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Design and Synthesis of Potent Cystine-Free Cyclic Hexapeptide Agonists at the Human Urotensin Receptor

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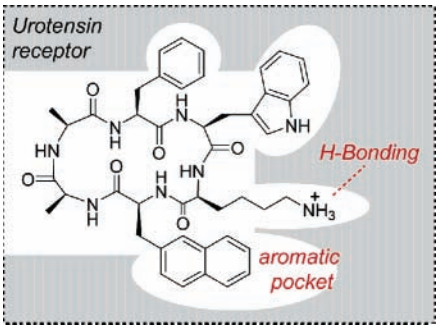
ABSTRACT



Cyclic hexapeptides, incorporating a dipeptide unit in place of the disulfide bond found in urotensin, were prepared and screened at the human urotensin receptor. The bridging dipeptide unit was found to influence dramatically the affinity for the urotensin receptor. Alanyl-*N*-methylalanyl and alanylprolyl dipeptide bridges failed to afford active ligands, while the alanyl-alanyl unit yielded a ligand with submicromolar affinity for the urotensin receptor. Further development led to a hexapeptide agonist with nanomolar affinity (2.8 nM).

Urotensin (U-II) is a urophysial peptide hormone originally isolated from goby *Gillichthy mirablis*. The cDNA encoding the U-II precursor has since been identified and sequenced in many species.[1] The overall length of mature U-II is variable, ranging from 11 residues in humans to 14 residues in mice; however, the core cyclic hexapeptide (CFWKYC) is conserved across all species and is the minimal fragment required for biological activity.[2] U-II is among the most potent known vasoconstrictors, inducing contraction of isolated arterial rings in rats and humans at nanomolar concentrations.[3] The endogenous target for U-II, the urotensin (UT) receptor, is a member of the G-protein-coupled receptor (GPCR) family.[4] The urotensin peptide and receptor are found in the heart, lungs, blood vessels, kidneys, and central nervous system.[5] U-II levels appear to be regulated post-

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translationally in a tissue-specific manner. In blood vessels, urotensin activity is influenced by anatomical location, species, and the surrounding endothelium. Thus, the physiology of urotensin is not straightforward.[6] Indeed, hU-II has been reported to behave as a vasodilator in some human tissues.[7] Despite the inherent complexity, the U-II/U-II receptor system possesses therapeutic potential. It regulates cardiovascular function at several levels including vascular tone, myocardial contraction, heart rate, and cell growth and proliferation.[8] Recent work also demonstrated up-regulated urotensin activity in disease states such as congestive heart failure,[9] pulmonary hypertension,[10] and chronic renal failure.[11]

The tissue and species variability of U-II response complicates direct comparison of structure−activity relationships, though some qualitative trends are apparent from the growing body of data (Figure 1).[12] The endocyclic residues

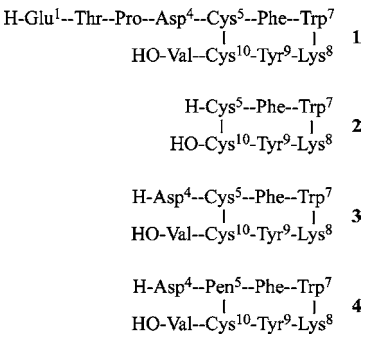

Figure 1. Urotensin SARs have identified the minimal active fragment as the core cyclic hexapeptide spanning Cys[5] through Cys[10]. Modification of the disulfide moiety in octapeptide derivatives of U-II has been used to construct potent agonists at the human urotensin receptor. The three letter abbreviation Pen represents penicillamine (β,β-dimethylcysteine).

of hU-II (**1**), bounded by the disulfide bridge between Cys[5] and Cys[10], are critical for biological activity. Alanine and D-amino acid scans of this fragment have shown that the side chains of Trp[7], Lys[8], and Tyr[9] comprise the minimal pharmacophore of hU-II. The above observations are supported by the moderate affinity of the truncated hexapeptide analogue **2** (K_i = 95 nM). Inclusion of the Asp[4] and Val[11] residues which flank the disulfide bridge in native U-II, affording octapeptide analogue **3**, dramatically enhances affinity (K_i = 0.25 nM), possibly by constraining the connected cysteine side chains.[12] Introduction of further conformational rigidity within **3**, by replacing Cys[5] with penicillamine (β,β-dimethyl cysteine), gave analogue **4**, the most potent urotensin agonist reported to date (K_i = 0.2 nM).[13] Disulfide-bridged octapeptide ligands for somatostatin and neuromedin receptors have shown moderate affinity for the urotensin receptor, indicative of similarities among these three receptors.[14]

The structural similarities between SRIF and hUII led us to ask if our previous experience with somatostatin SARs could be applied to urotensin. The endocyclic pharmacophore of somatostatin-14 (**5**) contains a β-turn between Trp[8] and Lys[9] with Thr[10] in the i + 3 position.[15] The analogous presumed β-turn of hU-II (**1**) spans Trp[7] and Lys[8] with Tyr[9] in the i + 3 position (Figure 2). Previous work has shown

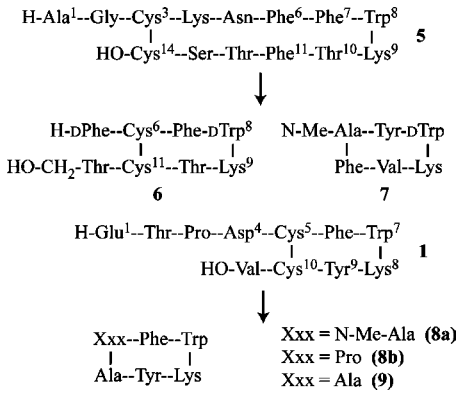

Figure 2. The demonstration by the Merck group that the cyclic hexapeptide **7** is a more potent mimic of SRIF-14 (**5**) led us to explore the possibility that a cysteine free cyclic hexapeptide could be designed to mimic urotensin (**1**). Indeed, **9** binds the hUT receptor.

that replacing the Phe[6]/Phe[11] residues of SRIF-14 with disulfide-linked cysteines maintains full potency,[16] as subsequently incorporated into sandostatin (**6**). Remarkably, it

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was found by Veber and colleagues that Phe[6]/Phe[11], which stabilize the bioactive conformation of SRIF-14, could be replaced not only by a cystine bridge but also by the phenylalanyl-*N*-methylalanyl dipeptide, affording the cyclic hexapeptide MK-678 (**7**).[17] This simple peptide showed enhanced potency at the somatostatin receptor.

Building on this research, we designed and synthesized c-hexapeptide **8a** in which the disulfide bridge of hUII is replaced by an alanyl-*N*-methylalanyl dipeptide, mimicking that of MK-678 (**7**). Unexpectedly, **8a** failed to bind that receptor. The fact that prolyl congener **8b** also failed to bind this receptor suggests that secondary amino acids such as N-Me-Ala or Pro induce conformationally unfavorable changes at the hUT receptor. We were encouraged, however, by the observation that congener **9**, containing an alanylalanyl dipeptide, showed modest binding affinity ($K_i = 221 \pm 11$ nM).

The combination of in vitro and in silico studies has yielded a putative binding model for hU-II peptides at the hUT receptor which proposes a charge-reinforced hydrogen bond between Lys[8] of U-II and an aspartic acid residue within the transmembrane domain of the receptor (Figure 3).[18] A similar model for interaction of hexapeptides with the somatostatin receptors was suggested previously.[19] Molecular modeling, wherein ligands were docked with the hUT receptor based upon the crystal structure of rhodopsin, suggests that the Asp[4] side chain of hU-II might form hydrogen bonds with extracellular residues of the receptor.[20] The landscape of the Tyr[9] binding pocket was probed in a previous structure−activity study which showed that replacement of Tyr[9] with a β-naphthylalanine residue enhanced affinity in full length U-II and derived octapeptides.[12a] We reasoned that if lead compound **9** bound the UT receptor according to the putative model, the above findings could be exploited in the construction of higher affinity cyclic hexapeptides targeting the human urotensin receptor (Figure 3).

Congener **10** was designed to incorporate an acidic side chain, spatially analogous to the Asp[4] residue of native hU-II, by replacing the Ala-Ala dipeptide of **9** with a Glu-Ala moiety. A noticeable gain in affinity was observed for **10** ($K_i = 109 \pm 20$ nM) relative to **9**; however, the acidic group of **10** might not be long enough to achieve optimal interaction with the receptor as for hU-II. Hexapeptide **11**, in which Tyr[9] of lead compound **9** is replaced with a β-naphthylalanine residue, bound the human urotensin receptor with much higher affinity ($K_i = 2.8$ nM) than the disulfide-bridged

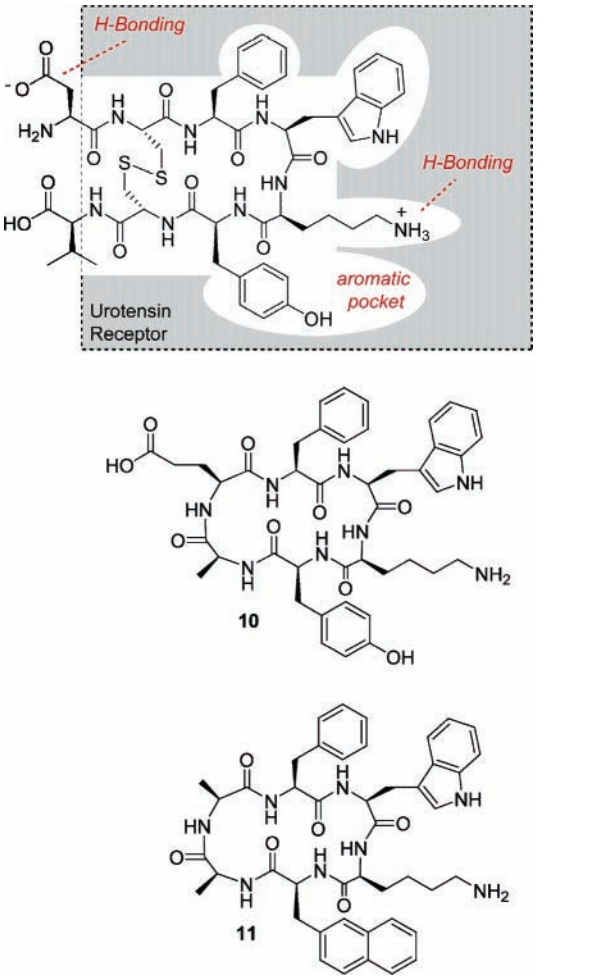

Figure 3. Putative binding model for peptides at the urotensin receptor based on the β-turn somatostatin binding model.[21]

hexapeptide counterpart (**2**; Table 2). Importantly both **2** and **11** proved to be urotensin agonists in the rat aortic ring assay. Equally significant, **11** was completely stable to chymotrypsin, while **2** displayed modest hydrolysis, perhaps suggesting that the larger macrocycle size of the disulfide-bridged peptide **2** facilitates enzymatic hydrolysis.

Finally, we determined the binding affinities of **2** and **11** at somatostatin receptor subtypes 2 and 5 ($hSST_2$ and $hSST_5$). Both **2** and **11** exhibited significantly lower binding affinities at somatostatin receptors than at the urotensin receptor. In contrast to MK-678 (**7**), cyclic hexapeptide **11** displayed higher affinities for $hSST_5$ relative to $hSST_2$ (Table 1).[21] The surprising lack of affinity shown by **8a** for the hUT receptor also stands in marked contrast to potent cyclic hexapeptides targeting the somatostatin receptor, typified by MK-678 (**7**).

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Table 1. Binding Affinities of Cyclic Hexapeptides at the Urotensin and Somatostatin Receptors

compd	K_i (hUT)	K_i ($hSST_2$)	K_i ($hSST_5$)
2	95 nM	1.7 μM	2 μM
11	2.8 nM	1.2 μM	120 nM
MK-678 (**7**)[21]	n.d.[a]	0.1 nM	13 nM

[a] n.d. indicates not determined.

Replacing the *N*-methylalanine residue of **8a** with proline also gave an inactive compound (**8b**), suggesting a subtle conformational effect induced by the secondary amino acids *N*-Me-Ala and Pro on the β-turn residues involved in binding the UT receptor not seen with MK-678 and L-363,301 [c-(Phe-Pro-Phe-D-Trp-Lys-Thr)] at the somatostatin receptor. Riviera and co-workers observed that the replacement of Trp^8 of SRIF-14 by the D-Trp diastereomer results in a 10 fold increase in potency. Subsequently, the Merck group pointed out that this affinity enhancement is accompanied by an upfield shift induced in the γ methylene protons of Lys^9 by the D-Trp. Table 2 lists the chemical shifts of the γ methylene protons of Lys, D-Trp-Lys, and for L-363,301 [c-(Phe-Pro-Phe-D-Trp-Lys-Thr)]. It can be seen that in every case the D-Trp compounds induce a shielding of the γ methylene protons. Interestingly, the urotensin congeners **9** and **11**, both of which contain L-Trp, nevertheless reveal a comparable upfield shift in the Lys γ-methylene protons suggesting either that the lysine is shielded by the aromatic amino acids Tyr or naphthylalanine in the $i + 3$ position, or that these two aromatic amino acids enable even the L-Trp to shield the lysine.

In summary, the rational optimization of lead compound **9** yielded a potent urotensin agonist **11**. This cyclic hexapeptide demonstrates greater affinity and stability relative to disulfide-bridged hexapeptides while maintaining lower molecular weight compared to nanomolar or subnanomolar octapeptide ligands. Further studies examining the conformational features of **11** underlying activity are planned. The cyclic hexapeptide scaffold might also prove valuable in the design of urotensin antagonists.

Table 2. Chemical Shifts of Lys γ-Methylene Protons in Lys, D-Trp-Lys, and Cyclic Hexapeptides

compd	K_i (hUT)	δ Lys γ-CH_2 (ppm)
Lys^{22}	n.d.	1.51
D-Trp-Lys^{22}	n.d.	0.58
L-363,301[23,b]	n.d.	0.6, 0.4
9[a]	221 nM	0.6, 0.3
11[a]	2.8 nM	0.5, 0.26

[a] Proton NMR spectra were obtained in D_2O at ambient temperature using a 500 MHz Bruker instrument. [b] c[Phe-Pro-Phe-D-Trp-Lys-Thr].

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Supporting Information Available: Experimental details and characterization data are provided for **2** and **8**−**11**. This material is available free of charge via the Internet at http://pubs.acs.org.

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