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Building bridges for highly selective, potent and stable oxytocin and vasopressin analogs

Rhiannon Beard [a], Andy Stucki [b], Muriel Schmitt [b], Gabrielle Py [b], Christophe Grundschober [b], Antony D. Gee [c], Edward W. Tate [a,*]

[a] Department of Chemistry, Imperial College London, Exhibition Road, London SW7 2AZ, UK
[b] Roche Pharma Research and Early Development, Discovery Neuroscience, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd, Grenzacherstrasse 124, 4070 Basel, Switzerland
[c] Division of Imaging Sciences, King's College London, 4th Floor, Lambeth Wing, St Thomas' Hospital, SE1 7EH London, UK

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ABSTRACT

Oxytocin (OT) is an exciting potential therapeutic agent, but it is highly sensitive to modification and suffers extensive degradation at elevated temperature and *in vivo*. Here we report studies towards OT analogs with favorable selectivity, affinity and potency towards the oxytocin receptor (OTR), in addition to improving stability of the peptide by bridging the disulfide region with substituted dibromo-xylene analogs. We found a sensitive structure-activity relationship in which meta-cyclized analogs (dOT_{meta}) gave highest affinity (50 nM K_i), selectivity (34-fold), and agonist potency (34 nM EC_{50}, 87-fold selectivity) towards OTR. Surprisingly, ortho-cyclized analogs demonstrated OTR and vasopressin V_{1a} receptor subtype affinity (220 nM and 69 nM, respectively) and pharmacological activity (294 nM and 35 nM, respectively). V_{1a} binding and selectivity for ortho-cyclized peptides could be improved 6-fold by substituting a neutral residue at position 8 with a basic amino acid, providing potent antagonists (14 nM IC_{50}) that displayed no activation of the OTR. Furthermore, xylene-bridged analogs demonstrated increased stability compared to OT at elevated temperature, demonstrating promising therapeutic potential for these analogs which warrants further study.

1. Introduction

Oxytocin (OT), a nonapeptide released from the pituitary gland, is a promising therapeutic agent that is heavily involved with lactation and uterine contraction in the peripheral system and is further linked with complex neurological disorders in the central nervous system. For example, OT is the World Health Organization's recommended drug to prevent postpartum hemorrhaging, which is one of the most common causes of maternal morbidity.[1,2] However, OT suffers from limited stability in aqueous solution that is especially problematic in subtropical climates where the majority of maternal deaths occur.[3–6] Further, OT is highly susceptible to metabolic degradation, having an *in vivo* half-life of 3 min, causing substantial loss of activity.[7]

Common approaches to overcome peptide degradation, including use of unnatural and D-amino acids, terminal capping and chemical modification or mutation of the proteolytic recognition sites, are often unsuitable for OT since its biological activity is highly sensitive to structural change. This is because OT shares a molecular structure closely related to vasopressin (also known as arginine vasopressin, AVP), making the development of a highly specific and stable oxytocin receptor (OTR) ligand challenging. Both OT and AVP contain a disulfide bridge between residues 1 and 6, resulting in a structure containing a cyclic core comprising six amino acids with a flexible three-residue amidated tail (Fig. 1, Table 1). The peptides differ by the amino acids at position 3, and at position 8 whereby OT-related peptides contain a neutral residue, while AVP peptides bear a basic amino acid. This subtle difference in polarity at position 8 is thought to confer the molecules' interaction with its receptor that, in turn, is related to its distinctive function.[8,9] However, due to their common structure, OT and AVP peptides bind to and act on multiple members of the G-protein coupled receptor family to exert their pharmacological effects, including OTR, and AVP receptor subtypes V_{1a}, V_{1b} and V_2.[10] For OT, Tyr2 and Asn5 are fundamental for activity in the uterus while residues Leu8, Pro7, Gln4 and Ile3 are key for receptor binding.[11]

Specifically, to improve the stability of OT two main strategies have been used (i) *N*-terminal deamination,[12,13] and (ii) disulfide bond engineering, since the disulfide bond is generally not implicated in OTR binding or activity. In the case of disulfide

* Corresponding author.
E-mail address: e.tate@imperial.ac.uk (E.W. Tate).

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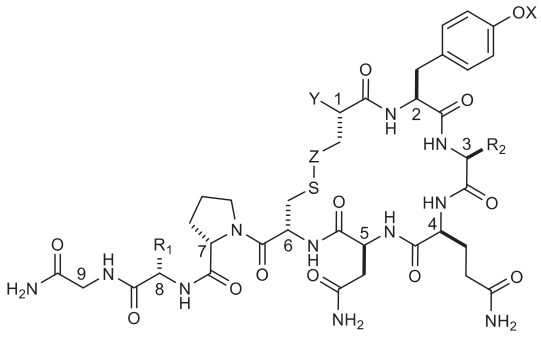

Fig. 1. Structure of therapeutic peptide OT, AVP and related analogs, with variables presented alongside in Table 1.

Table 1
Sequence residues for OT and related peptides.

Peptide	R_1	R_2	X	Y	Z
OT	Leu	Ile	H	NH_2	S
dOT	Leu	Ile	H	H	S
AVP	Arg	Phe	H	NH_2	S
dOT(L8R)	Arg	Ile	H	H	S
Carbetocin	Leu	Ile	CH_3	H	CH_2

engineering, a variety of modifications at this position have been investigated, typically replacing the disulfide bridge with alternatives including thioether,[14] carbon,[15,16] lactam,[17] and diselenide bridges.[18] Structure activity relationships (SARs) have identified that introduction of one alternative atom on the disulfide bond (such as selenium) causes a subtle change in the ring region of OT that is tolerated for OTR binding and efficacy,[15,19] while reductions in the ring size of OT can abolish OTR activity. However, the majority of studies omit determination of AVP receptor subtype activity, leaving a question over alterations in receptor selectivity induced by these chages.[19] Alternatively, carbetocin, an OT mimetic with an improved pharmacokinetic profile and prolonged uterotonic activity,[14] shows reduced selectivity and affinity towards the OTR (10-fold lower than OT), and is only a OTR partial agonist (Fig. 1).[20,21] In addition, recent reports have emerged that suggests carbetocin is also a V_{1a} and V_{1b} antagonist, complicating its pharmacological profile.[22]

In a complementary and relatively less studied approach to disulfide engineering, disulfide bridging represents an attractive alternative that circumvents the lengthy and complex synthesis of unnatural cyclized peptides through engineering strategies. For example, Collins et al. recently demonstrated that a maleimide-functionalized polymer could successfully bridge the disulfide region of OT, resulting in a conjugate with increased thermal stability.[23]

In the present study, we investigated the biological activity and stability of OT analogs bridged by dibromo-xylene molecules that offer an irreversible covalent modification. Previously, dibromoxylenes have been used to assist peptide cyclisation,[24] and increase proteolytic stability or helicity of peptides,[25,26] with further applications in the construction of protein mimics.[27] Furthermore, a range of substituted analogs are available commercially, enabling bridging distance structure-activity relationships (SAR) to be studied. We produced a library of cyclized OT analogs based on *N*-terminally deaminated OT (dOT), which has been reported to have increased stability compared to OT.[7] In addition, omission of a *N*-terminal amine on dOT prevented any complications from competing side reactions during cyclization. The peptide library was screened for binding affinity, biological activity and stability at elevated temperature using *in vitro* assays, revealing promising potency, selectivity and stability profiles driven by xylene-bridging in OT analogs.

2. Results and discussion

2.1. Synthesis of OT and peptide analogs

Native OT, deaminated OT (dOT) and dOT(L8R) were successfully synthesized *via* automated fluorenylmethoxycarbonyl (Fmoc) solid phase peptide synthesis (SPPS), and coupling with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU). In the case of dOT and dOT(L8R), a deaminated protected cysteine was made by reacting triphenylmethylchloride in dicholomethane with 3-mercaptopropionic acid[28], then coupled in the final position. Peptides were cleaved from the resin using trifluoroacetic acid (TFA) in the presence of 2.5% DTT to prevent oxidation of cysteine or deaminated cysteine residues. Crude peptides were purified by reverse phase (RP) liquid chromatography mass spectrometry (LC-MS) using either water/methanol or water/acetonitrile (MeCN). When required, cyclization of native disulfide bonds was achieved by stirring the peptide in ammonium bicarbonate buffer in the presence of oxygen for up to three days. Both purified peptides were lyophilized, and characterized by RP LC-MS (Supplementary Fig. 1.1), which is presented in Table 2.

2.2. Disulfide bridging of dOT with xylene analogs

Cyclization of dOT analogs using various isomers of dibromoxylene was first attempted in a mixture of ammonium bicarbonate and acetonitrile, at room temperature and at a final concentration of 1 mM peptide. While full conversion was seen after 10 min, a small proportion of polymerized product for both meta- (11%) and para- (21%) dibromoxylene-cyclized dOT was detected. Pleasingly, this side product was removed when the reaction was performed at higher dilution (0.5 mM) and lower dibromoxylene molar equivalents (1.1 equivalents vs. 3 equivalents), affording all peptides efficiently and in good overall yield (Table 2, Fig. 2). Xylene-bridged analogs showed reduced solubility in water; addition of 15% v/v DMSO solved this issue.

2.3. Binding affinity of peptides against OTR and AVP receptor subtypes

The OTR has a substantial reliance on cholesterol for proper functioning that has, in turn, presented severe challenges to attempts to generate a crystal structure of the OT-OTR complex.[29] Further, common problems facing solubilized OTR include reductions in affinity and loss of characteristic binding properties towards ligands. Therefore, SAR for OT analogs is best determined using *in vitro* receptor binding studies, with conclusive studies also testing against AVP receptor subtypes to address selectivity. Inhibitory constant (K_i) values for human receptors are presented in Table 3. Displacement of either [^{3}H]OT or [^{3}H]AVP by dOT analogs was measured over a concentration range of 0.95–30000 nM, while non-specific binding of peptide analogs was defined using the appropriate cold endogenous peptide.[30] Receptor specificity was calculated by comparing the affinity of ligands to members of the OT and the AVP receptor family member that demonstrated the highest binding capacity.

While all analogs screened suffered a decrease in binding affinity toward the OTR compared to native OT, selectivity over the AVP receptor subtypes was improved for dOT_{meta} and maintained for the dOT_{para} derivative (Table 3, Fig. 3a). Further, meta-cyclization demonstrated the highest affinity for OTR binding among the analogs tested. Interestingly, dOT_{ortho} showed high affinity and preferential binding towards V_{1a} receptor (Fig. 3b). The introduction of a