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Opioid receptor binding and antinociceptive activity of the analogues of endomorphin-2 and morphiceptin with phenylalanine mimics in the position 3 or 4

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Abstract—Endomorphin-2 (EM-2) and morphiceptin are the same class of putative μ-opioid receptor ligands. To investigate the effectiveness of phenylglycine (Phg, L or D) and homophenylalanine (Hfe) as the surrogates of phenylalanine in the position 3 and/or 4 of them, a series of their analogues were synthesized. Opioid receptor binding affinities were determined. Two analogues, [Hfe^3]EM-2 and [Phg^4] (EM-2/morphiceptin), showed different but potent antinociceptive activity in mouse hot-plate test, the results combined with their half-lives of degradation by mouse brain homogenate could also present some evidence to the in vivo degradative mechanism of EM-2.

In 1997, Zadina and his colleagues isolated Endomorphin-1 (EM-1: Tyr-Pro-Trp-Phe-NH_2) and Endomorphin-2 (EM-2: Tyr-Pro-Phe-Phe-NH_2) from bovine brain and human brain. They have shown high selectivity toward μ-opioid receptor with potent analgesic activity[1–3]. Morphiceptin (Tyr-Pro-Phe-Pro-NH_2) was isolated from an enzymatic digest of milk protein, β-casein, its structure differs from endomorphin-2 only by the amino acid in the fourth position (Pro and Phe, respectively).[4] Endomorphin-2 and morphiceptin are the same class of opioid peptides which have a unique N-terminal Tyr-Pro-Phe sequence different from the N-terminal tetrapeptide sequence Tyr-Gly-Gly-Phe, characteristic for typical opioid peptides, like enkephalins, endorphins, and dynorphins. The aromatic amino acids (Tyr^1, Phe^3, or Phe^4) of EM-2 and morphiceptin have been shown to be important structural elements that interact with the opioid receptors besides the proline residue at the second position conferring high selectivity on the μ-opioid receptor.[5] Unfortunately, the exogenous application of these opioid peptides generally met with failure, owing to their biological instability and inability to be transmitted through the blood–brain barrier (BBB).[6] In order to avoid these disadvantages and increase their affinity and selectivity, a number of their analogues were synthesized and evaluated. One major approach is introducing derivates of Tyr and Phe to opioid peptides. Replacement of Tyr^1 in EM-2 or its deleted fragments by 2′,6′-dimethyltyrosine (Dmt) could remarkably enhance their opioid receptor binding affinity and biological activity despite their selectivity on μ-opioid receptor decreased.[7] Dolle and his colleagues discovered that (*S*)-4-(carboxamido) phenylalanine (Cpa) could act as a surrogate for Tyr^1 in opioid peptide ligands.[8] Using 2′,6′-dimethylphenylalanine (Dmp) to substitute the Phe^3 in EM-2 increased both its binding affinity and selectivity to μ-opioid receptor and Dmp could also mimic the Tyr^1 in opioid peptides.[9,10] Cardillo and his colleagues introduced β-amino acids to scan the EM-1 sequence, only β-(R)-Pro^2 substitution acquired positive results; analogues containing β-Tyr^1, β-(S)-Pro^2, β-Trp^3, and β-Phe^4 all decreased the μ-opioid receptor binding affinity of EM-1.[11] N-methylation of Phe^3 in EM-2 attenuated

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Keywords: Endomorphin-2; Morphiceptin; Phenylalanine mimics; Structure–activity relationships; Opioid peptide analogues.

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its binding affinity and produced a rightward shift of [^{35}S]GTPγS binding curves.[12] Our group has first introduced α-aminoxy phenylalanine into EM-2 and obtained interesting N–O turn mimetics.[13] Our results on the bivalent analogues of EM-2 also suggested the importance of the Phe^3.[14] To morphiceptin, there are also some successful examples about introducing Phe derivates into it.[5,6]

Homophenylalanine (Hfe) and phenylglycine (Phg) have been introduced into deltorphin A, deltorphin B, and deltorphin C to study the role of phenylalanine in the interaction of these δ selective opioid peptides with their receptors.[15,16] Hfe and its isomer have also been introduced into the κ selective opioid peptide, [D-Ala^8]dynorphin A-(1–11), and good results were acquired.[17] Though Shimohigashi et al. had introduced side chain prolonged Phe mimics into morphiceptin, they had discovered the μ-opioid receptor binding affinities of these analogues decreased.[18] Except that, no one introduced both Hfe and Phg into the μ selective opioid peptides, EM-2 and morphiceptin, to study the structure–activity relationships. Hfe and Phg could not only keep the aromatic property of Phe but also resist to the enzymatic degradation owing to their profiles of non-protein amino acids and they could also help us to investigate the effects on activities by altering Phe's lipophilicity and length of side chain; these are the other reasons why we chose Hfe and Phg. Therefore, we designed and synthesized a series of EM-2 and morphiceptin analogues with Phe substituted by Hfe, Phg (L or D), respectively. We determined their binding affinities for μ- and δ-opioid receptors and chose two potent analogues to evaluate their antinociceptive activity and half-life of degradation by the mouse brain homogenate.

All the compounds in this study were synthesized by solution methodology for peptide synthesis using Boc-amino protection groups and using *N*-methyl morpholine (NMM) and isobutyl chloroformate (IBCF) as coupling reagents. TFA and anisole (v/v = 9:1) were used as deprotection reagents. All the analogues were synthesized by the modified methods reported previously.[13,19] After deprotection, the compounds were precipitated with ether, filtered, the crude peptides were obtained as TFA salts and then purified using RP-HPLC, purity of all peptides was greater than 95%. The analytical data are listed in Table 1.

The opioid receptor binding assays were performed in 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 0.5 mL containing 250–400 μg of protein (synaptosomal brain membrane P2 was prepared from Wistar rats). In competition experiments, the following conditions were used for incubations: [^{3}H]DAMGO (0.5 nM, 25 °C, 1 h), [^{3}H]DPDPE (1 nM, 25 °C, 3 h). Non-specific binding was determined in the presence of 10 μM naxolone. K_d values of [^{3}H]DAMGO and [^{3}H]DPDPE were 0.533 and 2.75 nM, respectively.[14] All reactions were repeated at least three times. K_i values were calculated according to the equation of Cheng and Prusoff.[20] The K_i values of EM-2 in [^{3}H]DPDPE and [^{3}H]DAMGO assays agreed with Okada et al.[7] and Tóth and co-workers,[21] respectively. The data are listed in Table 2.

The antinociceptive activity was determined by the hot-plate test according to the modified method of Tseng et al.[22] Mice were individually placed on the hot-plate (54.5 ± 0.5 °C), and the reaction time starting from the placement of the mouse on the hot-plate to the time of licking or quickly withdrawing the paw was measured. Control latencies were approximately 5–10 s. The latency of the hot-plate responses was measured before (T_0) and at various times after (T_1) intracerebroventricular (icv) injection of the testing compounds. The injection volume was 4 μl. The inhibition was expressed as a percentage of the maximum possible effect (% MPE), which was calculated as $[(T_1 - T_0)/(T_2 - T_0)] \times 100$, where the cut-off time, T_2, was set at 30 s. The data were expressed as means ± SEM ($n = 6$–10). The statistical significance of differences between groups of each dose of compounds and normal saline (NS) was assessed with a one-way ANOVA followed by Dunnett's test. The results are shown in Figures 1–3.

Mouse brains were isolated, pooled, and homogenized with cold Tris buffer, centrifuged twice at 49,000*g* for

Table 1. Analytical data of morphiceptin/EM-2 analogues

Compound		MS [M+H]$^+$		TLC[c] (R_f)	$[\alpha]_D$[d] (°)
		Calculated	Found		
Morphiceptin	Tyr-Pro-Phe-Pro-NH$_2$	522.3	522.6[b]		
1	Tyr-Pro-Hfe-Pro-NH$_2$	536.3	536.4[b]	0.50	−58
2	Tyr-Pro-Phg-Pro-NH$_2$	508.3	508.5[b]	0.48	+4
3	Tyr-Pro-D-Phg-Pro-NH$_2$	508.3	508.3[b]	0.45	−99
4	Tyr-Pro-Hfe-Phe-NH$_2$	586.3	586.3[a]	0.52	−12
5	Tyr-Pro-Phg-Phe-NH$_2$	558.3	558.3[b]	0.53	+16
6	Tyr-Pro-D-Phg-Phe-NH$_2$	558.3	558.2[b]	0.51	−37
7	Tyr-Pro-Phe-Hfe-NH$_2$	586.3	586.4[a]	0.56	−27
8	Tyr-Pro-Phe-Phg-NH$_2$	558.3	558.3[b]	0.55	+15
9	Tyr-Pro-Phe-D-Phg-NH$_2$	558.3	558.3[b]	0.52	−47
EM-2	Tyr-Pro-Phe-Phe-NH$_2$	572.3	572.3[a]		

[a] FAB-MS.

[b] ESI-MS.

[c] Solvent system: ethyl acetate/MeOH/ammonia (8:1:1).

[d] MeOH (20 °C), $c = 0.3$.

Table 2. Opioid receptors binding assay of morphiceptin/EM-2 analogues

Compound	[^{3}H]DAMGO (μ)		[^{3}H]DPDPE (δ)	Selectivity
	K_i ± SE (nM)	Relative potency	K_i ± SE (nM)	(K_i δ/K_i μ)
Morphiceptin	135 ± 18.4	1	>10,000	>74.1
1	586 ± 246	0.23	>10,000	>17.1
2	1042 ± 279	0.13	>10,000	>9.60
3	24.4%[a]		>10,000	
4	28.4 ± 11.5	4.75	>10,000	>352
5	268 ± 25	0.50	>10,000	>37.3
6	1347 ± 84	0.10	2100 ± 671	1.56
7	74.5 ± 18.1	1.81	>10,000	>134
8	26.9 ± 6.93	5.02	>10,000	>372
9	216 ± 22.9	0.63	>10,000	>46.3
EM-2	8.23 ± 0.48	16.4	8360 ± 1314	1016

[a] Percentage decrease of maximum binding at 10,000 nM.

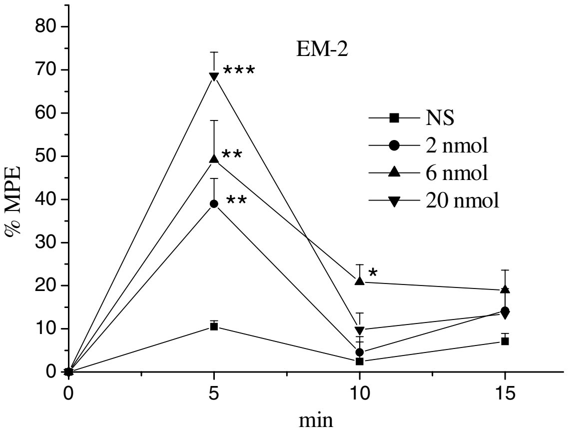

Figure 1. Time course of the changes in icv endomorphin-2 induced hot-plate inhibition. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

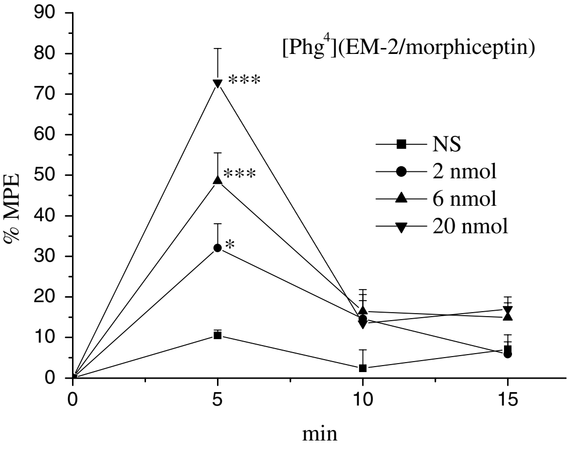

Figure 2. Time course of the changes in icv compound **8** induced hot-plate inhibition. $P < 0.05$ (*) and $P < 0.001$ (***).

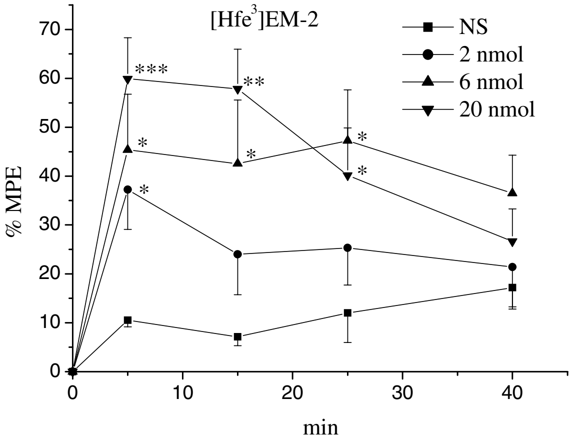

Figure 3. Time course of the changes in icv compound **4** induced hot-plate inhibition. $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

45 min, and stored at −80 °C until used. 10 μl of endomorphin-2 and its analogues (concentration of 5×10^{-4} M) was incubated with 190 μl of homogenate containing 2.15 mg protein/ml for 0, 15, 30, and 45 min at 37 °C. At the required time, 20 μl of the reaction mixture was taken out into a tube. The reaction was stopped by placing the tube on ice and adding 90 μl acetonitrile then acidifying with 90 μl of 0.5% aqueous HOAc solution. After centrifuging at 13,000*g* for 15 min, the obtained supernatants were filtered and analyzed by HPLC. All reactions were repeated twice. The half-life was calculated according to the literature.[23]

From the opioid receptor binding data shown in Table 2, we can see that all the analogues were selectively binding to the μ-opioid receptor. Compounds **1**, **2**, and **3** with substituted Phe^3 decreased the μ-opioid receptor binding affinity of their parent morphiceptin. The effectiveness order was Hfe > Phg > D-Phg. The compounds **4**, **5**, and **6** with substituted Phe^3 of EM-2 decreased the μ-opioid receptor binding affinity of their parent, but the effectiveness order was also Hfe > Phg > D-Phg. And the μ-opioid receptor affinity of all the three analogues of EM-2 was higher than that of the corresponding substituted analogues of morphiceptin. The μ-opioid receptor affinity of analogue **4** was more potent than morphiceptin for 4.75-fold. These results suggested that Hfe could be more effective than that of Phg and D-Phg in substitution of Phe^3 both in EM-2 and morphiceptin and Phe^3 might play the similar role in their binding to

Table 3. Amount of peptides remained after degradation by the mouse brain homogenate and their half-lives

Compound		Area [%]			Half-life (min)
		15 min	30 min	45 min	
EM-2	Tyr-Pro-Phe-Phe-NH_2	40.1	34.8	ND	19.7
4	Tyr-Pro-Hfe-Phe-NH_2	67.5	50.0	38.2	32.5
8	Tyr-Pro-Phe-Phg-NH_2	54.2	42.2	35.5	24.1

the μ-opioid receptor; aromatic group in position 4 might be important to μ-opioid receptor binding.

The parent of position 4 substituted analogues (compounds **7**, **8**, and **9**) can be considered as either EM-2 or morphiceptin. The effectiveness order was Phg > Hfe > D-Phg, which indicated the different role of aromatic groups in positions 3 and 4 combined with the results discussed above. Analogues **7** and **8** both increased the μ-opioid receptor binding affinity of morphiceptin for 1.81- and 5.02-fold, respectively. Though we believed that the N-terminal tripeptide of morphiceptin and endomorphin-2 might play the key role in their μ-opioid receptor binding, the aromatic group of position 4 can also improve the binding properties.[14,24,25]

We chose the compounds **4** and **8** to evaluate their antinociceptive activity by the mouse hot-plate test. From the curves shown in Figures 1 and 2, we can see that **8** had the similar potent antinociceptive activity as that of EM-2. After injection, they both reached their peak values at 5 min and declined fast. Their antinociceptive effects lasted for less than 15 min at the doses of 2, 6, and 20 nmol/kg. From Figure 3, we can see that compound **4** showed long-lasting antinociceptive effects at the same doses as that of EM-2 and compound **8**. The effects lasted for about 40 min. We also tested the effects of morphine (data are not shown); compound **4** had the similar antinociceptive properties (peak values and lasting times) as those of morphine. From the data of degradation by mouse brain homogenate shown in Table 3, the stability order was **4** > **8** > EM-2. Therefore, the compound **4** might be a good candidate to the opioid peptide analgesics. These results also presented evidence to the possible in vivo degradative mechanism of EM-2 which has been discussed by Tömböly and Péter et al. and Janecka and co-workers.[23,26,27] The Pro^2-Phe^3 bond might be the major in vivo enzymatic site of endomorphin-2 when injected intracerebroventricularly. Introducing unnatural amino acids or non-peptide structures into positions 2 and 3 could acquire improved stability and prolonged activity of endomorphin-2. The other related activities of the interesting compound **4** will be tested in our laboratory soon.

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