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Biological activity of endomorphin and [Dmt^1]endomorphin analogs with six-membered proline surrogates in position 2

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ABSTRACT

Endogenous μ-opioid receptor (MOR) selective peptides, endomorphin-1 (EM-1) and endomorphin-2 (EM-2), unlike so called 'typical opioids', are characterized by the presence of Pro^2 residue, which is a spacer connecting aromatic pharmacophoric residues. In order to investigate structural requirements for position 2, we synthesized endomorphin analogs incorporating, instead of Pro, unnatural amino acids with six-membered heterocyclic rings, such as piperidine 2-, 3- or 4-carboxylic acids (Pip, Nip and Inp, respectively). (*R*)-Nip residue turned out to be favourable for improving MOR affinity. Introduction of 2′,6′-dimethyltyrosine (Dmt) instead of Tyr^1 led to obtaining [Dmt^1, (*R*)-Nip^2]EM-2 which showed exceptional MOR affinity and high stability against enzymatic degradation in rat brain homogenate. In in vivo hot-plate test in mice, this analog given intracerebroventicularly (i.c.v.), produced profound supraspinal analgesia, being much more potent than EM-2. The antinociceptive effect of this analog lasted about 170 min and was almost completely reversed by β-funaltrexamine (β-FNA), a selective MOR antagonist.

1. Introduction

A major goal in opioid peptide chemistry and pharmacology is the development of novel analgesics that could replace morphine with its well-known side-effects, such as dependence, tolerance, breath-depression and reward-seeking behaviour.[1] Morphine and other related compounds that are clinically useful for relieving pain, act primarily at the μ-opioid receptor (MOR).[2] Endogenous ligands of this receptor remained unknown until 1997, when two tetrapeptides, named endomorphin-1 (EM-1, Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH_2), were isolated from bovine brain[3] and then from human cortex.[4] Even though morphine and endomorphins act at the same opioid receptor (MOR), the latter are thought to inhibit pain without some of the undesired side-effects of plant opiates. In particular, the rewarding effect of EM-1 can be separated from analgesia[5] and EM-1 is less prone than morphine to induce respiratory depression and cardiovascular effects at effective antinociceptive doses.[6] However, the use of endomorphins as analgesics is of little therapeutic value, since natural peptides are quickly degraded by enzymes. To improve biological activity of exogenously administered endomorphins, numerous chemical modifications of their structure have been proposed.[7]

Endomorphins, unlike so called 'typical opioids' with N-terminal Tyr-Gly-Gly-Phe sequence, incorporate Pro at the second position.[8] Pro is a spacer residue, connecting two pharmacophoric aromatic residues, Tyr^1 and Trp/Phe^3.[9,10] Substitution of Pro by D-Pro in EM-1 was shown to be detrimental for MOR affinity,[11] and indicated that Pro^2 plays an important role in the formation of the appropriate spatial orientation of aromatic residues. In the search for analogs of endomorphins with improved pharmacological profile different Pro surrogates have been introduced into position 2, such as 1-aminocyclopentane- and -cyclohexane-1-carboxylic acids (Cpn and Chx)[12] and also 2-aminocyclopentane- and cyclohexane-1-carboxylic acids (Acpc and Achc).[13]

In our previous report[14] we have described synthesis and binding affinities of novel EM-2 analogs, incorporating unnatural amino acids with six-membered heterocyclic rings, such as piperidine-2-, 3- and 4-carboxylic acids (Pip, Nip and Inp, respectively) (Fig. 1) instead of Pro in position 2. These compounds can be considered as α-, β- and γ-amino acids. Configuration of the chiral acids, Pip and Nip, was chosen to be *S* and *R*, respectively, to mimic the spatial arrangement of L-Pro present in the endogenous peptides. One

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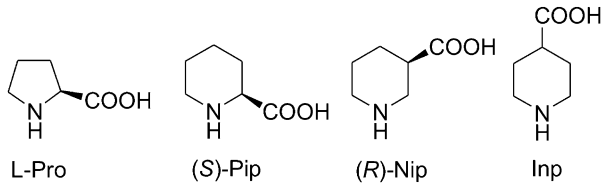

Figure 1. Structure of proline mimics incorporated in position 2 of endomorphin analogs.

of the analogs, [(*R*)-Nip^2]EM-2, displayed higher than the parent compound MOR affinity and greatly increased enzymatic stability.

In the present study we increased the number of analogs by the synthesis of similarly modified analogs of EM-1 and by introducing Dmt[1] to the most potent analogs. In vitro and in vivo tests allowed us to assess the biological profile of the most potent analogs with Pro mimics in position 2.

2. Results

2.1. Opioid receptor binding

Affinity and selectivity of new analogs (characterized in Table 1) were evaluated by radioligand binding assays using rat brain membranes. IC_{50} values at MOR and DOR determined against [^{3}H]DAMGO and [^{3}H]Ile^{5,6}deltorphin-2, respectively, are provided in Table 2. Both position 2 modified analogs incorporating (*R*)-Nip (**3** and **8**) showed high MOR affinity and selectivity, while two other substitutions in position 2 [(*S*)-Pip and Inp] were quite ineffective. Interestingly, peptide **8** displayed affinity that was about an order of magnitude higher than that of EM-2. Additional modification of analogs **3** and **8**, performed by replacing Tyr^1 by 2′,6′-dimethyltyrosine (Dmt), produced further enhancement of MOR affinity and a somewhat increase in DOR affinity, thus resulting in a slight decrease in MOR selectivity.

The kinetics of binding of the selected analogs to MOR was investigated using BIAcore X system. BIAcore X is a highly sensitive, semi-automated system for label-free studies of biomolecular binding, in samples ranging from small to macro molecules. The ability of EM-2 and selected analogs (**8** and **10**) to bind to the receptor protein immobilized on the biosensor was determined using this system. The binding and the subsequent dissociation of the peptides are presented in Figure 2 and the calculated parameters of the binding kinetics are given in Table 3. The kinetic association rate constants (k_a) indicate that both analogs bind to the receptor with equal rate, but 4–5 times faster than EM-2. The stability of peptide–receptor complexes, as judged by dissociation rate constants (k_d), is twofold higher for EM-2 and analog **10** than for analog **8**.

2.2. Enzymatic degradation

The stability of the new analogs towards enzymatic degradation was verified by measuring their hydrolysis rates in the presence of rat brain homogenate. Table 4 summarizes the half-lives ($t_{1/2}$) determined for EMs and the modified peptides. All new analogs showed an increased stability over their parent compounds, but despite the small structural differences, the obtained $t_{1/2}$ values differed significantly. Analogs **2** and **7** with (*S*)-Pip in position 2, which is a six-membered mimic of Pro, were only slightly more stable than EMs, analogs **3** and **8**, which can be considered β-amino acids, were about 5-times more stable, and analogs **4** and **9** which are γ-amino acids, were about 10-times more resistant to degradation than the parent compounds (Table 4). Additional modification with Dmt (analogs **5** and **10)** seemed to further enhance stability of the analogs.

2.3. Assessment of antinociception

Antinociceptive activities of EM-2 and analogs **8** and **10**, exhibiting the highest MOR affinities were studied in the hot-plate test in mice (supraspinally mediated analgesia), after i.c.v. administration of the peptides. Analgesic effect of analogs **8** and **10** was dose-dependent. Dose–response curves for EM-2 and analogs **8** and **10** were compared (Fig. 3). In case of analog **10**, the antinociceptive effect was observed at a dose as low as 3 ng, whereas for EM-2 and analog **8** much higher doses were required (about 100 ng/animal).

However, neither of the two tested analogs showed any analgesic effect after intravenous (iv) administration (data not shown).

The time-course curves for EM-2 (3 μg/animal) and analogs **8** and **10** (0.1 μg/animal) are shown in Figure 4. Analgesic effect was measured 5, 10, 20, 30, 45, 60, 90, 120, 150 and 180 min after administration of a peptide. The maximal response was obtained within 10 min after i.c.v. injection. The duration of the effect was 50 min for EM-2, about 150 min for analog **8** and over 170 min for analog **10**.

MOR selective antagonist, β-funaltrexamine (β-FNA), effectively reversed analgesic effect of EM-2 and analogs **8** and **10**, indicating that their action was mediated by MOR (Fig. 5).

3. Discussion

In the structure of EMs three aromatic amino acid residues, which play a crucial role in the recognition of the opioid receptor,

Table 1
Physicochemical data of endomorphin analogs

No.	Sequence	HPLC[a] (t_r)	FABS-MS		
			Formula	MW	$[M+H]^+$
1	Tyr-Pro-Trp-Phe-NH_2 (EM-1)	17.30	$C_{34}H_{37}N_6O_5$	610	611
2	Tyr-(*S*)-Pip-Trp-Phe-NH_2	17.52	$C_{35}H_{39}N_6O_5$	624	625
3	Tyr-(*R*)-Nip-Trp-Phe-NH_2	16.32	$C_{35}H_{39}N_6O_5$	624	625
4	Tyr-Inp-Trp-Phe-NH_2	16.82	$C_{35}H_{39}N_6O_5$	624	625
5	Dmt-(*R*)-Nip-Trp-Phe-NH_2	18.13	$C_{37}H_{43}N_6O_5$	652	653
6	Tyr-Pro-Phe-Phe-NH_2 (EM-2)[b]	16.21	$C_{32}H_{36}N_5O_5$	571	572
7	Tyr-(*S*)-Pip-Phe-Phe-NH_2[b]	17.18	$C_{33}H_{38}N_5O_5$	585	586
8	Tyr-(*R*)-Nip-Phe-Phe-NH_2[b]	16.41	$C_{33}H_{38}N_5O_5$	585	586
9	Tyr-Inp-Phe-Phe-NH_2[b]	16.47	$C_{33}H_{38}N_5O_5$	585	586
10	Dmt-(*R*)-Nip-Phe-Phe-NH_2	17.55	$C_{35}H_{42}N_5O_5$	613	614

[a] HPLC elution on a Vydac C_{18} column (5 μm, 4.6 × 250 mm) using the solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 0–100% solvent B over 25 min at flow rate of 1 ml/min.
[b] Data from Ref. 14.

Table 2
Opioid receptor binding assays of endomorphin analogs

No.	Sequence	IC_{50} (nM)		
		μ[a]	δ[b]	δ/μ
1	Tyr-Pro-Trp-Phe-NH_2 (EM-1)	0.430 ± 0.025	>1000	>1000
2	Tyr-(*S*)-Pip-Trp-Phe-NH_2	20.4 ± 0.9	>1000	>49
3	Tyr-(*R*)-Nip-Trp-Phe-NH_2	3.55 ± 0.25	>1000	>281
4	Tyr-Inp-Trp-Phe-NH_2	400.6 ± 52	860 ± 90	2.15
5	Dmt-(*R*)-Nip-Trp-Phe-NH_2	0.38 ± 0.04	89 ± 3.4	234
6	Tyr-Pro-Phe-Phe-NH_2 (EM-2)[c]	0.69 ± 0.03	>1000	>1000
7	Tyr-(*S*)-Pip-Phe-Phe-NH_2[c]	79.95 ± 3.8	>1000	>12.50
8	Tyr-(*R*)-Nip-Phe-Phe-NH_2[c]	0.04 ± 0.01	>1000	>1000
9	Tyr-Inp-Phe-Phe-NH_2[c]	360 ± 17	>1000	>5.36
10	Dmt-(*R*)-Nip-Phe-Phe-NH_2	0.035 ± 0.01	407 ± 28	>1000
	Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH_2 (deltorphin-II)		0.08 ± 0.004	

All values are expressed as mean ± SEM of three to six determinations.
[a] Determined against [^{3}H]DAMGO.
[b] Determined against [^{3}H][Ile^{5,6}]deltorphin-2.
[c] Data from Ref. 14.

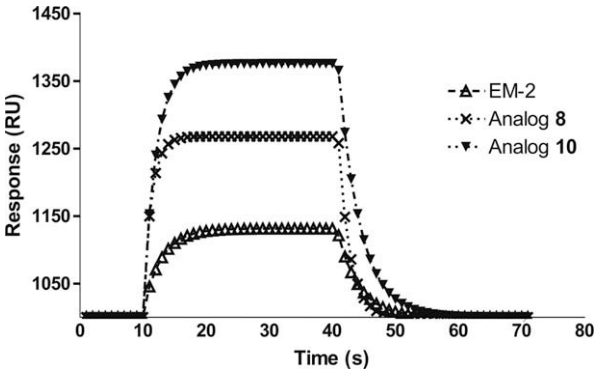

Figure 2. Representative sensogram for kinetic study of binding of EM-2 and analogs **8** and **10** to MOR protein immobilized on CM-5 biosensor.

Table 3
Kinetic parameters of EM-2 and analogs **8** and **10**

No.	Sequence	k_a (M^{-1} s^{-1})	k_d (s^{-1})
6	Tyr-Pro-Phe-Phe-NH_2 (EM-2)	120 ± 17	0.3 ± 0.01
8	Tyr-(*R*)-Nip-Phe-Phe-NH_2	570 ± 45	0.55 ± 0.01
10	Dmt-(*R*)-Nip-Phe-Phe-NH_2	515 ± 66	0.29 ± 0.03

Table 4
Degradation rates and half-lives of endomorphins and their analogs[a,b]

No.	Sequence	Brain homogenate	
		100 × k (per min)	$t_{1/2}$ (min)
1	Tyr-Pro-Trp-Phe-NH_2 (EM-1)	11.4 ± 0.57	6.02 ± 0.46
2	Tyr-(*S*)-Pip-Trp-Phe-NH_2	7.13 ± 0.33***	9.68 ± 0.63***
3	Tyr-(*R*)-Nip-Trp-Phe-NH_2	2.33 ± 0.2***	29.6 ± 1.56***
4	Tyr-Inp-Trp-Phe-NH_2	0.87 ± 0.03***	78.52 ± 6.3***
5	Dmt-Nip-Trp-Phe-NH_2	1.93 ± 0.04***	35.8 ± 1.8***
6	Tyr-Pro-Phe-Phe-NH_2 (EM-2)[c]	14.0 ± 0.68	4.8 ± 0.24
7	Tyr-(*S*)-Pip-Phe-Phe-NH_2[c]	10.1 ± 0.92***	6.80 ± 0.41***
8	Tyr-(*R*)-Nip-Phe-Phe-NH_2[c]	1.40 ± 0.10***	48.0 ± 2.9***
9	Tyr-Inp-Phe-Phe-NH_2[c]	0.80 ± 0.02***	86.0 ± 4.0***
10	Dmt-Nip-Phe-Phe-NH_2	2.23 ± 0.13***	30.9 ± 3.29***

[a] HPLC elution on a Vydac C_{18} column (5 μm, 4.6 × 250 mm) using the solvent system of 0.1% TFA in water (A)/80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 0–100% solvent B over 25 min at flow rate of 1 ml/min.
[b] Mean ± SEM of three independent experiments performed in duplicate.
[c] Data from Ref. 14.
*** $p < 0.001$ as compared to respective EM by using one-way ANOVA followed by the Student–Newman–Keuls test.

are connected by the Pro residue. Pro is considered a spacer, stabilizing the bioactive conformation of EMs.[15,16] In the present study we described analogs in which a five-membered pyrrolidine ring of Pro was substituted by a six-membered piperidine ring, with a carboxyl group in the position 2, 3 or 4. Rigid conformation of the six-membered ring and a well marked preference of the substituents (in this case a carboxyl group) to occupy equatorial positions, which is not the case in the five-membered ring, are the main differentiating factors which change the spatial orientation of aromatic pharmacophoric residues. Furthermore, in analogs **3** and **8** (incorporating (*R*)-Nip^2), and even to the greater extent in analogs **4** and **9** (incorporating Inp^2), the distance between Tyr^1 and Phe^3 residues is considerably bigger than in EMs due to the β or γ position, respectively, of the imine group in piperidinecarboxylic acid residues. Obviously, combination of all these factors caused in analogs **3** and **8** the proper orientation of Tyr^1 and Phe^3, which are believed to play the crucial role in the binding to the receptor. However, it is difficult to explain why the same (*R*)-Nip^2 substitution was less effective when introduced into EM-1. The obtained results once more give evidence of quite unpredictable role of different chemical modifications on the biological properties of modified peptides.

Studies on opioid peptides demonstrated that the replacement of Tyr^1 by Dmt results in an exceptional improvement in receptor affinity and functional bioactivity.[17] Analogs **3** and **8** with (*R*)-Nip^2 were further modified by incorporation of Dmt instead of Tyr at the N-terminus. As expected, this modification enhanced MOR affinity of the obtained analogs (**5** and **10**). The methyl groups on the phenolic ring in Dmt undoubtedly play a role in strengthening receptor binding, presumably by engaging in additional hydrophobic interactions.

Binding of the new analogs to the receptor protein immobilized on the biosensor allowed to observe significant differences in their binding kinetics. As deduced from the binding parameters calculated from the association and dissociation phases of the reaction, the exceptional activity of analog **10** can be a consequence of its high association rate (high k_a value) and high stability of the complex (low k_d value). Analog **8**, which has similar association rate as **10**, forms a less stable complex, while the complex receptor–EM-2 is formed more slowly. The receptor–peptide binding kinetics assessment may be a valuable tool in predicting the performance of new analogs at their respective receptors.

The in vivo activity of analogs **8** and **10** also demonstrated that these compounds were more potent than EM-2 in exerting analgesia. For both tested analogs much stronger antinociceptive activi-

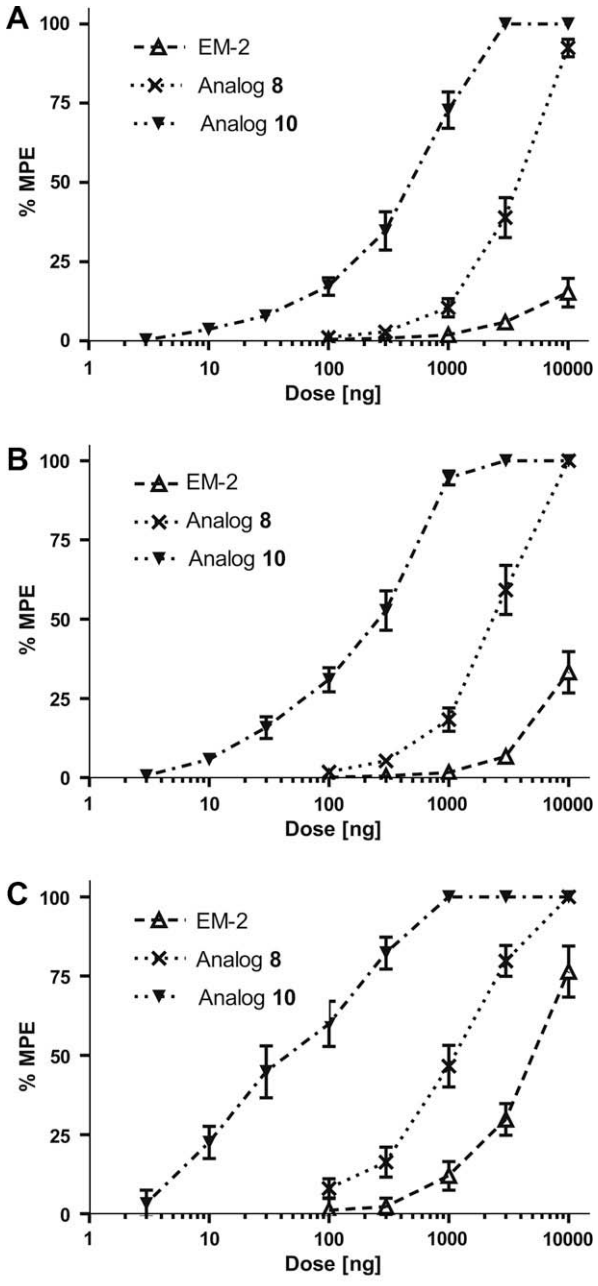

Figure 3. Dose–response curves determined in the hot-plate test for the inhibition of paw licking (A), rearing (B) and jumping (C) induced by i.c.v. injection of EM-2 and analogs **8** and **10**. The data represent the mean ± SEM of 10 mice per group.

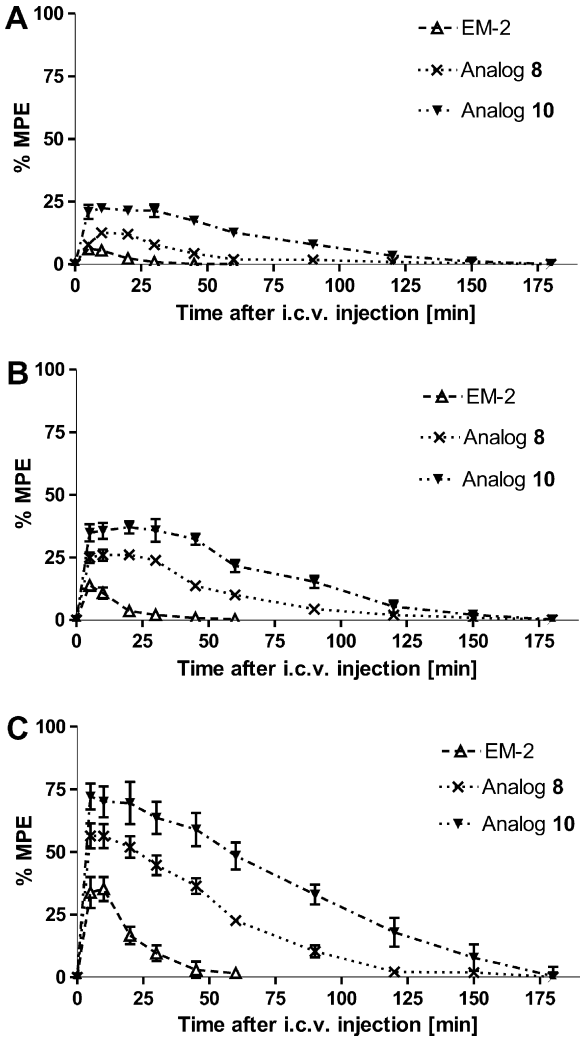

Figure 4. Time-course of the changes in inhibition of paw licking (A), rearing (B) and jumping (C) induced by i.c.v. injection of analogs **8** and **10** (0.1 μg) and EM-2 (3 μg), determined in the hot-plate test.

ties were observed. In a time-course study of analog **10** the duration of the analgesic effect was 4-times longer than for EM-2. However, no analgesic effect was observed when the same analogs were administered peripherally (iv). The majority of opioid peptides undergo rapid enzymatic degradation, particularly in blood, by exo- and endopeptidases.[7] Due to this reason and also low permeation of opioid peptides across blood-brain barrier (BBB), they cannot reach the central nervous system in an amount sufficient to elicit analgesia following peripheral administration.[18] Since it was demonstrated that all new analogs showed significantly enhanced stability in rat brain homogenate, the reason for their inactivity after peripheral administration can be explained by their poor BBB permeability. It has been shown that small peptides can slowly cross the BBB by simple diffusion,[19] but in the case of the tested analogs this process must have not been efficient enough. Further modifications of peptide **10** are planned in order to increase its permeation through BBB.

4. Experimental

4.1. Peptide synthesis

Peptides were synthesized by standard solid-phase procedures as described before[20] using techniques for 9-fluorenylmethoxycarbonyl (Fmoc)-protected amino acids on MBHA Rink-Amide peptide resin (100–200 mesh, 0.8 mM/g, Novabiochem) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as a coupling agent. Crude peptides were purified by preparative reversed-phase HPLC on a Vydac C_{18} column (10 μm, 22 × 250 mm) equipped with a Vydac guard cartridge. For purification a linear gradient of 0–100% acetonitrile containing 0.1% TFA over 15 min at a flow rate of 15 ml/min was used. The purity of the final peptides was verified by analytical HPLC employing a Vydac C_{18} column (5 μm, 4.6 × 250 mm) and the solvent system of 0.1% TFA in water (A) and 80% acetonitrile in water containing 0.1% TFA (B). A linear gradient of 0–100% solvent B over 25 min at a flow rate of 1 ml/min was used

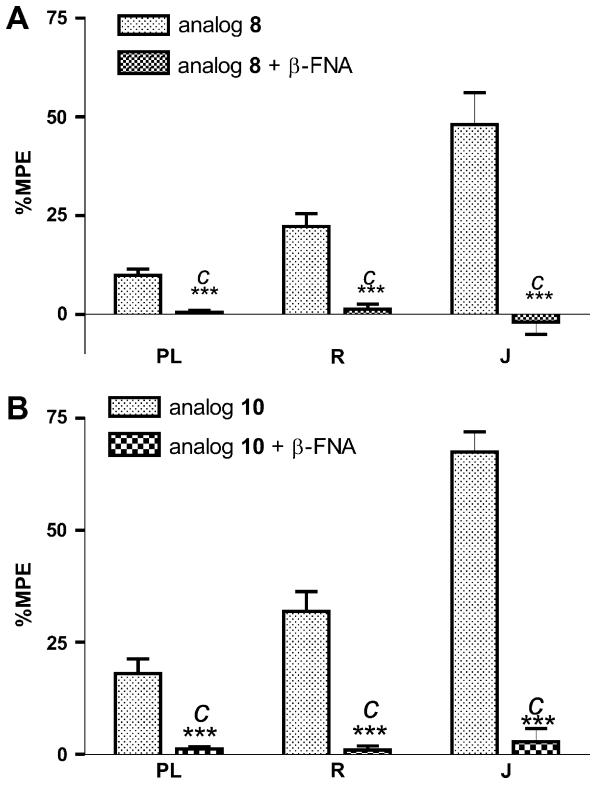

Figure 5. Antagonist effect of β-funaltrexamine (β-FNA, 1 μg) on the hot-plate inhibition of paw licking (PL), rearing (R) and jumping (J) induced by i.c.v. injection of A, analog **8** (1 μg) and B, analog **10** (0.1 μg), determined in the hot-plate test. Data represent mean ± SEM of 10 mice per group. $^{***}p < 0.001$, as compared to respective control by using two-way ANOVA, followed by the Student–Newman–Keuls test. A two-way ANOVA analysis revealed a significant interaction between β-FNA and analog **8**: $F(1,36) = 26{,}054$; $^{c}p<0.001$ (for paw licking), $F(1,36) = 31{,}616$; $^{c}p < 0.001$ (for rearing), $F(1,36) = 24{,}475$; $^{c}p < 0.001$ (for jumping); between β-FNA and analog **10**: $F(1,36) = 25{,}451$; $^{c}p < 0.001$ (for paw licking), $F(1,36) = 43{,}096$; $^{c}p < 0.001$ (for rearing), $F(1,36) = 62{,}088$; $^{c}p < 0.001$ (for jumping).

for the analysis. The absorbance was monitored at 214 nm. Final purity of all peptides was >98%. Calculated values for protonated molecular ions were in agreement with those determined by FAB mass spectrometry.

4.2. Radioligand binding assays

The MOR or δ-opioid receptor (DOR) binding studies were performed according to the modified method described elsewhere.[21] Briefly, crude membrane preparations, isolated from Wistar rat brains, were incubated at 25 °C for 120 min with appropriate concentration of a tested peptide in the presence of 0.5 nM [^{3}H]DAMGO (as a MOR-selective radioligand) or 0.5 nM [^{3}H][Ile^{5,6}]deltorphin-2 (as a DOR-selective radioligand), in a total volume of 0.5 ml of 50 mM Tris/HCl (pH 7.4), containing $MgCl_2$ (5 mM), EDTA (1 mM), NaCl (100 mM), and bacitracin (20 mg/l). Non-specific binding was determined in the presence of 1 μM naloxone. Incubations were terminated by rapid filtration through Whatman GF/B (Brentford, UK) glass fibre strips, which had been pre-soaked for 2 h in 0.5% polyethylamine, using Millipore Sampling Manifold (Billerica, USA). The filters were washed three times with 4 ml of ice-cold Tris buffer solution. The bound radioactivity was measured in Packard Tri-Carb 2100 TR liquid scintillation counter (Ramsey, MN, USA) after overnight extraction of the filters in 4 ml of Perkin Elmer Ultima Gold scintillation fluid (Wellesley, MA, USA). Three independent experiments for each assay were carried out in duplicate.

4.3. Interactions of endomorphin analogs with MOR assayed by the BIAcore system

Interactions between endomorphin analogs and the MOR protein (OPRM1 recombinant protein, Abnova, Taiwan) were investigated with the BIAcore X system (BIAcore AB, Uppsala, Sweden). The receptor protein was immobilized on the surface of CM5 biosensor by the amine-coupling procedure, according to the standard method delivered by the manufacturer. The instrument was set to maintain the temperature of 37 °C on the sensor surface.

Receptor protein, at the concentration of 10 μg/ml in 10 mM sodium acetate buffer, pH 5.0, was used for immobilization. Then each peptide analog was applied onto the sensor at the concentration of about 0.4 mM (in HBS buffer: 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.005% surfactant P20, pH 7.4) for 30 s with flow of 20 μl/min. Both, association and dissociation processes, observed as changes in the resonance response (RU), were recorded and used for calculations. The kinetic parameters k_a and k_d were calculated using the BIAcore evaluation software.

4.4. Degradation by brain homogenate

The degradation studies were performed on the rat brain homogenate, according to the modified method described elsewhere.[22] Briefly, rat brains were isolated, pooled, homogenized in a Polytron with 20 volumes of 50 mM Tris–HCl (pH 7.4), and stored at −80 °C until used. The aliquots (100 μl, 10 mg protein/ml) were incubated with 100 μl of a peptide (0.5 mM) over 0, 7.5, 15, 22.5, 30, and 60 min at 37 °C in a final volume of 200 μl. The reaction was stopped at the required time by placing the tube on ice and acidifying with 20 μl of 1 M aqueous HCl solution. The aliquots were centrifuged at 20,000*g* for 10 min at 4 °C. The obtained supernatants were filtered over Millex-GV syringe filters (Millipore) and analyzed by HPLC on a Vydac C_{18} column (5 μm, 0.46 × 250 mm), using the solvent system of 0.1% trifluoroacetic acid (TFA) in water (A) and 80% acetonitrile in water containing 0.1% TFA (B) and a linear gradient of 0–100% B over 25 min. Three independent experiments for each assay were carried out in duplicate. The rate constants of degradation (k) were obtained as described earlier,[22] by a least square linear regression analysis of logarithmic peak areas [$\ln(A/A_D)$], where A—amount of peptide remaining, A_D—initial amount of peptide, versus time. Degradation half-lives ($t_{1/2}$) were calculated from the rate constants as $\ln 2/k$.

4.5. In vivo antinociception test

Male Swiss albino CD1 mice (IFFA-CREDO/Charles River, Saint Germain sur L'Arbresle, France), weighing 20–22 g, were used for antinociception tests. The animals were housed 20 per Makrolon cage, with free access to standard semisynthetic laboratory diet and tap water ad libitum, under controlled environmental conditions (temperature 22 ± 1 °C, 7 am to 7 pm light-dark cycle). Mice were tested only once and sacrificed immediately thereafter by decapitation.

The procedures used in this study were in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were approved by the Local Ethical Committee for Animal Research with the following numbers: N/10-04-04-12 and N/12-04-04-14.

To determine the antinociceptive effects of the opioids, the hot-plate test was used. Intracerebroventricular (i.c.v.) injections were performed in the left brain ventricle of manually immobilized mice with a Hamilton microsyringe (50 μl) connected to a needle (diameter 0.5 mm), as described by Haley and McCormick.[23] All drugs for i.c.v. administration were dissolved in 0.9% NaCl.

The hot-plate test was performed according to the method of Eddy and Leimbach.[24] A transparent plastic cylinder (14 cm diameter, 20 cm height) was used to confine the mouse on the heated (55 ± 0.5 °C) surface of the plate. The animals were placed on the hot-plate 5 min after i.c.v. injection of saline or peptides and the latencies to paw licking, rearing and jumping were measured. A cut-off time of 240 s was used to avoid tissue injury.

To evaluate the hot-plate test responses detailed below, the control latencies (t_0) and test latencies (t_1) were determined after injection of saline and a peptide, respectively. The percentage of maximal possible effect (%MPE) was calculated as $\%MPE = (t_1 - t_0)/(t_2 - t_0) \times 100$, where the cut-off time (t_2) was 240 s.

4.6. Data analysis

Statistical and curve-fitting analyses were performed using Prism 4.0 (GraphPad Software Inc.). The data are expressed as means ± SEM. Differences between groups were assessed by one-way analysis of variance (ANOVA), followed by a post-hoc multiple comparison Student–Newman–Keuls test. Antagonist effects in the combination experiments were analyzed using two-way analysis of variance (ANOVA) and a post-hoc multiple comparison Student–Newman–Keuls test was used for multiple comparisons between groups. A probability level of 0.05 or lower was considered as statistically significant.

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References and notes

1. Olson, G. A.; Olson, R. D.; Vaccarino, A. L.; Kastin, A. J. *Peptides* **1998**, *19*, 1791.
2. Vaccarino, A. L.; Olson, G. A.; Olson, R. D.; Kastin, A. J. *Peptides* **1999**, *20*, 1527.
3. Zadina, J. E.; Hackler, L.; Ge, L. J.; Kastin, A. J. *Nature* **1997**, *386*, 499.
4. Hacker, L.; Zadina, J. E.; Ge, L. J.; Kastin, A. J. *Peptides* **1997**, *18*, 1635.
5. Wilson, A. M.; Soignier, R. D.; Zadina, J. E.; Kastin, A. J.; Nores, W. L.; Olson, R. D.; Olson, G. A. *Peptides* **2000**, *20*, 1871.
6. Czapla, M. A.; Gozal, D.; Alea, O. A.; Beckerman, R. C.; Zadina, J. E. *Am. J. Respir. Crit. Care Med.* **2000**, *162*, 994.
7. Janecka, A.; Staniszewska, R.; Gach, K.; Fichna, J. *Peptides* **2008**, *29*, 2066.
8. Janecka, A.; Fichna, J.; Janecki, T. *Curr. Top. Med. Chem.* **2004**, *4*, 1.
9. Yamazaki, T.; Ro, S.; Goodman, M.; Chung, N. N.; Schiller, P. W. *J. Med. Chem.* **1993**, *36*, 708.
10. Leitgeb, B. *Chem. Biodivers.* **2007**, *4*, 2703.
11. Paterlini, M. G.; Avitabile, F.; Ostrowski, B. G.; Ferguson, D. M.; Portoghese, P. S. *Biophys. J.* **2000**, *78*, 590.
12. Doi, M.; Asano, A.; Komura, E.; Ueda, Y. *Biochem. Biophys. Res. Commun.* **2002**, *297*, 138.
13. Toth, G.; Keresztes, A.; Tomboly, C.; Peter, A.; Fulop, F.; Tourwe, D.; Navratilowa, E.; Varga, E.; Roeske, W. R.; Yamamura, H. I.; Szucs, M.; Borsodi, A. *Pure Appl. Chem.* **2004**, *76*, 951.
14. Staniszewska, R.; Fichna, J.; Gach, K.; Toth, G.; Poels, J.; Vanden Broeck, J.; Janecka, A. *Chem. Biol. Drug. Des.* **2008**, *72*, 91.
15. Leitgeb, B.; Toth, G. *Eur. J. Med. Chem.* **2005**, *40*, 674.
16. Keresztes, A.; Szucs, M.; Borics, A.; Kover, K. E.; Forro, E.; Fulop, F.; Tomboly, C.; Peter, A.; Pahi, A.; Fabian, G.; Muranyi, M.; Toth, G. *J. Med. Chem.* **2008**, *51*, 4270.
17. Bryant, S. D.; Jinsmaa, Y.; Salvadori, S.; Okada, Y.; Lazarus, L. H. *Biopolymers* **2003**, *71*, 86.
18. Egleton, R. D.; Abbruscato, T. J.; Thomas, S. A.; Davis, T. P. *J. Pharm. Sci.* **1998**, *87*, 1433.
19. Kastin, A. J.; Pan, W.; Maness, L. M.; Banks, W. A. *Brain Res.* **1999**, *848*, 96.
20. Fichna, J.; Do-Rego, J. C.; Chung, N. N.; Lemieux, C.; Schiller, P. W.; Poels, J.; Vanden Broeck, J.; Costentin, J.; Janecka, A. *J. Med. Chem.* **2007**, *50*, 512.
21. Fichna, J.; Do-Rego, J. C.; Costentin, J.; Janecka, A. *Biochem. Pharmacol.* **2005**, *69*, 179.
22. Janecka, A.; Kruszynski, R.; Fichna, J.; Kosson, P.; Janecki, T. *Peptides* **2006**, *27*, 131.
23. Haley, T. J.; McCormick, W. G. *Br. J. Pharmacol.* **1957**, *12*, 12.
24. Eddy, N. B.; Leimbach, D. *J. Pharmacol. Exp. Ther.* **1953**, *107*, 385.