Bioorganic & Medicinal Chemistry 22 (2014) 4803–4809

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Antinociceptive and antidepressant-like action of endomorphin-2 analogs with proline surrogates in position 2

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ARTICLE INFO

Article history:
Received 25 April 2014
Revised 23 June 2014
Accepted 30 June 2014
Available online 9 July 2014

Keywords:
Unnatural amino acids
Opioid receptors
Binding studies
Hot-plate test
Forced-swimming test
Tail-suspension test
Locomotor activity

ABSTRACT

In our efforts to develop new candidate drugs with antinociceptive and/or antidepressant-like activity, two novel endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH_2) analogs, containing proline surrogates in position 2 were synthesized using commercially available racemic *trans*-4-phenylpyrrolidine-3-carboxylic acid (4-Ph-β-Pro). The obtained mixture of two diastereoisomeric peptides (**2a** and **2b**) was separated by HPLC and both enantiopure analogs were used in the in vitro and in vivo studies. To assign the absolute configuration to the 4-Ph-β-Pro residues in both peptides, the stereoselective synthesis of (3*R*,4*S*)-4-phenylpyrrolidine-3-carboxylic acid was performed and this enantiomer was introduced into position 2 of EM-2 sequence. Based on the HPLC retention times we were able to assign the absolute configuration of 4-Ph-β-Pro residues in both peptide analogs. Analog **2a** incorporating (3*R*,4*S*)-4-Ph-β-Pro residue produced strong analgesia in mice after intracerebroventricular (icv) administration which was antagonized by the μ-opioid receptor (MOR) antagonist, β-funaltrexamine (β-FNA). This analog also influenced an emotion-related behavior of mice, decreasing immobility time in the forced swimming and tail suspension tests, without affecting locomotor activity. The antidepressant-like effect was reversed by the δ-selective antagonist, naltrindole (NLT) and κ-selective nor-binaltorphimine (nor-BNI). Thus, the experiments with selective opioid receptor antagonists revealed that analgesic action of analog **2a** was mediated through the MOR, while the δ- and κ-receptors (DOR and KOR, respectively) were engaged in the antidepressant-like activity. Analog **2b** with (3*S*,4*R*)-4-Ph-β-Pro in position 2 showed no antinociceptive or antidepressant-like activity in animal studies.

1. Introduction

Two highly selective endogenous μ-opioid receptor (MOR) ligands, endomorphin-1 (EM-1, Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (EM-2, Tyr-Pro-Phe-Phe-NH_2)[1] differ structurally from the so called 'typical' opioids (enkephalins, dynorphins, endorphins) in their only tetrapeptide length, C-terminal amidation and the Pro residue in the second position. NMR spectroscopy and molecular modeling studies indicate that Pro induces the other residues to assume the proper spatial orientation for the ligand–receptor interaction and plays in EMs a role of a stereochemical spacer.[2–4]

In order to define structural requirements for position 2, various chemical modifications were evaluated, as reviewed by Liu and Wang.[5] Simple replacement of Pro by D-Pro in both EMs resulted in a drastic loss of affinity, indicating that the L-configuration of Pro must be vital for MOR activation.[6,7] Introduction of β-(*R*)-Pro, but not β-(*S*)-Pro residue into position 2 of EM-1 produced two orders of magnitude increased affinity for the MOR.[8,9] Incorporation of (*R*)-piperidine-3-carboxylic acid [(*R*)-Nip], a six-membered surrogate of Pro, into the structure of EM-2 produced a significant increase in the MOR affinity.[10,11] Giordano et al.[12] designed a series of EM-2 analogs with *R* and *S* isomers of β-homoproline, 2-pyrrolidinemethanesulphonic acid (HPrs) or 3-pyrrolidinesulphonic acid (βPrs) instead of Pro. Among them, the highest MOR affinity (only 2-fold lower comparing to EM-2) was produced by a β-sulfonamido analog, [(*S*)-βPrs^2]EM-2. In the study of Borics et al.,[13] replacement of Pro^2 in the sequence of EM-2 by 2-aminocyclopentene- or cyclohexene-1-carboxylic acid residues produced analogs with similar to the parent compound MOR affinity.

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http://dx.doi.org/10.1016/j.bmc.2014.06.056
0968-0896/

EMs and their receptors are present in brain regions known to be involved in mood disorders[14–17] in the proximity of monoamine neurotransmitters (serotonin, dopamine, and noradrenaline), which play a key role in the pathophysiology of depression. In fact, EMs have been shown to modulate serotoninergic,[18–20] dopaminergic,[18,21–23] and noradrenergic[18,20,24] transmissions.

In most of the behavioral models of depression animals are exposed to mildly aversive situations from which there is no possibility to escape and which induce recognizable behavioral changes. In the forced-swimming (FST) and tail-suspension (TST) tests, which are quite sensitive and relatively specific, a prolonged exposure to aversive situations induces immobility, interpreted as an expression of despair which could be related to the depression syndrome.[25,26]

There are many pharmacological observations suggesting the implication of the opioid system in the pathogenesis of depression and in the mechanism of antidepressant action.[27,28] Traditional antidepressants, which do not bind to the opioid receptors, could cause an indirect modulation of opioid neurotransmission.[27] There is evidence, that the effects of tricyclic antidepressants (TCAs), which for a long time were the first choice for pharmacological treatment of clinical depression, is antagonized by the blockade of the opioid receptors, indicating the possible participation of opioid neurotransmission in the antidepressant activity of these drugs.[29] Moreover, a common pathway in the analgesic effect of both TCAs and opioids has been described. Valvedere et al.[30] suggested that TCAs produce antinociception partly via the participation of the endogenous opioid system and partly by activation of noradrenergic, serotonergic or dopaminergic pathways.

Current treatments of depression either fail to produce complete recovery or induce unwanted side-effect.[31] Therefore, it is desirable to seek new antidepressant-like drug candidates.

In this study we investigated the antinociceptive and antidepressant-like action of new EM-2 analogs containing a modified β-Pro residue, that is, 4-phenylpyrrolidine-3-carboxilic acid (4-Ph-β-Pro). This synthetic amino acid combines the conformational rigidity of the pyrrolidine ring of the native Pro with the presence of a nonpolar phenyl ring, thus realizing the increased lipophilicity requirement for better bioavailability of a peptide. The influence of the introduced chemical modification on enzymatic stability, receptor binding, and in vivo activities are reported.

2. Results and discussion

2.1. Chemistry

4-Phenylpyrrolidine-3-carboxylic acid has two stereogenic centers and four possible stereoisomers. We used commercially available racemic (3*R*,4*S*)- and (3*S*,4*R*)-4-phenylpyrrolidine-3-carboxylic acid (4-Ph-β-Pro) (Fig. 1), supplied by Dr. Olczak (TriMen Chemicals Ltd, Lodz, Poland), as a Pro surrogate for the synthesis of EM-2 analogs. The obtained mixture of two diastereoisomeric peptides (**2a** and **2b**) was separated by HPLC and both enantiopure analogs were used in the in vitro and in vivo studies. To assign the absolute configuration to the 4-Ph-β-Pro residues in both peptides we performed the stereoselective synthesis of (3*R*,4*S*)-4-phenylpyrrolidine-3-carboxylic acid and introduced this enantiomer into position 2 of EM-2 sequence. Based on the HPLC retention times we were able to assign the absolute configuration of 4-Ph-β-Pro residues in both analogs.

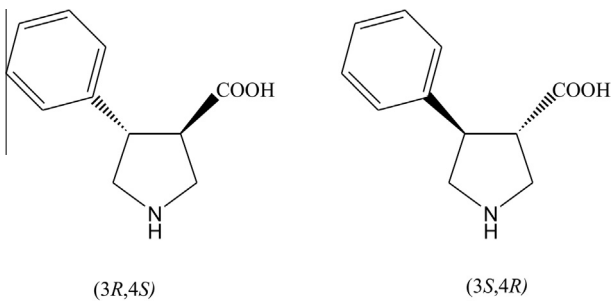


Figure 1. Structure of (3*R*,4*S*)- and (3*S*,4*R*)-4-phenylpyrrolidine-3-carboxylic acid (4-Ph-β-Pro).

Synthesis of N-Fmoc (3*R*,4*S*)-4-phenylpyrrolidine-3-carboxylic acid **5** was accomplished as shown in Scheme 1. Starting dimethyl [(1*S*)-2-nitro-1-phenylethyl]malonate (**1**) was obtained according to the literature procedure[32] by conjugate addition of dimethyl malonate to (*E*)-2-nitrostyrene with the use of de-methyl-quinine as a chiral catalyst. The subsequent reduction of the nitro group with concomitant intramolecular ring closure provided exclusively, thermodynamically more stable, methyl (3*R*,4*S*)-2-oxo-4-phenylpyrrolidine-3-carboxylate (**2**) with *trans* arrangement of the phenyl and methoxycarbonyl groups. This arrangement was confirmed by a characteristic J_{H3H4} coupling constant (9.6 Hz) in an ^{1}H NMR spectrum of **2**. This value is in full agreement with J_{H3H4} coupling constants observed in racemic *N*-substituted methyl *trans*-2-oxo-4-phenylpyrrolidine-3-carboxylates (J_{H3H4} = 9–10 Hz).[33] Treatment of pyrrolidine-3-carboxylate **2** with lithium diisopropylamide (LDA) resulted in reduction of both, amide and ester functionality, to give (3*R*,4*S*)-3-hydroxymethyl-4-phenylpyrrolidine (**3**). Finally, standard Fmoc protection of the amine functionality and subsequent oxidation of alcohol **4** yielded desired Fmoc-protected (3*R*,4*S*)-4-phenylpyrrolidine-3-carboxylic acid **5**, suitable for the solid phase peptide synthesis on the mild acid-labile support (e.g., Rink resin).

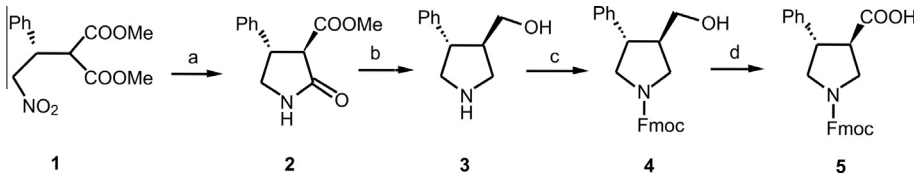
2.2. Peptide synthesis

Incorporation of the optically pure (3*R*,4*S*)-4-phenylpyrrolidine-3-carboxylic acid into the peptide sequence delivered peptide **2a**, whereas the use of the commercial racemic Fmoc-*trans*-4-phenylpyrrolidine-3-carboxylic acid provided two diastereomeric peptides that were resolved using preparative HPLC. Of these two peptides, one was identical (retention time, MS spectrum) to peptide **2a** and therefore the other one, peptide **2b**, contained 4-phenylpyrrolidine-3-carboxylic acid residue of (3*S*,4*R*) configuration. ^{1}H NMR spectra of both peptides revealed that they exist as a *E*/*Z* mixtures of conformational isomers due to restricted rotation around the Tyr-(4-Ph-β-Pro) amide bond. The ratio of isomers was 60/40 and 80/20 for **2a** and **2b**, respectively.

2.3. In vitro studies

The ability of the new EM-2 analogs to bind to opioid receptors was measured by displacement of [^{3}H]DAMGO and [^{3}H][Ile^{5,6}]deltorphin-2 from MOR and δ-opioid receptor (DOR) in rat brain membranes, and [^{3}H]nor-BNI from κ-opioid receptor (KOR) in guinea pig brain membranes, respectively. The binding results, expressed as equilibrium inhibition constants (IC_{50}) are provided in Table 1. Stereochemistry of the Pro surrogate played a critical role in the affinities of analogs. Peptide **2a** was equipotent with EM-2 at the MOR but showed also high affinity at the DOR and KOR. Analog **2b** was about three times less active at the MOR as compared with EM-2 and was inactive at the DOR and KOR.

The resistance of linear analogs to enzymatic degradation was tested using rat brain homogenate as a source of proteolytic enzymes. The analogs were incubated with the homogenate for 60 min and then the mixtures were analyzed by RP-HPLC. The obtained data are summarized in Table 2. The endogenous peptide,



Scheme 1. Reagents and conditions: (a) $CoCl_2 \times H_2O$, $NaBH_4$, MeOH, then EDTA, −10 °C, 26% yield. (b) $LiAlH_4$, THF, reflux, 79% yield. (c) Fmoc-OSu, Et_3N, DCM, rt, overnight 71% yield. (d) PDC, DMF, rt, overnight, 64% yield.

Table 1
Opioid receptor binding affinities of EM-2 and its analogs

No.	Sequence	IC_{50} ± SEM [nM]				
		MOR[a]	DOR[b]	KOR[c]	DOR/MOR	KOR/MOR
1	Tyr-Pro-Phe-Phe-NH_2 (EM-2)[d]	0.79 ± 0.05	>1000	>1000	>1000	>1000
2a	Tyr-(3*R*,4*S*)-4-Ph-β-Pro-Phe-Phe-NH_2	0.85 ± 0.01	9.06 ± 0.65	27.11 ± 1.85	10.66	31.89
2b	Tyr-(3*S*,4*R*)-4-Ph-β-Pro-Phe-Phe-NH_2	2.91 ± 0.65	>1000	>1000	>1000	>1000

All values are expressed as mean ± SEM of three independent experiments performed in duplicate.
[a] Displacement of [^{3}H]DAMGO.
[b] Displacement of [^{3}H][Ile^{5,6}]deltorphin-2.
[c] Displacement of [^{3}H]nor-BNI.
[d] Data from Ref. 44.

EM-2, was degraded rapidly, with half-life $t_{1/2}$ of about 6 min while both analogs were 5–6 times more stable.

2.4. In vivo studies

Antinociceptive activity of EM-2 and its two analogs was evaluated in the hot-plate test, in mice, after icv administration of the peptides at the dose range of 0.1–10 μg/animal. Analog **2a** produced a dose-dependent analgesic action, stronger than that of EM-2 (Fig. 2a), while analog **2b** failed to cause any antinociceptive effect which may suggest antagonist activity of this peptide at the MOR. To confirm that the analgesic action of **2a** was mediated through interaction with the opioid system, opioid receptor antagonists were used. The selective MOR, DOR and KOR antagonists, β-funaltrexamine (β-FNA), naltrindole (NLT) and nor-binaltorphimine (nor-BNI), respectively, (all in the dose of 1 μg/animal, icv, that was shown previously to produce no effect in the hot-plate test[34,35]), were used. β-FNA reversed the analgesic effect of analog **2a**, while the co-administration of **2a** with NLT or nor-BNI had no effect, indicating that the analgesic action of **2a** was mediated, exclusively by MOR (Fig. 2b).

In order to check a possible antidepressant-like activity of analogs **2a** and **2b**, these peptides were examined in two animal behavioral models of depression: FST and TST, as well as in locomotor activity assay (LMA).

In the FST, analog **2a** was tested in a dose range of 0.3–10 μg/animal following the icv injection. The obtained results are shown in Fig. 3a. Analog **2a** decreased, in a dose-dependent manner, the duration of immobility. The strongest effect ($p < 0.001$) was observed at a dose of 3 and 10 μg/animal. Analog **2b** was tested only at 3 μg/animal (in higher doses caused death) and did not produce any antidepressant effect, on the contrary, increased the immobility time. Yet another model of depression, the TST was performed for both analogs at the dose of 3 μg/animal (Fig. 3b). In the TST analog **2a** also produced antidepressant-like activity, reducing immobility time as compared with control. Analog **2b** did not influence animal behavior.

To establish which opioid receptor was engaged in mediating the observed antidepressant-like activity, analog **2a** was co-administered with selective antagonists of all three opioid receptors. The antagonists were used in the doses chosen on the basis of our previous studies[36] and alone did not produce any effects in the FST. As shown in Figure 3c NLT and nor-BNI, but not β-FNA, completely inhibited anti-depressant-like action of analog **2a**, indicating that the observed effect was mediated by DOR and KOR. The obtained results were in agreement with the binding studies, since **2a** had affinity also for these receptors.

In order to exclude that antidepressant-like activity of analog **2a** resulted from the stimulation of the horizontal motor activity, the LMA test was performed. The influence of the icv administration of analog **2a** on the locomotor activity of mice was measured over three consecutive 20 min periods (Fig. 4). Analog **2a** produced a small decrease in locomotor activity in the first two periods ($p < 0.05$). This is in agreement with other studies showing that classical antidepressants either do not affect or slightly decrease locomotor activity.[36,37]

Table 2
Degradation rates and half-lives of EM-2 and its analogs[a]

No.	Sequence	Brain homogenate	
		100 × k (per min)	$t_{1/2}$ (min)
1	Tyr-Pro-Phe-Phe-NH_2 (EM-2)	11.00 ± 0.58	6.00 ± 0.32
2a	Tyr-(3*R*,4*S*)-4-Ph-β-Pro-Phe-Phe-NH_2	2.12 ± 0.11***	32.55 ± 1.84***
2b	Tyr-(3*S*,4*R*)-4-Ph-β-Pro-Phe-Phe-NH_2	1.80 ± 0.09***	38.30 ± 1.42***

All values are expressed as mean ± SEM of three independent experiments performed in duplicate.
[a] RP-HPLC was performed on a Vydac C_{18} column (5 μm, 4.6 × 250 mm) using the solvent system of 0.1% TFA in water (A) and 0.1% TFA in acetonitrile/water (80:20, v/v) (B) and a linear gradient of 0–100% solvent B over 25 min at a flow rate of 1 mL/min.
*** $p < 0.001$ as compared to EM-2 by using one-way ANOVA followed by the Student-Newman–Keuls test.

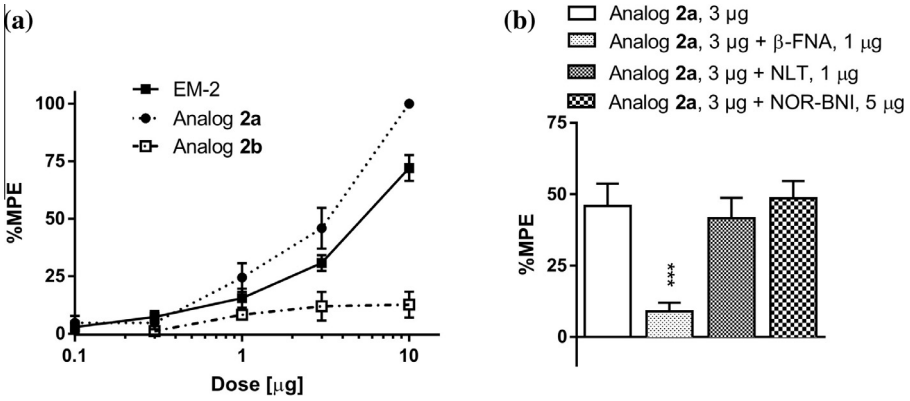


Figure 2. (a) Dose–response curves for the hot-plate inhibition of jumping induced by icv injection of EM-2 and analogs **2a** and **2b**. (b) Antagonist effect of β-FNA (1 μg/animal), NTL (1 μg/animal) and nor-BNI (5 μg/animal) induced by analog **2a** (3 μg/animal) determined in the hot-plate test in mice after icv injection. Data are mean ± SEM of 10 mice per group. $^{***}p < 0.001$ for analog + β-FNA versus analog alone using one-way ANOVA followed by the Student-Newman–Keuls' test.

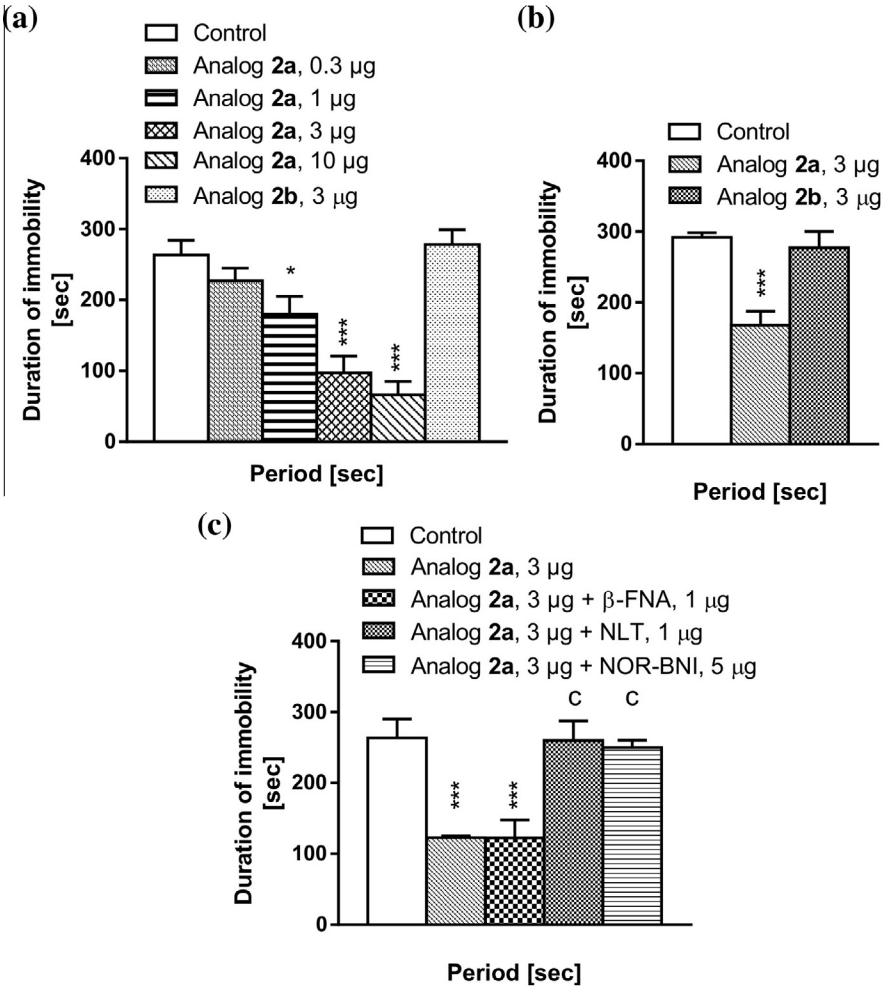


Figure 3. (a) Dose–response effect of analog **2a** (0.3–10 μg/animal) and **2b** (3 μg/animal) on the FST. (b) Effect of analog **2a** and **2b** (3 μg/animal) on the TST. (c) Comparison of antagonist action of β-FNA (1 μg/animal), NTL (1 μg/animal) and nor-BNI (5 μg/animal) on the analog **2a** (3 μg/animal)-induced effect on the FST. Mice were injected icv and submitted to the FST and TST 10 min after the injection. Data represent mean ± SEM of 10 mice per group. $^{*}p < 0.05$; $^{***}p < 0.001$, as compared to respective control, $^{c}p < 0.001$ as compared to analog alone by using one-way ANOVA followed by the Student-Newman–Keuls' test.

High opioid activity of EM analogs with modified Pro[2] residue may be, at least in part, the result of their increased stability against peptidases. Opioid peptides are known to undergo a rapid enzymatic degradation. The main enzyme responsible for EMs degradation is dipeptidyl peptidase IV (DPP IV) which liberates the N-terminal Tyr-Pro dipeptide with high afficiency.[38,39] DPP IV is unable to degrade peptide bonds in which Pro surrogates participate. Thus, introduction of a modified Pro into the sequence of EMs can play a special role in the peptide stability, both, in vitro and in vivo.

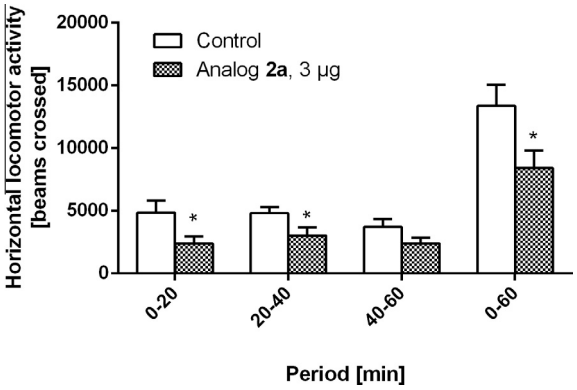


Figure 4. Effect of analog **2a** (3 μg/animal) on horizontal locomotor activity. Mice were injected icv with vehicle (control) or analog and placed in the actimeters 10 min after the injection. Horizontal displacements were measured for three consecutive periods of 20 min. Data represent mean ± SEM of 10 mice per group. $^{*}p < 0.05$ as compared to control by using one-way ANOVA followed by the Student-Newman–Keuls' test.

3. Conclusion

In our earlier study we have shown that EM-1 and EM-2 produced potent antidepressant-like effect after icv administration in mice in FST and TST depression tests and that this effect was mediated by the MOR.[36] In the present study we have tested two diastereoisomeric peptides, **2a** and **2b**, with (3*R*,4*S*)- and (3*S*,4*R*)-4-Ph-β-Pro residues in position 2, respectively. In vitro analog **2a** displayed high affinity for all three opioid receptors. In the hot-plate test only analog **2a** showed significant antinociception, even stronger than that produced by EM-2 and the effect was antagonized by the MOR-selective antagonist, β-FNA. In both, FST and TST, **2a** administered icv significantly decreased the duration of immobility, which could be related to the antidepressant-like responses. These effects were antagonized by DOR and KOR but not MOR-selective antagonists, indicating that analgesic and antidepressant-like effects can be dissociated.

In vitro analog **2b** showed an order of magnitude lower affinity for the MOR and did not bind to the other two opioid receptors. Analog **2b** failed to induce antinociception in mice at the dose up to 10 μg/animal and in the FST and TST, did not significantly influence the immobility time of animals.

Our observations are in accordance with the literature data indicating that the MOR is mostly involved in analgesia, while DOR, localized in the brain areas responsible for motor/motivational control,[27,40] and especially KOR present in the brain,[41] mediate antidepressant-like effects.[27,40]

The data obtained here confirm the results of earlier studies mentioned in the Introduction indicating that *R*-configuration of a β-Pro residue is necessary for the manifestation of agonist activity of EM analogs.

In many cases pain is accompanied by depression, but clinical treatment still focuses on the analgesic rather than mood altering effects.[42] Since we know that the opioid system is involved in both, the analgesic and mood-elevating effects, the analog **2a** with dual action, antinociceptive and antidepressant-like, could be considered an interesting structure in the search for new lead compounds in drug discovery.

4. Experimental

4.1. General

The analytical and semi-preparative RP-HPLC was performed on the Waters Breeze (Milford, MA, USA) with a Vydac C_{18} columns (5 μm, 4.6 × 250 mm and 10 μm, 22 × 250 mm), respectively. Mass spectra of peptides were recorded on Bruker Apex Ultra 7T FT-ICR mass spectrometer with electrospray ionization (ESI-MS; Billerica, MA, USA). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance II+ 700 spectrometer in $CDCl_3$ or DMSO-d_6 solutions. Chemical shifts were reported as δ values (ppm) related to tetramethylsilane (TMS). *J* values are given in Hz.

4.2. Chemistry

4.2.1. The preparation of methyl ((3*R*,4*S*)-2-oxo-4-phenylpyrrolidine-3-carboxylate (2)

The solution of dimethyl [(1*S*)-2-nitro-1-phenylethyl]malonate (**1**) (10.0 g, 35.5 mmol) in MeOH (130 mL) was cooled to −10 °C and stirred vigorously. The solution of $CoCl_2 \times H_2O$ (16.9 g, 71.1 mmol) was added, followed by a portion-wise addition of $NaBH_4$ (6.7 g, 177.8 mmol). After stirring for 3 h at −10 °C the reaction mixture was allowed to warm up to the ambient temperature and a solution of ethylenediaminetetraacetic acid disodium salt dihydrate (52.8, 141.2 mmol) in H_2O (300 mL) was added. The reaction mixture was extracted with Et_2O (3 × 100 mL), the combined organic extracts were dried ($MgSO_4$) and evaporated. Purification of the crude product by column chromatography (gradient elution CH_2Cl_2/MeOH: 500/1–300/1–100/1–9/1) yielded pure carboxylate **2** (2.02 g, 26%) as a white solid (mp 94–96 °C), $[\alpha]_D^{22}$ 104.8 (*c* 1.00 $CHCl_3$), IR 3369, 2920, 1730, 1694, 1432, 1281, 1158, 998 cm^{-1}. ^{1}H NMR (700 MHz, $CDCl_3$) 7.34–7.37 (m, 2H), 7.28–7.30 (m, 1H), 7.25–7.27 (m, 2H), 6.09 (br s, 1H), 4.14 (dt, *J* = 9.5 Hz, *J* = 8.7 Hz, 1H), 3.83 (dd, *J* = 9.4 Hz, *J* = 8.7 Hz, 1H), 3.79 (s, 3H), 3.59 (d, *J* = 9.5 Hz, 1H), 3.44 (dd, *J* = 9.4 Hz, *J* = 8.7 Hz, 1H). ^{13}C NMR (176 MHz, $CDCl_3$) 172.5, 169.6, 139.7, 129.0, 127.6, 127.0, 55.0, 52.8, 47.7, 44.3.

4.2.2. The preparation of (3*R*,4*S*)-3-hydroxymethyl-4-phenylpyrrolidine (3)

A solution of methyl 2-oxopyrrolidine-3-carboxylate **2** (3.90 g, 19 mmol) in THF (70 mL) was added to a stirred suspension of $LiAlH_4$ (1.47 g, 40 mmol) in THF (35 mL) at 60 °C and the reaction mixture was refluxed for 12 h. After cooling to 0 °C, H_2O (5 mL) was added, the mixture was stirred for additional 10 min and filtered through a Celite bed. The filtrate was evaporated and the crude product was purified by column chromatography (gradient elution with DCM/MeOH: 500/1–300/1–100/1–1/1) to give pure alcohol **3** (2.5 g, 79%) as a pale yellow oil, $[\alpha]_D^{22}$ 53.7 (*c* 0.50 $CHCl_3$), IR 2873, 1493, 1419, 1048, 906, 754 cm^{-1}. ^{1}H NMR (700 MHz, $CDCl_3$) 7.29–7.32 (m, 2H), 7.24–7.26 (m, 2H), 7.20–7.22 (m, 1H), 3.71–3.74 (m, 1H), 3.59–3.61 (m, 1H), 3.44–3.47 (m, 1H), 3.33–3.36 (m, 1H), 3.06–3.09 (m, 1H), 2.96–3.03 (m, 2H), 2.75 (br s, 2H), 2.40–2.42 (m, 1H). ^{13}C NMR (176 MHz, $CDCl_3$) 142.6, 128.7, 127.4, 126.6, 64.1, 55.5, 50.5, 49.8, 48.2.

4.2.3. The preparation of (3*R*,4*S*)-1-[(9-fluorenyl)methoxycarbonyl]-3-hydroxymethyl-4-phenylpyrrolidine (4)

A solution of pyrrolidine 3 (2.00 g, 11.3 mmol), 9-fluorenylmethyl *N*-succinimidyl carbonate (3.80 g, 11.3 mmol, 1 equiv) and Et_3N (3.12 mL, 33.9 mmol) in DCM (100 mL) was stirred overnight at rt. The reaction mixture was washed with 1 M HCl_{aq} (2 × 30 mL), brine (30 mL), dried ($MgSO_4$) and evaporated. Crude product was purified by column chromatography (gradient elution with DCM/MeOH: 500/1–300/1–100/1–1/1) to provide pure *N*-Fmoc-pyrrolidine 4 (3.2 g, 71%) as pale yellow oil, $[\alpha]_D^{22}$ 4.6 (*c* 0.50 $CHCl_3$), IR 2883, 1676, 1422, 1353, 1197, 1125 cm^{-1}. ^{1}H NMR (700 MHz, $CDCl_3$) 7.74–7.79 (m, 2H), 7.59–7.64 (m, 2H), 7.32–7.42 (m, 5H), 7.24–7.30 (m, 4H), 4.40–4.43 (m, 2H), 4.22–4.28 (m, 1H), 3.91–3.96 (m, 1H), 3.84–3.89 (m, 1H), 3.68–3.72 (m, 1H), 3.54–3.59 (m, 1H), 3.48–3.52 (m, 1H), 3.39–3.43 (m, 1H), 3.14–3.25 (m,

1H), 2.29–2.58 (m, 1H), 1.63 (br s, 1H). ^{13}C NMR (176 MHz, $CDCl_3$) 154.8, 144.1, 141.3, 140.0, 129.0, 128.9, 127.6, 127.5, 127.0, 125.1, 120.0, 67.3, 62.6, 54.8, 52.9, 49.3, 47.5, 45.7.

4.2.4. The preparation of (3*R*,4*S*)-1-[(9-fluorenyl)methoxycarbonyl]-4-phenylpyrrolidine-3-carboxylic acid (5)

Pyridinium dichromate (7.50 g, 20 mmol, 5 equiv) was added in one portion to a stirred solution of *N*-Fmoc-pyrrolidine **4** (1.6 g, 4 mmol) in DMF (40 mL) and the reaction mixture was stirred at rt overnight. Next, Et_2O/AcOEt (3/1, 100 mL) was added and the mixture was washed with 1 M HCl_{aq} (2 × 30 mL), brine (30 mL), dried ($MgSO_4$) and evaporated. Crude product was purified by column chromatography (gradient elution with DCM/MeOH: 200/1–100/1) to provide pure *N*-Fmoc-pyrrolidine-3-carboxylic acid **5** (1.05 g, 64%) as a white solid (mp 190–192 °C), $[\alpha]_D^{22}$ 29.9 (*c* 1.00 DMSO), IR 2888, 1694, 1418, 1323, 1224, 1127, 1022, 977 cm^{-1}. ^{1}H NMR (700 MHz, $CDCl_3$) 7.84–7.89 (m, 2H), 7.61–7.66 (m, 2H), 7.37–7.43 (m, 2H), 7.28–7.35 (m, 6H), 7.24–7.26 (m, 1H), 4.34–4.35 (m, 2H), 4.24–4.29 (m, 1H), 3.69–3.78 (m, 2H), 3.43–3.57 (m, 2H), 3.18–3.29 (m, 2H). ^{13}C NMR (176 MHz, $CDCl_3$) 173.2, 153.7, 143.9, 140.8, 139.6, 128.7, 127.8, 127.7, 127.4, 127.1, 125.1, 120.1, 66.5, 52.6, 49.4, 48.5, 47.2, 46.8.

4.2.5. Solid-phase peptide synthesis

Peptides were synthesized by standard solid phase procedure, using techniques for Fmoc-chemistry on the Rink amide 4-methylbenzhydrylamine (MBHA) resin (100–200 mesh, 0.8 mM/g) and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) as a coupling agent as described elsewhere.[11] Crude peptides were purified by RP-HPLC. using a linear gradient of 0–100% B over 40 min at the flow rate of 15 mL/min, with UV detection at 214 nm (injection volume 0.5 mL). Solvents: (A) 0.1% TFA in water and (B) 0.1% TFA in acetonitrile/water (80: 20, v/v). The purity of the final peptides was verified by analytical RP-HPLC in the same solvent system over 25 min with the flow rate 1 mL/min. The synthesized compounds were characterized by ESI-MS and ^{1}H NMR. The ^{1}H NMR spectra of both peptides revealed the presence of the mixtures of *E* and *Z* conformational isomers in 60/40 (for **2a**) and 80/20 (for **2b**) ratio, due to restricted rotation around the Tyr-(4-Ph-β-Pro) amide bond.

4.2.5.1. Tyr-(3*R*,4*S*)-4-Ph-β-Pro-Phe-Phe-NH$_2$ (2a). R_T: 16.350 min. ESI-MS calcd for $C_{38}H_{40}N_4O_6$ 647.70, found $[M+H]^+$ 648.30.

^{1}H NMR (700 MHz, DMSO-d_6) 2.66–3.08 (m, 10H + 10H, 60% + 40%), 3.17–3.22 (m, 1H + 1H, 60% + 40%), 3.35–3.44 (m, 1H + 1H, 60% + 40%), 3.59–3.65 (m, 1H + 1H, 60% + 40%), 4.36–4.43 (m, 1H + 1H, 60% + 40%), 4.51–4.57 (m, 1H + 1H, 60% + 40%), 6.69 (d, *J* = 8.5 Hz, 2H, 60%), 6.74 (d, *J* = 8.5 Hz, 2H, 40%), 6.98 (d, *J* = 8.5 Hz, 2H, 60%), 7.06 (d, *J* = 8.5 Hz, 2H, 40%), 7.07–7.36 (m, 15H + 15H, 60% + 40%), 8.01–8.04 (m, 1H + 1H, 60% + 40%), 8.10 (br s, 2H, 60% + 40%), 8.12 (d, *J* = 9.0 Hz, 1H, 40%), 8.18 (d, *J* = 8.5 Hz, 1H, 60%), 9.32 (s, 1H, 40%), 9.35 (s, 1H, 60%).

4.2.5.2. Tyr-(3*S*,4*R*)-4-Ph-β-Pro-Phe-Phe-NH$_2$ (2b). R_T: 17.191 min. ESI-MS calcd for $C_{38}H_{40}N_4O_6$ 647.70, found $[M+H]^+$ 648.30.

^{1}H NMR (700 MHz, DMSO-d_6) 2.27–2.32 (m, 1H + 1H, 80% + 20%), 2.62 (dd, *J* = 14.0 Hz, *J* = 8.5 Hz, 1H, 80%), 2.68 (dd, *J* = 14.0 Hz, *J* = 8.5 Hz, 1H, 20%), 2.78–3.02 (m, 6H + 6H, 80% + 20%), 3.22–3.27 (m, 1H + 1H, 80% + 20%), 3.36–3.41 (m, 1H + 1H, 80% + 20%), 3.66–3.72 (m, 1H + 1H, 80% + 20%), 3.85–3,92 (m, 1H + 1H, 80% + 20%), 4.19–4.25 (m, 1H + 1H, 80% + 20%), 4.40–4.44 (m, 2H + 1H, 80% + 20%), 4.51–4.55 (m, 1H, 20%), 6.72 (d, *J* = 8.0 Hz, 2H, 20%), 6.73 (d, *J* = 8.0 Hz, 2H, 80%), 6.86 (d, *J* = 8.0 Hz, 2H, 80%), 6.87 (d, *J* = 8.0 Hz, 20%), 6.93–7.32 (m, 15H + 15H, 80% + 20%), 8.03 (d, *J* = 7.5 Hz, 1H, 80%), 8.08 (br s, 2H, 80% + 20%), 8.12–8.16 (m, 1H + 2H, 80% + 20%), 9.37 (s, 1H, 20%), 9.47 (s, 1H, 80%).

4.3. Animals

Male Wistar rats (S3, Animal House, Faculty of Pharmacy, Lodz, Poland), weighing 200–250 g were used as a source of brain membranes and Male Swiss albino mice (CD1, JANVIER LABS, Le Genest-Saint-Isle, France), weighing 20–26 g, were used in the in vivo experiments. Animals were housed at a constant temperature (22 ± 1 °C) and maintained under a 12-h light/dark cycle in sawdust coated plastic cages with access to standard laboratory chow and tap water ad libitum.

4.4. In vitro experiments

4.4.1. Opioid receptor binding assays

Binding affinities of peptides for the MOR, DOR and KOR were determined by displacing [^{3}H]DAMGO, [^{3}H][Ile^{5,6}]deltorphin-2, or [^{3}H]nor-BNI, respectively, from the rat or guinea pig brain membrane binding sites, according to the method described by Misicka et al.[43]

4.4.2. Metabolic stability

Enzymatic degradation studies of the new analogs were performed using rat brain homogenate, following the method reported in detail previously.[44] Briefly, the analogs were incubated with brain homogenate over 0, 7.5, 15, 22.5, 30 and 60 min at 37 °C. The rate constants of degradation (*k*) were obtained by a least square linear regression analysis of logarithmic peak areas [ln(*A*/*A*$_0$)], where *A*—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*.

4.5. In vivo experiments

4.5.1. Assessment of antinociception

The analgesic activity of peptides was assessed in the hot-plate test in mice, as described earlier.[35] Tested peptides were dissolved in dimethyl sulfoxide (DMSO) and further diluted with saline to the desired concentrations. Control experiments showed that DMSO had no effect on the results. Intacerebroventricular injections (10 μl/mouse) of peptides or a vehicle, 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). The latencies to jumping were measured.

The percentage of the maximal possible effect (%MPE) was calculated as: $\%MPE = (t_1 - t_0)/(t_2 - t_0) \times 100$, where t_0–control latency, t_1–test latency and t_2–cut-off time (240 s).

4.5.2. The forced-swimming test (FST)

The FST was performed as described previously.[36,45] The apparatus consisted of two Plexiglas cylinders (20 cm height, 14 cm internal diameter), placed side-by-side in a Makrolon cage, separated by an opaque screen and filled with water (22 ± 1 °C) to a height of 12 cm. Twenty minutes before the test, the animals were placed in small individual cages (L: 25 cm, W: 9 cm, H: 8 cm) at an ambient temperature of 22 ± 1 °C. Ten minutes after the icv injection of a vehicle or an examined analog, groups of four mice were tested simultaneously for a 6-min period. The total duration of immobility was measured using automated FST for mice (FST X'PERT, BIOSEB, Vitrolles, France).

4.5.3. The tail-suspension test (TST)

The TST was performed as described previously.[36] Twenty minutes before the test animals were placed in small individual cages (as above). Ten minutes after the icv injection of a vehicle or an examined analog, mice were suspended by the tail, using an adhesive Scotch tape. Total duration of immobility was measured during the 6-min duration of the test.

4.5.4. Locomotor activity

Locomotor activity was assessed automatically in a computerized actimeter (Versamax, AccuScan Instruments, Inc., Ohio, USA) which monitored horizontal displacements.[36,45] Twenty minutes before the test the animals were placed in individual cages (as above). Ten minutes after the icv injection mice were placed individually in 20 × 20 × 30 cm compartments, in a dimly illuminated and quiet room, then motor activity was recorded. The responses were expressed as the number of crossed beams by mouse during three consecutive 20 min periods.

4.6. Statistical analysis

Statistical and curve-fitting analysis were performed using Graph Pad PRISM 5.0 (GraphPad Software In., San Diego, U.S.A.).

Results were 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. A probability level of 0.05 or lower was considered statistically significant.

Acknowledgments

This work was supported by a Grant from the Medical University of Lodz No 503/1-156-02/503-01, a Grant from the Medical University of Lodz for young researchers No 502-03/1-156-01/502-14-121 (to R.P.), Grants from the European Regional Development Fund (FEDER # 35233) (to S.C.A.C.) and from the EU INTERREG IVA 2 Seas Program (7-003-FR_TC2N) (to S.C.A.C.). R Perlikowska was a recipient of a fellowship from the Foundation for Polish Science ('Start') during the performance of this work.

The authors wish to thank Jozef Cieslak for his excellent technical assistance.

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