Journal of Peptide Science
J. Pept. Sci. 2007; **13**: 672–678
Published online 22 August 2007 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/psc.900

JOURNAL OF PEPTIDE SCIENCE

Opioid receptor-like 1 (ORL1) receptor binding and the biological properties of Ac-Arg-Tyr-Tyr-Arg-Ile-Arg-NH_2 and its analogs

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Received 20 October 2006; *Revised 30 May 2007*; *Accepted 4 June 2007*

Abstract: Hexapeptides such as Ac-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 and Ac-Arg-Tyr-Tyr-Arg-Trp-Arg-NH_2 have been isolated from a combinatorial peptide library as small peptide ligands for the opioid peptide-like 1 (ORL1) receptor. To investigate the detailed structural requirements of hexapeptides, 25 analogs of these hexapeptides, based on the novel analog Ac-Arg-Tyr-Tyr-Arg-Ile-Arg-NH_2 (1), were synthesized and tested for their ORL1 receptor affinity and agonist/antagonist activity on mouse vas deferens (MVD) tissues. Analog 1 and its Cit^6-analog (10) were found to possess high affinity to the ORL1 receptor, comparable to that of nociceptin/orphanin FQ, and exhibited potent antagonist activity (pA_2 values of 7.77 for 1 and 7.51 for 10, which are higher than that of [$NPhe^1$]nociceptin(1–13)-NH_2 (6.90) on MVD assay. It was also found that the amino acid residue in position 5 plays a key role in agonist/antagonist activity, i.e. an L-configuration aliphatic amino acid is required for potent antagonist activity, while a nonchiral or D-configuration residue produces potent agonist activity. These lines of evidence may provide insight into the mechanisms controlling agonist/antagonist switching in the ORL1 receptor, and may also serve to help developing more potent ORL1 agonists and antagonists. Copyright © 2007 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: opioid receptor-like 1 receptor; nociceptin; biological assay; mouse vas deferens

INTRODUCTION

The opioid receptor-like 1 (ORL1) receptor is a member of the G-protein coupled receptor family. The primary structure of the ORL1 receptor is very similar to those of opioid receptors, but classic opioid ligands cannot bind to the ORL1 receptor [1]. Nociceptin/orphanin FQ (NC) is an endogenous ligand for the ORL1 receptor isolated from the brain [2,3]. In rats, NC supraspinally suppresses opioid-mediated analgesia, but acts spinally as an analgesic. NC also suppresses spatial learning, impairs motor performance, induces the release of pituitary hormones, and induces feeding [1,4,5].

To date, several selective peptide ligands for the ORL1 receptor have been reported. For example, [$Nphe^1$]NC(1–13) amide competitively antagonizes the *in vitro* activity of NC on electrically evoked contractions in several isolated tissues, and forskolin-stimulated cAMP accumulation in Chinese hamster ovary (CHO) cells expressing the human ORL1 receptor [6]. Dooley *et al.* screened a synthetic peptide combinatorial library, and isolated and characterized several hexapeptides, including acetyl-Arg-Tyr-Tyr-Arg-Trp-Arg-NH_2 (Ac-RYYRWR-NH_2), acetyl-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 (Ac-RYYRWK-NH_2), and acetyl-Arg-Tyr-Tyr-Arg-Ile-Lys-NH_2 (Ac-RYYRIK-NH_2), that have high affinity for the ORL1 receptor [7]. In particular, the hexapeptide Ac-RYYRIK-NH_2 was shown to competitively inhibit NC-induced GTPγS binding to the ORL1 receptor [8,9].

These hexapeptides contain three positively charged basic residues similar to NC, which has four positively charged amino acids at the *C*-terminus. In an Ala-scanning study, two Arg residues, at positions 1 and 4, were found to be important for binding to the ORL1 receptor [10]. The two Arg residues would be expected to favorably interact with an acidic site in the second extracellular loop of the ORL1 receptor [2]. Recently, Judd *et al.* reported that pentanoyl-RYYRWR-NH_2 showed competitive antagonism against NC-induced GTPγS binding, without inducing GTP-binding itself [11].

According to the reports of Dooley *et al.* [7], each of the hexapeptides isolated from the peptide library exhibited high affinity for the ORL1 receptor, and potent agonist activity in the stimulation of GTPγS binding and inhibition of forskolin-stimulated cAMP accumulation in CHO cells transfected with the ORL1 receptor. These small peptides however behaved as partial agonists (lower maximal inhibition) in the inhibition of cAMP accumulation assay. Ac-RYYRWR-NH_2 showed the highest maximal inhibition of cAMP accumulation (75%), which is comparable to that of NC (84%), whereas Ac-RYYRWK-NH_2 showed significantly lower maximal inhibition (58%). These results suggest that the basic amino acid Arg in position 6 is superior to Lys for full NC agonist activity. In the present study,

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Table 1 Physicochemical data for synthetic peptides

No.	Peptides	$[\alpha]_D$[a] (°)	TLC[b]		FAB- MS[c]		Amino acid analysis[d]			
			Rf (A)	Rf (B)	Calcd	Found	Arg	Tyr	Ile	Other
1	Ac-RYYRIR-NH_2	−23.2	0.22	0.72	966.55	967	3.00	2.23	1.04	—
2	[Ala^1]	−29.7	0.32	0.80	881.49	882	2.00	2.27	1.04	1.04: Ala
3	[Ala^2]	−32.4	0.25	0.65	874.53	875	3.00	0.97	1.05	1.04: Ala
4	[Ala^3]	−39.8	0.21	0.76	874.53	875	3.00	1.10	1.04	1.05: Ala
5	[Ala^4]	−29.2	0.28	0.79	881.49	882	2.00	2.21	1.08	1.04: Ala
6	[Ala^5]	−24.4	0.27	0.40	924.50	925	3.00	2.16	—	1.08: Ala
7	[Ala^6]	−34.1	0.30	0.79	881.49	882	2.00	2.26	1.08	1.08: Ala
8	[Cit^1]	−25.0	0.25	0.79	967.54	968	2.00	2.24	1.06	1.06: Cit
9	[Cit^4]	−27.0	0.25	0.81	967.54	968	2.00	2.17	1.07	1.06: Cit
10	[Cit^6]	−24.2	0.25	0.79	967.54	968	2.00	2.18	1.07	1.07: Cit
11	[Val^5]	−21.2	0.17	0.65	952.54	953	3.00	2.23	—	1.02: Val
12	[Leu^5]	−21.6	0.23	0.84	966.55	967	3.00	2.22	—	1.37: Leu
13	[Nle^5]	−20.6	0.23	0.83	966.55	967	3.00	3.67[e]	—	+: Nle
14	[Tle^5]	−19.6	0.01	0.59	966.55	968	3.00	2.21	—	0.83: Tle
15	[Phe^5]	−18.2	0.24	0.72	1000.5	1001	3.00	2.22	—	0.97: Phe
16	[Tyr^5]	−16.3	0.21	0.81	1016.5	1017	3.00	3.57	—	—
17	[Tic^5]	−27.6	0.17	0.65	1012.5	1013	3.00	2.17	—	—
18	[Arg^5]	−16.9	0.06	0.72	1009.6	1010	4.00	2.45	—	—
19	[Aib^5]	−14.8	0.09	0.80	938.52	940	3.00	2.40	—	+: Aib
20	[D-Val^5]	−6.9	0.17	0.65	952.54	953	3.00	2.22	—	1.02: D-Val
21	[D-Leu^5]	−3.3	0.25	0.79	966.55	967	3.00	1.88	—	1.06: D-Leu
22	[D-Phe^5]	−17.3	0.22	0.73	1000.5	1001	3.00	2.14	—	1.02: D-Phe
23	[D-Tyr^5]	−20.0	0.21	0.73	1016.5	1017	3.00	3.73	—	—
24	[D-Trp^5]	−17.7	0.15	0.63	1039.6	1040	3.00	2.23	—	—
25	[D-Arg^5]	−8.9	0.27	0.78	1009.6	1010	4.00	1.72	—	—

[a] Optical rotation in 1% AcOH ($c = 0.5$) at 20 °C.
[b] Solvent systems: (A) *n*-BuOH/AcOH/H_2O (4/1/5, upper phase), (B) *n*-BuOH/AcOH/pyridine/H_2O (15/10/3/12).
[c] $[M + H]^+$.
[d] After hydrolysis with 6 N HCl at 110 °C for 24 h.
[e] Tyr + Nle.

we thus employed a novel hexapeptide analog, acetyl-Arg-Tyr-Tyr-Arg-Ile-Arg-NH_2 (Ac-RYYRIR-NH_2) (**1**), as a lead compound and synthesized 24 analogs, focusing mainly on the role of Arg residues in positions 1, 4, and 6 and various residues in position 5 (Table 1). The synthetic analogs were tested for ORL1 receptor affinity and for their agonist/antagonist potencies in the mouse vas deferens (MVD).

RESULTS AND DISCUSSION

Peptide Synthesis

Peptides **1**–**25** (Table 1) were prepared by solid phase synthesis using Fmoc chemistry, as described previously [12]. Peptides were assembled on Fmoc-NH SAL resin (0.61 mmol/g, 0.12 g) using Fmoc-amino acids in the presence of HBTU and HOBt as coupling reagents for 1 h for each coupling. Acetylation of the *N*-terminus was carried out by treatment with acetic anhydride and Et_3N (each 10 equiv.) for 30 min. All synthetic peptides gave satisfactory amino acid analytical and fast ion bombardment-mass spectrometry (FAB-MS) data, as summarized in Table 1.

ORL1 Receptor Affinity of Analogs 1–10

ORL1 receptor affinity of novel hexapeptide analog **1** and its analogs, in which each amino acid residue was substituted with Ala, is shown in Table 2. Analog **1** showed high affinity for the ORL1 receptor, comparable to those of NC and the previously reported hexapeptides Ac-RYYRWR-NH_2 and Ac-RYYRIK-NH_2 [7]. All Ala-scanning peptide analogs (**2**–**7**) showed reduced affinity. In particular, substitution of the three *N*-terminal residues Arg-Tyr-Tyr (**2**–**4**) resulted in a marked drop in affinity ($IC_{50} > 416$ nM), whereas modification of the three *C*-terminal residues Arg-Ile-Arg (**5**–**7**) led to analogs with moderately reduced affinity ($IC_{50} < 35.5$ nM). Only analog **7** showed a relatively high affinity, with an IC_{50} value of 3.37 nM, thus suggesting that the *C*-terminal Arg^6 is tolerant to

DOI: 10.1002/psc

Table 2 Binding affinities of nociceptin and synthetic hexapeptide analogs to human ORL1 receptor

No.	Peptide	$IC_{50} \pm SE$ (nM)[a]
	NC	0.436 ± 0.079
—	[Nphe^1]NC(1–13)NH_2	32.2 ± 5.9
—	Ac-RYYRIK-NH_2	0.592 ± 0.070
—	Ac-RYYRWR-NH_2	0.347 ± 0.090
1	Ac-RYYRIR-NH_2	0.664 ± 0.027
2	[Ala^1]	2363 ± 453
3	[Ala^2]	591 ± 166
4	[Ala^3]	416 ± 93
5	[Ala^4]	35.5 ± 3.7
6	[Ala^5]	30.5 ± 0.2
7	[Ala^6]	3.37 ± 0.65
8	[Cit^1]	3899 ± 777
9	[Cit^4]	12.9 ± 5.3
10	[Cit^6]	5.47 ± 2.53

[a] Data are means ± SE of 4–6 experiments.

Ala substitution. The *N*-terminal Arg residue appears to be the most critical for ORL1 receptor binding. These results are consistent with previously reported structure–activity relationship (SAR) studies of Ac-RYYRIK-NH_2 [10,11].

In order to assess the contribution of basicity to activity, the three Arg residues were substituted with citrulline (Cit) (**8, 9**, and **10**). Cit-substitution of Arg^1 resulted in a marked (over 5800-fold) decrease in ORL1 receptor affinity, similar to that observed upon substitution with Ala. In contrast, substitution at position 4 or 6 caused a moderate reduction in binding affinity (only 10.9-fold and 4.0-fold, respectively). These results suggest that the guanidino basicity of Arg^1 is critically important, but the basicity of Arg^4 and Arg^6 is not essential and plays only a supportive role in ORL1 binding.

MVD Activities of Analogs 1–10

MVD assay is convenient for evaluating NC activity and for making distinctions between NC agonism and antagonism [7,13–15]. The MVD preparation contains a functional site (ORL1 receptor) which is sensitive to NC and is not antagonized by compounds that interact with the opioid receptors [13–15]. In the present assay system, NC dose-dependently inhibited electrically evoked contraction of MVD tissue, with an IC_{50} value of 7.24 nM (maximal inhibition, 72%). As shown in Figure 1(a), the agonist potency of the hexapeptide analogs was evaluated at 10 μM because most analogs have relatively low activity on MVD assay. Analog **1** showed weak agonist activity (27% inhibition), similar to those of Ac-RYYRWR-NH_2 (21%) and Ac-RYYRIK-NH_2 (28%). Analog **2–7** also showed weak agonist activity, ranging from 13 to 24% inhibition. Cit-substituted analogs showed little or no agonist activity (less than 10% inhibition), which is apparently inconsistent with the observed ORL1 receptor affinities.

In order to screen antagonist activity, the percentage recovery of tissue contraction elicited by NC (40 nM) was determined by MVD assay (Figure 1(b)). Ac-RYYRWR-NH_2 showed very low antagonism, whereas Ac-RYYRIK-NH_2 and **1** showed potent antagonism with recovery rates of 70 and 52%, respectively. Interestingly, these three peptides have similar, high ORL1 receptor affinity; this strongly suggests that the hydrophobic, aliphatic side-chain residue of Ile^5 is important for antagonistic activity. Ala-substituted analogs **2–6** showed little or no antagonism, with recovery rates of less than 20%. Only the Ala^6 analog (**7**) retained potent antagonist activity, which suggests a weaker contribution of the Arg^6 residue to the antagonist potency. Among the Cit-substituted analogs, **9** and **10** showed potent antagonist activity (43–57%), thereby suggesting that the basicity of the guanidino function in Arg^4 and Arg^6 is not critical for antagonist activity. Figure 2 shows the antagonism of Ac-RYYRIK-NH_2, **1** and **10**, which have potent antagonism against NC in the MVD. Their antagonist activities were dose dependent and shifted the NC dose–response curve to the right. It is noteworthy that **1** and **10** have potent antagonist activity, greater than [Nphe^1]NC(1–13)-NH_2 (Figure 2(d)). Agonist and antagonist potencies of compounds **1**, **10**, and **24** were compared with those of Ac-RYYRIK-NH_2 and [NPhe^1]NC(1–13)-NH_2 in Table 3. The pA_2 values of Ac-RYYRIK-NH_2, **1**, and **10** were 7.98, 7.77, and 7.51, respectively, indicating that these hexapeptides have 4–12-fold higher antagonist activities than [NPhe^1]NC(1–13)-NH_2 (pA_2: 6.90), which is known to be a pure peptidic NC antagonist [6].

Table 3 ORL1 potency profiles of major peptide analogs

Peptides		MVD	
	ORL1 binding IC_{50} (nM)	Agonism IC_{50} (nM)	Antagonism pA_2
Ac-RYYRIK-NH_2	0.592	>10 000 (28%)[a]	7.98
Ac-RYYRWR-NH_2	0.347	>10 000 (21%)[a]	—
1 : Ac-RYYRIR-NH_2	0.664	>10 000 (27%)[a]	7.77
10 : Ac-RYYRI-Cit-NH_2	5.47	>10 000 (12%)[a]	7.51
24: Ac-RYYR-D-Trp-R-NH_2	12.4	4247 (53%)[a]	—
[Nphe^1]NC(1–13)-NH_2	32.2	>10 000	6.90
Nociceptin	0.436	7.24 (72%)[a]	—

[a] Percentages in parenthesis show the peak effects at a dose of 10 μM.

DOI: 10.1002/psc

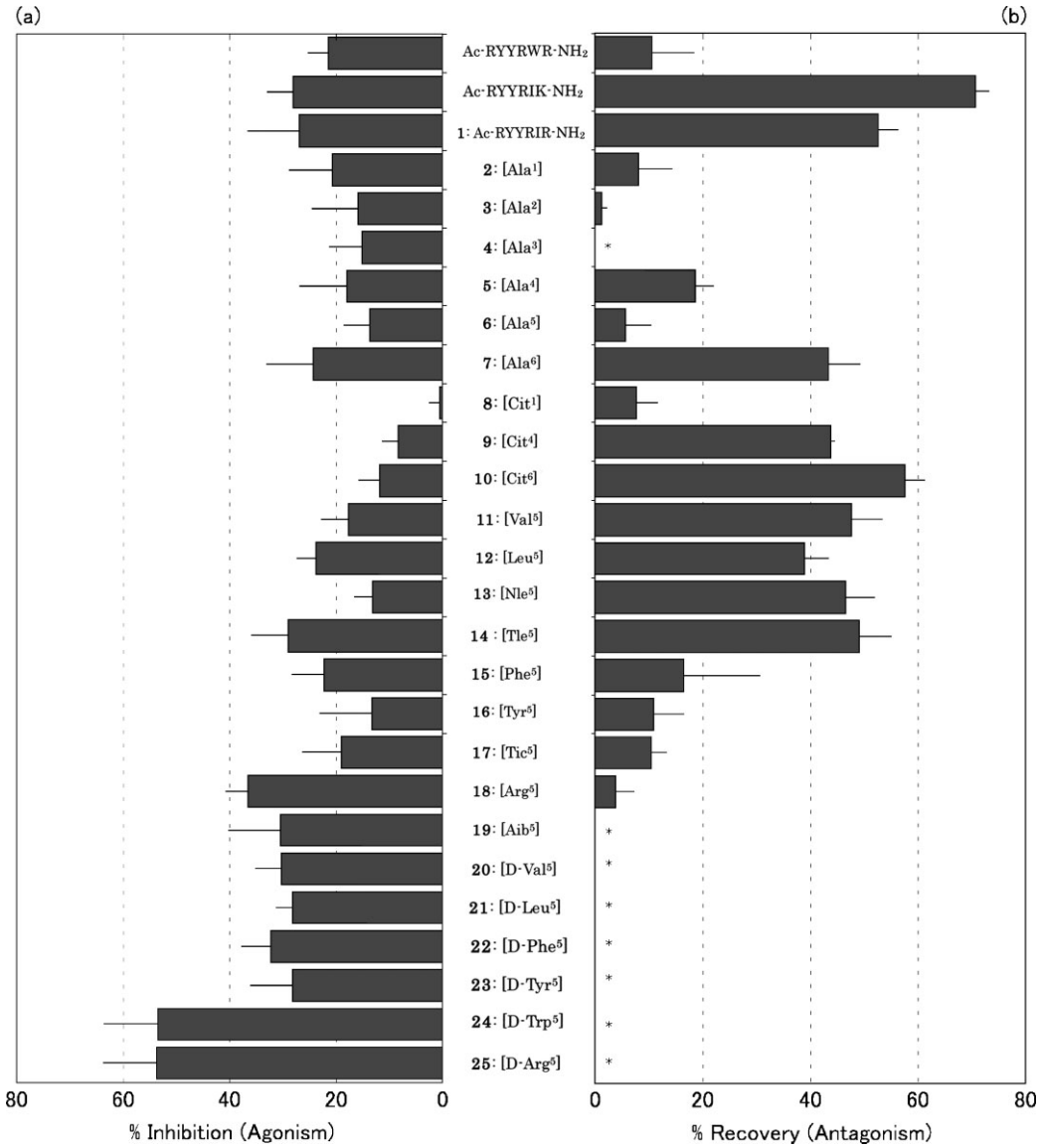

Figure 1 Agonistic and antagonistic potencies of hexapeptide analogs on MVD. (a) Inhibition of electrically induced MVD contraction by analogs at 10 μM. (b) Recovery of tissue contraction against 40 nM NC by analogs at 1 μM. Data are means ± SEM of $n > 3$ experiments. NC (40 nM) produced about 70% inhibition of contraction under these conditions. Asterisks indicate no recovery against inhibition of contraction by NC.

MVD Activity of Analogs with Amino Acid Replacements in Position 5 (11–25)

The sensitivity of the side chain in position 5 towards antagonist activity led us to further investigate the role of this residue by substitution with various amino acids. As shown in Figure 1, introduction of aliphatic residues [Val (**11**), Leu (**12**), Nle (**13**) and Tle (**14**)] in place of Ile^5 produced potent antagonism (40–50% recovery) with weak agonism (less than 30% inhibition). These results are consistent with those obtained with the Ile^5 analogs **1** and Ac-RYYRIK-NH_2. In contrast, introducing aromatic residues [Phe (**15**), Tyr (**16**), and Tic (**17**)] reduced antagonist activity, similar to that observed with the Trp^5 analog Ac-RYYRWR-NH_2. Carra' *et al.* reported similar properties for the peptide Ac-Arg-Tyr-Tyr-Arg-Trp-Lys-NH_2 [16]. These results strongly suggest that the L-configuration of aliphatic and hydrophobic side chains, but not aromatic side chains, at position 5 is needed to elicit potent antagonist activity. It is noteworthy that introduction of Arg^5 (**18**) produced potent agonist activity with only appreciable antagonism. Furthermore, lack of chirality (**19**) or introduction of D-isomers (**20**–**25**) at this position led to analogs with no antagonist activity but potent agonist activity. Among the hexapeptide analogs tested, analogs **24** and **25** showed the highest agonist potency (about 54%).

The results of the present SAR study of position 5 are in accordance with a recent study concerning

DOI: 10.1002/psc

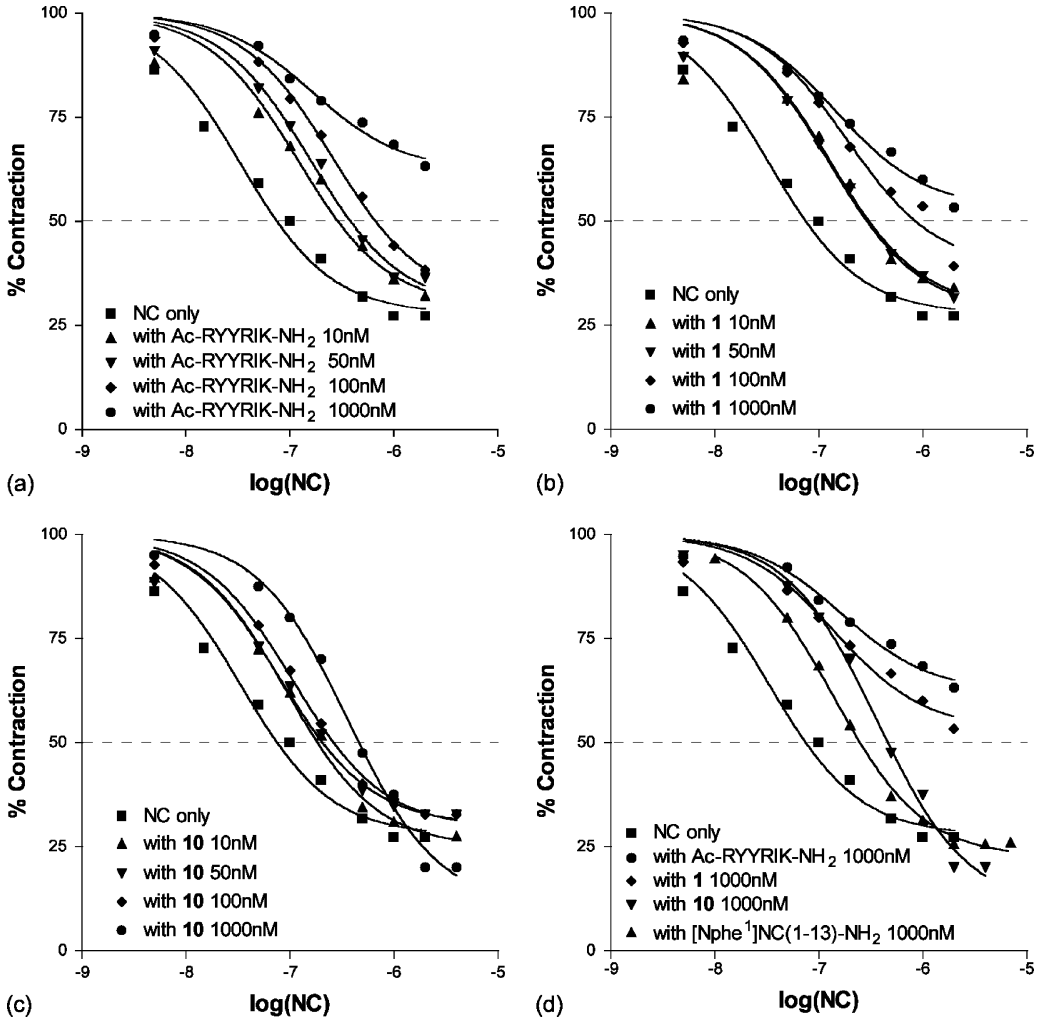

Figure 2 Rightward shift in dose–response curves of NC by Ac-RYYRIK-NH_2 (a), and hexapeptide analogs **1** (b) and **10** (c) at 10, 50, 100, and 1000 nM on MVD assay. Ac-RYYRIK-NH_2, **1**, and **10** were added to the organ bath 15 min prior to addition of NC. (d) Comparison of hexapeptide analogs with [$Nphe^1$]NC(1–13)-NH_2 at 1000 nM.

complement factor 5a (C5a) receptor, a member of the G-protein coupled receptor family; substitution of Trp^5 with Cha in a C5a receptor ligand, NMePhe-Lys-Pro-D-Cha-Trp-D-Arg-OH, converted the hexapeptide from an antagonist to an agonist [17]. These results suggest that the amino acid residue at position 5 in C5a-related hexapeptide analogs interacts with two residues, Ile^{116} and Val^{286}, of the C5a receptor, and acts as an activation switch [18].

Recently, Meunier *et al.* analyzed the interaction of the ORL1 receptor with the photo-labile ligands [Bpa^{10}, ^{125}I-Tyr^{14}]NC and Ac-Arg-Bpa-(^{125}I-Tyr)-Arg-Trp-Arg-NH_2 [19–23]. [Bpa^{10}, ^{125}I-Tyr^{14}]NC labeled the ORL1 receptor sequence (296–302), which comprises the *C*-terminus of the third extracellular loop and the *N*-terminus of transmembrane helix VII. Molecular modeling suggested that hexapeptides and NC interact with the ORL1 receptor in different ways, but their binding sites are thought to overlap [17,22]. Moreover, they identified Gln^{286} as a crucial switch residue at the membrane interface involved in the preferential stabilization of the active form of the receptor [23]. Taken together with these results, one could speculate that the side chain at position 5 in Ac-RYYRWR-NH_2 acts as an activation switch on ORL1 receptors. Thus, the results of the present study provide insight into this agonist/antagonist switching model. In addition, it seems likely that analogs with pure agonist activity (**24**–**25**) would be good templates for developing more potent ORL1 agonists.

CONCLUSIONS

The present structure–activity study revealed that the *N*-terminal Arg-Tyr-Tyr sequence is critical for ORL1 receptor binding and antagonist activity on MVD assay. Ac-RYYRIR-NH_2 (**1**) and its Cit^6 analog (**10**) were found to possess strong affinity for the ORL1 receptor, comparable to that of NC, and exhibited potent antagonist activity, superior to that

DOI: 10.1002/psc

of [NPhe^1]NC(1–13)-NH_2, on MVD assay. It was also found that the amino acid residue in position 5 played a key role for agonist/antagonist activities, i.e. an L-configuration aliphatic amino acid is required for potent antagonist activity, while a nonchiral or D-configuration residue produced potent agonist activity. These lines of evidence provide insight into the mechanisms controlling agonist/antagonist switching in the ORL1 receptor, and may also assist in developing pure ORL1 agonists and antagonists.

METHODS

Analytical Characterization

Optical rotation was measured in 1% acetic acid ($c = 0.5$) at 20 °C using a 10-cm path length cell in a JASCO DIP-40 polarimeter. TLC was carried out on silica gel plates (Merck, Kiesel gel 60, 5 × 10 cm) with 1-butanol : acetic acid : H_2O = (4 : 1 : 5, upper layer) as the solvent system. FAB-MS was conducted using a JEOL JMS-DX303 mass spectrometer. Amino acid analysis was performed on a HITACHI L-8500 amino acid analyzer on samples hydrolyzed in 6 N HCl at 110 °C for 24 h. Analyses by HPLC were performed on a Wakosil-II 5C18 AR (4 × 150 mm) column using the following solvent system: A, 0.06% trifluoroacetic acid; B, 80% acetonitrile containing 0.06% trifluoroacetic acid. A linear elution gradient from 10% B to 50% B over 40 min at a flow rate of 1.0 ml/min was used, and the eluent was monitored at 220 nm.

Solid Phase Synthesis of Peptides

All peptides were prepared by solid phase synthesis starting with Fmoc-NH SAL resin (0.61 mmol/g, 0.12g) using a SHIMADZU PSSM-8 peptide synthesizer and following the manufacturer's protocol. Fmoc-amino acids (Watanabe Chem. Ind., Ltd., Hiroshima, Japan) were used with the following side-chain protecting groups: Bu^t for Tyr; Boc for Trp; and ω-2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl for Arg. 2-(1*H*-Benzotriazole-1-yl)1,1,3,3-tetramethyluronium hexafluorophosphate and 1-hydroxybenzotirazole were used as coupling reagents; each coupling was conducted for 1 h. The *N*-terminus was acetylated with acetate anhydride and Et_3N (each 10 equiv.) for 30 min. Peptides were simultaneously deprotected and cleaved from the resin with 5% phenol/trifluoroacetic acid at room temperature for 3 h, and were then purified by medium-pressure HPLC using a Develosil LOP ODS column (30 × 300 mm) eluted with a linear gradient of 0.06% trifluoroacetic acid and 80% acetonitrile containing 0.06% trifluoroacetic acid. Peptide purity was verified by TLC and analytical HPLC (>95%). Amino acid analysis of the peptide acid hydrolysates gave the expected amino acid ratios.

Receptor Binding Assay for the ORL1 Receptor

HEK 293 cells expressing the human ORL1 receptor were used for the binding assay, as described previously [24]. [^{3}H]NC binding was conducted as follows: HEK 293 cell membranes (10.6 μg of protein), 0.2 nM [^{3}H]NC (5.62 TBq/mmol, Amersham), and the binding peptide were incubated in 50 mM HEPES buffer (pH 7.4) containing 1 mM EDTA and 10 mM $MgCl_2$ (total volume 100 μl), for 1 h at 25 °C in a siliconized tube. After incubation, each reaction mixture was filtered through a glass filter (Whatman, GF/B) and washed three times with 2 ml of ice-cold HEPES buffer. Filter-bound radioactivity was counted using a Beckman 9800 liquid scintillation counter. IC_{50} values were determined from log dose–response curves.

MVD Assay

ddY Mice (SLC, Shizuoka, Japan) weighing 25–30 g were used. MVD were removed as described by Hughes *et al.* [25] and mounted in a 10 ml organ bath in Krebs solution (Mg^{2+}-free) containing 0.1 mM ascorbic acid and 0.027 mM EDTA 4 Na. The Krebs solution was maintained at 37 °C and was continuously perfused with 95% O_2/5% CO_2. Tissue was stimulated transmurally with successive 'trains' of rectilinear pulses administered at 20 s intervals, with each train consisting of seven 1-ms stimuli at 10 ms intervals. Because reversal of the inhibited contraction height could not be observed with time, peptidase inhibitors were not used. To quantify agonist potency, percentage inhibition was calculated by: %Inhibition = $(A - B)/A \times 100$, where A is contraction before treatment and B is MVD contraction after treatment. The agonist potency of hexapeptide analogs was evaluated with a single dose of 10 μM because low concentrations of hexapeptide analogs did not have sufficient agonistic effects in the MVD tissue (Figure 1).

The antagonistic effect of the analogs was quantified by %Recovery, calculated as: %Recovery = $(C - B)/(A - B) \times 100$, where A is the MVD contraction after peptide treatment, B is the attenuated contraction by NC, and C is the original contraction. To quantify the antagonist potency of Ac-RYYRIK-NH_2, **1**, and **10**, the pA_2 value causing a twofold rightward shift of the dose–response curve of NC was determined. These peptides were added to the organ bath 15 min prior to addition of NC.

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DOI: 10.1002/psc