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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Kinetic studies of novel inhibitors of endomorphin degrading enzymes

Renata Perlikowska · Jakub Fichna · Jean Claude do-Rego · Katarzyna Gach · Anna Janecka

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Abstract Endomorphins (EMs), two endogenous μ-opioid receptor selective ligands, are attractive lead compounds for opioid-based pain management studies. However, these peptides are quickly degraded by peptidases, in particular by dipeptidylpeptidase IV (DPP IV) and aminopeptidase M (APM). Targeting enzymatic degradation is one approach to prolong endomorphin activity. In this study we characterized the action of two new inhibitors of similar to endomorphins structure, Tyr-Pro-Ala-NH_2 (EMDB-2) and Tyr-Pro-Ala-OH (EMDB-3), which were designed earlier in our laboratory. The presented data give evidence that EMDB-2 and EMDB-3 are potent inhibitors of enzymes responsible for endomorphin cleavage. These compounds are stable and easily synthesized. EMDB-2 and EMDB-3 are competitive inhibitors of both, DPP IV and APM, with K_i values in micromolar range. They are less potent than diprotin A in protecting EMs against DPP IV but more potent than actinonin in protecting these peptides against APM.

R. Perlikowska · J. Fichna · K. Gach · A. Janecka (✉)
Department of Biomolecular Chemistry, Medical University of Lodz, Mazowiecka 6/8, 92-215 Lodz, Poland
e-mail: anna.janecka@umed.lodz.pl

J. C. do-Rego
Institut Fédératif de Recherches Multidisciplinaires sur les Peptides (IFRMP 23), Laboratoire de Neuropsychopharmacologie Expérimentale, Faculté de Médecine et Pharmacie, Université de Rouen, EA 4359, Rouen, France

J. C. do-Rego
Centre National de la Recherche Scientifique (CNRS), Rouen, France

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Introduction

At present, the treatment of severe pain relies mostly upon administration of centrally acting opiates such as morphine and its surrogates, which target μ-opioid receptors in the brain. In spite of the powerful in vivo efficacy of these drugs, their long-term use is limited by a number of well-known side-effects, including tolerance, physical dependence, respiratory depression, and diverse gastrointestinal effects. Discovery of endogenous μ-opioid receptor ligands, endomorphin-1 (EM-1, Tyr-Pro-Trp-Phe-NH_2), and endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH_2) more than a decade ago (Zadina *et al.*, 1997) initiated extensive studies on the possible use of these peptides as analgesics instead of morphine. EMs exhibit outstanding potencies towards both, acute and chronic neuropathic pain, as was demonstrated in rodents in various types of pain tests (Narita *et al.*, 1999; Horvath *et al.*, 1999; Horvath, 2000; Przewłocki and Przewłocka, 2001; Grass *et al.*, 2002). Furthermore, potentially advantageous pharmacological properties of EMs are the possible dissociation of analgesic and rewarding effects in the rat (Wilson *et al.*, 2000) and the moderate respiratory depression when compared with morphine (Czapla *et al.*, 2000; Fichna *et al.*, 2007). However, the main limitations of the use of EMs as analgesics are short duration of action and lack of activity after oral administration, both due to the poor metabolic stability of these peptides (Shane *et al.*, 1999; Tomboly *et al.*, 2002). Applying chemical modifications to the structure of EMs is one strategy to obtain compounds with desired pharmacological profile.

Another strategy might be increasing the level of endogenous EMs by the use of peptidase inhibitors. The enzyme which is primarily involved in the first cleavage step of EMs is a serine peptidase, dipeptidyl peptidase IV (DPP IV), which liberates Tyr–Pro dipeptides from amino terminus of EMs (Mentlein, 1999; Tomboly *et al.*, 2002). Proline-specific aminopeptidase M (APM) further splits the obtained fragments of EMs (Sakurada *et al.*, 2003) (Fig. 1).

Degradation of EMs can be significantly blocked by protease inhibitors. The most often used inhibitors of DPP IV are tripeptides Ile-Pro-Ile (diprotin A) and Val-Pro-Leu (diprotin B) (Mentlein, 1999). The action of APM is inhibited by actinonin (Sugimoto-Watanabe *et al.*, 1999; Tomboly *et al.*, 2002). Sakurada *et al.* (2003) showed that simultaneous administration of EM-2 and diprotin A to the mouse brain resulted in a fivefold longer duration of analgesic action compared with EM-2 alone. Actinonin significantly blocked EM-1 degradation in rat spinal cord homogenate (Sugimoto-Watanabe *et al.*, 1999).

In the search for effective blockers of EM degrading enzymes, we have synthesized several tri- and tetrapeptides with similar to EMs structure but with low μ-opioid receptor affinities and tested them as possible inhibitors. Two of these peptides, Tyr-Pro-Ala-NH_2 (EMDB-2) and Tyr-Pro-Ala-OH (EMDB-3), turned out to be effective blockers of EM degradation by rat brain homogenate (Fichna *et al.*, 2006). The action of these two tripeptides was further investigated in rat ileum in vitro (Fichna *et al.*, 2010). They both significantly prolonged the inhibitory effect of EM-2 on smooth muscle contractility in rat ileum.

The aim of this study was to investigate how these tripeptides influence enzymatic cleavage of EMs by purified enzymes, DPP IV and APM, and what type of inhibition they represent.

Materials and methods

Peptide synthesis

Peptides were synthesized by a solid phase method on MBHA Rink amide resin for C-terminally amidated analogs and on Wang resin for peptide acids, using Fmoc strategy and were purified by HPLC, as described earlier (Fichna *et al.*, 2006).

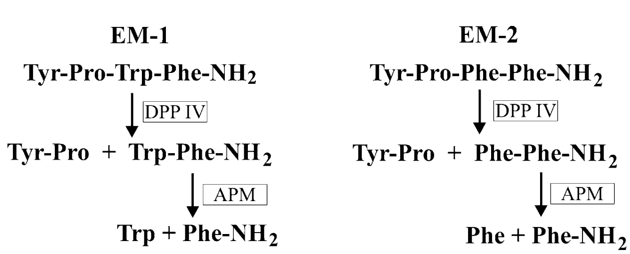

Fig. 1 Scheme of EM metabolism in the brain

Determination of EM degradation rates

The degradation studies were performed using pure, commercially available enzymes. DPP IV was used at a concentration of 0.002 mg protein/ml and APM at a concentration of 0.06 mg protein/ml. Solutions of EMs and inhibitors were made by dissolving them in Tris–HCl buffer (50 mM, pH 7.4) to obtain 1 mM concentrations. Enzymes, EMs and inhibitors were incubated over 0, 7.5, 15, 22.5, and 30 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 Millipore Millex-GV syringe filters (Millipore) and analyzed by RP-HPLC on a Vydac C_{18} column (5 µm, 4.6 mm × 250 mm), using the solvent system of 0.1% 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 (Tomboly *et al.*, 2002), by the least square linear regression analysis of logarithmic endomorphin peak areas ($\ln(A/A_0)$, where A the amount of peptide remaining, A_0 initial amount of peptide versus time. Degradation half-lives ($t_{1/2}$) were calculated from the rate constants as $\ln 2/k$.

Measurement of inhibition of proteolytic activity of DPP4 and APM

The inhibitory potency of each inhibitor was determined at five concentrations of substrate (1.25, 0.625, 0.25, 0.125, and 0.0625 mM). Reaction was initiated by addition of enzyme (DPP IV or APM) to solution containing substrate (EM-1 or EM-2) and inhibitor (EMDB-2 or EMDB-3). In each case, the reaction was allowed to proceed at 37°C for 0, 7.5, 15, 22.5, and 30 min as described in a previous section.

Statistical 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 Student–Newman–Keul's test.

Values of percentage inhibition of EM degradation were calculated using following formula, which was described earlier (Tomboly *et al.*, 2002):

$$\text{Inhibition}\ (\%) = (k_0 - k_i)/k_0 \times 100,$$

where k_0 the rate constant of degradation without inhibitor, k_i the rate constant of degradation with inhibitor.

Results

Effect of inhibitors on degradation of EMs by DPP IV

We evaluated EMDB-2 and EMDB-3 for their inhibitory effect on degradation of EMs by DPP IV. Diprotin A was included in the study for comparison. Degradation of EMs was analyzed by reversed phase HPLC. Effects of 30 min incubation of EM-2 with DPP IV in the absence and presence of inhibitors are shown in Fig. 2. The chromatographic peak area of EM-2 was found to decrease greatly in the sample without inhibitors. Diprotin A almost completely suppressed enzymatic cleavage of EM-2, while EMDB-2 and EMDB-3 only partially protected EM-2 against hydrolysis. Degradation rates and half-lives of EMs alone and in the presence of inhibitors are collected in Table 1. Different rates of degradation of EM-1 and EM-2 by DPP IV were observed. EM-1 was about 1.5 times more resistant to DPP IV than EM-2, which is in agreement with the data obtained by others (Tomboly *et al.*, 2002; Grass *et al.*, 2002; Fujita and Kumamoto, 2006; Keresztes *et al.*, 2010). EMDB-2 and EMDB-3 increased EM-1 and EM-2 half-lives two- to threefold. The effects of inhibitors on degradation of EMs after 30 min incubation with DPP IV

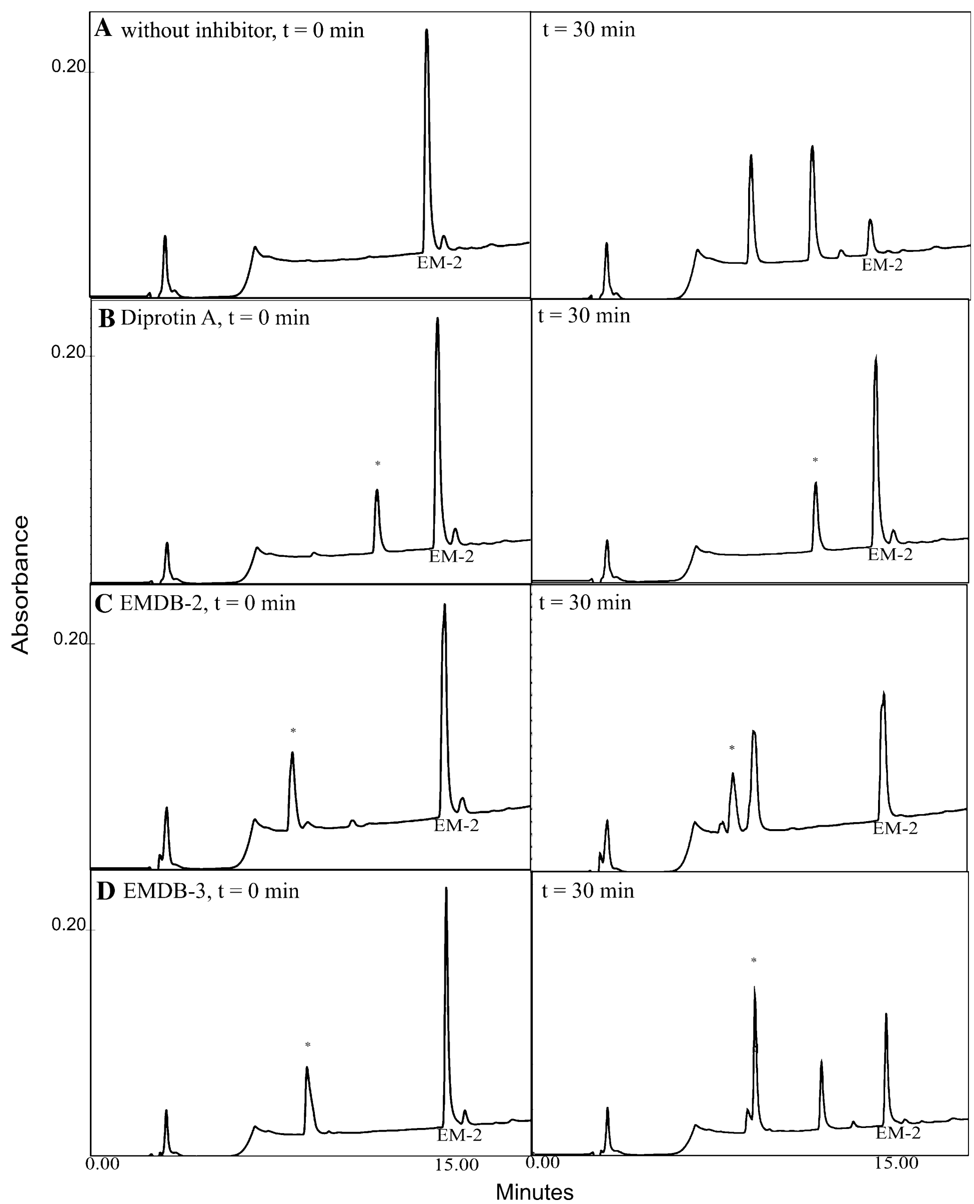

Fig. 2 Effect of inhibitors on the degradation of EM-2 by DPP IV. The reaction mixture was incubated at 37°C for 30 min in the absence (**a**) and presence of diprotin A (**b**), EMDB-2 (**c**), and EMDB-3 (**d**). *Asterisk* indicates the peak derived from the inhibitor added

Table 1 Degradation rates (k) and half-lives ($t_{1/2}$) of EMs incubated with DPP IV alone and in the presence of inhibitors

Inhibitor	DPP IV			
	EM-1		EM-2	
	$100 \times k$ (1/min)	$t_{1/2}$ (min)	$100 \times k$ (1/min)	$t_{1/2}$ (min)
Without inhibitor	4.12 ± 0.2	16.7 ± 0.52	6.30 ± 0.31	10.9 ± 0.64
Diprotin A	0.13 ± 0.01	530 ± 14.5***	0.18 ± 0.01	383 ± 20.2***
Tyr-Pro-Ala-NH_2 (EMDB-2)	3.02 ± 0.09	22.9 ± 1.14*	3.48 ± 0.13	19.8 ± 0.75*
Tyr-Pro-Ala-OH (EMDB-3)	2.51 ± 0.12	27.5 ± 1.21*	2.52 ± 0.13	27.4 ± 1.41*

* $P < 0.05$, *** $P < 0.001$ as compared to respective EM incubated in the absence of inhibitor by using one-way ANOVA followed by Student–Newman–Keul's test

Table 2 The effect of inhibitors on the degradation of EMs by DPP IV

Inhibitor	DPP IV			
	EM-1		EM-2	
	Inhibition (%)	K_i (μM)	Inhibition (%)	K_i (μM)
Diprotin A	96.8 ± 3.27	2.2[a]	97.1 ± 4.00	2.2[a]
Tyr-Pro-Ala-NH_2 (EMDB-2)	26.7 ± 1.20	420	44.8 ± 2.51	170
Tyr-Pro-Ala-OH (EMDB-3)	39.1 ± 1.41	270	60.0 ± 2.27	100

[a] Value taken from Ref. Umezawa *et al.* (1984)

are summarized in Table 2. EMDB-3 appeared to be a better DPP IV inhibitor than EMDB-2. The Lineweaver–Burk plots revealed that both tested compounds acted as competitive inhibitors of DPP IV (Fig. 3).

Effect of inhibitors on degradation of EMs by APM

EMDB-2 and EMDB-3 were then tested for their inhibitory effect on the degradation of EMs by APM. The known APM inhibitor, actinonin, was included for comparison. Degradation rates and half-lives of EMs alone and in the presence of inhibitors are collected in Table 3. EM-2 was slightly more resistant to APM degradation than EM-1, which is in agreement with earlier data by Peter *et al.* (1999). Both tested compounds turned out to be better inhibitors of EM degradation by APM than actinonin. The effect of inhibitors on degradation of EMs is summarized in Table 4. The Lineweaver–Burk plots revealed that both new compounds acted as competitive inhibitors of APM (Fig. 4).

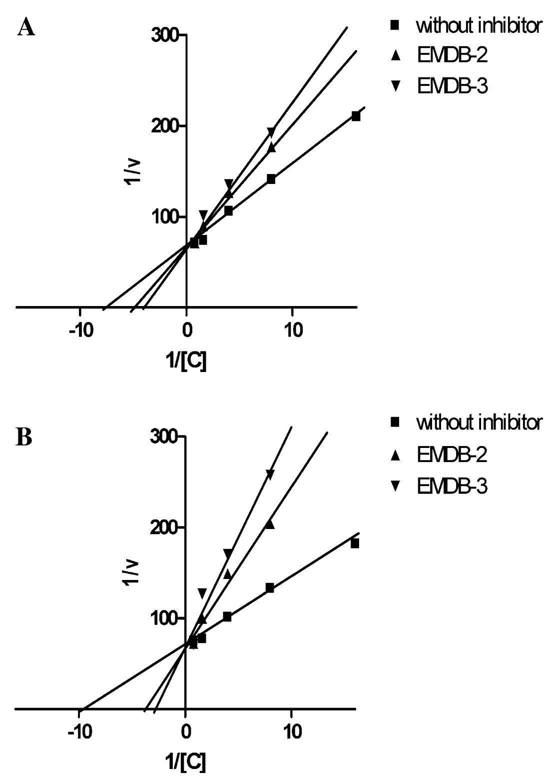

Fig. 3 Lineweaver–Burk diagrams for the inhibition of DPP IV by EMDB-2 and EMDB-3 in case of EM-1 (**a**) and EM-2 (**b**)

Discussion

The degradation of EMs is responsible for the fact that their analgesic activity decreases in time. Few inhibitors of DPP IV are described in the literature and all of them have limitations in terms of potency, stability or toxicity. Among them diprotin A and diprotin B are probably the best known and commercially available. They are competitive substrates that are slowly hydrolyzed and act as inhibitors for DPP IV at micromolar concentrations (Schon *et al.*, 1991).

Table 3 Degradation rates (k) and half-lives ($t_{1/2}$) of EMs incubated with APM alone and in the presence of inhibitors

Inhibitor	APM			
	EM-1		EM-2	
	100 × k (1/min)	$t_{1/2}$ (min)	100 × k (1/min)	$t_{1/2}$ (min)
Without inhibitor	3.51 ± 0.09	19.7 ± 0.50	2.96 ± 0.12	23.3 ± 0.98
Actinonin	1.88 ± 0.09	36.8 ± 2.10***	1.50 ± 0.05	46.3 ± 1.16**
Tyr-Pro-Ala-NH_2 (EMDB-2)	1.63 ± 0.06	42.3 ± 1.89***	1.28 ± 0.04	53.9 ± 1.53***
Tyr-Pro-Ala-OH (EMDB-3)	1.58 ± 0.05	43.7 ± 1.73***	1.44 ± 0.07	47.9 ± 2.14***

** $P < 0.01$, *** $P < 0.001$ as compared to respective EM incubated in the absence of inhibitor by using one-way ANOVA followed by Student–Newman–Keul's test

Table 4 The effect of inhibitors on the degradation of EMs by APM

Inhibitor	APM			
	EM-1		EM-2	
	Inhibition (%)	K_i (μM)	Inhibition (%)	K_i (μM)
Actinonin	46.2 ± 0.55	390	49.3 ± 0.90	300
Tyr-Pro-Ala-NH_2 (EMDB-2)	53.6 ± 1.21	130	56.8 ± 1.62	80
Tyr-Pro-Ala-OH (EMDB-3)	55.0 ± 1.10	100	51.4 ± 1.44	290

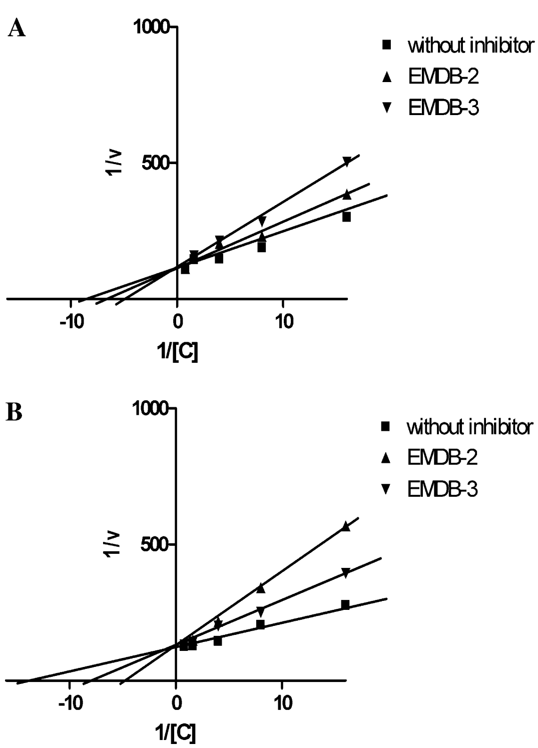

Fig. 4 Lineweaver–Burk diagrams for the inhibition of APM by EMDB-2 and EMDB-3 in case of EM-1 (**a**) and EM-2 (**b**)

The most potent DPP IV blockers so far reported are dipeptides containing boroPro, the boronic acid analog of Pro at the C-terminus (Flentke *et al.*, 1991). Although these compounds inhibit DPP IV action at nanomolar concentrations, they are quite unstable and that greatly limits their use. Dipeptide phosphonates described by Boduszek *et al.* (1994) are irreversible inhibitors of DPP IV, which are specific but not very potent. The series of aminoacylpyrrolidine-2-nitriles obtained by Li *et al.* (1995), that have K_i values in the micromolar range, are another group of specific DPP IV inhibitors with good potency and stability.

The studies presented here give evidence that EMDB-2 and EMDB-3 are potent inhibitors of enzymes responsible for EM cleavage. These compounds are stable and easily synthesized. EMDB-2 and EMDB-3 are competitive inhibitors of both, DPP IV and APM, with K_i values in submillimolar range. They are less potent than diprotin A in protecting EMs against DPP IV, but more potent than actinonin in protecting these peptides against APM.

So far we have shown that two new blockers of EM degrading enzymes, EMDB-2 and EMDB-3 significantly prolonged the inhibitory effects of EM-2 in gastrointestinal smooth muscle preparations (Fichna *et al.*, 2010). In vivo studies are under way to establish if these inhibitors can also prolong analgesic effect produced by exogenously administered EMs. Interestingly, preliminary results showed that EMDB-2 and EMDB-3 do not cross the blood–brain barrier, suggesting that their action is limited to the periphery after systemic administration.

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