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Design, synthesis, and evaluation of new endomorphin analogs with enhanced central antinociception after peripheral administration

Xin Liu, Long Zhao, Yuan Wang, Lingyun Mou, Junxian Yang, Yixin Zhang, Dan Wang, Rui Wang *

Department of pharmacology, Key Laboratory of Preclinical Study for New Drugs of Gansu Province, Institute of Biochemistry and Molecular Biology, School of Basic Medical Sciences, School of Life Science, Lanzhou University, Lanzhou 730000, PR China

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ABSTRACT

We synthesized two novel endomorphin-1 (EM-1) analogs by substituting the C-terminus residue with (thienyl)-α-methylene-β-amino acids (Map). Several in vitro and in vivo assays were used to determine the activity of the analogs. The two EM-1 analogs showed subnanomolar binding affinity and functional activity at the μ-opioid receptor in HEK293 cells. Tail-flick and formalin tests further revealed that the EM-1 analogs were very effective after intravenous administration. Our results indicate that compared to endomorphin-1, the (thienyl)Map modified peptides showed improved blood–brain barrier permeability.

The treatment of pain is a universal health problem and it is estimated that more than 1.5 billion people worldwide experience chronic pain. The annual cost of pain treatment is increasing, and therefore, the demands for novel pain medications to improve quality of life are rapidly increasing.[1,2] Opiates have been used for centuries by ancient cultures because of their significant analgesic effects. However, their side effects were soon recognized, which motivated the search for other safer and more effective pain therapies.[3–6]

The three opioid receptors (μ, δ, and κ) are extremely important clinical targets in the treatment of pain, and numerous opiates and endogenous ligands interact with these receptors to produce antinociception.[7,8] In 1997, Zadina discovered the most μ-opioid receptor (MOR) selective endogenous peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EM-1), and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM-2).[9] EMs are known to inhibit pain signals without exhibiting some of the undesirable side effects of opiates.[10,11] However, the clinical application of EMs is still hindered by their short duration of action and inability to cross the blood–brain barrier (BBB).[12–14] Many chemical strategies have been employed to improve the bioavailability of EMs.[4,15–17]

We previously developed a series of structurally constrained amino acids, α-methylene-β-amino acids (Map) and inserted them into the EMs sequence to increase the biological activity of the peptide.[18] Some of the resulting analogs showed improved MOR binding affinities and were more stable than the parent peptide was. Notably, the bioactivity of the analogs was varied by incorporating different side chain groups into the Map.[19] In the present study, we synthesized two new Map residues bearing the thiophene rings [(2-thienyl)Map and (3-thienyl)Map], and two novel EM-1 analogs were obtained by inserting the (thienyl)Map residues into the C-terminus of the peptide. Binding affinity and cAMP accumulation assays were performed to determine the in vitro activity of the EM-1 analogs. Furthermore, the in vivo activity and BBB permeability of the analogs following their peripheral administration were determined using tail-flick and formalin assays.

Peptides containing (thienyl)Map residues were synthesized using manual solution-phase methods with an active ester reaction using EDC/HOBt coupling. The (2-thienyl)Map and (3-thienyl)Map residues were derived from a product obtained by asymmetric addition of *N*-acyl pyrrole phosphonates to *N*-*tert*butyloxycarbonyl imines generated in situ via catalysis by a bifunctional thiourea catalyst (Fig. 1).[20] The crude products were purified using semipreparative reverse-phase high-performance-liquid chromatography (RP-HPLC) and were 95–99% pure, as determined by

Abbreviations: BBB, blood–brain barrier; Boc, *tert*-butyloxycarbonyl; cAMP, cyclic adenosine monophosphate; CNS, central nervous system; DAMGO, H-Tyr-D-Ala-Gly-NMePhe-Gly-ol; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DOR, δ-opioid receptor; DPDPE, Tyr-c(D-Pen-Gly-Phe-D-Pen); ED_{50}, median effective dose; EDC, *N*-ethyl-*N*-(3-(dimethylamino)propyl)-carbodiimide; EM-1, endomorphin-1; EM-2, endomorphin-2; HEK293, human embryonic kidney; HOBt, 1-hydroxybenzotriazole; i.c.v., intracerebroventricular; ip, intraperitoneally; iv, intravenous; Map, α-methylene-β-aminopropanoic acids; MOR, μ-opioid receptor.

* Corresponding author. Tel.: +86 931 8912567.
E-mail address: wangrui@lzu.edu.cn (R. Wang).

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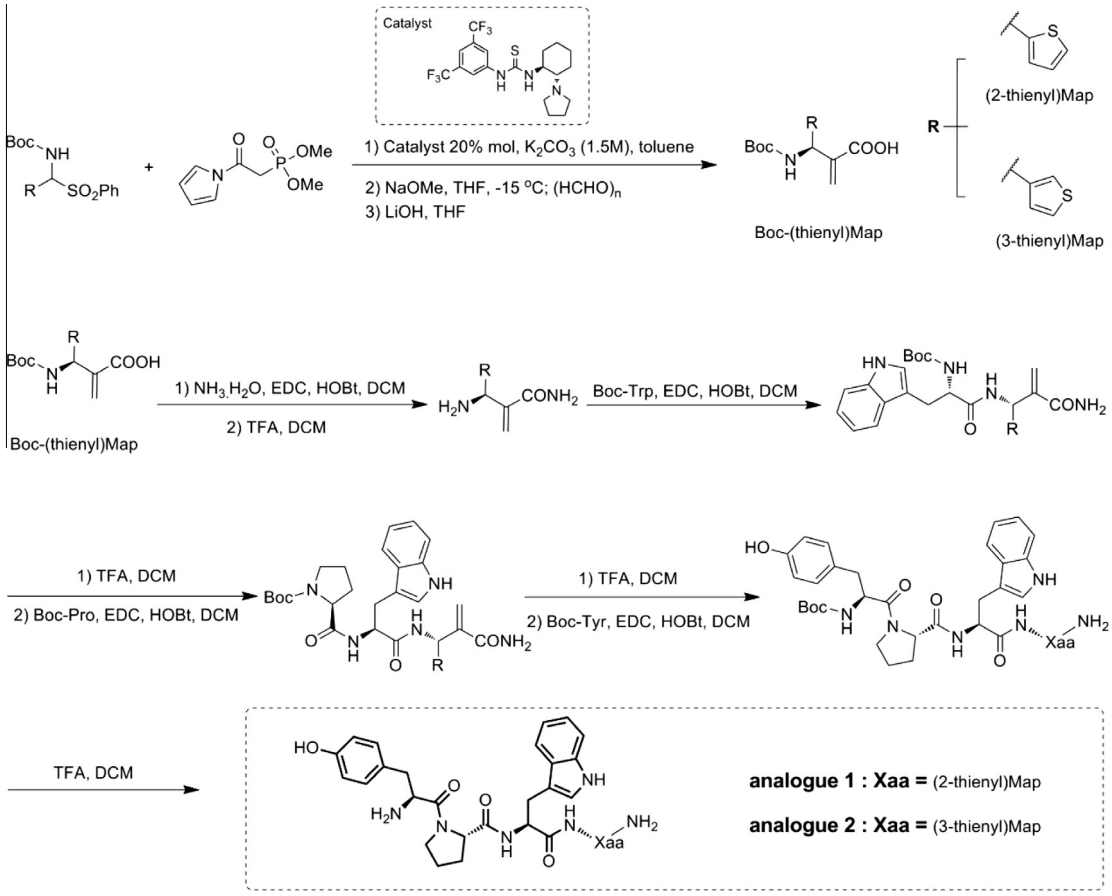

Figure 1. Synthesis and structure of EM-1 analog 1 and analog 2.

Table 1
Analytical data for EM-1 and its analogs

Peptide	Sequence	TOF MS [M+H]$^+$		TLC[a] (R_f)		Mp (°C)	Purity[b] (%)
		Calcd	Found	(I)	(II)		
EM-1	Tyr-Pro-Trp-Phe-NH_2	611	611.2833	0.47	0.61	160–162	98
1	Tyr-Pro-Trp-(2-thienyl)Map-NH_2	629	629.2288	0.55	0.76	135–138	97
2	Tyr-Pro-Trp-(3-thienyl)Map-NH_2	629	629.2518	0.52	0.78	141–143	97

[a] Retention factors on silica gel 60 F254 precoated glass plates; solvent system: (I) ethyl acetate–methanol–ammonia water (30:10:1), (II) acetone–glacial acetic acid–water (8:1:1).
[b] Purity determination based on analytical RP-HPLC.

analytical RP-HPLC. The molecular weight of the peptides was confirmed using an electrospray ionization mass spectrometer (Table 1).

The affinity and selectivity of the peptides were evaluated in whole-cell preparations of HEK293 cells expressing the MOR or δ-opioid receptor (DOR) by using a radioligand binding assay. [^{3}H] DAMGO and [^{3}H] DPDPE were used as MOR and DOR specific radioligands, respectively. Then, the agonist/antagonist profiles of these EM-1 analogs were assessed based on the accumulation of cyclic adenosine monophosphate (cAMP). The nociceptive response[21] was assessed using the warm water tail-flick[22] and formalin tests.[23]

The receptor-binding affinity of the EM-1 analogs was determined using the radioligand binding assay. As shown in Table 2, replacement of Phe^4 with (2-thienyl)Map or (3-thienyl)Map conferred the analogs with high MOR affinity in the subnanomolar range. Compared with the parent peptide, analogs 1 and 2 exhibited about 4.6- and 5.0-fold increased MOR affinity, respectively. In contrast to their increased MOR affinity, the DOR affinity of the two EM-1 analogs was markedly lower. As a result, the analogs showed significantly increased MOR versus DOR selectivity as compared to the EM-1 parent.

The functional behavior of the EM-1 analogs was evaluated based on forskolin-stimulated [^{3}H]cAMP accumulation. Each analog inhibited forskolin-stimulated cAMP production in a concentration-dependent manner. Potency (EC_{50}) and efficacy (E_{max}) values for the EM-1 analogs are summarized in Table 2. The results of functional assays were in agreement with those of the binding assays. Compared to EM-1, analogs 1 and 2 showed greatly increased potencies at the MOR (EC_{50} = 0.0629 and 0.0643 nM, respectively). Their functional activities at the DOR were also measured using the cAMP accumulation assay; however, the EM-1 analogs exhibited no detectable potencies. The results of the radioligand binding and cAMP accumulation assays indicated