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

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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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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.

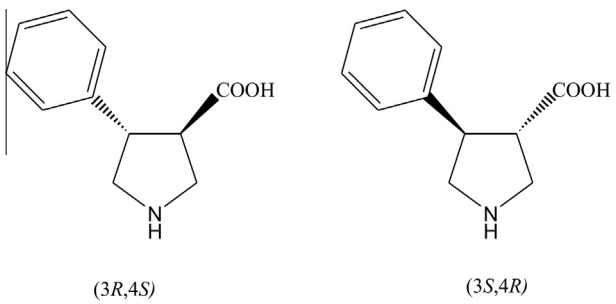

Figure 1. Structure of (3*R*,4*S*)- and (3*S*,4*R*)-4-phenylpyrrolidine-3-carboxylic acid (4-Ph-β-Pro).

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,