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In vitro and *in vivo* pharmacological characterization of the novel UT receptor ligand [Pen^5,DTrp^7,Dab^8]urotensin II(4–11) (UFP-803)

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1 The novel urotensin-II (U-II) receptor (UT) ligand, [Pen^5,DTrp^7,Dab^8]U-II(4–11) (UFP-803), was pharmacologically evaluated and compared with urantide in *in vitro* and *in vivo* assays.

2 In the rat isolated aorta, UFP-803 was inactive alone but, concentration dependently, displaced the contractile response to U-II to the right, revealing a competitive type of antagonism and a pA_2 value of 7.46.

3 In the FLIPR $[Ca^{2+}]_i$ assay, performed at room temperature in $HEK293_{hUT}$ and $HEK293_{rUT}$ cells, U-II increased $[Ca^{2+}]_i$ with pEC_{50} values of 8.11 and 8.48. Urantide and UFP-803 were inactive as agonists, but antagonized the actions of U-II by reducing, in a concentration-dependent manner, the agonist maximal effects with apparent pK_B values in the range of 8.45–9.05. In a separate series of experiments performed at 37°C using a cuvette-based $[Ca^{2+}]_i$ assay and CHO_{hUT} cells, urantide mimicked the $[Ca^{2+}]_i$ stimulatory effect of U-II with an intrinsic activity (α) of 0.80, while UFP-803 displayed a small ($\alpha = 0.21$) but consistent residual agonist activity. When the same experiments were repeated at 22°C (a temperature similar to that in FLIPR experiments), urantide displayed a very small intrinsic activity ($\alpha = 0.11$) and UFP-803 was completely inactive as an agonist.

4 *In vivo* in mice, UFP-803 (10 nmol kg^{-1}) antagonized U-II (1 nmol kg^{-1})-induced increase in plasma extravasation in various vascular beds, while being inactive alone.

5 In conclusion, UFP-803 is a potent UT receptor ligand which displays competitive/noncompetitive antagonist behavior depending on the assay. While UFP-803 is less potent than urantide, it displayed reduced residual agonist activity and as such may be a useful pharmacological tool.
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Abbreviations: $[Ca^{2+}]_i$, intracellular calcium concentration; CHO, Chinese hamster ovary cells; CRC, concentration–response curve; DMEM, Dulbecco's modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; FLIPR, fluorometric imaging plate reader; Fluo-4 AM, glycine, *N*-[4-[6-[(acetyloxy)methoxy]-2,7-difluoro-3-oxo-3*H*-xanthen-9-yl]-2-[2-[2-[bis[2-[(acetyloxy)methoxy]-2-oxoethyl]amino]-5-methylphenoxy]ethoxy]phenyl]-*N*-[2-[(acetyloxy) methoxy]-2-oxoethyl]-(acetyloxy)methyl ester; Fura 2-AM, 1-[2-(5-carboxyoxazol-2-yl)-6-amino-benzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane *N*,*N*,*N*′*N*′-tetraacetic acid pentaacetoxymethyl ester; G418, geneticin; HEK293, human embryonic kidney cells; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid; MEM, minimum essential medium; U-II, urotensin-II; UFP-803, [Pen^5,DTrp^7,Dab^8]urotensin-II (4–11); urantide, [Pen^5,DTrp^7,Orn^8]urotensin-II(4–11); UT, urotensin-II receptor

Introduction

Urotensin-II (U-II) is an undecapeptide, the actions of which are mediated by the G-protein-coupled receptor UT (Ames *et al*., 1999; Douglas & Ohlstein, 2000). U-II and the UT receptor are expressed in a variety of peripheral organs (kidney, liver, endocrine glands), and especially in cardiovascular tissues (cardiomyocyte, endothelium and vascular smooth muscle cell). In addition, both the peptide and the receptor have been detected in some areas of the central nervous system, such as the medulla oblongata and the spinal cord. In agreement with their localization, U-II and its UT receptor exert some important actions on the cardiovascular system, as suggested by the following lines of evidence. In

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primates, systemic administration of U-II causes hemodynamic changes that culminate in cardiac collapse and death (Ames *et al*., 1999). U-II seems to be the most potent vasoconstrictor known to date. However, U-II induces much lower maximal responses compared to other vasoconstrictors such as norepinephrine, angiotensin II and endothelin-1, and shows high response variability between species, vascular beds and even individuals (Douglas *et al*., 2000; Maguire *et al*., 2000; Camarda *et al*., 2002b). In humans, U-II induced vasoconstrictor responses in forearm brachial artery infusion studies (Bohm & Pernow, 2002); however, the peptide failed to induce hemodynamic changes using a similar protocol (Wilkinson *et al*., 2002) or after systemic infusion (Affolter *et al*., 2002). The increased levels of UT receptor and U-II in some pathological conditions suggest that the U-II/UT receptor system may be involved in congestive heart failure, renal diseases and diabetes mellitus (Totsune *et al*., 2001; 2003; Douglas *et al*., 2002; 2004). A possible role of endogenous U-II in renal ischemia has been recently proposed by Clozel *et al*. (2004), who showed that the nonpeptide UT receptor antagonist palosuran (ACT 058362) reduced tubular and glomerular dysfunction in a rat model of renal ischemia. Furthermore, some central effects of U-II have been described, namely stimulation of rat locomotor activity (Gartlon *et al*., 2001) and anxiogenic-like effects in mice (Matsumoto *et al*., 2004).

In order to clarify the physiological and pathological roles played by the U-II/UT receptor system, the development of selective UT receptor antagonists is essential. To date few selective UT receptor ligands have been identified; these are: [Orn^8]U-II, a partial agonist at UT receptor (Camarda *et al*., 2002a; Vergura *et al*., 2004), and [Pen^5,DTrp^7,Orn^8]U-II(4–11) (urantide) (Patacchini *et al*., 2003), a potent UT receptor antagonist in the rat aorta bioassay. However, urantide showed residual agonist activity at human recombinant UT receptors in a Ca^{2+} mobilization assay (Camarda *et al*., 2004). Further experiments by Guerrini *et al*. (2005) showed that the substitution of Orn^8 with diaminobutyric acid as in [Dab^8]U-II leads to a reduction in potency, but also attenuated efficacy of the peptide. This chemical modification has now been applied to the urantide sequence to generate the peptide [Pen^5,DTrp^7, Dab^8]U-II(4–11) (UFP-803).

The aim of the present study was to investigate the pharmacological profile of UFP-803 *in vitro* in the rat aorta bioassay (Camarda *et al*., 2002b) and in a FLIPR Ca^{2+} mobilization assay (Micheli *et al*., 2003) performed in HEK293 cells expressing human or rat UT receptors, as well as *in vivo*, in the mouse plasma extravasation assay (Vergura *et al*., 2004). Moreover, a detailed comparison between UFP-803 and urantide effects, especially in terms of residual agonist activity, has been performed at different temperatures (i.e. 22 and 37°C) using CHO_{hUT} cells and a standard cuvette-based [Ca^{2+}] assay system (Hirst *et al*., 1999).

Methods

Mice and rats were kept in a temperature-controlled environment (22°C, 12 h light/dark cycle) with standard laboratory food and water freely available. The animals were handled according to guidelines published in the European Communities Council directives (86/609/EEC) and National Regulations (DL116/92).

Rat aorta bioassay

Thoracic aortae were isolated from male Sprague–Dawley rats (200–250 g, Morini, Reggio Emilia, Italy) and cut into helical strips as described previously (Camarda *et al*., 2002b). The endothelium was mechanically removed, and the tissues were placed into organ baths containing Krebs solution of the following composition (in mM): NaCl 118, $NaHCO_3$ 25, KCl 4.7, $KHPO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.2, glucose 10, under standard conditions (pH 7.4, 37°C, bubbled with 5% CO_2 and 95% O_2). One g of preload was applied to the tissues that were washed every 20 min and allowed to stabilize for 1 h. After that time, their responsiveness was assessed by addition of 30 mM KCl. Norepinephrine (1 μM) was used as standard stimulus. Cumulative concentration–response curves (CRC) to U-II were performed in the absence and presence of UFP-803 (0.1, 1 and 10 μM) added to the organ baths 30 min before the agonist. Inhibition curves to UFP-803 were performed in tissues precontracted with 10 or 100 nM U-II. In addition, a separate series of experiments was performed by testing the contractile effects of noradrenaline, angiotensin II and endothelin-1 in the absence and presence of 10 μM UFP-803. Changes of isometric tension were measured by force transducers (GRASS FT03) and recorded by a multichannel polygraph (LINSEIS 2005).

Cell culture

HEK293 cells stably expressing the human or rat UT receptor ($HEK293_{hUT}$ and $HEK293_{rUT}$, respectively) were maintained in MEM with Earle's salt medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% nonessential amino-acids and geneticin (G418) (400 μg ml^{-1}). Wild-type HEK293 cells (HEK293) were maintained in the same medium in the absence of G418.

CHO cells stably expressing the human UT receptor (CHO_{hUT}) were cultured in DMEM/F12 (50/50) supplemented with 10% fetal calf serum, 100 IU ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Stock cultures were additionally supplemented with 800 μg ml^{-1} G418 to maintain receptor expression. Experimental cultures (G418 free) were harvested using EDTA 1.7 mM, NaCl 154 mM and HEPES 10 mM buffer, pH 7.4, and washed twice with Krebs/HEPES buffer (NaCl 143.4 mM, KCl 4.8 mM, HEPES 10 mM, $CaCl_2 \cdot 2H_2O$ 2.6 mM, KH_2PO_4 1.2 mM, $MgSO_4 \cdot 7H_2O$ 1 mM and glucose 11.7 mM, pH 7.4). All cells were cultured at 37°C in 5% CO_2 humidified air. Cells stably expressing recombinant receptors were generated in the laboratories of GlaxoSmithKline, PA, U.S.A.

Calcium mobilization assays

FLIPR experiments $HEK293_{hUT}$, $HEK293_{rUT}$ and wild-type HEK293 were seeded at a density of 50,000 cells well^{-1} into poly-D-lysine-coated 96-well black, clear-bottom plates (Biocoat, Becton Dickinson, NJ, U.S.A.). The following day, the medium was removed by aspiration and 100 μl well^{-1} of loading medium (cell culture medium supplemented with 20 mM HEPES, 2.5 mM probenecid, 3 μM of the calcium-sensitive fluorescent dye Fluo 4AM (Molecular Probes, Eugene, OR, U.S.A.) and 0.01% pluronic acid) was added. After 30 min at 37°C, the loading solution was aspirated and 100 μl well^{-1} of assay buffer Hank's Balanced Salt Solution (HBSS) supplemented with 20 mM HEPES, 2.5 mM probenecid

and 500 μM Brilliant Black (Sigma Aldrich, St Louis, MO, U.S.A.) was added. Peptides were dissolved in water at a concentration of 1 mM. Serial dilutions were carried out in HBSS/HEPES (20 mM) buffer (containing 0.02% BSA fraction V (w v^{-1})) in order to prepare a master plate at 3 × concentration. After placing both plates (cell culture and master plate) into an FLIPR (Molecular Devices, Sunnyvale, CA, U.S.A.), fluorescence changes were measured (excitation $\lambda = 488$ nm, emission $\lambda = 510$ nm) at room temperature. On-line additions were carried out in a volume of 50 μl well^{-1}. Maximum change in fluorescence, expressed in arbitrary units, over baseline was used to determine agonist response. Data are presented as mean ± s.e.m. from $n = 4$ experiments.

Cuvette-based $[Ca^{2+}]_i$ assay Freshly harvested CHO_{hUT} cells were incubated in Krebs–HEPES buffer containing Fura2-AM (5 μM), for 30 min at 37°C, followed by 20 min at room temperature to allow for complete dye de-esterification. Loaded cells were washed twice, resuspended in Krebs–HEPES buffer and maintained at room temperature in the dark. Volumes of 2 ml were pipetted into a quartz cuvette as required and equilibrated to 37 or 22°C (3 min) in the chamber of a Perkin-Elmer LS50B fluorimeter. Peptides were diluted from 2 mM stocks and added at various concentrations. Excitation λ was set to 340 and 380 nm, and emission to 510 nm. $[Ca^{2+}]_i$ was calculated from the 340/380 ratio, according to Grynkiewicz *et al*. (1985), where R_{max} and R_{min} were determined using Triton X-100 (0.1% v v^{-1}) and EGTA (4.5 mM, pH > 8.0). K_D of Ca^{2+} for Fura-2 was 225 nM at 37°C and 145 nM at 22°C (Simpson, 1999). Data are presented as change (Δ) in $[Ca^{2+}]_i$ (peak-prestimulus concentration) and are mean ± s.e.m. from $n = 5$.

Plasma extravasation assay

Male albino Swiss mice (25–30 g, Morini, Emilia Romagna, Italy) were used for these studies under the experimental conditions and protocols described previously (Vergura *et al*., 2004). Briefly, mice were anesthetized by i.m. administration of xylazine (5 mg 100 g^{-1}) and ketamine (10 mg 100 g^{-1}), UFP-803 (10 nmol kg^{-1}) or vehicle (100 μl saline) were injected in the tail vein time 0. Evans blue dye (30 mg kg^{-1}) and U-II (1 nmol kg^{-1}) were injected in the tail vein at 5 and 7 min later, respectively. After further 5 min, a cannula was placed in the left ventricle with its tip into the aorta, the right atrium opened, and the animal was perfused with saline (4 min, 10 ml min^{-1}). Tissues (trachea, bronchus, esophagus, stomach, colon, urinary bladder and kidney) were isolated, blotted, weighed and kept in formamide at room temperature for 24 h. Evans blue tissue concentration was determined by measuring the optical density of formamide extracts at 620 nm, and extrapolating the concentration from an Evans blue standard curve (0.05–25 μg ml^{-1}). Data are expressed as ng Evans blue mg^{-1} of fresh tissue.

Analysis of data

CRC were analyzed by a nonlinear curve-fitting equation, using Graph Pad 4.0 software. The equation used was as follows: $y = \text{bottom} + (\text{top} - \text{bottom})/(1 + 10\hat{}((\log EC_{50} - x) * \text{HillSlope}))$, 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. For data relative to UFP-803 in the rat aorta bioassay, the antagonist potency derived by Schild analysis was expressed as pA_2. Moreover, the same compound was also evaluated in inhibition experiments; in this case the antagonist potency was expressed as pK_B derived from the following equation:

$$K_B = IC_{50} / \left([2 + ([A]/EC_{50})^n]^{1/n} - 1 \right)$$

where IC_{50} is the concentration of antagonist that produces 50% inhibition of the agonist response, [A] is the concentration of agonist, EC_{50} is the concentration of agonist producing a 50% maximal response and n is the Hill coefficient of the CRC to the agonist (Kenakin, 2004).

In the FLIPR assay urantide and UFP-803 displayed an insurmountable/noncompetitive antagonism; their apparent pK_B values were obtained by the method of Gaddum *et al*. (1955). In practice, equiactive concentrations of the agonist in the absence ([A]) and presence ([A′]) of a noncompetitive antagonist ([B]) are compared in a double reciprocal plot describing a straight line, and pK_B was derived from the equation:

$$pK_B = \log_{10}[(\text{slope} - 1)/[B]$$

Data were expressed as mean ± s.e.m. of n experiments and analyzed statistically using one-way ANOVA followed by Dunnett's test for multiple comparisons or the Student's t-test for unpaired data.

Drugs and reagents

Urantide, UFP-803 and U-II were synthesized and purified at the Department of Pharmaceutical Sciences, University of Ferrara using the methods described in details by Guerrini *et al*. (2005). Tissue culture media and supplements were from Invitrogen (Paisley, Scotland). Other reagents were purchased by Sigma Chemical Co. (St Louis, MO, U.S.A.).

Results

Effects of UFP-803 on rat isolated aorta

In line with previous studies, U-II was able to induce contraction of the rat thoracic aorta in a concentration-dependent manner, with high potency (pEC_{50} 8.16 ± 0.10). UFP-803, up to 10 μM, did not evoke any contractile response in the thoracic aorta strips. UFP-803 (0.1, 1 and 10 μM) produced a parallel shift to the right of the CRC to U-II without reducing the maximal effects elicited by the agonist (Figure 1a). Schild analysis revealed a competitive type of antagonism (slope 1.11 ± 0.09) and a pA_2 value of 7.46 was derived from these experiments (Figure 1, inset panel). In tissues precontracted with 10 or 100 nM U-II, UFP-803 was able to reverse the effects of the natural peptide in a concentration-dependent manner with pIC_{50} values of 7.46 and 6.80, which were clearly dependent on the U-II concentration (10 and 100 nM, respectively) used for precontracting the tissues (Figure 1b). This confirms (Kenakin, 2004) a competitive type of interaction between UFP-803 and U-II in this preparation and a pK_B value of 7.55 ± 0.22 was derived from these data (see Methods for details).

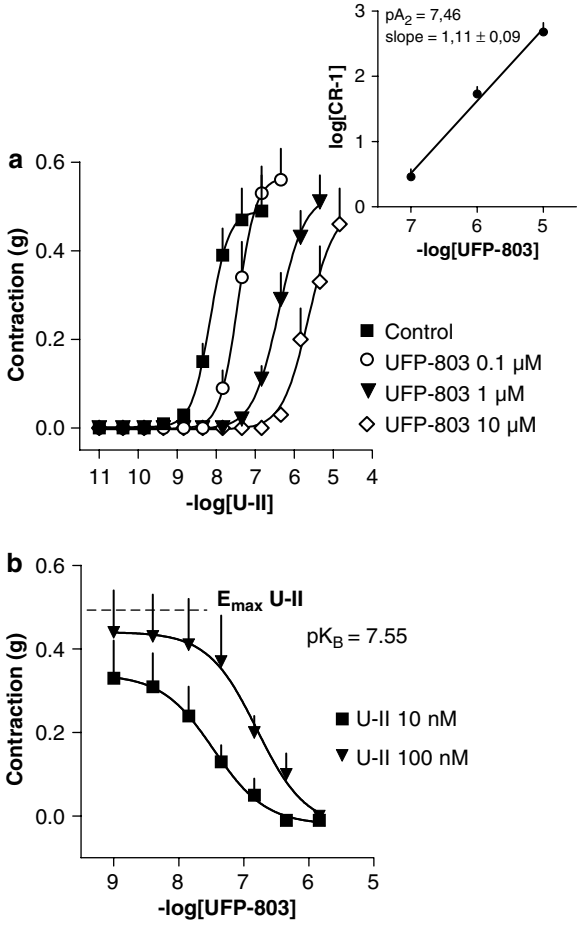

Figure 1 CRC to U-II obtained in the absence (control) and presence of increasing concentrations of UFP-803 (0.1, 1 and 10 μM) in the rat thoracic aorta (a). The corresponding Schild plot is shown in the inset panel. Inhibition CRC of UFP-803 in rat thoracic aorta precontracted with U-II (10 or 100 nM) (b). Data are mean $\pm$ s.e.m. of four experiments.

Table 1 Effects of noradrenaline, angiotensin II and endothelin-1 in the absence and in the presence of 10 μM UFP-803 in the rat aorta

	Control		*10* μM *UFP-803*	
	pEC_{50}	E_{max} (g)	pEC_{50}	E_{max} (g)
Noradrenaline	7.87 $\pm$ 0.13	0.67 $\pm$ 0.06	7.65 $\pm$ 0.23	0.74 $\pm$ 0.06
Angiotensin II	8.55 $\pm$ 0.29	0.55 $\pm$ 0.27	8.43 $\pm$ 0.31	0.70 $\pm$ 0.20
Endothelin-1	8.02 $\pm$ 0.15	0.88 $\pm$ 0.11	7.98 $\pm$ 0.11	0.78 $\pm$ 0.12

Data are means $\pm$ s.e.m. of at least four separate experiments.

In order to assess the selectivity of action of UFP-803, CRC to noradrenaline, angiotensin II and endothelin-1 was performed in the absence and presence of 10 μM of the peptide. As summarized in Table 1, UFP-803 did not affect the effects of the three contractile agents either in terms of potency or maximal effects.

Effects of urantide and UFP-803 in $[Ca^{2+}]_i$ assays

In the FLIPR assay, U-II stimulated a biphasic increase in $[Ca^{2+}]_i$, which was concentration-dependent in both $HEK293_{hUT}$ and $HEK293_{rUT}$ cells. Measured at the peak response, pEC_{50} values of 8.11 $\pm$ 0.05 and 8.48 $\pm$ 0.07 and E_{max} of 222 $\pm$ 14 and 565 $\pm$ 14%, respectively, were obtained (Figure 2a and b). In wild-type HEK293 cells, 10 μM U-II did not increase $[Ca^{2+}]_i$ (data not shown). In both $HEK293_{hUT}$ and $HEK293_{rUT}$ cells, urantide did not evoke an agonist response. In $HEK293_{hUT}$ cells, 1, 3 and 10 nM urantide antagonized the stimulatory action of U-II by inhibiting in a concentration-dependent manner the maximal effects induced by the agonist (Figure 2a). Very similar results were obtained in $HEK293_{rUT}$ (Figure 2b). Apparent pK_B values of 8.45 $\pm$ 0.31 and 8.90 $\pm$ 0.27 were derived from these experiments in $HEK293_{hUT}$ and $HEK293_{rUT}$ cells, respectively. In another series of experiments performed in both $HEK293_{hUT}$ and $HEK293_{rUT}$ cells, urantide inhibited in a concentration-dependent manner the Ca^{2+} mobilization induced by 10 or 100 nM U-II (Figure 2c and d). In these experiments, only slight (0.3 log units) differences in urantide pIC_{50} values (8.80 $\pm$ 0.18 and 9.04 $\pm$ 0.23 for $HEK293_{hUT}$ and 8.43 $\pm$ 0.33 and 8.78 $\pm$ 0.25 for $HEK293_{rUT}$ cells) were found by increasing the agonist concentration by 10-fold, thus suggesting a noncompetitive type of interaction.

Similar experiments were performed to pharmacologically characterize the novel UT receptor ligand UFP-803. In both $HEK293_{hUT}$ and $HEK293_{rUT}$, 10 μM UFP-803 did not increase $[Ca^{2+}]_i$, while at 1, 10 and 100 nM it shifted the CRC to U-II to the right and at the same time produced a concentration-dependent reduction of the maximal agonist effect (Figure 3a and b). Apparent pK_B values of 8.59 $\pm$ 0.33 and 9.05 $\pm$ 0.15 were calculated from these experiments for human and rat UT receptors, respectively. UFP-803 inhibited the effects of 10 or 100 nM U-II in a concentration-dependent manner, with pIC_{50} values of 8.42 $\pm$ 0.18 and 8.71 $\pm$ 0.25, and 8.33 $\pm$ 0.17 and 8.67 $\pm$ 0.23 in $HEK293_{hUT}$ and $HEK293_{rUT}$ cells, respectively (Figure 3c and d).

To more carefully investigate the possible residual agonist activity of urantide and UFP-803, a separate series of experiments were performed at different temperatures, namely 37 and 22°C (the latter temperature being close to that used in FLIPR experiments), using a standard cuvette based Ca^{2+} assay and CHO_{hUT} cells. In these cells, the response to U-II was also biphasic. As shown in Figure 4a, at 37°C urantide mimicked the stimulatory effects of U-II, showing slightly lower potency and maximal effects, while UFP-803 induced a small agonist effect approximately equal, in terms of maximal effects, to 20% of that elicited by U-II. No effects were recorded in response to U-II, urantide and UFP-803 in wild-type CHO cells (data not shown).

In similar experiments performed at 22°C (Figure 4b), U-II showed a decreased potency and maximal effects (note the difference in the scale of the *y*-axis), urantide displayed a very low residual agonistic activity, while UFP-803 was completely inactive as an agonist. Finally, in antagonism experiments performed at 22°C, both urantide and UFP-803 were able to decrease the maximum effect elicited by U-II, showing apparent pK_B values in the low nanomolar range (data not shown).

Effects of UFP-803 on mouse plasma extravasation

In line with previous findings (Vergura *et al*., 2004), 1 nmol kg^{-1} U-II was able to stimulate plasma extravasation in all mouse vascular beds investigated (Table 2). In most

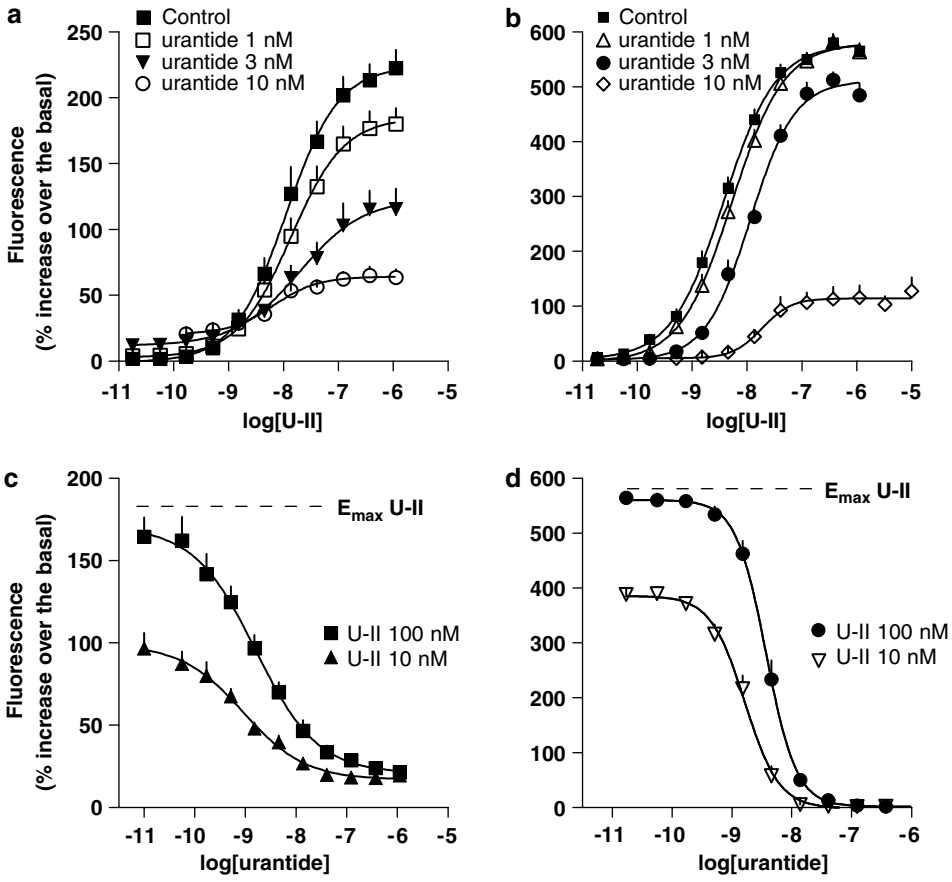

Figure 2 CRC to U-II in the absence and presence of urantide (1, 3 or 10 nM) in HEK293 cells expressing human (a) or rat (b) UT receptors. Inhibition CRC to urantide against the effects of 100 or 10 nM U-II in HEK293 cells expressing human (c) or rat (d) UT receptor. Changes in intracellular calcium levels were expressed as % increase of fluorescence intensity units over basal. Data are mean ± s.e.m. of five experiments performed in duplicate.

of the tissues, the stimulatory effect of U-II was about 200% of basal values. A 10-fold higher dose of UFP-803 (i.e. 10 nmol kg^{-1}) was selected on the basis of the rat aorta data (pA_2 UFP-803 7.46, pEC_{50} U-II 8.16) and tested alone and against the effects induced by U-II. As shown in Figure 5 (mouse stomach) and Table 2 (all the investigated tissues), UFP-803 did not produce any statistically significant effect *per se*, but fully prevented the stimulatory action of U-II on plasma extravasation in all the tissues tested.

Discussion

The present findings demonstrate that UFP-803 behaves as a potent UT receptor antagonist both *in vitro* and *in vivo*, with similar potency at rat and human receptors. In comparison with other molecules, UFP-803 displayed several advantages: it is more potent than palosuran and does not discriminate between species-related UT receptor orthologues (Clozel *et al*., 2004); its residual agonist activity is less than that of urantide (Patacchini *et al*., 2003; Camarda *et al*., 2004); contrary to SB-706375 (Douglas *et al*., 2005), it can be made available to the scientific community. These features make UFP-803 an important tool for future investigations on the role played by the U-II/UT receptor system in physiology and pathology.

In the rat aorta bioassay, UFP-803 did not show any residual agonist activity and competitively antagonized U-II contractile action. In addition, UFP-803 behaved as a selective UT receptor antagonist in this preparation since the peptide, even at high concentrations, that is, 10 μM, did not affect the contractile effects induced by other agents such as noradrenaline, angiotensin II and endothelin-1. Since U-II produced sustained contractions in this preparation (Itoh *et al*., 1987; Douglas *et al*., 2000; Camarda *et al*., 2002b), a series of inhibition experiments were performed. UFP-803 reversed U-II induced contractions in a concentration-dependent manner, with pIC_{50} values influenced by the U-II concentration used for precontracting the tissues. This confirmed competitive type of antagonism (Kenakin, 2004) was suggested by Schild analysis. Moreover, the estimated potency for this peptide in the two series of experiments (Schild analysis 7.46, inhibition experiments 7.55) is virtually superimposable. Thus, in the rat aorta bioassay the pharmacological behavior of UFP-803 is similar to that reported for urantide (Patacchini *et al*., 2003; Camarda *et al*., 2004), namely pure (no residual agonist activity), selective and competitive in terms of antagonism. However, UFP-803 appears to be about 10-fold less potent than urantide (pA_2 values $\approx$ 7.5 (present data) and $\approx$ 8.5 (Patacchini *et al*., 2003; Camarda *et al*., 2004), respectively). This is in line with our findings obtained by performing a structure activity study on position 8 of U-II (Guerrini *et al*.,

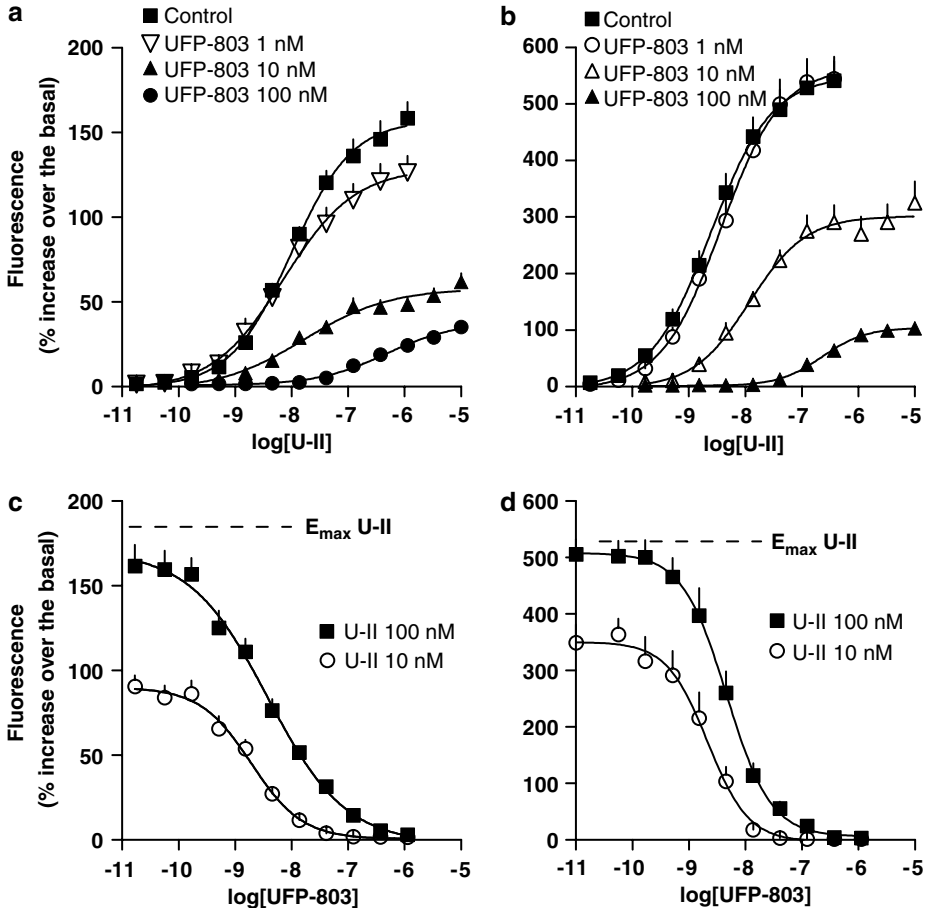

Figure 3 CRC to U-II in the absence and presence of UFP-803 (1, 10 or 100 nM) in HEK293 cells expressing human (a) or rat (b) UT receptors. Inhibition CRC to UFP-803 against the effects of 100 or 10 nM U-II in HEK293 cells expressing human (c) or rat (d) UT receptor. Changes in intracellular calcium levels were expressed as % increase of fluorescence intensity units over basal. Data are mean ± s.e.m. of four experiments performed in duplicate.

2005): indeed, the substitution of Orn with Dab produced an approximately 10-fold decrease in peptide potency (6.47 and 5.51, respectively).

In FLIPR studies, UFP-803 behaved as a pure and potent UT antagonist both at rat and human receptors. However, the antagonism exerted by UFP-803 against U-II was clearly insurmountable. Moreover, in inhibition experiments only slight (0.3 log units) differences in UFP-803 pIC_{50} values were found by increasing the agonist concentration by 10-fold (i.e. from 10 to 100 nM U-II). The reasons for the different type of antagonism displayed by this peptide in tissue (surmountable/competitive) and FLIPR (insurmountable/noncompetitive) studies are at present unknown. Although different pharmacological profiles have been reported for UT receptor ligands depending on the species under study (Behm *et al*., 2004), this aspect can be ruled out in the present case, because UT receptor of the same species, that is, the rat, has been investigated in tissue and FLIPR studies. It is worthwhile mentioning that peptide CRCs are measured at the peak response, which is clearly transient in nature. Importantly, the transient nature of this peak may not allow equilibrium between agonist–antagonist competition to be reached, thus generating depression of the agonist response in the presence of high concentrations of antagonist (Kenakin, 2004). Another point to be considered for interpreting these results is the fact that stirring is not possible in 96-well formats such as FLIPR. Unstirred water layers may be a factor causing exaggeration of apparent loss of drug potency due to adsorption; this can be relevant for detecting the agonist response in a system where hemiequilibrium could be a factor (Kenakin, 2004). However, the importance of this phenomenon seems to be limited in this case, since a noncompetitive type of interaction has been obtained also in antagonism experiments performed in the cuvette-based calcium assay under conditions of efficient stirring.

Very similar results have been obtained in the FLIPR studies with urantide, which, like UFP-803, behaves as an insurmountable, potent UT receptor antagonist in this assay. The same hypotheses made for UFP-803 can be proposed for interpreting the urantide insurmountable behavior. The lack of residual agonist activity of urantide in this assay is at variance to what has been reported previously (Camarda *et al*., 2004). However, there are several important differences between the experimental conditions used for generating the two sets of data, such as the type of cells expressing the recombinant receptor (HEK293 vs CHO), the equipment and the probe used for monitoring the calcium response (FLIPR- vs cuvette-based system, Fluo 4 vs Fura 2), and the temperature at which

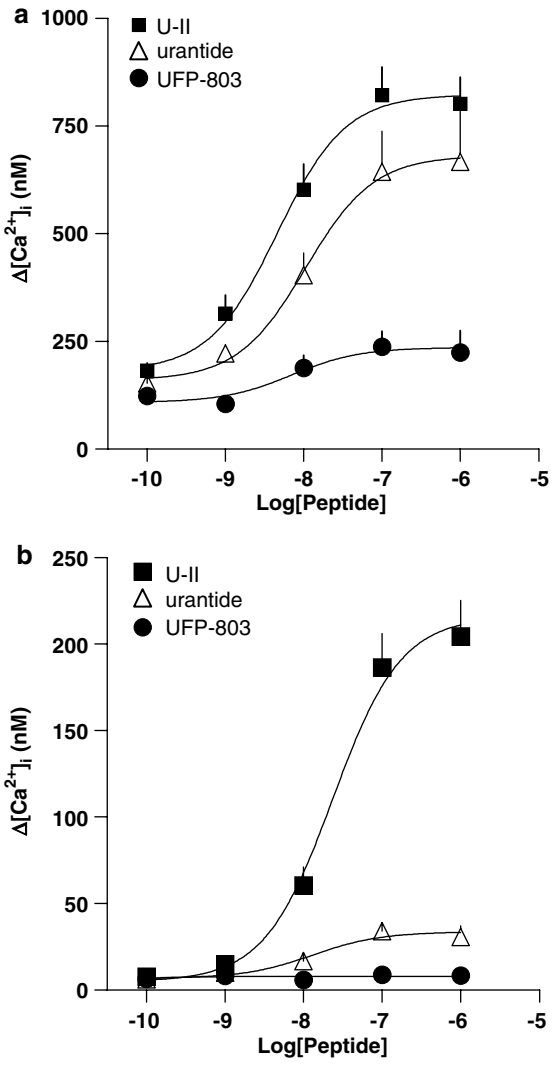

Figure 4 CRC to U-II, urantide and UFP-803 in CHO cells expressing human UT receptor, in a standard cuvette-based Ca^{2+} assay performed at 37°C (a) and 22°C (b). Effects are measured as change (Δ) in $[Ca^{2+}]_i$. Data are mean±s.e.m. of five experiments.

Table 2 Effects of 10 nmol kg^{-1} UFP-803 alone and against 1 nmol kg^{-1} U-II-stimulated plasma extravasation in various mouse vascular beds

	Saline	*U-II* 1 nmol kg^{-1}	*UFP-803* 10 nmol kg^{-1}	*UFP-803 + U-II*
Trachea	29.81±2.79	49.76±4.52*	24.64±1.53	32.66±3.23
Bronchus	33.59±1.99	51.82±4.61*	35.44±3.77	30.07±3.48
Esophagus	21.12±0.56	43.81±5.89*	28.63±3.05	28.28±2.01
Stomach	27.15±2.52	50.63±5.02*	19.68±2.21	22.16±4.04
Colon	16.38±1.44	35.17±4.09*	17.13±2.04	23.78±4.38
Bladder	20.76±1.15	47.48±4.38*	23.48±2.31	22.91±3.34
Kidney	9.74±0.58	25.15±3.61*	12.50±0.83	16.82±3.05

Values are expressed as ng Evans blue mg^{-1} fresh tissue. Means±s.e.m. of $n=4$–5 separate experiments. *$P<0.05$ vs saline, according to ANOVA followed by Dunnett's test.

the experiment has been performed (22 vs 37°C). These differences, especially the latter, may likely modify the efficiency of the stimulus–response coupling, which is a critical

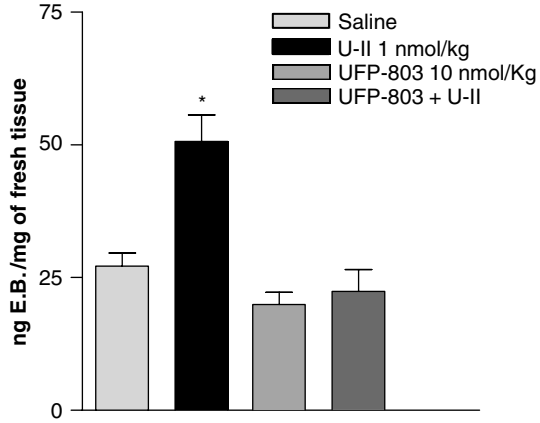

Figure 5 Effects of 10 nmol kg^{-1} UFP-803, alone and against 1 mol kg^{-1} U-II-stimulated plasma extravasation in the mouse stomach. Plasma extravasation is expressed as ng Evans blue mg^{-1} fresh tissue. Mean±s.e.m. of $n=4$ separate experiments. *$P<0.05$ vs saline (ANOVA followed by Dunnett's test).

parameter for estimating partial agonist efficacy (Kenakin, 2004). To investigate the possible effect of temperature on the estimation of UT receptor ligand efficacy, a separate series of experiments were performed in which CRCs to U-II, urantide and UFP-803 were generated both at 22 and 37°C using CHO_{hUT} cells in a cuvette-based system. The results of these experiments clearly demonstrated that (i) the estimation of the ligand efficacy is strongly temperature-dependent and (ii) the relative rank order of efficacy of these UT receptor ligands, U-II > urantide > UFP-803, is the same at 22 and 37°C. Thus, the Orn → Dab substitution in position 8 reduced at the same time both ligand potency and efficacy. This holds true both for the urantide sequence (present data) and for the U-II full sequence (Guerrini *et al*., 2005). Indeed, the intrinsic activity in the rat aorta bioassay of [Orn^8]U-II and [Dab^8]U-II were 0.25 and 0.07, respectively (Guerrini *et al*., 2005).

Finally, UFP-803 was evaluated *in vivo* in the mouse plasma extravasation assay. Based on previous findings (Vergura *et al*., 2004), we selected (i) a series of vascular beds of the mouse in which U-II produced a robust and consistent stimulation of plasma extravasation, (ii) the dose of U-II, that is, 1 nmol kg^{-1}, which produced the maximal stimulatory effect. UFP-803 was tested in a 10/1 antagonist/agonist dose ratio, which was chosen on the basis of the potency values obtained in the rat aorta bioassay. At 10 nmol kg^{-1}, UFP-803 fully prevented the increase in plasma extravasation elicited by U-II in all the tissues examined. These results confirm and extend *in vitro* findings demonstrating that UFP-803 behaves *in vivo* in the mouse cardiovascular system as a potent UT receptor antagonist whose residual agonist activity is negligible, that is, non detectable. These data also corroborate previous findings from our laboratory (Vergura *et al*., 2004) as well as from others (Gendron *et al*., 2004), indicating that the stimulatory effect of U-II on plasma extravasation is due to UT receptor activation.

In conclusion, the present *in vitro* and *in vivo* results demonstrate that UFP-803 is a potent and selective UT receptor ligand. Although UFP-803 retains some small residual agonist activity, it can be considered negligible since the peptide behaves as a pure antagonist in most of the *in vitro* assays and *in vivo*. The type of antagonism exerted by

UFP-803 competitive surmountable vs/noncompetitive insurmountable depends on the assay under study. Thus, we propose UFP-803 as a useful chemical template for identifying novel UT receptor ligands, as well as a pharmacological tool for *in vitro* and particularly *in vivo* studies aimed at clarifying the role(s) played by the U-II/UT receptor system in physiology and pathology. Moreover, UFP-803 can be used as a standard reference for comparative pharmacological studies aimed at characterizing the actions of nonpeptide (drug-like) molecules interacting with the UT receptor (e.g. the antagonists palosuran (Clozel *et al*., 2004) or SB-706375 (Douglas *et al*., 2005); see also patent literature reviewed in Douglas *et al*. (2004)), which are under development in industrial laboratories.

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