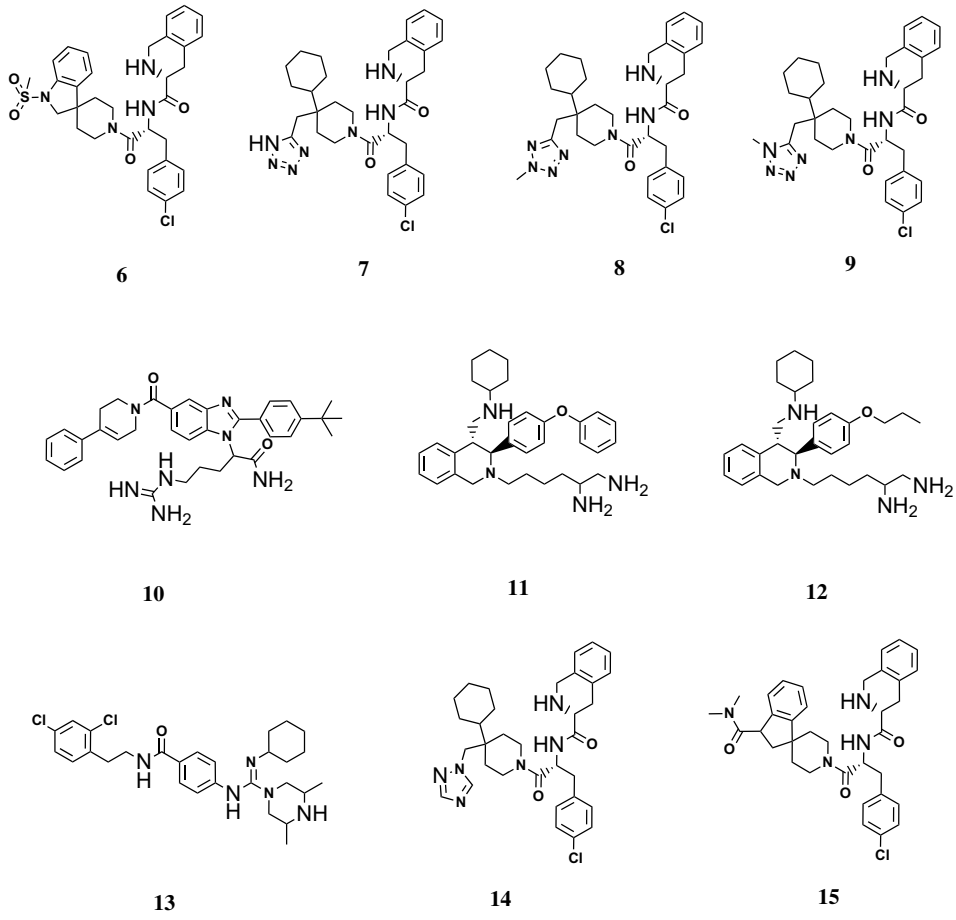

Figure 6. The structures of the 10 MCR ligands used in the virtual screening exercise.

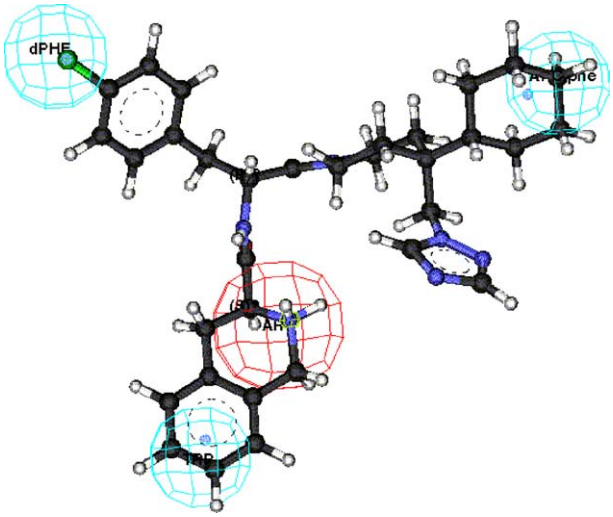

Figure 7. The mapping of compound **14** into the 4-point pharmacophore model of hMC4R.

that determine the finer differences in activity. One possible explanation of the detrimental effect of the tetrazole of compound **7** is that the site it hits is hydrophobic, corresponding to the binding site of the N-terminal pentyl group of cyclic peptide **5** (Fig. 8). This idea is supported by the observation that methylation of the tetrazole, in compounds **8** and **9**, restores activity.

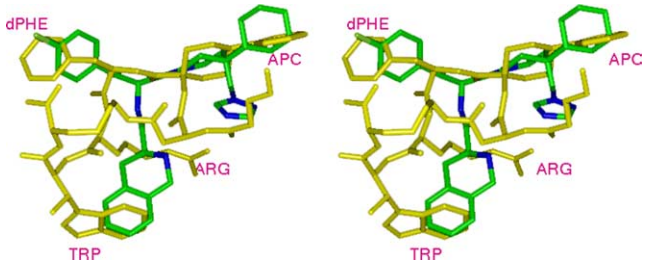

Figure 8. Stereopair illustrating the superposition of compound **14** (green, with nitrogens colored blue), in its calculated low-energy conformation, onto the NMR-determined solution structure of peptide **5** (yellow) (the lowest energy member of the ensemble is shown).

3. Summary

An exceptionally rigid cyclic peptide ligand (penta-cyclo(D-K)-Asp-Apc-(D)Phe-Arg-(2*S*,3*S*)-β-methylTrp-Lys-NH_2) for hMC4R has been developed. This novel peptide shows high hMC4R agonist activity, and over 60-fold selectivity against hMC1R. Its solution structure has been solved by NMR. The backbone is well defined, and features a β-turn encompassing the region Asp-Apc-(D)Phe-Lys, as has been observed for other peptide agonists. The key to selectivity is most likely the APC residue, which provides a rigid protrusion, that apparently is not compatible with the hMC1R binding site.

The other peptides we studied, whose side chains at this position were found to be more flexible, can apparently adapt properly to either the hMC1R or the hMC4R sites. We were able to fix the Trp side chain into its active conformation by introducing substitutions at the beta position with the optimal stereochemistry. The rigid structure of this potent, selective peptide was used to develop a well-defined pharmacophore model, designed for use in virtual screening with the intent of identifying small, nonpeptidic agonists. The model was validated by its ability to successfully select compounds reported to be potent ligands from a large library of random drug-like compounds.

4. Experimental section

4.1. Synthesis and assay

The linear precursors to the cyclic peptides were synthesized by SPPS using Fmoc/*t*-Bu strategy, deprotected from the resin by trifluoroacetic acid (TFA), purified by RP-HPLC, and characterized by LC/MS. The linear peptides were then cyclized in solution (DMF) using BOP as a cyclization agent. The crude cyclic peptides were purified again by RP-HPLC and characterized by LC/MS. The overall yield of the cyclization is about 50%.

HEK 293 cells transfected with hMC1 and hMC4 receptors were grown in 96 well plates. The cells were stimulated with either 100 nM α-MSH or the cyclic peptides. Cyclic AMP was extracted from the cells and concentration was determined using a c-AMP SPA assay.

More detailed description about the synthesis and assay methods for the cyclic peptides **1**–**5** can be found in Ref. 41.

4.2. NMR measurements

Each sample for NMR spectroscopy consisted of a 1.5 mM solution of peptide in DMSO-d_6 (99.96%; Cambridge Isotopes). NMR experiments were performed on a Varian Unity-500 spectrometer, operating at 499.825 MHz for the observation of protons. The sample was maintained at 20 °C. Resonances were assigned using standard procedures from DQ-COSY, TOCSY, and NOESY spectra.[34] The two-dimensional experiments were acquired in phase-sensitive mode and were collected as 600 (DQ-COSY, TOCSY) or 1024 (NOESY) t_1 increments of 2048 points per FID, with spectral widths of 6500 Hz. The acquisition time was 0.158 s per transient, which was preceded by a delay of 1.5 s. The total number of transients per FID was 48 (DQ-COSY, TOCSY) or 128 (NOESY). Mixing times were 75 ms (TOCSY) or 400 ms (NOESY). No significant secondary effects were observed in the NOESY spectrum at this mixing time. Data from the two-dimensional experiments were transformed using the program FELIX (Accelrys), by zero-filling to a final size of 2K × 2K real points. Data were multiplied by a sine bell function in t_2 extending to 300 (TOCSY), 600 (DQ-COSY), or 1024 (NOESY) points and shifted by 0° (DQ-COSY, TOCSY) or 90° (NOESY); and by a sine bell function in t_1 extending to 300 (DQ-COSY, TOCSY) or 600 (NOESY) points and shifted by 0° (DQ-COSY, TOCSY) or 90° (NOESY). NOEs were converted into distance constraints (r_0) using the approaches for volume measurement, calibration, and uncertainty assessments described by Wagner and co-workers[35] and outlined previously.[36] We applied uncertainty ranges to the distances by modifying the error function equations to the following forms: $r_{\text{lower}} = 1/(1.4/r_0^6 + 1/6.0^6)^{1/6} - 0.15$; $r_{\text{upper}} = 1/(0.7/r_0^6 - 1/6.0^6)^{1/6} + 0.15$. These equations resulted in uncertainty ranges about the distances of at least ±20%.

4.3. Structure calculations

Structure calculations were carried out using the X-PLOR program embedded in InsightII 98.0 package (Accelrys).[37] CHARMM force field was employed for all the energy calculations.[38] One hundred structures were initially computed by using DGSA (distance geometry simulated annealing) protocol,[39,40] and all the structures were generated from a random linear peptide structure, with a distance constraint of 1.32 ± 0.01 Å between amine nitrogen of Lys and carbonyl carbon of Asp. Each structure was then refined separately with a simulated annealing process with an initial annealing temperature of 2000 K, final temperature of 100 K, temperature step of 25 K, and dynamic time step of 1 ns. During the SA protocol, an amide bond was added between Lys and Asp, together with two new bond angles, three dihedral angles, and three new impropers to define the newly formed peptide bond. Each structure was finally minimized.

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