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Functional and binding characterizations of urotensin II-related peptides in human and rat urotensin II-receptor assay.

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Running Title : Molecular Pharmacology of Urotensin II-related Peptides

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ABBREVIATIONS: U-II, urotensin II; FMOC, fluorenylmethyloxycarbonyl; TFA, trifluoroacetic acid; EDT, ethanedithiol; TIS, triisopropylsilane; RP-HPLC, reverse-phase high performance liquid chromatography; MALDI-TOF, matrix assisted laser desorption and ionization – time of flight, EC_{50}, median effective concentration; FLIPR, fluorescence imaging plate reader.

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Abstract

Urotensin II (U-II : cyclo$^{5\text{-}10}$[H-Glu-Thr-Pro-Asp-Cys-Phe-Trp-Lys-Tyr-Cys-Val-OH]) is a potent vasoconstrictor in mammals and it is postulated that it plays a central role in cardiovascular homeostasis. Thus, we initiated a structure-to-function analysis of this peptide characterized by a N-terminal tail and a cyclic core formed through a disulfide bridging. A total of 41 analogues focusing on these characteristics were developed and evaluated using a binding assay on membranes from a stable HEK-293 cell line containing the human or rat U-II receptor, a functional assay for Ca^{2+} mobilization on transiently transfected CHO-K1 cells with the human or rat U-II receptor and, a rat thoracic aorta bioassay. At first, the focus was applied on peptide compounds containing exocyclic modifications. From this series, it appeared that only valine-11 played a significant role although it is not an essential amino acid. Similarly, endocyclic and ring transformations of hU-II were also studied. In most cases, a detrimental effect on affinity and biological activity was observed. A few exceptions however with [Tyr^{6}]hU-II and [Phe^{9}]hU-II, as both compounds retained affinity and activity. So far, our binding, functional and pharmacological data clearly demonstrated the minor contribution of the N-terminal segment and the essential role of the cyclic structure. More particularly, three residues within the loop *i.e.* Trp-7, Lys-8 and Tyr-9 are required for receptor recognition and activation. This 3-pole feature, kept by the disulfide bond in a correct spatial arrangement, appears as the key-pharmacophore for the U-II receptor.

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Urotensin II (U-II), a potent mammalian vasoconstrictor, was first isolated and characterized from the *Gillichthys mirabilis* (goby) urophysis, a neurohemal organ (Pearson et al., 1980). Recently, human cDNA was cloned and hU-II appeared as an 11-amino acid peptide with cysteine residues in positions 5 and 10, forming a bridge through their side chains (Coulouarn et al., 1998). A comparison of the U-II primary structures revealed a striking homology among species. In fact, from fish to man, evolution favored the conservation of residues 5 to 10, while the N-terminal segment varied in length and composition. In addition, position 4 is occupied by the acidic residue Asp or Glu, whereas position 11 is characterized by the presence of the hydrophobic residue Val or Ile.

The most striking effects of U-II are observed in the cardiovascular arterial system, where it is frequently identified as the most potent vasoconstrictor ever described. However, using tissues from several species, Douglas et al. (2000a) carried out a study on the vasoconstrictor activity of human U-II (hU-II) and they showed that both potency and efficacy vary significantly between species, individuals and vessels. As such, hU-II was described as the most potent vasoconstricting compound on the rat thoracic aorta but in mouse, whatever the vessels tested, no vasoconstriction was recorded. On the other hand, hU-II was a potent vasoconstrictor of non-human primate arteries (Ames et al., 1999, Douglas et al., 2000b). In absence of endothelium, radial, coronary and mammary human arteries demonstrated a moderate, yet inconsistent, constriction to hU-II (Maguire et al., 2000; Paysant et al., 2001). Removal of endothelium from specific human arteries appeared to unmask the contractile respond to hU-II (Hillier et al. 2001). In contrast, the abdominal and small pulmonary arteries showed a potent vasodilation to hU-II in presence of endothelium (Stirrat et al., 2001). Additional in vitro

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studies have shown that hU-II is a potent inotropic agent on human cardiac muscle (Russell et al., 2001) and that it induces vascular smooth muscle cell proliferation (Watanabe et al., 2001). In vivo reports regarding U-II vascular effects appeared inconclusive since two independent studies using identical procedure showed opposite results (Böhm and Pernow 2002; Wilkinson et al., 2002). Nevertheless, the potency of hU-II, as a vasoconstricting, vasodilating or inotropic agent strongly suggests a key-role of this peptide in the cardiovascular homeostasis.

These observations are well-matched with the hypothesis that U-II might be involved in various diseases, including cardiovascular pathologies such as hypertension and arteriosclerosis. Thus, the U-II biological system exhibits a remarkable potential for the development of novel therapeutic strategies, and especially of those related to the treatment of cardiovascular diseases. Such developments require a precise knowledge of the pharmacophoric elements within U-II essential for the affinity and the activity of this peptide. So far, because of its resemblance to somatostatin (SST), it is conceivable that some of the critical structural features already described for SST are shared with U-II. Accordingly, both peptides contain the tripeptide Phe, Trp, Lys followed by the hydroxylated residue Thr or Tyr. Moreover, it was demonstrated in SST that an interaction between the aromatic moities of residues Phe-6 and Phe-11 stabilizes the orientation of residues Phe-7, Trp-8, Lys-9 and Thr-10. Similarly, the disulfide bridge in U-II would act in a similar manner. Structure-activity relationship (SAR) studies of SST already showed that these pharmacophoric features are essential for the SST biological activity (Janecka et al., 2001). Furthermore, previous fish U-II (Itoh et al., 1987) and human U-II (Flohr et al., 2002; Kinney et al., 2001, 2002) SAR studies showed that the highly conserved C-terminal segment CFWKYCV is the minimal sequence required to

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maintain a high potency and that Lys-8 appeared as an important residue for U-II activity (Carmada et al., 2002). In addition, substitution of the U-II disulfide bridge with lactam bridges of various lengths showed that the biological activity of U-II is dependent upon the size of the cyclic structure (Grieco et al., 2002). Therefore, we initiated a SAR study of the human and rat urotensin-II, by targeting the essential structural features already pinpointed in the somatostatin and fish urotensin-II molecules. We report the evaluation of 41 human or rat urotensin II-derived peptides, using two different functional assays on HEK293 cells stably expressing the human and rat U-II receptor: (i) mobilization of intracellular calcium, and (ii) constriction of the rat thoracic aorta. In addition, all peptides were tested in binding assays on membranes prepared from HEK293 cells stably expressing the rat and human U-II receptor for their ability to compete with [^{125}I]U-II binding.

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Materials and Methods

Reagents and solvents

The following Fmoc-protected amino acids were purchased from Chem-Impex International (Wood Dale, IL) : Ala, Cys(Trt), His(Trt), Phe, Trp, Lys(Boc), Tyr(tBu), Asp(OtBu), Glu(OtBu), Pro, Gln(Trt), Thr(tBu), Arg(Pbf), Orn(Boc), HomoCys(Trt), Cys(Acm) and D-Trp. Biograde TFA was obtained from Halocarbon (River Edge, NJ). Diisopropylethylamine was from Aldrich Chemical Company (Milwaukee, WI). Wang resin and BOP were purchased from Albatross Chemical (Montreal, QC).

Basal ISCOVE medium and fetal calf serum (FCS) were from Biochrom, (Berlin, Germany). The GC-melt PCR-kit as well as the human and rat genomic DNA were purchased from Clontech (Palo Alto, CA). The pEAK8 mammalian episomal expression vector and the selection marker puromycin were from Edge Biosystems (Gaithersburg, MD). Dulbecco's modified Eagles medium (DMEM), L-glutamine, HEPES, LipofectAMINE reagent and penicillin-streptomycin were from GIBCO BRL (Rockville, MD). The pCDNA3.1(+) mammalian expression vector was from Invitrogen (Carlsbad, CA). The calcium-sensitive fluorescence dye Fluo-4 and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR). Flashplates PLUS and monoiodinated human [^{125}I-Tyr^{9}]-urotensin II for radioligand binding assays were from PerkinElmer Life Sciences (Boston, MA). Gentamicine, the transfection reagent FuGene 6 and Complete Protease Inhibitor were purchased from Roche (Basel, Switzerland). Bacitracin, EDTA-disodium salt, probenecid, $MgCl_2$, NaCl and sucrose were obtained from Sigma (St.-Louis, MO).

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Peptide synthesis and cleavage

The peptides hU-II(5-10), hU-II(2-11), hU-II(3-11), hU-II(4-11), hU-II(1-10), [Ala^{5}]hU-II, [D-Phe^{6}]hU-II, [D-Lys^{8}]hU-II, [D-Tyr^{9}]hU-II, [des-Phe^{6}]hU-II, [des-Trp^{7}]hU-II, [des-Lys^{8}]hU-II, [des-Tyr^{9}]hU-II, [des-Lys^{8},Val^{11}]hU-II and [des-Tyr^{9},Val^{11}]hU-II were custom-designed and purchased from EMC Microcollections (Tübingen, Germany). All the other analogues were synthesized using a solid phase procedure based on a Fmoc chemistry/BOP coupling strategy designed for a home-made manual multireactor synthesizer. Wang resin was used as the solid support and each coupling step was monitored using a ninhydrin test. Cleavage from the resin was achieved with a mixture of TFA:EDT:TIS:phenol:water (50ml/g 80:5:5:5:5 v/v) for 2 hours. After TFA evaporation, peptides were precipitated and washed using diethyl ether. Crude peptides were dried and kept at -20°C until purification.

Peptide purification and characterization

About 700 mg of the crude material was dissolved in water before being injected onto a RP-HPLC Jupiter C_{18} (15 µm; 300Å) column (250 x 21.20 mm) (Phenomenex, Torrance, CA). The purification step was carried out using a Waters Prep 590 pump system connected to a Waters Model 441 absorbance detector. The flow rate was fixed at 40 ml/min and the peptide was eluted with a solvent gradient of 0 to 40% solvent B where solvent A is aqueous NH_4OH 0.05% and solvent B is CH_3CN 40% in NH_4OH 0.05%. Fractions homogeneity was evaluated using analytical RP-HPLC with a Jupiter C_{18} (5 µm; 300 Å) column (250 x 4.60 mm) (Phenomenex, Torrance, CA) connected to a Beckman 128 solvent module coupled to a Beckman 168 PDA detector. The flow rate

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was at 1.0 ml/min and the elution of the peptide was carried out with a linear gradient of 20 to 60% B where A was TFA 0.06% and B was CH_3CN 40% in aqueous TFA 0.06%. Aliquots of 20 µl were injected and analyzed. Homogeneous fractions were pooled, lyophilized and then analyzed by MALDI-TOF mass spectrometry (Voyager DE spectrometer – Applied Biosystems, Foster City, CA). The laser was set at 337 nm and an acceleration voltage of 25 kV was applied. The compound α-cyano-4-hydroxycinnamic acid was used as a matrix for peptide inclusion and ionization. The purified peptide precursor with the right mass was cyclized using $K_3Fe(CN)_6$ as an oxidant. Thus, each peptide was dissolved in water at a concentration of 0.5 mg/ml and 20 eq of oxidant was added. After one hour, the reaction mixture was injected onto a preparative column and, as described above, purified using HPLC. The final pure peptides were characterized by analytical HPLC and MALDI-TOF mass spectrometry.

Biological activity study

All peptides were tested pharmacologically using the rat thoracic aorta preparation. Sprague-Dawley male rats from Charles River (St-Constant, QC), weighing 250-300g, were anesthetized with Somnotol™(pentobarbital-MTC Pharmaceuticals, Cambridge, ON) and killed by exsanguination. The thoracic aorta was quickly removed and placed in oxygenated Krebs-Henseleit buffer (118 mM NaCl, 25 mM $NaHCO_3$, 4.7 mM KCl, 1.18 mM KH_2PO_4, 2.5 mM $CaCl_2$, 22.2 mM glucose, pH 7.2) at 37°C. The endothelium was not removed as preliminary studies showed that no differences in the data were recorded with endothelium-denuded tissues. Aorta were cleaned of their connective tissues and cut into rings (6) of ~4 mm. Rings were suspended in 5 ml

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oxygenated (95% O_2 and 5% CO_2) bioassay vessels and maintained at 37°C. The tissues were allowed to equilibrate for 1 h under a resting tension of 1 g before being exposed to 60 mM KCl for normalization. Mechanical responses were recorded isometrically on a Grass 7D polygraph equipped with force-displacement transducers. All peptides were dissolved in water. Concentrations of 1.0×10^{-10} M to 1.0×10^{-5} M of hU-II or analogues were used to establish the concentration-response curves. All results were normalized as a percentage of the contraction obtained with KCl 60 mM. The median effective concentrations (EC_{50}) are expressed as mean ± s.e.m. and the *n* values refer to the number of rats. Differences between data were tested for significance using the Student's *t*-test for unpaired samples. Values of $p < 0.05$ were considered as significant.

Cloning of human and rat urotensin-II receptor

As the putative human urotensin II receptor sequence is intronless, we cloned this protein from human genomic DNA via polymerase chain reaction (PCR). PCR conditions, established to amplify the human GPR14 sequence were 94°C, 10 min followed by 35 cycles of 94°C, 1 min, 60°C, 1 min, 72°C and 2 min using the GC-melt kit. Primers designed to amplify the coding sequence contained a *Bam*HI site in the forward, and a *Xba*I-site in the reverse primer, respectively. The urotensin receptor coding region, flanked by *Bam*HI/*Xba*I sites, was cloned into the pCDNA3.1(+) mammalian expression vector and sequenced in both directions. For generation of stable cell lines, the human U-II receptor coding sequence flanked by a 5' *Eco*RI site

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and a 3' *Eco*RV site was cloned into the mammalian episomal expression vector pEAK8.

The rat U-II receptor coding sequence flanked by a 5' *Eco*RI and 3' *Not*I site was amplified via PCR from rat kidney cDNA and cloned into the mammalian pEAK8 expression vector. Sequences of all urotensin II receptor expressing plasmids were verified by dideoxy sequencing in both directions.

Cell culture and transfection

CHO K1-cells were grown in basal ISCOVE medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, penicillin-streptomycin (10,000 IU/ml – 10,000 µg/ml), 25 mg/ml gentamicin, at 37°C in a humidified 5% CO_2 incubator. Cells were transiently transfected with the U-II-receptor cDNAs using the LipofectAMINE™ reagent according to the manufacturer's protocol. After 18-24 h following the transfection, cells were split into black-wall 96-well plates at a density of 50 000 cells per well and cultured for an additional 18-24 h period before being used in the functional FLIPR-assay (described below in details), measuring intracellular Ca^{2+} release upon receptor activation.

Fluorescence imaging plate reader (FLIPR) assay

Cells were loaded in 96-well plates for 1 h (37°C, 5% CO_2) with 100 µl of PBS (without Ca^{2+}, Mg^{2+} and $NaHCO_3$) containing 4 µM of the fluorescent calcium indicator Fluo-4, 0.22% Pluronic F-127 in DMSO, 2.5 mM probenecid, 1 mM EGTA and 1% FCS. Cells were then washed three times with PBS (without Ca^{2+}, Mg^{2+} and $NaHCO_3$)

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containing 1 mM EDTA, 0.5 mM $MgCl_2$ and 2.5 mM probenecid. After the final wash, 100 µl residual volume remained on the cells. Peptides were aliquoted as 2X solutions in 96-well plates and transferred by the instrument from the ligand plate to the cell plate. Fluorescence was recorded with the fluorometric imaging plate reader FLIPR™, (Molecular Devices, Sunnyvale, CA) over a period of 3 minutes. Fluorescence was recorded simultaneously in all wells in 3-second intervals during the first minute and in 10-second intervals during the last two minutes. Fluorescence data were generated in duplicate and repeated for at least 3 times. Data were analyzed by non-linear curve fitting using GraphPad Prism version 3.0 (GraphPad Software Inc., San Diego, CA).

Generation of stable human and rat U-II receptor expressing cell lines

For the generation of stable cell lines expressing the human and rat urotensin II receptor, HEK293 cells were transfected with human- and rat-pEAK8 constructs using the FuGene 6 transfection reagent according to the supplier's protocol. Two days after transfection, cells were selected in DMEM, supplemented with 10% fetal calf serum, 20 mM HEPES, penicillin-streptomycin (10,000 IU/ml – 10,000 µg/ml) and 1µg/ml puromycin for a period of 4 weeks (37°C, 5% CO_2, 95% relative humidity). Functional activity of the urotensin II receptor-expressing cell population was verified with a FLIPR assay, recording urotensin II-mediated intracellular Ca^{2+} release, as described above.

Membrane preparation and radioliogand binding assays

HEK293 cells stably expressing human or rat urotensin II receptor were cultured up to 80% confluency in DMEM, supplemented with 10% fetal calf serum, 20 mM HEPES, penicillin-streptomycin (10,000 IU/ml – 10,000 µg/ml) and 1µg/ml puromycin

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(37°C, 5% CO_2, 95% relative humidity). Cells were washed once with ice-cold PBS and a second time with PBS containing the Protease Inhibitor Cocktail (Complete™). Cells were scraped off and centrifuged gently. The pellet was resuspended in a buffer containing 5mM HEPES, 1 mM EDTA-disodium salt and the cocktail of protease inhibitors Complete™ and then incubated on ice for 15 minutes. Cells were pelleted again and resuspended with a homogenizer (Unit F8B, Constant cell disruption systems, Honiley, Warwickshire, UK). The supernatant and resuspended pellet were combined and centrifuged at 50,000 *g* (Beckman Avanti J251, Fullerton CA). The cell membrane pellet was resuspended in a buffer consisting of 20 mM HEPES, 1 mM EDTA-disodium salt, 150 mM NaCl and 10% sucrose. Membrane aliquots were stored at -80°C. One day prior to the binding assay, membranes were thawed, pelleted and resuspended in the assay buffer consisting of 20 mM HEPES, 150 mM NaCl, 1 mM EDTA-disodium salt, 160 µg/ml bacitracin and Complete Protease Inhibitor (2 tablets/100 ml).

Membranes were distributed into 96-well wheatgerm-agglutinin Flashplates PLUS, incubated overnight for adsorption and then, the Flashplates PLUS were washed twice with the assay buffer. For equilibrium binding assays, 0.2 nM human [^{125}I-Tyr^{9}]urotensin II (initial specific activity 2200 Ci/mmol) was incubated with the indicated amounts of unlabeled competitors during 4 h and radioactivity counted in a 1450 Microbeta Wallac Jet (Wallac, Turku, Finland). Nonspecific binding was determined in the presence of 10 µM human U-II and corresponded to 31 ± 2 % (n=5, mean ± SEM) of the total binding for membranes expressing the human urotensin II receptor, and to 8 ± 1 % (n=5, mean ± SEM) of the total binding for membranes expressing the rat urotensin II receptor. Data analysis was performed with GraphPad Prism 3.0 (GraphPad Software, San Diego, CA).

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Results

Pharmacological studies (rat thoracic aorta bioassay)

A series of 41 analogues (Table 1) was developed and evaluated for their biological activity, using the rat thoracic aorta paradigm (Figure 1 for representative curves). The focus was first applied on peptide compounds containing exocyclic modifications (Table 2). Thus, the fragments obtained by shortening the N-terminal segment of hU-II, such as hU-II(2-11) (compound 3), hU-II(3-11) (compound 4), hU-II(4-11) (compound 5), hU-II(5-11) (compound 6) and hU-II(5-10) (compound 2) appeared as partial agonists with EC_{50} values of 3.3×10^{-9} M, 2.8×10^{-10} M, 3.0×10^{-8} M, 6.8×10^{-7} and 1.5×10^{-6} M, respectively. Interestingly, hU-II(3-11) was 10 times more potent than the whole molecule (0.28 nM *vs* 3.3 nM for hU-II), but displayed only partial agonism. In addition, the removal of valine-11 (compounds 2 and 7), led to a dramatic loss of activity as compared to the parent molecule. The Ala-scan series related to the exocyclic amino acid residues of hU-II showed that most derivatives are potent analogues of hU-II. Only the substitution of valine-11 (compound 17) gave rise to a 50-fold decrease in potency, accompanied with a reduced efficacy. On the other hand, an analogue with a tetra-alanine N-terminal segment (compound 31: [$Ala^{1,2,3,4}$]hU-II) or with the C-terminal carboxylic function transformed into an amidated form (compound 27) were partial agonists with an efficacy about half of that of hU-II but with EC_{50} values close to that of the parent molecule (#31: 6.0 ± 0.6 nM, #27: 2.0 ± 0.2 nM *vs* #1: 3.3 ± 0.3 nM).

Endocyclic and ring transformations of hU-II were also studied using the rat thoracic aorta bioassay (Table 3 and 4). So far, except for [Ala^6]hU-II (compound 13) which was a weak agonist, all alanine substitutions of the endocyclic amino acids led to

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inactive peptides. Similarly, the successive deletion or the inversion of chirality of the endocyclic residues also exhibited, in most cases, a detrimental effect on the biological activity. One exception though with [D-Trp^7]hU-II (compound 23) which showed a significant but weak effect (EC_{50}) on the aorta. Substitutions with isofunctional residues were carried out at various positions. For instance, lysine-9 was replaced with arginine (compound 32) and this analogue was only 20 times less potent than hU-II. However, when the lysine-8 side chain was shortened by a distance equivalent to a methylene group, as obtained with ornithine-8 (compound 33), the new analogue exhibited a complete loss of constricting activity. The role of the Phe-6 and Tyr-9 side chains were also investigated (compounds 35 and 36) using another aromatic residue for the substitution. Potent analogues with only a 10-fold decrease in activity were produced with this alteration.

Ring modifications were introduced in a few analogues (Table 4). The results showed that an increase of the ring size (compound 37) caused a 100-fold decrease in activity while linearization and substitution of the sulfhydryl moiety with acetamidomethyl groups (compound 34) gave a weakly active analogue.

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Binding assays on human and rat receptors

[^{125}I-Tyr^9]hU-II was used as a probe for evaluating the binding characteristics of the peptides on the human and rat U-II receptors (Figure 2 for representative competition curves; Tables 5, 6 and 7). Nonspecific binding was determined in the presence of 10 µM human U-II and corresponded to 31 ± 2 % (n=5, mean ± SEM) of the total binding for membranes expressing the human urotensin II receptor, and to 8 ± 1%

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(n=5, mean ± SEM) of the total binding for membranes expressing the rat urotensin II receptor. The binding of [^{125}I-Tyr^{9}]urotensin II was characterized by a K_D of 1.4 ± 0.2 nM and a B_{max} of 1.0 ± 0.1 pmol/mg protein for human U-II receptor-expressing membranes (n=4, mean ± SEM) , and a K_D of 1.25 ± 0.06 nM and B_{max} of 3.4 ± 0.2 pmol/mg protein for rat U-II receptor-expressing membranes (n=5, mean ± SEM). As in the rat thoracic aorta bioassay, human and rat U-II exhibited very similar affinities in the binding experiments. The magnitude of binding for both isoforms was superior on the human than on the rat receptor. Successive N-terminal deletions (Table 5 – compounds 3 to 6) had only minor effects on the binding properties on both receptor types. Surprisingly, an exception was observed with hU-II(5-11) (compound 6) on the rat receptor since no binding was measured although this peptide appeared as a weak agonist on the rat thoracic aorta. The removal of valine-11 in the whole molecule (compound 7) or in the hU-II(5-11) fragment (compound 2) led to molecules with poor affinities for the human and rat receptors. On the other hand, the substitution of residue 11 with alanine (compound 17) exhibited a 30-fold decrease on the rat receptor affinity and a 10-fold reduction of binding on the human receptor. All peptides with any other Ala substitution in the exocyclic segment of U-II (compounds 8 to 11), and even [Ala^{1,2,3,4}]hU-II (compound 31), appeared as very potent ligand of the U-II receptors. Similarly, the C-terminal carboxylic function can be replaced with a neutral amide group without losing affinity for both human and rat receptors. Except for hU-II(5-11) (compound 6) that did not attach to the rat receptor but appeared as equipotent to hU-II in binding to the human receptor, all compounds containing exocyclic modifications exhibited a Hill slope close to -1 thus signifying the presence of a single U-II receptor population.

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Endocyclic modifications confirmed the key role of the residues 6 to 9 of hU-II (Table 6). Successive deletions, inversion of amino acid configuration or Ala substitutions gave rise to detrimental effects on the binding properties related to the human or rat U-II receptors. A few alterations were rather well tolerated as seen with the replacement of Tyr-9 with Phe (compound 35) or the substitution of Phe-6 with Tyr (compound 36). So far, both compounds exhibited very significant affinity for the U-II receptors.

Poor affinities were usually measured with peptide analogues containing cysteinyl modifications (Table 7). Nevertheless, the cyclic compound [HomoCys$^{5-10}$]hU-II (compound 37) was described as a 100-fold weaker agonist than the parent molecule (EC_{50}: 348 ± 23 *vs.* 3.3 ± 0.3) in the rat thoracic bioassay. Strikingly, this analogue showed no significant affinity for the rat receptor expressed in the HEK293 cell line but appeared well recognized by the human U-II receptor with an affinity about 100-fold lower than that of hU-II.

Functional FLIPR assay on Ca^{2+} mobilization

Using the functional FLIPR-assay, the effects of exocyclic, endocyclic and ring modifications on Ca^{2+} mobilization were measured on transfected CHO-K1 cells expressing the human or rat U-II receptors (Tables 8, 9 and 10). In contrast to the biological and binding results obtained with some exocyclic-altered analogues, the functional data showed that such modifications had in most cases only little effects on Ca^{2+} mobilization. In fact, EC_{50} values ranged from 1.8 to 193 nM with the human receptor while it was between 0.24 to 735 nM with the rat receptor preparation. When endocyclic substitutions or mutations were introduced in hU-II, the absence of Ca^{2+}

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mobilization was, as expected, frequently encountered. However, some transformations had little detrimental impacts on the calcium assay. This was particularly noticeable with the inversion of configuration of Trp-7 (compound 23, Table 9), the substitution of Tyr-9 with Phe (compound 35, Table 9) and the substitution of Phe-6 with Tyr (compound 36, Table 9). These analogues, as observed with the two other paradigms, retained on Ca^{2+} mobilization a very large activity. Surprisingly, analogues containing respectively a histidine residue at position 7 (compound 28, Table 9) or an ornithine moiety substituting Lys-8 (compound 33, Table 9), still exhibited a very significant potency in promoting calcium movements in the transfected cells. Similarly, the weak rat thoracic aorta agonist [HomoCys$^{5-10}$]hU-II (compound 37) appeared as a very potent agonist on Ca^{2+} mobilization in CHO-K1 cells.

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Discussion

Three series of urotensin II analogues were designed to elucidate the role of the exocyclic, endocyclic and ring-related amino acids in three different paradigms *i.e.* the rat thoracic aorta bioassay, binding assays with HEK293 cells expressing the human or rat U-II receptor and functional assays on Ca^{2+} mobilization using CHO-K1 cells expressing the human or rat U-II receptor. The data revealed disparities depending of the assay or the species. For instance, the Ca^{2+} mobilization assay, whether carried out with the human or rat receptor (Table 8 – compounds 2 to 6) suggested, likewise to the report of Itoh et al. (1987), that the C-terminal part of U-II contains the pharmacophoric elements for giving agonists. However, the contractile effects evaluated in the rat aorta and the binding values measured with the human and rat receptors showed that the C-terminal fragments hU-II(5-11) (compound 6) and hU-II(5-10) (compound 2) were rather weak ligands or agonists. The structure of the cyclic core of urotensin II is similar to that of other peptides such as somatostatin (SST) (Rohrer et al., 1998) and vasopressin (VP) (Manning et al., 1999). Therefore, it is not excluded that some U-II analogues and fragments might interact with receptors other than GPR14. As a matter of fact, although SST itself is not recognized by the U-II receptor, the group of Coy (Coy et al., 2000; Rossowski et al., 2002) screened a series of SST ligands in the rat aorta bioassay and for competitive binding in a rat preparation. They discovered that several analogues had moderate affinity for GPR14 and were able to block U-II induced phasic contractions in rat thoracic aorta rings. Coy et al. concluded that their data support the suggestion that U-II receptor and SST type 2/5 receptors display similar surface topologies.

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Nevertheless, the rat aorta pharmacological assay and the binding studies, showed correlations allowing the identification of useful pharmacophores. Hence, as suggested by Perkins et al. (1990), the "capping" of the C-terminal heptapeptide appears as a useful feature for obtaining potent peptides (compounds 3 to 5, 38, 8 to 11, 31 and rat U-II). Whether one amino acid or a short segment of residues is linked to the C-terminal heptapeptide, an effective analogue can be obtained in such conditions. This strategy was utilized by a few groups (Kinney et al., 2001, 2002; Grieco et al., 2001), including us (Flohr et al., 2002), by acetylating the N-terminal function of some U-II analogues. In contrast, as shown with [*des*-Val^{11}]hU-II (compound 7) and [Ala^{11}]hU-II (compound 17), the valine residue would play an important role in activity and affinity. It is worth mentioning that this amino acid is highly conserved throughout species and the rare substitution observed for instance in the rat or carp is made with the isofunctional residue isoleucine (Conlon, 2000). Finally, it was demonstrated that the C-terminal negative charge of U-II is not important for the GPR14 receptor since its amidation (compound 27) did not significantly alter the affinity and biological activity of the U-II ligand.

Endocyclic modifications revealed the major role played by the Phe^{6}-Trp^{7}-Lys^{8}-Tyr^{9} segment of hU-II. All assays (Tables 3, 6 and 9), including the Ca^{2+} mobilization paradigm, showed that deletions or substitutions in the cyclic core of the peptide gave rise frequently to a severe loss of activity and affinity and, as reported before (Flohr et al., 2002), this is particularly evident with the amino acid triade WKY. In fact, the substitution with Ala of the endocyclic residues led in most cases to the abolition of the contractile activity in the rat thoracic aorta. An exception though was noticed with the evolutionarily highly conserved Phe-6 since [Ala^{6}]hU-II retained some biological activity

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in the rat aorta. In agreement with these results, Phe-6 can be replaced with Tyr without losing much activity nor affinity.

To explore the importance of the correct conformation of the message sequence, we inverted the chirality of the amino acids found in the cyclic part of hU-II (compounds 22 to 25). The incorporation of D-residues caused a major decrease of the agonistic activities and binding affinities, except for Trp-7. The corresponding analogue [D-Trp^7]hU-II exhibited significant activity and affinities thus suggesting that the orientation is not critical as it was with the three other endocyclic residues. It is known that amino acids with D-configuration support the formation of β-turn type structures resulting in conformationally restricted peptides (Freidinger et al., 1984). Our previous NMR studies (Flohr et al., 2002) demonstrated, using the cyclic hexapeptide Ac-CFwKYC-NH_2, that the introduction of a D-Trp residue in position *i* + 1 gave a structure characterized by a distorted β-II' turn. Therefore, we tried to favor the stabilization of an intramolecular β-turn conformation by introducing, according to the Chou-Fasman parameters (Chou and Fasman, 1978), a Pro moiety at position *i* + 1 (compound 41) or a sequential rearrangement of the endocyclic residues (compound 40). Although the Chou-Fasman $\langle P_t \rangle$ parameter of these analogues was close to 1 and their respective Chou-Fasman p_t value largely above the theoretical threshold limit of 0.75×10^{-4} (1.3×10^{-4} and 1.6×10^{-4} for [Lys^7,Tyr^8,Trp^9]hU-II and [Pro^7]hU-II, respectively), thus exhibiting a strong probability of β-turn structure, both compounds were inactive in the biological and binding assays.

A few other alterations were also investigated. In occurence, lysine-8 was replaced with the isofunctional amino acids Arg and Orn. It was observed that the rat thoracic aorta was very sensitive to those alterations since [Arg^8]hU-II was 20-fold less potent than the parent molecule and [Orn^8]hU-II was inactive. As concluded before

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(Flohr et al., 2002), it seems that the position and the distance of the positive ionizable feature, in relation to the two hydrophobic – aromatic poles formed by Trp-7 and Tyr-9, are key characteristics of U-II ligands. It is likely, as described for Lys-9 of SST-14 (Strnad and Hadcock, 1995; Marchese et al., 1995), that Lys-8 of U-II would be involved in an interaction with GPR14, probably with the Asp-130 residue positioned in the TM3 of the receptor. Interestingly, when Lys-8 is substituted with Orn, the new derivative behaves as a competive and selective antagonist in the rat aorta bioassay (Camarda et al., 2002).

In the third series of compounds, we explored the impact of modifications of the ring-related amino acids (Tables 4, 7 and 10). Hence, linearization of hU-II (analogues 12 and 34), caused a major loss of affinity and activity. Moreover, a peptide containing a Phe pair (compound 39), designed in order to mimic the π-π overlap of the aromatic rings observed in somatostatin, did not exhibit any significant binding nor activity. As demonstrated with [HomoCys$^{5-10}$]hU-II, an analogue with a disulfide-bridged ring containing two extra methylene moieties (compound 37), the ring size also appeared as a critical feature since this analogue was about 100 times less active than U-II in the rat thoracic aorta assay. Similar conclusions were reached by Grieco et al. (2002) after exploring the ring size of hU-II analogues containing a lactam bridge. Interestingly, the affinity of compound 37 was significant in only one of the two binding paradigms. In this case, [HomoCys$^{5-10}$]hU-II was only about 100-fold less potent than the parent molecule towards the human receptor whereas it showed no affinity for rat GPR14.

In summary, this SAR study revealed the role of the amino acids of U-II in aorta contraction and human – rat receptor affinity, and more particularly, of the peptide triade

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WKY that appeared as the essential part of the physiological ligand. A few other amino acids might also be key-residues for activity and/or affinity. However, the occasional lack of relationship between the results of the rat aorta paradigm and the Ca^{2+} mobilization functional assay led sometimes to difficult interpretations. This situation might be a consequence of the differences in Bmax values of the cell systems expressing human or rat receptors, as different expression levels may influence agonist potencies and efficacies. This phenomenon, described as the stimulus response coupling (Kenakin, 2002), was reported before (Kenakin and Beek, 1984 ; Rizzi et al., 1999) and could apply to our paradigms because the rat aorta expresses 250-1000 times less receptors than our transfected cell preparations (Bmax for the rat aorta according to Ames et al., 1999: 2-20 fmol/mg protein ; Bmax for our human U-II receptor-expressing membranes : 1 pmol/mg protrein and Bmax for our rat U-II receptor-expressing membranes : 3.4 pmol/mg protein). On the other hand, as suggested by the low Hill coefficients measured with some U-II analogues, the divergence between the bioactivity and the Ca^{2+} might also be related to effects induction through more than one receptor/binding site, not necessarily related to GPR14. Such an hypothesis has been also postulated previously by Coy et al. (2000) after observing a clear dichotomy between the phasic and tonic rat aorta contractions induced with U-II. Consequently, the analysis of functional data of U-II and analogues, using a Ca^{2+} mobilization assay developed with transfected mammalian cells expressing the human or rat GPR14 receptor, must be carried out with care as it might lead to ambiguous SAR conclusions.

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Footnotes

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Legends for Figures

Fig. 1. Representative concentration-response curves obtained with rat thoracic aorta rings after adding cumulative concentrations of hU-II and its analogues. Data represent mean ± SEM and *n* = 5 to 10 animals.

Fig. 2. Representative curves of inhibition of the [^{125}I-Tyr^{9}]hU-II binding to the human GPR14 receptor. Panel A illustrates the effect of some deletions of amino acids in hU-II : ■, hU-II; □, hU-II(5-10) (compound 2); ▲, hU-II(4-11) (compound 5); △, [Glu^{4}]hU-II(5-11) (compound 38). Panel B illustrates the effect of some single substitutions using Ala : ■, hU-II, □, [Ala^{1}]hU-II (compound 8); ▲, [Ala^{2}]hU-II (compound 9); △, [Ala^{3}]hU-II (compound 10); ▼, [Ala^{11}]hU-II (compound 17). Panel C illustrates the effect measured with some ring-altered hU-II derivatives : ■, hU-II; □, [Ala^{5}]hU-II (compound 12); ▲, [Cys(Acm)^{5,10}]hU-II (compound 34); △, [Phe^{5,10}]hU-II (compound 39). Data represent mean ± SEM of three independent experiments each performed in duplicates.

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Table 1. Structure and molecular weight of the various analogues and fragments of urotensin II.

Peptide	Structure[a]	Mr [g/mol]	Peptide	Structure[a]	Mr [g/mol]
1	ETPDCFWKYCV (hU-II)	1389	22	ETPDC**f**WKYCV	1389
2	CFWKYC	847	23	ETPDCF**w**KYCV	1389
3	TPDCFWKYCV	1260	24	ETPDCFW**k**YCV	1389
4	PDCFWKYCV	1158	25	ETPDCFWK**y**CV	1389
5	DCFWKYCV	1061	26	$(ETPDAFWKYCV)_2$ [b]	2715
6	CFWKYCV	946	27	ETPDCFWKYCV-NH_2	1397
7	ETPDCFWKYC	1290	28	ETPDCF**H**KYCV	1339
8	**A**TPDCFWKYCV	1331	29	ETPDC**A**FWKYCV	1461
9	E**A**PDCFWKYCV	1359	30	QHGTAPECFWKYCI (rU-II)	1661
10	ET**A**DCFWKYCV	1363	31	**AAAA**CFWKYCV	1230
11	ETP**A**CFWKYCV	1345	32	ETPDCFW**R**YCV	1445
12	ETPD**A**FWKYCV	1359	33	ETPDCFWK**Orn**CV	1374
13	ETPDC**A**WKYCV	1313	34	ETPDC(Acm)FWKYC(Acm)V[c]	1561
14	ETPDCF**A**KYCV	1274	35	ETPDCFWK**F**CV	1401
15	ETPDCFW**A**YCV	1331,5	36	ETPDC**Y**WKYCV	1404
16	ETPDCFWK**A**CV	1297	37	ETPD**Hcy**FWKY**Hcy**V	1416
17	ETPDCFWKYC**A**	1361	38	ECFWKYCV	1075
18	ETPDCWKYCV	1241	39	ETPD**F**FWKY**F**V	1478
19	ETPDCFKYCV	1202	40	ETPDCF**KYW**CV	1388
20	ETPDCFWYCV	1260	41	ETPDCF**P**KYCV	1299
21	ETPDCFWKCV	1225			

[a] Unless otherwise stated, all peptides exhibit free N- and C-terminus. Unless otherwise stated, all peptides containing cysteines or homocysteines are cyclic.

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[b] Dimer obtained through a disulfide bridge.

[c] Linear hU-II analogue with an acetamidomethyl group on the cysteine side-chains.

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Table 2: Effects of exocyclic modifications in U-II on the rat thoracic aorta.

Peptide	Structure	Rat thoracic aorta bioassay		
		EC_{50} ± SEM [nM][a]	E_{max} ± SEM [%][b]	*n*[c]
1	ETPDCFWKYCV (hU-II)	3.3 ± 0.3	98 ± 3	12
30	QHGTAPECFWKYCI (rU-II)	2.0 ± 0.2	48 ± 2	8
2	CFWKYC	1500 ± 200	61 ± 8	10
3	TPDCFWKYCV	3.3 ± 0.3	49 ± 1	7
4	PDCFWKYCV	0.28 ± 0.04	37 ± 2	5
5	DCFWKYCV	30 ± 3	67 ± 3	7
6	CFWKYCV	680 ± 50	31 ± 2	6
7	ETPDCFWKYC	1900 ± 300	65 ± 2	8
38	ECFWKYCV	10 ± 1	81 ± 2	10
8	**A**TPDCFWKYCV	3.0 ± 0.1	106 ± 3	12
9	E**A**PDCFWKYCV	3.0 ± 0.2	99 ± 4	8
10	ET**A**DCFWKYCV	13.0 ± 0.2	93 ± 13	6
11	ETP**A**CFWKYCV	9.3 ± 0.6	69 ± 4	10
17	ETPDCFWKYC**A**	160 ± 15	62 ± 8	5
31	**AAAA**CFWKYCV	6.0 ± 0.6	44 ± 5	10
27	ETPDCFWKYCV-**NH_2**	2.0 ± 0.2	48 ± 2	8

[a] Concentration producing 50% of the maximum effect.

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[b] Percentage of efficacy as compared to the value obtained with 60 mM KCl.

[c] Number of animals.

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Table 3: Effects of endocyclic modifications in U-II on the rat thoracic aorta.

Peptide	Structure	Rat thoracic aorta bioassay		
		EC_{50} ± SEM [nM][a]	E_{max} ± SEM [%][b]	*n*[c]
1	ETPDCFWKYCV (hU-II)	3.3 ± 0.3	98 ± 3	12
13	ETPDC**A**WKYCV	1900 ± 300	67 ± 12	4
14	ETPDCF**A**KYCV	>10 000	nd	4
15	ETPDCFW**A**YCV	>10 000	nd	4
16	ETPDCFWK**A**CV	>10 000	nd	4
18	ETPDC**-**WKYCV	>1000	nd	4
19	ETPDCF-KYCV	>10 000	nd	4
20	ETPDCFW-YCV	>10 000	nd	3
21	ETPDCFWK-CV	>10 000	nd	4
22	ETPDC**f**WKYCV	>1000	nd	6
23	ETPDCF**w**KYCV	170 ± 50	37 ± 3	8
24	ETPDCFW**k**YCV	>10 000	nd	4
25	ETPDCFWK**y**CV	>10 000	nd	4
28	ETPDCF**H**KYCV	2600 ± 420	52 ± 13	6
32	ETPDCFW**R**YCV	69 ± 15	64 ± 14	8
33	ETPDCFW**Orn**YCV	>10 000	nd	4
35	ETPDCFWK**F**CV	35 ± 2	64 ± 14	8
36	ETPDC**Y**WKYCV	40 ± 4	83 ± 5	9
41	ETPDCF**P**KYCV	>10 000	69 ± 3	4
29	ETPDC**A**FWKYCV	2660 ± 1300	34 ± 4	7
40	ETPDCF**KYW**CV	>10 000	nd	5

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[*a, b, c*] As in Table 2.

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Table 4: Effects of ring modifications in U-II on the rat thoracic aorta.

Peptide	Structure	Rat aorta bioassay		
		EC_{50} ± SEM [nM][a]	E_{max} ± SEM [%][b]	*n*[c]
1	ETPDCFWKYCV (hU-II)	3.3 ± 0.3	98 ± 3	12
12	ETPD**A**FWKYCV	> 1000	nd	5
34	ETPD**C(Acm)**FWKY**C(Acm)**V	603 ± 7	64 ± 7	8
39	ETPD**F**FWKY**F**V	>10 000	nd	3
37	ETPD**Hcy**FWKY**Hcy**V	348 ± 23	95 ± 6	8
26	$(ETPDAFWKYCV)_2$ [d]	>10 000	nd	3

[a, b, c] As in Table 2.

[d] Dimer obtained through a disulfide bridge.

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Table 5: Effects of exocyclic modifications in U-II on the binding of [^{125}I-Tyr^{9}]hU-II on the human and rat U-II receptor.[a]

Peptide	Structure	human U-II receptor			rat U-II receptor		
		K_i ± SEM [nM][b]	n_H ± SEM	n[c]	K_i ± SEM [nM][b]	n_H ± SEM	n[c]
1	ETPDCFWKYCV (hUT-II)	12 ± 5	-1.11 ± 0.26	4	63 ± 17	-1.04 ± 0.06	4
30	QHGTAPECFWKYCI (rUT-II)	14 ± 4	-0.76 ± 0.06	3	28 ± 8	-1.09 ± 0.05	3
2	CFWKYC	530 ± 164	-0.90 ± 0.06	3	4550 ± 421	-1.1 ± 0.03	3
3	TPDCFWKYCV	27 ± 17	-0.97 ± 0.11	3	63 ± 17	-1.04 ± 0.06	4
4	PDCFWKYCV	4.6 ± 1.2	-0.95 ± 0.11	3	28 ± 8	-1.09 ± 0.05	3
5	DCFWKYCV	5.5 ± 0.4	-0.68 ± 0.13	4	13 ± 3	-1.26 ± 0.21	4
6	CFWKYCV	13 ± 7	-0.51 ± 0.04	3	> 10 000	nd	3
7	ETPDCFWKYC	> 10 000	nd	3	2672 ± 407	-0.87 ± 0.03	4
38	ECFWKYCV	2.6 ± 0.8	-0.78 ± 0.14	3	51 ± 29	-0.77 ± 0.24	3

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8	**A**TPDCFWKYCV	17 ± 5	-0.91 ± 0.47	4	30 ± 8	-0.97 ± 0.03	4
9	E**A**PDCFWKYCV	5.4 ± 2.5	-1.15 ± 0.30	3	52 ± 30	-0.75 ± 0.08	3
10	ET**A**DCFWKYCV	12 ± 5.3	-0.54 ± 0.05	4	66 ± 28	-0.70 ± 0.08	3
11	ETP**A**CFWKYCV	11 ± 3.5	-0.80 ± 0.18	5	19 ± 5	-1.11 ± 0.05	3
17	ETPDCFWKYC**A**	171 ± 29	-1.19 ± 0.28	4	1718 ± 542	-1.22 ± 0.17	4
31	**AAAA**CFWKYCV	7.3 ± 1.8	-1.39 ± 0.39	3	31 ± 4	-1.29 ± 0.11	3
27	ETPDCFWKYCV-**NH$_2$**	10 ± 4	-0.81 ± 0.13	4	31 ± 3	-0.98 ± 0.09	3

[a] Binding assay using membranes isolated from a stable HEK293 cell line expressing the U-II receptor.

[b] Concentration producing a 50% inhibition of binding.

[c] Number of experiments made in triplicate.

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Table 6: Effects of endocyclic modifications in U-II on the binding of [^{125}I-Tyr^{9}]hU-II on the human and rat U-II receptor.[a]

Peptide	Structure	human U-II receptor			rat U-II receptor		
		K_i ± SEM [nM][b]	n_H ± SEM	n[c]	K_i ± SEM [nM][b]	n_H ± SEM	n[c]
1	ETPDCFWKYCV (hUT-II)	12 ± 5	-1.11 ± 0.26	4	63 ± 17	-1.04 ± 0.06	4
13	ETPDC**A**WKYCV	1744 ± 544	-0.25 ± 0.01	5	> 10 000	nd	3
14	ETPDCF**A**KYCV	> 10 000	nd	3	> 10 000	nd	3
15	ETPDCFW**A**YCV	4403 ± 2027	-0.58 ± 0.09	3	> 10 000	nd	3
16	ETPDCFWK**A**CV	> 10 000	nd	3	> 10 000	nd	3
18	ETPDC-WKYCV	> 10 000	nd	3	> 10 000	nd	3
19	ETPDCF-KYCV	> 10 000	nd	3	> 10 000	nd	3
20	ETPDCFW-YCV	5082 ± 1991	-1.21 ± 0.53	3	> 10 000	nd	3
21	ETPDCFWK-CV	> 10 000	nd	3	> 10 000	nd	3
22	ETPDC**f**WKYCV	6680 ± 621	-0.88 ± 0.06	3	> 10 000	-0.72 ± 0.12	3

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23	ETPDCF**w**KYCV	918 ± 8	-0.93 ± 0.21	3	3044 ± 445	-1.04 ± 0.05	3
24	ETPDCFW**k**YCV	> 10 000	nd	3	> 10 000	nd	3
25	ETPDCFWK**y**CV	2247 ± 231	-1.55 ± 0.21	3	> 10 000	nd	3
28	ETPDCF**H**KYCV	5053 ± 429	-1.32 ± 0.06	3	> 10 000	-0.86 ± 0.24	3
32	ETPDCFW**R**YCV	684 ± 185	-1.04 ± 0.08	5	3068 ± 259	-0.89 ± 0.05	3
33	ETPDCFW**Orn**YCV	848 ± 107	-1.25 ± 0.28	3	4014 ± 542	-1.96 ± 0.76	3
35	ETPDCFWK**F**CV	4.0 ± 2.5	-0.87 ± 0.16	3	663 ± 114	-0.92 ± 0.04	3
36	ETPDC**Y**WKYCV	187 ± 69	-1.14 ± 0.17	4	145 ± 11	-0.91 ± 0.04	3
41	ETPDCF**P**KYCV	> 10 000	nd	4	> 10 000	nd	5
29	ETPDC**A**FWKYCV	> 10 000	-1,13 ± 0,38	4	> 10 000	-0.45 ± 0.08	4
40	ETPDCF**KYW**CV	> 10 000	nd	4	> 10 000	nd	5

[a, b, c] As in Table 5.

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Table 7: Effects of ring modifications in U-II on the binding of [^{125}I-Tyr^9]hU-II on the human and rat U-II receptor.[a]

Peptide	Structure	human U-II receptor			rat U-II receptor		
		$K_i \pm$ SEM [nM][b]	$n_H \pm$ SEM	n[c]	$K_i \pm$ SEM [nM][b]	$n_H \pm$ SEM	n[c]
1	ETPDCFWKYCV (hUT-II)	12 ± 5	-1.11 ± 0.26	4	63 ± 17	-1.04 ± 0.06	4
12	ETPD**A**FWKYCV	3779 ± 1338	-1.00 ± 0.19	3	> 10 000	nd	3
34	ETPD**C(Acm)**FWKY**C(Acm)**V	1607 ± 488	-1.04 ± 0.13	5	> 10 000	nd	3
39	ETPD**F**FWKY**F**V	2857 ± 871	-0.96 ± 0.19	5	> 10 000	nd	4
37	ETPD**Hcy**FWKY**Hcy**V	1299 ± 184	-0.79 ± 0.11	5	> 10 000	nd	5
26	(ETPDAFWKYCV)$_2$[d]	> 10 000	nd	3	> 10 000	nd	3

a, b, c As in Table 5.

d Dimer obtained through a disulfide bridge.

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Table 8: Effects of exocyclic modifications in U-II on Ca^{2+} mobilization following activation of the human and rat U-II receptor.[a]

Peptide	Structure	human U-II receptor			rat U-II receptor		
		EC_{50} ± SEM [nM][b]	E_{max} ± SEM [%][c]	n[d]	EC_{50} ± SEM [nM][b]	E_{max} ± SEM [%][c]	n[d]
1	ETPDCFWKYCV (hUT-II)	2.5 ± 0.2	100	17	1.5 ± 0.1	100	3
30	QHGTAPECFWKYCI (rUT-II)	16 ± 2	118 ± 9	6	12 ± 1	96 ± 3	5
2	CFWKYC	2.3 ± 1.1	95 ± 7	4	10 ± 1	92 ± 10	5
3	TPDCFWKYCV	2.4 ± 0.1	103 ± 4	3	1.0 ± 0.1	92 ± 3	3
4	PDCFWKYCV	3.6 ± 0.6	100 ± 6	3	2.1 ± 0.4	97 ± 20	3
5	DCFWKYCV	3.0 ± 0.9	102 ± 5	4	0.24 ± 0.04	106 ± 10	4
6	CFWKYCV	1.8 ± 0.3	106 ± 4	6	4 ± 1	104 ± 9	6
7	ETPDCFWKYC	1.8 ± 0.1	99 ± 4	4	5.4 ± 0.5	66 ± 10	3
38	ECFWKYCV	44 ± 9	105 ± 4	4	10 ± 2	91 ± 8	4
8	**A**TPDCFWKYCV	1.8 ± 0.3	101 ± 2	6	0.8 ± 0.2	84 ± 8	7
9	E**A**PDCFWKYCV	3.3 ± 0.8	101 ± 1	5	1.4 ± 0.3	107 ± 14	7

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10	ET**A**DCFWKYCV	1.9 ± 0.8	102 ± 3	4	1.5 ± 0.1	99 ± 15	3
11	ETP**A**CFWKYCV	2.7 ± 0.5	101 ± 3	8	1.8 ± 0.2	98 ± 16	3
17	ETPDCFWKYC**A**	3.2 ± 0.3	99 ± 2	3	0.7 ± 0.2	88 ± 6	4
31	**AAAA**CFWKYCV	193 ± 28	147 ± 27	4	735 ± 343	99 ± 8	4
27	ETPDCFWKYCV-$\mathbf{NH_2}$	117 ± 21	133 ± 56	3	5 ± 2	94 ± 9	3

[a] Functional FLIPR-assay on Ca^{2+} mobilization using transiently transfected CHO-K1 cells expressing the U-II receptor.

[b] Concentration producing 50% of the maximal effect.

[c] Percentage of efficacy as compared to the value obtained with human U-II.

[d] Number of experiments made in duplicate.

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Table 9: Effects of endocyclic modifications in U-II on Ca^{2+} mobilization following activation of the human and rat U-II receptor.[a]

Peptide	Structure	human U-II receptor			rat U-II receptor		
		EC_{50} ± SEM [nM][b]	E_{max} ± SEM [%][c]	n[d]	EC_{50} ± SEM [nM][b]	E_{max} ± SEM [%][c]	n[d]
1	ETPDCFWKYCV (hUT-II)	2.5 ± 0.2	100	17	1.5 ± 0.1	100	3
13	ETPDC**A**WKYCV	5.7 ± 0.3	98 ± 1	5	5 ± 2	75 ± 5	4
14	ETPDCF**A**KYCV	1303 ± 98	45 ± 2	7	906 ± 92	67 ± 9	6
15	ETPDCFW**A**YCV	14 800 ± 800	10.2 ± 0.1	5	>10 000	nd	4
16	ETPDCFWK**A**CV	193 ± 9	75 ± 3	3	180 ± 48	78 ± 7	4
18	ETPDC-WKYCV	53 ± 2	67 ± 1	4	438 ± 83	69 ± 4	4
19	ETPDCF-KYCV	>10 000	nd	5	>10 000	nd	3
20	ETPDCFW-YCV	>10 000	nd	5	>10 000	nd	6
21	ETPDCFWK-CV	>10 000	nd	4	>10 000	nd	4
22	ETPDC**f**WKYCV	1003 ± 98	87 ± 1	3	245 ± 46	73 ± 11	3
23	ETPDCF**w**KYCV	4 ± 1	98 ± 3	4	3 ± 1	79 ± 11	4

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24	ETPDCFW**k**YCV	>10 000	nd	3	385 ± 75	47 ± 3	3
25	ETPDCFWK**y**CV	>10 000	nd	4	>10 000	nd	6
28	ETPDCF**H**KYCV	19 ± 3	84 ± 1	4	8 ± 5	78 ± 7	4
32	ETPDCFW**R**YCV	13 ± 1	103 ± 10	6	1.4 ± 0.2	97 ± 13	3
33	ETPDCFW**Orn**YCV	17 ± 3	107 ± 14	3	14 ± 3	80 ± 2	3
35	ETPDCFWK**F**CV	39 ± 9	122 ± 5	7	1.2 ± 0.1	91 ± 10	5
36	ETPDC**Y**WKYCV	6 ± 1	150 ± 14	5	0.10 ± 0.01	75 ± 9	3
41	ETPDCF**P**KYCV	>10 000	nd	8	1752 ± 238	75 ± 6	8
29	ETPDC**A**FWKYCV	3298 ± 1034	33 ± 3	5	999 ± 151	51 ± 4	7
40	ETPDCF**KYW**CV	>10 000	nd	6	1226 ± 199	46 ± 3	4

[*a, b, c, d*] As in Table 8.

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Table 10: Effects of ring modifications in U-II on Ca^{2+} mobilization following activation of the human and rat U-II receptor.[a]

Peptide	Structure	human U-II receptor			rat U-II receptor		
		EC_{50} ± SEM [nM][b]	E_{max} ± SEM [%][c]	n[d]	EC_{50} ± SEM [nM][b]	E_{max} ± SEM [%][c]	n[d]
1	ETPDCFWKYCV (hUT-II)	2.5 ± 0.2	100	17	1.5 ± 0.1	100	3
12	ETPD**A**FWKYCV	233 ± 16	96 ± 4	3	687 ± 226	93 ± 12	8
34	ETPD**C(Acm)**FWKY**C(Acm)**V	1165 ± 101	71 ± 7	3	274 ± 100	86 ± 9	3
39	ETPD**F**FWKY**F**V	189 ± 10	116 ± 7	4	107 ± 2	75 ± 5	3
37	ETPD**Hcy**FWKY**Hcy**V	5 ± 1	104 ± 4	4	0.7 ± 0.1	85 ± 3	3
26	$(ETPDAFWKYCV)_2$[e]	>10 000	nd	3	>10 000	nd	3

[a, b, c, d] As in Table 8.

[e] Dimer obtained through a disulfide bridge.

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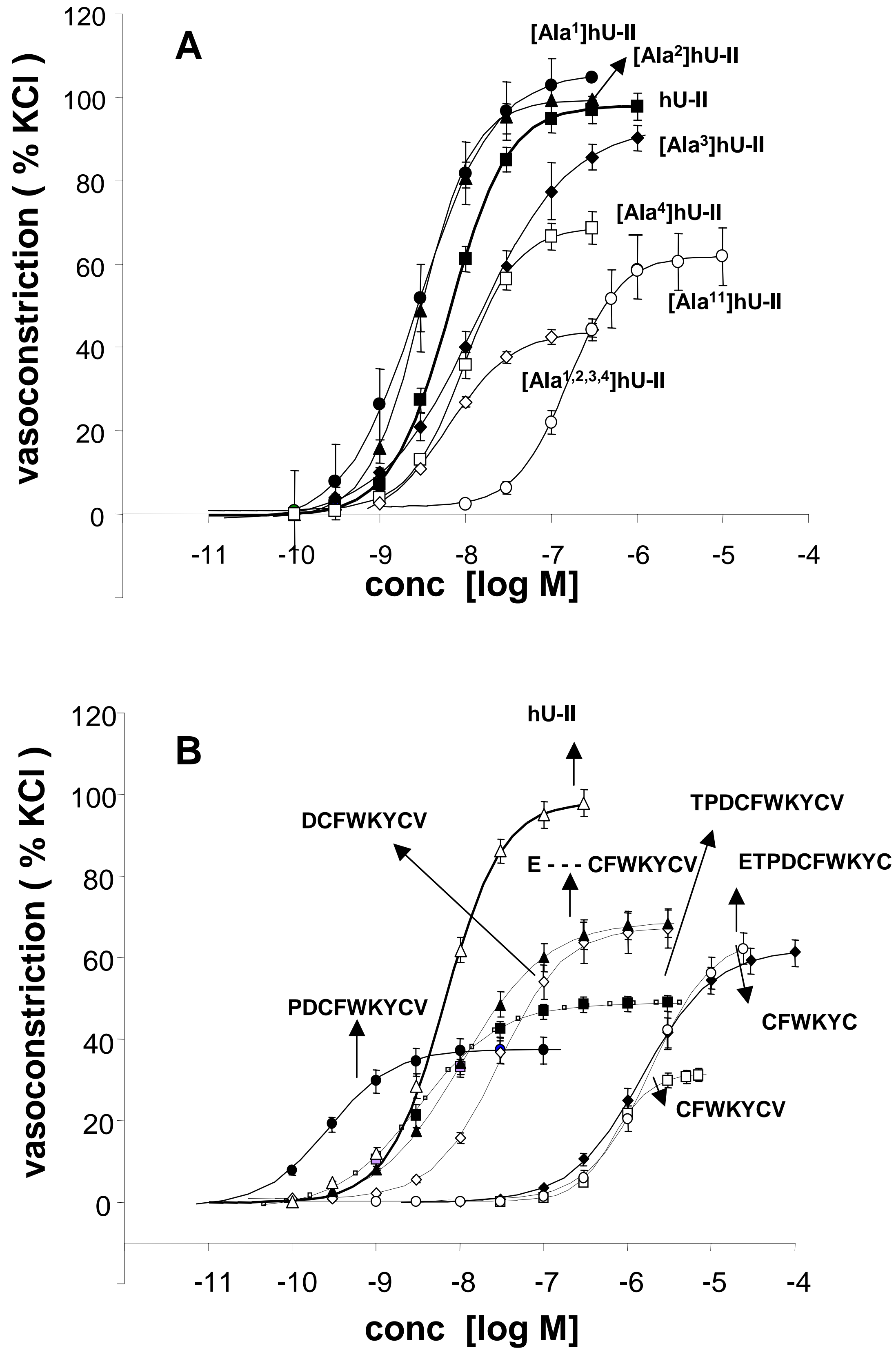

Figure 1

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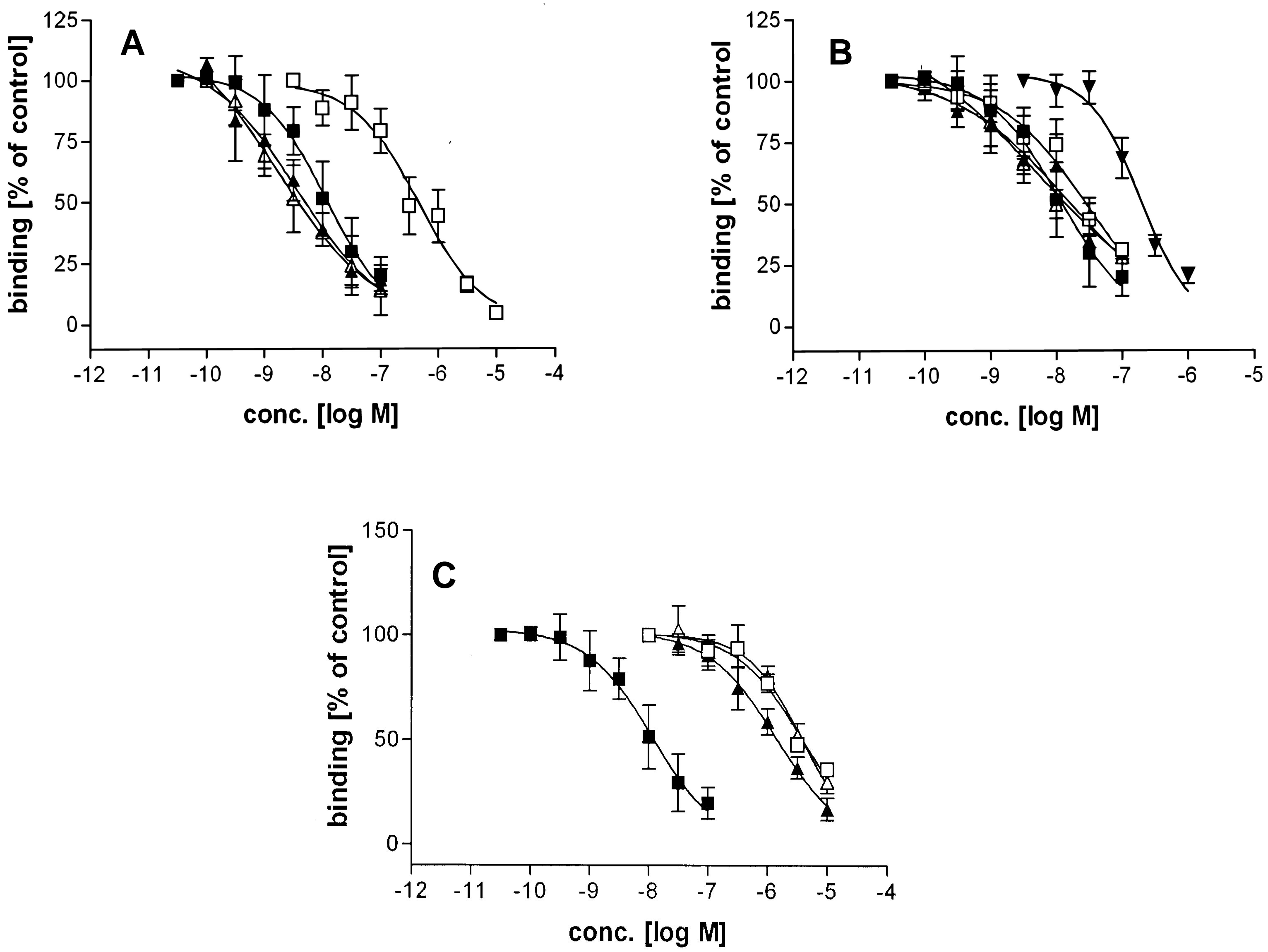

Figure 2

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