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Endomorphin 1[ψ] and endomorphin 2[ψ], endomorphins analogues containing a reduced (CH_2NH) amide bond between Tyr^1 and Pro^2, display partial agonist potency but significant antinociception

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Abstract

Endomorphin 1 (EM1) and endomorphin 2 (EM2) are highly potent and selective μ-opioid receptor agonists and have significant antinociceptive action. In the μ-selective pocket of endomorphins (EMs), Pro^2 residue is a spacer and directs the Tyr^1 and Trp^3/Phe^3 side chains into the required orientation. The present work was designed to substitute the peptide bond between Tyr^1 and Pro^2 of EMs with a reduced (CH_2NH) bond and study the agonist potency and antinociception of EM1[ψ] (Tyr[ψ(CH_2NH)]Pro-Trp-Phe-NH_2) and EM2[ψ] (Tyr[ψ(CH_2NH)]Pro-Phe-Phe-NH_2). Both EM1[ψ] and EM2[ψ] are partial μ opioid receptor agonists showing significant loss of agonist potency in GPI assay. However, EMs[ψ] exhibited potent supraspinal antinociceptive action in vivo. In the mice tail-flick test, EMs[ψ] (1, 5, 10 nmol/mouse, i.c.v.) produced potent and short-lasting antinociception in a dose-dependent and naloxone (1 mg/kg) reversed manner. At the highest dose of 10 nmol, the effect of EM2[ψ] was prolonged and more significant than that of EM2. In the rat model of formalin injection induced inflammatory pain, EMs[ψ] (0.1, 1, 10 nmol/rat, i.c.v.), like EMs, exerted transient but not dose-dependent antinociception. These results suggested that in the μ-selective pocket of EMs, the rigid conformation induced by the peptide bond between Tyr^1 and Pro^2 is essential to regulate their agonist properties at the μ opioid receptors. However, the

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increased conformational flexibility induced by the reduced (CH_2NH) bond made less influence on their antinociception.

Keywords: Endomorphins; Analogues; Reduced amide bond; Antinociception; Intracebroventricular; Tail-flick; Formalin-model

Introduction

Endomorphin 1 (EM1, Tyr-Pro-Trp-Phe-NH_2) and endomorphin 2 (EM2, Tyr-Pro-Phe-Phe-NH_2) are two endogenous opioid peptides with high affinity and selectivity for μ-opioid receptors (Zadina et al., 1997). Since the μ opioid receptors mediate the most prominent pharmacological effects of morphine, the discovery of EM1 and EM2 in the mammalian brain (Hackler et al., 1997; Zadina et al., 1997, 1999) has encouraged the application of natural and synthetic peptides as analgesics instead of morphine (Cardillo et al., 2000; Czapla et al., 2000; Foran et al., 2000; Wilson et al., 2000). Indeed, both EM1 and EM2 exhibit potent antinociceptive effects in acute, inflammatory and neuropathic pain models without some of the undesirable side effects of morphine (Carrigan et al., 2000; Czapla et al., 2000; Przewlocka et al., 1999; Sakurada et al., 2002; Soignier et al., 2000; Vaccarino et al., 1999; Wilson et al., 2000). However, EMs as well as opioid peptides in general, have a limited in vivo efficacy, since they are easily degraded by different proteases (Péter et al., 1999; Shane et al., 1999; Szatmari et al., 2001; Tömböly et al., 2002). To resolve these problems, the structure-activity study of EMs and the search for new peptidomimetics or more stable peptide analogues are of particular interest (Cardillo et al., 2002; Keller et al., 2001; Leitgeb et al., 2003; Okada et al., 2000; Paterlini et al., 2000). NMR spectroscopy and molecular modeling have indicated that proline which fixes the peptide shape and induces the other residues to assume the proper spatial orientation for ligand-receptor interaction, is a key residue and has a stereochemical spacer role in EMs (Leitgeb et al., 2003; Okada et al., 2000; Paterlini et al., 2000; Podlogar et al., 1998; Yang et al., 1999). Replacement of Pro^2 with other amino acids produced a vastly exchanged receptor profile, such as TIPP (Tyr-Tic-Phe-Phe-OH) and TAPP (Tyr-D-Ala-Phe-Phe-NH_2). The L-configuration of Pro was considered vital for μ opioid activity and selectivity (Cardillo et al., 2000, 2002; Okada et al., 2000; Paterlini et al., 2000). Stereochemical inversion of Pro resulted in drastic loss of activity in [D-Pro^2]EM1 (Paterlini et al., 2000) and [D-Pro^2]EM2 (Huo et al., 2001). In EMs, the Tyr-Pro peptide bond exists as a mixture of the cis and trans isomers (Paterlini et al., 2000; Keller et al., 2001). The amino acid side chains in EMs exhibit considerable conformational flexibility (Tömböly et al., 2004), this flexibility probably makes EMs and some of their analogues interact with μ opioid receptor in a similar way (Cardillo et al., 2002). Previous studies were designed to constrain the conformational mobility of the Tyr^1 and Pro^2 side chain to investigate their bioactive conformation (Keller et al., 2001; Olma and Tourwé, 2000; Harrison et al., 2003). However, the Pro residue at second position of EMs is a rigid spacer that fixes the conformation of the other residues (Cardillo et al., 2002). The conformational importance of this combined flexible-rigid N-terminal dipeptide is not well known. The reduced (CH_2NH) amide bond is a useful peptide bond surrogate in the design of opioid mimetics because it could enhance conformational flexibility and metabolic stability (Chen et al., 2002b; Schiller et al., 1999). In the present work, using this

pseudopeptide bond surrogate, we synthesized two new tetrapeptides EM1[ψ] (Tyr[ψ(CH_2NH)]Pro-Trp-Phe-NH_2) and EM2[ψ] (Tyr[ψ(CH_2NH)]Pro-Phe-Phe-NH_2) containing the same residue sequence as the parent EMs. We tested their agonist potency and antinociceptive activity and the formed conclusions concerning the possible conformational importance of this peptide bond between Tyr^1 and Pro^2 in EMs.

Materials and methods

Chemistry

Peptides were synthesized by solid-phase method on a p-methylbenzhydrylamine resin (MBHA) using tert-butyloxycarbonyl (Boc)-protected amino acids (Sigma Chemical Co.) and dicyclohexylcarbodiimide (DCC)/1-hydroxybenzotriazole (HOBT) as coupling agent. Introduction of the (CH_2NH) peptide bond isostere was accomplished in solid phase. The free N-terminal amino group of the resin-bound peptide was reductively alkylated by the requisite Boc-protectd α-aminoaldehyde in the presence of sodium cyanoborohydride ($NaBH_3CN$) in DMF containing 1% AcOH. The aldehydes Boc-Tyr-H was prepared by $LiALH_4$ reduction of their corresponding N,O-dimethy hydroxamates at 0–10°C in dried THF (Sasaki and Coy, 1987). Peptides were deprotected by 33% TFA/DCM and cleaved from the resin by HF/anisole. The crude peptides were obtained in solid form by reversed-phase HPLC on a DELTA PAK C_{18} column (Waters, 15u C_{18} 300 Å 7.8 × 300mm) with a linear gradient of 10–100% B in 60 min at flow rate of 3 ml/min (A = 0.05% trifluroacetic acid in water and B = acetonitrile). Each peptide was >95% pure as determined by analytical reverse-phase HPLC on a DELTA PAK C_{18} column (Waters, 5u 300Å 3.9 × 150mm). The identity of purified peptides was verified by mass spectrometry (Mariner 5074). Naloxone hydrochloride was purchased from Sigma Chemical Co.

Animals

Male ddY mice (30–35 g) were used for the preparation of MVD. The analgesic effects of EMs and EMs[ψ] were studied in ddY mice weighing 18–22 g and Wistar rats (250–300 g) in vivo. These animals were supplied by the Animal Center of Lanzhou Medical College. Guinea pigs (400–600 g) were obtained from National Institute of the Biological Products (Gansu, China). They had free access to food and water in an animal room which was maintained at 22 ± 1°C with a 12 h light/dark cycle. Each animal was used only once. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

In vitro experiments

Opioid activity of the compounds was tested in the GPI and MVD bioassays as reported previously (Xu et al., 2003). Agonists (EMs and EMs[ψ]) were evaluated for their ability to inhibit

the electrically evoked twitch. Evaluation of a possible antagonist component of EMs[ψ] was accomplished by incubating the peptide (1 μM) for 15min with the preparation before testing with EMs in the GPI assay. The twitch intention was recorded isometrically via a force-displacement transducer (JZ101 muscle force transducer, Xinhang, HeBei, China) connected to a computer with Bl-420E system (TAIMENG, Chengdu, China). The results are expressed as the IC_{50} values obtained from concentration-response curves. Reported IC_{50} values represent the mean (± S.E.M.) of not less than eight tissue samples.

Analgesic test

Tail flick test

The acute nociceptive sensitivity was assessed by the tail-flick technique described by Chen et al (Chen et al., 2002a). Drugs were administrated in a volume of 5 μl at a constant rate of 10 μl/min. Proper injection site was verified in pilot experiments by administration and localization of methylene blue dye. Effects of drugs were tested with tail-flick reflex every 5 min for 10–30 min post-injection and every 10 min for 30–60 min post-injection. (47.5°C water, cut-off time: 15 s). Analgesic latencies were converted to percentage maximum possible effects (MPE%) by using the formula MPE% = [(observed latency-baseline latency)/(cut-off time-baseline latency)] × 100%. Naloxone (1 mg/kg, s.c.) was pre-injected respectively 5 min before the i.c.v. administration of EMs[ψ].

Inflammatory pain test

Wistar rats, anesthetized with chloral hydrate (350 mg/kg, i.p.), were implanted with i.c.v. permanent PE 10 catheters for drug administration. Coordinates for i.c.v. implantation were the following (Chen et al., 2001): 1.5 mm lateral and posterial to bregma, 4.5 mm below the skull surface. Cannulae were fixed in place with acrylic dental cement and one skull screw. After surgery, rats were individually housed for 4 days to allow for recovery, and only animals not showing neurological or motor deficits during this period of observation were used for the experiments.

The rats were placed in a clear glass box (width: 50 cm; length: 100 cm; height: 60 cm) and allowed to habituate for 30 min before testing. After that, the rats were lightly anesthetized with ether and drugs were administrated in a volume of 10 μl in 5 min. 150 μl of a 10% formalin solution was injected subcutaneously (s.c.) into the dorsal surface of the right hind paw respectively 10 min after the beginning of administrating of drugs (Przewlocki et al., 1999). The rat was then placed in the box for observation the time spent on touchdown, lifting, shaking or licking of the injected paw every 5 min. Since these behaviors represented different degrees of injury, the antinociceptive effects of drugs were converted to pain identify score (PIS) by using the formula PIS = (touchdown time + lifting time × 2 + shaking or licking time × 3) /300. The time was measured for 60 min for each animal and was finally scored for two characteristic time points: 0–5 min (first phase) and 20–40 min (second phase) after formalin administration. Control animals

were injected i.c.v. with sarline and were tested according to the same time schedule as experimental groups.

Statistics

All results are expressed as mean ± S.E.M. One-way ANOVA, Bonferroni test for data were used as appropriate to evaluate the statistical significance of differences with a computer program (SPSS 11.0). P values less than 0.05 were considered to indicate statistical significance.

Results

Agonist potency of GPI and MVD assay

As shown in Table 1, EMs had significant μ-opioid receptor agonist potency in agreement with a previous report (Huo et al., 2001). Compared to EMs, both EM1[ψ] and EM2[ψ] behaved as partial μ-opioid receptor agonists. The maximal inhibition of the electrically evoked contractions that could be achieved amounted to 15% of EM1[ψ] and 25% of EM2[ψ] respectively, at concentration of 10 μM. Furthermore, EMs[ψ] were not antagonists in the GPI assay as they were unable to shift the dose-response curve of EMs. Compared to parent EMs, EM1[ψ] and EM2[ψ] showed significantly reduced μ-receptor agonist potency in GPI assay, 3.2 and 2.1 fold reduced δ agonist potency in MVD assay respectively. The selectivity of agonist potency of EMs[ψ] turned to δ-receptor.

The antinociceptive effects of EMs[ψ] and EMs in tail-flick test

I.c.v. administration of EMs induced significant dose-dependent (0.1, 1, 10 nmol/mouse) analgesic response with the most potent effect at 15 min after the injection. The ED_{50} values were 2.75 nmol for EM1 and 3.76 nmol for EM2. Both EM1[ψ] and EM2[ψ] (1, 5, 10 nmol/mouse) dose-dependently increased the tail-flick latency with ED_{50} of 8.13 nmol and 3.65 nmol, respectively. The most potent effect was observed at 20 min. EM2[ψ] at the highest doses (10 nmol) had a more significant effect and longer time interval than EM2. The antinociceptive effects of EMs[ψ] at the highest dose (10 nmol) were antagonized by naloxone (1 mg/kg, s.c.), demonstrating that the effects were mediated by opioid-receptor (Figs. 1 and 2).

Table 1
GPI and MVD assay data of EMs and EMs[ψ]

Peptides	IC_{50} ± SE (nM)[a]		Ratio	
	GPI (μ)	MVD (δ)	MVD/GPI	GPI/MVD
EM1	3.41 ± 0.3	47.40 ± 9.75	13.9	0.072
EM2	5.68 ± 1.44	23.30 ± 6.57	4.102	0.244
EM1[ψ]	>10000 (<15%)	151.94 ± 53.25	<0.0151	>65.82
EM2[ψ]	>10000 (<25%)	49.51 ± 15.63	<0.0050	>201.98

[a] Values are the mean of 8 experiments ± S.E.M.

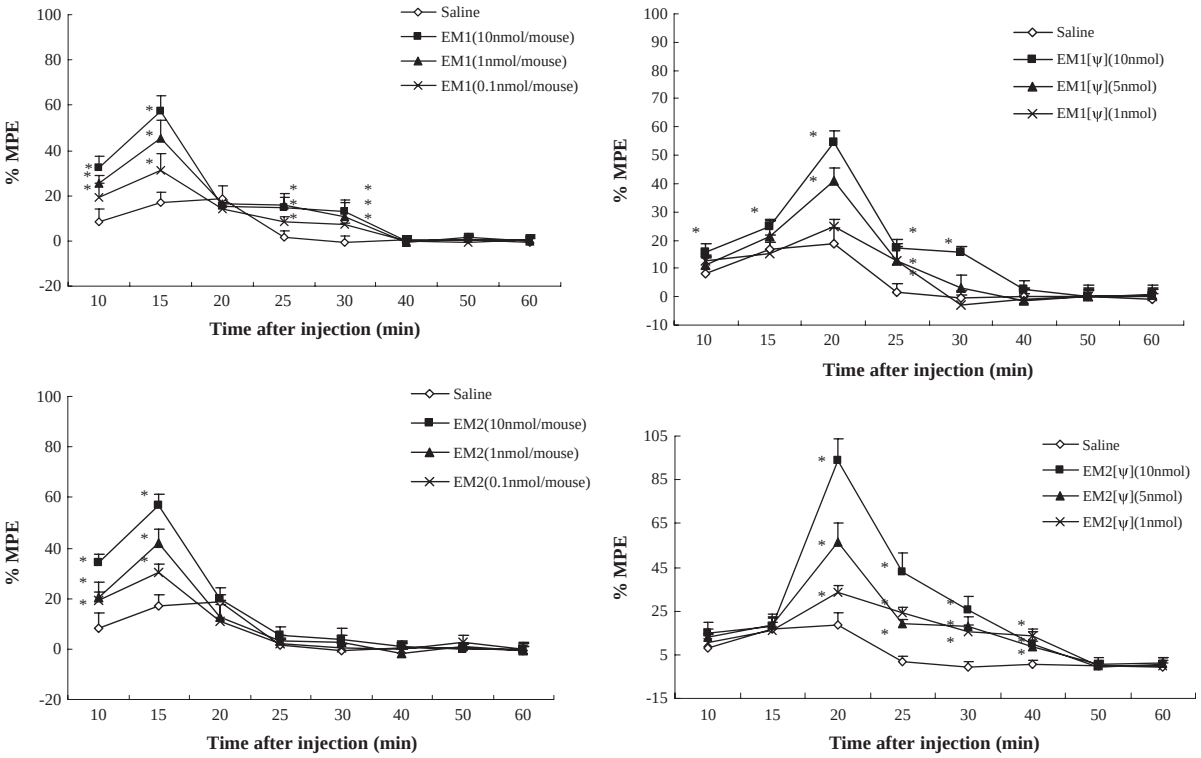

Fig 1. The antinociceptive effect of EMs and EMs[ψ] on the nociceptive threshold measured by tail-flick test in mice. The results are presented as % of the maximal positive effect (MPE). 8–10 animals were used at each dose level. Asterisk (*) represent significant results ($P<0.05$) in comparison with the control.

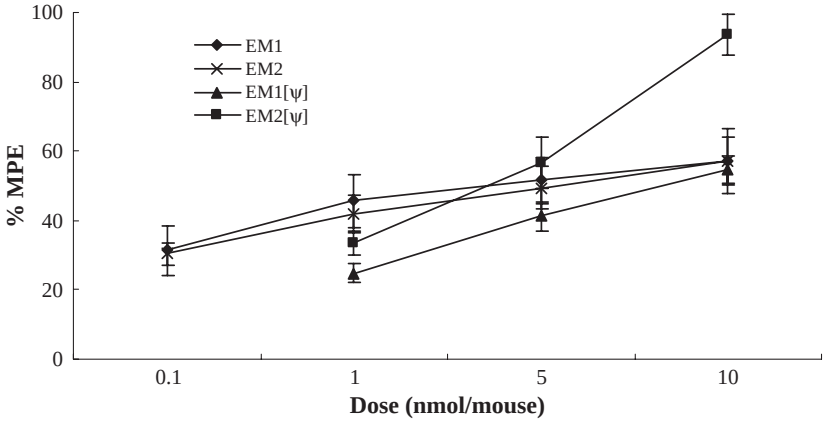

Fig 2. The analgesic dose-response curves of i.c.v. administration of EMs and EMs[ψ]. %MPE was calculated from the peaks of individual time-response curves. 8–10 animals were used at each dose level.

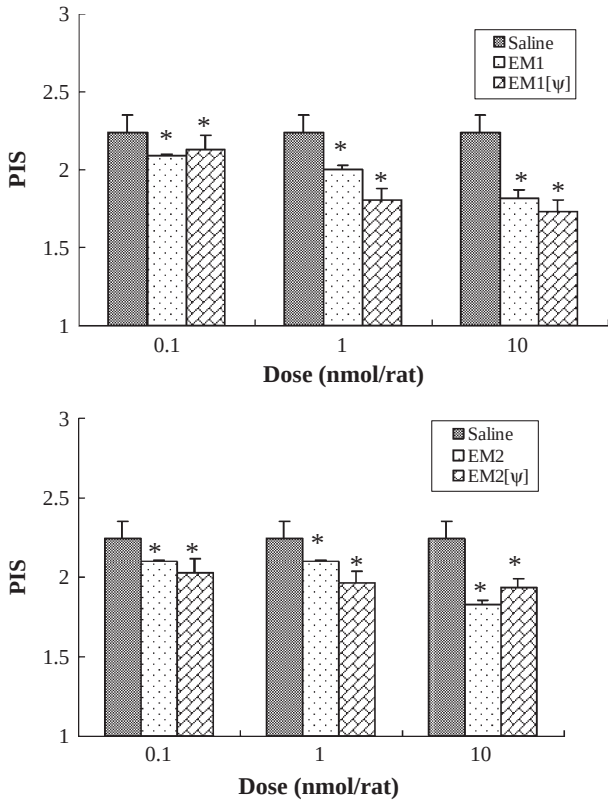

Fig 3. The antinociceptive effect of EMs and EMs[ψ] on formalin induced first phase pain. The results are presented as % of the pain identical score (PIS) of the formalin-injected paw for the first 5 min interval after formalin injection. 8-10 animals were used at each dose level. Asterisk (*) represent significant results ($P<0.05$) in comparison with the control.

The antinociceptive effects of i.c.v. injection of EMs[ψ] and EMs in formalin test

The formalin-induced pain-related behavior was significantly, but not dose-dependently, reduced by both EMs[ψ] (0.1, 1, 10 nmol/rat.) and EMs (0.1, 1, 10 nmol/rat) in the first phase (0–5 min period after formalin injection, Fig. 3). No significant effect was observed in second phase (data not shown).

Discussion

The introduction of a reduced (CH_2NH) bond between Tyr^1 and Pro^2 in EMs caused a significant loss of agonist potency in GPI assay compared with parent EMs. These results can be explained by considering that the rigid conformation caused by peptide bond between Tyr^1 and Pro^2 contributes to EMs bioactivity. Thus the enhanced conformational flexibility introduced by the reduced (CH_2NH) bond is detrimental for ligand-receptor fit. NMR data suggested that in [D-Pro^2]EM1 the loss of activity upon inversion of chirality at Pro occurred as a result of the Trp

residue being directed to different spatial regions (Paterlini et al., 2000) whereas the chirality of the Pro residue did not change the conformational preference of Tyr. Since the reduction of the amide bond increased local flexibility at the site of the peptide bond surrogate (Chen et al., 2002b), the dramatic loss of agonist potency of EMs[ψ] suggested that N-terminal dipeptide portion of EMs contained key factors for binding to the μ-opioid receptor. In this region, not only Pro^2, but the rigid conformation between Tyr^1 and Pro^2 were required for their μ opioid receptor agonist potency.

EMs[ψ] were effective both in the model of acute pain in mice and in the model of formalin-induced inflammatory pain in rats, thus extending their analgesic effects at the supraspinal level. Naloxone antagonism provided evidence that EMs[ψ] act via opioid receptor system. It has been reported that antinociception induced by EM1 and EM2 is mediated by the stimulation of μ-opioid receptor (Narita et al., 1998; Przewlocka et al., 1999; Stone et al., 1997; Tseng et al., 2000; Zadina et al., 1997). Neither EM1 nor EM2 activates μ-opioid receptor-coupled G proteins in μ-opioid receptor knockout mice, and the antinociception induced by EM1 and EM2 is attenuated in heterozygous knockout mice (Mizoguchi et al., 1999). However, both EM1[ψ] and EM2[ψ] displayed significantly decreased μ-opioid receptor agonist potency in GPI assay, and they were partial agonist compared with EMs. Their potent antinociceptive effects were unexpected. Interestingly, this kind of discrepancy was also observed in [D-Pro^2]EMs. These two stereoisomeric analogues of EMs had significant antinociceptive activity (Hung et al., 2002; Huo et al., 2001; Sakurada et al., 2002; Shane et al., 1999). However, both [D-Pro^2]EM1 and [D-Pro^2]EM2 had dramatic decreased μ-opioid receptor agonist potency (Huo et al., 2001; Paterlini et al., 2000) and no antagonist potency in GPI assay (Huo et al., 2001). These findings imply receptor agonist potency and antinociceptive activity may not be a simple corresponding relationship. It has been known that antinociception induced administered EM2 involves κ- and δ_2-opioid receptors for the production of antinociception (Sakurada et al., 2001) whereas EMs has a very low affinity for δ-and κ-opioid receptors in the opioid receptor binding assays (Zadina et al., 1997). The mechanisms for this discrepancy are unclear and maybe involve undiscovered subtypes splice varnance or physical states of receptor (Sakurada et al., 2001).

EMs significantly attenuate the thermal hyperalgesia with a short lasting course which suggests tachyphylaxis or fast degradation of the peptides (Péter et al., 1999; Shane et al., 1999; Sakurada et al., 2003). The reduced (CH_2NH) bond would enhance metabolic stability by excluding the possibility of chemical degradation (Chen et al., 2002b; Schiller et al., 1999). However, the present data indicate that EMs[ψ] do not produce prolonged antinociception. Therefore, simple substitution of a reduced peptide bond between Tyr^1 and Pro^2 is not sufficient to enhance EMs stability. We propose there are two reasons for this effect. Firstly, the catabolism to liberate Tyr from EMs did not occur or was too weak to influence the analgesic potency in vivo. Secondly, reduced amino bond was not stable enough to prevent the degradation of EMs if Tyr was really liberated in vivo.

Conclusion

The N-terminal dipeptide portion of EMs contains key factors for binding to the μ opioid receptor. In this portion, not only Pro^2 itself but the rigid conformation induced by peptide bond between Tyr^1 and Pro^2 is substantial for their μ-opioid receptor agonist potency. A reduced

(CH_2NH) bond with conformationally flexibility between Tyr^1 and Pro^2 makes EM1[ψ] and EM2[ψ] partial μ opioid agonists. However the reduced agonist potency of these two analogues resulted in less influence on their antinociceptive activity. These findings imply that there may be a complex relationship between receptor agonist potency and antinociception of EMs and their analogues. Our results will help to better delineate the structure-activity relationship for the N-terminal dipeptide of EMs in the μ-selective pockets.

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