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COMMUNICATION

Endomorphin-1 analogs containing α-methyl-β-amino acids exhibit potent analgesic activity after peripheral administration

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This study describes the design and synthesis of endomorphin-1 analogs containing a C-terminal aromatic α-methyl-β-amino acids and an N-terminal native tyrosine or 2,6-dimethyl-tyrosine. We show that, in comparison with the parent peptide, these analogs exhibit improved bioactivity and blood–brain barrier penetration after intravenous administration, and have a lower tendency to induce constipation and sedation than morphine.

Pain significantly affects life quality, requiring the development of safe and efficient pain killers. Despite the ongoing search for new treatments, opioids are still the golden standard for alleviating moderate to severe pain. However, the chronic use of opioids causes undesired side effects, including respiratory depression, constipation, tolerance development, and addiction.[1-3] Opioid drugs primarily exert their analgesic effect by binding to μ- (MOP), δ- (DOP) and κ-opioid receptors (KOP). Among them, MOP is the main pharmacological target for alleviating pain, with a large number of available opioid analgesics binding to it.[4,5] As alternative pain killers, endogenous opioid peptides exhibit better side-effect profiles, opening up new possibilities for analgesic drug development.[6-9] Endomorphins (EMs) are opioid peptides isolated from bovine brain and human frontal cortex, which have attracted the attention of peptide chemists/pharmacologists,[10,11] due to their high MOP affinities and specificities as compared with those for DOP and KOP. Furthermore, EMs are believed not to exhibit some of the undesirable side effects of morphine. However, similar to other peptides, EMs have poor metabolic stability and are unable to cross the blood–brain barrier (BBB), with peripheral EMs administration thus producing almost no detectable analgesic effects.[12,13] Thousands of EM analogs have been synthesized to understand the corresponding structure-activity relationships (SARs) and thus improve their pharmacological profiles,[13-18] with β-amino acid substituted opioid peptides attracting increased attention due to their improved metabolic stability, remarkable physicochemical properties and bioactivities.[19-24] As a subclass of β-amino acids, $\beta^{2,3}$-amino acids are important building blocks of natural products, which makes their incorporation into opioid peptides a practical strategy for bioactivity and bioavailability improvement.[25-28] Previously, we have developed a series of novel $\beta^{2,3}$-amino acids (2-methylene-3-aminopropanoic acids) containing a methylene group at the C_α position, with SAR studies demonstrating that their incorporation at the C-terminus of endomorphin-1 (EM-1) increases the biological activity of the corresponding peptides.[29]

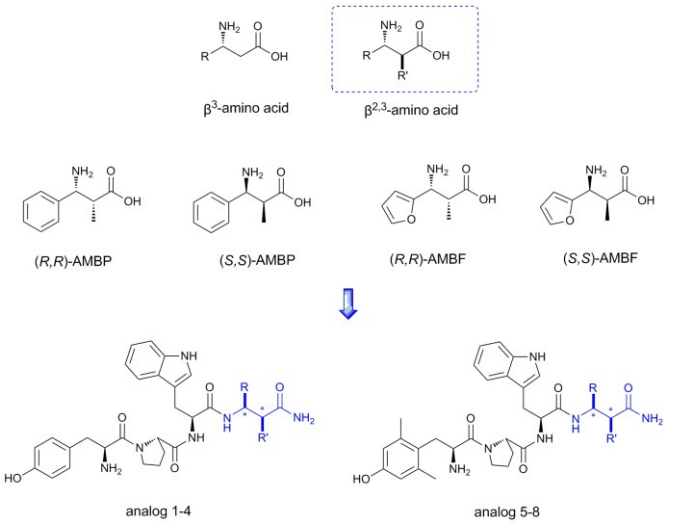

Figure 1. Unnatural amino acids and structure of EM-1 analogs.

Herein, we describe the synthesis and pharmacological evaluation of novel EM-1 analogs containing α-methyl-β-phenylalanine (AMBP) or α-methyl-β-furylalanine (AMBF) by investigating the influence of introducing these residues at C-terminus and determining the binding affinity and functional activity of the synthesized analogs. *In vivo* activities after

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Table 1. Analytical Data for EM-1 Analogs.

no.	sequence	TOF MS $[M+H]^+$		retention time[a] (min)	mp (°C)	purity[b] (%)
		calcd	found			
analog 1	H-Tyr-Pro-Trp-(*R*,*R*)-AMBP-NH_2	625.3094	625.3192	17.1	133-135	98
analog 2	H-Tyr-Pro-Trp-(*S*,*S*)-AMBP-NH_2	625.3094	625.3045	17.0	129-133	97
analog 3	H-Tyr-Pro-Trp-(*R*,*R*)-AMBF-NH_2	615.2886	615.2964	17.9	138-140	96
analog 4	H-Tyr-Pro-Trp-(*S*,*S*)-AMBF-NH_2	615.2886	615.2834	18.0	131-133	99
analog 5	H-Dmt-Pro-Trp-(*R*,*R*)-AMBP-NH_2	653.3407	653.3489	17.5	149-152	99
analog 6	H-Dmt-Pro-Trp-(*S*,*S*)-AMBP-NH_2	653.3407	653.3501	17.5	152-155	99
analog 7	H-Dmt-Pro-Trp-(*R*,*R*)-AMBF-NH_2	643.3199	643.3255	17.4	143-147	98
analog 8	H-Dmt-Pro-Trp-(*S*,*S*)-AMBF-NH_2	643.3199	643.3284	17.3	151-154	98

[a] t_R 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.

intracerebroventricular (i.c.v.) and intravenous (i.v.) administration were evaluated by tail-flick and formalin test, and BBB permeability was determined using a peripherally restricted opioid antagonist. Furthermore, the effects of the above analogs on locomotor and gastrointestinal functions were evaluated (experimental details are provided in the Supplementary Information).

The (*S*,*S*)/(*R*,*R*)-AMBP and (*S*,*S*)/(*R*,*R*)-AMBF (Fig. 1) were obtained by addition of titanium enolates to *tert*-butane-sulfinyl imine,[30,31] and subsequently incorporated into EM-1 to afford analogs 1-8. Other non-natural amino acids were sourced from commercially suppliers. All compounds were prepared by solution-phase synthesis, with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and hydroxybenzotriazole (HOBt) used for peptide coupling (experimental details are provided in the Supplementary Information).[32] Crude peptides were purified by semi-preparative reversed-phase high-performance liquid chromatography (RP-HPLC) and identified by electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS), with their purities determined as >95%. Detailed properties of the above analogs are provided in Table 1.

A radioligand binding assay was employed to examine the binding affinities and receptor selectivities of the synthesized peptides in whole-cell preparations from HEK293 cells, which stably expressed MOP, DOP or KOP. [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]U69,593 were used as the radioligand for MOP, DOP and KOP, respectively. The results are summarized in Table 2. Analogs 1–4 exhibited significant MOP affinities, with the corresponding K_i values ranging from 6.3 to 21.2 nM. Among these four species, analogs 2 and 4 containing (*S*,*S*)-AMBP[4]/AMBF[4] exhibited higher MOP binding affinities than their (*R*,*R*)-stereoisomers did. Replacement of Phe[4] with AMBP[4]/AMBF[4] resulted in decreased DOP binding affinity and thus greatly increased MOP selectivity. Subsequent replacement of Tyr[1] with 2, 6-dimethyl-Tyr[1] (Dmt[1]) afforded analogs 5–8 with subnanomolar MOP affinities. Specifically, analog 8 exhibited the highest MOP affinity among all synthesized compounds, corresponding to a K_i value of 0.12 nM. Although analogs containing Dmt[1] exhibited increased DOP affinities, their MOP-over-DOP selectivity was still acceptable. Interestingly, Dmt[1] substitution was reported to enhance affinity to all three opioid receptor subtypes, resulting in decreased selectivity.[14,33] All synthesized analogs exhibited very low KOP binding affinities.

The functional activity profiles of EM-1 analogs were assessed by an accumulation of cyclic adenosine monophosphate (cAMP) assay using MOP-expressing HEK293 cells. The tested analogs inhibited forskolin-stimulated cAMP in a dose-dependent manner, as shown in Table 2, with cAMP accumulation assay results being in good agreement with those of the radioligand binding assay. Analogs 1–4 exhibited potencies between 10.5 and 22.9 nM, whereas combination of Dmt[1] and AMBP[4]/AMBF[4] substitution produced analogs 5–8 with improved subnanomolar-range potencies. The cAMP accumulation assay indicated that all investigated species behaved as MOP agonists, with the highest potency observed for analogs 7 and 8.

The metabolic stability of EM-1 and its analogs was assessed in mouse brain homogenate, with the corresponding half-lives listed in Table 2. While EM-1 showed a short half-life of 16.9 min, substitution of Phe[4] with AMBP[4]/AMBF[4] resulted in improved stability, with analogs 1–4 exhibiting half-lives ranging from 112 to 142 min. Moreover, subsequent Dmt[1] substitution increased the metabolic stability even further (analog 5-8), e.g. analog 5 exhibited a ~16-fold enhanced metabolic stability compared with that of EM-1. Thus, insertion of unnatural amino acids into EM-1 probably impedes protease recognition of peptide cleavage sites. [34]

In vivo antinociceptive effects of analog 7 and 8 were further evaluated in the mouse tail-flick test after i.c.v. administration and expressed as a percentage of maximum possible effect (%MPE). As shown in Figure 2a–2c, all analogs exhibited dose- and time-dependent inhibition of heat-induced analgesia and exhibited better antinociceptive activities than the parent peptide did. The ED_{50} value of EM-1 equalled 0.304 nmol,[29] whereas analog 7 and 8 were

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Table 2. Opioid Receptor Binding Affinities, Functional Activity and Half-Lives of EM-1 and its Analogs.

No.	Binding Affinities[a]				Functional Activity[e]		Half-lives (min)
	K_i^{μ} (nM)[b]	K_i^{δ}(nM)[c]	K_i^{κ} (nM)[d]	K_i ratio (μ/δ)	EC_{50}(nM)	E_{max} (%)	
EM-1	2.60 ± 0.21	6080 ± 640	>10000	1/2338	14.4 ± 0.6	83.1 ± 4.1	16.9 ± 2
analog 1	21.2 ± 3.71	>10000	>10000	-	22.9 ± 1.14	68.9 ± 3.3	112 ± 9
analog 2	7.7 ± 1.94	>10000	>10000	-	18.8 ± 2.01	70.1 ± 5.4	142 ± 11
analog 3	9.3 ± 2.58	>10000	>10000	-	13.7 ± 1.42	72.7 ± 6.2	133 ± 12
analog 4	6.3 ± 0.31	>10000	>10000	-	10.5 ± 0.67	79.2 ± 5.2	127 ± 8
analog 5	0.75 ± 0.03	551 ± 38	>10000	1/735	0.28 ± 0.039	88.2 ± 7.3	276 ± 21
analog 6	0.31 ± 0.02	273 ± 12	>10000	1/881	0.23 ± 0.026	90.9 ± 4.2	254 ± 15
analog 7	0.47 ± 0.02	297 ± 14	>10000	1/632	0.16 ± 0.021	90.6 ± 3.9	163 ± 7
analog 8	0.12 ± 0.008	165 ± 3.4	>10000	1/1375	0.12 ± 0.011	93.1 ± 2.7	202 ± 6

[a] Displacement was done using whole cell preparations from transfected HEK293 cells expressing μ-opioid receptor, δ-opioid receptor or κ-opioid receptor, respectively. [b]Displacement of [^{3}H]DAMGO (K_d = 0.6 nM, μ-selective). [c] Displacement [^{3}H]DPDPE (K_d = 2.8 nM, δ-selective). [d] Displacement [^{3}H]U69,593 (K_d = 2.9 nM, κ-selective). K_i values were calculated according to the Cheng−Prusoff equation: $K_i = EC_{50}/(1 + [ligand]/K_d)$, where the shown K_d values were taken from isotope saturation experiments. Data are expressed as the mean ± SEM, each performed in triplicate. [e]Effects of peptides on forskolin stimulated cyclic AMP accumulation by μ opioid receptor. HEK293 cells expressing MOR were stimulated with increasing concentrations of the indicated peptides. EC_{50} and E_{max} values were calculated by using the GraphPad Prism software. Data are expressed as the mean ± SEM, each performed in triplicate.

significantly more potent, showing ED_{50} values of 0.019 (0.006−0.036) and 0.016 (0.005−0.032) nmol, respectively. Pretreatment of opioid antagonist naloxone (Nlx, 0.2 nmol, i.p.) significantly reduced the activities of tested compounds, confirming the central role of the opioid system in determining the effect. In addition, pretreatment with a MOP antagonist β-funaltrexamine (β-FNA, 0.2 nmol, i.c.v.) significantly reduced the analgesic activity of EM-1 analogs, indicating that they mainly targeted the MOP (Figure 2D). Thus, these results were in good agreement with binding affinity and functional activity assays.

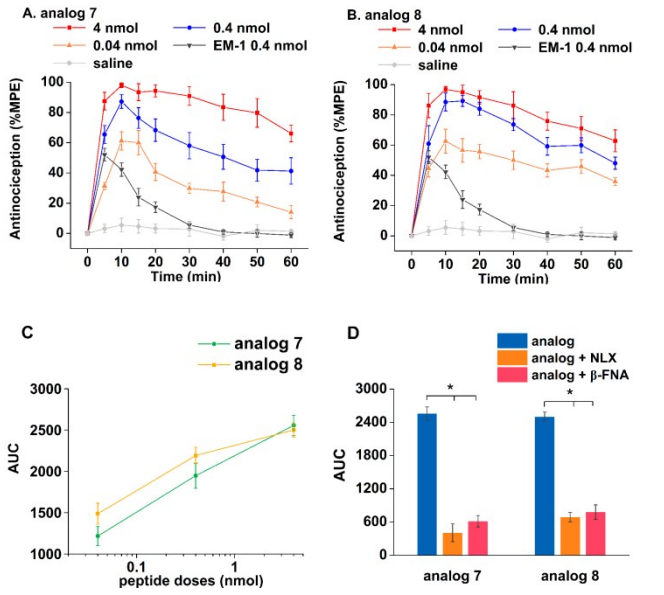

Figure 2. Tail-flick test versus time curves of the antinociceptive effect of analog 7 (A) and analog 8 (B) after i.c.v. administration. Dose-response curves for the analgesic effects were presented as AUC data over the period 0 to 30 min (C). The antinociceptive effect induced by analogs after injection of naloxone (i.p.) and β-FNA (i.c.v.) (D). Each value represents the mean ± SEM for 8−12 mice. The asterisk indicates that the response is significantly different from control ($p < 0.05$).

The antinociceptive effect of EM-1 analogs was tested upon peripheral administration by i.v. injection and was re-examined using the mouse tail-flick test. As shown in Figure 3a−3c, the tested analogs exhibited potent antinociceptive activities, with ED_{50} values for analogs 7 and 8 equalling 0.089 (0.048−0.13) and 0.016 (0.009−0.024) mg, respectively. Conversely, EM-1 displayed no obvious analgesic effect under the same conditions. Naloxone methiodide (Nlx-M), a peripherally restricted opioid antagonist, was used to determine the action site of the tested analogs. Pretreatment with Nlx-M (0.2 nmol, i.c.v.) significantly reduced the antinociceptive effect of the above two compounds, whereas the i.v. injection (0.2 mg) of this antagonist did not attenuate their activity (Figure 3d). These results indicate that analogs 7 and 8 can penetrate the highly selective BBB and elicit their activity in the central nervous system (CNS). The promising pharmacological properties of EMs are offset by their lack of activity upon peripheral administration and short action duration, which limits their potential clinical usage. This work, relying on the use of AMBP/AMBF as a building block, indicated that incorporation of $\beta^{2,3}$-amino acids may improve the BBB penetration ability of peptides, in agreement with our previous reports.[35] In addition, the multiple site modification method used in this study may serve as a useful strategy for future EM optimization: the aromatic $\beta^{2,3}$-amino acids substituted at the C-terminus afford the peptide with BBB permeability, and the Dmt[1] replacement at the N-terminus provids increased binding affinity and functional activity.

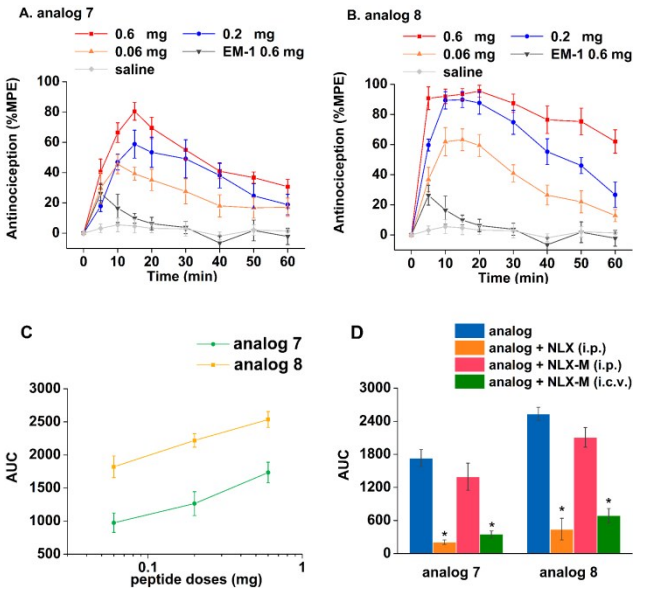

Figure 3. Tail-flick test versus time curves of the antinociceptive effect of analog 7 (A) and analog 8 (B) after i.v. administration. Dose-response curves for the analgesic effects were presented as AUC data over the period 0 to 30 min (C). The antinociceptive effect induced by analogs after injection of naloxone (i.p.) and naloxone methiodide (i.p. and i.c.v.). Each value represents the mean ± SEM for 8−12 mice. The asterisk indicates that the response is significantly different from control ($p < 0.05$).

The warm water tail flick assay was used to test the acute pain-killing activity, with analog 7 and 8 subsequently evaluated in the formalin test (persistent pain model). As shown in Figure 4, analog 8 exhibited higher analgesic activity upon intravenous administration than analog 7 did in both first (0-5 min) and second phase (15-30 min) of formalin test, with the respective ED_{50} values in phase I equalling 0.021 (0.004−0.042) and 0.11 (0.07−0.151) mg, and ED_{50} values in phase II equalling 0.013 (0.002−0.029) 0.061 and (0.041−0.08) mg, respectively.

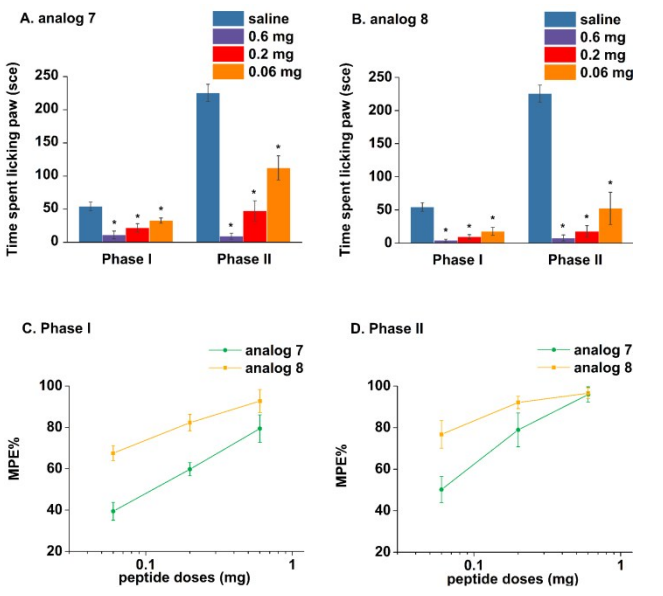

Figure 4. Dose-related analgesic effects of i.v. administration of analog 7 (A) and analog 8 (B) in the formalin test. Dose-response curves for the analgesic effects induced by i.v. administration the tested analogs at different doses in the phases I (C) and phases II (D). Each value represents the mean ± SEM for 8-12 mice. The asterisk indicates that the response is significantly different from the saline-treated group ($p < 0.05$).

Although previous reports showed that the introduction of β-Phe/β-homo-Phe into EM-1 results in low MOP affinity,[20,21] the current work indicates that additional methyl group modification at the α-carbon of the β-amino acid imparts nanomolar MOP activity and improved bioavailability after peripheral administration. Besides, it is reported that incorporation of α-hydroxy-β-phenylalanine into EMs afforded peptides with prolonged stability and varied activity toward opioid receptors.[27] Thus, these findings suggest that tuning the C_α site structure of β-amino acids may afford EM analogs with improved pharmacological profiles.

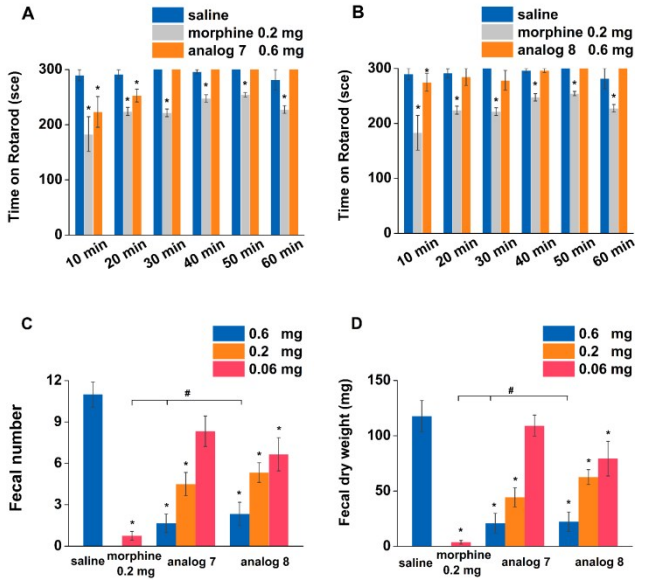

Figure 5. The effect of i.v. administrations of analog 7, analog 8 and morphine on the locomotor activity in the rotarod test, and are expressed as the endurance time (A and B). The effect of i.v. administrations of analog 7, analog 8 and morphine on the fecal number (C) and fecal dry weight (D). Data are the mean ± SEM of 8–12 mice. The asterisk indicates that the response is significantly different from control group ($p < 0.05$).

Short-term side effects of analogs 7 and 8 were examined and compared with those of morphine, using rotarod test to determine the locomotor behaviour of mice after i.v. administration of these compounds. Equal antinociceptive doses affording MPE >95% were used for morphine (0.2 mg) and EM-1 analogs (0.6 mg). As shown in Figure 5, morphine administration significantly impaired the motor function throughout the course of the examination, in contrast, analogs 7 and 8 only interfered with motor function up to 20 min and 10 min after administration, respectively. These results indicated that the analogs exhibited a lower tendency to induce sedation effect. Since opioid-induced constipation (OIC) is a common side effect of classical opioids, the

propensity of the synthesized analogs to induce OIC was determined by assessing the fecal pellet output of mice, showing that their i.v. administration reduced the pellet number and dry weight in a dose-dependent manner. Compared with morphine (0.2 mg), analogs 7 and 8 (0.6 mg) exhibited a significantly lower influence on gastrointestinal mobility.

In summary, a number of EM-1 analogs containing aromatic $\beta^{2,3}$-amino acids were synthesized and characterized by several *in vitro* and *in vivo* assays, which showed that such modification can improve the bioactivity and bioavailability of these peptides. The prepared EM-1 analogs were effective in alleviating pain after peripheral administration, with *in vivo* assays employing naloxone methiodide showing that they may elicit their antinociceptive effect in the CNS. Additionally, the above analogs displayed a low propensity to induce locomotor impairment and constipation. Thus, this study demonstrates the potential application of aromatic $\beta^{2,3}$-amino acids in EMs optimization, with their further modification possibly affording effective novel building blocks for the development of EMs compounds with better pharmacological profiles.

Acknowledgments

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