Journal of Medicinal Chemistry

Article

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J. Med. Chem., **Just Accepted Manuscript** • DOI: 10.1021/jm500365s • Publication Date (Web): 29 May 2014

Journal of Medicinal Chemistry is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

New, Potent and Selective Peptidic Oxytocin Receptor Agonists

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ABSTRACT

Mothers of preterm babies frequently have difficulty establishing or maintaining lactation, thought to be due to interference with the milk ejection reflex. Administration of exogenous oxytocin can produce alveolar contraction and adequate breast emptying resulting in establishment of successful lactation. The natural hormone oxytocin is not receptor-selective and may cause hyponatremia via V_2 receptor mediated antidiuresis. We have designed a series of potent oxytocin analogues containing N-alkylglycines in position 7 with excellent selectivity versus the related V_{1a}, V_{1b}, and V_2 vasopressin receptors and short half-life: agonists **31** ([2-ThiMeGly^7]dOT), **47** (carba-6-[Phe^2,BuGly^7]dOT), **55** (carba-6-[3-MeBzlGly^7]dOT) and **57** (carba-1-[4-FBzlGly^7]dOT) have EC_{50} values at the hOTR < 0.1 nM, selectivity ratios versus related human vasopressin receptors > 2000, IC_{50} at $hV_{1a}R$ > 500 nM, and total clearance in rats in the range of 60-80 mL/min/kg. Compound **57 (**FE 202767) is currently in clinical development for the treatment of preterm mothers requiring lactation support.

INTRODUCTION

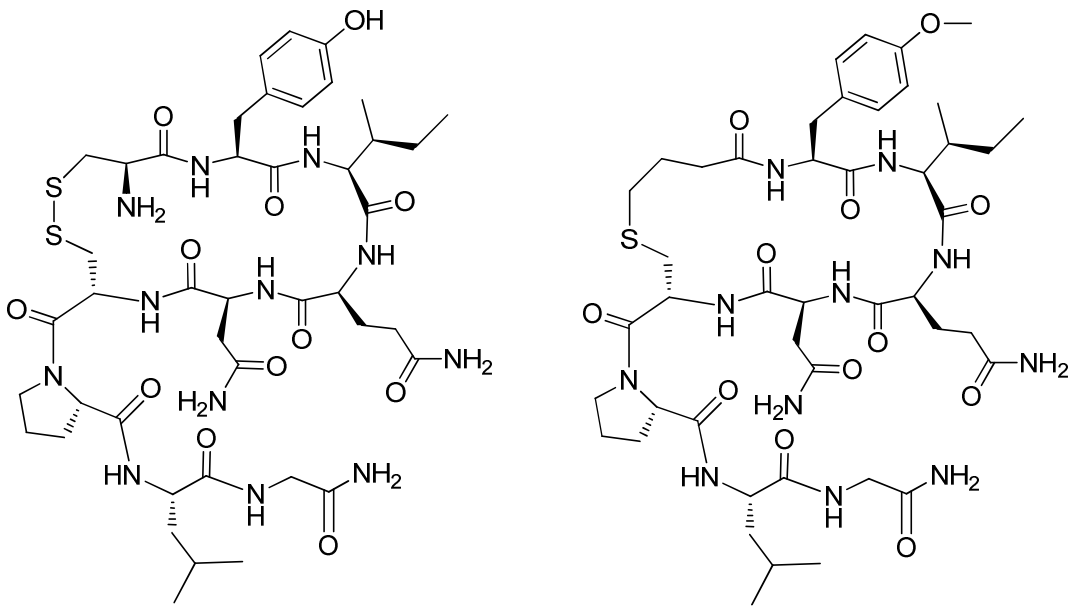
Of the four million births annually in the U.S.A, approximately 12% are preterm (born prior to 37 weeks) with 1.4% being very low birthweight;[1] incidences of 4 - 6 % are reported in some European countries, and higher rates occur in less developed countries.[2] Babies born preterm are at risk for a variety of complications resulting in morbidity and mortality and in most cases require specialized treatment in hospital and may be subject to adverse long term health and neurodevelopmental outcomes.[2] It is well established that early feeding of the preterm infant with mother's own milk has significant advantages over formula, and even over pasteurized donor milk, with lower incidence of necrotizing enterocolitis, sepsis, infections, and readmissions[3, 4] and shorter length of hospital stay.[5] However, a significant proportion of preterm mothers experience difficulty or fail to establish successful lactation.[6] One contributing factor is that preterm infants are typically treated in an intensive care unit and cannot nurse at the breast, requiring that their mothers initiate and maintain lactation by manual and mechanical milk expression. Although milk expression and frequent emptying of the breast may not be necessary to initiate lactation, it is necessary for its successful maintenance. The hormone oxytocin (OT), **1**, is released into the circulation by the posterior pituitary in short pulses in response to suckling and acts on the OTR on myoepithelial cells surrounding the breast alveoli, causing them to contract and empty their contents (the "milk ejection reflex"). It is believed that stress and other factors are responsible for the inhibition of oxytocin release from the pituitary resulting in incomplete removal of milk and eventual down-regulation of milk production.[7] Administration of exogenous oxytocin by the intranasal route to preterm mothers produced significant increase in milk production in one study,[8]

but not in another at a lower dose.[6] A nasal spray containing oxytocin at a concentration of 40 IU/mL, Syntocinon, is approved for lactation support in a small number of European countries. However, **1** is not selective versus the related receptors hV_{1a} and hV_2 (Table 1). It is markedly potent at the hV_2 receptor of the kidneys to produce antidiuresis, which may result in inappropriate water conservation[9] and hyponatremia. Guidelines for OT use have been created to prevent hyponatremia during labor induction[10] and several cases of severe hyponatremia by use of the nasal spray have been reported.[11-13]

We set out to design a new peptidic oxytocin agonist which would be safe and effective for lactation support in preterm mothers. As the compound would ideally be self-administered multiple times a day before each breast pumping session, the intranasal route of administration appeared the most convenient. Because only low doses can be absorbed by this route, the agonist should have high potency to be efficacious. It should also be selective versus the hV_2 receptor in order to minimize the risk of hyponatremia in the event of excessive or too frequent drug use by patients. Eliminating activity at the V_{1a} receptor would prevent local vasoconstriction at the site of application, which would be exposed to a relatively high concentration of drug in the administered solution. Finally, although the effects of peripheral V_{1b} receptors are less well characterized, we decided to introduce selectivity against this receptor in order to avoid potential peripheral effects on the hypothalamic-pituitary-adrenal axis or on the pancreas.[14] The compound should have high clearance and very short half-life to mimic the very short duration of contractile action of **1** on the alveolar myoepithelium sufficient to cause milk let-down while avoiding accumulation of drug in the circulation after multiple daily administrations. Thus, we aimed at obtaining compounds with human clearance similar to that of

oxytocin, around 20 mL/min/kg.[15] Consideration of basic allometric relationship between body weight and clearance suggested that the desired compounds should have much higher clearance in rats (see Results section).

Extensive research has been focused on identifying OTR antagonists suitable for treatment of premature labor,[16-20] while efforts to design new, clinically useful OTR agonists have been relatively sparse[21, 22] and have resulted in a sole synthetic peptide analogue of **1,** carbetocin (carba-1-[Tyr(Me)^2]dOT, **2**, Fig. 1) approved in several countries for the treatment of postpartum bleeding.[23, 24] The Gly^7 modified analogues proposed by Manning's group[25] are used as rOTR-selective pharmacological tools. Recently, orally available, non-peptidic antagonists[26, 27] and agonists[28] for the OTR have been discovered.



1 (OT) **2** (carbetocin)

Figure 1. Structures of **1** (OT) and, **2** (carbetocin)

The structure of **1** (Fig.1) was elucidated by du Vigneaud and his research team in the early 1950's,[29, 30] and it was shown to be a nonapeptide comprising a 20-membered ring, formed by a disulfide bridge between the Cys^1 and Cys^6 residues, and a C-terminal tripeptide amide. The cyclic part of the hormone molecule is believed to interact with the second extracellular loop and upper portions of transmembrane domains 3, 4 and 6 of the OTR whereas the C-terminal part of **1** binds to the first extracellular loop and the N-terminal domain of the receptor.[31-33] A similar binding profile has been postulated for **2** but not for antagonist molecules.[34]

Indeed, the biological activity of **1** has been shown to be very sensitive to changes in the C-terminal part of the molecule. OT analogues with Gly^7 or Sar^7 displayed an improved selectivity profile versus the related vasopressin receptors[25, 35, 36] while the introduction of bulkier and conformationally restricted residues in position 7 resulted in compounds with different degrees of antagonistic activity.[37-39] The replacement of the Leu^8 residue with basic amino acids[17] or the Gly^9 residue with a variety of natural or unnatural amino acids[40] led to potent OTR antagonists when combined with D-Aaa^2 and other modifications.[41, 42]

Here we report the synthesis, *in vitro* pharmacological and pharmacokinetic evaluation of a series of peptidic OT agonists with N-alkylglycine residues (-NR^2-CH_2-CO-) in position 7 as the key modification which resulted in the discovery of potent and selective analogs with high systemic clearance that could be used clinically for lactation support. All new compounds were prepared as position 1 desamino analogs containing either an unaltered disulfide bridge or its monocarba modifications. Some compounds were also prepared with a Phe^2 modification known to improve the selectivity profile of **1**.[43, 44]

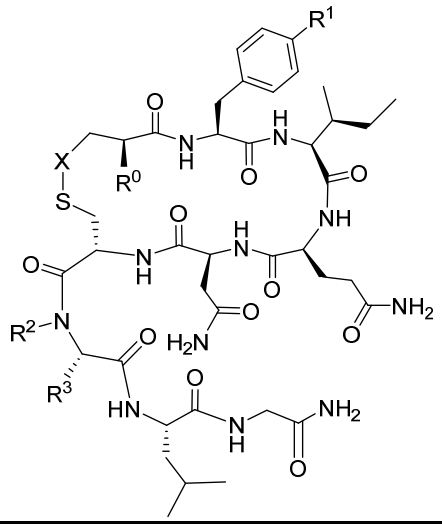
RESULTS AND DISCUSSION

The peptides were assembled on resin by standard solid phase peptide synthesis methods using either Fmoc or hybrid Boc/Fmoc strategies. To prepare compound **5**, its cisHyp[7] analogue was assembled and the hydroxyl group was replaced on resin with the S-acetyl moiety under Mitsunobu reaction conditions.[45] After cleavage and cyclization, the acetyl group was removed by hydrolysis[46] and the resulting sulfhydryl function was methylated *in situ*. For compounds **9-50**, **52-56**, **58**, **59** and **61-65** the N-alkylglycine residues in position 7 were introduced by a two-step procedure used in the chemistry of peptoids:[47] the resin-bound C-terminal Leu-Gly dipeptide was acylated with bromoacetic acid and the product treated with an appropriate primary amine. Analogues **51, 57** and **60** were prepared with Fmoc-N-alkylglycines synthesized by a modified literature procedure.[48] Peptides **1**, **3** and **9-36** were cleaved from the Rink amide resin and cyclized with iodine in aqueous TFA. Analogues **2**, **4**, **6-8** and **51-65** were cleaved from the Rink amide resin and cyclized in DMF with HBTU/DIPEA. Compounds **37-50** were cyclized on MBHA resin with BOP/DIPEA and cleaved with HF/anisole. All analogues were purified by preparative HPLC as described in the experimental section.

Compounds **1**, **2,** and desaminooxytocin (**3**) were chosen as reference compounds for this study. To assess potency and selectivity the compounds were tested in *in vitro* transcriptional reporter gene assays at the human OT/vasopressin family of receptors as described in the Experimental Section. Compounds **1-3** were tested as agonists at the OTR, $V_{1a}R$, $V_{1b}R$ and V_2R receptors, and as antagonists at the $V_{1a}R$, $V_{1b}R$ and V_2R. Consistent with published data,[49, 50] **3** exhibited higher potency and improved selectivity

for the OTR as compared to **1**. Compound **2**[51] was less potent at the OTR, but more selective versus related receptors than **3**. Analogues **1-3** did not exhibit antagonism at the $hV_{1a}R$ (Table 1), $hV_{1b}R$ and hV_2R (data not shown). To assess their pharmacokinetic properties the compounds were injected by the intravenous route to male Sprague-Dawley rats, plasma concentrations determined by LC-MS, and the values of systemic clearance calculated as described in the Experimental Section. The CL value of **1** obtained, 21±2.2 mL/min/kg, is similar to that reported previously in rats by Lundin.[52] The CL of reference compounds **2** and **3** was only slightly higher (Table 1).

Table 1. Structure and pharmacological profiles of reference compounds **1-3** and initial leads **4-8**.



analogue	Structure[a]				*In vitro* biological activity								Rat iv clearance (mL/min/kg)
					EC_{50} receptor (nM)				Selectivity vs. receptor[b]			IC_{50} (nM)[c]	
	X	R^1	R^2	R^3	hOT	hV_2	hV_{1a}	hV_{1b}	hV_2	hV_{1a}	hV_{1b}	hV_{1a}	
1[a]	S	OH	-$(CH_2)_3$-		2.3	7.3	10	240	3	4	100	>10000[d]	21
2[a]	CH_2	OMe	-$(CH_2)_3$-		0.70	170	41[e]	>10000[f]	240	58	>14000	>10000[d]	22
3[a]	S	OH	-$(CH_2)_3$-		0.10	3.5	21[e]	180	35	210	1800	>10000[d]	27
4[a]	CH_2	OMe	-CH_2-CH(OMe)-CH_2-[g]		0.98	690	>10000[f]	>10000	700	>10000	>10000	1300	21

5[a]	CH_2	OMe	-CH_2-CH(SMe)-CH_2-[g]		0.82	670[e]	>1000[h]	>10000[f]	810	>1200	>12000	670	38
6[a]	CH_2	Et	-CH_2-CH(OH)-CH_2-[i]		0.06	40	>1000[h]	1100	660	>16000	18000	17	25
7[a]	CH_2	Et	-CH_2-CH(OMe)-CH_2-[g]		0.21	450	>100	>10000[f]	2100	>470	>47000	55	20
8[a]	CH_2	OMe	H	H	0.37	450[e]	>1000[h]	>10000	1200	>2700	>27000	1400	45
AVP					22	0.05	0.24	4.3	0.002	0.01	0.20	NT[j]	NT[j]
dDAVP					72[e]	0.20	>1000[h]	11	0.003	>13	0.15	NT[j]	7.5

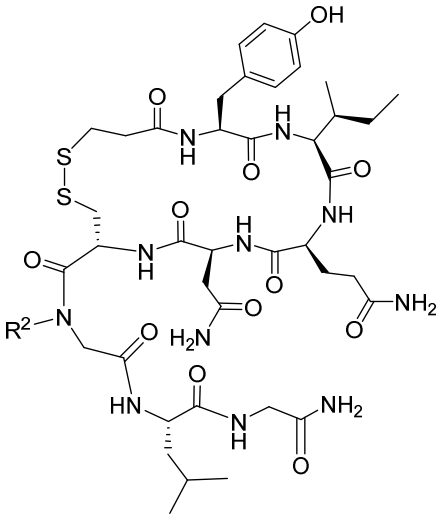
[a] For compound **1** R^0 is NH_2 and for compounds **2-8** R^0 is H; [b] EC_{50} (receptor)/EC_{50} (hOTR) ratio. If no significant agonism was observed at the highest concentration tested selectivity is >highest conc. tested/EC_{50} (hOTR); the selectivity ratios are rounded down to the nearest values with two significant figures; [c] IC_{50} at hV_{1a} receptor stimulated with 2 nM AVP; [d] no significant antagonism up to 10000 nM, the highest concentration tested; [e] partial agonist, efficacy < 70%; [f] no significant agonism up to 10000 nM, the highest concentration tested; [g] the middle carbon atom is of R configuration; [h] no significant agonism up to 1000 nM, the highest concentration tested; [i] the middle carbon atom is of S configuration; [j] not tested.

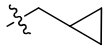
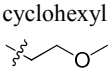
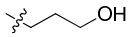
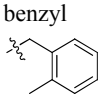
Using compound **2** as a starting point, our initial effort was focused on obtaining compounds with high potency at the hOTR, improved *in vitro* selectivity versus the other human receptors, and higher CL in rats by introducing a substituent on the pyrrolidine ring in position 7. Compounds were prepared with O-methyl tyrosine (**4**, **5**, R^1 = OMe) or 4-ethylphenylalanine (**6**, **7**, R^1 = Et) in position 2. The addition of a substituent in position 4 of the Pro^7 pyrrolidine ring led to analogs **4**-**7** with no activity at the hV_1R, and somewhat improved receptor selectivity profile versus the hV_2R as compared to **2**, but with only modest if any increase in rat CL.

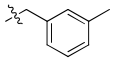
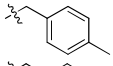
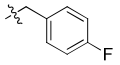
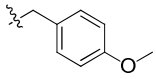
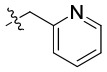
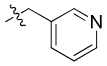
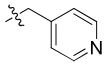
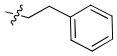
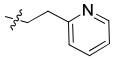
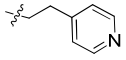
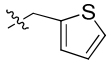
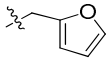
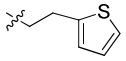
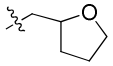
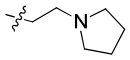
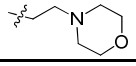
Both the Gly^7 and the Sar^7 modifications of OT have been reported to yield analogues with good potency and selectivity for the OTR.[35, 36, 53, 54] Indeed, when we prepared compound **8**, the Gly^7 analogue of **2**, it displayed an *in vitro* selectivity profile comparable to those of compounds **4-7,** but interestingly showed substantially higher CL in male rats (45 mL/min/kg) than compounds **1-7**. Preliminary work in our laboratories indicated that replacement of Pro^7 by various N-alkyl α-substituted amino acids was not

well tolerated (data not shown). Therefore, a new drug discovery program was initiated based on **3**, which is more potent at the hOTR than **2**, where Pro^7 was replaced by glycine N-alkylated with an array of chemically diverse R^2 groups to produce open ring compounds **9-36** (Table 2).

Table 2. Structures and pharmacological profiles of disulfide bridge compounds **9-36**.



Compound	R^2	*In vitro* biological activity								Rat iv clearance (mL/min/kg)
		EC_{50} receptor (nM)				Selectivity vs. receptor[a]			IC_{50} (nM)[b]	
		hOT	hV_2	hV_{1a}	hV_{1b}	hV_2	hV_{1a}	hV_{1b}	hV_{1a}	
9	cyclopropyl	0.05	4.2	>10000[c]	700	84	>200000	14000	>10000[d]	36
10		0.01	9.9	>10000[c]	660	990	>1000000	66000	>10000[d]	59
11	n-butyl	0.06	35	>10000[c]	1000[e]	580	>160000	16000	>10000[d]	63
12	cyclobutyl	0.16	1.8	85[e]	500	11	530	3100	>10000[d]	NT[f]
13	pentyl	0.01	77	19[e]	370	7700	1900	37000	>10000[d]	94
14	cyclopentyl	0.13	11	>10000[c]	980[e]	84	>76000	7500	>10000[d]	61
15	hexyl	0.01	100	12[e]	470	10000	1200	47000	>10000[d]	46
16	cyclohexyl	1.3	57	>10000[c]	>10000[c]	43	>7600	>7600	2000	NT[f]
17		0.03	80	>10000[c]	2800[e]	2600	>330000	93000	1800	47
18		0.39	100	>10000[c]	>10000[c]	250	>25000	>25000	3400	28
19	benzyl	0.11	34	>10000[c]	260	300	>90000	2300	>10000[d]	83
20		0.12	68	>10000	290	560	>83000	2400	1900	81

21		0.01	30	>10000[c]	110	3000	>1000000	11000	>10000[d]	91
22		0.15	84	>10000[c]	200	560	>66000	1300	1400	67
23		0.01	82	>10000[c]	140	8200	>1000000	14000	880	24
24		0.06	78	>10000[c]	440	1300	>160000	7300	970	73
25		1.3	180	>10000[c]	1800[e]	130	>7600	1300	2200	NT[f]
26		1.3	170	>10000[c]	1300[e]	130	>7600	1000	1300	NT[f]
27		0.46	380	>10000[c]	2600	820	>21000	5600	3200	43
28		0.12	66	>10000[c]	590	550	>83000	4900	270	94
29		0.12	180	>10000[c]	770	1500	>83000	6400	730	63
30		0.02	47	>10000[c]	720	2300	>500000	36000	350	48
31		0.01	57	>10000[c]	240	5700	>1000000	24000	1200	71
32		0.11	62	>10000[c]	930[e]	560	>90000	8400	390	67
33		0.02	86	>10000[c]	600	4300	>500000	30000	280	65
34[g]		0.07	230	>10000[c]	>10000[c]	3200	>140000	>140000	1500	39
35		19	1400[e]	>10000[c]	>10000[c]	73	>520	>520	1300	NT[f]
36		10	1400	>10000[c]	>10000[c]	140	>1000	>1000	>10000[d]	NT[f]

[a] EC_{50} (receptor)/EC_{50} (hOTR) ratio. If no significant agonism was observed at the highest concentration tested selectivity is >highest conc. tested/EC_{50} (hOTR); the selectivity ratios are rounded down to the nearest values with two significant figures; [b] IC_{50} at hV_{1a} receptor stimulated with 2 nM AVP; [c] No significant agonism at the highest concentration tested – 10000 nM; [d] No significant antagonism at the highest concentration tested - 10000 nM; [e] partial agonist, efficacy < 70%; [f] Not tested; [g] Compound **34** is the faster running diastereoisomer separated by preparative HPLC.

***In vitro* profile of position 7 modified desaminooxytocin analogues 9-36 (Table 2).**

Replacement of Pro^7 in **3** by N-alkylated glycines produced remarkable and very different changes in potency in *in vitro* functional assays at the four receptors studied. The change resulted in complete elimination of activity at the $hV_{1a}R$ for all compounds in Table 2 with notable exceptions when R^2 was cyclobutyl (**12),** n-pentyl (**13**), and n-hexyl (**15**),

which exhibited potencies similar to the reference **3**. Compound **3** is already fairly selective for the hOTR over $hV_{1b}R$; replacement of Pro^7 in **3** by N-alkylated glycines yielded compounds with similar or lower potency at the $hV_{1b}R$ and consequently similar or better selectivity. The R^2 groups producing the largest loss in potency at the $hV_{1b}R$ were the bulky cyclohexyl in **16**, and some but not all of the polar groups, for example in compounds **18**, **25 - 27**, and **34** – **36**.

Compound **3** is not very selective for hOTR (EC_{50} 0.10 nM), over hV_2R (EC_{50} 3.5 nM), and replacement of Pro^7 by N-alkylated glycines resulted in modest to good improvements in selectivity (Table 2). Considerable reductions in potency at hV_2R occurred only with the pyridylmethyl R^2 substituents in **25 - 27**, the charged R^2 groups in **35** and **36**, and with the 2-tetrahydrofurylmethyl group in **34**. Potency at the hV_2R was maintained as in **3**, only with the small cycloalkyl substituents in **9**, **12**, and **14**.

Replacement of Pro^7 in **3** by N-alkylated glycines resulted in compounds with similar potency at the hOTR (Table 2) for most R^2 groups tested, with some interesting exceptions. The small linear alkyl R^2 groups n-butyl (**11**), n-pentyl (**13**), and n-hexyl (**15**) increased potency at the hOTR whereas the corresponding cyclic substituents (**12**, **14**, **16**) did not. However, the small cyclic cyclopropyl (**9**) and cyclopropylmethyl (**10**) R^2 groups were well tolerated and actually also improved potency. The insertion of an oxygen atom into a straight alkyl substituent resulted in analogue **17** (R^2 = 3-oxabutyl) with higher potency at the hOTR as compared to **11** (R^2 = n-butyl). The location of the oxygen atom in R^2 seemed to be important as the 3-hydroxypropyl compound in **18** was about 10-fold less potent than **17**.

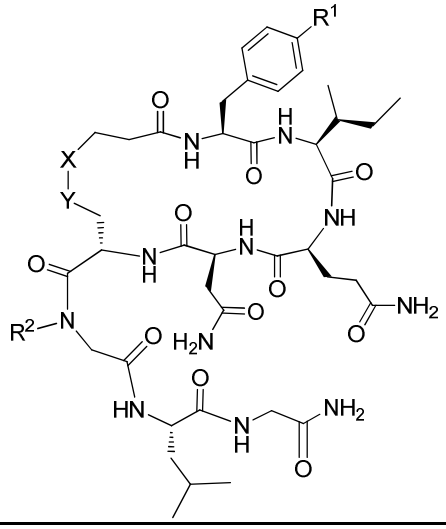
N-benzyl compounds **19-24** were generally equipotent to **3** at the hOTR, but the 4-fluorobenzyl (**23**, EC_{50} 0.01 nM) and 3-methylbenzyl (**21,** EC_{50} 0.01 nM) analogues were the most potent in this series. The pyridylmethyl analogues of **25-27** were less potent agonists at the hOTR than the benzyl analogue of **3** (**19**), paralleling the effect observed at the hV_2R. Increasing the distance from the phenyl group to the glycine nitrogen with an additional methylene group (R^2 = 2-phenylethyl) in **28** resulted in no loss of potency at the hOTR relative to **19** (R^2 = Bzl). Surprisingly, the compounds in which the pyridyl ring was also separated from the peptide backbone by two carbon atoms (**29, 30**) were markedly more potent than their picolyl counterparts **25-27**, with the 4-pyridylethyl analogue **30** being substantially more potent (EC_{50} = 0.02 nM) than **3**. The introduction of the heterocycloalkyl R^2 groups 2-thiophenylmethyl (**31**) and 2-thiophenylethyl (**33**) yielded potent hOTR agonists whereas the oxygen containing analogues with R^2 2-furylmethyl (**32**) and 2-tetrahydrofurylmethyl (**34**) had similar potency to **3**. The presence of a positive charge/tertiary amino group in the non-aromatic heterocyclic R^2 substituents had a deteriorating effect on potency at the hOTR of compounds **35** and **36,** as seen for the hV_2R.

The above observations on the potency of the various analogues at the hOTR can be interpreted as resulting from specific interactions between the R^2 group and a lipophilic binding site in the receptor. It is also possible that an additional conformational factor might contribute to the potency of the analogues. The conformation of the Cys^6-Pro^7 bond in **1** in water at pH 3 is 90% *trans,*[55] and it has been suggested that agonists at the OTR require a *trans* conformation around this bond with potency increasing proportionally to their *trans* content.[56] In the sarcosyl[7] derivative of OT the proportion of

Cys^6-Pro^7 *trans* conformer is somewhat decreased to about 75%.[57] Work in the peptoid field showed that in acetyl N-alkylglycine model compounds the proportion of *cis* conformer is not very different when the N-alkyl group is methyl, ethyl or benzyl, but it increases when it is branched in the α-carbon to the nitrogen.[58] Preliminary examination of the ^{1}H-NMR spectra of compounds **10**, **16**, **23** and **57** suggests that all the analogues contain similar proportions of *cis* and *trans* isomers and that this is an unlikely cause of the differences in potency obtained (data not shown). Overall, the strategy of replacing Pro^7 by N-alkylated glycines resulted in considerable progress towards obtaining selective hOTR agonists. Activity at the $hV_{1a}R$ was eliminated in most analogues, and potency at the $hV_{1b}R$ and hV_2R decreased, which when coupled to the substantial increase in potency in some analogues resulted in compounds with considerably increased selectivity for the hOTR.

The R^2 modifications that resulted in the most potent and/or most selective (especially versus the hV_2R) dOT analogues were 3-oxabutyl, 3-hydroxypropyl, n-butyl, n-pentyl, benzyl, 3-methylbenzyl, 4-fluorobenzyl, phenethyl, and 2-thienylmethyl. A final series of compounds **37-65** (Table 3) was prepared where these R^2 groups were combined with one of the two possible replacements of sulfur by a methylene group ("carba" alterations) in the disulfide bridge, and in some cases, with Tyr^2 replaced by Phe^2 in an attempt to further improve selectivity versus the hV_2R.[59]

Table 3. Structures and pharmacological profiles of monocarba analogues **37-65**.



Compound	Structure				*In vitro* biological activity								Rat iv clearance (mL/min/kg)
					EC_{50} receptor (nM)				Selectivity vs. receptor[a]			IC_{50}[b] (nM)	
	X	Y	R^1	R^2	hOT	hV_2	hV_{1a}	hV_{1b}	hV_2	hV_{1a}	hV_{1b}	hV_{1a}	
37	CH_2	S	OH		0.11	70	>10000[c]	3200	630	>90000	29000	>10000[d]	20
38	S	CH_2	OH		0.16	52	>10000[c]	830	320	>62000	5100	990	22
39	CH_2	S	H		0.96	1100	>10000[c]	>10000[c]	1100	>10000	>10000	>10000[d]	32
40	S	CH_2	H		0.85	1300	>10000[c]	>10000[c]	1500	>11000	>11000	2500	21
41	CH_2	S	OH		0.01	500[e]	>10000[c]	>10000[c]	50000	>1000000	>1000000	1400	18
42	S	CH_2	OH		0.13[e]	150	>10000[c]	>10000[c]	1100	>76000	>76000	1800	13
43	S	CH_2	H		0.86	2600	>10000[c]	>10000[c]	3000	>11000	>11000	>10000[d]	22
44	CH_2	S	OH	n-butyl	0.12	73	>10000[c]	820	600	> 83000	6800	>10000[d]	36
45	S	CH_2	OH	n-butyl	0.14	18	>10000[c]	450	120	>71000	3200	>10000[d]	NT[f]
46	CH_2	S	H	n-butyl	0.23	2000[e]	>10000[c]	>10000[c]	8600	>43000	>43000	300	NT[f]
47	S	CH_2	H	n-butyl	0.09	1200[e]	>10000[c]	>10000[c]	13000	>110000	>110000	740	61
48	CH_2	S	OH	n-pentyl	0.11	110	86[e]	500	1000	780	4500	>10000[d]	45
49	S	CH_2	OH	n-pentyl	0.25	2100[e]	>10000[c]	3600[e]	8400	>40000	14000	>10000[d]	95
50	S	CH_2	H	n-pentyl	0.07	1000[e]	>10000[c]	1000	14000	>140000	14000	>10000[d]	83
51	CH_2	S	OH	benzyl	0.04	140	>10000[c]	100	3500	>250000	2500	1400	51
52	S	CH_2	OH	benzyl	0.05	36	>10000[c]	100	720	>200000	2000	>10000[d]	68
53	S	CH_2	H	benzyl	0.40	720	>10000[c]	440	1800	>25000	1100	1600	43
54	CH_2	S	OH		0.08	210	>10000[c]	84	2600	>120000	1000	>10000[d]	70
55	S	CH_2	OH		0.02	55	71[e]	87	2700	3500	4300	>10000[d]	61
56	S	CH_2	H		0.56	1600[e]	>10000[c]	620	2800	>17000	1100	480	63
57	CH_2	S	OH		0.08	330	>10000[c]	180	4100	>120000	2200	1200	65
58	S	CH_2	OH		0.04	140	>10000[c]	91[e]	3500	>250000	2200	>10000[d]	58

59	S	CH_2	H		0.23	1400	>10000[c]	750[e]	6000	>43000	3200	1300	68
60	CH_2	S	OH		0.04	160	>10000[c]	450	4000	>250000	11000	490	75
61	S	CH_2	OH		0.05	100	>10000[c]	160	2000	>200000	3200	640	52
62	S	CH_2	H		0.78	3000[e]	>10000[c]	1500[e]	3800	>12000	1900	330	62
63	CH_2	S	OH		0.09	250	>10000[c]	420	2700	>110000	4600	2100	48
64	S	CH_2	OH		0.14	30	>10000[c]	190	210	>71000	1300	380	53
65	S	CH_2	H		0.30	760	>10000[c]	850	2500	>33000	2800	1200	49

[a] EC_{50} (receptor)/EC_{50} (hOTR) ratio. If no significant agonism was observed at the highest concentration tested selectivity is >highest conc. tested/EC_{50} (hOTR); the selectivity ratios are rounded down to the nearest values with two significant figures; [b] IC_{50} at hV_{1a} receptor stimulated with 2 nM AVP; [c] No significant agonism at the highest concentration tested - 10000 nM; [d] No significant antagonism at the highest concentration tested - 10000 nM; [e] partial agonist, efficacy < 70%; [f] Not tested.

***In vitro* profile of position 7 modified monocarba analogues 37-65 (Table 3).**

In the Tyr^2 series (R^1 = OH), the potency of the carba analogues at the hOTR generally decreased slightly relative to their disulfide bridge counterparts, but there were substantial gains in selectivity versus the hV_2R, especially in the carba-1 (X = CH_2, Y = S) analogues. The most striking improvement over a disulfide analogue was seen for the 3-hydroxypropyl compound **41** that showed remarkably higher potency as hOTR agonist (EC_{50} 0.01 nM) and notably lower potency as hV_2R agonist (EC_{50} 500 nM) than compound **18**. The benzyl analogues **51** and **52** and the phenethyl analogues **60** and **61** showed only a slightly better *in vitro* pharmacological profile than their disulfide counterparts **19** and **28**, respectively, while the 2-thienylmethyl carba analogues **63** and **64** were less potent and selective than **31**. As expected in the Phe^2 series, the potency at the hOTR was markedly reduced as compared to the corresponding Tyr^2 compounds with the noteworthy exception of **47** and **50** where R^2 was a straight hydrocarbon chain. The EC_{50} values at the hV_2R for all Phe^2 compounds were in the micromolar range resulting in high hV_2 selectivities (>1000) with peptide **47** showing the most remarkable *in vitro*

pharmacological profile among Phe[2] analogues. None of the analogues in Table 3 were active at the $hV_{1a}R$ (EC_{50} > 10000 nM) except for the combination of carba-6 and 3-methylbenzyl R^2 in **55** (EC_{50} 71 nM) and of carba-1 and n-pentyl in **48** (EC_{50} 86 nM), illustrating the exquisite sensitivity of this receptor to subtle changes in structure. Overall, compounds **9-65** showed superior *in vitro* pharmacological profile as compared to **1**. Dose-response curves for compound **57** and **1** in reporter gene assays at the hOTR, hV_2R, $hV_{1a}R$ and $hV_{1b}R$ are shown in Figure 2.

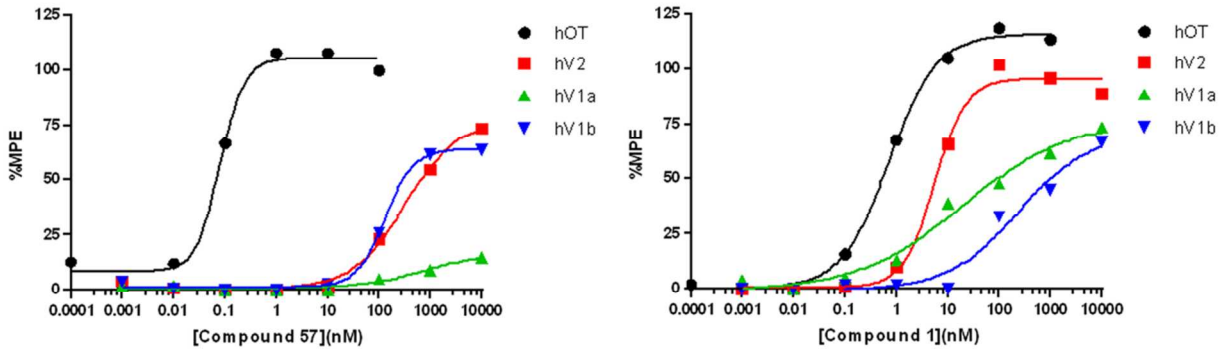


Figure 2. Representative *in vitro* dose-response curves for functional activation of the hOTR and related receptors by compound **57** (left) and parent hormone oxytocin (**1**, right)**.** Data are expressed as percentage of the maximum response (%MPE) to the internal control agonist used in each reporter gene assay (carbetocin for hOTR, AVP for $hV_{1a}R$ and $hV_{1b}R$, dDAVP for hV_2R).

Compounds 9-65 as vasopressin receptors antagonists.

With the exception of compounds **12**, **13**, **15**, **48** and **55**, none of the analogues in Tables 2 and 3 exhibited agonism at the $hV_{1a}R$ up to the highest concentration tested (10,000 nM). To rule out the possibility that the compounds might still have affinity for

the $hV_{1a}R$ they were tested as antagonists against 2 nM concentration of AVP. Although none of the compounds was found to be a potent $hV_{1a}R$ antagonist, some peptides where an aromatic or heteroaromatic ring was linked to the peptide backbone by an ethyl spacer (**28**, **30**, **33**, **60**, and **62**) or a methylene spacer (**56** and **64)** displayed IC_{50} values below 500 nM. A subset of compounds **9-65** was also tested as hV_2R and $hV_{1b}R$ antagonists and none of the compounds showed any significant antagonistic activity at either receptor (data not shown).

Structure-pharmacokinetics relationship.

In addition to high potency and selectivity for the hOTR, our objective was to identify peptides which would likely have short duration of action in humans. The half-life of endogenous oxytocin, **1,** released during nursing appears to be very short[60] and obviously adequate for its purpose. Therefore, the clearance of **1** in non-pregnant women, about 20 mL/min/kg[15, 61], was selected as the target value for the desired compound in humans. Assuming that the CL of (at least some) the analogues prepared follows classic allometric scaling (whole body CL = $a \cdot BW^{0.75}$ where a is allometric coefficient and BW is body weight),[62] the target CL in rats falls in the range of 60-80 mL/min/kg. However, the CL of **1** itself does not scale allometrically as its value is similar in various species (rat, Cynomolgus monkey, humans, data not shown). Indeed, not only **1**, but reference compounds **2** and **3** had CL value in male rats in the range of 21-27 mL/min/kg (Table 1), similar to **1** in humans. The CL values in female and postpartum rats (data not shown) were not different from male rats, and therefore these were used throughout the program. The CL values in male rats for compounds **9** – **65** are shown in Tables 2 and 3. Seventeen

analogues had rat CL in the desired range of 60 – 80 mL/min/kg and of those, compounds **24**, **31**, **33**, **47**, **55**, **57**, **59**, **60** and **62** showed the best combined *in vitro*/PK pharmacological profiles and were deemed potential drug candidates.

Figure 3 shows the correlation between total male rat CL and the capacity factor k' determined by reversed phase HPLC on a C18 column using a TFA acetonitrile-water based gradient, a surrogate of compound lipophilicity (see Experimental for details). For all four compound series, disulfide bridged (Table 2), carba-1, carba-6, and [Phe^2, carba-6] analogues (Table 3), there is a reasonably good direct correlation between CL and lipophilicity. In the disulfide bridged series, the compounds with the highest k' values containing hydrophobic R^2 groups such as n-pentyl (**13**), benzyl (**19**), methylbenzyl (**20**, **21**) and 2-phenylethyl (**28**) displayed the highest CL values, with the maximum value of 94 mL/min/kg for **13** and **28**. At the other extreme, compound **18** with the polar hydroxypropyl R^2 group had the lowest retention time (k' = 7.60) and the lowest CL of 28 mL/min/kg in the series. Compounds **27**, **29** and **30** followed a different trend line, with higher CL and low k' values. Unlike all other compounds in Figure 3, which are neutral peptides, these contain a pyridyl ring that is protonated in the TFA HPLC buffer, resulting in much shorter retention times. Under physiological conditions the compounds are not expected to be protonated and their CL values are indeed similar, but somewhat lower, than their non-pyridyl counterparts **11** and **28**. Two compounds in the disulfide bridged series are outside the general correlation, the N-hexyl analogue **15** and the fluorobenzyl analogue **23**, which have lower CL than expected from their k' value.

For otherwise identical sequences, the CL values increased in the order: CL (carba-6 analogues) ≤ CL (carba-1 analogues) < CL (disulfide bridge analogues). The CL values

for compounds where R^2 is 2-phenylethyl (**61**, **60** and **28**), 3-methylbenzyl (**55**, **54**, **21**) and 3-hydroxypropyl (**42**, **41**, **18**) followed the trend remarkably well. Peptide **42**, with the lowest k' value of all compounds tested, had CL 13 mL/min/kg, which is lower than that of the reference compounds **1 - 3**. On the other hand, the disulfide analogue **23** (R^2 = 4-fluorobenzyl), displayed a considerably lower CL (24 mL/min/kg) than its carba counterparts **57** and **58**, which otherwise follow the trend within their respective series.

The relationship of pharmacokinetic parameters such as CL and volume of distribution with lipophilicity and/or charge is relatively well described in the literature for small molecules.[63, 64] There appears to be a positive correlation between the lipophilicity of compounds expressed as octanol-water distribution coefficient logD and the extra-renal clearance of free, plasma protein-unbound fraction; and there is an inverse relationship between the lipophilicity and the renal component of the free-fraction clearance.[59, 60] On the other hand, for compounds with high plasma protein binding, CL negatively correlates with hydrophobicity.[65] These correlations reflect passive partitioning of the molecules into hepatocytes where they undergo metabolism by cythochrome P450 enzymes as the main mechanism of disposition. Such systematic correlations between pharmacokinetic parameters and lipophilicity appear not to have been previously performed for peptides. Peptides are not expected to diffuse passively into cells, and typically are not metabolized by cytochromes. The fairly good correlation between rat clearance and capacity factor shown in Figure 3 was actually surprising. The disposition of peptides is generally considered to occur by passive renal filtration, proteolysis by enzymes in plasma and in tissues, and by carrier mediated uptake by the liver and other tissues. We expected to find that the SAR for the CL of the analogues would be complex

and exhibit features reflecting recognition by proteases and/or transporters. For small molecules, very good correlations have been found between LogP and the logarithm of the reversed phase HPLC capacity factor k', as both reflect the change in free energy for partition between an aqueous and a non-polar phase.[66]

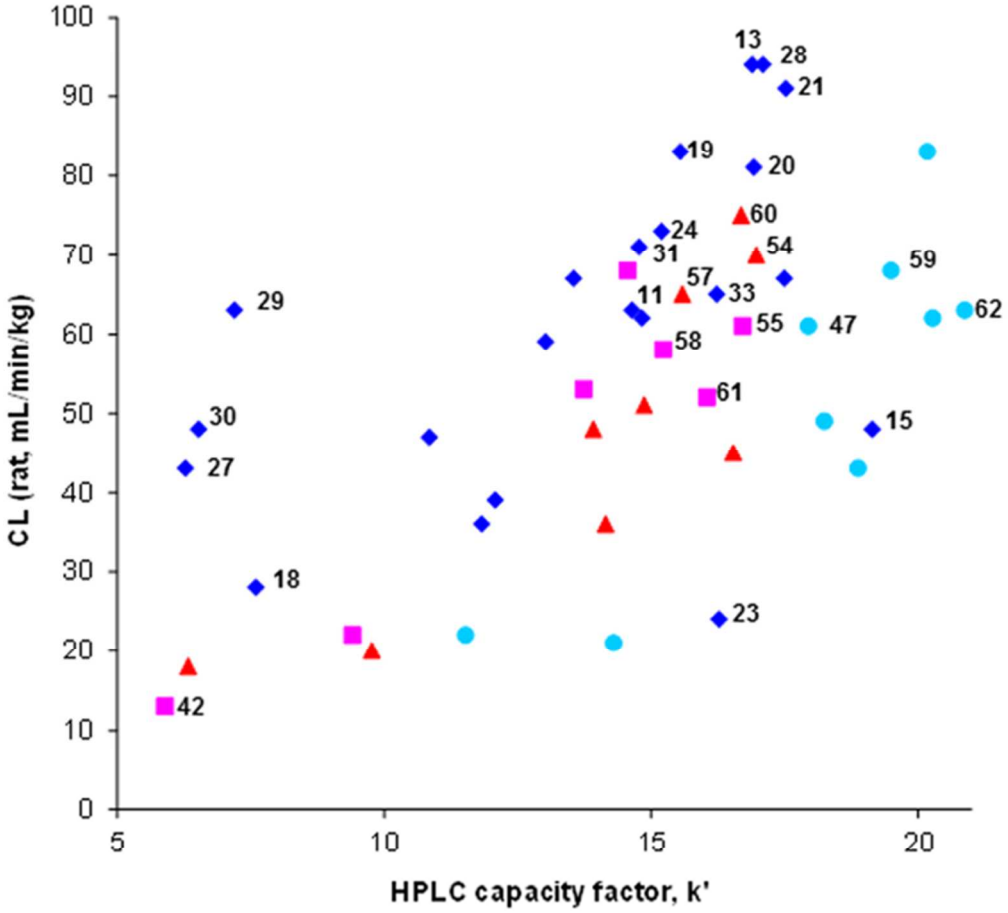


Figure 3. Effect of lipophilicity expressed as k' (k' = HPLC capacity factor, see experimental section) on systemic clearance (CL) in male rats for different series of OT analogues: disufide bridge analogues (diamonds), carba-1 analogues (triangles), carba-6 analogues (squares) and Phe[2] carba-6 analogues (circles). Compound numbers for analogues mentioned in the PK discussion are shown in the graph.

Figure 3 shows an approximately linear correlation between CL and k', and *a priori* there is no reason to assume that a more complex function of k' would provide a better correlation. Since at this time the main mechanism of disposition of the peptides is unknown, any rationale for the empirical relationship found would be speculative. Binding to plasma proteins can probably be excluded as this would result in an inverse correlation between CL and lipophilicity by any mechanism that eliminates or metabolizes the free peptide fraction. A full pharmacokinetic characterization of selected compounds from this collection in several animal species will be reported elsewhere.

CONCLUSION

We have designed, synthesized and pharmacologically characterized a series of hOTR agonists modified in position 2, 7 and at the disulfide bridge. Replacement of Pro^7 by N-alkyl glycines resulted in potent hOTR agonists devoid of $hV_{1a}R$ activity and with improved selectivity against the $hV_{1b}R$ and hV_2R, and high rat clearance. Replacement of one sulfur atom in the disulfide bridge further improved selectivity versus the hV_2R. A correlation between rat clearance and lipophilicity was found and exploited to obtain compounds with high rat clearance, which would be expected to have short half-life in humans suitable for multiple daily administrations. Several compounds such as **31** ([2-ThiMeGly^7]dOT, **47** (carba-6-[Phe^2,BuGly^7]dOT), **55** (carba-6-[3-MeBzlGly^7]dOT) and **57** (carba-1-[4-FBzlGly^7]dOT), showing the desired pharmacological profile (high potency as OTR agonists, high selectivity for the hOTR particularly versus the hV_2R, no antagonist activity at the vasopressin receptors and high CL in rats) have been identified. Based on the *in vitro* and pharmacokinetic data reported in this paper and other

considerations, compound **57,** FE 202767, has been selected as a clinical candidate for the lactation support indication.

EXPERIMENTAL SECTION

Synthesis

General. Amino acid derivatives and resins were purchased from Novabiochem, Bachem, Peptide International and RSP Amino Acids. Fmoc-Cys($(CH_2)_3$COOtBu)-OH (the carba-1 derivative)[67] and Fmoc-Hcy($(CH_2)_2$COOtBu)-OH (the carba-6 derivative)[68] were synthesized according to published procedures. Fmoc-N-alkylglycines: Fmoc-BzlGly-OH, Fmoc-4FBzlGly-OH and Fmoc-PhEtGly-OH were prepared from ethyl bromoacetate by an improved (i.e., without chromatography) literature method.[48] Fmoc-Hyp(Me)-OH was synthesized from Boc-Hyp-OtBu by a three step procedure consisting of O-methylation with MeI in DME in the presence of NaH, removal of protecting groups with neat TFA and the Fmoc group introduction with Fmoc-OSu.

Other chemicals and solvents were purchased from Sigma-Aldrich and VWR.

Analytical HPLC was performed on a Waters 600 Liquid Chromatograph with Empower 2 software package using a Vydac C18, 5 μm, 4.6 x 250 mm column at a flow rate of 2 mL/min. Preparative HPLC was performed on a Waters 2000 Liquid Chromatograph using a 15 μm PrepPak 47 x 300 mm cartridge at a flow rate of 100 mL/min. Final purity of analogues was assessed on a 1100 Agilent Liquid Chromatograph using the following analytical method: column – Vydac C18, 5 μm, 2.1 x 250 mm; column temperature – 40°C; flow rate – 0.3 mL/min; solvent A – 0.01% aqueous TFA; solvent B – 70% CH_3CN, 0.01% TFA; gradient – 0-20% B in 1 min., then

20-40% B in 20 min., then held at 100% B for 5 min.; when necessary the first two segments of the gradient were adjusted for compound lipophilicity; UV detection at 214 nm. The purity of all analogues exceeded 95% (see Table 4 in Supporting Information). HPLC capacity factors (k') were calculated using the following equation: $k' = (t - t_0)/t_0$ where t is retention time of a test compound and t_0 is retention time of unretained species. For capacity factor calculations the retention times were determined on a 1200rr Agilent Liquid Chromatograph using an Agilent Zorbax SB-C18, 1.8 μm, 4.6 x 50 mm column at a flow rate of 1.5 mL/min. Solvent A was 0.05% aqueous TFA and solvent B was 90% CH_3CN, 0.045% TFA. Mass spectra were recorded on a Finnigan MAT spectrometer.

Peptide synthesis.

Disulfide bridge analogues 9-36. All compounds were assembled on Rink amide resin by Fmoc strategy. The following derivatives were employed: Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Cys(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH and Fmoc-Tyr(tBu)-OH and Mpa(Trt)-OH. The Cys derivative was coupled using DIC in DCM with a 4-fold excess and all other couplings were mediated by DIC/HOBt with a 3-fold excess of reagents. The N-alkylglycine residues in position 7 were introduced by a two-step procedure comprising the acylation of the resin-bound dipeptide with bromoacetic acid/DIC and the displacement of the bromine atom with a suitable primary amine.[47] The linear peptides were cleaved with the TFA/TIS/H_2O 95/2.5/2.5 (v/v/v) cocktail and the ring formation was achieved by an iodine oxidation of crude analogues dissolved in 10% aqueous TFA containing up to 20% acetonitrile. The crude products

were purified by preparative HPLC in acetic acid buffer. If necessary, an additional purification using a triethylammonium phosphate buffer was performed prior to the acetic acid run. The fractions with purity exceeding 97% were pooled and lyophilized. Compound **34** was synthesized using racemic 2-aminomethyl furane and was separated by preparative HPLC as a faster running diastereoisomer. No attempt to determine the absolute configuration of the C1 carbon in the furane ring was undertaken.

Carba analogues 37-50. All compounds were synthesized by a hybrid Boc/Fmoc strategy on MBHA resin. The following derivatives were employed: Boc-Gly-OH, Boc-Leu-OH, Fmoc-Cys($(CH_2)_3$COOtBu)-OH (for carba-1 analogues) or Fmoc-Hcy($(CH_2)_2$COOtBu)-OH (for carba-6 analogues), Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH and Boc-Tyr(tBu)-OH or Boc-Phe-OH. The carba derivatives were coupled using DIC in DCM with a 4-fold excess and all other couplings were mediated by DIC/HOBt with a 3-fold excess of reagents. The N-alkylglycine residues in position 7 were introduced by the two-step procedure as described above for compounds **9-36**. The resin bound peptides were treated with the TFA/TIS/H_2O 95/2.5/2.5 (v/v/v) cocktail to remove the acid sensitive protecting groups and were subsequently cyclized with BOP/DIPEA in DMF. The crude cyclic peptides were cleaved from the resin with HF/anisole 10/1 (v/v) and purified by preparative HPLC as described above.

Carba-1 analogues 51, 57 and 60. The peptides were assembled by Fmoc chemistry on Rink amide resin. The following derivatives were employed: Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-BzlGly-OH (for compound **51**) or Fmoc-4FBzlGly-OH (for compound

57) or Fmoc-PhEtGly-OH (for compound **60**), Fmoc-Cys((CH$_2$)$_3$COOtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH and Boc-Tyr(tBu)-OH. The couplings were performed as described above. The linear peptides were cleaved with the TFA/TIS/H$_2$O 95/2.5/2.5 (v/v/v) cocktail and cyclized with HBTU/DIPEA in DMF. The crude products were purified as described above.

Compounds 52-56, 58, 59 and 61-65. These compounds were prepared by methods comprising various steps used in the synthesis of analogues **9-51**, **57** and **60**.

Analogues 2, 4 and 6-8. The peptides were assembled by Fmoc chemistry on Rink amide resin. The following derivatives were employed: Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Pro-OH (for compound **3**) or Fmoc-Hyp(Me)-OH (for compounds **4, 6** and **7**) or Fmoc-Gly-OH (for compound **8**), Fmoc-Cys((CH$_2$)$_3$COOtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH and Boc-Tyr(Me)-OH (for compounds **2**, **4** and **8**) or Fmoc-Phe(Et)-OH (for compounds **6** and **7**). The couplings were mediated by DIC/HOBt with 3-fold excess of reagents. The cleavage, cyclization and purification were performed as described for analogues **51**, **57** and **60**.

Analogue 5. The peptide was assembled by Fmoc chemistry on Rink amide resin using the following derivatives: Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-cisHyp-OH, Fmoc-Cys((CH$_2$)$_3$COOtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Ile-OH and Boc-Tyr(Me)-OH. The couplings were mediated by DIC/HOBt with 3-fold excess of reagents. The resin bound peptide was converted to the Pro(SAc)[7] analogue upon

treatment with CH_3COSH/TPP/DIAD[45] in DME and cleaved with the TFA/TIS/H_2O 95/2.5/2.5 (v/v/v) cocktail. The crude linear peptide was cyclized with PyBOP/DIPEA in DMF and purified by HPLC. The S-acetyl group was converted to the S-methyl group by hydrolysis in MeOH with an equivalent of 1M NaOH[46] and in situ methylation of the resulting sulfhydryl group with methyl iodide. The peptide was then purified as described above.

Biological methods

***In vitro* receptor assays.** Agonist activity of compounds at the human oxytocin OTR was determined in a transcriptional reporter gene assays (RGA) using CHO-K1 cells stably expressing the human OTR transfected with a reporter DNA construct containing intracellular calcium responsive promoter elements regulating expression of the firefly luciferase. Two days following transfection, cells were treated with appropriate doses of peptides, incubated at 37°C for 5 hours, lysed in the presence of luciferin and the total luminescence was measured.

To determine receptor selectivity, compounds were also tested in luciferase-based transcriptional reporter assays in HEK293 cells expressing the human V_2R, human $V_{1a}R$ or human $V_{1b}R$.[69, 70] AVP was used as an internal control for the vasopressin $V_{1a}R$ and $V_{1b}R$ assays, dDAVP was used as an internal control for the V_2R assays and carbetocin, was used as an internal control for the OTR assays. The assays were standardized by including these controls in every experiment. Dose-response curves were analyzed using a one-site, four parameter model from Xlfit (IDBS) to estimate EC_{50} and efficacy values.

Agonist potency determined from multiple independent experiments is reported as the geometric mean EC_{50}, first calculating pEC_{50} values (pEC_{50} = -log(EC_{50} in M) for each individual value, and then determining the arithmetic mean, and standard error of the mean (SEM) of pEC_{50} values. Average pEC_{50} values are transformed back to the normal numeric scale to report as EC_{50}. Compounds in these assays were diluted with logarithmic spacing between concentrations, requiring the use of the geometric mean for potency values.

Selectivity values are given as ratios of the EC_{50} values at the receptor of interest to the corresponding EC_{50} values at the OTR.

Antagonist activity of compounds at human $V_{1a}R$, human V_2R and human $V_{1b}R$ were determined in luciferase-based transcriptional reporter assays in cells expressing the human $V_{1a}R$, human $V_{1b}R$ and human V_2R. Antagonism was tested against receptors activated with reference agonists at concentrations which lie between the respective EC_{50} and EC_{90} (2 nM AVP at human $V_{1a}R$, 50 nM AVP at human $V_{1b}R$, 1 nM dDAVP at human V_2R). Interassay reproducibility was controlled using known receptor antagonists (HO-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH_2[71] for human $V_{1a}R$ and human $V_{1b}R$, and AdAc-D-Tyr(Et)-Phe-Val-Asn-Abu-Pro-Arg-Arg-NH_2[72] for human V_2R) as internal controls. Compounds were tested in at least three independent experiments. Dose-response curves were analyzed using a one-site, four parameter model from Xlfit (IDBS) to estimate IC_{50} and efficacy values. pIC_{50} values are calculated from IC_{50} (pIC_{50} = -log (IC_{50} in M) for each individual value, then determining the arithmetic mean and SEM of pIC_{50}. Average pIC_{50} values are then transformed into the normal numeric scale to report the geometric mean IC_{50}. Compounds in these assays were diluted with

logarithmic spacing between concentrations, requiring the use of the geometric mean for potency values. Antagonist potency is presented as geometric means of IC_{50} values in nanomol/L (nM).

Determination of clearance in rat

Animals. Single-dose pharmacokinetic profiles of OT analogues were investigated following i.v. bolus adminstration in male Sprague-Dawley rats (230 – 300 g). Animals were obtained from Harlan Laboratories Inc. (Indianapolis USA) with chronic jugular vein and carotid artery catheters inserted surgically. The rats were given free access to food (18% protein Rodent Diet, Harlan Teklad, Madison USA) and water. They were housed in a conventional animal facility in either static caging (Alternative Design Manufacturing & Supply Inc., Siloam Springs USA) or individually ventilated caging (LabProducts Inc., Seaford USA) with appropriate air flow under controlled environmental conditions (20-22ºC, 12hr light/dark cycle).

All animal procedures were approved by the Ferring Research Institute Institutional Animal Care and Use Committee and were in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the National Research Council.

Dosing and sampling. Analogues were dissolved in water for injection or sterile saline. Each rat was given a single dose of 0.1 to 0.2 mg/kg of test compounds in cassette mode through the jugular vein.[73] Cassettes were constituted of 1 to 8 analogues with each compound at a concentration of 0.1 to 0.2 mg/mL. Blood samples (250 μL) were collected from the carotid artery catheter into Microtainer EDTA Tubes (BD Diagnostics,

Franklin Lakes NJ, USA) at nominal times of 1, 3, 5, 8, 12, 20, 30, 45 and 60 min after administration. Blood was replaced with an equal volume of saline. The samples were centrifuged and plasma portions were separated. All samples were immediately frozen on dry ice and stored at -50ºC until analysis.

Bioanalysis of the analogues in plasma. The concentrations of analogues were determined using a liquid chromatography tandem mass spectrometry (LC/MS/MS) method. The dynamic range of the assays was generally between 0.5 and 1000 ng/mL. Briefly, aliquots of rat plasma containing internal standards were extracted after mixing with equal volume of 0.1% TFA in acetonitrile. Supernatant solutions were obtained by filtration of the mixture through 0.22 μm Amicon Ultrafree-MC filters (Millipore, Bedford USA). The filtered samples were diluted (1:1, v/v) with 0.01% trifluoroacetic acid in water prior to injection. Samples were injected into a Jupiter C12 4 μm Proteo 90A 150 x 2.0 mm column (Phenomenex, Torrance USA) coupled to an Agilent 1100 series LC (Agilent Technologies, Santa Clara USA). The analytes were eluted by a mobile phase gradient containing 0.01% trifluoroacetic acid and acetonitrile and detected using a Finnigan TSQ Quantum Ultra (Thermo Electron, Waltham USA) triple quadrupole mass spectrometer in the positive electrospray ionization mode. Analyte concentrations were calculated by linear regression analysis using the peak area ratio of analyte to the internal standard on the Thermo Xcalibur software.

Pharmacokinetic data analysis. Pharmacokinetic parameters were calculated using noncompartmental curve stripping methods (PK Solutions 2.0, Summit Research

Services, Ashland, USA). The area under the plasma concentration-time curve to infinity (AUC∞) was calculated by adding the area under the curve from zero to the last time point measured (AUC0-t) and the extrapolated AUC from the last time point measured to infinity. The extrapolated part of AUC was calculated using the slope of the terminal phase and did not exceed 5% for any compound tested. Body weight normalized clearance (CL) values (mL/min/kg) for each animal were calculated as dose divided by AUC∞ and rat body weight. The arithmetic mean and standard error values from all animals in the group (N = 3 or 4) are reported in the tables.

SUPPORTING INFO AVAILABLE

Detailed synthetic procedures, additional pharmacological data and physicochemical properties of compounds are available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGEMENTS

The authors thank Marlene Brown and John Kraus for their excellent technical assistance. We also thank Denise Riedl for auditing the data and critical reading of the manuscript.

ABBREVIATIONS USED

AVP, 8-arginine vasopressin; BuGly, N-(n-butyl)glycine; BOP, benzotriazol-1-yloxytrisdimethylaminophosphonium hexafluorophosphate; cisHyp, *cis*-hydroxyproline, (2S,4S) 4-hydroxyproline; CL, systemic clearance; dDAVP, 8-D-arginine desaminovasopressin; DIC, diisopropylcarbodiimide; DIPEA, N',N'-diisopropylamine; 4-FBzlGly, N-(4-fluorobenzyl)glycine; Fmoc-OSu, N-(9-fluorenylmethylcarbonyloxy)succinimide; HBTU, 2-(1H-bezotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; hOTR, human oxytocin receptor; logD, logarithm of octanol – water distribution coefficient; logP, logarithm of octanol – water partition coefficient; 3-MeBzlGly, N-(3-methylbenzyl)glycine; OT, oxytocin; OTR, oxytocin receptor; PK, pharmacokinetics; 2-ThiMeGly, N-(2-thienylmethyl)glycine; TIS, triisopropylsilane; TPP, triphenylphosphine; $hV_{1a}R$, human vasopressin type 1a receptor; $hV_{1b}R$, human vasopressin type 1b receptor; hV_2R, human vasopressin type 2 receptor;

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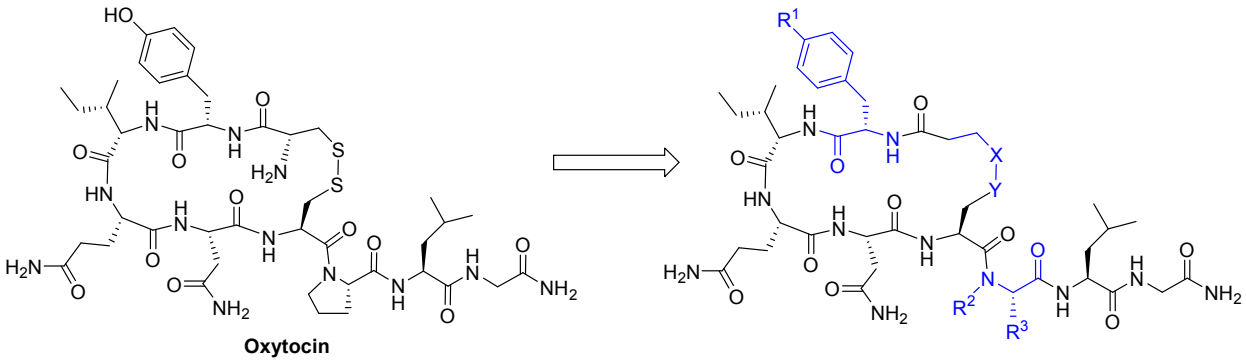
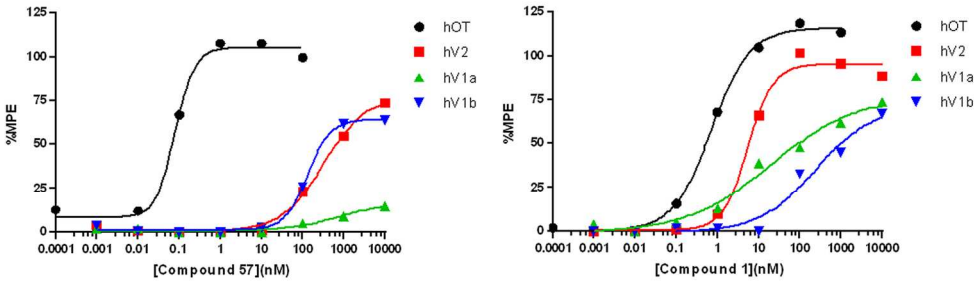
TABLE OF CONTENT GRAPHIC



Oxytocin



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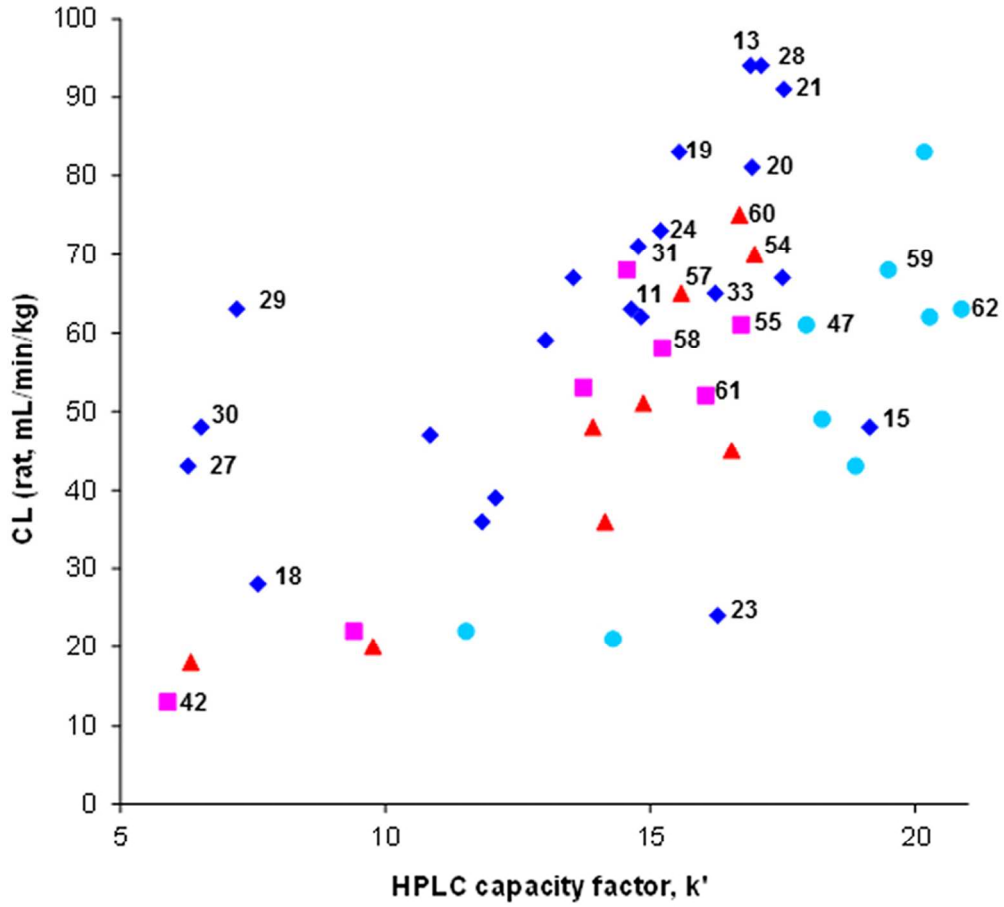

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