ACS Medicinal Chemistry Letters

Letter

pubs.acs.org/acsmedchemlett

Truncated Orexin Peptides: Structure–Activity Relationship Studies

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Supporting Information

ABSTRACT: Orexin receptors are involved in many processes including energy homeostasis, wake/sleep cycle, metabolism, and reward. Development of potent and selective ligands is an essential step for defining the mechanism(s) underlying such critical processes. The goal of this study was to further investigate the structure–activity relationships of these peptides and to identify the truncated form of the orexin peptides active at OX_1. Truncation studies have led to OXA (17–33) as the shortest active peptide known to date with a 23-fold selectivity for OX_1 over OX_2. Alanine, D-amino acid, and proline scans have highlighted the particular importance of Tyr^{17}, Leu^{20}, Asn^{25}, and His^{26} for agonist properties of OXA(17–33). The conformation of the C-terminus might also be a defining factor in agonist activity and selectivity of the orexin peptides for the OX_1 receptor.

KEYWORDS: *Orexin, peptide, structure–activity relationship*

Orexin A and B (or hypocretin-1 and -2) are two neuropeptides discovered in 1998.[1,2] Both peptides bind to two G protein-coupled receptors (GPCRs), orexin 1 (OX_1) and orexin 2 (OX_2). OX_1 selectively binds orexin A, whereas OX_2 is activated by both peptides nonselectively.[2] The orexin system has been shown to play an important role in energy homeostasis,[3] wake/sleep cycle,[4] metabolism,[5] modulating nonsleep energy consumption,[6] and reward processing.[7] Therefore, these receptors have been implicated as potential targets for treatment of a variety of conditions such as insomnia, narcolepsy, obesity, and drug addiction.[8−10] Additionally, OX_1 and OX_2 receptors may modulate different physiological responses as suggested by their different distribution patterns in brain tissues.[2,11,12] Although a list of subtype selective or dual orexin antagonists have been reported,[13−15] progress on agonist development has been limited, and no small molecule agonists have been reported to date. Orexin A and B have been widely used for research purposes, but they display minimal subtype selectivity. Hence, identification of peptide analogues with increased selectivity is needed to further elucidate the respective functions of these receptors.

Orexin A consists of 33 amino acids with C-terminal amidation, an N-terminal pyrolgutamyl residue, and two intrachain disulfide bonds [Cys^6-Cys^{12} and Cys^7-Cys^{14}]. Orexin B is a 28 amino acid linear peptide with an amidated C-terminus. While orexin A has a completely conserved sequence among several mammalian species, human orexin B has variations at two amino acid residues compared to the rodent sequence.[1] High homology is present at the C-termini of orexin A and B, whereas the N-termini are more divergent in structure (11% homology),[16] suggesting that the C-terminal region might be important for activity.

Structure–activity relationship (SAR) studies on orexin A and B have been performed by several groups. The importance of the C-terminus for agonism has been confirmed.[16−19] Several

Figure 1. Orexin A and B peptide sequence. Q* corresponds to the pyroglutamoyl group.

key amino acids in human orexin A and B required for binding to OX_1 and OX_2 have also been identified. Truncation studies have identified OXA (15–33) and OXB (10–28) as the shortest active peptides at OX_1 and OX_2 receptors, respectively.[17,19] The two-point substituted analogue of orexin B, [Ala^{11}, D-Leu^{15}]orexin B (SB-668875-DM), was reported to have 400-fold selectivity for OX_2 over OX_1[20,21] although the selectivity was much weaker when studied by another group.[22] Truncation of orexin B followed by one-point mutation studies resulted in orexin B (6–28) analogues with good selectivity for OX_2 (>1000 fold).[17] Among reported orexin A analogues, orexin A (2–33) is the only one that shows modest selectivity for OX_1 (3-fold).[17] We report herein our work on further studying the SAR of the orexin peptides in an effort to understand the structural requirements for peptides with minimal sequences that are selective for the OX_1 receptor.

All target peptides were synthesized following conventional solid-phase technique using Fmoc/tBu strategy on an Apptec Focus XC peptide synthesizer (Supporting Information). Activity of the target compounds at the human OX_1 and OX_2 receptors was determined utilizing CHO-RD-HGA16 cells (Molecular Devices) engineered to stably express either the human OX_1 or the human OX_2 receptor. Test compound EC_{50} values were determined by running 8-point half-log concentration response curves.

Received: August 29, 2013
Accepted: October 15, 2013
Published: October 15, 2013

As the first step of the SAR studies, truncated analogues of both orexin A and B were prepared and tested in our assays (Table 1). Since the C terminus has been shown to be crucial for activity,[16−19] truncation was carried out at the N terminus. The truncation of orexin A resulted in several analogues having agonist activity at the OX_1 receptor (Table 1). OXA (19−33) displayed modest potency (EC_{50} = 1380 nM) at the OX_1 receptor. Two peptides OXA (15−33) with EC_{50} of 64 nM and OXA (17−33) with EC_{50} = 8.29 nM displayed the highest potency among all analogues. In contrast to previous reports,[19] orexin A (15−33) showed modest selectivity for the OX_1 receptor (∼15-fold) in our hands. OXA (17−33) had slightly higher selectivity for OX_1 (∼23-fold), which represents the shortest analogue with OX_1 selectivity to date. Although the reason for this discrepancy is unclear, it has been proposed that orexin agonist discrimination is dependent on the expression system.[23] Indeed, the EC_{50} of orexin A and B varies across several assay systems.[17,19,20] Truncation on orexin B resulted in OXB (10−28) that showed activity at both receptors, confirming the previous notion that at least 19 amino acids are required for orexins to retain activity at orexin receptors.[17,19]

Since orexin A is capped with a pyroglutamoyl group at the N-terminus and acylation may be important for activity, as is observed with Ghrelin,[24] the corresponding N-acyl analogues were also synthesized (Supporting Information). No significant effect on agonist properties of truncated peptides was detected with the presence of the N-terminus "cap".

OXA (17−33) was chosen for further SAR studies because of its good potency and OX_1 preference. An alanine scan was first performed to determine the contributions of the side chains to the overall activity (Table 2). Substitution of the first six N-terminal amino acids (17−22) partially retained the agonist activity at the OX_1 receptor, except for [Ala^{20}] (EC_{50} > 3 μM), whose analogue with the acetyl cap had no OX_1 activity at up to 10 μM. [Ala^{18}] substitution showed the least effect on activity (EC_{50} 79 nM). Alanine substitution at the 24−28 positions resulted in a more significant drop in activity, whereas changes in the last 5 amino acids (C-terminal) led to a complete loss in activity, confirming that modifications on the C-terminal region are not tolerated. A similar trend is present at the OX_2 receptor. The preference for the OX_1 receptor was generally maintained with substitution at 17−22 positions but was abolished with substitution at the 24−26 positions. Acylation on the N-terminus displayed minimal effects on agonist activity in this series of peptides (see Supporting Information).

Table 1. Biological Activity of Orexin A and B and Their Truncates

peptides	EC_{50} (nM)[a] OX_1	EC_{50} (nM)[a] OX_2	OX_2/OX_1
orexin A	0.22 ± 0.06	1.07 ± 0.3	4.9
orexin A (15−33)	64.4 ± 6.9	976[b]	15
orexin A (17−33)	8.29 ± 1.1	187[b]	23
orexin A(19−33)	1380 ± 510	947 ± 300	0.7
orexin A(21−33)	<50%[c]		
orexin B (10−28)	144 ± 35	99.3 ± 27	0.7
orexin B (12−28)	<50%[c]	1040 ± 300	

[a]Values are the mean ± SEM of at least three independent experiments in duplicate. [b]Values are the mean of two independent experiments in duplicate. [c]Percentage of <50% means activity at 10 μM was less than 50% of the control orexin A E_{max}.

Table 2. Biological Activity of Alanine Substituted OXA (17−33)

peptides	EC_{50} (nM)[a] OX_1	EC_{50} (nM)[b] OX_2	OX_2/OX_1
orexin A (17−33)	8.29 ± 1.1	187	23
[Ala^{17}]OXA (17−33)	1910 ± 620	4820	2.5
[Ala^{18}]OXA (17−33)	78.9 ± 22	439	5.6
[Ala^{19}]OXA (17−33)	212 ± 57	1890 ± 610[a]	8.9
[Ala^{20}]OXA (17−33)	3230 ± 1080	7850	2.4
[Ala^{21}]OXA (17−33)	493 ± 49	1910	3.9
[Ala^{22}]OXA (17−33)	422 ± 55	1440	3.4
[Ala^{24}]OXA (17−33)	1040 ± 300	1360	1.3
[Ala^{25}]OXA (17−33)	3070 ± 1180	2320	0.8
[Ala^{26}]OXA (17−33)	3060 ± 940	1580	0.5
[Ala^{29}]OXA (17−33)	<50%[c]	<50%[c]	
[Ala^{30}]OXA (17−33)	inactive at 10 μM[b]	inactive at 10 μM	
[Ala^{31}]OXA (17−33)	inactive at 10 μM[b]	inactive at 10 μM	
[Ala^{32}]OXA (17−33)	<50%[b,c]	<50%[c]	
[Ala^{33}]OXA (17−33)	<50%[b,c]	inactive at 10 μM	

[a]Values are the mean ± SEM of at least three independent experiments in duplicate. [b]Values are the mean of at least two independent experiments in duplicate. [c]Perecentage of <50% means activity at 10 μM was less than 50% of the control orexin A E_{max}.

The effect of side-chain chirality on the activity was examined by a D-amino acid scan on OXA (17−33) (Table 3). Similarly, changes on the C-terminal side of the peptide (28−33) led to a complete loss of activity. Changes in the remaining positions (17−27) revealed that [D-Glu^{18}], [D-Leu^{19}], and [D-Leu^{20}] analogues retained some of the activity, whereas all other analogues had noticeable decreases in agonist potency. Interestingly, substitution to D-amino acids at positions 19−

Table 3. Biological Activity of D-Amino Acid Substituted OXA (17−33)

peptides	EC_{50} (nM)[a] OX_1	EC_{50} (nM)[b] OX_2	OX_2/OX_1
[D-Tyr^{17}]OXA (17−33)	1360 ± 540[b]	3220	2.4
[D-Glu^{18}]OXA (17−33)	739 ± 190[b]	2140	2.9
[D-Leu^{19}]OXA (17−33)	526 ± 100	263	0.5
[D-Leu^{20}]OXA (17−33)	535 ± 110	156	0.3
[D-His^{21}]OXA (17−33)	1560 ± 310	610	0.4
[D-Ala^{23}]OXA (17−33)	3070 ± 970	1920	0.6
[D-Asn^{25}]OXA (17−33)	inactive at 10 μM[b]	<50%[c]	
[D-His^{26}]OXA (17−33)	5700 ± 3420	1760	0.3
[D-Ala^{27}]OXA (17−33)	1900 ± 530	1250	0.7
[D-Ala^{28}]OXA (17−33)	inactive at 10 μM	<50%[c]	
[D-Ile^{30}]OXA (17−33)	inactive at 10 μM[b]	inactive at 10 μM	
[D-Leu^{31}]OXA (17−33)	inactive at 10 μM[b]	<50%[c]	
[D-Thr^{32}]OXA (17−33)	inactive at 10 μM[b]	inactive at 10 μM	
[D-Leu^{33}]OXA (17−33)	inactive at 10 μM[b]	inactive at 10 μM	

[a]Values are the mean of at least three independent experiments in duplicate. [b]Values are the mean of two independent experiments in duplicate. [c]Percentage of <50% means activity at 10 μM was less than 50% of the control orexin A E_{max}.

dx.doi.org/10.1021/ml400333a | ACS Med. Chem. Lett. 2013, 4, 1224−1227

27 seemed to reverse the binding preference to favor OX_2. For example, [D-Leu^{20}] orexin (17−33) analogue had no change in activity at OX_2 and a 64-fold drop in activity at OX_1; [D-His^{26}] OXA (17−33) had 9-fold reduction in activity at OX_2 and almost 700-fold drop in activity at OX_1. These results suggest a more pronounced impact of chain orientation on orexin receptor binding in this region. [Tyr^{17}] and [Glu^{18}] retained slight selectivity for OX_1, although agonist activity of both analogues significantly decreased.

The effect of more dramatic changes on secondary structure of peptides was investigated using proline scans of OXA (17−33). Given that modification in the C-terminus resulted in total loss of activity in previous scans, only the first half from the N-terminus (17−22) was investigated. The analogues resulting from proline substitution at 17−19 position retained some of the original activity, with [Pro^{18}] OXA (17−33) being the least affected by change (Table 4). A more significant drop in activity was observed for analogues at 20−22 positions. Selectivity of OX_1 was slightly maintained or abolished in this series.

Table 4. Biological Activity of Proline Substituted OXA (17−33)

peptides	EC_{50} (nM)[a] OX_1	EC_{50} (nM)[b] OX_2	OX_2/OX_1
[Pro^{17}]OXA (17−33)	1370 ± 140	3250	2.4
[Pro^{18}]OXA (17−33)	143 ± 16	729	5.1
[Pro^{19}]OXA (17−33)	865 ± 350	1010	1.2
[Pro^{20}]OXA (17−33)	1130 ± 450	1610	1.4
[Pro^{21}]OXA (17−33)	1200 ± 360	1990	1.7
[Pro^{22}]OXA (17−33)	2480 ± 1125	2180	0.9

[a]Values are the mean ± SEM of at least three independent experiments in duplicate. [b]Values are the mean of two independent experiments in duplicate.

NMR and computational studies performed on orexin A (in aqueous[25] and in membrane mimetic micellar solutions[26]) identified a highly conserved hydrophobic region on the C-terminus. This region contains two α-helices that are connected by a short linker.[16,25] Similarly, solution structure of orexin B (in aqueous[27] and in micellar solution[28]) also shows two α-helices at similar positions. These α-helices seem to differ in their length, the position of the loops, and their relative orientation between orexin A and B. The observed selectivity of OX_1 toward orexin A was suggested to be due to specific recognition of the overall peptide conformation, especially the hydrophobic side chain in the C-terminal region of the receptor.[16] In our study, two residues, His^{21} and Gly,[22] were shown to be of moderate importance in the alanine scan; however, their proline analogues had more pronounced reduction in potency, especially at OX_1. These two residues and Asn^{25} are bordering the two helices and the linker, according to Kim et al., who suggested the helices to be Cys^{14}-His^{21} and Asn^{25}-Leu^{31},[25] and Takai et al., who proposed Leu^{16}-Ala^{23} and Asn^{25}-Thr^{32}.[16] Considering their close proximity to the linker our results may indicate that these three residues are important for orientation of the two α-helices.[16] Since OX_2 has lesser recognition requirements, the effect of these mutations on activity of corresponding peptides was less pronounced.

In the proposed orexin A structure, the residues that were deemed important for potency in truncated peptide OXA (15−33) (Leu^{16}, Leu^{19}, Leu^{20}, His^{26}, Gly^{29}, Ile^{30}, Leu^{31}, Thr^{32}, and Leu^{33}) seemed to exist on one side of orexin A, forming a hydrophobic surface.[16,25] This surface might also be important in binding to the cell membrane.[26] Residues that were less important (Arg^{15}, Tyr^{17}, Glu^{18}, His^{21}, Gly^{22}, Gly^{24}, and Asn^{25}) formed a hydrophilic surface on orexin A, which were thought to play a minimal role in potency. In our study, alanine and proline scans of OXA (17−33) have confirmed the importance of the hydrophobic surface residues Leu^{20} and His^{26}, together with C-terminal residues 29−33. Leu^{18} showed no significant contribution to OXA (17−33) binding to OX_1, agreeing with an earlier report on other truncated orexin A analogues.[16] Interestingly, hydrophilic surface residues Tyr^{17} and Asn^{25} were also shown to be important for potency in our study.

It was previously suggested that OX_1 had more strict recognition requirements than OX_2 in binding endogenous ligands and their analogues.[17] The potency of our truncated orexin A analogues showed more dramatic change in response at OX_1 for all performed mutations. Both OXA (15−33) and OXA (17−33) showed some selectivity for OX_1 in our hands (15- and 23-fold, respectively). Although mutations at Tyr^{17}, one of the key amino acid residues, led to dramatically reduced potency at both receptors, the preference for OX_1 was preserved. Interestingly, D-amino acid substitution at Leu^{20}, His^{21}, and His^{26} had not only abolished preference to OX_1, but seemed to reverse it. Together, these results confirm that the binding pocket on OX_1 might have more specific interactions with ligands than that on OX_2.

In summary, truncated OXA (17−33) showed preferential agonist activity at OX_1 in our study and is the shortest peptide with such selectivity. Given its shorter sequence and higher selectivity toward OX_1, OXA (17−33) may serve as an improved biological probe of the OX_1 receptor function. During single amino acid mutagenesis, OXA (17−33) seems to maintain structural requirements similar to those reported for orexin A and OXA (15−33). Our data suggest that Tyr^{17}, Leu^{20}, Asn^{25}, and His^{26} play a key role in interaction of OXA (17−33) with OX_1, whereas His^{21} and Gly^{22} might be important in the orientation of its two α-helices. Also in the majority of the active analogues studied to date, inversion of configuration at positions 19−27 has resulted in dramatic drops in potency at OX_1, whereas potency at OX_2 changed moderately. Finally, our results on OXA (17−33) suggest that its spatial conformation might be similar to that of human orexin A peptide and is important for binding preference of OX_1.

■ ASSOCIATED CONTENT

Supporting Information

Peptide synthesis, calcium assays, HPLC analysis of target peptides, and agonist activity of capped peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This work was support by National Institute of Health/National Institute on Drug Abuse grant DA032837 and Research Triangle Institute. We are grateful for the help from Drs. Scott Runyon and Yanyan "Jasmine" Zhang in peptide synthesis. We thank Tiffany Langston, Allyson Smith, Keith Warner, and Dr. Elaine Gay for technical assistance.

■ ABBREVIATIONS

OX_1, orexin 1 receptor; OX_2, orexin 2 receptor; OXA, orexin A; GPCR, G protein-coupled receptor; SAR, structure−activity relationship; F-moc, Fluorenylmethyloxycarbonyl; tBu, *t*-butyl; HPLC, high performance liquid chromatography

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