A New, Potent Urotensin II Receptor Peptide Agonist Containing a Pen Residue at the Disulfide Bridge

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Abstract: Replacing Cys[5] by Pen (penicillamine, *β*,*β*-dimethylcysteine) in the cyclic C-terminal U-II octapeptide, U-II(4−11), we have obtained a potent urotensin II (U-II) receptor agonist. Conformational analysis of solution NMR data indicated that the putative biologically active conformation of U-II is stabilized by introduction of a Pen residue. To the best of our knowledge, this is the most potent U-II receptor agonist reported to date.

Urotensin II (U-II) is a cyclic peptide composed of 11 amino acid residues initially isolated from the fish neurosecretory system[1] and subsequently found in different species, including human.[2] The C-terminal sequence, characterized by a highly conserved cyclic heptapeptide (CFWKYCV), is essential for biological activity.[3] Recently, human urotensin II (hU-II) was cloned[2] and shown to be the endogenous ligand for the orphan G-protein-coupled receptor GPR14.[4] Following this seminal observation, an increasing number of biological studies indicate that hU-II is the most potent mammalian peptide vasoconstrictor reported to date, and it appears to be involved in the regulation of cardiovascular homeostasis and pathology.[5] Consequently, hU-II synthetic analogues acting as agonists or antagonists would be extremely important tools for exploring the (patho)physiological role of the U-II/GPR14 system. A recent paper disclosed a family of nonpeptide U-II receptor antagonists,[6] but to the best of our knowledge, no potent U-II receptor agonist has been described. We have obtained a highly potent GPR14 receptor agonist, introducing a conformational constraint in the sequence of the hU-II C-terminal octapeptide (DCFWKYCV) by replacement of Cys[5] residue with penicillamine (Pen, *β*,*β*-dimethylcysteine).

Table 1 shows the biological activity of three hU-II-(4−11) analogues in which Cys residues in positions 5 and 10 have been replaced by Pen singularly or simultaneously: peptide **1**, H-Asp-*c*[**Pen**-Phe-Trp-Lys-Tyr-**Cys**]-Val-OH; peptide **2**, H-Asp-*c*[**Cys**-Phe-Trp-Lys-Tyr-**Pen**]-Val-OH; peptide **3**, H-Asp-*c*[**Pen**-Phe-Trp-Lys-Tyr-**Pen**]-Val-OH. Peptides were synthesized manually using a conventional Fmoc-based solid-phase strategy (see Supporting Information). Disulfide bridges were obtained by potassium ferricyanide oxidation, using the syringe pump method.[7] Both reduced linear precursors and final products were purified to homogeneity by semipreparative RP-HPLC. Analytical HPLC indicated a purity greater than 98%, and molecular weights were confirmed by FAB-MS (Fisons mod. Prospec) or HR-MS (Kratos Analytical mod. Kompact). The peptides were tested for their ability to displace the radioligand [^{125}I]-Tyr[9]-hU-II (Amersham Bioscience, U.K.) from human recombinant U-II receptors stably transfected into membranes of CHO-K1 cells (Euroscreen, Brussels, Belgium). Contractile activity was measured in the rat isolated thoracic aorta, a preparation that has been reported to be the most sensitive and reliable one over a broad range of human and nonhuman vessels for evaluating biological activities of hU-II and related peptides.[8] Radioligand binding experiments showed that iodinated hU-II bound the human U-II receptor saturably with high affinity ($pK_D = 9.2 \pm 0.14$). In competition experiments, hU-II displaced the iodinated radioligand with comparable affinity ($pK_i = 9.1$, Table 1). Replacement of Cys[5] by Pen yielded a peptide (**1**, termed P5U) showing a 3-fold higher affinity for the receptor (measured in competition experiments) than the parent peptide, similar to the unmodified C-terminal octapeptide hU-II(4−11). In contrast, replacement of Cys[10] with Pen (peptide **2**) or replacement of both Cys residues with Pen (peptide **3**) yielded analogues that were 1000- or 10-fold less potent than the parent peptide, respectively (Table 1). In functional experiments on the rat aorta (Figure 1), P5U was the most active contractile peptide,

Table 1. Receptor Affinity and Biological Activity of Urotensin-II Analogues of General Formula H-Asp-c[Xaa-Phe-Trp-Lys-Tyr-Yaa]-Val-OH

peptide	Xaa	Yaa	pK_i[a]	pD_2[b]
hU-II	Cys	Cys	9.1 ± 0.08	8.3 ± 0.06
hU-II(4−11)	Cys	Cys	9.6 ± 0.07	8.6 ± 0.04
1 (P5U)	Pen	Cys	9.7 ± 0.07	9.6 ± 0.07
2	Cys	Pen	7.9 ± 0.04	6.7 ± 0.06
3	Pen	Pen	8.9 ± 0.11	8.2 ± 0.12

[a] $pK_i = -\log K_i$. [b] $pD_2 = -\log EC_{50}$. Each value in the table is the mean ± SEM of at least four determinations.

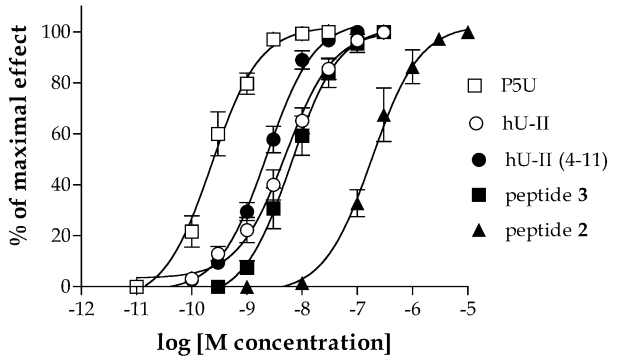

Figure 1. Concentration−response curves in the rat isolated thoracic aorta. Each point is a mean of four to eight experiments. Vertical lines show the standard error of the mean (SEM).

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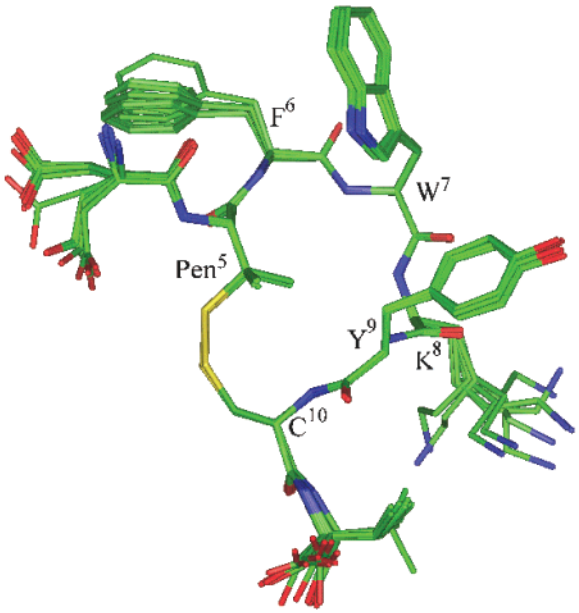

Figure 2. Superposition of the 10 lowest energy structures of P5U (**1**). Heavy atoms are shown with different colors (carbon, green; nitrogen, blue; oxygen, red; sulfur, yellow).

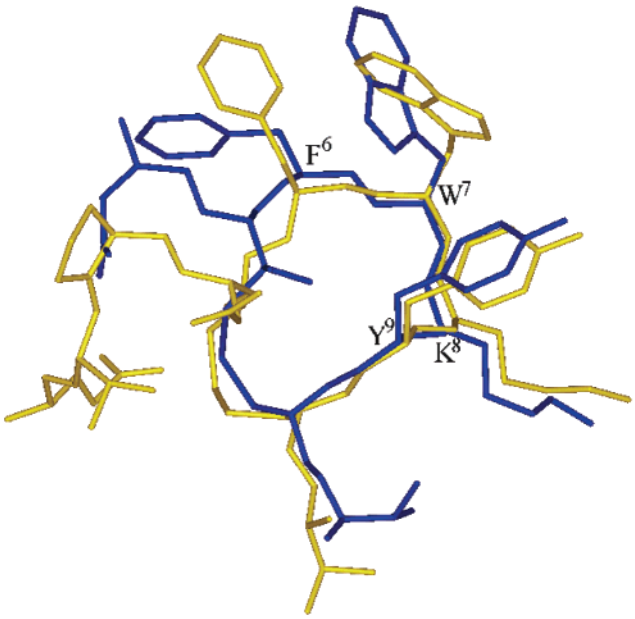

Figure 3. Superposition of the most representative structure (i.e., the most similar to the mean structure) of **1** (blue), with the corresponding one of hU-II (yellow). Structures were superimposed by fitting heavy atoms of cyclic residues.

being 20-fold more potent than hU-II and 10-fold more potent than hU-II(4−11), whereas the other derivatives were 2−3 orders of magnitude less potent (Table 1). The present results show that P5U bears a subnanomolar affinity for the human urotensin II receptor and it is by far the most potent U-II analogue in the rat thoracic aorta bioassay.

We subsequently performed a conformational analysis, using nuclear magnetic resonance (NMR) and molecular modeling techniques, to study the solution structure of peptide P5U. NMR analysis was performed using one-dimensional and two-dimensional proton homonuclear techniques. Double-quantum-filtered correlation spectroscopy (DQF-COSY),[9] total correlation spectroscopy (TOCSY),[10] and nuclear Overhauser enhancement spectroscopy (NOESY)[11] experiments were recorded on a Bruker 600 MHz spectrometer at 300 K. To check for the absence of an aggregation state of the peptide, spectra were acquired in the concentration range of 0.2−2 mM. No significant changes were observed in the distribution and in the shape of the ^{1}H resonances, indicating that no aggregation phenomena occurred in this concentration range. Complete ^{1}H chemical shift assignments were effectively achieved (see Supporting Information). Interresidual nuclear Overhauser effect (NOE) between C$^\beta$H's of Pen^5 and Cys^{10} confirmed the presence of the disulfide bridge in P5U.

The preferred conformations of peptide P5U in DMSO solution were derived from the analysis of NMR experimental data (NOEs, $^3J_{H^\alpha-HN}$ coupling constants, and temperature coefficients of amide protons; see Supporting Information). From the NOESY spectra, a total of 100 NOEs were collected (31 intraresidual, 38 sequential, 30 medium range, and 1 long range). From a qualitative evaluation of the NOE connectivities (data not shown), no evidence for ordered α-helix or β-sheet structures could be detected. The values of the temperature coefficients of the observed amide protons (Supporting Information) indicate that all the HNs are solvent-exposed, with the exception of the HN of Trp^7 ($-\Delta\delta/\Delta T = 0.5$ ppb/K). NMR-derived constraints were used as input data for a torsion angle dynamics structure calculation as implemented in the DYANA program.[12] NOEs were translated into interproton distances and used as constraints in subsequent annealing procedures to produce 200 conformations. The 50 structures whose interproton distances best fitted NOE-derived distances were then refined through successive steps of unrestrained energy minimization (EM) calculations using the program Discover (Biosym, San Diego). An ensemble of 30 structures satisfying the NMR-derived constraints (violations smaller than 0.5 Å) were chosen for further analysis. Although no classical turn structure could be observed, the hexacyclic region of P5U was well defined, possessing an average root-mean-square (rms) deviation of the backbone heavy atoms of 0.22 Å (Figure 2). Side chain orientations of residues 5−10 were also highly defined (the average rms deviation for all cyclic heavy atoms was 0.84 Å). In particular, the χ_1 angle of Pen^5 was structurally defined, showing a preferred trans conformation ($\chi_1 = 175° \pm 6°$). Asp^4 and Val^{11} were more flexible (average rms deviation for all non-hydrogen atoms rose to 1.0 Å).

We have recently investigated the NMR structure of hU-II in DMSO solution.[13] Figure 3 shows the superposition of representative structures of P5U and hU-II. As can be observed, Lys^8 and Tyr^9 residues can be very efficiently superimposed, considering both the backbone and the side chain atoms, while the Trp^7 residues of the two peptides share almost undistinguishable backbone atom positions, but the side chain orientation is different. In particular, amide protons of Trp^7, which show low-temperature coefficients for both peptides ($-\Delta\delta/\Delta T = 0.3$ and 0.5 ppb/K for hU-II and P5U, respectively), share similar spatial dispositions. In the calculated structures, the amide proton of Trp^7 is not engaged in

any hydrogen bond. The low value of the temperature coefficient of this proton found for both hU-II and P5U comes out from its spatial location, which results in not being accessible to the solvent. The Phe[6] residues display different backbone and side chain spatial disposition. As expected, the substitution of the Cys[5] residue of hU-II with Pen mainly influences the proximal Phe[6] residue, leaving Trp[7], Lys[8], and Tyr[9] nearly unaffected. Interestingly, Flohr and co-workers[6] have recently reported a detailed structure−activity relationship study on hU-II, showing that the WKY sequence in the cyclic portion of the peptide is the most important for full agonist activity of hU-II, whereas Phe[6] plays only a minor role. The conformational analysis of peptide P5U reported herein clearly indicates that in this analogue the putative pharmacophore of hU-II maintains the same spatial orientation as in the native peptide. The enhanced pharmacological properties observed in the case of P5U can be ascribed to the conformational restriction obtained by replacement of Cys with Pen,[14] a modification that apparently favors the selection of a bioactive conformation. The higher conformational rigidity of the cyclic portion of P5U compared to hU-II can be established from a decrease in the rms deviation of the cyclic atoms upon Cys to Pen substitution. Actually, considering the 30 lowest energy structures of both peptides, the rmsd decreases from 0.50 Å (hU-II) to 0.22 Å (P5U) for backbone heavy atoms and from 1.40 Å (hU-II) to 0.84 Å considering all cyclic heavy atoms. This enhancement of the conformational rigidity observed in the calculated structures of P5U, compared to hU-II, results from an increase of the number of medium- and long-range NOE contacts among the proton signals of the cyclic region observed in the NOESY spectra of P5U (24 NOEs) compared to those observed for hU-II (10 NOEs).

Chemical modifications are commonly used to restrict a residue or group of residues in a peptide to a small region of the three-dimensional space so that the peptide can interact with the specific receptor in an appropriate conformation.[15] Among these modifications, the use of unusual amino acid residues is common. In particular, the use of Pen, a β,β-dimethyl-substituted cysteine residue, enables the exploration of the importance of χ space and the χ_1 torsional angle in the interaction of the peptide under study with the cognate receptor.[16] Indeed, replacement of Cys by Pen was successfully used 35 years ago by Du Vigneaud in the design of oxytocin antagonists.[17] Similarly, replacement of Cys[7] by Pen in somatostatin yielded a μ-selective opioid antagonist,[18] while the well-known δ-selective opioid antagonist DPDPE was obtained by cyclization of enkephalin via two D-Pen residues.[19] Recently, the biological and conformational effects of the replacement of Cys by Pen was also described in the case of h-CGRP.[20]

In conclusion, we have shown that replacement of Cys[5] residue by Pen in the cyclic C-terminal hU-II octapeptide hU-II(4−11) yielded a potent urotensin II receptor agonist termed P5U. The biological data, along with conformational analysis in solution, indicated that the putative biologically active conformation of U-II is stabilized by introduction of a Pen residue. P5U, which, to the best of our knowledge, is the most potent U-II receptor agonist reported to date, is indeed a useful pharmacological tool for the study of the hU-II/GPR14 system.

Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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