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Endomorphin 2 Analogues Containing Dmp Residue as an Aromatic Amino Acid Surrogate with High μ-Opioid Receptor Affinity and Selectivity

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Abstract—To investigate the effectiveness of a 2′,6′-dimethylphenylalanine (Dmp) residue as an aromatic amino acid surrogate, endomorphin 2 (EM_2: Tyr-Pro-Phe-Phe-NH_2) analogues were prepared, in which the constitutive aromatic amino acids (Tyr^1, Phe^3, or Phe^4) were replaced by Dmp or its isomer, D-Dmp. Replacement of Phe^3 by Dmp increased the affinity over 10-fold for both μ- and δ-opioid receptors, without affecting receptor selectivity. In contrast, replacement of Phe^4 considerably reduced the μ-receptor affinity and selectivity. These data indicated that the Dmp-substitution of Phe^3, but not Phe^4, in EM_2 is favorable for improving μ-receptor specificity. Inversion of the chirality of the substituted Dmp residue resulted in marked decrease in the μ-receptor affinity. Replacement of Tyr^1 by Dmp yielded an analogue that exhibited only a limited decrease in μ-receptor affinity and GPI potency, despite the lack of a phenolic hydroxyl group at the N-terminal residue. In contrast, D-Dmp^1- or Phe^1-substitution of Tyr^1 resulted in a significant decrease in μ-receptor affinity and GPI potency. These results suggested that the Dmp residue can mimic Tyr^1, which is one of the critical structural elements of opioid peptides.

Introduction

The aromatic amino acids of opioid peptides (Tyr^1, Phe^3, or Phe^4) have been shown to be important structural elements that interact with the opioid receptors. Recent studies of opioid peptides have demonstrated that the introduction of 2′,6′-dimethyltyrosine (Dmt) in place of Tyr^1 of opioid peptides resulted in great improvement in receptor affinity and functional bioactivity.[1,2] The introduction of Dmt residues led to the discovery of various Dmt-Tic pharmacophore peptides as potent δ-opioid receptor antagonists.[2] We have recently reported on the use of 2′,6′-dimethylphenylalanine (Dmp) in place of Phe^3 or Phe^4 of opioid peptides to produce novel analogues with antagonist[3] or potent agonist activity.[4] Notably, in the cases of dermorphin (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2) and deltorphin II (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2) heptapeptides, Dmp-substitution for the Phe^3 residue significantly improved both preferential receptor affinity and selectivity.[4] Furthermore, we found that Dmp can mimic Tyr^1, as well as Phe^3, in the potent μ-receptor ligand Tyr-D-Arg-Phe-βAla-NH_2 (YRFB). Substitution of Tyr^1 with Dmp produced an analogue with high μ-receptor affinity and biological activity, which were comparable to the potency of YRFB itself.[5] It is interesting to note that this analogue lacks the phenolic hydroxyl group at the N-terminal, which was considered to be a critical element for opioid activity. Similarly, some cyclic somatostatin- or enkephalin-based analogues, in which the N-terminal Tyr was replaced by Phe, have been reported as potent μ-receptor ligands.[6] The present study was undertaken to further investigate the use of the Dmp residue as an aromatic amino acid surrogate in an endogenous μ-receptor ligand, endomorphin 2 (EM_2: Tyr-Pro-Phe-Phe-NH_2).[7] Herein, we describe the syntheses of a series of EM_2 analogues, in which one of the three aromatic amino acids (Tyr^1, Phe^3 and Phe^4) was replaced by Dmp or D-Dmp, and the subsequent studies of their binding properties to μ- and δ-opioid receptors, and their in vitro biological activities.

Results and Discussion

The Dmp analogues were prepared using solid phase peptide synthesis via Fmoc chemistry. Dmp was initially

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prepared as a racemic mixture,[3] which was converted to the Fmoc-Dmp racemate. Subsequent solid-phase synthesis afforded diastereoisomeric peptides, which were easily separated using medium-pressure HPLC. Following acid hydrolysis, the configuration of the Dmp residue of the peptides was determined by using chiral TLC separation,[3] or by digestion with D-amino acid oxidase.[8] Purity of all peptides was over 96%, as determined by analytical HPLC. The analytical data for the peptides are listed in Table 1.

Table 2 lists the values of the receptor binding affinity of the analogues toward the μ- and δ-opioid receptors, in comparison with the parent peptide EM_2, which were obtained using rat brain synaptosomal fractions as previously reported.[4,9] EM_2 showed a high affinity and selectivity for the μ-opioid receptors. Interestingly, peptide **1** (Dmp^3-EM_2) displayed affinity that was over an order of magnitude higher for both μ- and δ-receptors than that of EM_2, and furthermore, retained high μ-receptor selectivity that was comparable to that of EM_2. In contrast, peptide **3** (Dmp^4-EM_2) resulted in a roughly 23-fold decrease in μ-receptor affinity, and a somewhat increase in δ-receptor affinity, thus resulting in a significant decrease in μ-receptor selectivity. These results suggested that Phe^3 but not Phe^4 of EM_2 is favorable for Dmp-substitution towards improving μ-receptor specificity. The introduction of D-Dmp at either positions 3 or 4 (peptide **2** or **4**, respectively) resulted in significant decreases in μ-affinity and selectivity, which coincided with the results of D-Phe-replaced analogues.[10] However, peptide **2** still retained a moderate affinity toward μ receptors with a K_i value of 2.4 nM whereas peptide **3** or **4** decreased μ-receptor affinity significantly, suggesting that Phe^3 is apparently more tolerant to Dmp or its D-isomer substitution than Phe^4 (peptide **3** or **4**). On the other hand, replacement of Tyr^1 by Dmp led to peptide **5**, which showed a 4-fold reduction, retaining significant μ-receptor affinity that is comparable to peptide **2**. It should be noted that peptide **5** retained a moderate potency at μ-receptors despite the lack of a phenolic hydroxyl group at the aromatic N-terminal residue. Substitution of Tyr^1 with D-Dmp (peptide **6**) or Phe (peptide **7**) resulted in a distinct decrease of μ-receptor affinity. These results suggested that the Dmp residue can partially compensate for the Tyr^1 residue in EM_2 to interact with the μ-receptor. More effectiveness of Dmp^1-substitution was observed in a series of YRFB analogues.[5]

The in vitro biological activities were evaluated using isolated guinea pig ileum (GPI) and mouse vas deferens (MVD) tissue samples (Table 3). GPI tissues contain predominantly μ-receptors, whereas MVD tissues include δ-receptors.[11] Results obtained using these two assays were comparable to those obtained from the receptor binding assays. As expected, peptide **1** exhibited considerably higher GPI potency than EM_2; however, this analogue produced higher than expected MVD potency for δ receptor affinity. This may be due to μ-receptors, which coexist concomitantly in the MVD tissues, because the high MVD potency was strongly inhibited by a specific μ-receptor antagonist D-Phe-cyclo(Cys-Tyr-D-Trp-Arg-Thr-Pen)-Thr-NH_2 (CTAP)[12] (data not shown). Similar phenomenon was also observed with other high μ-receptor ligands, such as [Dmp^3]dermorphin,[4] Tyr-D-Arg-Dmp-βAla-NH_2[5] and even EM_2 itself (Table 3). Compared to the parent peptide,

Table 1. Physico-chemical properties of synthetic EM_2 analogues

Peptide	$[\alpha]_D$[a] (°)	HPLC[b] (t_R, min)	Amino acid analysis				HR-FABMS		
			Tyr	Pro	Phe	Dmp[c]	Formula	Calcd	Found
Tyr-Pro-Dmp-Phe-NH_2(**1**)	−39.8	16.55	0.98	0.94	1.00	0.99	$C_{34}H_{42}N_5O_5$	600.3186	600.2979
Tyr-Pro-D-Dmp-Phe-NH_2(**2**)	−41.1	26.38	0.99	0.94	1.00	0.83	$C_{34}H_{42}N_5O_5$	600.3186	600.3457
Tyr-Pro-Phe-Dmp-NH_2(**3**)	−35.7	18.11	0.98	0.94	1.00	1.00	$C_{34}H_{42}N_5O_5$	600.3186	600.2979
Tyr-Pro-Phe-D-Dmp-NH_2(**4**)	−18.3	22.46	0.98	0.96	1.00	0.99	$C_{34}H_{42}N_5O_5$	600.3186	600.2979
Dmp-Pro-Phe-Phe-NH_2(**5**)	−6.20	24.88	—	1.11	2.00	0.76	$C_{34}H_{42}N_5O_5$	584.3237	584.3268
D-Dmp-Pro-Phe-Phe-NH_2(**6**)	−94.2	25.54	—	1.04	2.00	0.76	$C_{34}H_{42}N_5O_5$	584.3237	584.3268
Phe-Pro-Phe-Phe-NH_2(**7**)	−31.3	18.43	—	1.12	3.00	—	$C_{32}H_{38}N_5O_4$	556.2924	556.2964

[a]Measured in 50% AcOH (*c* 0.2) at 20 °C.
[b]See Experimental for conditions.
[c]Eluted at the position of His using an analyzer (Hitachi L-8500) and calculated as His.

Table 2. Opioid receptor binding assay of EM_2 analogues

Peptide	[^{3}H]DAMGO K_i±SE (nM)	[^{3}H]Deltorphin II K_i±SE (nM)	Selectivity (δ/μ)
Tyr-Pro-Phe-Phe-NH_2 (EM_2)	0.557±0.306	14,070±3346	25,260
Tyr-Pro-Dmp-Phe-NH_2(**1**)	0.0304±0.0208	1063±336	34,967
Tyr-Pro-D-Dmp-Phe-NH_2(**2**)	2.40±0.56	4169±954	1737
Tyr-Pro-Phe-Dmp-NH_2(**3**)	13.2±1.9	7624±2571	578
Tyr-Pro-Phe-D-Dmp-NH_2(**4**)	106±20	1765±834	17
Dmp-Pro-Phe-Phe-NH_2(**5**)	2.48±1.46	6762±590	2727
D-Dmp-Pro-Phe-Phe-NH_2(**6**)	40.4±2.6	9714±3820	241
Phe-Pro-Phe-Phe-NH_2(**7**)	54.1±23.4	18,851±10,487	348

Table 3. In vitro biological assay of EM_2 analogues

Peptide	GPI (μ) IC_{50} ± SE (nM)	MVD (δ) IC_{50} ± SE (nM)	Ratio (MVD/GPI)
Tyr-Pro-Phe-Phe-NH_2 (EM_2)	10.5 ± 1.2	317 ± 65	30.2
Tyr-Pro-Dmp-Phe-NH_2(**1**)	0.378 ± 0.104	1.39 ± 0.17	3.68
Tyr-Pro-D-Dmp-Phe-NH_2(**2**)	30.4 ± 2.80	187 ± 30	6.15
Tyr-Pro-Phe-Dmp-NH_2(**3**)	196 ± 40	320 ± 55	1.63
Tyr-Pro-Phe-D-Dmp-NH_2(**4**)	587 ± 119	2267 ± 603	3.86
Dmp-Pro-Phe-Phe-NH_2(**5**)	76.9 ± 20.7	661 ± 316	8.61
D-Dmp-Pro-Phe-Phe-NH_2(**6**)	1392 ± 221	2329 ± 943	1.67
Phe-Pro-Phe-Phe-NH_2(**7**)	1073 ± 309	5199 ± 2584	4.85

Dmp[1] peptide (**5**) showed only a 7-fold drop in GPI potency, which was expected from the binding affinity; in contrast, peptides **6** and **7** were 132- and 102-fold less potent than EM_2, respectively.

Conclusion

The present study demonstrated that Dmp-substitution for Phe[3] but not for Phe[4] in EM_2 is effective in enhancing μ-receptor affinity and selectivity. This study also demonstrated an interesting aspect of the Dmp residue, that is it can substitute for the N-terminal Tyr residue, which was considered as indispensable for the opioid activity. The Dmp-substitution for Tyr[1] residue in EM_2 (peptide **5**) caused only a limited reduction in μ-receptor affinity despite the lack of a phenolic hydroxyl group on the N-terminal residue. It is conceivable that the loss of a phenolic hydroxyl oxygen atom can be overcome, at least partly, by the enhanced hydrophobicity and/or the reduced side-chain flexibility of the Dmp side chain. These characteristics of the Dmp residue would allow for the molecule to bind to the hydrophobic pocket of the μ-receptor, such that a phenolic hydroxyl oxygen atom at the N-terminal residue, presumed to be a hydrogen bond partner of a conserved His residue (TMH VI) of opioid receptors,[6c,13] is apparently not required. Results of this study have provided additional support that a Dmp residue can mimic the N-terminal Tyr of opioid peptides,[5] and thus Dmp-substitution can provide insights into the development of novel opioid mimetics with high receptor specificity.

Experimental

Peptide synthesis

EM_2 and its analogues were synthesized via a diisopropylcarbodiimide/1-hydroxybenztriazole-mediated Fmoc strategy, starting with 9-fluorenylmethyloxycarbonyl (Fmoc)-NH-SAL-resin, as previously described.[9] For the incorporation of the Dmp residue, Fmoc-Dmp racemate[3] was used to construct the peptides on the resin. Cleavage of the peptides from the resin was carried out by treatment with a solution of TFA–phenol (95:5) for 1 h, to afford a pair of diastereoisomeric peptides (**1** and **2**, **3** and **4**, or **5** and **6**), which was subsequently separated using medium-pressure HPLC as described previously.[9] The absolute configuration of the Dmp residue of the separated peptides was determined by the analysis of the HCl hydrolysate of the peptides using chiral TLC plates (Macherey-Nagel, 5 × 20 cm, MeCN/MeOH/H_2O = 4:1:1)[3] or by enzymatic digestion of the hydrolysate with D-amino acid oxydase.[8] The purity of all peptides was determined as > 96% using analytical HPLC (YMC-Pack ODS-AM-302 column, 150 × 4.6 mm). The column was eluted at a flow rate of 1 mL/min, using the following solvents: A, 0.06% TFA in H_2O; B, 0.06% TFA in 80% MeCN (with a linear gradient: 25% B to 55% B in 40 min). The elution was monitored using UV detection (220 nm). The analytical data of synthesized peptides are shown in Table 1.

Receptor binding assay

The opioid receptor-binding assays were performed using methods as described previously.[4,9] [^{3}H]Tyr-D-Ala-Gly-MePhe-Gly-ol (DAMGO) and [^{3}H]deltorphin II are radioligands for the μ- and δ-opioid receptors, respectively. Synaptosomal fraction (600 μg of protein), 2 nM radioligand, synthetic peptide sample, bovine serum albumin (500 μg), bacitracin (50 μg), bestatin (10 μg) and soybean trypsin inhibitor (20 μg) were incubated in Tris–HCl buffer (50 mM ; pH 7.40; total volume = 500 μL) for 1 h at 25 °C. Bound- and free-radioactivities were separated by passage through Whatman GF/B glass filters that had been soaked in 0.5% polyethyleneimine. The filters were then washed twice using ice-cold Tris–HCl buffer. Nonspecific bindings were determined in the presence of 1 μM unlabeled ligand. The amounts of radioactivity bound to the filter were determined after overnight extraction with 3 mL of Creasol I (Nacalai Tesque, Japan) using a Beckman 9800 liquid scintillation counter. The IC_{50} values were determined from logarithmic dose-displacement curves, and the values of the inhibitory constant (K_i) of peptides were calculated according to the equation of Cheng and Prusoff.[14] The K_d values of [^{3}H]DAMGO and [^{3}H]deltorphin II were 0.86 and 0.79, respectively.

In vitro bioactivity assay

Biological activities towards the μ- and δ-receptors were evaluated using guinea pig ileum (GPI) and mouse vas deferens (MVD) tissues, respectively. For GPI assays, the myenteric plexus-longitudinal muscle was obtained from male Hartley strain guinea pig (250–300 g) ileum, and the tissue was mounted in a 10-mL organ bath that

contained Krebs–Henseleit solution at 35 °C. The tissue was stimulated transmurally between the platinum wire electrodes using pulses of 0.5 ms duration with a frequency of 0.1 Hz at the supramaximal voltage. Longitudinal contractions were recorded using an isometric transducer. For MVD assays, the vas deferens of male ddY strain mouse (25–35 g) were prepared, as described by Hughes et al.[15] A pair of *vasa* was mounted in a 10-mL organ bath that contained modified Mg^{+}-free Krebs solution at 35 °C. The tissue was stimulated transmurally using trains of rectilinear pulses of 1 ms, in which the stimulation trains were given at intervals of 20 s, and consisted of seven stimuli of 1-ms duration at intervals of 10 ms. For both assays, the tissues were washed four times at intervals of 20 min between each dose. Using the data, dose–response curves were plotted, and the IC_{50} values were determined graphically.

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