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Structure−Activity Study of the ORL1 Antagonist Ac-Arg-D-Cha-Qaa-D-Arg-D-*p*-ClPhe-NH_2

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The structure−activity requirements of the ORL1 antagonist Ac-Arg-D-Cha-Qaa-D-Arg-D-*p*-ClPhe-NH_2 **4** were investigated by varying the position, structure, and charge of the Arg residues. Attempts to abridge the peptide by removal of the Arg, D-Cha, and D-*p*-ClPhe residues abolished affinity for the ORL1 receptor, whereas deletion of the acetamido N-terminus maintained receptor affinity and selectivity. This series of analogues has provided an improved potent and selective ORL1 receptor antagonist, Ac-Cit-D-Cha-Qaa-D-Arg-D-*p*-ClPhe-NH_2.

Introduction

The opioid receptor like 1 (ORL1) is a G-protein-coupled receptor structurally related to μ-, δ-, and κ-opioid receptors. However, it has been shown to display poor affinity for opioid receptor ligands.[1−4] The endogenous ligand of ORL1 was shown to be a heptadecapeptide known as nociceptin/orphanin FQ.[5,6] Although the physiological role for the ORL1 receptor is still poorly defined, nociceptin/orphanin FQ has exhibited a broad spectrum of pharmacological actions, including pain modulation, anxiolytic-like effects, stimulation of food intake, and modulation of spontaneous locomotor activity.[7] 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 this receptor.

From screening of a synthetic combinatorial constrained peptide library on the human μ-, δ-, and κ-opioid receptors, and the ORL1 receptor, a hexapeptide analogue (**1**) was identified as a ligand exhibiting good affinity and modest selectivity for the ORL1 receptor (Figure 1).[8] Hexapeptide analogue **1** contains a thiaindolizidinone β-turn mimic and acts as a competitive ORL1 receptor antagonist. At the opioid receptors, analogue **1** also exhibited agonist activity at higher concentrations. Replacement of the thiaindolizidinone β-turn mimic in **1** with indolizidinone and quinolizidinone amino acids produced a series of related peptides **2**−**4**. Peptide **4**, which contained the quinolizidinone dipeptide mimic, Qaa, displayed similar affinity and antagonist potency as **1** at the ORL1 receptor; moreover, it exhibited enhanced receptor selectivity (Table 2).[9]

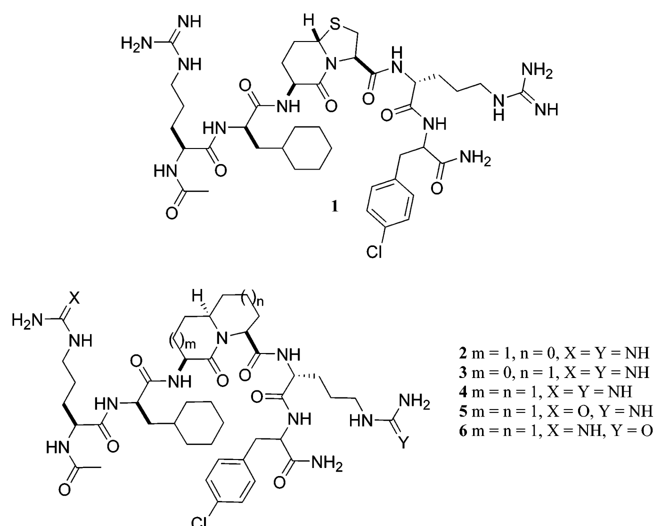

Figure 1. Structure of peptide III−BTD **1** and peptides containing azabicyclo[*X.Y.*0]alkane amino acids **2**−**6**.

Probing the structural and electronic requirements for antagonist activity and selectivity for the ORL1 receptor, we have now synthesized and evaluated a new series of analogues of Ac-Arg-D-Cha-Qaa-D-Arg-D-*p*-ClPhe-NH_2 (**4**). In particular, we have varied the position, structure, and charge of the arginine residues in **4** in order to better understand their importance for activity and selectivity. The neutral amino acid citrulline (Cit) has been used to replace each of the Arg residues in **4** to study the importance of their positive charge for interaction with the ORL1 receptor (analogues **5** and **6**).[10] Furthermore, the size of the hexapeptide analogue was reduced by systematic deletions of the Arg, D-Cha, and D-*p*-ClPhe residues as well as the acetamido N-terminus in analogues **7**−**10** in order to assess their importance for biological activity (Tables 1 and 2).

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Table 1. Qaa-Containing Peptides to Investigate the Structure−Activity Relationship of Hexapeptide **4** at the ORL1 Receptor

	peptide	formula	calcd: (M + H$^+$)	MS: (M + H$^+$)	HPLC,[a] min	HPLC,[b] min	TLC, R_f
4	Ac−Arg-D-Cha-Qaa-D-Arg-D-*p*-ClPhe-NH_2	$C_{42}H_{66}ClN_{13}O_7$	900.5	900.5	15.5	20.67	0.64
5	Ac−Cit-D-Cha-Qaa-D-Arg-D-*p*-ClPhe-NH_2	$C_{42}H_{65}ClN_{12}O_8$	901.5	901.5	14.7	20.56	0.70
6	Ac−Arg-D-Cha-Qaa-D-Cit-D-*p*-ClPhe-NH_2	$C_{42}H_{65}ClN_{12}O_8$	901.5	901.5	14.6	20.11	0.63
7	Ac−Arg-Qaa-D-Arg-D-*p*-ClPhe-NH_2	$C_{33}H_{51}ClN_{12}O_6$	747.4	747.6	11.8	17.05	0.50
8	Ac−Arg-D-Cha-Qaa-D-Arg-NH_2	$C_{33}H_{58}N_{12}O_6$	719.5	719.6	13.9	17.55	0.48
9	Ac-D-Cha-Qaa-D-Arg-NH_2	$C_{27}H_{46}N_8O_5$	563.4	563.6	15.3	19.29	0.56
10	$H_2NC(=NH)NH(CH_2)_4CO$-D-Cha-Qaa-D-Arg-D-*p*-ClPhe-NH_2	$C_{40}H_{63}ClN_{12}O_6$	843.5	843.7	15.7	21.06	0.69

[a] HPLC conditions 1. [b] HPLC conditions 2. See Supporting Information.

Scheme 1. Synthesis of the Guanidine End of Peptide **10** by Guanylation

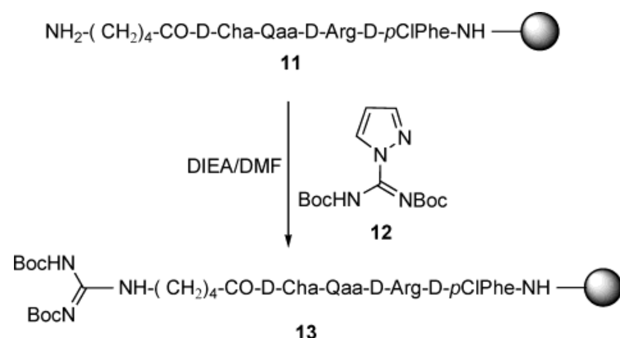

Table 2. Binding Affinities for hMOR, hKOR, and hORL1 of Peptides

peptide	K_i (nM)[a] hORL	hMOR	hKOR	hDOR
1	34 ± 8[b]	53 ± 20[b]	78 ± 14[b]	222 ± 44[b]
4	27 ± 7	90 ± 12	105 ± 18	>5000
5	72 ± 21	2555 ± 635	4270 ± 100	>5000
6	>5000	>5000	>5000	>5000
7	>5000	833 ± 187	779 ± 176	>5000
8	>5000	>5000	>5000	>5000
9	>5000	>5000	>5000	>5000
10	45 ± 18	85 ± 25	153 ± 55	>5000

[a] K_i values were determined using [^{3}H]diprenorphine for hKOR, hMOR, hDOR, and [leucyl-^{3}H]nociceptin. Experiments were conducted on hMOR and hDOR transiently transfected into COS-1 cells, and hORL1 and hKOR stably expressed into CHO cells. Values are means ± SEM from two or more separated experiments, performed in duplicate. [b] From Becker et al., 1999 (ref 8).

Results and Discussion

Chemistry. Enantiopure *N*-(Boc)aminoquinolizidinone acid was synthesized from aspartic and pyroglutamic acids as inexpensive chiral educts according to the literature procedure.[9,11] Peptides **5**−**9** were synthesized using the solid-phase method of Merrifield[12] on MBHA resin in a semiautomatic apparatus as described in the Experimental Section. Elongation of the peptide involved coupling of *N*-Boc protected amino acids in the presence of HOBt and DIC as coupling reagent in a CH_2Cl_2/DMF solution. Amine deprotection was performed with TFA and anisole in CH_2Cl_2. Before the peptide was cleaved from the resin, the N-terminal end was acetylated by treatment with acetic anhydride and pyridine in a CH_2Cl_2/DMF solution. Cleavage of the peptide and deprotection of the tosyl groups of the arginyl residues was accomplished simultaneously by treatment of the resin with anhydrous liquid HF in the presence of anisole. Crude peptide was purified by reverse-phase HPLC. Peptide purity and composition were respectively ascertained by analytical HPLC and mass spectrometry (Table 1). Peptide **10** was synthesized by a similar protocol involving an additional guanylation of resin-bound 5-aminovaleryl peptide **11** using *N*,*N*′-bis(*tert*-butyloxycarbonyl)-1-guanylpyrazole **12**[13] and excess of DIEA in DMF (Scheme 1).[14]

Receptor Binding Activity. The receptor binding affinities of peptides **5**−**10** were compared with the parent peptide, Ac-Arg-D-Cha-Qaa-D-Arg-D-*p*-ClPhe-NH_2 (**4**), in assays on membrane homogenates of COS-1 or CHO cells expressing recombinant human μ-, δ-, and κ-opioid receptors (hMOR, hDOR, hKOR) and the human opioid receptor-like (hORL1, Table 2). Like peptide **4,** peptides **5**−**10**, all displayed affinities higher than 5000 nM at the DOR. Peptides **6**−**9** exhibited drastically reduced binding affinities for the ORL1 receptor, indicating the importance of a positively charged D-Arg residue as well as the difficulty in abridging peptide **4** by removal of the Arg, D-Cha, and D-*p*-ClPhe residues. On the other hand, peptide **10** exhibited similar affinity as peptide **4** (45 and 27 nM, respectively) for hORL1 as well as similar modest selectivity for the hORL1 versus the hMOR and hKOR, indicating that the N-terminus acetamido group could be removed without influencing binding and receptor specificity. Furthermore, peptide **5**, in which the N-terminal Arg residue was replaced by Cit, showed similarly good affinity for ORL1 as **4** (72 and 27 nM, respectively). In addition, peptide **5** displayed a greatly enhanced selectivity for hORL1 versus hMOR and hKOR (1:36:59 K_i ratio of hORL1/hMOR/hKOR) relative to that observed for peptide **4** (1:3:4 K_i ratio of hORL1/hMOR/hKOR). The maintained activity and improved selectivity of peptide **5** relative to the parent peptide **4** demonstrated that the positively charged guanidine at the N-terminus of **4** was more important for maintaining interactions with the μ- and κ-opioid receptors relative to the ORL1 receptor.

[^{35}S]GTPγS Binding Assay. Because they both exhibited submicromolar affinities for hORL1, peptides **5** and **10** were further characterized in a functional assay consisting of agonist promoted stimulation of [^{35}S]-GTPγS binding to hORL1, hMOR, and hKOR cell membranes. At high concentrations (up to 50 μM), neither peptide **5** nor **10** increased nor decreased the [^{35}S]GTPγS binding to cell membranes expressing these receptors (data not shown). Peptide **5** (7 μM) shifted the concentration−effect curve of orphanin FQ/nociceptin (100 K_i concentration) to the right by about 55-fold, confirming its antagonist activity toward hORL1 (Figure 2). Moreover, peptide **5** displayed higher antagonist potency (K_e = 136 ± 32 nM) than was previously observed for peptide **4** (K_e = 300 ± 60 nM).

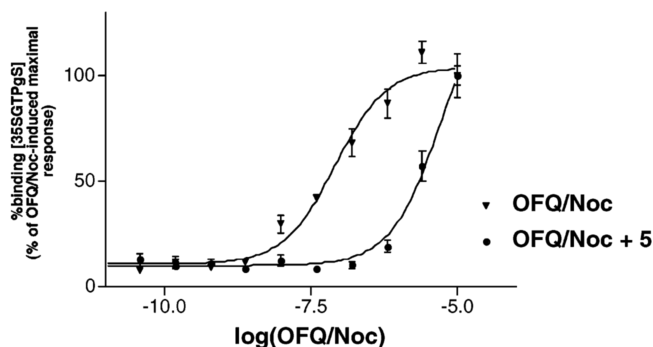

Figure 2. Stimulation of [^{35}S]GTPγS binding by orphanin FQ/nociceptin on hORL1 in the presence of putative antagonist peptide **5**. CHO-hORL1 membranes (5 μg of protein) were incubated 1 h at 37 °C with [^{35}S]GTPγS (0.2 nM) and GDP (40 μM), with increasing concentrations of ligands: nociceptin/orphanin FQ (▼) and 7 μM of peptide **5** (●). Peptide **5** shifted the concentration-effect curve of orphanin FQ/nociceptin to the right by about 55-fold. Data are expressed as percentage Nociceptin/Orphanin FQ-induced maximal [^{35}S]GTPγS binding and represent mean ± SEM from at least two separated experiments.

Conclusions

A better insight into the structural and electronic requirements for affinity and selectivity at the ORL1 receptor has been obtained by the synthesis and analysis of a series of analogues of the ORL1 antagonist **4**. Contraction of the peptide caused typically a dramatic loss of biological activity; however, the N-terminal acetamido group could be removed with little influence on affinity and selectivity. Replacement of each of the guanidine moieties with a urea, by substitution of Arg with Cit, demonstrated the importance of positive charge for affinity and selectivity. In particular, replacement of the C-terminal D-Arg residue with D-Cit in peptide **6** abolished affinity at the ORL1 receptor and indicated an essential electrostatic interaction. On the other hand, replacement of the charged moiety at the N-terminus by a neutral hydrogen-bonding surrogate had little effect on affinity, yet improved antagonist potency and selectivity for the ORL1 receptor. Peptide **5** (Ac-Cit-D-Cha-Qaa-D-Arg-D-*p*-ClPhe-NH_2) represents a new improved potent and selective ORL1 receptor antagonist.

Experimental Section

Solid-phase synthesis was conducted using a semiautomatic peptide synthesizer by a DIC/HOBt-mediated BOC-protection strategy on a 4-methylbenzhydrylamine resin (MBHA, 1.10 mmol/g). Before the coupling was started, the resin was washed with DIEA (2 ×) and CH_2Cl_2 (2 ×). The following schedule was employed: (1) Boc-protected amino acid (3 equiv)/DIC (3 equiv)/HOBt (3 equiv) in DMF/CH_2Cl_2 (1:1) for 3 h; (2) the coupling reaction was monitored by Kaiser ninhydrin test[15] (in cases of incomplete couplings, the resin was resubmitted to the same coupling conditions); (3) DMF wash (3 ×); (4) ^{i}PrOH wash (3 ×); (5) CH_2Cl_2 wash (3 ×); (6) BOC-deprotection with TFA/CH_2Cl_2/anisole (49/49/2) (2 ×, 5 and 15 min); (7) CH_2Cl_2 wash (2 ×); (8) DIEA wash (2 ×); (9) CH_2Cl_2 wash (2 ×). N-Terminal acetylation of the peptides was accomplished by treating the resin with Ac_2O (1.5 eq) and pyridine (1.5 equiv) in DMF/ CH_2Cl_2 (1/1) for 1 h. The side chain protection group used for Arg was Tos (toluene-4-sulfonyl). The peptides were cleaved from the resin and side chain groups were deprotected by treating the peptide on solid support with liquid HF and anisole at 0 °C for 90 min. After HF evaporation, the residue was treated with diethyl ether (2 ×), to precipitate the peptide, and filtered. Separation of the peptide from the resin was performed by adding acetic acid to obtain a solution that was lyophilized. The crude material was purified by semipreparative RP-HPLC, with UV-detection at 215 nm, on a Supelco, Discovery BIO Wide Pore column (C18, 25 cm × 21.2 mm, 10 μm particle size) using a flow rate of 20 mL/min and a gradient from 97:3 to 80:20 water/CH_3CN containing 0.1% TFA over 30 min, followed by a 10 min isocratic run with the final eluant.

Abreviations

BTD ((3*S*,6*S*,9*R*)-2-oxo-3-amino-7-thia-1-azabicyclo-[4.3.0]nonane-9-carboxylic acid); CI-977, [5*R*-(5α,7α,8β)-*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; MBHA, 4-methylbenzhydrylamine; Qaa, (3*S*,6*R*,10*S*)-2-oxo-3-amino-1-azabicyclo[4.4.0]decane-10-carboxylate.

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Supporting Information Available: Experimental details as well as protocols for the biological testing of **4**−**10** 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.

References

(1) Bunzow, J. R.; Saez, C.; Mortrud, M.; Bouvier, C.; Williams, J. T.; Low, M.; Grandy, D. K. Molecular cloning and tissue distribution of a putative member of the rat opioid receptor gene family that is not a mu, delta or kappa opioid receptor type. *FEBS Lett.* **1994**, *347*, 284−288.

(2) Lachowicz, J. E.; Shen, Y.; Monsma, F. J.; Sibley, D. R. Molecular cloning of a novel G protein coupled receptor related to the opioid receptor family. *J. Neurochem.* **1995**, *64*, 34−40.

(3) Mollereau, C.; Parmentier, M.; Mailleux, P.; Butour, J. L.; Moisand, C.; Chalon, P.; Caput, D.; Vassart, G.; Meunier, J. C. ORL1, a novel member of the opioid receptor family − Cloning, functional expression and localization. *FEBS Lett.* **1994**, *341*, 33−38.

(4) Wang, J. B.; Johnson, P. S.; Imai, Y.; Persico, A. M.; Ozenberger, B. A.; Eppler, C. M.; Uhl, G. R. cDNA cloning of an orphan opiate receptor gene family member and its splice variant. *FEBS Lett.* **1994**, *348*, 75−79.

(5) Meunier, J. C.; Mollereau, C.; Toll, L.; Suaudeau, C.; Moisand, C.; Alvinerie, P.; Butour, J. L.; Guillemot, J. C.; Ferrara, P.; Monserrat, B.; Mazarguil, H.; Vassart, G.; Parmentier, M.; Costentin, J. Isolation and structure of the endogenous agonist of opioid receptor like ORL1 receptor. *Nature* **1995**, *377*, 532−535.

(6) Reinsheid, R. K.; Nothacker, H. P.; Bourson, A.; Ardati, A.; Henningsen, R. A.; Bunzow, J. R.; Grandy, D. K.; Langen, H.; Monsma, F. J., Jr.; Civelli, O. Orphanin FQ: a neuropeptide that activates an opioid-like G protein-coupled receptor. *Science* **1995**, *270*, 792−794.

(7) Mogil, J. S.; Pasternak, G. W. The molecular behavioural pharmacology of the orphanin FQ/nociceptin peptide and receptor family. *Pharmacol. Rev.* **2001**, *53*, 381−415.

(8) Becker, J. A. J.; Wallace, A.; Garzon, A.; Ingallinella, P.; Bianchi, E.; Cortese, R.; Simonin, F.; Kieffer, B. L.; Pessi, A. Ligands for κ-opioid and ORL1 receptors identified from a conformationally constrained peptide combinatorial library. *J. Biol. Chem.* **1999**, *274*, 27513−27522.

(9) Halab, L.; Becker, J. A. J.; Darula, Z.; Tourwé, D.; Kieffer, B. L.; Simonin, F.; Lubell, W. D. Probing Opioid Receptor Interactions with Azacycloalkane Amino Acids. Synthesis of a potent and selective ORL1 antagonist. *J. Med. Chem.* **2002**, *45*, 5353−5357.

(10) Rudinger, J. In *Drug Design*; Ariëns E. J., Ed.; Academic: New York, 1971; Vol II, pp 319−419.

(11) Gosselin, F.; Lubell, W. D. Rigid Dipeptide Surrogates: Syntheses of Enantiopure Quinolizidinone and Pyrroloazepinone Amino Acids from a Common Diaminodicarboxylate Precursor. *J. Org. Chem.* **2000**, *65*, 2163−2171.
(12) Merrifield, R. B. Solid-Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide. *J. Am. Chem. Soc.* **1963**, *85*, 2149−2154.
(13) Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. Urethane Protected Derivatives of 1-Guanylpyrazole for the Mild and Efficient Preparation of Guanidines. *Tetrahedron Lett.* **1993**, *34*, 3389−3392.
(14) Bernatowicz, M. S.; Wu, Y.; Matsueda, G. R. 1H−Pyrazole-1-carboxamidine Hydrochloride: An Attractive Reagent for Guanylation of Amines and Its Application to Peptide Synthesis. *J. Org. Chem.* **1992**, *57*, 2497−2502.
(15) Kaiser, E.; Colescott, R. L.; Bossinger, C. D.; Cook, P. L. Color test for Detection of Free Terminal Amino Groups in the Solid-Phase Synthesis of Peptides. *Anal. Biochem.* **1970**, *34*, 595−598.

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