Journal of Peptide Science
J. Peptide Sci. **11**: 593–608 (2005)
Published online 3 May 2005 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.667

JOURNAL OF PEPTIDE SCIENCE

Design of peptide oxytocin antagonists with strikingly higher affinities and selectivities for the human oxytocin receptor than atosiban‡

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Received 7 January 2005; Revised 4 February 2005; Accepted 7 February 2005

Abstract: The peptide oxytocin (OT) antagonist atosiban, approved for tocolytic use in Europe (under the tradename Tractocile), represents an important new therapeutic advance for the treatment of premature labor. This paper presents some new peptide OT antagonists which offer promise as superior tocolytics. The solid phase synthesis is reported of four pairs of L and D-2-naphthylalanine (L/D-2Nal) position-2 modified analogs of the following four oxytocin (OT) antagonists: des-9-glycinamide [1-(β-mercapto-β,β-pentamethylene propionic acid), 2-O-methyltyrosine, 4-threonine]ornithine-vasotocin (desGly-NH_2,d$(CH_2)_5$[Tyr(Me)^2,Thr^4]OVT) (**A**); the Tyr-$NH_2{}^9$ analog of (**A**), d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Tyr-$NH_2{}^9$]OVT (**B**); the Eda^9 analog of (**A**), d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Eda^9]OVT (**C**); and the retro $COCH_2$Ph(4-0H)^{10} modified analog of (**C**), d$(CH_2)_5$[Tyr(Me)^2,Thr^4,Eda^9 ← $COCH_2$Ph(4-0H)^{10}]OVT (**D**). The eight new analogs of **A–D** are (**1**) desGly-NH_2,d$(CH_2)_5$[D-2Nal^2,Thr^4]OVT, (**2**) desGly-NH_2,d$(CH_2)_5$[2-Nal^2,Thr^4]OVT, (**3**) d$(CH_2)_5$[D-2Nal^2,Thr^4,Tyr-$NH_2{}^9$]OVT, (**4**) d$(CH_2)_5$[2Nal^2,Thr^4,Tyr-$NH_2{}^9$]OVT, (**5**) d$(CH_2)_5$[D-2Nal^2,Thr^4,Eda^9]OVT, (**6**) d$(CH_2)_5$[2Nal^2,Thr^4,Eda^9]OVT, (**7**) d$(CH_2)_5$[D-2Nal^2,Thr^4,Eda^9 ← $COCH_2$Ph(4-0H)^{10}]OVT, (**8**) d$(CH_2)_5$[2Nal^2,Thr^4,Eda^9 ← $COCH_2$Ph(4-OH)^{10}]OVT. Peptides *1–8* were evaluated for agonistic and antagonistic activities in *in vitro* and *in vivo* rat bioassays, in rat OT receptor (rOTR) binding assays and in human OT receptor (hOTR) and human vasopressin (VP) vasopressor (V_{1a}) receptor (hV_{1a}R) binding assays. Also reported are the hOTR and hV_{1a}R affinity data for atosiban and for *B*. None of the eight peptides exhibit oxytocic or vasopressor agonism. Peptides *1–8* exhibit weak antidiuretic agonism (activities in the range 0.014–0.21 U/mg). Peptides *1–6* exhibit potent *in vitro* (no Mg^{2+}) OT antagonism (anti-OT pA_2 values range from 7.63 to 8.08). Peptides *7* and *8* are weaker OT antagonists. Peptides *1–6* are all OT antagonists *in vivo* (estimated *in vivo* anti-OT pA_2 values in the range 6.94–7.23). Peptides *1–8* exhibit vasopressor antagonism, anti-V_{1a} pA_2 values in the range 5.1–7.65. Peptides **1**–**8** exhibit high affinities for the rOTR (K_i values = 0.3–7.8 nM). Peptides *1–4* and *B* exhibit surprisingly very high affinities for the hOTR; their K_i values are 0.17, 0.29, 0.07, 0.14 and 0.59 nM, respectively. Peptides *1–4* and *B* exhibit respectively 449, 263, 1091, 546 and 129 times greater affinity for the hOTR than atosiban (K_i = 76.4 nM). Peptides *1–4* exhibit high affinities for the hV_{1a}R (K_is = 1.1 nM, 1.3 nM, 0.19 nM and 0.54 nM, all higher than the hV1$_a$R affinities exhibited by atosiban (K_i = 5.1 nM) and by *B* (K_i = 5.26 nM). Because of their strikingly higher affinities for the hOTR than atosiban, peptides *1–4* and *B* exhibit gains in anti hOT/anti hV_{1a} receptor selectivity compared with atosiban of 93, 64, 39, 56 and 127, respectively. These OT antagonists are thus promising candidates for development as potential new tocolytic agents. Copyright © 2005 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: oxytocin; antagonists; human OT receptor; vasopressin; V_{1a} receptor; atosiban; barusiban

Abbreviations: As recommended in *J. Pept. Sci.* 2003; **9**:1–8 and references there cited, and as follows. All amino acids are in the L-configuration unless otherwise noted. Other abbreviations used are: VT, vasotocin; OVT, ornithine vasotocin; D-Tyr(Et), O-ethyl-D-tyrosine; D-Tyr(Me), O-methyl-D-tyrosine; 2Nal, L-2-naphthylalanine; D-2Nal, D-2-naphthylalanine; Eda, ethylenediamine; desGly-NH_2, desglycineamide [carboxy terminus is Orn (OH)]; –$COCH_2$Ph(4-OH), 4-hydroxyphenylacetyl; Eda ← $COCH_2$Ph(4-OH), Eda retro-$COCH_2$Ph(4-0H); d$(CH_2)_5$-, β-mercapto-β,β-pentamethylenepropionic acid; d$(CH_2)_5$(Mob), β-(4-methoxybenzyl)mercapto-β,β-pentamethylenepropionyl; desGly- NH_2, d$(CH_2)_5$[Tyr(Me)^2, Thr^4]OVT(**A**), des-9-glycinamide [1-(β-mercapto-β,β-pentamethylenepropionic acid), 2-O-methyl-tyrosine, 4-threonine] ornithine vasotocin; d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr-$NH_2{}^9$]OVT (**B**), the Tyr-$NH_2{}^9$ analog of (A); d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Eda^9]OVT (**C**); the Eda^9 analog of (**A**); d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Eda^9 ← $COCH_2$Ph(4-0H)^{10}] OVT (**D**), the retro-$COCH_2$Ph(4-0H)^{10} analogue of (**C**); d[D-Tyr(Et)^2, Thr^4]OVT, [1-deamino, 2-O-ethyl-D-tyrosine 4-threonine] ornithine vasotocin [generic name: atosiban; trade name: Tractocile]; carba-6-[D-Trp^2, alle^4, MeOrn^7]dOT-(1–7)-oL, $C^{4,6}$, S^1-cyclo[*N*-(3-sulfanylpropanoyl)-D-tryptophyl-isoleucyl-alloisoleucyl-asparaginyl-2-aminobutanoyl-*N*-methyl-ornithinol] [generic names: barusiban; FE220 440]; d, deamino: 6-carba, methylene group is substituted for sulfur atom in cysteine residue at position 6; DIPEA, *N*,*N*-diisopropylethylamine; CH_3CN, acetonitrile; V_2, antidiuretic; V_{1a}, vasopressor; Z[2Cl], 2-chlorobenzyloxycarbonyl; ESMS, electrospray mass spectrometry; HO-LVA, linear vasopressin antagonist, (4-OH)PhCH_2CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2; [^{125}I]HO-LVA, (3-^{125}I-4-OH)PhCH_2CO-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2; OTA, d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr-$NH_2{}^9$]OVT; [^{125}I]OTA, position 9 ^{125}I-labeled d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr-$NH_2{}^9$]OVT; ED, effective dose; K_i, inhibition constants.

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‡ This paper is dedicated to the memories of Professors Arno Spatola and Murray Goodman.

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INTRODUCTION

Preterm birth affects about 10% of all births and is the major cause of perinatal morbidity and death [1–3]. Although the factors that bring about preterm births have not been clearly defined [3–6], there is strong evidence suggesting that the uterine contracting hormone oxytocin may play a role as a trigger for preterm labor [7–10]. Thus the design of synthetic peptide and non-peptide OT antagonists as potential tocolytic agents for the prevention of preterm births has been and continues to be an area of intensive investigation [3,5,6,11–62]. [For reviews see: 63–70]. Of the many OT antagonists (peptide and non-peptide) reported to date, only one, atosiban, has been approved (in Europe) under the Tradename Tractocile for the treatment of preterm labor [70]. This peptide OT antagonist, d[D-Tyr(Et)^2, Thr^4]OVT, first reported in 1986 [26], was based on earlier lead peptide OT antagonists from this and other laboratories [11–22]. After many years of extensive clinical trials, atosiban is being widely used as the therapy of choice for preterm labor [3,5,6,70–73]. Atosiban is, however, far from being an ideal OT antagonist [54]. It is highly non-selective for OT receptors versus VP V_{1a} receptors [47,54,74–75]. For reviews of the known VP V_{1a} (vascular), V_{1b} (pituitary) and V_2 (renal) receptors and the OT uterine receptor see [76–78]. In *in vivo* rat bioassays, atosiban is only eight times more potent as an OT antagonist than as a V_{1a} antagonist [47]. In human receptor binding assays reported here, atosiban exhibits 15 times greater affinity for the VP V_{1a} receptor than for the OT uterine receptor. It is thus a more potent V_{1a} antagonist than an OT antagonist in humans. It was previously shown that V_{1a} antagonism is an undesirable side effect in OT antagonists [79]. Thus there is a pressing need for OT antagonists with superior potency, and selectivity than atosiban. As part of a longstanding program aimed at the design and synthesis of potent and selective OT antagonists [17–22,29,30,46–48,54–56], a series of OT antagonists was reported that is more potent and selective than atosiban in *in vivo* assays in the rat [47]. These were designed by replacing the Tyr(Me) residue at position 2 in one of our early, somewhat selective OT antagonists: desGly-NH_2, d$(CH_2)_5$[Tyr(Me)^2, Thr^4]OVT (**A**) [30] with D-Tyr(Me), D-Tyr, D-Phe and D-Trp. Our more recent study [54] reported a series of OT antagonists designed by replacing the Tyr(Me)^2 residue in (**A**) and in three position 9 modified analogs of (**A**) with L and D-β-thienylalanine (Thi). These OT antagonists exhibit 3–18-fold gains in anti-OT (*in vivo*)/anti V_{1a} selectivity with respect to atosiban in rat bioassays [54]. When subsequently examined in human OT and V_{1a} receptor binding assays, a number of these OT antagonists exhibited marked gains in human OT receptor/human V_{1a} receptor selectivity compared with atosiban [55 and unpublished]. Although these Thi^2/D-Thi^2-OT antagonists exhibit good gains in anti-OT/anti-V_{1a} selectivity relative to atosiban [55], their binding affinities for the human OT receptor were in the same range as that exhibited by atosiban. In seeking to enhance human OT receptor affinity, it was decided to investigate the use of L and D-naphthylalanine as a position 2 substituent in four of our previously reported OT antagonists [30,29,54,68]. 2Nal had previously been utilized in structure/activity studies on tetragastrin and somatostatin analogs [80,81]. Subsequently D-2Nal was utilized in the design of superagonist LHRH analogs [82]. It may be recalled also that replacement of the Tyr^2 residue in OT and in AVP agonists with D-2Nal and 2Nal resulted in analogs which exhibited potent OT antagonism *in vitro* and *in vivo* in rat bioassays [45,52,53,83–85]. It was subsequently found that [D-2Nal^2]AVP and d[D-2Nal^2]AVP exhibited high affinities for the human OT receptor [86]. These later findings, thus pointed to the usefulness of the D-2Nal^2/2Nal^2 modification for the design of OT antagonists with high affinity for the human OT receptor. To our knowledge, neither modification had been incorporated into an OT antagonist. Thus, using the D-2Nal^2/Nal^2 modifications with a view to enhancing human receptor affinity and selectivity relative to atosiban, the following four Tyr(Me)^2 containing oxytocin antagonists **A–D** were selected: desGly-NH_2, d$(CH_2)_5$[Tyr(Me)^2, Thr^4]OVT (**A**) [30]; d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr-NH_2^{9}]OVT (**B**) [29]; d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Eda^9]OVT (**C**) [54]; d$(CH_2)_5$ [Tyr(Me)^2, Thr^4, Eda^9 $\leftarrow$ $COCH_2$Ph4-(OH)^{10}] OVT (**D**) [68]. Replacement of the Tyr(Me)^2 residue in these four peptides by D-2Nal^2 and by 2Nal^2 residues resulted in the following eight new peptides: **(1)** des-9-glycinamide [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-D-2-naphthylalanine, 4-threonine]ornithine-vasotocin (desGly-NH_2, d$(CH_2)_5$ [D-2Nal^2, Thr^4]OVT); **(2)** des-9-glycinamide [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-L-2-naphthylalanine, 4-threonine]ornithine-vasotocin (desGly-NH_2, d$(CH_2)_5$, [2Nal^2, Thr^4]OVT); **(3)** [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-D-2-naphthylalanine, 4-threonine, 9-tyrosinamide] ornithine-vasotocin (d$(CH_2)_5$[D-2Nal^2, Thr^4, Tyr-NH_2^{9}] OVT); **(4)** [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-L-2-naphthylalanine, 4-threonine, 9-tyrosinamide] ornithine-vasotocin (d$(CH_2)_5$[2Nal^2, Thr^4, Tyr-NH_2^{9}]OVT); **(5)** [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-D-2-naphthylalanine, 4-threonine, 9-ethylenediamine] ornithine-vasotocin (d$(CH_2)_5$ [D-2Nal^2, Thr^4, Eda^9]OVT); **(6)** [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-L-2-naphthylalanine, 4-threonine, 9-ethylenediamine] ornithine-vasotocin (d$(CH_2)_5$[2Nal^2, Thr^4, Eda^9]OVT); **(7)** [1-(β-mercapto-β,β-cyclopentamethylenepropionic acid), 2-D-2-naphthylalanine, 4-threonine, 9-ethylenediamine, retro 10-(4-hydroxy)phenylacetyl] ornithine-vasotocin (d$(CH_2)_5$[2-D-Nal^2, Thr^4, Eda^9 $\leftarrow$ $COCH_2$Ph $(4-OH)^{10}$); **(8)** [1-(β-mercapto-β,β-cyclopenta-

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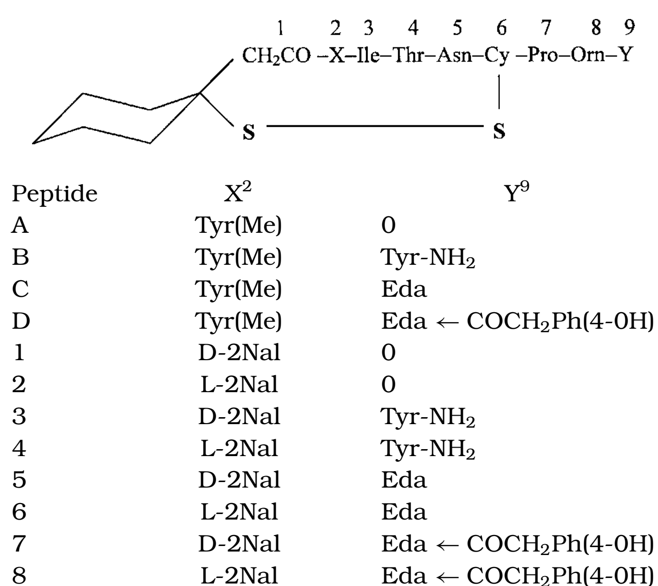
methylenepropionic acid), 2-L-2-naphthylalanine, 4-threonine, 9-ethylenediamine, retro 10-(4-hydroxy) phenylacetyl] ornithine-vasotocin ($d(CH_2)_5[2Nal^2, Thr^4, Eda^9 \leftarrow COCH_2Ph_2(4\text{-}OH)^{10}]OVT$). Preliminary rat bioassay and human receptor affinity data on peptides *1–6* have been reported [87]. Those studies [87] used human OT receptor and human V_{1a} receptor affinity data for atosiban reported by others [76,88]. However, a review of the literature for this manuscript, revealed wide discrepancies in atosiban affinities for the human OT receptor [58,62,75,88–90]. It was thus decided to measure the human OT receptor and human V_{1a} receptor affinities for the atosiban previously resynthesized in our laboratories [47]. These values together with those from other laboratories [58,62,74,75,88–90] are reported here (Table 3).

Peptides **A–D** and *1–8* have the following general structure:



Peptide	X^2	Y^9
A	Tyr(Me)	0
B	Tyr(Me)	Tyr-NH_2
C	Tyr(Me)	Eda
D	Tyr(Me)	Eda ← $COCH_2Ph$(4-0H)
1	D-2Nal	0
2	L-2Nal	0
3	D-2Nal	Tyr-NH_2
4	L-2Nal	Tyr-NH_2
5	D-2Nal	Eda
6	L-2Nal	Eda
7	D-2Nal	Eda ← $COCH_2Ph$(4-0H)
8	L-2Nal	Eda ← $COCH_2Ph$(4-0H)

Peptide Synthesis

The synthesis of the free peptides *1–8* (Table 5) was carried out utilizing the Merrifield solid-phase method [91–93] with the modifications previously described [54,94–96]. It was previously shown that the Na/Liq NH_3 procedure cannot be utilized with naphthylalanine containing peptides [85]. Thus the synthetic strategy used for the synthesis of peptides *1–8* was modified accordingly [85]. The protected retromodified peptides VII and VIII (Table 4) were obtained by coupling the protected peptides V, VI (Table 4) with 4-hydroxyphenylacetic acid in DMF using the DCC/HOBt procedure [97], as described in [98]. Boc-Orn[Z(2Cl]-resin was converted to the corresponding protected acylheptapeptidyl resin and Boc-Tyr(Bzl)-resin was converted to the corresponding acyloctapeptidyl resin in seven or eight cycles of deprotection, neutralization and coupling, respectively. A HCl (1 M)/AcOH mixture was used in all the deprotection steps [54,94–96]. Neutralizations were carried out with 10% Et_3N/CH_2Cl_2. Coupling reactions were mediated mainly by DCC/HOBt [97] in CH_2Cl_2/DMF except for Boc-Asn which was incorporated as its 4-nitrophenyl ester [99] in DMF. The acylpeptide resins were cleaved using the following procedures: (i) ammonolysis in methanol with DMF extraction [95,100] to give the protected peptide amides III, IV (Table 4); (ii) aminolysis with Eda in methanol with DMF extraction [98,101] to give the protected Eda peptides V, VI (Table 4); (iii) the HF procedure [93] for simultaneous cleavage of the peptides from the resin and their deprotection to give the free peptides *1* and *2* (Table 5). The TFMSA procedure [102,103] was used to deblock the protected precursors III–VIII. The resulting disulfhydryl compounds from the HF treatment and the TFMSA treatment were oxidatively cyclized with $K_3[Fe(CN)_6]$ [104] using the modified reverse procedure [105]. The free peptides were desalted and purified by gel filtration on Sephadex G-15 and Sephadex LH-20 mainly in a two-step procedure [106] using 50% AcOH and 2 M AcOH as eluents, respectively, as previously described [54,94–96]. When necessary, an additional purification on Sephadex G-15 or/and Sephadex LH-20 with 0.2 M AcOH as eluent was carried out. The purity of the free peptides *1–8* (Table 5) was checked by TLC, HPLC and electron spray mass spectrometry (ESMS).

Bioassays

Peptides were assayed for agonistic and antagonistic activities in *in vitro* and *in vivo* rat oxytocic assays, in the rat vasopressor assay, and in the rat antidiuretic assay. For agonists, the four-point assay design [107] was used and for antagonists, the Schild's pA_2 method [108] was employed. The pA_2 is the negative logarithm of the molar concentration of the antagonist that will reduce the response to $2\times$ units of the agonist to equal the response to $1\times$ unit of the agonist in the absence of antagonist. In practice, this dose is estimated by finding doses above and below the pA_2 dose and interpolating on a logarithmic scale. In the rat *in vivo* assays, the pA_2 dose (effective dose, ED) is divided by an arbitrarily assumed volume of distribution of 67 ml/kg to derive the molar concentration of the pA_2 dose [109]. Thus, *in vivo* pA_2 values are estimates. Synthetic oxytocin and arginine-vasopressin which had been standardized in oxytocic and vasopressor units against the USP Posterior Pituitary Reference Standard were used as working standards in all bioassays. *In vitro* oxytocic assays were performed on isolated uteri from diethylstilbestrol-primed rats in a Mg^{2+}-free van Dyke-Hasting's solution [110]. *In vivo* anti-OT potencies were determined in urethane anesthetized diethylstilbestrol-primed rats as previously described [111]. Vasopressor assays were performed on urethane-anesthetized and phenoxybenzamine-treated rats as

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described by Dekanski [112]. Antidiuretic assays were on water-loaded rats under ethanol anesthesia as described by Sawyer [113]. When standard errors are presented in Table 1, the means reflect results from at least four independent assay groups.

Cell culture. CHO cell lines expressing the vasopressin receptor subtypes or the oxytocin receptor [114] were maintained in Dulbecco's modified Eagle's medium supplemented with 10% decomplemented fetal calf serum, 4 mM glutamine and 500 units/ml penicillin and streptomycin, in an atmosphere of 95% air and 5% CO_2 at 37 °C.

Membrane preparation. The cells were treated overnight with 5 mM sodium butyrate to increase receptor expression [115]. This treatment does not modify the pharmacological properties of the receptors. Culture dishes of CHO cells expressing one receptor subtype were washed twice with PBS without Ca^{2+} and Mg^{2+}. Lysis buffer (15 mM Tris: HCl, 2 mM $MgCl_2$, 0.3 mM EDTA, pH 7.4) was added and the cells were scraped, polytron homogenized and centrifuged at $100 \times \mathbf{g}$ for 5 min. The supernatant was then centrifuged at $44\,000 \times \mathbf{g}$ for 20 min at 4 °C. The pellets were resuspended in buffer A (50 mM Tris: HCl, 5 mM $MgCl_2$, pH 7.4) and centrifuged a second time at $44\,000 \times \mathbf{g}$ for 20 min at 4 °C. Pellets were then resuspended in an appropriate volume of buffer A. The protein concentration was estimated. The membranes were immediately used or aliquoted and stored in liquid nitrogen.

Binding experiments. Affinities of the various ligands for the vasopressin and oxytocin receptor subtypes were determined as previously described [56] by competition experiments using 170–270 pM [^{125}I]OTA [29] for the OT receptor; 60–80 pM [^{125}I]HO-LVA antagonist [116] for the V_{1a} vasopressin receptor as radioligands. Nonspecific binding was determined in the presence of 1 µM unlabeled ligand. Membranes were used at 0.5–1.5 µg of protein/assay for competition with iodinated ligands. Binding assays were performed at 30 °C for 1 h. Ligand binding data were analysed with the computer program Ligand, and the inhibition constants, K_i, were determined with a nonlinear least-squares regression from at least three independent competition experiments, each performed in triplicate [117].

RESULTS AND DISCUSSION

The antioxytocic (anti-OT) (*in vitro*, no Mg^{2+} and *in vivo*) potencies, antivasopressor (anti-V_{1a}) potencies, antidiuretic (V_2 receptor) agonistic and/or antagonistic activities, of peptides *1–8*, their parent peptides **A–D** and atosiban are presented in Table 1. Affinities for the rat OT receptor are also given in Table 1. The affinities of peptides *1–7*, (**B**) and atosiban for the human OT and V_{1a} receptors, all obtained for this study, are presented in Table 2. The human OT receptor and human V_{1a} receptor affinity data for atosiban together with previously reported data are shown in Table 3. None of the eight new peptides (*1–8*) exhibited oxytocic or vasopressor agonism. All eight D-2Nal^2/2Nal^2 substituted peptides are very weak antidiuretic agonists. Six of the eight D-2Nal^2/2Nal^2 substituted peptides are potent *in vitro* OT antagonists. The same six peptides exhibit OT antagonism *in vivo*. They also exhibit potent antivasopressor (anti-V_{1a}) potencies *in vivo*. Seven of the D-2Nal^2/2Nal^2 peptides exhibit very high affinities for the rat OT receptor; all much higher than the affinity reported for atosiban [58] (Table 1). Surprisingly, seven of the eight new peptides (*1–7*) have higher affinities for the human OT receptor than atosiban. Four of the eight new peptides (*1–4*) exhibit very high affinities for the human OT receptor, strikingly enhanced with respect to that of atosiban (Table 2). All but one (*7*) exhibit higher affinities for the human V_{1a} receptor than atosiban. Peptides *1–7* exhibit significant gains in human OT receptor/V_{1a} receptor selectivity relative to atosiban.

Effects of D-2-Nal^2/2Nal^2 Modifications in OT Antagonists A–D

Effects on in vitro (no Mg^{++}) OT antagonism (Table 1). With the exception of peptides *7* and *8*, both the D-2Nal^2 and 2Nal^2 substitutions in the OT antagonists **A–D** were very well tolerated with complete retention of *in vitro* OT antagonism in peptides *1–6* ranging from slightly diminished to slightly enhanced. Replacement of the Tyr(Me)^2 residue in desGly-NH_2, d$(CH_2)_5$[Tyr(Me)^2, Thr^4]OVT **(A)** (anti-OT *in vitro* $pA_2 = 7.89$ [30]) by D-2-Nal^2 and by 2Nal^2 gave peptides **1** and **2** with anti-OT *in vitro* $pA_2 = 7.66$ and 7.82, respectively. Thus, while the 2Nal^2 analog of **A** (peptide **2**) retains the full *in vitro* OT antagonism of **A**, the D-2Nal^2 analog of **A** (peptide **1**) exhibits slightly diminished *in vitro* OT antagonism. This is analogous to what was found previously for a D-Thi^2/Thi^2 interchange in **A** (54). Replacement of the Tyr(Me)^2 residue in d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr-NH_2^{9}]OVT (**B**) (anti-OT $pA_2 = 7.63$ [29]) by D-2-Nal^2 and 2Nal^2 to give peptides **3** and **4** led to enhanced *in vitro* OT antagonism in both instances. With anti-OT pA_2 of 7.73 and 8.08, peptides **3** and **4** are more potent than **B**. The D-2Nal^2 and 2Nal^2 analogs of **C** (peptides *5* and *6*), with anti-OT *in vitro* pA_2 values of 7.83 and 7.63, respectively, both exhibit *in vitro* OT antagonism in the same range as **C** (anti-OT *in vitro* pA_2 of 7.75 [54]). d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Eda^9 ← $COCH_2$Ph(4-OH)^{10}]OVT (**D**) exhibits an anti-OT *in vitro* pA_2 of 7.55 [68]. The D-2Nal^2 and 2Nal^2 analogs of

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Table 1 Pharmacological Properties of D-2Nal^2 and 2Nal^2 substituted Oxytocin Antagonists (1–8), the Parent Tyr(Me)^2 peptides (A–D) and Atosiban (Rat Bioassays and Rat OT receptor Affinities)

No	Peptide	Antioxytocic (Anti-OT)			Antivasopressor (Anti-V_{1a})		Antidiuretic activity(V_2)	Rat OTR
		In vitro pA_2[a]	*In vivo* pA_2		ED	pA_2[c]	U mg	K_i(nM)[d]
		NO Mg++	ED[b]	pA_2				
	Atosiban, d[D-Tyr(Et)^2, Thr^4]OVT[e,f,g]	7.71 ± 0.05[h] [M] = 1.94^{-8}	5.95 ± 0.65	7.05 ± 0.05	48.5 ± 2.7 [M] = 7.24^{-7}	6.14 ± 0.02	Antagonist pA_2 5.9	76[i]
A	desGly-NH_2, $d(CH_2)_5$[Tyr(Me)^2, Thr^4]OVT[j]	7.89 ± 0.04 [M] = 1.26^{-8}	1.3 ± 0.2 [M] = 2.0^{-8}	7.69 ± 0.07	23 ± 4 [M] = 3.74^{-7}	6.48 ± 0.08	Antagonist pA_2 5.3	
1	desGly-NH_2, $d(CH_2)_5$[D-2Nal^2, Thr^4]OVT[k]	7.66 ± 0.04 [M] = 2.26^{-8}	8.73 ± 1.19 [M] = 1.3^{-7}	6.89 ± 0.05	17.9 ± 0.2 [M] = 2.67^{-7}	6.59 ± 0.05	pA_2 6.99	0.03
2	desGly-NH_2, $d(CH_2)_5$[2Nal^2, Thr^4]OVT[k]	7.82 ± 0.05 [M] = 1.63^{-8}	4.08 ± 0.51 [M] = 6.09^{-8}	7.23 ± 0.06	2.71 ± 0.21 [M] = 4.05^{-8}	7.40 ± 0.03	0.21 ± 0.02	0.10
B	$d(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr-NH_2^{9}]OVT[l]	7.63 ± 0.07 [M] = 2.43^{-8}	1.0 ± 0.1 [M] = 1.5^{-8}	7.83 ± 0.04	6.6 [M] = 9.8^{-8}	7.02 ± 0.07	~0.015	0.47 ± 0.03
3	$d(CH_2)_5$[D-2Nal^2, Thr^4, Tyr-NH_2^{9}]OVT[k]	7.73 ± 0.04 [M] = 1.9^{-8}	5.37 ± 0.64 [M] = 8.01^{-8}	7.10 ± 0.05	1.36 ± 0.12 [M] = 2.03^{-7}	6.70 ± 0.04	pA_2 7.17	0.11
4	$d(CH_2)_5$[2Nal^2, Thr^4, Tyr-NH_2^{9}]OVT[k]	8.08 ± 0.06 [M] = 8.84^{-9}	7.66 ± 0.58 [M] = 1.14^{-7}	6.94 ± 0.03	2.88 ± 0.22 [M =]4.30^{-8}	7.38 ± 0.04	~0.03	0.09
C	$d(CH_2)_5$[Tyr(Me)^2, Thr^4, Eda^9]OVT[m]	7.75 ± 0.04 [M] = 1.85^{-8}	—		5.5 ± 0.51 [M] = 7.9^{-8}	7.10 ± 0.04	~0.03 also antagonist pA_2 ~ 6	—

(*continued overleaf*)

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Table 1 (*Continued*)

No	Peptide	Antioxytocic (Anti-OT)			Antivasopressor (Anti-V_{1a})		Antidiuretic activity(V_2)	Rat OTR
		In vitro pA_2[a]	*In vivo* pA_2		ED	pA_2[c]	U mg	K_i(nM)[d]
		NO Mg++	ED[b]	pA_2				
5	$d(CH_2)_5$[D-2Nal^2, Thr^4, Eda^9]OVT[k]	7.83 ± 0.096 [M] = 1.73^{-8} very long acting	5.95 ± 0.59 [M] = 8.9^{-8}	7.05 ± 0.04	4.79 ± 0.36 [M] = 7.15^{-8}	7.15 ± 0.03	0.24 ± 0.02	0.15
6	$d(CH_2)_5$[2Nal^2, Thr^4, Eda^9]OVT[k]	7.63 ± 0.07 [M] = 2.49^{-8} very long acting	6.05 ± 0.74 [M] = 9.01^{-8}	7.05 ± 0.05	1.61 ± 0.23 [M] = 2.39^{-8}	7.65 ± 0.06	0.12 ± 0.01	1.60
D	$d(CH_2)_5$[Tyr(Me)^2, Thr^4, Eda^9 ← $COCH_2$(4-OH)^{10}]OVT[n]	7.55 ± 0.03				6.98 ± 0.06	~5.5	
7	$d(CH_2)_5$[D-2Nal^2, Thr^4, Eda^9 ← $COCH_2Ph$(4-OH)^{10}]OVT[k]	<5 very weak mixed agonist/ antagonist			5.51 ± 1.25 [M] = 8.22^{-6}	5.1 ± 0.09	0.066 ± 0.0003	7.8
8	$d(CH_2)_5$[2Nal^2, Thr^4, Eda^9 ← $COCH_2Ph$(4-OH)^{10}]OVT[j]	6.58 ± 0.078 [M] = 2.86^{-7} very long acting			9.01 ± 0.56 [M] = 1.35^{-6}	5.88 ± 0.03	0.014 ± 0.0009	

[a] *In vitro* pA_2 values represents the negative logarithm to the base 10 of the average molecular concentration [M] of antagonist which reduces the response to 2× units of agonist to equal the response 1× of the agonist in the absence of antagonist.
[b] The effective dose (ED) is defined as the dose (in nanomoles/kilogram) of antagonist that reduces the response to 2× units of agonist to equal the responses seen with 1× unit of agonist administered in the absence of antagonist.
[c] Estimated *in vivo* pA_2 values represent the negative logarithms of the 'effective dose' divided by the estimated volume of distribution (67 ml/kg).
[d] K_i = concentration of peptide leading to half- maximal specific binding deduced from competition experiments.
[e] Atosiban is the trade name for [1-deamino, 2-O-ethyl-D-tyrosine, 4-threonine] ornithine-vasotocin.
[f] Original synthesis is reported in ref. [26].
[g] Pharmacological data here are from repeat synthesis: ref. [47].
[h] Mean ± SE.
[i] Data from ref. [58].
[j] Data from ref. [30].
[k] This publication.
[l] Data from ref. [29].
[m] Data from ref. [54].
[n] Data from ref. [68].

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Table 2 Affinity Constants for Human OT and VP V_{1a} Receptors of D-2Nal^2 and 2Nal^2 OT-Antagonists (*1–7*), OTA (B) and Atosiban

	Peptide	hOTR	$hV_{1a}R$	hReceptor Selectivity	hOT Receptor Affinity vs Atosiban	hReceptor Selectivity Vs Atosiban
		K_i(nM)[b]	K_i(nM)[b]	$V_{1a}R$/OTR		
	Atosiban, d[D-Tyr(Et)^2, Thr^4]OVT[a,c,d]	76.4 ± 11.3	5.1 ± 1.3	0.07	1	1
1	desGly-NH_2, d$(CH_2)_5$[D-2Nal^2, Thr^4]OVT[d]	0.17 ± 0.03	1.1 ± 0.7	6.5	449	93
2	desGly-NH_2, d$(CH_2)_5$[2Nal^2, Thr^4]OVT[d]	0.29 ± 0.19	1.3 ± 0.1	4.5	263	64
B	d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr-NH_2^{9}]OVT[d]	0.59	5.26	8.9	129	127
3	d$(CH_2)_5$[D-2Nal^2, Thr^4, Tyr-NH_2^{9}]OVT[d]	0.07 ± 0.01	0.19 ± 0.04	2.7	1091	39
4	d$(CH_2)_5$[2Nal^2, Thr^4, Tyr-NH_2^{9}]OVT[d]	0.14 ± 0.07	0.54 ± 0.28	3.9	546	56
5	d$(CH_2)_5$[D-2Nal^2, Thr^4, Eda^9]OVT[d]	2.5 ± 1.9	3.0 ± 3.8	1.2	31	17
6	d$(CH_2)_5$[2Nal^2, Thr^4, Eda^9]OVT[d]	7.3 ± 4.4	3.2 ± 3.8	0.44	11	6
7	d$(CH_2)_5$[D-2Nal^2, Thr^4, Eda^9 ← $COCH_2$Ph(4-OH)^{10}]OVT[d]	16.0 ± 1.4	39.5 ± 33.4	2.4	5	34

[a] Original synthesis reported in ref. [26].
[b] K_i = concentration of peptide leading to half-maximal specific binding deduced from competition experiments.
[c] Data reported here was obtained with atosiban resynthesized in the Manning laboratory (ref. [47]).
[d] This publication.

Table 3 Re-evaluation of Human OT Receptor and VP V_{1a} Receptor Affinities for Atosiban. Cell Type or Tissue Type in Brackets

Atosiban (d[D-Tyr(Et)^2, Thr^4]OVT)[a]		
hOTR, K_i(nM)[b]	$hV_{1a}R$, K_i(nM)[b]	Reference
76.4 ± 11.3[c] (CHO cells)	5.1 ± 1.3[c] (CHO cells)	This publication
81 (CHO cells)	3.5 (CHO cells)	[58] (Cirillo R, *et al.*)
41 ± 12 (human uterus)	0.4 ± 0.1 (human uterus)	[88] (Maggi M, *et al.*)
34 ± 2.0 (human myometrium)	—	[89] (Jasper JR, *et al.*)
	0.4 (human liver)	[74] (Pettibone DG, *et al.*)
397 ± 87 (Ltk cells)	4.7 ± 0.5 (CHO cells)	[90] (Akerlund M, *et al.*)
895 ± 238 (Ltk cells)	4.7 ± 0.5 (CHO cells)	[62] (Serradeil-Le Gal C, *et al.*)
3.5 ± 0.5 (uterine smooth muscle cells)	—	[75] (Tahara A, *et al.*)

[a,b,c] See corresponding footnotes in Table 2.

D (peptides *7* and *8*), with anti-OT *in vitro* pA_2 values of <5 and 6.58 are significantly less potent than **D** as *in vitro* OT antagonists. Thus with the exception of peptides *7* and *8*, replacement of the Tyr(Me)^2 residue in peptides **A, B** and **C** by D-2Nal^2 and by 2Nal^2 was generally well tolerated with full retention or enhancement of *in vitro* OT antagonism in peptides *2–6* and only a slight decrease in peptide *1*.

Effects on in vivo OT antagonism (Table 1). Because peptides *7* and *8* exhibited significantly lower *in vitro* anti-OT potencies *in vitro*, they were not examined in *in vivo* anti-OT assays. Peptides *1–6* all exhibit *in vivo* OT antagonism. However, replacement of the Tyr(Me)^2 residue in peptides **A** and **B**, by D-2Nal^2 and by 2Nal^2 to give peptides *1–4* (Table 1) appears to have resulted in reductions of *in vivo* OT antagonistic potencies. Whereas, desGly-NH_2, d$(CH_2)_5$[Tyr(Me)^2, Thr^4]OVT (**A**) exhibits an anti-OT *in vivo* $pA_2 = 7.69$ [30], the corresponding pA_2 values for peptides *1* and *2* are 6.89 and 7.23, respectively. Likewise, d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr-NH_2^{9}]OVT (**B**) possesses an anti-OT *in vivo* pA_2 of 7.83 [29]. The respective D-2Nal^2 and 2Nal^2 analogs (peptides *3* and *4*) exhibit anti-OT pA_2 values of 7.1 and 6.94, respectively. However, it should be noted that since the *in vivo* anti-OT potencies of the parent peptides **A** and **B** were assayed by a slightly different quantitative method [29,30], these reductions in *in vivo* anti-OT potencies for peptides *1–4* may not be as substantial as they appear here. Since *in vivo* pA_2 values are not available for peptide **C**, it was not possible to evaluate whether the anti-OT *in vivo* pA_2 values of 7.05 and 7.05 exhibited by the corresponding D-2Nal^2 and 2Nal^2 analogs (peptides *5–6*) are higher or lower than those of peptide **C**.

Relative Effects of D-2Nal^2 and 2Nal^2 Substitutions on *in vivo* OT Antagonistic Potencies (Table 1)

The six new analogs of the OT antagonists **A–C** (peptides *1–6*) constitute a series of three pairs of D-2Nal^2/2Nal^2 substituted OT antagonists. Examination of the ED and pA_2 values of each pair shows that for one of these three pairs, the 2Nal^2 analog is more potent than its D-2-Nal^2 counterpart. Thus peptide *2*,

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the 2Nal^2, analog of **A** with an anti-OT *in vivo* pA_2 of 7.23 is almost twice as potent as peptide *1*, the D-2Nal^2 analog of **A**, $pA_2 = 6.89$. The findings are opposite to those found for the relative effectiveness of the D-Thi^2/Thi^2 interchange in **A** [54].

For the two remaining pairs, peptides *3* and *4* and *5* and *6*, the D-2Nal^2 and 2Nal^2 substitutions resulted in peptides which exhibit virtually identical anti-OT potencies *in vivo*: with anti-OT *in vivo* pA_2 values of 7.10 ± 0.05 and 6.94 ± 0.03, peptides *3* and *4* are statistically undistinguishable. Peptides *5* and *6* exhibit identical anti-OT pA_2 *in vivo* values of 7.05. This contrasts with our previous findings showing that a D-Thi^2 substitution was more effective than an Thi^2 substitution in three of four pairs of peptides in leading to enhanced *in vivo* OT antagonism [54]. The findings with three pairs of D-2Nal^2/2Nal^2 OT antagonists reported here clearly show that the D-2Nal^2 substitution is not more effective than the 2Nal^2 substitution in effecting enhanced *in vivo* antagonism in the rat in any of the three pairs of peptides reported here.

Effects of D-2Nal^2 and 2Nal^2 Substitutions on Antivasopressor Potencies (Table 1)

With anti-V_{1a} pA_2 values of 5.10 and 5.88, peptides *7* and *8* exhibit significantly diminished antivasopressor potencies relative to the parent peptide **D** (anti $pA_2 = 6.98$ [68]). For the three remaining pairs, the 2Nal^2 analogs (peptides *2*, *4* and *6*) with anti V_{1a} pA_2 values of 7.40, 7.38 and 7.65 exhibited potent vasopressor antagonism greatly enhanced relative to the three parent peptides **A, B, C** (anti V_{1a} $pA_2\text{s} = 6.48$ [30], 7.03 [29] and 7.10 [54]). By contrast the three D-2Nal^2 containing peptides, with anti-V_{1a} pA_2 values = 6.59, 6.70 and 7.15 exhibit antivasopressor pA_2 values substantially in the same range as the parent peptides **A, B** and **C**. These findings on the effects of the 2Nal^2/D-2Nal^2 substitutions on the antivasopressor potencies of peptides **A–C** are in striking contrast to those reported for the effects of Thi^2/D-Thi^2 substitution in peptides **A–C**. The Thi^2/D-Thi^2 substitutions brought about in all cases drastic reductions in antivasopressor potencies with respect to the parent peptides **A–C** and thus consequently led to peptides which exhibit significant gains in anti-OT/anti-V_{1a} selectivity in the rat [54].

Comparisons of Properties of OT Antagonists 1–8 in Rat Bioassays with Those of Atosiban (Table 1)

Antioxytocic (in vitro). With *in vitro* pA_2 values in the range 7.63–8.08, peptides *1–6* exhibit anti-OT potencies *in vitro* in the same range as those exhibited by atosiban (anti-OT $pA_2 = 7.71$ [47]). With *in vitro* pA_2 values of <5 and 6.58 peptides *7* and *8* are clearly less potent than atosiban.

Antioxytocic (in vivo). With *in vivo* pA_2 values of 6.94–7.23, the antioxytocic *in vivo* potencies of peptides *1–6* are in the same range as the *in vivo* antioxytocic potency of atosiban in the rat ($pA_2 = 7.05$) [47]. Thus the striking differences in affinities for the rat and human OT receptors between peptides *1–7* and atosiban noted below were very much unexpected.

Antivasopressor. With the exception of peptide *7* and *8*, which have an anti-V_{1a} $pA_2 = 5.10$ and 5.88, peptides *1–6*, with anti-V_{1a} pA_2 ranging from 6.48 to 7.65 are significantly more potent in the antivasopressor assay in the rat than atosiban ($pA_2 = 6.14$) [47].

Rat OT Receptor Affinities for D-2Nal^2/2Nal^2 OT Antagonists are Significantly Higher than Atosiban (Table 1)

Five of the seven D-2Nal^2/2Nal^2 peptides examined in rat OT receptor binding assays exhibit very high subnanomolar affinities (0.03–0.15 nM) for the rat OT receptor. All five peptides *1–5* exhibit strikingly enhanced rat OT receptor affinities with respect to atosiban ($K_i = 76$ nM) [58]. These findings are particularly unexpected considering that in *in vivo* antioxytocic assays in the rat, all six peptides *1–6* are virtually equipotent as atosiban. Furthermore, all have higher affinities for the rat OT receptor than the widely used radioiodinatable OT antagonist peptide (**B**) ($K_i = 0.47$ nM) [29]. Considering that all six peptides *1–6* exhibit greatly diminished anti-OT pA_2 values *in vivo* (6.89–7.23) relative to the OT antagonist (**B**) (anti-OT $pA_2 = 7.83$), these findings are truly surprising and suggest that rat *in vitro* and *in vivo* antagonistic assays do not always correlate with rat OT receptor affinities. These findings point to the need for caution in concluding that high receptor affinities correlate with antagonistic effectiveness *in vivo* in the rat. Whether this also applies to humans for these new OT antagonists needs to be established.

Re-evaluation of Human OT Receptor and Human V_{1a} Receptor Affinities for Atosiban (Table 3)

Table 3 presents the human OT receptor and human V_{1a} receptor affinities for atosiban previously synthesized in this laboratory [47], together with those for atosiban from seven previously reported studies [58,62,74,75,88–90]. With the exception of the high affinity (0.40 nM) for the human V_{1a} receptor on the human uterus and on the human liver reported by Maggi *et al.* [88] and Pettibone *et al.* [74] respectively, the K_i value obtained for the human V_{1a} receptor = 5.1 ± 1.3 nM using CHO cells, is virtually identical to the human V_{1a} receptor affinity values reported by others [58,62,90]. The reported affinities for the human OT receptor for atosiban (Table 3) vary widely, depending on cell type and tissue type; ranging from a high affinity of 3.5 nM on uterine smooth muscle cells [75] to a low affinity of 895 ± 238 nM on LtK cells [62]. Using CHO

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cells the human OT receptor affinity for atosiban was 76.4 ± 11.3 nM. This is very much in the same range as the value of 81 nM recently reported, also with CHO cells, by Cirillo *et al.* [58]. This study utilized the human OT receptor affinity of 76.4 ± 11.3 nM and the human V_{1a} receptor affinity of 5.1 ± 1.3 nM for atosiban for comparison with the corresponding affinities of the new OT antagonists reported here (peptides *1–7*) (Table 2).

Human OT Receptor Affinities and OT/V_{1a} Selectivities of D-2Nal^2/2Nal^2 OT Antagonists are Strikingly Higher than those of Atosiban (Table 2)

With a K_i value of 76.4 nM atosiban has a relatively low affinity for the human OT receptor. By contrast, all of the D-2Nal^2/2Nal^2 OT antagonists reported here and the previously reported OT antagonist **(B)** [29] exhibit high affinities for the human OT receptor (Table 2). With K_i values of 0.17 nM, 0.29 nM, 0.07 nM, 0.14 nM, 0.59 nM peptides *1–4* and **B** exhibit respectively 449, 263, 1091, 546 and 129 times greater affinity for the human OT receptor than atosiban. With K_i values of 1.1 nM, 1.3 nM, 0.19 nM and 0.54 nM, peptides *1–4* have high affinities for the human V_{1a} receptor. These are 2–25 times greater than the human V_{1a} receptor affinity reported here and that others have reported [58,62,90] for atosiban ($K_i = 5.1$ nM) and are also higher than that reported here for **B** [29] ($K_i = 5.26$ nM). However, because of their greatly enhanced affinities for the human OT receptor relative to atosiban, peptides *1–4* and **B** exhibit significant gains in selectivity for the human OT receptor versus the human V_{1a} receptor relative to atosiban. Thus the OT antagonists *1–4* and **B** exhibit, respectively, the following gains in human OT receptor/human V_{1a} receptor selectivity relative to atosiban of $1 = 93$, $2 = 64$, $3 = 39$, $4 = 56$ and $B = 127$. Since peptides *1–4*, and **B** have much higher affinities and selectivities for the human OT receptor than atosiban, they clearly possess a superior and safer pharmacological profile as potential tocolytic agents than atosiban.

Finally, with affinities for the human OT receptor of 0.17 nM, 0.29 nM, 0.07 nM and 0.14 nM, respectively, the OT antagonists *1–4* (Table 2) compare very favorably with the selective peptide OT antagonist barusiban (also known as FE200 440), now in preclinical development [2,118–120]. The reported binding affinities of barusiban for the human OT and VP V_{1a} receptors are, respectively, 0.31 nM/l and 85.3 nM/l [118].

CONCLUSION

In attempts to design OT antagonists with higher affinity and selectivity than atosiban in human receptor assays, we have explored, for the first time in OT-antagonist design, the use of D-2Nal^2/2Nal^2 replacements for Tyr(Me)^2 in the OT antagonists: desGly-NH_2, d$(CH_2)_5$[Tyr(Me)^2, Thr^4]OVT (**A**) [30]; d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Tyr-NH_2^{9}]OVT (**B**) [29]; d$(CH_2)_5$ [Tyr(Me)^2, Thr^4, Eda^9]OVT (**C**) [54] and d$(CH_2)_5$[Tyr(Me)^2, Thr^4, Eda^9 $\leftarrow$ $COCH_2$Ph(4-OH)^{10}]OVT **(D)** [68]. In rat bioassays, six of the resulting eight D-2Nal^2/2Nal^2 analogs, peptides *1–6*, exhibit *in vivo* OT antagonism (EDs = 4.08–8.73 nM) in the same range as atosiban (anti-OT ED = 5.95 nM) (Table 1). However, the anti-V_{1a} potencies of peptides *1–6* (EDs = 1.61–17.9 nM) are significantly higher than the anti-V_{1a} potency of atosiban (ED = 48.5 nM). Thus in rat bioassays, all six new OT antagonists are less selective for OT versus V_{1a} receptors than atosiban. In rat OT receptor binding assays, peptides *1–6* exhibit much higher affinities than atosiban. Because the literature values for atosiban affinities for the human OT receptor and human V_{1a} receptor [58,62,74,88,89,90] vary so widely, they were reexamined (Table 3). The human OT receptor and human V_{1a} receptor affinities for atosiban, (previously synthesized in our laboratories [47]) = 76.4 nM and 5.1 nM, were used as standards for comparisons with the human OT receptor and human V_{1a} receptor affinities of peptides *1–7*. Surprisingly, peptides *1–6* exhibit strikingly enhanced affinities for the human OT receptor relative to atosiban; particularly peptides *1–4*, which possess respectively 449, 263, 1091 and 546, times greater affinity for the human OT receptor than atosiban (Table 2). All six new OT antagonists possess high affinities for the human V_{1a} receptor, all higher than those reported here for atosiban. However, due to their greatly enhanced affinities for the human OT receptor relative to atosiban, four of these OT antagonists peptides *1–4*, exhibit gains in selectivity for the human OT receptor versus the human V_{1a} receptor that are 93, 64, 39 and 56 times greater than that exhibited by atosiban.

Since the OT antagonists *1–4* and **B** [29] exhibit strikingly higher affinities and selectivities for the human OT receptor than atosiban, they clearly possess a superior and safer pharmacological profile as potential tocolytic agents than atosiban [2–6,70–73]. They also compare very favorably with the selective peptide OT antagonist barusiban [FE200 440) now in preclinical development [2, 118–120). They are thus promising candidates for development as potential tocolytic agents in humans.

Finally, these findings offer promising clues for the design of more potent and selective peptide OT antagonists for development as potential tocolytic agents for the prevention of premature labor, for use as pharmacological tools and new radioligands and as possible diagnostic and therapeutic agents for the treatment of tumors which express OT receptors [121].

EXPERIMENTAL PART

All reagents used were analytical grade. The Boc-Orn[Z(2CL)] and Boc-Tyr(Bzl)-resins were prepared by the cesium salt

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method [122]. The 4-hydroxyphenylacetic acid was purchased from Aldrich Chemical Co., Inc. (St Louis, MO). The β-(4-methoxybenzyl)mercapto-β,β-pentamethylenepropionic acid was purchased from Bachem Bioscience, Inc. (King of Prussia, PA). The Boc-2Nal and Boc-D-2Nal were purchased from Synthetech, Inc. (Albany, OR). All other amino acid derivatives were purchased from Bachem (Torrance, CA) or Chem-Impex International, Inc. (Wood Dale, IL). For HF cleavage/deprotection, the HF Apparatus (Immuno-Dynamics Inc., Division of Peptide Chemistry, La Jolla, CA) was used. TLC was run on precoated silica gel plates (60F-254, E. Merck) with the following solvent systems: (a) 1-butanol : AcOH : H_2O (4 : 1 : 5, upper phase) (b) 1-butanol : AcOH : H_2O (4 : 1 : 1); (c) 1-butanol : AcOH : H_2O: pyridine (15 : 3 : 3 : 10); (d) 1- butanol : AcOH : H_2O (4 : 1 : 2); (e) 1-butanol : AcOH : H_2O (2 : 1 : 1); (f) chloroform : methanol (7 : 3). Loads of 10–15 µg were applied and chromatograms were developed at a minimal length of 10 cm. The chlorine gas procedure for the KI-starch reagent was used for detection [93]. Optical rotations were measured with a Rudolph Autopol III polarimeter. Analytical HPLC was performed on a Waters 810 instrument under the following conditions: 90 : 10 to 30 : 70 0.05% aqueous TFA : 0.05% TFA in CH_3CN, linear gradient over 60 min at 1.0 ml/min ($\lambda = 210$ nm), on a Microsorb C_{18} column (Rainin Instrument Co., Inc.). All peptides were at least 95% pure. ESMS were done by the University of Oklahoma Health Science Center Molecular Biology Resource Facility on PE Sciex Q-STAR Quadropole TOF Mass Spectrometer using 50 : 50 CH_3CN/H_2O with 0.5% AcOH as a solvent. ESMS spectra of the free peptides were in agreement with the composition of each peptide.

Solid-phase Synthesis Procedures

Peptides *1–8* (Table 5) were synthesized using the standard Merrified method [91–93] with the modifications previously described [54,94–96]. The protected retro-modified peptides VII and VIII (Table 4) were prepared by coupling the protected peptides V and VI (Table 4) with 4-hydroxyphenylacetic acid by the DCC/HOBt procedure [97], as described in [98]. For the synthesis of protected peptidyl resins, starting with 1.0 mM of Boc-Orn[Z(2Cl)]-resin and with 0.6 mM Boc-Tyr(Bzl)-resin, seven and eight cycles respectively of deprotection, neutralization and coupling, were performed. For deprotections, a HCl (1 M)/AcOH mixture was used [54,94–96]. Neutralizations were carried out with 10% Et_3N/CH_2CL_2. Coupling reactions were mediated primarily by DCC/HOBt [97] except for the Boc-Asn which was incorporated as its 4-nitrophenyl ester [99] in DMF. The acylpeptide resins were cleaved using the following procedures: (i) ammonolysis in methanol with DMF extractions [95,100] to give the protected peptide amides III, IV (Table 4); (ii) aminolysis with Eda in methanol with DMF extraction [98,101] to give the protected Eda peptides V, VI (Table 4); acidolysis with HF/anisole [93] for simultaneous cleavage of the peptides from the resin and their deprotection to give the free peptides *1* and *2* (Table 5). The TFMSA procedure (a mixture of TFMSA, TFA, thioanisole and 1,2-ethanedithiol) [102,103] was used to deblock the protected precursors III–VIII. The resulting disulfhydryl compounds from the HF treatment and the TFMSA treatment were oxidatively cyclized with $K_3[Fe(CN)_6]$ [104] using the modified reverse procedure [105]. The free peptides were desalted and purified by gel filtration on Sephadex G-15 and Sephadex LH-20 mainly in a two-step procedure [106] using 50% AcOH and 2 M AcOH as eluents, respectively, as previously described [54,94–96]. When necessary, an additional purification on Sephadex G-15 or/and Sephadex LH-20 with 0.2 M AcOH as eluent was carried out. The purity of the free peptides *1–8* (Table 5) was checked by TLC, HPLC and ESMS. The physicochemical data for the free peptides *1–8* are given in Table 5.

Table 4 Physicochemical Properties of Protected Peptides III–VIII

No	Peptide	Yield %[b]	mp °C	$[\alpha]_D^{25}$ c = 1.0, DMF	TLC, R_f[c] a	b	c	d	f
III	$d(CH_2)_5$(Mob)-D-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2Cl)]-Tyr(Bzl)-NH_2	51.5	203–205	−7.0	0.94	0.75	0.79	0.94	0.96
IV	$d(CH_2)_5$(Mob)-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2Cl)]-Tyr(Bzl)-NH_2	37.8	223–225	−25.2	0.87	0.74	0.78	0.96	0.96
V	$d(CH_2)_5$(Mob)-D-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2Cl)]-Eda	88.9	190–192	−13.3	0.54	0.36	0.65	—	0.94
VI	$d(CH_2)_5$(Mob)-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2Cl)]-Eda	68.8	212–214	−23.4	0.54	0.36	0.65	—	0.94
VII	$d(CH_2)_5$(Mob)-D-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2Cl)]-Eda ← $COCH_2Ph$(4-OH)	77.6	187–189	−7.7	0.95	0.78	0.85	0.92	—
VIII	$d(CH_2)_5$(Mob)-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2Cl)]-Eda ← $COCH_2Ph$(4-OH)	87.9	210–212	−17.0	0.93	0.77	0.78	0.93	—

[a] The protected peptides III–VIII are the immediate protected precursors for the peptides *3–8* given in Tables 1 and 2.
[b] Yields are calculated on the basis of the Boc-Orn[Z(2Cl)], or the Boc-Tyr(Bzl) content on the resin except for VII and VIII, which were calculated on the theoretical yield expected from the solution coupling.
[c] Solvent systems are described in the Experimental.

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β-(4-methoxybenzyl)mercapto-β,β-pentamethylenepropionyl-D-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn(Z(2CL))-Tyr(Bzl)-NH_2 (III, Table 4)

Boc-Tyr(Bzl)-resin (0.8 g, 0.6 mM) was converted to the protected acyloctapeptidyl resin in eight cycles of deprotection, neutralization and coupling (mediated by DCC/HOBt or active ester) with Boc-Orn[Z(2CL)], Boc-Pro, Boc-Cys(Mob), Boc-Asn-ONp, Boc-Thr(Bzl), Boc-Ile, Boc-D-2Nal and β-(4-methoxybenzyl)mercapto-β,β-pentamethylenepropionic acid, respectively, by the manual method of solid-phase synthesis as previously described [54,94–96]. The protected peptide III was split from the resin by ammonolysis with DMF extraction [95,100] as follows. The resin was suspended in anhydrous methanol (50 ml) in a 250 ml round bottom flask, cooled at ca. −70 °C and NH_3 (ca. 30 ml) was bubbled through the suspension for ca. 30 min. The tightly stoppered flask was kept at room temperature in the hood for 2 days. The suspension was recooled, the stopper removed and the methanol and NH_3 allowed to evaporate at room temperature. The protected peptide was extracted with warm (ca. 50 °C) DMF (ca. 30 ml) and precipitated with warm (ca. 50 °C) water (ca. 500 ml). Following overnight storage at 4 °C, the product was collected, dried *in vacuo* over P_2O_5, reprecipitated from warm (ca. 50 °C) DMF (ca. 5 ml) with ethyl ether (ca. 300 ml), collected and dried *in vacuo* over P_2O_5 to give the protected acyloctapeptide amide III Table 4). This procedure was used for the preparation of the protected peptide amide IV (Table 4) by replacing Boc-D-2Nal with Boc-2Nal in the penultimate coupling step.

β-(4-methoxybenzyl)mercapto-β,β-pentamethylenepropionyl-D-2-Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn(Z(2CL))-Eda (V, Table 4)

Starting from $d(CH_2)_5$(Mob)-D-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2CL)]-resin (2.86 g, 1 mM), prepared as described above, the protected Eda-peptide V was obtained by aminolysis with Eda/MeOH and DMF extraction [98,101] as follows. The protected peptidyl resin was replaced in a 250 ml round bottomed flask, 75 ml of anhydrous methanol was added, the suspension was cooled at ca. 0 °C and 30 ml of ethylenediamine (Eda, 99.5 + %, redistilled; Aldrich) was added with stirring. After 30 min, the cooling bath was removed and the suspension stirred at room temperature for 2 days. The solvents were removed on a rotary evaporator and the protected peptide V (Table 4) was further extracted and purified as described above for III). The protected Eda-peptide VI (Table 4) was prepared by the same procedure starting from $d(CH_2)_5$(Mob)-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2CL)]-resin.

β-(4-methoxybenzyl)mercapto-β,β-pentamethylenepropionyl-D-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn(Z(2CL))-Eda←$COCH_2$Ph(4-OH) (VII, Table 4)

The retro-modified peptide VII was synthesized by a DCC/HOBt mediated coupling [98] of protected peptide V and 4-hydroxyphenylacetic acid as follows. To a cooled (0 °C) solution of 4-hyroxyphenylacetic acid (0.19 g, 1.25 mM) and HOBt (0.26 g, 1.9 mM) in 3 ml of anhydrous DMF was added 0.95 ml (1.9 mM) of a 2 M solution of DCC in DMF. The reaction mixture was stirred for 1 h, whereupon the dicyclohexylurea (DCU) was removed by filtration. The filtrate was added to a solution of $d(CH_2)_5$(Mob)-D-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2CL)]-Eda (V) (0.54 g, 0.35 mM) in 2 ml anhydrous DMF. DIPEA was added to give a pH ≈ 7.5. After the mixture was stirred for 18 h at room temperature (TLC monitoring), MeOH (20 ml) was added followed by ether (250 ml). The precipitated product was collected following overnight storage at 4 °C. Washing with warm MeOH gave the required protected peptide VII, 0.45 g, yield 77.6% (Table 4). The protected retromodified peptide VIII (Table 4) was prepared by the same procedure utilizing the protected Eda peptide VI.

β-mercapto-β,β-pentamethylenepropionyl-D-2Nal-Ile-Thr-Asn-Cys-Pro-Orn-Tyr-NH_2 ($d(CH_2)_5$(D-$2Nal^2$,Thr^4,Tyr-$NH_2{}^9$)OVT) (3, Table 5)

The deprotection of the acyloctapeptide III (Table 4) was performed by the standard TFMSA procedure [102,103] as follows. The protected peptide III (150 mg) was placed in a round bottom flask with a stirring bar and 0.5 ml of thioanisole and 0.25 ml of 1,2-ethanedithiol were added. The mixture was chilled with an ice bath and 5 ml of TFA was added. After stirring for 5 min at 0 °C, 0.5 ml of TFMSA was added slowly, dropwise with vigorous stirring. Following additional stirring for 30 min at room temperature, 50 ml of anhydrous ether was added dropwise. The precipitated peptide was filtered, washed with ether and dried overnight *in vacuo* over P_2O_5 and NaOH. Oxidation of the resulting residue of the deblocked disulfhydryl precursor of peptide *3* was performed with potassium ferricyanide [104] by the modified reverse procedure [105] as follows. The residue (ca. 120 mg) was dissolved in degassed 50% AcOH (25 ml) and the solution diluted with 50 ml of H_2O. The peptide solution was added dropwise with stirring over a period of 15–30 min to an 600 ml aqueous solution which contained 20 ml of a 0.01 M solution of potassium ferricyanide. Meanwhile, the pH was adjusted to ca. 7.0 with concentrated ammonium hydroxide. The yellow solution was stirred for an additional 20 min. Following oxidation, the free peptide (*3*, Table 5) was isolated and purified as follows: after acidification with AcOH to pH 4.5 and stirring for 20 min with an anion exchange resin (Bio-Rad, AG 3 × 4, $C1^-$ form, 5 g damp weight), the suspension was slowly filtered and washed with 0.2 M AcOH (3 × 30 ml), the combined filtrate and washings were lypophilized. The resulting powder was desalted on a Sephadex G-15 column (110 × 2.7 cm) eluting with aqueous AcOH (50%) with a flow rate of 5 ml/h [106]. The eluate was fractionated and monitored for absorbance at 254 nm. The fractions making up the major peak were checked by TLC, pooled and lyophilized. The residue was further subjected to two consecutive gel filtrations on Sephadex LH-20 (100 × 1.5 cm) eluting with aqueous AcOH (2 M and 0.2 M) respectively, with a flow rate of 4 ml/min. The peptide was eluted in a single-peak (absorbance at 254 nm). Lyophilization of the pertinent fractions gave the desired OT antagonist *3* (Table 5). With minor modifications, the same procedure was utilized for the deprotection, oxidation and purification of protected peptides IV–VIII (Table 4) to give the OT antagonists *4*–*8* (Table 5).

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Table 5 Physicochemical Properties of Free Peptides *1–8*

No	Peptide	Yield (%)[a,b]	$[\alpha]_D^{25}$ c = 0.1′ 1N,AcOH	TLC, R_f[c] a	b	c	d	e	HPLC[d] t_R (min)	Formula	MW(Calc) MS(Found)
1	desGly-NH$_2$, d(CH$_2$)$_5$[D-2Nal^2, Thr^4]OVT	25.9	−55.0	0.34	0.24	0.10	0.42	0.38	39.5	$C_{48}H_{69}O_{11}N_9S_2$	1012.3 1012.3
2	desGly-NH$_2$, d(CH$_2$)$_5$[2Nal^2, Thr^4]OVT	21.2	−87.0	0.31	0.19	0.16	0.41	0.36	39.7	$C_{48}H_{69}O_{11}N_9S_2$	1012.3 1012.3
3	d(CH$_2$)$_5$[D-2Nal^2, Thr^4, Tyr-NH$_2$^{9}]OVT	10.0	−66.0	0.36	0.24	0.41	0.49	0.49	38.4	$C_{57}H_{80}O_{12}N_{11}S_2$	1174.4 1174.5
4	d(CH$_2$)$_5$[2-Nal^2, Thr^4, Tyr-NH$_2$^{9}]OVT	6.0	−44.0	0.38	0.26	0.42	0.52	0.51	37.8	$C_{57}H_{80}O_{12}N_{11}S_2$	1174.4 1174.5
5	d(CH$_2$)$_5$[D-2Nal^2, Thr^4, Eda^9]OVT	13.7	−38.0	0.22	—	—	0.37	0.44	36.2	$C_{50}H_{75}O_{10}N_{11}S_2$	1054.4 1054.5
6	d(CH$_2$)$_5$[2Nal^2, Thr^4, Eda^9]OVT	13.7	−80.0	0.41	—	—	0.36	0.42	35.3	$C_{50}H_{75}O_{10}N_{11}S_2$	1054.4 1054.5
7	d(CH$_2$)$_5$[D-2Nal^2, Thr^4, Eda^9 ← COCH$_2$Ph(4-OH)^{10}]OVT	14.5	−35.0	0.29	—	—	0.40	0.53	46.8	$C_{58}H_{81}O_{12}N_{11}S_2$	1188.5 1188.7
8	d(CH$_2$)$_5$[2Nal^2, Thr^4, Eda^9 ← COCH$_2$Ph(4-OH)^{10}]OVT	7.7	−49.0	0.24	—	—	0.39	0.52	44.7	$C_{58}H_{81}O_{12}N_{11}S_2$	1188.5 1188.6

[a] For peptides *1* and *2*, yields are based on the amount of the protected peptide on the resin in the cleavage-oxidation step in each case and are uncorrected for acetic acid and water content.
[b] For peptides *3–8*, yields are based on the amount of the protected peptide (III–VIII) in the deprotection-oxidation step in each case and are uncorrected for acetic acid and water content.
[c] Solvent systems and conditions are given in the Experimental.
[d] All peptides were at least 95% pure. For elution, a linear gradient; 90 : 10 to 30 : 70 (0.05% aqueous TFA: 0.05% TFA in CH_3CN) over 60 min with flow rate of 1.0 ml/min was applied.

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β-mercapto-β,β-pentamethylenepropionyl-D-2Nal-Ile-Thr-Asn-Cys-Pro-Orn-OH (desGly-NH_2, $d(CH_2)_5$(D-2-Nal^2,Thr^4)OVT) (1, Table 5)

Starting from $d(CH_2)_5$(Mob)-D-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2CL)]-resin (0.28 g, 0.1 mM) prepared as described above, the free peptide *1* was obtained by simultaneous cleavage/deprotection utilizing the standard HF/anisole procedure [93]. The protected peptidyl resin was placed in the reaction vessel of the HF apparatus containing a stirring bar. Dry anisole (0.5 ml) was added, the mixture was cooled with a dry ice-ethanol bath and 5 ml of liquid HF was redistilled into the reaction vessel. The dry ice bath was replaced with an ice bath and the mixture was stirred for 45 min. After the reaction was completed, the HF was evaporated *in vacuo*, the peptide was extracted from the resin with (3 × 2 ml of TFA) precipitated with ether, filtered and dried overnight *in vacuo* over P_2O_5 and NaOH. The reoxidation of the resulting residue of the deblocked disulfhydryl precursor of OT antagonist *1* (by the reverse procedure [105]) and its purification was performed as described above for OT antagonist *3* to give the OT antagonist *1* (Table 5).

Starting from $d(CH_2)_5$(Mob)-2Nal-Ile-Thr(Bzl)-Asn-Cys(Mob)-Pro-Orn[Z(2CL)]-resin, the same approach was used for cleavage/deprotection, oxidation and purification of the OT antagonist *2* Table 5.

Acknowledgements

We thank Ms Ann Chlebowski for expert help in the preparation of this manuscript. Supported by NIH Grant GM-25280 and the Institut National de la Santé et de la Recherche Médicale.

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