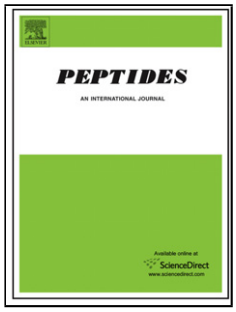

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Stapled truncated orexin peptides as orexin receptor agonists

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Highlights

Peptide stapling of the neuropeptide orexin-$A_{15–33}$ lowers the potency significantly.

The peptide C-terminus is crucial for activity, and modifications were not allowed.

Central and N-terminal modifications yielded bioactive peptides.

Abstract

The peptides orexin-A and -B, the endogenous agonists of the orexin receptors, have similar 19-amino-acid C-termini which retain full maximum response as truncated peptides with only marginally reduced potency, while further N-terminal truncations successively reduce the activity. The peptides have been suggested to bind in an α-helical conformation, and truncation beyond a certain critical length is likely to disrupt the overall helical structure. In this study, we set out to stabilize the α-helical conformation of orexin-$A_{15–33}$ via peptide stapling at four different sites. At a suggested hinge region, we varied the length of the cross-linker as well as replaced the staple with two α-aminoisobutyric acid residues. Modifications close to the peptide C-terminus, which is crucial for activity, were not allowed. However, central and N-terminal modifications yielded bioactive peptides, albeit with decreased potencies. This provides evidence that the orexin receptors can accommodate and be activated by α-helical peptides. The decrease in potency is likely linked to a stabilization of suboptimal peptide conformation or blocking of peptide backbone–receptor interactions at the hinge region by the helical stabilization or the modified amino acids.

Abbreviations

Aib, α-aminoisobutyric acid; Fmoc, fluorenylmethyloxycarbonyl; HPLC, high-performance liquid chromatography; HBM, Hepes-buffered medium

Keywords

peptide stapling; pseudopeptide; orexin; G protein-coupled receptor

1. Introduction

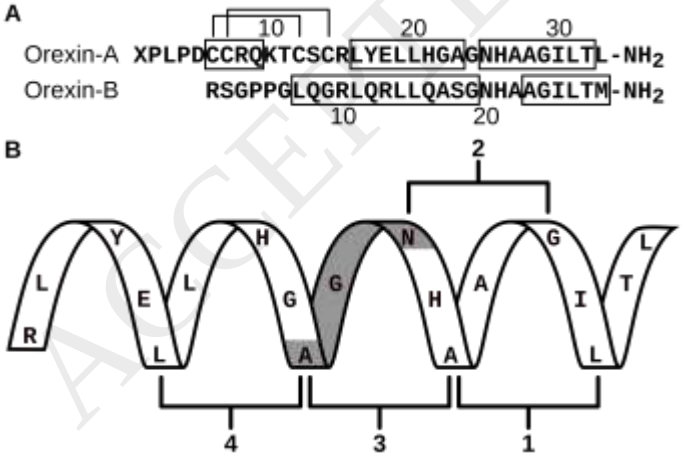
The neuropeptides orexin-A and -B regulate energy homeostasis, the reward system, and the sleep–wake patterns through the G protein-coupled OX_1 and OX_2 receptors [1–4]. Drug discovery for the orexinergic system has mainly concentrated on insomnia [3]. Several small molecule orexin receptor antagonists have been developed as hypnotics; thus far one, suvorexant, has reached the market [5]. In contrast, only few non-peptide agonists have been reported [6–9]. Both orexin receptors have recently been crystallized with non-peptide antagonists [10,11].

Human orexin-A is a 33-amino-acid peptide with C-terminal amidation, N-terminal pyroglutamyl cyclization and two disulfide bridges (Cys6–Cys12 and Cys7–Cys14) [1]. Orexin-B is a linear 28-amino-acid peptide with C-terminal amidation [1]. The C-termini of the peptides are highly conserved as 13 of the 19 C-terminal amino acids are identical (Fig. 1A). In aqueous solution, orexin-A exhibits three helical segments (residues 6–9, 16–23 and 25–32) [12,13], and orexin-B two helical segments (residues 7–19 and 23–28) [14](Fig. 1A). In orexin-A, the turn between the N-terminal helix and the middle helix is stabilized by the disulfide bridges. Both peptides feature a flexible “hinge” between the C-terminal helix and the adjacent helix (Fig. 1A). NMR studies have identified two distinct bent conformations and one straight conformation for orexin-A [12,13], and one bent conformation for orexin-B [14]. It has been postulated that receptor binding would also take place in one of these conformations [15,16], yet despite efforts, the bioactive conformation and the binding mode remain to be experimentally confirmed.

The conserved C-terminus is critical for the biological activity, and while N-terminal truncation down to 19 residues is tolerated with only a modest reduction in potency, further shortenings lead to successive loss of activity [17–20]. We speculate this to be due to disruption of the secondary structure, especially since the residues eliminated in this further truncation (orexin-A: Arg15–Leu19; orexin-B: Arg10–Leu14) can be individually mutated to alanine without a similar loss of biological activity [18–21]. We set out to investigate whether an introduction of a conformational constraint in orexin-$A_{15–33}$ was allowed, and if successful, whether this could allow further truncation of the peptide. We employed the hydrocarbon stapling technology, a macrocyclization strategy that involves an introduction of two non-natural amino acids, each having a side chain with a terminal alkene group, followed by a ring-closing metathesis reaction between these side chains [22]. Successful stapled ligands towards protein–protein interaction targets such as Bcl-2 and the p53-inactivating proteins MDM2 and MDMX, and even towards G protein-coupled receptors such as neuropeptide Y and galanin receptors, highlight that the position and length of the hydrocarbon staple are critical for successful stabilization of an α-helical conformation [23]. In addition to

prospects of successful further truncation of the orexin peptides, macrocyclization of linear peptides can enhance proteolytic stability of peptides, which would be beneficial for drug development.

We selected four sites in orexin-$A_{15–33}$ (Fig. 1B, Table 1) to probe the effects of helical stabilization and the available space for the cross-linker. Orexin-A features distinctive hydrophobic and hydrophilic sides, which may be required for membrane interactions and receptor binding [13,20,24]. To avoid disturbing this, we preferred placing the hydrophobic staple at the hydrophobic side, even though many hydrophobic residues are important for bioactivity [17–21]. Our first choice for introducing a staple was a pair of alanine residues (Ala23 and Ala27) at the peptide hinge region (site 3 in Fig. 1B). Secondly, we selected Ala27 and Leu31 near the C-terminus (site 1 in Fig. 1B), as a staple near the end of the helix might reduce helical fraying and result in stabilization. The peptide C-terminus is expected to penetrate into the receptor cavity [16–20], but as leucine has a bulky side chain, we supposed that there might be sufficient space for the hydrocarbon staple (Fig. 2A). Thirdly, we placed a staple at Leu19 and Ala23 at the N-terminus of the peptide fragment (site 4 in Fig. 1B). This part of the peptide is expected to reside beside the extracellular loops of the receptor [16], where there should be more space and flexibility to accommodate the staple. However, this staple would not permit us to truncate further than to orexin-A_{19-33}, thus countering our secondary aim of producing shorter biologically active orexin peptide fragments. Our fourth stapling site was at Asn25 and Gly29, on the hydrophilic face of the helix (site 2 in Fig. 1B). Previous studies suggest that Asn25 could be replaced with alanine without a marked loss on activity [17,18]. However, we were doubtful whether the introduction of hydrophobic bulk at a polar side of the helix would be tolerated, especially as replacing Gly29 with alanine is not allowed



[17,19–21].

Fig. 1. (A) Sequence alignment of the orexin peptides. Lines show the disulfide bridges, and boxes the helical segments [13,14]. X = pyroglutamic acid. (B) Schematic representation of the sites for helical stapling in orexin-$A_{15–33}$. Peptide N-terminus (R15) is on the left, and modification sites are numbered starting from the C-terminus. The flexible hinge region is shaded in grey.

Table 1

Sequences of the synthetic peptides

Compound	Stapling		Amino acid position in orexin-A																			
			15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	
Ac-orexin-$A_{15–33}$	Linear	Ac-	R	L	Y	E	L	L	H	G	A	G	N	H	A	A	G	I	L	T	L	-NH_2
Orexin-$A_{15–33}$	Linear		R	L	Y	E	L	L	H	G	A	G	N	H	A	A	G	I	L	T	L	-NH_2
1A	Stapled	Ac-	R	L	Y	E	L	L	H	G	A	G	N	H	**X^5**	A	G	I	**X^5**	T	L	-NH_2
$1A^L$	Linear		R	L	Y	E	L	L	H	G	A	G	N	H	**X^5**	A	G	I	**X^5**	T	L	-NH_2
2A	Stapled	Ac-	R	L	Y	E	L	L	H	G	A	G	**X^5**	H	A	A	**X^5**	I	L	T	L	-NH_2
3A	Stapled	Ac-	R	L	Y	E	L	L	H	G	**X^5**	G	N	H	**X^5**	A	G	I	L	T	L	-NH_2
3B	Stapled	Ac-	R	L	Y	E	L	L	H	G	**X^4**	G	N	H	**X^7**	A	G	I	L	T	L	-NH_2
3C	Stapled	Ac-	R	L	Y	E	L	L	H	G	**X^7**	G	N	H	**X^4**	A	G	I	L	T	L	-NH_2
3D	Stapled	Ac-	R	L	Y	E	L	L	H	G	**X^4**	G	N	H	**X^5**	A	G	I	L	T	L	-NH_2
$3D^L$	Linear		R	L	Y	E	L	L	H	G	**X^4**	G	N	H	**X^5**	A	G	I	L	T	L	-NH_2
$3E^L$	Linear	Ac-	R	L	Y	E	L	L	H	G	**Aib**	G	N	H	**Aib**	A	G	I	L	T	L	-NH_2
4A	Stapled	Ac-	R	L	Y	E	**X^5**	L	H	G	**X^5**	G	N	H	A	A	G	I	L	T	L	-NH_2

Modified residues are in bold. X^N: a stapling residue with side-chain length of N atoms

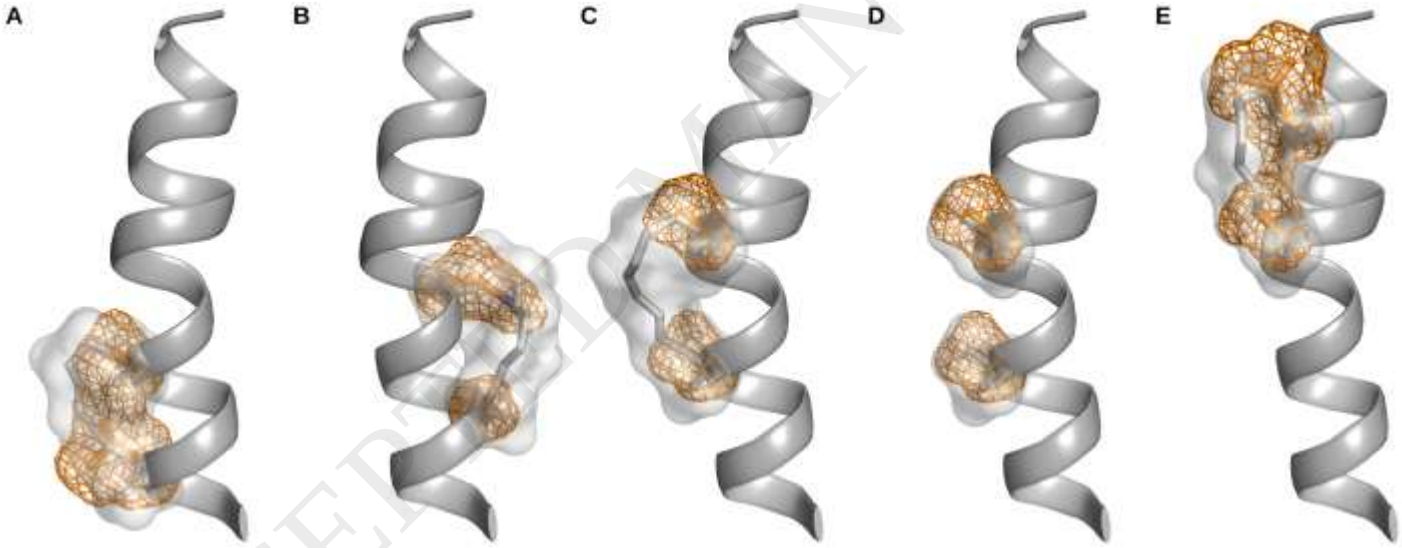


Fig. 2. Size comparison of the wild-type residues (in orange) and the modifications (grey) in orexin-$A_{15–33}$. (A–C and E) Sites 1–4 with an eight-atom staple, (D) site 3 with an Aib-modification. The peptide conformation shown here is the straight helical conformation determined by Takai et al. [13] for this region of the full-length orexin-A. The left-hand face of the peptide features a hydrophobic wall.

2. Materials and Methods

2.1. Stapling site design and images

Stapling sites were designed, three-dimensional space requirements estimated, and the 3D figures in the article generated with Discovery Studio 4.5 (Dassault Systèmes BIOVIA, San Diego, CA, USA) and PyMOL 1.7.0.0. (Schrödinger, LLC, New York, NY, USA).

2.2. Peptide synthesis and purification

The peptides were synthesized manually in a plastic syringe fitted with a filter on Rink Amide AM resin in 0.05 mmol scale using fluorenylmethyloxycarbonyl (Fmoc) -chemistry. The alkene-containing side chains for ring-closing metathesis reaction were introduced by using Fmoc-(*S*)-*N*-Fmoc-2-(3'-butenyl)alanine, (*S*)-*N*-Fmoc-2-(4'-pentenyl)alanine and (*S*)-*N*-Fmoc-2-(6'-heptenyl)alanine as amino acids in the peptide synthesis, depending on the desired length of the cross-linker; two (4-pentenyl)alanines gives the canonical α-helix-stabilizing staple. The ring-closing metathesis reaction was performed on the Fmoc-protected on-resin peptide with Hoveyda-Grubbs 2nd generation catalyst in anhydrous 1,2-dichloroethane in a microwave tube. The reaction mixture was bubbled with argon for 30 min at room temperature, and then the sealed tube was heated in the microwave cavity at 100 °C for 60 min without stirring; for these peptides, standard reaction conditions with argon-bubbling at room temperature did not lead to a high conversion of the ring-closing metathesis reaction. The Fmoc-group was removed after ring-closing metathesis and the peptides were acetylated with dimethylformamide:acetic anhydride:diisopropylethylamine (90:10:1) prior to cleavage from the resin with trifluoroacetic acid:H_2O:1,2-ethanedithiol:triisopropylsilane (94:2.5:2.5:1). Those peptides that were not ring-closed or acetylated did not undergo these reactions. The yield of crude peptides after cleavage from resin, followed by precipitation with diethyl ether, was 39–72%. The peptides were purified to a minimum purity of 90% by preparative high-performance liquid chromatography (HPLC) followed by identity verification by mass spectrometry and purity analysis by analytical HPLC. After purification, the total yield for linear peptides was 4.4–14% and for ring-closed 1.0–6.7%. More detailed description of the synthesis is available in the Supplemental Material. The resulting peptides are summarized in Table 1.

2.3. Cell culture

CHO-hOX_1 and -hOX_2 cells [18,25] were cultured in Ham's F12 medium (Gibco/Life Technologies, Paisley, UK) supplemented with 10% (v/v) fetal calf serum, 100 U/ml penicillin G (Sigma-Aldrich, St. Louis, MO, USA) and 80 U/ml streptomycin (Sigma) at 37°C in 5% CO_2 in an air-ventilated humidified incubator on plastic culture dishes (56 cm^2 bottom area; Greiner Bio-One GmbH, Frickenhausen, Germany). For Ca^{2+} measurements, the cells were plated on polyethyleneimine-coated (25 μg/ml for 1 hour at 37°C; Sigma-Aldrich) black, clear-bottom half-area Cellstar μClear 96-well cell culture plates (Greiner).

2.4. Experimental medium

Hepes-buffered medium (HBM) was used as the experimental medium for cell experiments. It was composed of 137 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 0.44 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1 mM $CaCl_2$, 10 mM glucose, and 20 mM HEPES, pH 7.4 with NaOH. Stripped bovine serum albumin [0.1% (w/v)] [26] was added to reduce possible attachment of the ligands to the plastic, and 1 mM probenecid [*p*-(dipropylsulfamoyl)benzoic acid] to inhibit extrusion of the Ca^{2+} indicator.

2.5. Drugs

Probenecid [*p*-(dipropylsulfamoyl)benzoic acid] was from Sigma-Aldrich and human orexin-A from NeoMPS (Strasbourg, France).

2.6. Pharmacological analysis

The ability of the synthetic ligands to elevate intracellular Ca^{2+} concentration in human OX_1 or OX_2 receptor-expressing cells was used as a measure of agonistic activity on orexin receptors [8,18,27], and the ability of the synthetic ligands at subeffective concentrations to inhibit orexin-A response as a (semiquantitative) measure of antagonistic activity. Ca^{2+} elevations were measured as described [8]. The cells, 1.5×10^4 per well, were plated on black, clear-bottom half-area 96-well plates. Twenty-four hours later, cell culture medium was removed and the cells were loaded with the loading solution composed of FLIPR Calcium 4 Assay Kit (Molecular Devices, Sunnyvale, CA, USA) dissolved in and diluted with HBM + 1 mM probenecid, for 60 min at 37 ºC. Then, the plate was placed in a FlexStation 3 fluorescence plate reader (Molecular Devices) and the intracellular Ca^{2+} levels were measured as fluorescence changes (excitation at 485 nm, emission at 525 nm) at 37 ºC. A recording was made approximately every 1.9–2.4 s. Each well was measured for 180–210 s with 30 s of baseline before stimulation (orexin-A or other ligands in 10-fold dilution series). This experimental setup gave the agonistic property of the ligands. Orexin-A was always tested in parallel to the synthetic ligands to allow evaluation of cell responsiveness and normalization to the orexin response (Fig. 3). Every concentration-response curve in each independent experiment was separately curve-fitted to Equation (1) [28].

$$\text{response} = \frac{[\text{compound}]^n \times \text{response}_{max}}{[\text{compound}]^n + EC_{50}{}^n} \qquad (1)$$

The EC_{50}-values obtained were converted to $logEC_{50}$-values and normalized to the $logEC_{50}$-value of orexin-A [$logEC_{50}$(compound) – $logEC_{50}$(orexin-A)] in each batch of cells before averaging the values from different experiments (Fig. 3). However, there was not much variation in the EC_{50} of orexin-A between the independent experiments (see Results).

Secondly, we assessed the antagonistic ability of the synthetic ligands [8,18]. Each plate was rerun in FlexStation 3 (approximately 60 min after the first run). The scheme was as above, but all wells with the synthetic ligands were stimulated with 0.1–0.3 nM orexin-A. The concentration of orexin-A was chosen close to the EC_{50}-value to give a robust response yet sensitive to antagonism.

Calcium measurements were performed in triplicate or quadruplicate. All the values are given as mean ± sem. *n* indicates number of independent experiments; each experiment was repeated at least 3 times. Statistical significance was evaluated using Student's two tailed paired *t* test. All data analyses, including curve-fitting and *t* test, were performed with Microsoft Excel (Microsoft Co., Redmond, WA, USA).

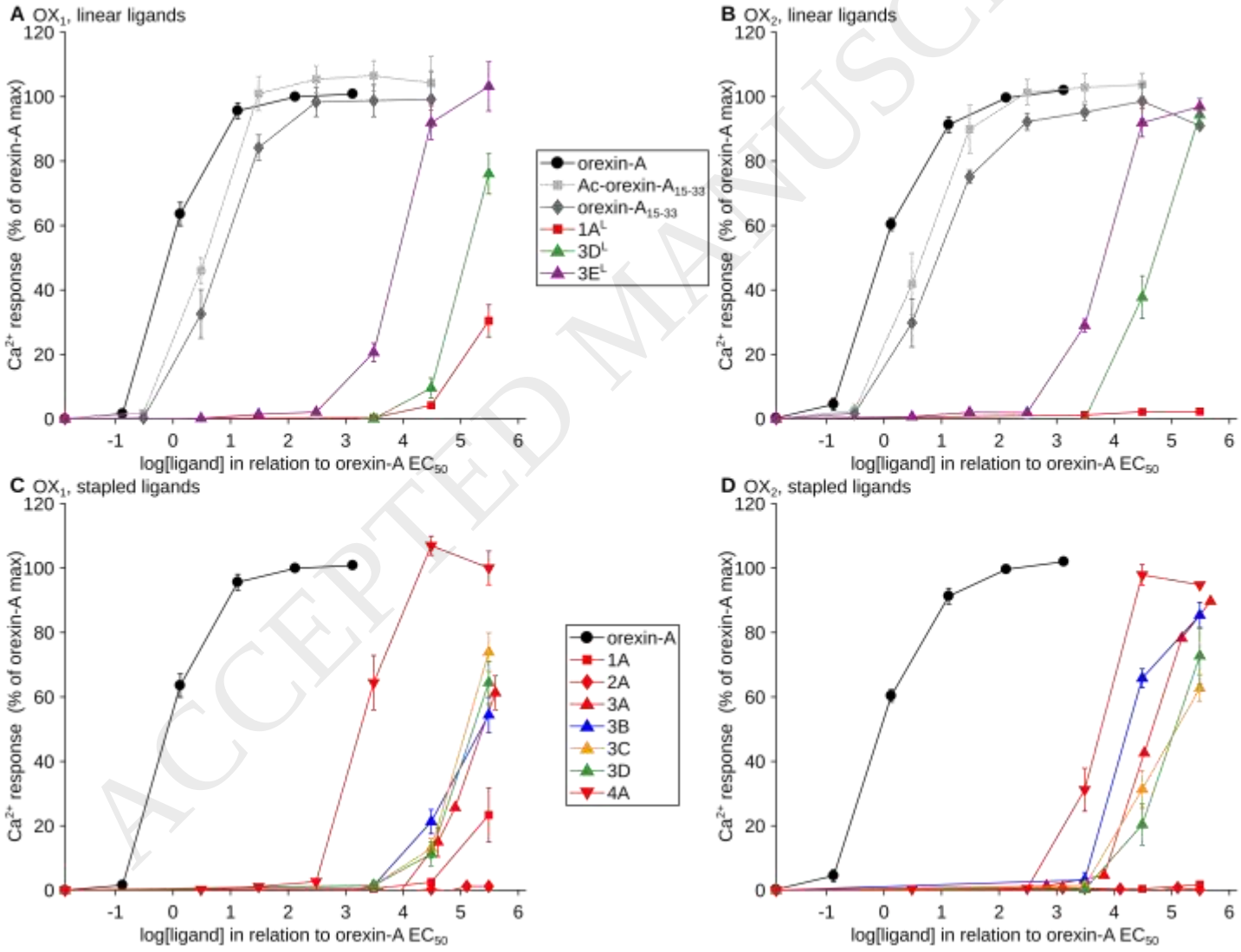
3. Results

Full-length orexin-A showed a robust Ca^{2+} response with an EC_{50} of 0.12 nM in both OX_1- and OX_2-expressing cells ($logEC_{50} = -10.0 \pm 0.08$, $n = 8$, for both OX_1 and OX_2). Data with all other ligands were normalized to orexin-A responses measured in parallel. Truncation to orexin-$A_{15–33}$ resulted in a 6-fold and 7-fold (5.9 ± 2.0 and 6.9 ± 2.5 -fold, $n = 3$) reductions in potency for OX_1 and OX_2, respectively, while the N-terminally acetylated truncated peptide (Ac-orexin-$A_{15–33}$) was slightly more potent ($p < 0.05$) with a 4-fold (3.5 ± 0.74 and 4.1 ± 1.7 -fold, $n = 3$) decrease in potency in comparison to orexin-A (Fig. 3B). Both fragments produced the same maximal response as orexin-A (Fig. 3A).

Introduction of the typical α-helix-stabilizing staple of eight atoms into Ac-orexin-$A_{15–33}$ (compounds 1A, 2A, 3A and 4A; Table 1) was unfavorable for the biological activity (Figs. 3 and 4). Within the tested concentration range (up to 20 μM), only compound 4A was able to elicit the same maximal response as the full agonists orexin-A and orexin-$A_{15–33}$, with 610–980-fold decrease in potency in relation to Ac-orexin-$A_{15–33}$. Compounds 1A and 3A did not reach response saturation at the maximum concentration of 20 μM; for these, responses at 20 μM are given in Fig. 3. Compound 1A was extremely weak at OX_1 and inactive at OX_2, while compound 2A was inactive at both receptors. Assuming that compound 3A is a full agonist, it would show approximately 57 000-fold (OX_1) and 6700-fold (OX_2) reductions in potency in relation to Ac-orexin-$A_{15–33}$.

At site 3, we tested cross-linkers that were either one carbon atom longer (compounds 3B and 3C) or shorter (compound 3D) than the typical α-helix-stabilizing staple of eight atoms. None of these compounds reached response saturation at 20 μM (Fig. 4), but if one assumes that these are full agonists, these compounds show 4800–76 000-fold decreases in potency in relation to Ac-orexin-$A_{15–33}$ (Fig. 3).

Fig. 3. Potency as $logEC_{50}$ (A) and maximal response (B) of the compounds in relation to orexin-A. Error bars show standard error of the mean. n = 3, except for orexin-A (n = 8), and for 1A, 3D, $3D^L$, and 4A (n = 4).



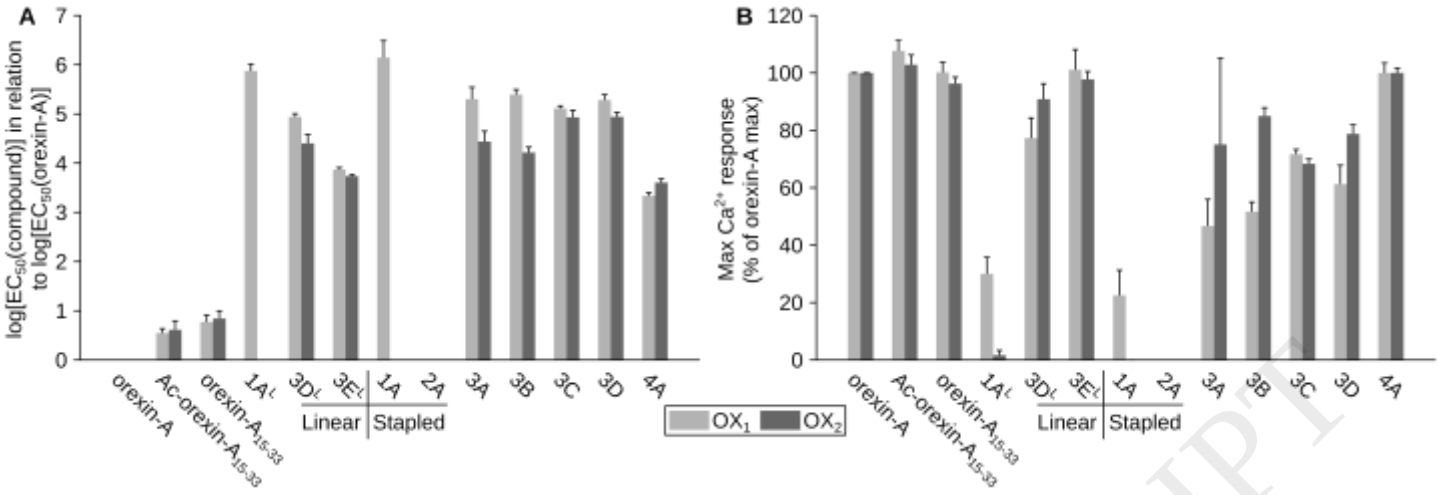


Fig. 4. Concentration-response curves for the tested ligands. *n* = 3, except for orexin-A (*n* = 8), and for 1A, 3D, 3DL, and 4A (*n* = 4). Non-normalized data were averaged and then normalized to the logEC_{50} of orexin-A.

To assess the effect of the cross-linking between the unnatural amino acids, we set out to purify the reaction intermediates prior to the ring-closing metathesis reaction for biological testing. From samples originally taken aside for analysis, we purified two linear intermediates: precursors of compounds 1A and 3D, which were named $1A^L$ and $3D^L$, respectively (Table 1). Compound $1A^L$ appeared slightly more potent than compound 1A at OX_1 (Figs. 3 and 4), but the difference was not significant. Like compound 1A, compound $1A^L$ was inactive at OX_2. Site 3 showed a similar trend, with compound $3D^L$ being slightly more potent than the stapled 3D at both receptors (Figs. 3 and 4), but the small difference was only significant ($p<0.05$) at OX_2.

To test if the problem lay with the unnatural side chains of the cross-linking residues, we synthesized a derivative of Ac-orexin-$A_{15–33}$ in which Ala23 and Ala27 (site 3) were replaced by α-aminoisobutyric acid (Aib) residues (compound $3E^L$; Fig. 2D, Table 1). Aib features the same disubstituted α-carbon as the stapling residues – in essence it has the side chains of both L- and D-alanine – and it too promotes helicity [29]. Compound $3E^L$ was a full agonist with 2200-fold (OX_1) and 1600-fold (OX_2) decreases in potency in relation to Ac-orexin-$A_{15–33}$ (Figs. 3 and 4).

To assess whether the structural modifications affect the binding itself (affinity) or ability to activate the receptor (efficacy), we also analyzed the synthetic peptides for possible antagonistic properties. Cells pre-treated with subeffective concentrations (10-fold below the first concentration giving a response) of modified peptides and then exposed to 0.1–0.3 nM orexin-A showed that none of the modified peptides inhibited orexin-A response (data not shown), which indicates that the structural modifications impair the binding affinity.

4. Discussion

The potency of full-length orexin-A was in line with previous studies [27], and also the 4–7-fold reduction in potency associated with truncation to orexin-$A_{15–33}$ is well in line with some previous reports [18,19], while greater decrease in potency has also been reported in other studies [17,30].

The typical eight-atom stapling was detrimental to the biological activity of orexin-$A_{15–33}$, and consistent with the results of the site-directed mutagenesis studies [17,19–21], the effect was greater towards the peptide C-terminus. The staples in compounds 1A and 2A were clearly not allowed, as these compounds were (virtually) inactive. For compound 2A, this was to be expected, as it introduced a hydrophobic staple at a hydrophilic site, and also because the replaced Gly29 is important for bioactivity [17,19–21]. In compound 1A, the staple replaces Leu31, which also is required – in correct orientation – for the activity [17,19–21]. While the staple should mostly occupy the same space as the wild-type residue (Fig. 2A), it clearly is not a suitable replacement. However, in compounds 3A and 4A, the replaced residues should have been non-essential for receptor activation [18,21], but we still observed marked decreases in potency. We reasoned that there were three likely alternatives: the stabilized conformations were suboptimal, or the conformational rigidity or the added bulk of the staples was detrimental to receptor binding or activation.

As the NMR-studies on orexin peptides have pointed out different conformations, especially in the "hinge" region (site 3 in Fig. 1B) [12–14], we decided to probe them with cross-linkers that were either one carbon atom longer (compounds 3B and 3C) or shorter (compound 3D). As the potencies of these compounds were of the same magnitude with the compound 3A, the conformations were apparently still unsuitable or the problem indeed lay in the added bulk of the cross-linker. To assess this further, we tested whether the linear precursor peptides, which carried the same unnatural amino acid substitutions but were not cross-linked, would have been more potent. We reasoned that although the unnatural amino acid residues with two alkyl substituents on the α-carbon promote helicity even without the cross-linking, the unlinked side chains can rotate freely and adopt better to the receptor's binding site. As the linear compounds $1A^L$ and $3D^L$ were slightly more potent than their ring-closed counterparts 1A and 3D, it appears that the ring closure was indeed unfavorable. However, as the linear compounds were still much weaker than Ac-orexin-$A_{15–33}$, the main cause for the potency decrease at these sites was clearly not the cross-linking step, but the introduction of the cross-linking residues per se.

Since the unnatural amino acid residues used in the cross-linking were poorly tolerated, we synthesized the Aib-derivative $3E^L$, in which the wild-type alanines at site 3 were replaced by Aib residues, which – like the cross-linking residues – feature disubstituted α-carbons to promote

helicity [29]. The compound $3E^L$ was the most potent among compounds that carried a modification at site 3 – though still weaker than compound 4A. In comparison to the linear $3D^L$ with 4-pentenyl and 3-butenyl side chains, $3E^L$ with methyl side chains is 5–12-fold more potent. This suggests that, at least at this site, one of the problems with stapling lies with the bulk of the side chains, which may interfere with the peptide–receptor interactions. However, as also $3E^L$ was significantly less potent than the parent peptide Ac-orexin-$A_{15–33}$, something in the double Ala-to-Aib replacement must be detrimental to bioactivity, even though Aib should only take a little more space than alanine (Fig. 2D). Since the only difference as compared to the wild-type L-alanine is a methyl group instead of a hydrogen atom at the α-carbon, there is a limited number of possible causes for the decrease in potency (see below).

Our original hypothesis was that the C-terminus of the orexin peptides would interact with the cognate receptors in a straight α-helical conformation, and that stabilization of that conformation would be beneficial for biological activity. However, it now seems likely that this hypothesis might be incorrect, and that the stabilization, at least with the methods we have used here, in fact impairs the peptide's ability to bind to, or to activate their receptors. While the stapled peptides 3A, 3B, 3C, 3D and 4A show that the receptor can accommodate and be activated by α-helical peptides, the stapling clearly decreases the potency. The linear compounds $1A^L$ and $3D^L$ show that the unnatural stapling amino acids themselves are poorly tolerated, and the Aib-variant $3E^L$ highlights the disubstituted α-carbon as a likely culprit. It is possible that the peptide needs to adopt a specific bent conformation or that a certain degree of conformational flexibility is beneficial for binding to or activation of the receptor, while our modifications stabilize the α-helix and restrict flexibility. It is also possible that the additional methyl groups at the α-carbons present steric hindrance for peptide–receptor interactions involving the peptide backbone.

We only used Ca^{2+}-elevation as a measure for orexin-receptor-mediated activity. While this G_q-mediated pathway is suggested to reflect the main physiological coupling [31], we cannot rule out biased signaling; i.e. even though the modified peptides show marked drops in potency for Ca^{2+}-elevation, they might be more potent in activating other pathways. However, as our assay for antagonistic properties implies that our modified peptides are weak binders, it seems implausible that they would be very potent with respect to another signal pathway either.

All the staples we introduced into the orexin-$A_{15–33}$ spanned one helical turn (*i*, *i*+4), whereas for some peptides, *i*, *i*+7 stapling spanning two helical turns is better [22]. Given that modifications in the peptide C-terminus were poorly tolerated, *i*, *i*+7 stapling could be tried closer to peptide N-terminus, perhaps at Leu19–His26 or Leu20–Ala27, even though Leu20 is important for receptor

activation. However, if the helical stabilization itself is detrimental to the binding or efficacy of the peptide, the location or configuration of the staple should not alleviate this.

Peptide stapling has mostly been utilized to interfere with protein–protein interactions, which often take place over large surface areas, where potential binding sites are shallow grooves easily accessible from the solvent. In these cases, one side of the peptide helix is often not in contact with the target protein, leaving ample space for the staple. In only few cases stapled peptides, namely those based on galanin and neuropeptide Y, have been reported to activate G protein-coupled receptors [32]. The bioactive N-terminal fragment of galanin, galanin$_{1–16}$, was stabilized with central or C-terminal staples together with increased cationization at the C-terminus, and neuropeptide Y as the bioactive N-terminal fragment NPY$_{25–36}$ with central staple or with C-terminal addition of spacer and a stapled poly-Lys sequence. These compounds had lower in vitro potencies (NPY) or affinities (galanin) than their parent peptides but retained their *in vivo* activities, perhaps due to improved metabolic stability.

5. Conclusions

The *i*,*i*+4 stapling of the truncated orexin-A$_{15–33}$ peptide at four different positions (Leu19–Ala23, Ala23–Ala27, Asn25–Gly29, and Ala27–Leu31) lowers the potency of the peptide significantly. Modifications close to the peptide C-terminus are not tolerated. At the central position (Ala23–Ala27) spanning the putative small bend, cross-linkers one carbon atom shorter or longer, or simple Aib replacements at the same position, also result in decreased potency. The introduced modifications clearly lead to poor peptide–receptor recognition and/or impaired activation. This might be due to suboptimal peptide conformation or the lack of space for the cross-linker and the additional methyl substituent at the α-carbons, or alternatively, the modifications block important peptide–receptor interactions either directly or due to helical stabilization, which shields the peptide backbone from receptor residues. However, although this does not confirm the active conformation of the wild-type orexin-A, our stapled peptides provide evidence that the orexin receptors can be activated by α-helical peptides.

Our study shows that successful conformational stabilization of orexin-A with the stapled peptide methodology or other similar cross-linkers, or by introduction of Aib is challenging. However, as two of our compounds (3E^{L} and 4A) show, certain modifications are better tolerated. Stapled or Aib-modified peptides could be further studied especially for their pharmacokinetic properties such as membrane penetration (mainly gut wall and blood–brain barrier), plasma half-life and metabolic

stability, as these factors also affect biological activity; however, such studies would require development of peptides with higher in vitro activity than the current ones.

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Declaration of interest

The authors declare no conflicts of interest.

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