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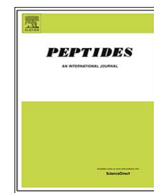

Stapled truncated orexin peptides as orexin receptor agonists

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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.

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.

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

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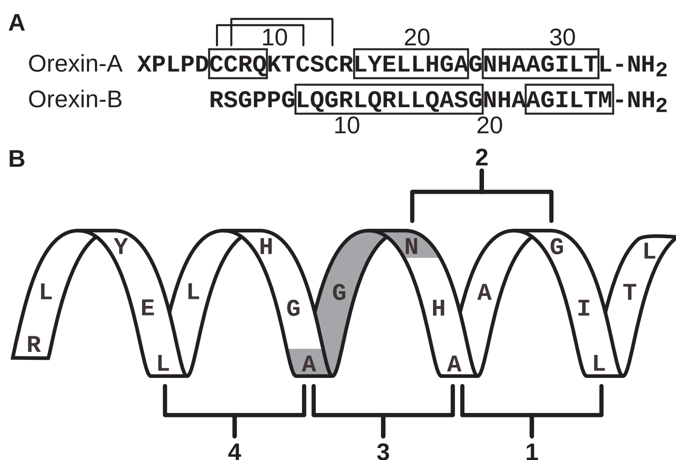

Fig. 1. (A) Sequence alignment of the orexin peptides. Lines show the disulfide bridges, and boxes the helical segments [13,14]. X = pyroglutamic acid. (B) Schematic representation of the sites for helical stapling in orexin-$A_{15–33}$. Peptide N-terminus (R15) is on the left, and modification sites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

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 crosslinker. 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].

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

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	$\mathbf{X}^{5}$	A	G	I	$\mathbf{X}^{5}$	T	L	$-NH_2$
$1A^L$	Linear		R	L	Y	E	L	L	H	G	A	G	N	H	$\mathbf{X}^{5}$	A	G	I	$\mathbf{X}^{5}$	T	L	$-NH_2$
2A	Stapled	Ac-	R	L	Y	E	L	L	H	G	A	G	$\mathbf{X}^{5}$	H	A	A	$\mathbf{X}^{5}$	I	L	T	L	$-NH_2$
3A	Stapled	Ac-	R	L	Y	E	L	L	H	G	$\mathbf{X}^{5}$	G	N	H	$\mathbf{X}^{5}$	A	G	I	L	T	L	$-NH_2$
3B	Stapled	Ac-	R	L	Y	E	L	L	H	G	$\mathbf{X}^{4}$	G	N	H	$\mathbf{X}^{7}$	A	G	I	L	T	L	$-NH_2$
3C	Stapled	Ac-	R	L	Y	E	L	L	H	G	$\mathbf{X}^{7}$	G	N	H	$\mathbf{X}^{4}$	A	G	I	L	T	L	$-NH_2$
3D	Stapled	Ac-	R	L	Y	E	L	L	H	G	$\mathbf{X}^{4}$	G	N	H	$\mathbf{X}^{5}$	A	G	I	L	T	L	$-NH_2$
$3D^L$	Linear		R	L	Y	E	L	L	H	G	$\mathbf{X}^{4}$	G	N	H	$\mathbf{X}^{5}$	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	$\mathbf{X}^{5}$	L	H	G	$\mathbf{X}^{5}$	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.

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