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ORL1 and opioid receptor preferences of nociceptin and dynorphin A analogues with Dmp substituted for N-terminal aromatic residues☆

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Abstract—Nociceptin (NOC) and dynorphin A (DYN) analogues containing 2′,6′-dimethylphenylalanine (Dmp) in place of Phe or Tyr in position 1 and/or 4 were synthesized and their metabolic stability and receptor-binding properties were investigated. [Dmp^1]NOC(1–13)-NH_2 (**1**) possessed high ORL1 receptor affinity comparable to that of the parent peptide with substantially improved affinities for κ-, μ-, and δ-opioid receptors. However, Dmp^4 substitution of NOC peptide (**2**) reduced ORL1 receptor affinity. [Dmp^1]DYN(1–13)-NH_2 (**4**) and its Dmp^4 analogue (**5**) possessed a 3-fold greater κ-opioid receptor affinity and improved κ-receptor selectivity compared to the parent peptide. Analogue **4** however exhibited an unexpectedly low in vitro bioactivity (GPI assay), suggesting, the phenolic hydroxyl group at the N-terminal residue in DYN peptide is extremely important for activation of the κ-opioid receptor. Analogue **5** possessed an improved κ-opioid receptor selectivity with an IC_{50} ratio of 1(κ)/509(μ)/211598(δ); thus, this peptide may serve as a highly selective κ-receptor agonist for pharmacological study. Dmp^1 substitution in both the NOC and DYN peptides improved metabolic stability toward these peptides, while Dmp^4 substitution provided no additional metabolic stability.

1. Introduction

Nociceptin[2] or orphanin FQ[3] (NOC: Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) is a ligand for the opioid receptor-like-1 (ORL-1) receptor, which structurally resembles dynorphin A (DYN: Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln), the endogenous agonist of the κ-opioid receptor. Despite the sequence homology, NOC and DYN have different pharmacological profiles.[2–5] Structural similarities between the two peptides include the N-terminal tetrapeptide sequence, Phe-Gly-Gly-Phe (NOC) and Tyr-Gly-Gly-Phe (DYN), and the existence of basic residues, although with different distributions, at the C-terminus. Recent structure–activity studies revealed that two aromatic amino acids at the N-terminus, Phe^{1,4} in NOC[6–9], and Tyr^1 and Phe^4 in DYN,[10–12] are required for receptor binding and/or biological activity and are needed for discriminating between them. Furthermore, N-terminal Phe^1 and the C-terminal half of NOC may serve as a domain that prevents binding to opioid receptors.[13] We recently demonstrated that an artificial amino acid, 2′,6′-dimethylphenylalanine (Dmp), is an effective surrogate not only for the Phe^3 but also for the Tyr^1 residue in some opioid peptides[14–17] and produced analogues with extremely potent opioid activities.[15,16] Therefore, evaluation and comparison of the effect of Dmp replacement of N-terminal aromatic residues in both peptides are of interest. Here, we describe the synthesis of NOC and DYN analogues containing Dmp in position 1 and/or 4 of the template structures, NOC(1–13)-NH_2 and DYN(1–13)-NH_2, which represent the minimum sequence required to maintain high receptor affinity[8,9] and receptor-binding profiles for ORL1 and opioid receptors. In addition, metabolic stability of the Dmp analogues was investigated to determine the effects of Dmp substitution (see Table 1).

Keywords: 2′,6′-Dimethylphenylalanine; Nociceptin analogue; Dynorphin A analogue; Receptor-binding property.
☆ See ref. 1.
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Table 1. Analytical data of Dmp-containing nociceptin (NOC) and dynorphin A (DYN) analogues

Peptide	$[\alpha]_D$[a] (°)	HPLC[b] (t_R, min)	ESI-MS ($[M+H]^{2+}$)		Amino acid ratio										
			Found	Theoretical	Thr	Ser	Gly	Ala	Ile	Leu	Tyr	Phe	Lys	Arg	Pro	Dmp[c]
[Dmp^1]NOC(1–13)NH_2 (**1**)	−6.0	16.01	705.85	705.409	0.92	0.87	2.95	2.00				1.00	1.96	2.19		0.72
[Dmp^4]NOC(1–13)NH_2 (**2**)	−52.5	15.39	705.80	705.409	0.93	0.96	3.01	2.00				1.09	2.01	1.97		0.65
[$Dmp^{1,4}$]NOC(1–13)NH_2 (**3**)	−220.0	18.73	719.90	719.425	0.99	0.98	2.78	2.00					1.93	1.68		1.27
[Dmp^1]DYN(1–13)NH_2 (**4**)	−36.0	24.67	808.61	808.025			2.07		1.00	2.13		1.00	2.02	2.94	1.10	0.82
[Dmp^4]DYN(1–13)-NH_2 (**5**)	−11.0	23.39	816.50	816.022			1.96		1.00	2.16	0.90		2.02	2.94	0.89	0.75
[$Dmp^{1,4}$]DYN(1–13)-NH_2 (**6**)	−6.5	27.37	822.78	822.040			2.11		1.00	2.14			1.99	2.85	0.85	1.35

[a] Measured in 1% AcOH ($c = 0.5$) at 20 °C.
[b] See Experimental for conditions.
[c] Eluted at position of His using an analyzer and calculated as His.

2. Results and discussion

All synthetic analogues were tested for their binding affinity to membrane preparations derived from HEK-cells expressing the human ORL1 receptor and to opioid receptors derived from rat (μ and δ receptors) and guinea pig (κ receptor) brains. Receptor-binding results of NOC analogues are presented in Table 2 with those of reference peptides. NOC(1–13)-NH_2 possessed high ORL1 receptor affinity and poor affinity for κ, μ, and δ-opioid receptors. Dmp^1-NOC peptide (**1**) showed high ORL1 receptor affinity comparable to that of that of the parent peptide NOC(1–13)-NH_2. The high ORL1 receptor affinity of **1** agrees with recent findings that the Phe^1 can be replaced with aromatic or aliphatic amino acids without loss of activity.[9] Interestingly, this analogue exhibited improved affinity toward the three opioid receptors, with 5- and 16-fold improved affinities for κ and μ receptors, respectively, perhaps due to the effect of Dmp^1, which can mimic Tyr^1 in some opioid peptides without a substantial decrease in receptor affinity.[16,17] The Dmp^4-NOC analogue (**2**), however, showed a 70-fold decrease in ORL1 affinity without significant changes in affinity toward the opiate receptors. These results indicate the critical importance of the Phe^4 residue for interactions with the ORL1 receptor. Similar studies have suggested that aromaticity and specific structures are required in position 4 of the NOC peptide for ORL1 receptor binding and biological activity.[8,9] A Dmp residue at this position appears to influence conformation of the NOC peptide by 2′,6′-dimethylation of the Dmp side-chain aromatic nucleus. According to the proposed model of the ORL1 receptor and its complex with NOC, the Phe^4 residue of NOC located at a hydrophobic pocket in a cavity formed by TM helices 3, 5, 6, and 7, and the Phe^4 side chain interact with Phe^{220} of the ORL1 receptor by an edge-face interaction.[18] Two methyl groups on Dmp^4 may interfere with the interaction with the receptor due to a reduction in conformational flexibility and/or enhanced lipophilicity Dmp substitutions in positions 1 and 4 afforded **3** with a moderate decrease in affinity toward the ORL1 and opioid receptors, indicating that a Dmp residue in position 1 can compensate for the decrease caused by the Dmp^4 substitution.

Table 3 shows receptor binding results of a series of DYN(1–13)-NH_2 analogues. The parent peptide showed high affinity toward κ-, μ-, and δ-opioid receptors with κ-receptor selectivity, that is, IC_{50} ratio 1/15.6/40.1 and considerably low affinity toward the ORL1 receptor, which is consistent with an observation of intact DYN.[5,6,13] Interestingly, Dmp^1 or Dmp^4 replacement afforded **4** or **5** with very high κ opioid receptor affinity (ca. 3-fold higher than that of the parent peptide) and a significantly improved κ-receptor selectivity [i.e., IC_{50} ratios: **4**, 1(κ)/293(μ)/180(δ); **5**, 1(κ)/509(μ)/21159(δ) vs DYN(1–13)-NH_2, 1(κ)/15.6(μ)/40.1(δ)]. The results of analogue **4**, which lacks the N-terminal side chain phenolic hydroxyl group essential in the opioid peptide, support our recent finding that Dmp is an effective surrogate for the Tyr^1 residue in opioid peptides.[16,17] Note that **4** has 3-fold greater affinity toward the

Table 2. Receptor-binding affinity of NOC analogues for ORL1 and opioid receptors

Peptides	IC_{50} ± SEM (nM)			
	ORL1 receptor	Opioid receptor		
	[^{3}H]NOC[a]	[^{3}H]U-69593 (κ)[b]	[^{3}H]DAMGO (μ)[c]	[^{3}H]DLT (δ)[c]
NOC	0.151 ± 0.058	643 ± 218	1540 ± 601	>10,000
NOC (1–13)-NH_2	0.743 ± 0.125	193 ± 54	319 ± 88	>10,000
[Dmp^1]NOC (1–13)-NH_2 (**1**)	0.814 ± 0.090	38.8 ± 16.7	25.0 ± 6.5	292 ± 61
[Dmp^4]NOC (1–13)-NH_2 (**2**)	51.6 ± 12.9	299 ± 63	629 ± 433	>10,000
[Dmp^{1,4}]NOC (1–13)-NH_2 (**3**)	21.3 ± 3.20	100 ± 29	56.8 ± 12.3	3407 ± 990

[a] Using cell membrane expressing human ORL1 receptor in Hek-293 cells.
[b] Using guinea pig brain homogenate.
[c] Using rat brain homogenate.

Table 3. Receptor-binding affinity of DYN analogues for ORL1 and opioid receptors

Peptides	IC_{50} ± SE (nM)				
	ORL1	Opiod receptor			
	[^{3}H]NOC	[^{3}H]U-69593 (κ)[a]	[^{3}H]DAMGO (μ)[b]	[^{3}H]DLT[c] (δ)[b]	κ/μ/δ
DYN (1–13)-NH_2	18.8 ± 3.01	0.162 ± 0.049	2.53 ± 0.38	6.49 ± 1.11	1/15.6/40.1
[Dmp^1] DYN (1–13)-NH_2 (**4**)	6.60 ± 0.952	0.056 ± 0.026	16.4 ± 2.35	10.1 ± 6.02	1/293/180
[Dmp^4] DYN (1–13)-NH_2 (**5**)	188 ± 18.2	0.044 ± 0.035	22.4 ± 10.2	931 ± 723	1/509/21159
[Dmp^{1,4}] DYN (1–13)-NH_2 (**6**)	51.5 ± 1.62	5.45 ± 1.65	251 ± 56.3	415 ± 185	1/46/76.1

[a] Using guinea pig brain homogenate.
[b] Using rat brain homogenate.

ORL1 receptor, whereas **5** exhibited an order of magnitude decreased affinity, indicating that the Dmp^4 modification in DYN peptides is detrimental to ORL1 receptor affinity, as was observed with NOC peptides. Simultaneous Dmp replacements in positions 1 and 4 (**6**), however, resulted in a two order of magnitude decrease in κ-receptor affinity and selectivity.

To examine the usefulness of DYN peptides as κ-opioid receptor ligands, the in vitro bioactivity of DYN peptides was determined using guinea pig ileum preparations (GPI assay) that contain κ- and μ-opioid receptors predominantly[19] (Table 4). Contrary to the high κ-opioid receptor-binding profile, **4** and **5** exhibited an unexpectedly low GPI potency, two and one orders of magnitude lower, respectively, than the parent peptides. In particular, **4** possessed an unexpectedly low GPI potency, two orders of magnitude less active than DYN(1–13)-NH_2. Low K_e values of κ-receptor antagonist nor-BNI and high K_e values of μ-antagonist CTAP and δ-against *N*,*N*(Me)$_2$Dmt-Tic-OH against **4** and **5** suggest that the GPI activity of these analogues occurred mainly via the κ-opioid receptor. Analogue **6** also showed dramatically reduced GPI potency and loss of receptor selectivity. The discrepancy between κ-opioid receptor binding and GPI potency observed with **4** can be attributed to the lack of hydroxyl side chains on the N-terminal residue because [2,6-dimethyltyrosine^1]DYN peptide is as active as the parent peptide in a GPI assay.[20] Similar results have been reported with Phe^1-DYN(1–11) peptide.[21] Taken together, results indicate that the N-terminal phenolic hydroxyl group of the DYN peptide is not mandatory for κ-receptor binding but is critically important for receptor activation.

Substitution of the peptide with an artificial or non-coded amino acid often improves metabolic stability useful for in vivo and in vitro studies. Therefore, we examined the effect of Dmp analogues using aminopeptidase M (AP-M) and rat brain synaptosomal membrane fractions, and compared the results with those of experiments involving the parent peptides. As shown in Table 5, Dmp^1-substituted analogues **1** and **4** showed greater stability toward AP-M and brain enzymes than did the parent peptides. These results suggest the involvement of aminopeptidase(s) in the brain that break down these analogues as observed with intact NOC[22,23] and DYN.[24,25] However, the stability of the Dmp^4-substituted analogues (**2** and **5**) was similar to or somewhat less than that of the parent peptide toward rat brain enzymes. These

Table 4. GPI assay and opioid receptor preference of DYN analogues

Peptide	IC_{50} ± SEM (nM)	K_e (nM) value of receptor selective antagonist		
		nor BNI (κ)	CTAP (μ)	*N*,*N*(Me)$_2$Dmt-Tic-OH (δ)
DYN(1–13)-NH_2	3.14 ± 1.13	1.1	99	98
[Dmp^1]DYN(1–13)-NH_2 (**4**)	306 ± 68	10	115	>1000
[Dmp^4]DYN(1–13)-NH_2 (**5**)	32.2 ± 9.16	0.63	108	198
[Dmp^{1,4}]DYN(1–13)-NH_2 (**6**)	1341 ± 303	80.9	59.5	>1000

Table 5. Comparison of stability of Dmp-peptides toward enzymatic degradation

Peptide	Half-lifetime (min)[a]	
	Aminopeptidase-M	Rat brain homogenate
NOC(1–13)-NH_2	12	41.5
[Dmp^1]NOC(1–13)-NH_2 (**1**)	28	60.3
[Dmp^4]NOC(1–13)-NH_2 (**2**)	n.d.[b]	33.6
[$Dmp^{1,4}$]NOC(1–13)-NH_2 (**3**)	n.d.[b]	27.1
DYN(1–13)-NH_2	15.5	435
[Dmp^1]DYN(1–13)-NH_2 (**4**)	>30	577
[Dmp^4]DYN(1–13)-NH_2 (**5**)	n.d.[b]	315
[$Dmp^{1,4}$]DYN(1–13)-NH_2 (**6**)	n.d.[b]	770
Met-enkephalin	<5	8.5

[a] See Section 4 for experimental conditions.
[b] Not determined.

results imply that a Dmp residue in position 4 offers no additional metabolic stability in either peptide and that endopeptidases play a major role in brain metabolism. A doubly Dmp-replaced NOC analogue **3** also possesses no additional stability, whereas its counterpart DYN analogue **6** demonstrates improved metabolic stability. Comparison of the metabolism of Dmp-containing NOC and DYN analogues suggests that the NC peptides generally are more susceptible to aminopeptidases and endopeptidases, although the opposite result in human blood has been reported.[24]

3. Conclusions

The present study revealed that the noncoded amino acid Dmp is a useful surrogate for Phe^1 and Tyr^1 residues in NOC and DYN peptides, respectively, in producing analogues with novel receptor-binding profiles. Dmp^1-NOC analogue (**1**) possessed high affinity toward the ORL1 receptor comparable to that of the parent peptide as well as greater affinity for κ, μ, and δ-opioid receptors. However, Dmp^4 substitution of the NOC peptide (**2**, **3**) decreased ORL1 receptor affinity. Dmp^1-substitution of DYN peptide (**4**) produced higher κ-opioid receptor affinity and selectivity, and a slightly improved ORL1 receptor affinity as compared to those of the parent peptides. However, the unexpectedly low potency of this analogue in a GPI assay suggests the importance of the phenolic hydroxyl group at the N-terminus for κ-opioid receptor activation. It is noteworthy that the Dmp^4-DYN peptide (**5**) had an exclusively low ORL1 affinity and high κ-receptor affinity and selectivity. Although the GPI potency of **5** was somewhat lower than that of the parent peptide, it may function as a κ-receptor agonist because of its very low K_e value (0.63 nM) toward κ-antagonist nor-BNI. Thus, this peptide may serve as a highly selective κ-receptor agonist for pharmacological study. Owing to the high affinity profiles for both ORL1 and the opioid receptors, **1** and **4** may become lead compounds for developing 'universal' ligands that interact with both ORL1 and opioid receptors.

4. Experimental

4.1. Peptide synthesis

Peptides were synthesized by a diisopropylcarbodiimide/1-hydroxybenztriazole-mediated Fmoc strategy according to a procedure described previously,[26] starting with 9-fluorenylmethyloxycarbonyl (Fmoc)-NH-SAL-resin. The side-chain-protecting groups used included 2,2,5,7,8-pentamethylchroman-6-sulfonyl group for Arg, benzyloxycarbonyl group for Lys, and *tert*-butyl group for Ser and Thr. Fmoc-Dmp was prepared as described previously.[27] Peptides were cleaved from the resin and deprotected by treatment with Reagent K[28] at room temperature for 1–1.5 h. The crude peptides obtained were purified on a Develosil LOP ODS column as described previously.[26] Analytical HPLC used for synthesizing all peptides and for determining enzymatic stability was performed on a Wakopak column (Wakosil-II 5C18 AR, 4 × 150 mm) using the following solvent systems: A, 0.06% TFA; B, 0.06% TFA in 80% CH_3CN. A linear gradient elution from 10 to 50 B% over 40 min was used at a flow rate of 1 mL/min with monitoring of the column eluate at 220 nm. Amino acid analysis was performed using a HITACHI L-8500 amino acid analyzer after 6 N HCl hydrolysis of peptide at 110 °C for 22 h. ESI-MS was performed on a Finnigan TSQ Quantum Discovery instrument (ThermoElectron, Massachusetts, USA). The doubly charged ion was detected as a major ion mass peak in all synthetic peptides in the ESI-MS analysis. Purity of all peptides was ~95% as determined by analytical HPLC. Analytical data of the synthetic peptides are shown in Table 1.

4.2. Enzymatic stability

Peptide (1 μmol/ml, 150 μL) was incubated with a solution of AP-M (1 mg/mL, 80 μL) or a rat brain synaptosomal fraction[29] (protein content: 2.0 mg/mL, 400 μL) in 10 mM Tris–HCl buffer (pH 7.60, 500 μL for AP-M or 200 μL for rat brain enzymes) at 37 °C for varying times. A portion of the mixture was withdrawn and 0.2 N HCl (20 μL) was added. After centrifugation at 5 °C for 10 min (4000 rpm), the supernatant was analyzed by HPLC. The degradation rate was estimated from the relative peak area of residual intact peptide to that of the peptide at zero time by HPLC using a Chromatocorder 12 integrator (System Instruments). The half-life time was obtained from the time-course curve of the degradation rate. Data indicate the average of two experiments.

4.3. ORL1 receptor-binding assay

The ORL1 receptor-binding assay was conducted using cell membranes expressing human ORL1 receptors in HEK-293 cells (Receptor Biology Inc.) as described previously.[30] The cell membranes (10.62 μg protein), 2 nM [^{3}H]NOC (2.78 TBq/mmol) and peptide sample were incubated in 50 mM Hepes buffer (pH 7.4) containing 1 mM EDTA and 10 mM $MgCl_2$ in a total volume of 100 μL, for 1 h at 25 °C in a siliconized tube. The reaction was terminated by filtration through a Whatman GF/B glass filter previously soaked in 0.5%

polyethyleneimine. The glass filter was washed three times with 2 mL of the Hepes buffer. Nonspecific binding was determined in the presence of 1 μM of unlabeled NOC. Filter-bound radioactivity was counted after overnight extraction with 3 ml Creasol I using a Beckman 9800 liquid scintillation counter. The IC_{50} values were determined from log dose–displacement curves.

4.4. Opioid receptor-binding assay

Opioid receptor-binding assays were performed as described previously.[16] Receptor binding was measured using rat brain homogenate for the μ- and δ-opioid receptors or guinea-pig brain homogenate for the κ-opioid receptor.[31] [^{3}H]DAMGO, [^{3}H]deltorphin II, and [^{3}H]U69593 (Amersham Corp.) were used for the μ-, δ-, and κ-radioligands, respectively. Competitive-binding experiments were performed in the presence of peptidase inhibitors, bacitracin, bestatine, and soybean trypsin inhibitor in 50 mM Tris–HCl buffer (pH 7.40). IC_{50} values were determined from log dose–displacement curves.

4.5. GPI assay

In vitro biological activity was evaluated using guinea pif ileum tissues as described previously.[15] A log dose–response curve was constructed and IC_{50} values (concentration causing 50% reduction in electrically induced twitches in the tissues) were determined. K_e values for the antagonists were obtained from the ratio of IC_{50} values in the presence and absence of antagonist. The antagonist concentrations used were nor-BNI, 50 nM; CTAP, 100 nM; *N*,*N*(Me)$_2$DMT-Tic-OH, 100 nM.

References and notes

1. Amino acids and peptides were of L-configuration unless otherwise noted. Amino acids and peptides were those recommended by the IUPAC-IUB Commission on Biochemical Nomenclature in *Eur. J. Biochem.* **1984**, *139*, 9. Other abbreviations used are as follows: NOC, nociceptin/orphaninFQ; DYN, dynorphin A; ORL1, opioid receptor-like 1; Dmp, 2′,6′-dimethylphenylalanine; AP-M, aminopeptidase M; TFA, trifluoroacetic acid; SAL-resin, 4-(2′,4′-dimethoxyphenyl-hydroxymethyl)-phenoxy resin; Fmoc-NH-SAL-resin, 4-(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin; DAMGO, [D-Ala^2, MePhe^4, Gly-ol^5]enkephalin; DLT, [D-Ala^2]deltorphin II(Tyr-D-Ala-Phe-Glu-Val;-Val-Gly-NH$_2$); GPI, guinea pig ileum; MVD, mouse vas deferens; t_R, retention time.
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