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# Development of an Orexin-2 Receptor Selective Agonist, [Ala$^{11}$, D-Leu$^{15}$]orexin-B

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**Abstract**—Investigation of L-alanine and D-amino acid replacement of orexin-B revealed that three L-leucine residues at the positions of 11, 14, and 15 in orexin-B were important to show selectivity for the orexin-2 receptor ($OX_2$) over the orexin-1 receptor ($OX_1$). L-Alanine substitution at position 11 and D-leucine substitution at positions 14 and 15 maintained the potency of orexin-B to mobilize $[Ca^{2+}]_i$ in CHO cells expressing the $OX_2$, while their potency for the $OX_1$ was significantly reduced. In combined substitutions, we identified that [Ala$^{11}$, D-Leu$^{15}$]orexin-B showed a 400-fold selectivity for the $OX_2$ ($EC_{50} = 0.13$ nM) over $OX_1$ ($EC_{50} = 52$ nM). [Ala$^{11}$, D-Leu$^{15}$]orexin-B is a beneficial tool for addressing the functional roles of the $OX_2$.


Recently, two novel hypothalamic neuropeptides, orexin-A and orexin-B, have been isolated from rat brain extracts as ligands for orphan G protein coupled receptors, namely, orexin-1 receptor ($OX_1$) and orexin-2 receptor ($OX_2$), which are exclusively expressed in the brain.[1] Both peptides are derived from the same precursor protein by proteolytic processing and are mainly presented in the lateral and posterior sections of the hypothalamus, which is known to be the center for feeding regulation.[1,2] Orexin-A, which is a 33-residue peptide with two intramolecular disulfide bridges, shows similar potency for both the $OX_1$ and the $OX_2$, whereas orexin-B, which is a 28-amino acid-linear peptide, showed about a 10-fold selectivity for the $OX_2$ over the $OX_1$.[1]

Several investigations have shown that orexins are involved in a variety of physiological functions, such as the regulation of locomotor activity, the sleep/awake cycle, blood pressure, metabolic rate, gastric acid secretion, pituitary luteinizing hormone secretion, and feeding regulation.[3–9] Different expression patterns of $OX_1$ and $OX_2$ mRNAs support the diverse functions of orexins.[10] Using potent and selective antagonists, the roles of $OX_1$ are going to be elucidated.[12] However, the lack of selective ligands for the $OX_2$ makes it difficult to address the role of the $OX_2$.

To develop potent and selective $OX_2$ ligands, we conducted L-alanine and D-amino acid replacements to emphasize the intrinsic selectivity of orexin-B (Fig. 1).

## Peptide Preparation

Orexin-A and -B were purchased (Peptide Institute, Inc, Osaka, Japan). All orexin-B analogue peptides were synthesized on the solid support using a Fmoc/$^t$Bu strategy on Pioneer™ peptide synthesizer (Applied Biosystems, Foster City, CA, USA) or ACT357MPS (Advanced ChemTech, Louisville, KY, USA). Fmoc-NovaSyn® TGR resin (Novabiochem, Laufelfingen, Switzerland) was used as a starting material. All crude peptides were cleaved from resins with TFA/thioanisole/ethandithiol/*m*-cresol within 2 h, followed by the addition of cold diethyl ester for solidification. The resulting crude peptides, obtained after centrifugation, were purified with preparative reversed-phase HPLC (Hitachi D-6000 system, Hitachi, San Jose, CA, USA) with a YMC-Pack ODS-AQ column (20 × 250 mm; YMC, Kyoto, Japan). All peptides were characterized by analytical HPLC (HP 1100LC system, Agilent Technologies, Palo Alto, CA, USA) with a YMC-Pack ODS-AQ column (2.0 × 150 mm; YMC, Kyoto, Japan) and by electrospray mass

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spectrometry (Waters Platform ZMD2000, Waters, Millford, MA, USA).

### Measurement of Intracellular Calcium Ion Concentrations

CHO-K1 cells expressing each subtype of orexin receptors ($OX_1$ and $OX_2$) were maintained by a general method. The intracellular calcium ion concentrations were measured by the method previously reported.[3] In brief, CHO–K1 cells stably expressing human $OX_1$ or $OX_2$ receptors ($OX_1$–CHO or $OX_2$–CHO) were seeded into 96-well plates and incubated with a cytoplasmic calcium indicator, Fluo-3 AM. After free dye was washed away, the intracellular $Ca^{2+}$ mobilization evoked by orexins and related peptides was monitored as the change in cell fluorescence intensity by FLIPR (Molecular Devices, Sunnyvale, CA, USA). The lack of agonistic activities of synthesized peptides in the intact CHO–K1 cells were confirmed to address their specificity for orexin receptors (data not shown).

### L-Alanine Scan of Orexin-B

To address the importance of each residue with minimal impact to the three-dimensional structure, alanine-scanned analogues of orexin-B were synthesized. Three alanine residues at positions 17, 22, and 23 in the orexin-B sequence were replaced with glycine. Their potency to mobilize intracellular $Ca^{2+}$ concentrations in $OX_1$–CHO and $OX_2$–CHO cells were measured (Table 1). The substitutions at positions 1–9, 12–14, 16, and 17 did not significantly affect the intrinsic potency of orexin-B for both the $OX_1$ and the $OX_2$. In contrast, replacement at positions 10, 15, and 18–28 considerably decreased the potency for both receptors. Among these alanine-scanned analogues, [Ala$^{11}$]orexin-B showed unique profile. The potency of [Ala$^{11}$]orexin-B was selectively reduced for the $OX_1$ (EC50 = 8.1 nM), but not the $OX_2$ (EC50 = 0.066 nM), and resulted in a 120-fold selectivity for the $OX_2$. Additional substitutions using 12 usual and 10 unusual amino acids at position 11 showed that serine and tryptophan were also efficacious in emphasizing the selectivity of orexin-B to a similar extent of alanine (Table 1).

### D-Amino Acid Replacement of Orexin-B

To change the orientation of the amino acid side chain with minimal impact on the overall hydrophobicity and dipole moment, we conducted D-amino acid replacement (Table 2). D-Alanine was used as a substitute for glycine in the sequence of orexin-B. D-Amino acid substitutions at positions 23–28 showed significant reduction of the potency for both orexin receptors. In addition, D-leucine substitutions at positions 14 and 15 significantly improved the selectivity of orexin-B by 5- and 8-fold, respectively.

### Combination Study on the Alanine Scan and D-Amino Acid Replacement

Each positional scan showed that three leucine residues, Leu$^{11}$, Leu$^{14}$, and Leu$^{15}$, in the orexin-B sequence were important for the activation of the $OX_1$, and that modification of these sites was relatively tolerable for the activation of the $OX_2$. Based on these results, combination replacements of orexin-B were performed to determine whether or not synergistic effects would be observed (Table 3). Among the synthesized peptides, the combination of an L-alanine

**Table 1.** Biological activity and receptor selectivity of L-alanine or glycine substituted orexin-B analogues[a]

| Peptide | $EC_{50}$ value (nM) | | Selectivity |
|---|---|---|---|
| | $OX_1$ | $OX_2$ | $OX_1/OX_2$ |
| Orexin-B | 0.83 ± 0.041 | 0.081 ± 0.0091 | 10 |
| [Ala$^{1}$]orexin-B | 2.3 ± 0.24 | 0.20 ± 0.055 | 12 |
| [Ala$^{2}$]orexin-B | 2.4 ± 0.55 | 0.22 ± 0.063 | 11 |
| [Ala$^{3}$]orexin-B | 2.3 ± 0.81 | 0.18 ± 0.072 | 13 |
| [Ala$^{4}$]orexin-B | 3.2 ± 1.2 | 0.23 ± 0.096 | 14 |
| [Ala$^{5}$]orexin-B | 3.1 ± 0.68 | 0.29 ± 0.10 | 11 |
| [Ala$^{6}$]orexin-B | 1.6 ± 0.21 | 0.12 ± 0.025 | 14 |
| [Ala$^{7}$]orexin-B | 1.2 ± 0.13 | 0.12 ± 0.0083 | 9.2 |
| [Ala$^{8}$]orexin-B | 2.7 ± 0.20 | 0.46 ± 0.10 | 5.9 |
| [Ala$^{9}$]orexin-B | 1.1 ± 0.20 | 0.20 ± 0.029 | 5.5 |
| [Ala$^{10}$]orexin-B | 9.1 ± 1.6 | 0.77 ± 0.086 | 12 |
| [Ala$^{11}$]orexin-B | 8.1 ± 0.72 | 0.066 ± 0.011 | 120 |
| [Ala$^{12}$]orexin-B | 3.4 ± 0.53 | 0.23 ± 0.045 | 15 |
| [Ala$^{13}$]orexin-B | 1.7 ± 0.30 | 0.13 ± 0.026 | 13 |
| [Ala$^{14}$]orexin-B | 0.82 ± 0.18 | 0.065 ± 0.021 | 13 |
| [Ala$^{15}$]orexin-B | 39 ± 4.6 | 2.2 ± 0.33 | 18 |
| [Ala$^{16}$]orexin-B | 1.5 ± 0.33 | 0.13 ± 0.042 | 12 |
| [Gly$^{17}$]orexin-B | 0.41 ± 0.087 | 0.091 ± 0.035 | 4.5 |
| [Ala$^{18}$]orexin-B | 6.0 ± 2.4 | 1.6 ± 0.97 | 3.8 |
| [Ala$^{19}$]orexin-B | 14 ± 3.0 | 1.1 ± 0.43 | 13 |
| [Ala$^{20}$]orexin-B | 32 ± 7.4 | 2.3 ± 1.1 | 14 |
| [Ala$^{21}$]orexin-B | 5.7 ± 1.7 | 0.72 ± 0.41 | 7.9 |
| [Gly$^{22}$]orexin-B | 7.5 ± 1.1 | 0.27 ± 0.083 | 28 |
| [Gly$^{23}$]orexin-B | 22 ± 4.3 | 0.84 ± 0.13 | 26 |
| [Ala$^{24}$]orexin-B | 680 ± 73 | 290 ± 92 | 2.3 |
| [Ala$^{25}$]orexin-B | > 10,000 | 390 ± 92 | — |
| [Ala$^{26}$]orexin-B | 710 ± 91 | 140 ± 34 | 5.1 |
| [Ala$^{27}$]orexin-B | 110 ± 27 | 9.7 ± 1.8 | 11 |
| [Ala$^{28}$]orexin-B | 220 ± 40 | 15 ± 2.6 | 15 |
| [Ser$^{11}$]orexin-B | 150 ± 41 | 1.2 ± 0.57 | 120 |
| [Trp$^{11}$]orexin-B | 4.8 ± 1.4 | 0.15 ± 0.095 | 32 |

[a]Data represent the mean of more than three independent determinations performed in duplicate.

| Peptide | Sequence |
|---|---|
| orexin-A | <EPLPDCCRQKTCSCRLYELLHGAGNHAAGILTL-$NH_2$ |
| orexin-B | RSGPPGLQGRLQRLLQASGNHAAGILTM-$NH_2$ |

**Figure 1.** Amino acid sequences of orexins.

**Table 2.** Biological activity and receptor selectivity of corresponding D-amino acid substituted orexin-B analogues[a]

| Peptide | $EC_{50}$ value (nM) | | Selectivity |
|---|---|---|---|
| | $OX_1$ | $OX_2$ | $OX_1/OX_2$ |
| Orexin-B | 0.83±0.041 | 0.081±0.0091 | 10 |
| [D-Arg[1]]orexin-B | 1.6±0.80 | 0.19±0.15 | 8.2 |
| [D-Ser[2]]orexin-B | 1.2±0.60 | 0.15±0.10 | 8.3 |
| [D-Ala[3]]orexin-B | 1.3±0.49 | 0.16±0.090 | 8.4 |
| [D-Pro[4]]orexin-B | 0.89±0.35 | 0.15±0.092 | 5.8 |
| [D-Pro[5]]orexin-B | 0.85±0.39 | 0.16±0.12 | 5.3 |
| [D-Ala[6]]orexin-B | 1.4±0.51 | 0.20±0.16 | 7.1 |
| [D-Leu[7]]orexin-B | 1.4±0.37 | 0.084±0.044 | 16 |
| [D-Gln[8]]orexin-B | 4.0±1.3 | 0.32±0.18 | 13 |
| [D-Ala[9]]orexin-B | 1.4±0.63 | 0.13±0.089 | 10 |
| [D-Arg[10]]orexin-B | 9.8±3.6 | 0.87±0.62 | 11 |
| [D-Leu[11]]orexin-B | 7.0±0.71 | 0.60±0.24 | 12 |
| [D-Gln[12]]orexin-B | 0.77±0.26 | 0.11±0.078 | 6.8 |
| [D-Arg[13]]orexin-B | 3.8±1.2 | 0.41±0.29 | 9.4 |
| [D-Leu[14]]orexin-B | 34±3.0 | 0.44±0.060 | 85 |
| [D-Leu[15]]orexin-B | 2.7±0.26 | 0.052±0.0051 | 52 |
| [D-Gln[16]]orexin-B | 5.8±1.6 | 0.41±0.27 | 14 |
| [D-Ala[17]]orexin-B | 2.6±0.77 | 0.26±0.19 | 10 |
| [D-Ser[18]]orexin-B | 7.9±0.87 | 0.46±0.22 | 17 |
| [D-Ala[19]]orexin-B | 0.52±0.12 | 0.16±0.069 | 3.2 |
| [D-Asn[20]]orexin-B | 0.61±0.14 | 0.26±0.14 | 2.4 |
| [D-His[21]]orexin-B | 7.3±3.9 | 1.0±0.75 | 6.9 |
| [D-Ala[22]]orexin-B | 22±4.4 | 2.6±1.7 | 8.3 |
| [D-Ala[23]]orexin-B | 230±12 | 14±6.7 | 16 |
| [D-Ala[24]]orexin-B | 400±66 | 67±23 | 6.0 |
| [D-Ile[25]]orexin-B | >10,000 | 1400±1200 | — |
| [D-Leu[26]]orexin-B | >10,000 | 200±170 | — |
| [D-Thr[27]]orexin-B | >10,000 | 260±52 | — |
| [D-Met[28]]orexin-B | >10,000 | 290±110 | — |

[a]Data represent the mean of more than three independent determinations performed in duplicate

substitution at Leu[11] and a D-leucine substitution at Leu[15] showed a good selectivity (400-fold) without significant reduction of the potency for the $OX_2$.

Recently, the 3-D structure of human [Phe[1]]orexin-B was determined using NMR analysis.[11] The structure of orexin-B consisted of a random coil (positions 1–7), kink region (positions 20–22), and two α-helices (positions 7–19 and 23–28). These two helices form a bent structure. Our results from L-alanine and D-amino acid substitutions suggest that modification at positions 23–28 significantly reduced the potency of orexin-B for both $OX_1$ and $OX_2$. Amino acid residues in the C-terminal portion were well conserved between orexin-A and orexin-B. In addition, C-terminal truncated orexins drastically lost their potency for both orexin receptors (ref 13, unpublished our observations). Therefore, the second helix, consisting of amino acid residues 23–28, might be a common core needed to recognize and activate orexin receptors. Moreover, the changes at position 11 (L-leucine to L-alanine) and at positions 14 and 15 (L-leucine to D-leucine) located in the 1st helix selectively reduce the potency for the $OX_1$. Thus, the 1st helix might play a key role in determining the selectivity of orexin analogues. In addition, interestingly, the side chains of Leu[11] and Leu[15] faced to the same direction in the 1st helix.[11] To consider the drastically decreased potency of [Ala[11], D-Leu[15]]orexin-B for the $OX_1$, the surface of the 1st helix constructed by Leu[11] and Leu[15] might be of interest.

**Table 3.** Biological activity and receptor selectivity of two-point substituted orexin-B analogues[a]

| Peptide | | $EC_{50}$ value (nM) | | Selectivity |
|---|---|---|---|---|
| | | $OX_1$ | $OX_2$ | $OX_1/OX_2$ |
| Orexin-B | | 0.83±0.041 | 0.081±0.0091 | 10 |
| [Ala[11],D-Leu[14]] | orexin-B | 270±13 | 1.9±0.28 | 140 |
| [Ala[11],D-Leu[15]] | orexin-B | 52±3.1 | 0.13±0.012 | 400 |

[a]Data represent the mean of more than three independent determinations performed in duplicate

In conclusion, [Ala[11], D-Leu[15]]orexin-B identified in the course of the L-alanine and D-amino acid replacements of orexin-B is a potent and selective $OX_2$ agonist. A novel agonist, [Ala[11], D-Leu[15]]orexin-B should be a promising tool for addressing the roles of $OX_2$.

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