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Blood–brain transfer and antinociception of linear and cyclic *N*-methyl-guanidine and thiourea-enkephalins

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ABSTRACT

Enkephalins are active in regulation of nociception in the body and are key in development of new synthetic peptide analogs that target centrally located opioid receptors. In this study, we investigated the *in vivo* blood–brain barrier (BBB) penetration behavior and antinociceptive activity of two cyclic enkephalin analogs with a thiourea (CycS) or a *N*-methyl-guanidine bridge (CycNMe), and their linear counterparts (LinS and LinNMe) in mice, as well as their *in vitro* metabolic stability. ^{125}I-LinS had the highest blood–brain clearance (K_1 = 3.46 μL/g min), followed by ^{125}I-LinNMe, ^{125}I-CycNMe, and ^{125}I-CycS (K_1 = 1.64, 0.31, and 0.11 μL/g min, respectively). Also, these peptides had a high metabolic stability ($t_{1/2}$ > 1 h) in mouse serum and brain homogenate, and half-inhibition constant (K_i) values in the nanomolar range with predominantly μ-opioid receptor selectivity. The positively charged NMe-enkephalins showed a higher antinociceptive activity (LinNMe: 298% and CycNMe: 205%), expressed as molar-dose normalized area under the curve (AUC) relative to morphine, than the neutral S-enkephalins (CycS: 122% and LinS: 130%).

Abbreviations: AUC, area under the curve; Alloc, allyloxycarbonyl; BBB, blood–brain barrier; Boc, tertButyloxycarbonyl; BSA, bovine serum albumin; tBu, tertButyl; CD, capillary depletion; CNS, central nervous system; CycNMe, H-Tyr-c[D-Ala-Gly-Phe-*Nω*-methylguanidino-Ala]-NH_2; CycS, H-Tyr-c[D-Ala-Gly-Phe-thioureaAla]-NH_2; DADLE, [D-Ala^2, D-Leu^5]-Enkephalin; DAMGO, ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; Dap, diaminopropionyl; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, dimethylformamide; DMSO, dimethylsulfoxide; DOR, δ-opioid receptor; DPDPE, [d-Pen^2,d-Pen^5]-Enkephalin; Fmoc, fluorenylmethyloxycarbonyl; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; HBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high-performance liquid chromatography; ^{125}I-CycNMe, radiolabeled CycNMe; ^{125}I-CycS, radiolabeled CycS; ^{125}I-LinNMe, radiolabeled iodinated LinNMe; ^{125}I-LinS, radiolabeled LinS; I-CycNMe, "cold" (non-radioactive) iodinated CycNMe; I-CycS, "cold" (non-radioactive) iodinated CycS; I-LinNMe, "cold" (non-radioactive) iodinated LinNMe; I-LinS, "cold" (non-radioactive) iodinated LinS; LC-MS, liquid chromatography coupled mass spectrometer; LinNMe, H-Tyr-D-Ala-Gly-Phe-*Nω*-methylguanidino-Ala-NH_2; LinS, H-Tyr-D-Ala-Gly-Phe-thioureaAla-NH_2; LNAA, large neutral amino acids; LR, Lactated Ringer; MeI, methyl iodide; MOR, μ-opioid receptor; MPE, maximum possible effect; MTR, multiple time regression; NMM, *N*-methylmorpholine; NMP, *N*-methylpyrrolidone; OATP-A, organic anion-transporting polypeptide A; PS, polystyrene; PTS, peptide transport system; RP-HPLC, reversed-phase high-performance liquid chromatography; SEM, standard error on mean; TFA, trifluoroacetic acid; TIS, triisopropylsilane.

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Introduction

Peptides serve many functions of the central nervous system (CNS), including the perception and modulation of pain. It was suggested that these substances may exert many of their effects by crossing the blood–brain barrier (BBB), directly influencing the CNS [5]. The use of these small molecules as pharmacological agents is attractive because of the generally low toxicity of their metabolites, and the enhanced potency and selectivity. Despite the growth in understanding of neurological disorders, at this point, peptide-based therapeutics are still rare [45]. This is largely due to inadequate delivery of active peptides to their receptors, located in specific brain and cellular regions. The delivery of peptide-based drugs to their brain target is limited by several factors, *i.e.* the general bioavailability and the existence of a BBB [24]. The BBB is a complex anatomical, physiological, and biochemical defense mechanism, protecting the brain from toxic substances and serving to maintain brain homeostasis [3,24,56]. Although the delivery of (therapeutic) peptides to the brain is limited, there is evidence that peptides cross the BBB by one of two main mechanisms, *i.e.* direct membrane permeation by passive diffusion, or saturable, active or facilitated, transport systems, or both [5].

The pentapeptides methionine (Met-) and leucine (Leu-) enkephalin are naturally occurring small neuropeptides distributed in both the central and peripheral nervous systems of vertebrates [2,18,19,21]. These endogenous opioid peptides are involved in regulating nociception in the body. The presence of the N-terminal tyrosine residue appears to be an absolute requirement for their pharmacological opioid activity and essential for active transport from brain-to-blood of some small peptides, *e.g.* Met- and Leu-enkephalins, by peptide transport system 1 (PTS-1) [6,56,67]. Moreover, enkephalins, especially Leu-enkephalin, are relatively lipid soluble, making them, at least partly, capable of crossing the BBB by passive diffusion.

The bioavailability of peptides to the brain is limited, due to poor metabolic stability and/or the inability to cross the BBB. Besides a physical barrier, the BBB is also a metabolic barrier, containing a number of enzymes, including enkephalinases (such as aminopeptidases and endopeptidases), carboxypeptidases, and angiotensin-converting enzyme [27,63]. *In vitro*, inhibition of these enzymes increased the penetration of opioid peptides [13]. However, enzyme inhibition generally is not a practical means of increasing the BBB entry of peptides, because of the multiplicity of enzymes that degrade peptides and toxicity issues. Other physical and chemical methodologies to increase BBB penetration have been investigated, including a more favorable lipophilicity by chemical modification [14,15,65], targeting of known transport mechanisms [1,42], glycosylation [26], and/or co-administration with compounds which improve BBB entry [27,63,64]. Therefore, synthetic modifications promise to rationally improve key pharmacokinetic characteristics [14]. As such, peptide cyclization is a recognized and powerful tool of peptide chemistry for generating analogs with improved pharmacokinetic properties [37]. Recently, a structural study and synthesis of a new kind of cyclic peptides, which incorporated either a thiourea bridge or a *N*-alkyl substituted guanidine bridge, have been reported [52]. This approach has been applied for the first time to enkephalins, yielding analogs with nanomolar affinity and moderate selectivity towards the μ-opioid receptor [51]. In this study, we investigated the effect of structural variation on BBB penetration behavior and antinociceptive activity using four synthetic amidated enkephalin analogs: two cyclic enkephalins, incorporating either a thiourea bridge (CycS) or a *N*-methyl guanidine bridge (CycNMe), and their two linear counterparts H-Tyr-D-Ala-Gly-Phe-thioureaAla-NH_2 (LinS) and H-Tyr-D-Ala-Gly-Phe-*Nω*-methylguanidino-Ala-NH_2 (LinNMe) (Fig. 1). We tested the hypothesis that both cyclization and the nature of the bridge interactively influence the biological characteristics.

Materials and methods

Materials

Male ICR-CD-1 (Institute for Cancer Research, Caesarean Derived-1) mice (Harlan Laboratories), weighing 25–30 g, were used according to the Ethical Committee principles of laboratory animal welfare as approved by Ghent University (Faculty of Veterinary Medicine, EC: 2009/052). The animals were group-housed in a temperature/humidity controlled room with a light/dark (day/night) cycle for at least 5 days before the experiment. Food (Harlan Laboratories) and water were available ad libitum.

Fmoc-protected amino acids, HBTU, DIEA, TFA, piperidine, solvents, and other reagents were purchased from Iris-Biotech, Novabiochem, Riedel-de Haën, Carlo Erba or Acros organics, and used without further purification. Fmoc Rink amide polystyrene resins (100–200 mesh, 0.14 mmol/g for the cyclic peptides; 0.45 mmol/g for the linear peptides) were purchased from Iris-Biotech. Solvents used for RP-HPLC and LC–MS were of HPLC grade.

Bovine serum albumin (BSA), calcium chloride dihydrate, D-glucose, HEPES, magnesium sulfate heptahydrate, potassium chloride, disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, sodium chloride, sodium lactate, urethane, Krebs–Henseleit (KH) buffer, and sodium iodide were all purchased from Sigma and Merck KGaA. Dextran was obtained from AppliChem GmbH, sodium bicarbonate from UCB and formic acid (99%) from Acros Organics. Pierce pre-coated Iodogen tubes and $Na^{125}I$ (3700 MBq/mL) were obtained from Thermo Scientific and Perkin Elmer, respectively.

[^{3}H]DAMGO ([D-Ala^2, NMePhe^4, Gly^5-ol]-enkephalin; 1.517E + 06 MBq/mmol) and [^{3}H]DIDI ([Ile^{5,6}]-deltorphin-II, 1.776E + 06 MBq/mmol) were synthesized in the Isotope Laboratory of BRC.

Methods

Peptide synthesis

The synthesis of the two cyclic enkephalin analogs, CycS and CycNMe, was performed on solid phase and is published in detail elsewhere [51,52]. Another solid phase synthetic strategy, according to the synthesis of *N*ω-mono- and di-alkylated-arginine containing compounds, was used for the synthesis of the two linear enkephalin analogs LinS and LinNMe [33]. The protected peptide Boc-Tyr(tBu)-D-Ala-Gly-Phe-Dap(Alloc) was first assembled by manual solid-phase synthesis on a Rink amide PS resin following Fmoc chemistry with HBTU/DIEA as coupling agents [51,52]. After peptide assembly, the Alloc group was removed by treatment of the resin with $Pd[PPh_3]_4$ (0.2 equiv.) and phenylsilane (24 equiv.) in anhydrous DCM. After 4 h of stirring at room temperature, the resin was filtered and washed twice with DCM, DMF, DCM, after which the treatment was repeated. The deprotected side-chain amino group reacted with Fmoc-isothiocyanate (5 equiv.) in DCM for 3 h at room temperature to give the Fmoc-protected thiourea-Ala residue. The Fmoc group was removed by two consecutive treatments with piperidine/DMF (20/80) for 10 and 25 min and the resin was separated into two portions: one was submitted to TFA cleavage to yield compound LinS, the second was treated three times for 1 h with a 2 M solution of MeI in DMF to methylate the thiourea moiety. Guanidinylation of the S-methyl-isothiourea intermediate was performed by reaction with a 2 M methylamine HCl/NMM solution in DMSO overnight at 80 °C, followed by TFA cleavage to yield compound LinNMe.