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Original endomorphin-1 analogues exhibit good analgesic effects

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ABSTRACT

A new class of endomorphin-1 analogues was synthesized by combining successful chemical modifications including N-terminal guanidino modification, Phe[4] was chlorinated, D-Ala-Gly Substituted L-Pro[2]. Their bioactivities were measured by radioligand binding assay, metabolic stability and the tail-flick test. In radioligand binding assays, analogue GAGPC (N^{α}-Amidino-Tyr-D-Ala-Gly-Trp-p-Cl-Phe-NH_2), shown a μ-opioid receptor affinity about 1.42-fold higher and a 2.51-fold higher δ-opioid receptor affinity than EM-1. In the metabolic stability assays, GAGPC had the longest half-lives which was 284 min and 53-fold higher than that of EM-1. In the tail-flick test in mice, GAGPC chloride modification increases the lipid content of the drug, thus increases the permeability of the blood brain barrier, and has a higher analgesic activity. It might be of importance in potential application as drug candidates as analgesic.

Pain is an unpleasant emotion and feeling caused by tissue damage. Opioids occupy a very important position in all analgesic drugs, and it consists of three subtype receptors: the μ-opioid receptor (MOR), the δ-opioid receptor (DOR), and the κ-opioid receptor (KOR).[1] The MOR endogenous tetrapeptides, endomorphin-1 (EM-1, H-Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, H-Tyr-Pro-Phe-Phe-NH_2), were isolated from the bovine brain and the human cortex in 1997.[2,3] Endomorphins (EMs), containing EM-1 and EM-2, showed the greatest affinity for MOR and the selectivity of MOR was higher than DOR.[3] EMs displayed profound antinociceptive effects in neuropathic pain and inflammatory pain.[4,5] Yamaguchi found that intracisternal injection of EM-1 and EM-2 produced the same analgesic effect as morphine in a tail-press experiment.[6] Furthermore, EMs exhibit potent antinociceptive effects without some of the undesirable side effects of morphine, for example, the rewarding effect, respiratory depression and cardiovascular effects at effective antinociceptive doses.[7–9] Tseng reported that an analgesic effect of endomorphins in mouse hot-plate and tail-flick experiments, and they found that the analgesic effect of EM-1 was 2 to 3 times greater than that of EM-2.[10] However, EM-1 still suffers from serious limitations, especially poor metabolic stability, short duration of action, relative inability to cross the blood-brain barrier (BBB) into the central nervous system (CNS).[11] Therefore, it is essential to enhance their metabolic stability and the ability of access CNS in order to therapeutic use.

For the past several years, numerous successful modifications have been developed for enhancing peptide delivery to the CNS and metabolic stability.[12–16] (1) Hau designed guanidine modification on EM-2 and morphiceptin, found its analogues in the enzymatic of stability and the ability to appear a BBB has significantly improved.[17] In 2006, Liu reported that synthesis of guanidination EM-1 and its analogues, not only improve the stability of enzyme solution, but also produced significant analgesic effect compared with EM-1 under the condition of subcutaneous (s.c.) administration.[18] Thus N-terminal guanidine can promoted the stability, at the same time, improved its ability to penetrate the BBB. (2) In recent years, it has been shown that the halogenated modification of the phenyl ring of Phe of opioid peptides enhances the overall lipid solubility of the compounds and enhances the ability of the drug to pass through the BBB, due to the increased lipid solubility of the compounds which can make the drugs more easily diffused through the BBB.[19,20] Previous studies have shown that addition of halogens to enkephalin analogues may enhance overall lipophilicity of the compound resulting in greater BBB permeability.[19–22] (3) A series of 2 position substituted analogues were synthesized by Liu,[18] D-Ala, Sar and D-Pro-Gly replace the L-Pro[2], its stability of analogues have greatly improved in vitro. In previous studies, we found that the analogue Guanidino-[D-Ala[2]] EM-1 shows longest metabolic half-lives in the serum, however, the analogues with the replacement of L-Pro[2] by D-Pro-Gly at position 2 and guanidino-addition at N-terminal shows longest metabolic half-lives in

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the brain homogenate.[18] Thus we try to design analogues containing D-Ala-Gly replace L-Pro at position 2.

On the basis of the above-mentioned strategies, we designed and synthesized a series of EM-1 analogues(guanidino-addition at N-terminal, C-terminal chloro-halogenation and D-Ala-Gly substitutions in position 2) to enhance its resistance to enzymatic degradation and its BBB permeability for future therapeutic use. Moreover, we determined their opioid receptor affinity and selectivity as well as stability. We further characterized their antinociceptive activities by a tail-flick test after i.c.v. and s.c. administrations. These analogues could help identify the best possible drug candidate for clinical pain management.

Endomorphin-1 and their analogues were obtained by solution-phase methods with segment-coupling peptide synthesis strategy, the sequences and MS, RP-HPLC, purity(%) of EM-1 and all its analogues shown in Tables 1 and 2.

The affinity and selectivity of EM-1 and all its analogues were evaluated, using rat brain membranes. Their binding affinities for μ- and δ-opioid receptors are shown in Table 3. In the radioligand binding assay, EM-1 was characterized as comparison. Its affinity value (Ki(μ) = 4.53 nm) consisted with literature reports.[18] The EM-1 analogue HAGDC shown a Ki (μ) being about 2.92-fold higher and a Ki (δ) being 1.26-fold lower compared to the EM-1. Analogue GAGPC shown a μ-opioid receptor affinity about 1.42-fold higher than EM-1 and a 2.51-fold higher δ-opioid receptor affinity. The HAGPC displayed a Ki (μ) being about 1.7-fold higher and a Ki (δ) being about 1.25-fold lower compared to the EM-1 parent peptide. GAGDC displayed 2.5-fold higher μ-opioid receptor affinity and 1.31-fold higher δ-opioid receptor affinity than EM-1.

Table 1
Amino acid sequences of EM-1 and all its analogues.

Petptides	Sequences
EM-1	Tyr-Pro-Trp-Phe-NH_2
HAGDC	Tyr-D-Ala-Gly-Trp-Phe-NH_2
GAGPC	N^{α}-Amidino-Tyr-D-Ala-Gly-Trp-*p*-Cl-Phe-NH_2
GAGDC	N^{α}-Amidino-Tyr-D-Ala-Gly-Trp-Phe-NH_2
HAGPC	Tyr-D-Ala-Gly-Trp-*p*-Cl-Phe-NH_2

Table 2
TOF MS, RP-HPLC and purity data of all analogues.

Peptide	TOF MS $[M+H]^+$		RP-HPLC	Purity (%)[b]
	Calcd	Found	t[a] (min)	
EM-1	611	611.1	16.239	100
HAGDC	642	642.5	13.205	96.18
GAGPC	717	718.4	18.475	98.97
HAGPC	675	676.3	17.371	96.26
GAGDC	683	684.4	17.091	99.67

[a] With Delta-Park C18 column (4.6 mm × 250 mm, 5 μm), A:B = 10:90 to A:B = 90:10 for 30 min, A:B = 90:10 to A:B = 10:90 for 5 min.
[b] Purity determination based on analytical RP-HPLC.

Table 3
μ-opioid and δ-opioid receptor affinity and selectivity, half-lives in the plasma of EM-1 and all its analogues in vitro.

Peptides	Ki(μ)(nM)	Ki(δ)(nM)	Ki(δ)/Ki(μ)	Half-life (min)	
				15% brain homogenate	Plasma
EM-1	4.53 ± 0.21	5093 ± 660	1119.34	13.5 ± 0.2	5.4 ± 1.0
HAGDC	13.3 ± 0.71	4051 ± 359	304.59	219.3 ± 21.6	150.8 ± 29.3
GAGPC	3.2 ± 0.31	2031 ± 148	634.68	434.9 ± 27.2	284.0 ± 45.5
HAGPC	7.7 ± 0.4	4063 ± 312	527.66	251.9 ± 4.9	170.3 ± 9.8
GAGDC	1.81 ± 0.29	3872 ± 213	2139.23	443.8 ± 58.2	244.7 ± 7.3

The results are expressed as mean ± SEM for 8–12 measurements.

The metabolic stability of EM-1 and its analogues was assessed in the 15% brain homogenate and plasma of mice. The half-lives determined for all the test peptides in brain homogenate and serum were summarized as shown in Table 2. In the15% brain homogenate, EM-1 shown only 13.5 min half-life. HAGDC had a longer half-life with 219 min. GAGPC displayed 434.9 min half-life which was 32.2-fold higher than EM-1. HAGPC showed a half-life about 251.9 min. Half-life of GAGDC was about 443.8 min. In the plasma, EM-1 was also degraded rapidly, with half-life being only 5.4 min. All analogues showed significant increase in half-lives than EM-1. HAGDC had the half-life which was 150.8 min. GAGPC had the longest half-lives which was 284 min and 53-fold higher than that of EM-1. But HAGPC showed a 1.7-fold lower half-lives than GAGPC. GAGDC had a half-life about 244.7 min.

Antinociceptive activities of EM-1 and its analogues were tested in the tail-flick test in mice intracerebroventricular (i.c.v.) and subcutaneous (s.c.) administration. Time course of the antinociceptive effect in i.c.v. and s.c. and area under the curve (A.U.C) of EM-1 and its analogues were shown in Figs. 1 and 2 respectively. We were measured analgesic effect at a fixed dose of 20 nmol/kg following i.c.v administration and 10 mg/kg following s.c. administration. In i.c.v. administration, among them, EM-1 showed poor analgesia, which was A.U.C = 270.75. HAGDC had poor analgesia with A.U.C = 335.5. GAGPC showed better analgesia with A.U.C = 649.75 than EM-1. HAGPC showed the lowest analgesic effect with A.U.C = 246.25. Yet, GAGDC showed best analgesia with A.U.C = 1164.25. There were some different outcomes following s.c. and i.c.v. administration. In s.c. administration, EM-1 showed better analgesia, which was A.U.C = 692.99. HAGDC had poor analgesia which was A.U.C about 303.85. GAGPC displayed best analgesia with A.U.C = 1396.08, which was 2-fold higher than EM-1. HAGPC and GAGDC showed the lower analgesic effect than EM-1 with A.U.C = 569.63 and 630.95, respectively. The analgesic activity of subcutaneously injected GAGPC was evaluated by the use of naloxone. The results are shown in Fig. 3. The results showed that naloxone can completely antagonize the analgesic activity of drugs, we can see that analogue GAGPC is opioid receptor by analgesic effect and mainly through μ-opioid receptor function.

The EM-1 displayed profound antinociceptive effects in neuropathic pain and inflammatory pain via MOR.[5,3,27–30] Antinociceptive effect of analogues after s.c. administration was depended on the number of activated MOR and metabolic stability in the plasma and the ability to cross the BBB. EM-1 expressed short acting antinociceptive activities, due to low metabolic stability. Besides, EM-1 was with the lower ability to penetrate the BBB. Therefor EM-1 didn't exert the desired pharmacological effects by s.c administration.

In the present study, it is our hope to improve the biological activity of EM-1 analogues by combining chemical modifications. The previous results revealed that the increase in serum stability seemed to mainly arise from the replacement of L-Pro^2 by unnatural amino acids (D-Ala as well as D-Pro-Gly).[18] Meanwhile the analogues, replaced of L-Pro^2 by D-Pro-Gly at position 2 and N-terminal guanidino-addition, shown good stability in the brain homogenate.[18] Thus, we try to design analogues containing D-Ala-Gly replace L-Pro at position 2. The EM-1 analogue HAGDC, L-Pro^2 was Substituted by D-Ala-Gly shown the half-lives was 16-fold higher than EM-1 in the15% brain homogenate and 28-fold higher than EM-1 in the plasma. In others studies, the analogue NR-amidino-Tyr-D-Arg-Phe-X with N-terminal Guanidino-addition was shown good pharmacokinetic.[31] In our studies, GAGDC