High Affinity Conformationally Constrained Nociceptin/Orphanin FQ(1−13) Amide Analogues

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Abstract: A series of cyclic analogues with a lactam linkage were prepared by solid phase peptide synthesis to explore possible biologically active conformation(s) of nociceptin/orphanin FQ (N/OFQ). *cyclo*[D-Asp^7,Lys^{10}]- and *cyclo*[Asp^6,Lys^{10}]N/OFQ(1−13)NH$_2$ exhibit high affinity (K_i = 0.27 and 0.34 nM, respectively) and high potency in the GTPγS assay (EC_{50} = 1.6 and 4.1 nM, respectively) at human nociceptin/orphanin FQ peptide (NOP) receptors. These analogues exhibit 2- to 3-fold higher affinity and 2- to 5-fold higher potency than the parent peptide.

A novel receptor which possesses sequence similarity to the opioid receptors was identified and cloned from human, rat, and mouse brains in the 1990s.[1–3] The endogenous ligand, nociceptin[4,5] or orphanin FQ[5] (N/OFQ),[a] was subsequently identified by two research groups. Both ligand and receptor are widely distributed in the central nervous system and spinal cord.[6,7] Pharmacological studies of the nociceptin/orphanin FQ system reveal important roles in several physiological and pharmacological effects.[8,9] Despite the sequence similarity to opioid peptides, N/OFQ exhibits distinct physiological and pharmacological profiles different from opioids.[8–10] While early studies clearly demonstrated that N/OFQ modulates nociception, the effects of N/OFQ in nociception are complex and appear to be dependent on a number of factors, including the route of administration (see ref 8). Initially, N/OFQ was reported to induce hyperalgesia in the hot plate and tail flick assays when administered intracerebroventricularly (icv)[4,5] that were subsequently reclassified as antianalgesic effects.[10,11] N/OFQ administered icv can also functionally antagonize the analgesic effects of morphine and other opiates.[8,10,11] After intrathecal administration, however, N/OFQ has been reported to produce analgesia and/or to potentiate morphine analgesia.[8–10,12] N/OFQ also produces anxiolytic-like effects in several behavioral paradigms of different types of anxiety states in animals,[9,13,14] and thus agonists for this receptor could have potential therapeutic applications as novel anxiolytic agents.

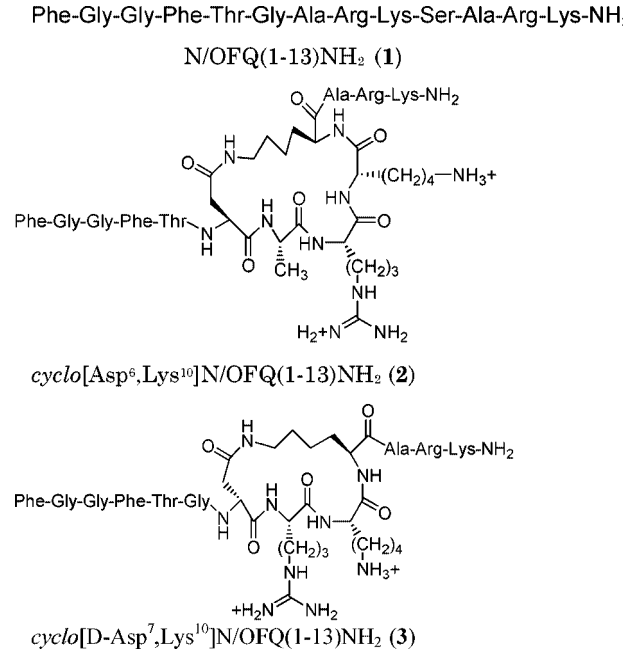

Figure 1. Structures of N/OFQ(1−13)NH$_2$, *cyclo*[Asp^6,Lys^{10}]-, and *cyclo*[D-Asp^7,Lys^{10}]N/OFQ(1−13)NH$_2$ (**1**, **2**, and **3**).

Like opioid receptors, the opioid receptor-like 1 (ORL1) receptor or nociceptin/orphanin FQ peptide (NOP) receptor belongs to the G-protein coupled receptor family, and when N/OFQ interacts with its receptor, it triggers effector systems in a similar manner to the opioid receptors. Agonist binding to the NOP receptor inhibit adenylyl cyclase[4,5] and Ca^{2+} channels, and activate protein kinases and K^+ channels.[9] However, N/OFQ has low affinity and negligible activity at opioid receptors, and similarly opioid ligands have low affinity at the NOP receptor.[15]

To understand the structural requirements and the physiological roles of the nociceptin/orphanin FQ system, attempts have been made to develop high affinity and potent ligands for the NOP receptor. A variety of structure−activity relationship studies of N/OFQ have been performed (see refs 9, 16, and 17 for reviews). An alanine scan examined the effects of functional groups at each position and found that the residues in positions 1, 4, and 8 are important for receptor binding and activation.[18,19] D-Amino acid substitution indicated that an amino acid with the D-configuration was well tolerated at positions 2 and 7.[19] In addition, a C-terminal truncation study indicated that OFQ/N(1−13)NH$_2$ (**1**, Figure 1) was the smallest fragment which was as potent as the endogenous ligand,[18,20] and thus **1** was the parent peptide in this study.

Because the endogenous ligand is a linear peptide, it exhibits considerable conformational flexibility. Cyclization is one approach to reduce the flexibility of linear peptides and may also increase receptor affinity, potency, and/or metabolic stability.[21] Also potent cyclic analogues may provide information on possible biologically active conformations of the peptide. Only a limited number of cyclic analogues of N/OFQ have been reported to date,[22–25] and these have all involved cyclization via a disulfide bond; *cyclo*[Cys^{10},Cys^{14}]N/OFQ(1−14)NH$_2$[23,24] and *cyclo*[Cys^7,Cys^{10}]N/OFQ(1−13)NH$_2$[25] exhibit the highest NOP receptor affinity and agonist potency of the reported cyclic analogues. We prepared conformationally constrained analogues of **1**, along with their corresponding linear analogues. The

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[a] Abbreviations: Fmoc, fluorenylmethoxycarbonyl; CHO, Chinese hamster ovary; GTPγS, guanosine 5′-[γ-S]thiotriphosphate; HOAt, 1-hydroxy-7-azabenzotriazole; Mtt, 4-methyltrityl; N/OFQ, nociceptin/orphanin FQ; NOP, nociceptin/orphanin FQ peptide; ORL1, opioid receptor-like 1; PAL-PEG-PS, peptide amide linker-polyethylene glycol-polystyrene; Pip, 2-phenylisopropyl; PyAOP, 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate.

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Table 1. NOP Receptor Binding Affinity and Potency of Cyclic N/OFQ(1−13)NH_2 Analogues and the Corresponding Linear Peptides

	[^{35}S]GTPγS binding		
peptide	affinity K_i ± SEM (nM)[a]	EC_{50} ± SEM (nM)[b]	% stimulation[c]
1 N/OFQ (1−13)NH_2	0.62 ± 0.23	7.82 ± 1.88	100 ± 1
2 *cyclo* [Asp^6,Lys^{10}]-	0.34 ± 0.10	4.12 ± 1.21	80 ± 5
3 *cyclo* [D-Asp^7,Lys^{10}]-	0.27 ± 0.03	1.60 ± 0.45	92 ± 6
4 [Asp^6,Lys^{10}]-	0.54 ± 0.05	22.3 ± 5.9	122 ± 12
5 [D-Asp^7,Lys^{10}]-	1.79 ± 0.10	106 ± 23	100 ± 3

[a] K_i values are the average ± SEM of 3−4 independent determinations performed in duplicate. [b] EC_{50} values are the average ± SEM of 3−5 independent determinations performed in triplicate. [c] Relative to **1**.

constrained analogues were cyclized via a lactam linkage between side chain functional groups of noncritical residues and were prepared by solid phase peptide synthesis. We identified two cyclic analogues, *cyclo*[Asp^6,Lys^{10}]- and *cyclo*[D-Asp^7,Lys^{10}]N/OFQ(1−13)NH_2 (**2** and **3**, Figure 1), with very high affinity and potency at the NOP receptor. The synthesis and pharmacological evaluation of these analogues are described below.

The cyclic analogues were prepared by solid phase synthesis using the Fmoc (fluorenylmethoxycarbonyl)/*tert*-butyl protection strategy. The linear peptides were prepared on a PAL-PEG-PS resin (peptide amide linker–polyethylene glycol-polystyrene resin, Applied Biosystems, Foster City, CA) using Fmoc-protected amino acids.[26] Hyperacid labile side-chain protecting groups (2-phenylisopropyl (Pip) ester for D/L-Asp and 4-methyltrityl (Mtt) for Lys) were used for the amino acids involved in the lactam ring. The linear peptides were elongated to the position where Fmoc-D-Asp(Pip) or Fmoc-Asp(Pip) was incorporated, and the hyperacid labile protecting groups removed using 5% trifluoroacetic acid in dichloromethane. The cyclization to yield the lactam linkage was performed using 7-azabenzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate (PyAOP), 1-hydroxy-7-azabenzotriazole (HOAt), and *N,N*-diisopropylethylamine (5:5:10) in dicholoromethane and *N,N*-dimethylformamide (1:1).[27] The peptides were cleaved from the solid support using Reagent B[28] and purified by reversed-phase HPLC to yield the final peptides in high purity (≥98%, as determined by HPLC and capillary electrophoresis); the peptides gave the expected molecular weights by electrospray mass spectrometry.

The affinity and potency of these analogues were determined in Chinese hamster ovary (CHO) cells expressing the NOP receptor using radioligand binding[29,30] and GTPγS assays[31] (Table 1). Cyclic peptides **2** and **3** and their corresponding linear analogues exhibit high affinity (K_i = 0.27−1.8 nM) for the NOP receptor. These analogues stimulate GTPγS binding with moderate to high potency (EC_{50} = 1.6−106 nM) and act as full agonists (80−122% maximum stimulation relative to **1**).

The cyclic lactam peptides **2** and **3** exhibit 2- to 3-fold higher affinity for the NOP receptor than the parent peptide **1**. Both of the cyclic peptides **2** and **3** also exhibit higher potency (2- to 5-fold) than the parent peptide **1** in the GTPγS assay. In spite of the differences in ring size and position and stereochemistry of the Asp residue, the binding affinity and potency in the functional assay are very similar for the two cyclic analogues. These results indicate that a cyclic constraint in the middle of the peptide sequence is compatible with receptor interaction, and suggest that both cyclic structures are compatible with the bioactive conformation of the peptide in spite of the differences in the lactam rings. The related cyclic analogue *cyclo*[Cys^6,Cys^{10}]N/OFQ(1−13)NH_2 exhibits slightly (2-fold) lower NOP receptor affinity than the parent peptide **1** (K_i = 0.83 vs 0.35 nM, respectively), but it exhibits a much larger (16-fold) decrease in potency (EC_{50} = 31 vs 2.0 nM, respectively) in the GTPγS assay.[23] The recently reported analogue *cyclo*[Cys^7,Cys^{10}]N/OFQ(1−13)NH_2 exhibits high affinity (K_i = 0.10 nM) and potency in the GTPγS assay (EC_{50} = 0.69 nM) comparable to the parent peptide **1** (K_i = 0.15 nM and EC_{50} = 0.78 nM).[25] The linear N/OFQ-NH_2 analogue containing α-aminoisobutyric acid (Aib) residues, which can promote an α-helical conformation, in positions 7 and 11 also exhibits high NOP receptor affinity and potency.[31]

The cyclic peptide **2** exhibits only slightly higher binding affinity than its corresponding linear peptide **4**, while the cyclic analogue **3** has 6.6-fold higher binding affinity than its corresponding linear analogue **5**. Both of the cyclic peptides stimulate the NOP receptor with higher potency (5- to 66-fold) than the corresponding linear analogues **4** and **5**. The linear peptide **4** has binding affinity indistinguishable from the parent peptide **1**, while the other linear peptide **5** exhibits 3-fold lower binding affinity. These results suggest that a charged residue in positions 6 and/or 10 did not interfere with receptor binding. The lower affinity of the linear peptide **5** could be due to differences in conformation and/or introduction of a negative charge at position 7. In contrast to the high potency of the two cyclic analogues, these linear peptides exhibit significant decreases (3- to 13-fold) in potency in the GTPγS assay compared to the parent peptide **1**. The disulfide cyclized analogue *cyclo*[Cys^6,Cys^{10}]N/OFQ-(1−13)NH_2 also exhibits increased NOP receptor affinity (3.6-fold), but no increase in potency in the GTPγS assay, compared to the corresponding linear peptide [Cys^6,Cys^{10}]N/OFQ(1−13)NH_2.[23] (The linear peptide [Cys^7,Cys^{10}]N/OFQ(1−13)NH_2 was not reported for comparison to *cyclo*[Cys^7,Cys^{10}]N/OFQ-(1−13)NH_2.[25]) These results suggest that a lactam or disulfide constraint in the middle region may facilitate binding of the rest of the N/OFQ sequence with the NOP receptor in an agonist conformation.

In conclusion, this paper reports the first high affinity and potent cyclic lactam N/OFQ(1−13)NH_2 analogues **2** and **3**. These results indicate that incorporation of a lactam constraint in the middle of the N/OFQ sequence is compatible with the biologically active conformation(s) of the peptide. While the binding affinities of the constrained peptides and their corresponding linear analogues were generally similar, the differences in potency in the functional assay between the cyclic and linear peptides were significantly larger. These results suggest that the conformational constraints may help stabilize conformation(s) which are compatible with binding to the agonist conformation of the NOP receptor, and that the flexibility of the linear analogues may contribute to their lower potency in the functional assay. The cyclic analogues reported here will be useful pharmacological tools for studying the NOP receptor and possible bioactive conformations of N/OFQ. They also are new lead compounds which can be further modified to gain further insight into the interactions of OFQ with the NOP receptor.

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Supporting Information Available: Characterization of compounds by HPLC, capillary electrophoresis, and electrospray mass spectrometry. This material is available free of charge via the Internet at http://pubs.acs.org.

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(29) The affinities of the peptides were determined in a radioligand binding assay using [^{3}H]N/OFQ and CHO cells stably expressing hNOP receptor. hNOP-CHO cells were harvested in TEM buffer (50 mM Tris HCl, 10 mM EGTA, 5 mM $MgCl_2$), pH 7.4, homogenized and centrifuged. The pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1% protease inhibitors (0.02 mM PMSF, 3.2 μg/mL benzamidine, 1 μg pepstatin A). Each incubation mixture (0.25 mL, in duplicate) consisted of 30 μg membrane protein. Saturation and competition binding assays were performed in TEM buffer with 0.1% BSA, pH 7.4, and incubated for 2.5 h. Nonspecific binding was determined in the presence of 1 μM N/OFQ. For saturation binding studies, 0.125−5 nM [^{3}H]N/OFQ was used. Nonlinear regression analysis was performed to determined K_d (0.86 ± 0.28 nM, $n = 3$) and B_{max} (1.23 ± 0.14 pmol/mg protein, $n = 3$) using Graphpad (Prism) software (San Diego, CA). For competition experiments, the ability of serial dilutions (10^{-11}−10^{-6} M) of the peptides to displace the binding of 1 nM [^{3}H]N/OFQ was assessed. IC_{50} values were also determined by nonlinear regression analysis using Graphpad (Prism) software. K_i values were calculated using the Cheng and Prusoff equation.

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