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Structure–activity relationship study on human urotensin II

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Abstract: The vasoactive cyclic undecapeptide urotensin-II (U-II) has been identified as an endogenous ligand for the G-protein coupled receptor now referred to as the UT receptor. The U-II/UT receptor system might be relevant for cardiovascular functions. A structure-activity study of human U-II investigating 31 peptides in the rat aorta bioassay is reported. Ala- and D-scan investigations indicated that the sequence Phe^6-Trp^7-Lys^8-Tyr^9 is essential for biological activity and that Lys^8 and Tyr^9 are particularly important. These two residues were substituted with a series of coded and non-coded amino acids. These studies demonstrated that the positive charge of the primary aliphatic amine at position 8 and its relative spatial orientation is crucial for both receptor occupation and activation, while the only chemical requirement at position 9 is the presence of an aromatic moiety. Moreover, this study led to the identification of UT receptor partial agonists (compounds **23** and **24**) which can be used as chemical templates for further investigations aimed at the identification of selective antagonists.

Keywords: ligand efficacy; rat aorta bioassay; SAR studies; urotensin-II; UT receptor

INTRODUCTION

Human urotensin-II (U-II) is a cyclic undecapeptide (H-Glu-Thr-Pro-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH) containing a disulphide bridge between Cys^5 and Cys^{10}. This peptide, isolated originally from fish urophyses [1], has now been identified in different species, including man [2,3]. The cyclic region c[Cys-Phe-Trp-Lys-Tyr-Cys] is fully conserved among species, suggesting that it may play a crucial role in biological activity [2,3]. U-II was recognized as the natural ligand of an orphan G-protein coupled receptor, GPR14 [4] (see also [5–7]), now referred to as the UT receptor [8]. This novel peptide/receptor system appears to be involved particularly in the regulation of cardiovascular homeostasis [9]. Both the receptor and the peptide are expressed abundantly in cardiovascular tissues (vascular smooth muscle, endothelium, myocardium) and U-II exerts sustained contractile actions in isolated veins and arteries from different species, generally showing very high potency but low maximal effects [2,3]. *In vivo* studies investigating the systemic and regional haemodynamic effects of U-II in various animal species and recently also in humans generated variable and sometimes contradictory results (see for an updated review [9]). Very recently, mice lacking the UT receptor gene have been generated; these knockout mice, however, did not show any obvious cardiovascular phenotype suggesting a negligible cardiovascular role of the U-II/UT receptor system in this species, at least under physiological conditions [10].

The identification of novel UT receptor selective ligands, especially antagonists, is mandatory for investigating the biological functions of this system and its role(s) in pathologies. To this end, a classical structure–activity study of U-II was performed by (i) identifying the peptide sequence which may be important for biological activity, (ii) performing the Ala- and D-scans of this region for investigating the relative importance of the amino acid side chains and their spatial orientation, respectively, and (iii) substituting the amino acid residues crucial for biological activity. Thus, 31 peptides were synthesized and their pharmacological activities were evaluated on the rat isolated aorta bioassay [11].

MATERIALS AND METHODS

Amino acids, protected amino acids and chemicals were purchased from Bachem, Novabiochem, Fluka (Switzerland) or Chem-Impex International (USA). The resin [4-hydroxymethylphenoxyacetic acid (PAC)] on the polyethyleneglycol (PEG)/ polystyrene (PS) support, loaded with Fmoc-Val-(Fmoc-Val-PAC-PEG-PS) or Fmoc-Cys(Trt)-[Fmoc-Cys(Trt)-PAC-PEG-PS] were from Applied Biosystems (Foster City, CA, USA). Stock solutions (1 mM) of peptides were made in distilled water and kept at −20 °C until use. Krebs solution (gassed with 95% O_2 and 5% CO_2, pH 7.4) was of the following composition (in mM): NaCl 118.5, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5, glucose 10. All other reagents were from Sigma (Poole, UK) or Merck (Darmstadt, Germany) and were of the highest purity available.

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General Procedures for the Solid-phase Synthesis

As an illustrative example, the synthesis of the natural peptide U-II (**1**) is described. Fmoc-Val-PAC-PEG-PS resin (0.23 mmol/g, 0.5 g) was treated with 20% piperine/DMF (N,N-dimethylformamide) and linked with Fmoc-Cys(Trt)-OH, Fmoc-Tyr(*t*Bu)-OH, Fmoc-Lys(Boc)-OH, Fmoc-Trp(Boc)-OH, Fmoc-Phe-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asp(O*t*Bu)-OH, Fmoc-Pro-OH, Fmoc-Thr(*t*Bu)-OH and Fmoc-Glu(O*t*Bu)-OH (4 equiv) by using [O-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate] HATU [12] (4 equiv) as the coupling reagent. The coupling reaction time was 1 h and piperidine (20%)/DMF was used to remove the Fmoc group at every step. The peptide resin was washed with methanol and dried *in vacuo* to yield the protected U-II-resin. The other peptides (**2–31**) were synthesized in a similar manner. The protected peptide-resin was treated with reagent K [13] [TFA (trifluoroacetic acid)/H_2O/phenol/ethanedithiol/thioanisole, 82.5 : 5 : 5 : 2.5 : 5, *v*/*v*] (10 ml/0.5 g of resin) for 1 h at room temperature. After filtration of the exhausted resin, the solvent was concentrated *in vacuo* and the residue triturated with diethyl ether. The crude linear peptide was purified by preparative reverse-phase HPLC to yield a powder after lyophilization.

Disulphide Bond Formation

The corresponding linear, purified peptide analogues were dissolved in a mixture of H_2O/DMSO (dimethylsulfoxide)/TFA (75 : 25 : 0.1, *v*/*v*) at a concentration of 1 mg/ml. The cyclization reactions proceeded completely within a day and were monitored by analytical HPLC and Ellman test [14]. After partial evaporation of the solvent, the products were purified by preparative HPLC as mentioned above to yield the desired cyclic compounds after lyophylization.

Peptide Purification and Analytical Determinations

Crude peptides were purified by preparative reversed-phase HPLC using a Waters Delta Prep 4000 system with a Waters PrepLC 40 mm Assembly column C_{18} (30 × 4 cm, 300 A, 15 μm spherical particle size column). The column was perfused at a flow rate of 40 ml/min with solvent A (5%, v/v, acetonitrile in 0.1% aqueous TFA), and a linear gradient from 0 to 50% of solvent B (80%, v/v, acetonitrile in 0.1% aqueous TFA) over 25 min was adopted for the elution of peptides. Analytical HPLC analyses were performed on a Beckman 125 liquid chromatograph fitted with an Alltech C_{18} column (4.6 × 150 mm 5 μm particle size) and equipped with a Beckman 168 diode array detector. The analytical purity and retention time (t_R) of each peptide were determined using HPLC conditions in the above solvent system (solvents A and B) programmed at a flow rate of 1 ml/min using a linear gradient from 5% to 40% B over 25 min. All analogues showed >97% purity when monitored at 220 nm. Molecular weights of the compounds were determined by a MALDI-TOF (Matrix Assisted Laser Desorption Ionization-Time of Flight) analysis using a Hewlett Packard G2025A LD-TOF system mass spectrometer and α-cyano-4-hydroxycinnamic acid as the matrix. Values are expressed as $[M + H]^+$.

Bioassay

U-II and its analogues were tested for their ability to contract rat aorta strips prepared as described previously [11]. Briefly, the rat thoracic aorta was isolated from male Sprague Dawley rats weighing 200–250 g (Morini, Reggio Emilia, Italy) and cut into helical strips. The endothelium was mechanically removed by gently rubbing the internal surface with moistened filter paper; the absence of functional endothelium was confirmed by a lack of relaxation in response to 1 μM Ach (acetylcholine) in tissues precontracted with noradrenaline 1 μM. The tissues were placed in organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs solution maintained at 37°C and at pH 7.4. Contractions elicited by various compounds were measured with isometric transducers (Grass FT03) and recorded on a multichannel polygraph (Linseis L2005). Cumulative concentration-response curves to the peptides were performed and the potencies were expressed as pEC_{50}. A single concentration-response curve was performed in each tissue. When peptides were found to be inactive as agonists, they were left in contact with the tissue for 15 min before performing a concentration-response curve to U-II. Antagonist potencies were expressed in terms of pA_2, calculated using the Gaddum-Schild equation: $pA_2 = \log (CR-1)/[\text{antagonist}]$, assuming a slope value equal to unity, where CR (concentration ratio) indicates the ratio between the EC_{50} of the agonist in the presence and in the absence of antagonist.

RESULTS AND DISCUSSION

Compounds **1–31** were prepared by solid-phase peptide synthesis. The chemical characteristics of these new peptides are presented in Table 1 while the chemical structure of the side chains of the non-coded amino acids employed in this study are reported in Figure 1.

In line with previous findings [11], U-II (**1**) induced a concentration-dependent contraction of rat aorta strips with high potency (pEC_{50} 8.0–8.5) but relatively small maximal effects (E_{max} = 0.6–0.8 g). To evaluate the role of the cyclic region c[Cys-Phe-Trp-Lys-Tyr-Cys] in the biological activity, the truncated peptide U-II(5–10) (**2**) and the linear form (containing $Cys^{5,10}$ as reduced, -SH side chains) of U-II (**3**) were synthesized and tested. U-II(5–10) mimicked the contractile action of the natural peptide behaving as a full agonist (E_{max} 0.65 ± 0.11) but showing a 50-fold lower potency [pEC_{50} 6.58 ($CL_{95\%}$ 6.38–6.78)]. This finding is in line with the results

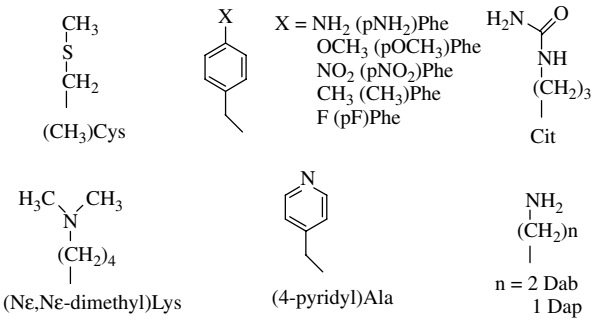

Figure 1 Chemical structure of the side chains of some of the non-coded amino acids employed in this study.

Table 1 Abbreviated Names and Analytical Properties of Urotensin-II (U-II: H-Glu-Thr-Pro-Asp-c[Cys-Phe-Trp-Lys-Tyr-Cys]-Val-OH) Analogues

Compound	Abbreviated name	Purity[a] %	t_R[b] (min) I	$[M+H]^{+c}$	
				Calcd	Found
1	U-II	>99	10.02	1389.6	1389.5
2	U-II(5–10)	>99	10.33	848.1	847.9
3	linear U-II	>98	10.27	1391.6	1391.8
4	[(CH_3)Cys^{5,10}]U-II	>97	10.42	1419.6	1420.2
5	[Ala^6]U-II	>98	9.48	1313.5	1313.1
6	[Ala^7]U-II	>98	9.20	1274.5	1274.5
7	[Ala^8]U-II	>98	10.88	1332.5	1332.6
8	[Ala^9]U-II	>98	9.87	1297.5	1298.2
9	[D-Phe^6]U-II	>98	10.13	1389.6	1389.1
10	[D-Trp^7]U-II	>98	10.61	1389.6	1389.6
11	[D-Lys^8]U-II	>98	9.18	1389.6	1389.8
12	[D-Tyr^9]U-II	>98	10.47	1389.6	1389.3
13	[Nle^8]U-II	>98	10.75	1374.6	1375.2
14	[Nva^8]U-II	>98	10.68	1360.6	1361.1
15	[Leu^8]U-II	>98	10.97	1374.6	1374.2
16	[Cit^8]U-II	>98	10.26	1420.5	1420.1
17	[Gln^8]U-II	>98	10.19	1389.6	1389.3
18	[Lys^8(Ac)]U-II	>98	10.38	1431.6	1432.2
19	[Arg^8]U-II	>98	9.98	1417.6	1417.5
20	[(N,N-dimethyl)Lys^8]U-II	>98	10.13	1417.6	1417.1
21	[(4-pyridyl)Ala^8]U-II	>98	10.31	1409.6	1409.6
22	[(pNH_2)Phe^8]U-II	>98	10.43	1423.6	1423.3
23	[Orn^8]U-II	>99	9.97	1375.6	1375.4
24	[Dab^8]U-II	>98	9.86	1361.6	1362.3
25	[Dap^8]U-II	>98	9.69	1347.6	1346.9
26	[$(pOCH_3)$Phe^9]U-II	>98	10.24	1403.6	1403.5
27	[(pNO_2)Phe^9]U-II	>98	10.73	1418.6	1418.2
28	[(pCH_3)Phe^9]U-II	>99	10.66	1387.6	1387.8
29	[(pF)Phe^9]U-II	>99	9.71	1391.6	1392.1
30	[Phe^9]U-II	>99	10.39	1373.6	1374.1
31	[(pNH_2)Phe^9]U-II	>98	9.57	1388.6	1388.3

[a] Determined by analytical HPLC.
[b] t_R is the retention time determined by analytical HPLC.
[c] The mass ion $[M+H]^+$ was obtained by MALDI-TOF mass spectrometry.

obtained by Itoh *et al.* [15]: in fact, the goby fish U-II(6–11) was inactive up to 100 nM in the rat aorta bioassay. The linear form of U-II also contracted the rat aorta strips; however, HPLC and mass spectrometry analyses (data not shown) demonstrated that the linear peptide underwent a spontaneous and rapid cyclization when exposed to the organ bath conditions. This rapid conversion, probably due to the high pO_2 of the medium, did not allow the evaluation of the biological activity of the linear form of the peptide. Thus, (4) was synthesized in which cyclization was prevented by S-methylation of the Cys^5 and Cys^{10} residues; this peptide was found to be completely inactive (both as agonist and as antagonist) at concentrations up to 10 μM (data not shown). These results suggest that the -Cys-Phe-Trp-Lys-Tyr-Cys- sequence and the Cys^5-Cys^{10} bridge are crucial for UT receptor activation. The fact that U-II isopeptides from different vertebrate species (which share the -Cys-Phe-Trp-Lys-Tyr-Cys- sequence and the cyclic structure [2,3]) behave in the same way (equipotent, equi-efficacious) in mammalian bioassays [16,17] and that monkey and mouse UT receptors expressed in HEK-293 cells are reported to exhibit the same affinity for human, fish, rat mouse and pig U-II isopeptides [18] further strengthens the idea that the conserved cyclic hexapeptide part of the U-II isopeptide family represents the bioactive core of the naturally occurring molecule.

The importance of the disulphide (Cys^5-Cys^{10}) bridge has been recently documented by Grieco *et al.* [19] who demonstrated that substitution of the disulphide with a lactam bridge produces loss of biological activity, while

Table 2 Effects of U-II and its Cyclic Region Ala-/D-scan Analogues in the Rat Aorta Bioassay

Compound		Agonist		Antagonist
		pEC_{50} ($CL_{95\%}$)	E_{max} (g)	pA_2 ($CL_{95\%}$)
1	U-II	8.21 (8.02–8.40)	0.60 ± 0.10	ND
5	[Ala^6]U-II	5.83 (5.68–5.98)	0.48 ± 0.05	ND
6	[Ala^7]U-II	Inactive		Inactive
7	[Ala^8]U-II	Inactive		Inactive
8	[Ala^9]U-II	Inactive		Inactive
9	[D-Phe^6]U-II	5.77 (5.17–6.37)	0.46 ± 0.17	ND
10	[D-Trp^7]U-II	7.09 (6.73–7.45)	0.54 ± 0.13	ND
11	[D-Lys^8]U-II	Inactive		Inactive
12	[D-Tyr^9]U-II	Inactive		Inactive

ND, not determined since the compound behaves as a full agonist. pEC_{50} the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. pA_2 antagonist-potency calculated using the Gaddum-Schild equation. $CL_{95\%}$ 95% confidence limits. E_{max} the maximal effect elicited by the agonist expressed as grams (g) of contraction. Inactive up to 10 μM.

replacement of Cys^5 with penicillamine is well tolerated and even increases the peptide potency [20].

Based on these data, attention was focused on the -Phe^6-Trp^7-Lys^8-Tyr^9- U-II sequence by performing an Ala- and D-scan analysis of these residues. As shown in Table 2, the Ala replacement in positions 7–9 produced inactive derivatives (**6–8**) while, in position 6, it generated a low potency agonist (**5**, $pEC_{50} = 5.83$). Similar results were obtained by amino acid replacement with the corresponding D-enantiomer. In fact, [D-Lys^8]U-II and [D-Tyr^9]U-II (**11**,**12**) were inactive while [D-Phe^6]U-II (**9**) and [D-Trp^7]U-II (**10**) behaved as low ($pEC_{50} = 5.77$) and moderate ($pEC_{50} = 7.09$) potency agonists, respectively. Collectively these results point to the basic and aromatic side chains of Lys^8 and Tyr^9 and their spatial orientation as important pharmacophores for UT receptor occupation and activation. Similar findings were reported by other groups who tested U-II derivatives on CHO cells transfected with the human [21] or the rat [22] UT receptor. In particular, Flohr *et al.* [21], used the Ala- and D-scan data, together with NMR analysis, for generating a pharmacophore model of U-II/UT receptor interaction. This model which proposes as a crucial U-II message sequence the two aromatic side chains of Trp^7 and Tyr^9 and the basic side chain of Lys^8 has been experimentally validated: the preselection of non-peptide ligands based on this model produced a 2% hit rate (number of active compounds/number of compounds tested × 100) which is about 20-fold higher than the hit rate of normal high throughput screens for GPCR ligands [21].

The relative role of Lys^8 (Table 3, **13–25**) and Tyr^9 (Table 4, **26–31**) were investigated for UT receptor interaction by replacement with coded and non-coded amino acids. As shown in Table 3, substitution of Lys^8 with lipophilic amino acids (**13**–**15**) produced inactive peptides. Similar results were also obtained by the substitution with hydrophilic but not basic residues (**16**–**18**), indicating that the basic character of the Lys side chain is essential for biological activity. The replacement of Lys^8 with other basic residues produced different results depending on the side chain of the amino acid. Substitution of Lys^8 with Arg (**19**) produced a 50-fold decrease in potency ($pEC_{50} = 6.56$), while the dimethylation of Lys side chain (**20**) was relatively well tolerated ($pEC_{50} = 7.60$). Reduction of the amino acid side-chain basic character, as in (**21**,**22**), is not compatible with biological activity and produced inactive analogues. These data suggested the presence of a primary aliphatic amine as an essential requirement for U-II position 8. The relative importance of the distance of the primary aliphatic amine from the peptide backbone has been investigated with compounds (**23**–**25**) in which this distance was progressively reduced from 4 carbon atom (Lys, **1**) to 3 (Orn, **23**), 2 (Dab, **24**) and 1 (Dap, **25**). [Orn^8]U-II produced a weak contraction (corresponding to about 20% of the U-II maximal effect) of rat aorta strips only at micromolar concentrations; however, it antagonized U-II effects showing a pA_2 value of 6.47 (see also [23]). Recent studies confirmed the antagonist properties of (**23**) also *in vivo* where it prevented U-II induced plasma extravasation in mice [24]. Interestingly, the Orn residue is present in the same position of the 6-amino acid cyclic structure of BIM 23 127, a somatostatin related peptide which has been recently described as UT receptor antagonist [25].

Similar results were obtained with (**24**), which, however, showed lower residual agonist activity (7% of the U-II maximal effect) and potency (pA_2 5.51). On the other hand, further shortening of the amino acid side chain as in (**25**) produced a low potency (pEC_{50} 6.77) full agonist. Thus, data obtained with compounds

Table 3 Effects of U-II and [Xaa^8]U-II Analogs in The Rat Aorta Bioassay

Compound		Agonist		Antagonist
		pEC_{50} ($CL_{95\%}$)	E_{max} (g)	pA_2 ($CL_{95\%}$)
1	U-II	8.22 (8.06–8.38)	0.72 ± 0.06	
13	[Nle^8]U-II	Inactive		Inactive
14	[Nva^8]U-II	Inactive		Inactive
15	[Leu^8]U-II	Inactive		Inactive
16	[Cit^8]U-II	Inactive		Inactive
17	[Gln^8]U-II	Inactive		Inactive
18	[Lys^8(Ac)]U-II	5.69 (5.39–5.99)	0.67 ± 0.11	ND
19	[Arg^8]U-II	6.56 (6.12–7.00)	0.68 ± 0.09	ND
20	[(N,N-dimethyl)Lys^8]U-II	7.60 (7.47–7.63)	0.67 ± 0.12	ND
21	[(4-pyridyl)Ala^8]U-II	10 μM: 0.29 ± 0.06		Inactive
22	[(pNH_2)Phe^8]U-II	Inactive		Inactive
23	[Orn^8]U-II	10 μM: 0.15 ± 0.06		6.47 (6.23–6.71)
24	[Dab^8]U-II	10 μM: 0.05 ± 0.02		5.51 (5.32–5.70)
25	[Dap^8]U-II	6.77 (6.67–6.97)	0.66 ± 0.06	ND

ND, not determined since the compound behaves as a full agonist. pEC_{50} the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. pA_2 antagonist-potency calculated using the Gaddum-Schild equation. $CL_{95\%}$ 95% confidence limits. E_{max} the maximal effect elicited by the agonist expressed as grams (g) of contraction. Inactive, up to 10 μM.

Table 4 Effects of U-II and [Xaa^9]U-II Analogs in the Rat Aorta Bioassay

Compound		Agonist	
		pEC_{50} ($CL_{95\%}$)	E_{max} (g)
1	U-II	8.35 (8.22–8.48)	0.59 ± 0.11
26	[($pOCH_3$)Phe^9]U-II	8.18 (8.04–8.28)	0.61 ± 0.08
27	[(pNO_2)Phe^9]U-II	8.19 (7.93–8.44)	0.85 ± 0.10
28	[(pCH_3)Phe^9]U-II	8.43 (8.16–8.70)	0.62 ± 0.06
29	[(pF)Phe^9]U-II	7.82 (7.65–7.99)	0.59 ± 0.11
30	[Phe^9]U-II	7.74 (7.54–7.92)	0.66 ± 0.05
31	[(pNH_2)Phe^9]U-II	7.56 (7.25–7.87)	0.58 ± 0.12

pEC_{50} the negative logarithm to base 10 of the molar concentration of an agonist that produces 50% of the maximal possible effect. $CL_{95\%}$ 95% confidence limits. E_{max} the maximal effect elicited by the agonist expressed as grams (g) of contraction.

23 and **24** indicate that the reduction of the distance of the primary aliphatic amine from the peptide backbone produces a progressive reduction of both potency (Lys 8.22; Orn 6.47; Dab 5.51) and efficacy (Lys 1.00; Orn 0.21; Dab 0.07). On the other hand, further shortening of the amino acid side chain as in (**25**) led to an increase in potency (6.77) and, most importantly, fully restored efficacy. Taken together these data suggest that the positive charge of the primary aliphatic amine in position 8 and its relative spatial orientation is crucial for both receptor occupation and activation. It is worth mentioning that Kinney *et al.* [22] recently proposed a model of U-II/UT receptor binding in which the interaction between the amino function of Lys^8 of the peptide and the carboxylic group of Asp^{130} of the receptor TM3 plays an 'essential' role.

As shown in Table 4, substitution of the -OH group of Tyr^9 with -OCH_3 (**26**), -NO_2 (**27**), -CH_3 (**28**), -F (**29**), -H (**30**) and -NH_2 (**31**) did not produce any substantial change of potency (pEC_{50} ranging from 7.56 to 8.43) or efficacy (all derivatives behaved as full agonists). These results are in agreement with those obtained by Kinney *et al.* [22], who demonstrated that Tyr^9 can be substituted with the more bulky non-coded amino acids 1-naphthylalanine, 2-naphthylalanine or 1,1-biphenylalanine without modifying the peptide biological activity. Interestingly, the replacement of Tyr^9 with 3-iodo-Tyr has been reported recently to generate a full UT receptor agonist, 6-fold more potent than the natural peptide [26]. These results, together with those from the literature, suggest that the only chemical requirement at position 9 is the presence of an aromatic moiety. Indeed, several different chemical groups can be added to the phenyl ring without significantly modifying the peptide biological activity.

CONCLUSIONS

The present findings confirm and extend knowledge regarding U-II pharmacophore requirements for UT receptor occupation and activation demonstrating that the cyclic region of the peptide and in particular the primary aliphatic amine of the Lys^8 side chain are

crucial. Moreover, novel peptides (**23** and **24**) were identified acting as low efficacy (partial) agonists at the UT receptor. They can be used in future studies as chemical templates for the design and identification of selective UT receptor pure antagonists.

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