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Original article

Synthesis and biological evaluations of novel endomorphin analogues containing α-hydroxy-β-phenylalanine (AHPBA) displaying mixed μ/δ opioid receptor agonist and δ opioid receptor antagonist activities

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ABSTRACT

A novel series of endomorphin-1 (EM-1) and endomorphin-2 (EM-2) analogues was synthesized, incorporating chiral α-hydroxy-β-phenylalanine (AHPBA), and/or Dmt[1]-Tic[2] at different positions. Pharmacological activity and metabolic stability of the series was assessed. Consistent with earlier studies of β-amino acid substitution into endomorphins, multiple analogues incorporation AHPBA displayed high affinity for μ and δ opioid receptors (MOR and DOR, respectively) in radioligand competition binding assays, and an increased stability in rat brain membrane homogenates, notably Dmt-Tic-(*2R,3S*) AHPBA-Phe-NH_2 (compound **26**). Intracerebroventricular (i.c.v.) administration of **26** produced antinociception (ED_{50} value (and 95% confidence interval) = 1.98 (0.79–4.15) nmol, i.c.v.) in the mouse 55 °C warm-water tail-withdrawal assay, equivalent to morphine (2.35 (1.13–5.03) nmol, i.c.v.), but demonstrated DOR-selective antagonism in addition to non-selective opioid agonism. The antinociception of **26** was without locomotor activity or acute antinociceptive tolerance. This novel class of peptides adds to the potentially therapeutically relevant collection of previously reported EM analogues.

1. Introduction

Opioids are currently the gold standard for the clinical treatment of pain [1]. Opioid receptors belong to the superfamily of G-protein coupled receptors (GPCRs) [2], and are classified into three subtypes: the μ opioid receptor (MOR), the δ opioid receptor (DOR), and the κ opioid receptor (KOR). Agonist activation of each opioid receptor produces potent antinociception, but also results in significant side effects including tolerance and physical dependence, as well as respiratory depression and drug abuse (MOR), seizure activity (DOR) and psychomimetic effects (KOR) which complicate their clinical use [3]. Morphine, a potent MOR-preferring agonist, has been used clinically as an analgesic for decades, but possesses significant liabilities of use, notably dependence, tolerance, constipation, nausea, respiratory depression and abuse behavior, driving the search for safer alternatives. Endomorphin-1 (EM-1, YPWF-NH_2) and endomorphin-2 (EM-2, YPFF-NH_2) are two endogenous peptides first isolated from the bovine brain and the human cortex in 1997 [4], displaying high affinity and selectivity for the MOR. Of interest, both endomorphins produced potent MOR-mediated antinociception without some of the undesirable side effects of morphine [4]. In particular, the rewarding effect of endomorphins are readily separated from the therapeutic doses producing analgesia, and they are less likely than morphine to induce respiratory depression and cardiovascular effects at efficacious antinociceptive doses [5]. However, like many endogenous opioid peptides, endomorphins are susceptible to rapid proteolysis and metabolism, low lipid solubility and limited ability to cross the blood–brain barrier (BBB) [6]. These effects greatly reduce the magnitude and duration of endomorphin activity, demonstrated by

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less than 30 min' antinociception after direct administration into the CNS, and a failure to elicit antinociception following peripheral administration in rodents [7]. Notably, several studies have attempted to increase endomorphin activity through chemical modification of the peptide, seeking to overcome the problems of rapid enzymatic degradation and BBB impermeability by utilizing the incorporation of unnatural amino acids [8,9], various types of cyclization [10–13], change of peptide backbone [14], modification of the pharmacophore groups [15], glycosylation [7,16,17], lipidation [17], and introduction of conformational constraints [18]. The resultant peptide mimics are designed to retain or improve the biological selectivity and activity of natural endomorphins while overcoming the undesirable poor bioavailability and rapid metabolism.

In several cases, an α-amino acid has been substituted with its β-isomer in biologically active peptides, resulting in an increased activity and enzymatic stability [19,20]. It has been reported that the replacement of Pro^2 (in EM-1/EM-2) with alicyclic β-amino acids, such as β-proline [21], piperidine-3-carboxylic acid [22], azetidine-3-carboxylic acid [23], 2-aminocyclopentane-1-carboxylic acid, 2-aminocyclohexane-1-carboxylic acid [24], 2-aminocyclopentene-1-carboxylic acid and 2-aminocyclohexene-1-carboxylic acid [25], leads to compounds with increased affinity for the MOR and improved metabolic stability. Notably, β-amino acids have also been successfully utilized as substitutes for Trp^3 or Phe^4 in EM-1 analogues [26]. More generally, inclusion of the dipeptide fragment Dmt^1-Tic^2 is known to introduce a versatile opioid pharmacophore, adding a wide range of activities by interaction with MOR and/or DOR [27]. Dmt promotes DOR/MOR agonist activity, while Tic is reported to promote DOR-antagonist activity when incorporated alone into position 2 of opioid peptides [28].

In the present study, we introduced several β-amino acids into endomorphins, including the novel unnatural chiral 3-amino-2-hydroxy-4-phenylbutanoic acid (AHPBA) and the commercially available 3-amino-4-phenylbutyric acid (β-HoPhe-OH) (Scheme 1). The use of AHPBA was a key interest in this study, as it places a free hydroxyl group onto the back-bone of the peptide which could be utilized for future modifications such as the introduction of a lipid or sugar group, potentially improving BBB penetration and facilitating oral administration of drugs. The protected chiral AHPBA building blocks used in the peptide synthesis are shown in Scheme 2. These were obtained from chiral phenylalanine (Boc-*D*-Phe-OH) and *N*,*O*-Dimethyl hydroxylamine hydrochloride. The corresponding coupling adducts were further reacted with vinylmagnesium chloride to generate the elongation of the carbon chain. The subsequent reduction with sodium borohydride and cerium chloride heptahydrate generated a new chiral center at position 3. The resulting crude product was used directly in a reaction with benzyl bromide. The chiral products were then separated by chromatography and oxidized to afford the desired benzyl protected AHPBA (Scheme 3). The same procedure was repeated with Boc-*L*-Phe-OH. The benzyl group of these building blocks was removed by HF during the cleavage of the final peptides from the solid support generating the free hydroxyl.

In previous studies, β-HoPhe-OH was incorporated into EM-1 analogues, but resulted in low affinity for MOR [21c]. Chiral AHPBA has been found in various natural products as well as synthetic biological active compounds in drug discovery efforts [29]. In the present study EM analogues in which these β-amino acids were employed at positions 3 or 4 were synthesized and evaluated. Herein, we described a detailed pharmacological characterization *in vitro* and *in vivo*, as well as enzymatic degradation studies of these endomorphin analogues. The opioid receptor affinity for all analogues was first determined by radioligand competition binding experiments, and functional activity of selected lead compounds evaluated *in vitro* using a label free assay to monitor cell movement in response to receptor activation. The degradation rate of the endomorphin analogues was examined in the presence of rat brain homogenate. Antinociception and the opioid receptor selectivity of agonist and antagonist effects of the most promising compounds was characterized *in vivo* with the mouse 55 °C warm-water tail-withdrawal test after intracerebroventricular (i.c.v) administration. Finally, initial liabilities of the lead compounds were evaluated in mouse assays of coordinated locomotor activity and acute antinociceptive tolerance.

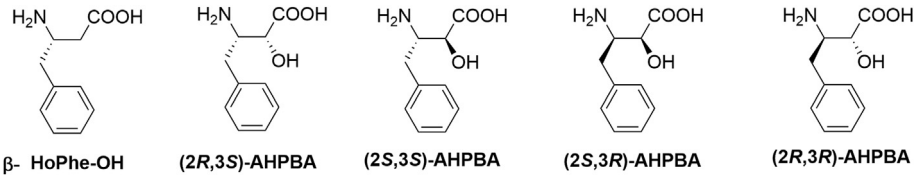
2. Chemistry

Chiral Boc-3-amino-2-(benzyloxy)-4-phenylbutanoic acids were prepared in solution-phase (see Experimental for details). EMs and their analogues were synthesized by standard solid-phase methods on MBHA resin using Boc-protected amino acids and DIC/HOBt as coupling reagents. Peptides, as well as the benzyl group of the protected AHPBA building blocks, were cleaved from the resin by HF in the usual manner. Peptides were purified by using preparative RP-HPLC and characterized by RP-HPLC, ESI-MS, HRMS and ^{1}H NMR. Purities were determined to be at least 97% in all cases. Analytical properties of EMs and their analogues are summarized in tabular form (as Table S1) with accompanying NMR spectra (as Section S2) in the Supplementary data.

3. Results

3.1. Evaluation of opioid receptor affinity and selectivity for the endomorphin analogues

Affinities of EM-1, EM-2 and their analogues for MOR, DOR, and KOR were determined by measuring the IC_{50} values for the inhibition of [^{3}H]DAMGO, [^{3}H]DPDPE and [^{3}H]U69,593 respectively, from their rat brain membrane binding sites. Calculated inhibitory constants (*Ki*) and opioid receptor selectivity (Ki^{δ}/Ki^{μ}, Ki^{κ}/Ki^{μ}) of the analogues are listed in Table 1. The affinity and MOR selectivity of EM-1 and EM-2 agreed well with previously published results [30]. The Ki^{μ} values ranged from 3–2083 nM for the set of analogues tested. The EM-1 analogues, in which Phe^4 was replaced by the four isomers of AHPBA (**2**–**5**), all showed decreased (2.5–61 fold lower) MOR affinity, and a lower MOR selectivity (as compared vs DOR). [(*3S*)-AHPBA^4]EM-1 (**2** and **3**) showed much higher affinity for MOR as compared to the corresponding [(*3R*)-AHPBA^4]EM-1 (**4** and **5**). Among these four analogues, [(*2S*,*3S*)-AHPBA^4]EM-1 (**3**) displayed



Scheme 1. Structures of β-amino acids incorporated into endomorphins.