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SPECIAL REPORT

A new ligand for the urotensin II receptor

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The cyclic peptide human urotensin II (U-II) has been recently recognized as the endogenous ligand of an orphan GPCR, subsequently named the UT receptor. No synthetic ligands are available for investigating this novel peptide-receptor system. A novel UT receptor ligand, [Orn^8]U-II, was synthesized and evaluated in calcium functional assays performed on HEK293 cells expressing the recombinant rat and human UT receptor and in the rat aorta bioassay. [Orn^8]U-II behaves as a full agonist ($pEC_{50} \approx 8$) at both human and rat UT receptors in the FlipR calcium assay eliciting similar maximal effects as the natural ligand U-II. On the contrary, in the rat aorta bioassay, [Orn^8]U-II behaves as a competitive and selective antagonist ($pA_2 = 6.56$) showing however a small but consistent residual agonist activity. It is therefore proposed that [Orn^8]U-II is a partial agonist at UT receptors.

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Abbreviations: hUT, human urotensin II receptor; rUT rat urotensin II receptor; U-II, Urotensin II

Introduction Urotensin II (U-II) is a cyclic peptide that was isolated from the urophysis of the teleost fish (Pearson *et al*., 1980). The gene coding for the human peptide was cloned (Coulouarn *et al*., 1998) and U-II was demonstrated to selectively bind and activate a G-protein coupled receptor (Ames *et al*., 1999; Nothacker *et al*., 1999), subsequently named the UT receptor (Douglas & Ohlstein, 2000b). This receptor is mainly expressed in the heart, vascular smooth muscle, and central nervous system (spinal cord and cerebellum) (Douglas & Ohlstein, 2000a). The vascular activity of U-II is the most studied action of this peptide. Several groups reported contractile effects of U-II in some vascular smooth muscle preparations with huge regional, species, and interindividual variations (Camarda *et al*., 2002; Douglas *et al*., 2000c; Maguire *et al*., 2000). In the few vessels sensitive to U-II, the peptide always displayed very high potency but low maximal effects. Worthy of mention is the recent demonstration of vasoconstrictive properties of U-II in humans *in vivo* (Bohm & Pernow, 2002). Altogether, the biological actions mediated by the U-II / UT receptor system are poorly known due to the lack of selective receptor ligands (particularly antagonists) and knockout or transgenic animal models (Douglas & Ohlstein, 2000a).

In the frame of a structure-activity study on U-II, we identified [Orn^8]U-II and we report herein on the *in vitro* pharmacological profile of this novel UT receptor ligand.

Methods Experiments were performed on HEK293 cells stably expressing the recombinant human (hUT) or rat (rUT) UT receptor and in the rat isolated aorta. Details about hUT receptor cloning and generation of stable cell lines expressing the hUT protein have been previously reported (Flohr *et al*., 2002). The rat UT 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. Correctness of the construct was verified by dideoxy sequencing in both directions. The pEAK8 construct was used for generation of cell lines stably expressing the rUT receptor.

Saturation studies performed in our laboratories indicated that the binding of [^{125}I]Tyr^9-U-II was characterized by a K_D of 1.66 nM and a B_{max} of 1100 fmol mg protein^{-1} for the hUT receptor, and a K_D of 1.23 nM and B_{max} of 3700 fmol mg protein^{-1} for rUT receptor membranes.

Functional experiments were performed on HEK293-hUT and HEK293-rUT cells by measuring $[Ca^{2+}]_i$ levels with the fluorometric imaging plate reader FlipR™, (Molecular Devices, Sunnyvale CA, U.S.A.) and 4 μM of the fluorescent calcium indicator Fluo-4 as described previously in detail (Flohr *et al*., 2002). Fluorescence data were generated in duplicate.

Bioassay experiments were performed as previously described (Camarda *et al*., 2002). Briefly, thoracic aorta were taken from male Sprague–Dawley rats (200–250 g) decapitated under ether anaesthesia. The tissues were suspended in organ baths containing oxygenated Krebs solution at 37°C and at pH 7.4, stretched to a resting tension of 1 g and allowed to equilibrate for 1 h. Contractions were measured with isometric transducers (GRASS FT03) and recorded by a multichannel polygraph (LINSEIS L2005). Cumulative concentration response curves to U-II, noradrenaline, angiotensin-II and endothelin-1 were performed in the absence and in the presence of [Orn^8]U-II. A few experiments were also performed in the

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electrically stimulated guinea-pig ileum where the effects of [Orn^8]U-II were tested against the inhibitory effect of somatostatin.

Concentration response curve data were analysed by non-linear curve fitting using Graph Pad Prism version 3.02; the equation used was as follows:

$$y = \text{bottom} + \frac{\text{top} - \text{bottom}}{1 + 10^{\log EC50 - x}}$$

where y = effect; bottom = baseline; top = maximal effect; EC_{50} = concentration of an agonist that produces 50% of the maximal effect; x = log of agonist concentration.

The peptides used in this study were synthesized and purified in house at the Department of Pharmaceutical Sciences of the University of Ferrara. All other substances and reagents were from Sigma (St. Louis, MO, U.S.A.) or Tocris (Bristol, U.K.).

The data are expressed as means ± s.e.mean of *n* experiments. Data were statistically analysed using the Student's *t*-test for unpaired data or one way ANOVA followed by Dunnett test for multiple comparison. All experimental procedures complied with the standards of the European Communities Council directives (86/609/EEC).

Results U-II increased intracellular calcium levels in HEK293 hUT and rUT cells with similar high potencies (pEC_{50} 8.51 ± 0.18 and 8.54 ± 0.14, respectively) and maximal effects (Figure 1), and evoked concentration dependent contractions (pEC_{50} 8.17 ± 0.18) in the rat isolated aorta (Figure 2, top panel). These results are in line with several published reports (Ames *et al*., 1999; Camarda *et al*., 2002; Douglas *et al*., 2000c; Nothacker *et al*., 1999). The synthetic analogue, [Orn^8]U-II, behaved as a full agonist in the calcium assay in HEK293 hUT and rUT cells, inducing maximal effects similar to those of U-II. The potency of the analogue at both the hUT and rUT receptor (pEC_{50} 7.93 ± 0.16 and 8.06 ± 0.22, respectively) was, however, 3 fold lower than that of U-II. Quite different results have been obtained in the rat aorta bioassay, where [Orn^8]U-II produced negligible contractile effects at 0.1 and 1 μM, and caused at 10 μM a stable contraction of 0.12 ± 0.04 g (corresponding to about 25% of the maximal effect of U-II). In this range of concentrations, however, [Orn^8]U-II produced a concentration dependent rightward shift of the concentration response curve to U-II, without significantly modifying the maximal effects induced by the natural ligand (Figure 2, top panel). Although the analysis of these data is biased by the residual agonist activity of [Orn^8]U-II, Schild plot is compatible with a competitive

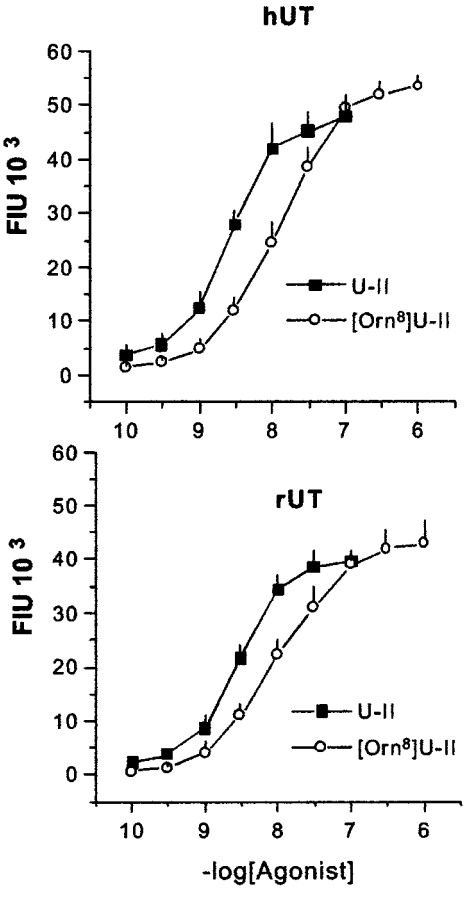

Figure 1 Concentration-response curves to human urotensin II (U-II) and [Orn^8]U-II in HEK293 cells expressing human (hUT, top panel) or the rat (rUT, bottom panel) UT receptor. Intracellular calcium levels were expressed as fluorescence intensity units (FIU) x 10^3. Points indicate the means and vertical bars the s.e.mean of at least three experiments performed in duplicate.

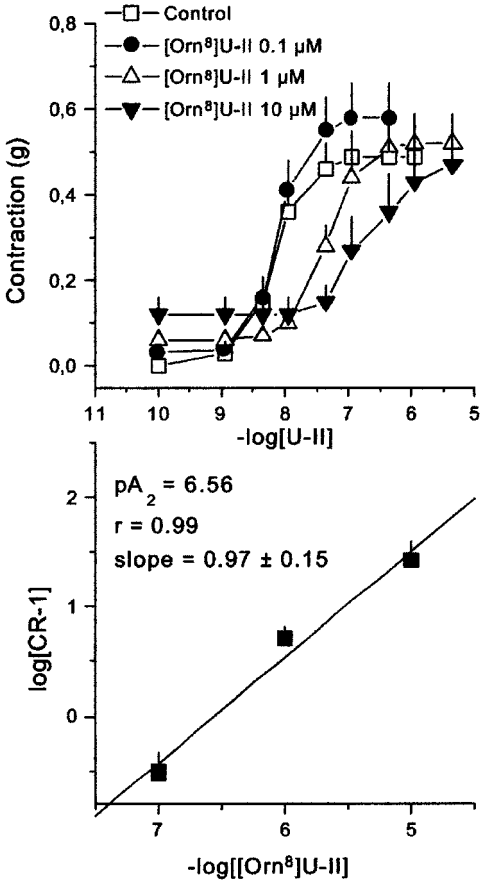

Figure 2 Concentration-response curves to human urotensin II (U-II) obtained in the absence (control) and in presence of increasing concentrations (0.1, 1, and 10 μM) of [Orn^8]U-II. The bottom panel shows the corresponding Schild plot. Points indicate the means and vertical bars the s.e.mean of 6–8 separate experiments.

type of antagonism (the slope of the regression line is not significantly different from unity) and a pA_2 value of 6.56 ± 0.15 was calculated (Figure 2, bottom panel). Ten μM [Orn^8]U-II did not modify (either in terms of potency or of maximal effects) the concentration response curves to noradrenaline, angiotensin II and endothelin-1 in the rat aorta or that to somatostatin in the electrically stimulated guinea-pig ileum (data not shown).

Discussion The natural peptide U-II selectively activates the UT receptor modulating cardiovascular functions (Douglas & Ohlstein, 2000a). Selective UT receptor antagonists are required for investigating the (patho)physiological relevance of this novel peptide-receptor system. Here we described the identification and *in vitro* characterization of [Orn^8]U-II, a novel peptide ligand for the UT receptor. The chemical modification (Lys^8→(Orn) used for generating this peptide was suggested by previous structure-activity studies demonstrating that the Lys^8 residue in U-II sequence is the most important for biological activity (Flohr *et al*., 2002). In the calcium functional assay [Orn^8]U-II behaves as a full agonist, inducing similar maximal effects as U-II. In contrast, different results were obtained in the rat aorta bioassay where the compound mainly behaved as a competitive antagonist showing however at the highest concentration tested (10 μM) a small ($\approx$25%) but consistent residual agonist activity. The discrepancy between the results obtained in the cell assay *vs* tissue bioassay can be interpreted assuming that [Orn^8]U-II is actually a partial agonist (low efficacy agonist) whose final behaviour (agonist *vs* antagonist) strongly depends on the preparation under study. In preparations like our HEK293 cells that express a very high number of receptors (B_{max} 1–4 pmol mg protein^{-1}) the efficiency of the stimulus-response coupling is probably very high and the maximal effects can be elicited even by low efficacy agonists. On the contrary, in preparations like the rat aorta that express a low number of receptors (B_{max} 1–20 fmol mg protein^{-1} (Ames *et al*., 1999)), the efficiency of the stimulus-response coupling is so low that low efficacy agonists behave mainly as competitive antagonists. For further details on the importance of stimulus-response coupling on the estimation of ligand efficacy see Kenakin (2002). Such a preparation-dependent pharmacological behaviour displayed by [Orn^8]U-II at UT receptors is not unique, since other examples have been reported in the GPCR field as for instance the nociceptin/orphanin FQ receptor ligand [F/G]N/OFQ(1-13)NH_2 (Calo *et al*., 2000), the kinin B_2 receptor ligand FR 190997 (Rizzi *et al*., 1999), or the β_1 adrenoceptor ligand prenalterol (Kenakin & Beek, 1984). Based on these considerations we propose to classify the novel UT receptor ligand [Orn^8]U-II as a partial agonist. However, further investigations on the pharmacological behaviour of [Orn^8]U-II, especially at mammalian UT receptors from different species (i.e. mouse, monkey), are mandatory. Despite limitations related to the residual agonist activity of [Orn^8]U-II, we expect that this molecule can be useful for at least two purposes: (1) as a template for future SAR studies aimed to reduce the residual agonistic activity thus leading to the identification of pure antagonists, (2) as a selective UT receptor antagonist to be used in pharmacological studies performed *in vitro* on tissues and organs expressing native receptors or *in vivo* in intact animals. In these preparations, the number of UT sites is generally very low and [Orn^8]U-II may mainly behave as a receptor antagonist.

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