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Nature-inspired dimerization as a strategy to modulate neuropeptide pharmacology exemplified with vasopressin and oxytocin†

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Vasopressin (VP) and oxytocin (OT) are cyclic neuropeptides that regulate fundamental physiological functions *via* four G protein-coupled receptors, $V_{1a}R$, $V_{1b}R$, V_2R, and OTR. Ligand development remains challenging for these receptors due to complex structure–activity relationships. Here, we investigated dimerization as a strategy for developing ligands with novel pharmacology. We regioselectively synthesised and systematically studied parallel, antiparallel and N- to C-terminal cyclized homo- and heterodimer constructs of VP, OT and dVDAVP (1-deamino-4-valine-8-D-arginine-VP). All disulfide-linked dimers, except for the head-to-tail cyclized constructs, retained nanomolar potency despite the structural implications of dimerization. Our results support a single chain interaction for receptor activation. Dimer orientation had little impact on activity, except for the dVDAVP homodimers, where an antagonist to agonist switch was observed at the $V_{1a}R$. This study provides novel insights into the structural requirements of VP/OT receptor activation and spotlights dimerization as a strategy to modulate pharmacology, a concept also frequently observed in nature.

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Introduction

Vasopressin (VP, CYFQNCPRG) and oxytocin (OT, CYIQNCPLG) are closely-related multifunctional neuropeptides consisting of a six-residue macrocyclic structure that is cyclized by a disulfide bond and a three-residue amidated C-terminal tail. VP and OT act *via* four receptors ($V_{1a}R$, $V_{1b}R$, V_2R, OTR) belonging to the rhodopsin-like/class A G protein-coupled receptor (GPCR) family.[1–8] VP/OT receptors are drug targets for cardiovascular and bleeding conditions, fluid and electrolyte disorders, anxiety, aggression, stress, depression, autism, schizophrenia, nephrogenic diabetes insipidus, nocturnal enuresis, prevention of preterm labour, pain and cancer.[4,6,9–11] The study of these receptors relies on a good structural understanding of the ligand–receptor interactions as well as on molecular probes with defined pharmacology. Despite considerable efforts in probe development,[12–15] we still do not have a complete pharmacological toolbox to study this signalling system.[16–18] To address this limitation, we have pursued alternative ligand discovery and modification strategies ranging from innovative medicinal chemistry[11,18–21] to natural product discovery.[22–26] The latter led us to peptide dimerization, a concept frequently observed in nature[27–36] and also studied using a variety of conjugation strategies.[37–48] Disulfide-linked homo- and heterodimeric peptides are commonly found in venoms, underpinning the evolutionary relevance of this strategy.[31,49–56] Dimerization is also integral for human physiology: for instance, β-human atrial natriuretic peptide (β-hANP), a dimer of α-hANP, elicits cardiovascular responses reminiscent of monomeric α-hANP, but with distinct pharmacokinetics and pharmacodynamics, providing slower onset and longer duration.[28,57–59] Insulin is another example for a heterodimer controlling glucose homeostasis, with the monomeric A and B

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chains also able to regulate this function, but to a lesser extent.[60–62] We were further encouraged by a naturally occurring antiparallel inotocin (insect version of VP/OT) homodimer that regulates fluid secretion in the migratory locust.[63,64]

We thus set out to systematically study VP/OT dimerization to establish (a) if and how receptor activation occurs, (b) if dimer directionality plays a role, and (c) whether this strategy can be used to modulate pharmacology.

Results and discussion

Dimer design and synthesis

We devised several regioselective synthetic strategies to produce the parallel and antiparallel VP/OT homo- and heterodimers as well as N- to C-terminal cyclized VP/OT dimers (Fig. 1 and S1†). We used Fmoc-SPPS and orthogonal cysteine protecting groups (combinations of S-Trt, S-Acm and S-Npys groups) to access the individual homo- and heterodimers in the desired orientations (parallel and antiparallel) and Fmoc-SPPS in combination with intramolecular native chemical backbone ligation[65,66] and directed folding to produce the N- to C-terminal cyclized analogues (Fig. 1).

Pharmacological characterization

We evaluated the activity of these dimeric constructs in cells overexpressing each receptor of interest using well-established IP-1 ($V_{1a}R$, $V_{1b}R$, OTR) and cAMP (V_2R) quantitative second messenger-detecting assays (Fig. 2a–c, S3 and S4†).

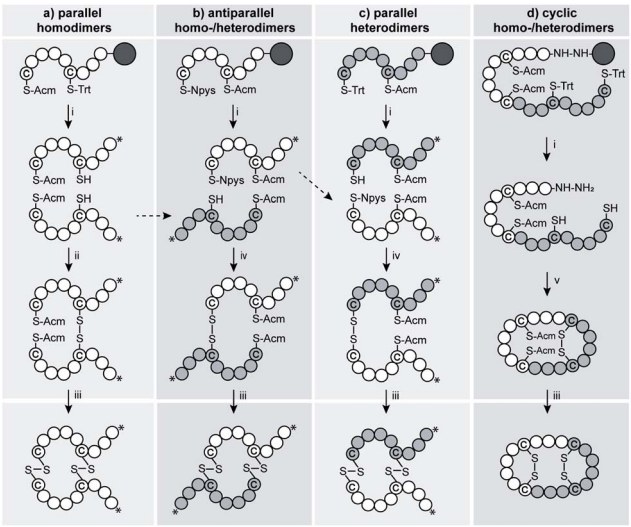

Fig. 1 Synthetic strategies towards parallel, antiparallel and cyclic two-disulfide bond homo- and heterodimers of VP/OT. Orthogonal protecting groups used: Acm, acetamidomethyl; Npys, 3-nitro-2-pyridylsulfenyl; Trt, trityl. Amino acids are represented as circles, cysteine residues are highlighted. Dark grey circle represents rink-amide- (strategy a–c) and 2-chlorotrityl-resin (d). *indicates C-terminal amide. Conditions: (i) TFA:H_2O:TIS, rt, 1.5 h; (ii) air, 6 M GdnHCl/0.2 M NH_4HCO_3, pH 8.2; 24 h; (iii) I_2, 20 mM HCl, 80% MeOH (aq); (iv) argon, 6 M GdnHCl/0.2 M NH_4OAc, pH 4.5; (v) $NaNO_2$, pH 3, 6 M GdnHCl/0.2 M NaH_2PO_4, 2 mM, −20 °C, 15 min then MesNa, pH 7.5, 0.2 mM, rt, o.n.

Interestingly, both parallel and antiparallel VP homodimers activated all four receptors, with only 5–15-fold reduced potency (EC_{50}) compared to VP, yet presented no significant change in selectivity or intrinsic efficacy (E_{max}). Parallel and antiparallel OT homodimers also activated all four receptors, yet with 10–100-fold reduced potencies compared to OT. No significant differences in selectivity or directionality were observed. The lack of directionality dependence coupled with the potency differences between VP and OT dimers suggested that activation occurs through a single chain of the dimer and that the VP chain is structurally better tolerated than the OT chain. This finding was further supported by the data of the antiparallel OT-VP heterodimer, which was slightly more potent than the OT dimers at OTR and less potent than the VP dimers at the VPRs.

The observed pico- to nanomolar potencies were striking considering the switch from a single intramolecular disulfide bond in the monomers to two intermolecular disulfide bonds in the dimers, particularly when put in context with past studies where even minor modifications (*e.g.*, shortening of the OT ring structure by one sulfur atom, or disulfide bond replacement by a dimethylene bridge) led to substantial decrease in activity.[18,20,67] We thus ensured that this observation was not due to monomer formation during the assays (confirmed by HPLC before and after, Fig. S5†). Moreover, we determined the EC_{50} using a fluorescent imaging plate reader (FLIPR) that measured Ca^{2+} responses within seconds after the addition of freshly prepared dimers, further confirming that signalling was indeed induced by intact dimers (Fig. S6 and Table S2†).

Considering the potent agonism of the VP dimers, we also designed and synthesized N- to C-terminal cyclized VP and OT dimers with the aim of creating metabolically more stable analogues. The cyclic analogues were however largely inactive across the four receptors (Fig. 2a and S4†), underpinning the importance of the N-terminus and the free three-residue C-terminus for activity.

We then expanded this strategy to an improved analogue of the drug desmopressin, namely dVDAVP (dCYFVNCPrG, 1-deamino-4-valine-8-D-arginine-VP), a well-known $V_2R/V_{1b}R$ agonist and $V_{1a}R$ antagonist.[14,68–72] Interestingly, during the synthesis of monomeric dVDAVP, we observed spontaneous dimerization during folding (60% dimer, 40% monomer), even at a relatively low peptide concentration of 100 μM, which was not observed with VP or OT. MS/MS experiments revealed that the observed product was exclusively the antiparallel homodimer (Fig. S7 and S8†) with no parallel homodimer formed. We also synthesized the dVDAVP homodimers *via* directed disulfide bond formation to access the parallel homodimer and to confirm the MS/MS experiments *via* analytical HPLC co-elution study (Fig. S9†). Both dVDAVP homodimers were potent V_2R agonists that also displayed some activity at $V_{1b}R$, but no activity at OTR (Fig. 2a and c). Interestingly, in this case, directionality played a role at $V_{1a}R$, where the antiparallel homodimer was an antagonist ($K_i = 53$ nM, similar to dVDAVP monomer:[14,68–72] $K_i = 17$ nM, Fig. S2 and Table S1†) whereas the parallel homodimer was a full $V_{1a}R$ agonist ($EC_{50} = 78$ nM, $K_i = 76$ nM). This finding illustrates that dimeric design can lead to significant functional (antagonist/agonist) switches (Fig. 2a, c and S2, Table S1†).

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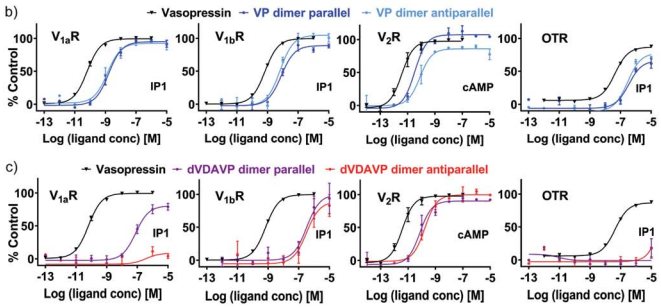
a)

Ligand	hV1aR EC50(nM)	hV1aR Emax	hV1bR EC50(nM)	hV1bR Emax	hV2R EC50(nM)	hV2R Emax	hOTR EC50(nM)	hOTR Emax
VP	**0.14**±0.05	100%	**0.81**±0.10	100%	**0.003**±0.001	100%	**45.6**±6.3	86%±2
p(VP)2	**1.68**±0.40	95%±2	**8.10**±0.67	89%±3	**0.04**±0.006	105%±2	**380**±159	66%±6
ap(VP)2	**1.87**±0.96	93%±3	**6.73**±1.53	105%±3	**0.06**±0.03	98%±3	**278**±46	78%±6
ap(OT-VP)	**10.1**±3.05	95%±4	**34.3**±6.8	103%±5	**0.69**±0.18	100%±2	**100**±34	93%±6
p(dVDAVP)2	**78.0**±16.3	80%±3	**322**±92	100%±7	**0.08**±0.04	101%±3	>10000	–
ap(dVDAVP)2	**Ki=53 nM antagonist**		**300**±53	90%±9	**0.14**±0.06	101%±4	>10000	–
OT	**19.4**±3.3	94%±5	**119**±18	103%±2	**0.36**±0.05	99%±2	**13.4**±6.4	100%
p(OT)2	**512**±105	91%±6	**964**±310	103%±7	**16.6**±7.4	99%±4	**320**±40	100%±6
ap(OT)2	**1100**±657	77%±9	**3752**±1,843	–	**37.8**±5.7	98%±3	**189**±63	97%±5
c(VP)2	>10000	–	>10000	–	**63.9**±10.4	104%±1	>10000	–
c(OT)2	>10000	–	>10000	–	>10000	–	>10000	–
c(OT-VP)	>10000	–	>10000	–	**1494**±529	70%±3	>10000	–



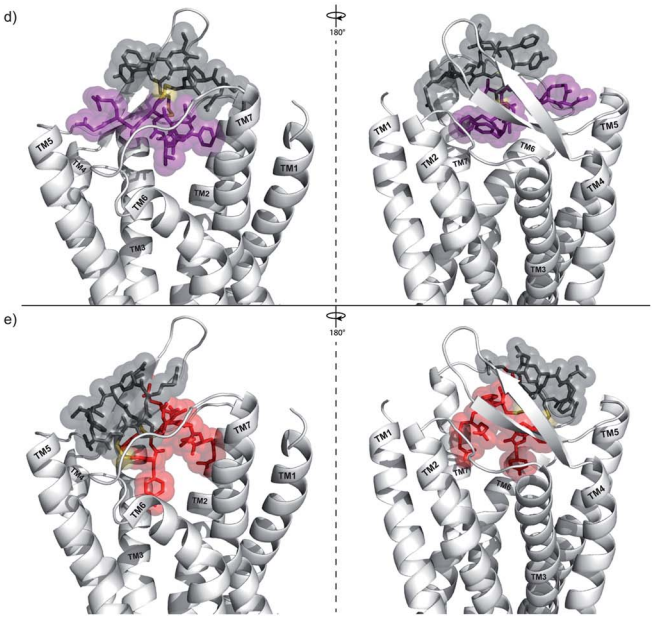


Fig. 2 EC_{50}, E_{max} and representative full-dose response curves of the most potent dimers, $(VP)_2$ and $(dVDAVP)_2$. (a) Table summarising EC_{50} and E_{max}; green colour highlighting noteworthy activity (5–15-fold of OT/VP), and blue improved selectivity. K_i determined by radioligand displacement assays (Table S1†). (b) Concentration response curves of VP homodimers with respect to VP across $V_{1a}R$, $V_{1b}R$, V_2R and OTR. (c) Concentration response curves of dVDAVP dimers with respect to VP across OTR, $V_{1a}R$, $V_{1b}R$, V_2R and OTR. $N = 3$ independent experiments; errors presented as standard error of the mean (SEM), some error bars are smaller than the symbol; for concentration response curves of all dimers, please refer to ESI Fig. S3, S4 and S6.† (d) & e) Possible binding mode derived from *in silico* docking of $p(dVDAVP)_2$ (d) and $ap(dVDAVP)_2$ (e) to $hV_{1a}R$. Receptor docking was performed using a recently reported homology model of $hV_{1a}R$. Please refer to the ESI† for details on structure modelling and binding simulation. The individual strands of the dimers are indicted in grey/purple or grey/red, respectively; interstrand disulfide bond connections are highlighted in yellow; the seven transmembrane (TM) $V_{1a}R$ helices are labelled.

Structural characterization

NMR-based structure determination protocols were not applicable to the dimers due to a lack of long-range nuclear Overhauser effects and assignment ambiguities due to the sequence homology of the dimer chains. Hence, we modelled both, the parallel and antiparallel dVDAVP dimer, by adapting well-established protocols and docked these dVDAVP dimer structures into the $hV_{1a}R$ binding pocket (see ESI† for details on the modelling and docking procedure).[22,73–75] The resulting ligand-receptor-complex models supported our experimental *in vitro* activity data and hypothesis that only a single dimer subunit/chain fits and interacts with the receptor binding pocket (Fig. 2d and e). In qualitative agreement with previously described binding modes of VP and related variants,[22,76–78] we observed energetically low-lying complex conformations where Y^2 and F^3 of the interacting dimer chain, in both parallel and antiparallel $(dVDAVP)_2$, insert into the hydrophobic core of $hV_{1a}R$.

We also carried out circular dichroism (CD) studies (Fig. 3) to provide further insights into overall secondary structures. VP and OT homodimers yielded CD spectra that were highly similar to their corresponding monomeric ligands VP and OT (Fig. 3a and b). The data suggest high flexibility and a structure of intrinsically disordered nature, corresponding well with literature[79] and bioactivity data. The OT-VP heterodimer also displayed a CD profile similar to VP and OT (Fig. 3c), while the inactive N- to C-terminal cyclized analogues had a similar, but less pronounced profile (Fig. 3a–c). By contrast, the dVDAVP homodimers displayed considerable differences, not only in comparison with VP and dVDAVP monomers, but also between the parallel and antiparallel orientation (Fig. 3d). We used BeStSel software tool[80,81] to estimate the secondary structures and found that the parallel dVDAVP homodimer featured a CD spectrum reminiscent of a β-turn structure (38% structural contribution), while the antiparallel dVDAVP homodimer had a spectrum reminiscent of an antiparallel β-strand (58% structural contribution). The CD data also aligned well with our ligand models as two distinct structures were observed for the parallel and antiparallel dVDAVP homodimer (Fig. 3e and f): The $p(dVDAVP)_2$ model features two stable β-turn motifs between residues F^3 and C^6 of both subunits, which were less pronounced for $ap(dVDAVP)_2$. These structural differences, might also account for the spontaneous formation of the antiparallel dimer during the undirected folding event, indicating an energetically favoured antiparallel orientation. This was supported by energy calculations for the antiparallel and parallel model structures, which yielded a considerably lower conformational energy (-4501 kJ mol^{-1}) for the antiparallel homodimer *versus* the parallel dimer (-2364 kJ mol^{-1}).

Our work furthermore clarified the mechanism of action of earlier efforts investigating neuropeptide dimers:[63,82–86] OT dimers displayed weak but protracted and long-lasting uterotonic activity in rats, which the authors speculated was due to

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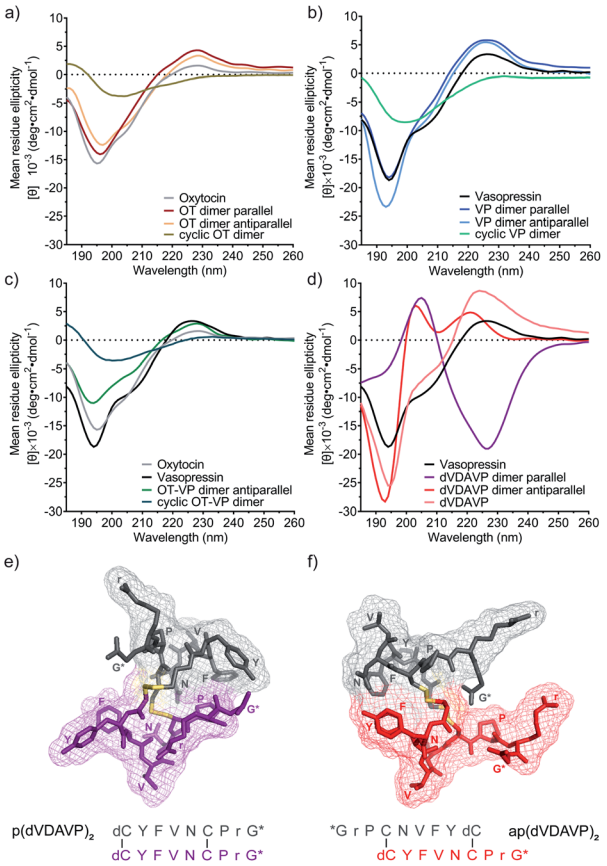

Fig. 3 CD spectra of homo and heterodimeric constructs and *in silico* models of parallel and antiparallel dVDAVP dimers. CD spectra of (a) OT dimers in comparison with OT. (b) VP dimers in comparison with VP. (c) OT-VP antiparallel and cyclic dimers in comparison with OT and VP. (d) dVDAVP dimers in comparison with VP and dVDAVP. CD spectra were recorded in 10 mM aqueous sodium phosphate at pH 7.4 and 25 °C. Ligand concentrations were 50 μM for monomers and 25 μM for dimers. (e) *In silico* modelled 3D structure of p(dVDAVP)$_2$. (f) *In silico* modelled 3D structure of ap(dVDAVP)$_2$. Single chains are indicted in different colours; interstrand disulfide bond connections are highlighted in yellow. The structures represent energetically low-lying snapshots of a highly dynamic conformational ensemble. Please refer to the ESI† for details on the structure simulation procedure.

the dimers slowly reverting to monomers.[83,86] Here we, however, demonstrated that monomer formation is not required for receptor activation. This also highlights that dimerization can modulate *in vivo* pharmacokinetics and pharmacodynamics, as observed with the more protracted and longer-lasting uterotonic activity compared to OT.[86] As such, dimerization could play a valuable role in optimizing therapeutic effects or in the development of bifunctional heterodimers to generate synergistic effects by targeting two distinct receptors.

Conclusions

We demonstrated that VP and OT homo- and heterodimers retain their abilities to potently activate their receptors despite the structural rearrangement imposed through dimerization. We provided novel structural and mechanistic insights into the concept of dimerization which is commonly observed in nature and adds a new design strategy to the development of ligands with distinct pharmacology. Considering the common activation mechanism across class A GPCRs,[1] our findings may also be relevant for other receptors of this family.

Author contributions

Z. D. and T. K. contributed equally to this work. Z. D., T. K. and M. G. synthesised the compounds. Z. D. and T. K. performed the CD analysis. P. K., N. T. and L. D. performed the functional assays. L. D., H. X., D. G. and C. B. carried out the radioligand binding assays. R. H., M. G., M. V. conducted the structure determination *via* mass spectrometry. D. K. and T. K performed the molecular modelling. M. M., C. W. G., P. F. A., and F. A. conceived, funded, and supervised the project. M. M., C. W. G., D. K. and T. K. prepared the manuscript. All authors read the manuscript and provided feedback.

Conflicts of interest

There are no conflicts to declare.

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