Probing Opioid Receptor Interactions with Azacycloalkane Amino Acids. Synthesis of a Potent and Selective ORL1 Antagonist

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Azacycloalkane turn mimics **6**−**9** were used to explore the relationship between conformation and biological activity of peptide ligands to the opioid receptor-like (ORL1) receptor. Three azabicyclo[*x.y.*0]alkane amino acids and a 5-*t*BuPro type VI β-turn mimic were introduced into peptides **10**−**13** by solid-phase synthesis on MBHA resin. Biological examination of peptides **10**−**13** showed two new antagonists (**10** and **12**) exhibiting increased selectivity for the ORL1 receptor.

Introduction

The opioid receptor like (ORL1) receptor was identified through cloning experiments and shown to have high homology (∼60%) with known opioid receptors (μ, κ, and δ) without having high affinity for the common opioid ligands.[1−4] The heptadecapeptide orphanin FQ/nociceptin (NC) was soon shown to be the endogenous ligand of the ORL1 receptor (Figure 1).[5,6] The shorter C-terminal fragment of NC, $NC_{1-13}NH_2$, was found to be a selective agonist, and the analogue [Nphe[1]]$NC_{1-13}NH_2$ was identified to be an antagonist.[7−9] Hexapeptide and nonpeptide ligands were later synthesized and shown to bind to ORL1 receptor with high affinity.[10−13] Among the small peptide analogues, from a positional scanning library of 2×10^7 β-turn-constrained peptides, the thiaindolizidinone analogue III−BTD (**1**, Figure 1) was recently selected because it displayed good affinity and a modest selectivity for the ORL1 receptor (1:5:22:6 K_i ratio of hORL1/hMOR/hDOR/hKOR) with competitive ORL1 receptor antagonist activity and agonist activity at the other opioid receptors at higher concentrations.[14,15]

The development of new ORL1 ligands with high selectivity and bioavailability remains an important challenge for the elucidation and control of the physiological role of the ORL1 receptor. A broad spectrum of potential therapeutic applications have been reported for the ORL1 receptor system.[16] For example, ORL1 agonists have been identified as anxiolytics, stimulants of food-intake, analgesics, suppressants of drug abuse, antiepileptics, and for the management of hyponatemic and water-retaining syndromes.[16] Furthermore, ORL1 receptor antagonists have been evaluated as anorectics, analgesics, and nootropic agents.[16] The nonpeptide antagonist JTC-801, which exhibits high affinity and selectivity to the ORL1 receptor, is currently undergoing evaluation in clinical trials as a novel analgesic (Figure 1).[12] Selective ligands for the ORL1 receptor possessing agonist and antagonist activity are thus desired as tools for characterizing the physiology of ORL1.

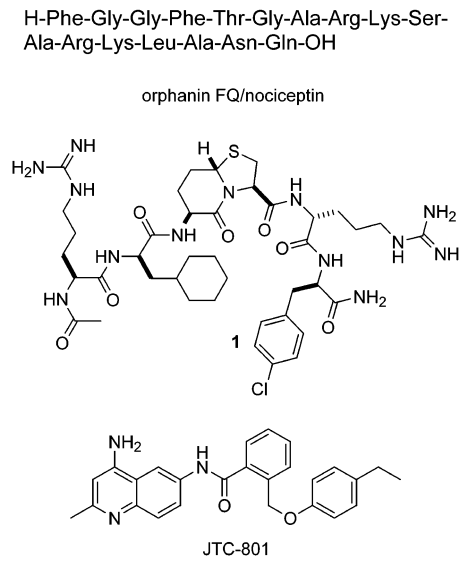

Figure 1. Structures of orphanin FQ/nociceptin, peptide III-BTD (**1**) and JTC-801.

The exploration of opioid peptides with azabicycloalkane amino acids has improved understanding of the conformational requirements at the opioid receptors.[14,17−19] For example, in a combinatorial approach to study the ORL1 receptor, peptide **1** possessing the thiaindolizidinone amino acid **2** demonstrated greater selectivity for hORL1 relative to related analogues incorporating the turn mimics **3**−**5** which showed higher affinities for hKOR (Figure 2).[14] Because a deeper understanding of the conformational requirements for receptor interaction may arrive through a systematic examination of the turn region of analogue **1**, a series of related scaffolds have now been used to study structure−activity relationships at the ORL1 (Figure 3).

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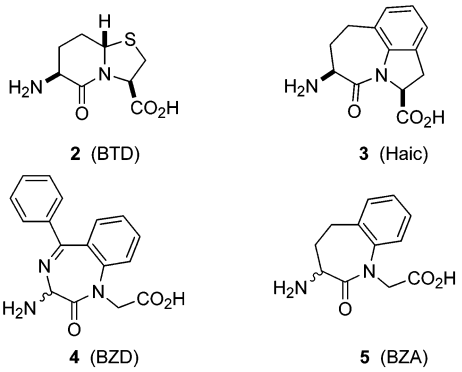


Figure 2. Structures of alternative turn mimics incorporated at residues 3 and 4 of hexapeptide ligands.

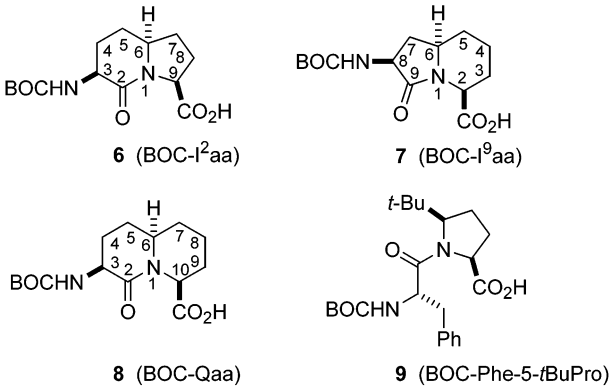


Figure 3. Structures of dipeptide mimics used in the present study of the ORL1 receptor.

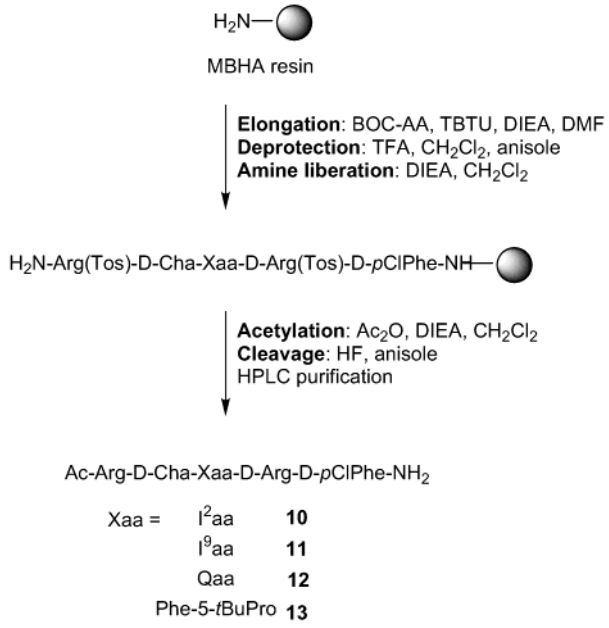
Three different azabicycloalkane amino acids (**6**−**8**), (3*S*,6*S*,9*S*)-indolizidin-2-one, (2*S*,6*R*,8*S*)-indolizidin-9-one, and (2*S*,6*R*,8*S*)-quinolizidinone amino acids (I^2aa, I^9aa, and Qaa, respectively), as well as the phenylalanyl-(2*S*,5*R*)-5-*tert*-butylproline (Phe-5-*t*BuPro, **9**) dipeptide, all were employed in complementary strategies featuring the use of structural links and steric interactions for inducing turn conformations.

Comparison of the X-ray crystal structures of esters of azabicycloalkane amino acids has demonstrated that the heterocycle ring size influenced significantly the preferred geometry of the internal ψ and ϕ dihedral angle values of the peptide backbone contained within the bicyclic system.[20−23] The I^2aa and I^9aa systems, for example, mimic different features of the central residues in an ideal type II′ β-turn.[21,22] X-ray analysis of Ac-L-Leu-5-*t*BuPro-NHMe and Ac-L-Tyr-5-*t*BuPro-NHMe have shown that the steric interactions of the 5-*t*BuPro residue induced the backbone to adopt dihedral angles characteristic of the central $i + 1$ and $i + 2$ residues of an ideal type VIa β−turn.[24,25] Employing these dipeptide mimics to study the relationships between dihedral angle geometry, conformation and activity, we have maintained the potency and improved the selectivity of the parent peptide **1**. Two new potent antagonists have been synthesized that exhibit increased selectivity for the ORL1 receptor.

Results

Synthesis. Enantiopure turn-inducing amino acids, *N*-BOC-Xaa, were synthesized according to procedures developed in our laboratory from aspartic and glutamic acids as inexpensive chiral educts as described in the literature.[21−24] Azabicyclo[*x*.*y*.0]alkane amino acids, I^2-aa, I^9aa, and Qaa, were made by procedures involving reactions of the ω-carboxylates of the aminodicarboxylates to provide a linear ketone intermediate, followed by intramolecular reductive amination and lactam cyclization to give the bicycle.[21−23] 5-*tert*-Butylproline was synthesized from glutamic acid via our acylation/decarboxylation/reductive amination sequence and converted to the dipeptide by coupling BOC-Phe to 5-*t*BuPro benzyl ester in solution, followed by hydrogenolysis of the benzyl ester.[24,26] The set of peptides **10**−**13** incorporating I^2aa, I^9aa, Qaa, and Phe-5-*t*BuPro were synthesized using the solid-phase strategy of Merrifield on MBHA resin as detailed in the Supporting Information (Scheme 1).[27] Sequential elongation of the peptides involved couplings of *N*-BOC-protected amino acids using TBTU as coupling reagent in DMF followed by deprotections using TFA in CH_2Cl_2. The peptides were cleaved and the tosyl groups of the arginyl residues were deprotected by treatment of the resin with anhydrous liquid HF in the presence of anisole. The crude peptides were purified by reverse-phase HPLC and lyophilized. The purity of the peptides was assessed by analytical HPLC, which with NMR spectroscopy showed a mixture of conformers for **12**, and peptide composition was determined by fast atom bombardment mass spectrometry (FAB-MS) as discussed in detail in the SI.

Scheme 1 Synthesis of Peptides **10**−**13**



Biological Activity. Binding Characterization. The K_i values of peptides incorporating I^2aa (**10**), I^9aa (**11**), Qaa (**12**), and Phe-5-*t*BuPro (**13**) were determined on membrane homogenates of COS-1 or CHO cells expressing recombinant human μ-, δ-, and κ-opioid receptors (hMOR, hDOR, and hKOR) and the human opioid receptor-like (hORL1) receptor, and compared to the K_i values of the original peptide, peptide III-BTD (**1**, Table 1). Peptides **11** and **13** did not exhibit any improvement in affinity nor of selectivity relative to peptide **1**. Peptides **10** and **12**, like peptide **1**, showed high affinity for hORL1 (44 and 35 nM, respectively). Peptide **10** displayed an improved selectivity for hORL1 versus hDOR (1:1.7:118:4 K_i ratio of hORL1/hMOR/

Table 1. Binding Affinities for HKOR, HMOR, HDOR, and HORL1 of Peptides 10−13

ligands	Ac-Arg-D-Cha-Xaa-D-Arg-D-*p*ClPhe-NH_2	K_i (nM)[a] hKOR	hMOR	hDOR	hORL1
1	BTD	78 ± 14	53 ± 20	222 ± 44	34 ± 8
10	I^2aa	190 ± 1	75 ± 21	5200 ± 50	44 ± 2
11	I^9aa	1085 ± 5	2700 ± 20	10900 ± 370	2130 ± 10
12	Qaa	441 ± 85	496 ± 89	7050 ± 850	35 ± 10
13	Phe-5-*t*BuPro	282 ± 81	368 ± 80	2340 ± 260	1700 ± 300
nociceptin/orphaninFQ		1500[b]	270[b]	3000[b]	0.10[c]
JTC-801		1057[d]	102[d]	8647[d]	8.2[d]

[a] K_i values were determinated using [^{3}H]diprenorphine for hKOR, hMOR, hDOR, and [leucyl-^{3}H]nociceptin. Data taken: [b]from Shimohigashi et al.;[29] [c]from Lapalu et al.;[30] [d]from Shinkai et al.[12] Experiments were conducted on hKOR, hMOR, hDOR transiently transfected into COS-1 cells and hORL1 stably expressed into CHO cells. Values are means ± SEM from three or more separate experiments, performed in duplicate.

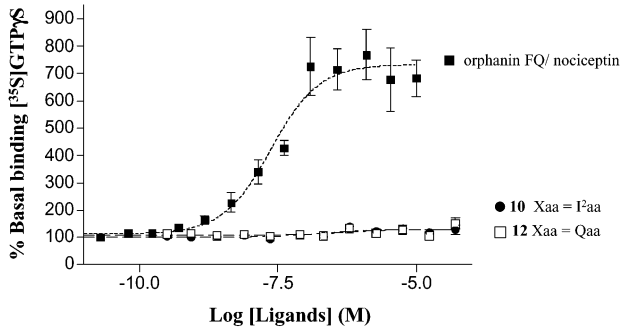


Figure 4. Stimulation of [^{35}S]GTPγS binding to hORL1 by orphanin NQ/nociceptin and peptides 10 and 12. CHO-hORL1 membranes (5 μg proteins) were incubated one hour at 37 °C with [^{35}S]GTPγS (0.2 nm) and GDP (40 μM), with increasing concentrations of ligands: orphanin FZ/nociceptin (■), peptide **10** (●), peptide **12** (□). Data are expressed as percentage of basal [^{35}S]GTPγS binding and represent mean ± SEM from at least two separate experiments.

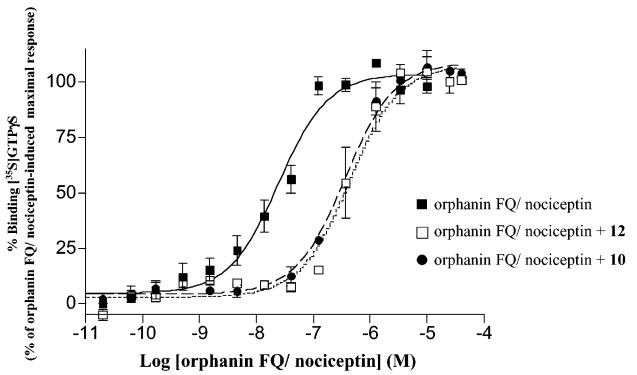


Figure 5. Stimulation of [^{35}S]GTPγS binding by orphanin FQ/ nociceptin on hORL1 in presence of putative antagonist peptides. CHO-hORL1 membranes (5 μg proteins) were incubated 1 h at 37 °C with [^{35}S]GTPγS (0.2 nm) and GDP (40 μM), with increasing concentrations of orphanin FQ/nociceptin (■) and 4.4 μM of peptide **10** (●) and 3.5 μM of peptide **12** (□). Peptides **10** and **12** shifted the concentration effect-curve of orphanin FQ/nociceptin to the right by 15−16-fold. Data are expressed as percentage orphanin FQ/nociceptin-induced maximal [^{35}S]GTPγS binding and represent mean ± SEM from at least two separated experiments.

hDOR/hKOR) relative to that previously observed with peptide **1** (1:1.5:6.5:2 K_i ratio of hORL1/hMOR/hDOR/hKOR). A greater improvement in selectivity for hORL1 over the other opioid receptors was exhibited by peptide **12** (1:14:201:13 K_i ratio of hORL1/hMOR/hDOR/hKOR).

[^{35}S]GTPγS Binding Assay. We further characterized the peptides having I^2aa (**10**) and Qaa (**12**) residues in a functional assay consisting of agonist promoted stimulation of [^{35}S]GTPγS binding to hORL1, hKOR, and hMOR cell membranes,[14] because they exhibited submicromolar affinities for these receptors. Figure 4 shows the results obtained with CHO-hORL1 membranes. In this experiment, orphanin FQ/nociceptin, a potent agonist of hORL1, stimulated the [^{35}S]GTPγS binding with an EC_{50} value of 24 ± 2 nM and a maximal activity corresponding to 733 ± 23% that of the basal level of [^{35}S]GTPγS binding. Peptides **10** and **12** neither increased nor decreased significantly the [^{35}S]GTPγS binding at high concentration. To confirm the antagonist activity of the two latter peptides, we performed concentration-effect curves of orphanin FQ/nociceptin in the presence of 100 K_i of each competitor peptide (Figure 5). Peptide **10** (4.5 μM) and peptide **12** (3.5 μM) shifted the concentration−effect curve of orphanin FQ/nociceptin to the right by about 15- and 16-fold, respectively, indicating that they exhibit antagonist activity toward hORL1. Both compounds displayed higher K_e values (400 ± 140 nM and 300 ± 60 nM) compared to that previously observed for peptide **1** (40 ± 10 nM).[14] These values are in the same range than that were recently reported for [Nphe^1]nociceptin(1−13)NH_2, another peptide antagonist, in the same functional assay.[28]

The activity of peptides **10** and **12** was also assessed in the [^{35}S]GTPγS binding assay using COS-hKOR membranes. Under our conditions, CI-977 stimulated the [^{35}S]GTPγS binding with an EC_{50} value of 3.9 ± 0.7 nM and a maximal activity corresponding to 190 ± 3% that of the basal level of [^{35}S]GTPγS binding. Peptides **10** and **12** stimulated the [^{35}S]GTPγS binding to COS-hKOR membranes with EC_{50} values > 1 μM and maximal activity of about 120% that of the basal level of [^{35}S]GTPγS binding (Table 2), indicative of their partial agonist activity at hKOR.

The two peptides with I^2aa (**10**) and Qaa (**12**) residues also displayed submicromolar affinities for hMOR. We therefore tested their activity at this receptor in the [^{35}S]-GTPγS binding assay (Table 2). Peptides **10** and **12** stimulated the [^{35}S]GTPγS binding to COS-hMOR membranes with EC_{50} values of 1.4 ± 0.7 μM and 2.8 ± 0.5 μM, respectively. Maximal activation values obtained with peptide **10** (207%) and peptide **12** (140%) on COS-hMOR membranes were lower than that obtained with DAMGO (239%), a classical peptidic MOR-agonist, indicative of their partial agonist activity at hMOR.

Discussion

The spectrum of biological activity exhibited by peptides **10**−**13** suggested that the ring size of the bicyclic system and the steric interactions of the 5-*t*BuPro

Table 2. Stimulation of [^{35}S]GTPγS Binding by Prototypical Receptor Agonists and Peptide **10** and **12**[a]

	hKOR		hMOR	
	EC_{50} (nM)	maximal activation (%)	EC_{50} (nM)	maximal activation (%)
CI-977	3.9 ± 0.7	190 ± 3		
DAMGO			11.8 ± 0.2	239 ± 10
peptide **10**	>1000	122 ± 3	1400 ± 700	207 ± 14
peptide **12**	>1000	117 ± 4	2800 ± 500	141 ± 11
peptide III-BTD[b]	434 ± 2	110 ± 1	611 ± 116	158 ± 6

[a] Activity of peptides **10** and **12** was tested on membrane preparations expressing hKOR and hMOR. COS-hKOR and COS-hMOR membranes (5 μg) were incubated 1 h at 30 °C with [^{35}S]GTPγS (0.2 nM) and GDP (see Supporting Information) with increasing concentrations of ligands: CI-977, DAMGO, peptide **10**, and peptide **12**. Maximal activation is expressed as percentage of basal [^{35}S]GTPγS binding and values represents mean ± SEM from at least two separate experiments, performed in triplicate. [b] Values for this peptide are from Becker et al.[14]

residue caused significant modification of the peptide conformation. Peptides **11** and **13** exhibited significantly reduced affinity and selectivity at the ORL1 receptor relative to peptide **1** which demonstrated that replacement of the BTD residue by I^{9}aa and Phe-5-*t*BuPro caused structural modifications that decreased the activity. Antagonist potency at the ORL1 receptor was maintained and ORL1 versus DOR selectivity was enhanced on replacement of the thiaindolizidinone BTD by I^{2}aa which introduced a methylene for sulfur and flipped the ring fusion stereochemistry. Peptide **12** incorporating the Qaa residue revealed the importance of a 6,6-bicyclic system for selectivity at the ORL1 receptor. Conversion of the sulfur in BTD to an ethylene group and flipping the ring fusion stereochemistry in Qaa produced a peptide that exhibited similarly potent antagonist activity and enhanced selectivity for the ORL1 receptor. Replacement of the BTD residue by azabicycloalkane amino acids **6**−**8** demonstrated that ligands with 6,5- and 5,6-bicyclic lactams manifested less selectivity for the ORL1 receptor than the 6,6-bicycle. The 7,5-bicyclic lactam **3** was previously incorporated into peptide **1** and exhibited decreased selectivity for the ORL1 receptor.[14] X-ray analysis of analogues of BTD and azabicycloalkane amino acids **6**−**8** has revealed that the dihedral angle ψ was similar (−141 ± 35°) for all three residues; however, the ϕ torsion angle for the Qaa residue was significantly different.[20−23] Thus, the ψ and ϕ dihedral angles of −163° and 48° that were observed for the central peptide bonds in the X-ray structure of 3-*N*-(Boc)amino quinolizidin-2-one 10-carboxylic *tert*-butyl ester (BOC-Qaa-O*t*Bu) may be important for the peptide conformation to exhibit activity and specificity at the ORL1 receptor.

In conclusion, we have employed structurally related and complementary turn inducing amino acids to provide information on the relationship between conformation and activity at the ORL1 receptor. We have synthesized two new antagonists for the ORL1 receptor. Peptide **10,** synthesized with the indolizidin-2-one amino acid **6**, exhibited improved ORL1/DOR selectivity relative to the parent BTD ligand. Peptide **12**, synthesized with quinolizidinone amino acid **8**, showed an increased overall selectivity relative to ligand **1**. This methodology has enhanced the pharmacological profile of the parent ligand and advanced the understanding of the conformational requirements for ORL1 receptor affinity. This methodology may be similarly applied to study other peptide ligands that possess turn conformations. The quinolizidinone amino acid is presently serving as a scaffold to provide potent and selective antagonists for the ORL1 receptor possessing improved bioavailability.

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Abbreviations

BTD, (3*S*,6*S*,9*R*)-2-oxo-3-amino-7-thia-1-aza-bicyclo-[4.3.0]nonane-9-carboxylic acid; Haic, 5-amino-1,2,4,5,6,7-tetrahydroazepino[3,2,1-*hi*]indol-4-one-2-carboxylate; BZA, 3-amino-1-carboxymethyl-2,3,4,5-tetrahydro-1*H*-[1]-benzazepin-2-one; BZD, 3-amino-*N*-1-carboxymethyl-2-oxo-5-phenyl-1,4-benzodiazepine; I^{2}aa, (3*S*,6*R*,10*S*)-3-amino indolizidin-2-one-9-carboxylate; I^{9}aa, (2*S*,6*R*,8*S*)-8-aminoindolizidin-9-one-2-carboxylate; Qaa, (3*S*,6*R*,10*S*)-3-amino quinolizidin-2-one-10-carboxylate; 5-*t*BuPro, (2*S*,5*R*)-5-*tert*-butylproline; CI-977, [5*R*-(5α,7α,8*β*)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzo[*b*]furan-4-acetamide; DAMGO, [D-Ala^2,*N*-Me-Phe^4,-Gly-ol^5]enkephalin; hDOR, human δ-opioid receptor; hKOR, human κ-opioid receptor; hMOR, human μ-opioid receptor; hORL1, human opioid receptor like; TBTU, benzotriazol-1-yl-1,1,3,3-tetramethyluronium tetrafluoroborate.

Supporting Information Available: Experimental details for the peptide synthesis of **10**−**13**, listings of spectroscopic, chromatographic, and analytical data for **10**−**13**, as well as protocols for the biological testing of **10**−**13** in cell culture, cell transfections, and cell membrane preparations, receptor binding assay, and [^{35}S]GTPγS binding assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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