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The improved blood–brain barrier permeability of endomorphin-1 using the cell-penetrating peptide synB3 with three different linkages

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ABSTRACT

Endomorphins, although they have high analgesic activity and few undesirable side effects, are not in clinical use because of the blood–brain barrier (BBB). One promising solution is to use cell-penetrating peptides (CPPs). CPPs have the ability to translocate cell membranes and have been successfully applied for delivery of therapeutic molecules across the BBB. However, little is known about the transport efficiency of different conjugation strategies between cargo and CPPs. In this study, endomorphin-1 (EM-1) was conjugated with SynB3, an efficient CPP-carrier, via amide, maleimide and disulfide linkages. The delivery efficiency of three linkers was compared in terms of pharmacodynamics and *in vitro* metabolic stability. Near-infrared fluorescent and fluorescent microscopy experiments were applied to detect the brain uptake and distribution of CPP delivery qualitatively and quantitatively. After the most successful linkage was screened out, the further mechanisms were discussed. We concluded that compared with the other two linkages, the disulfide bond was the most efficient linkage to deliver EM-1 across the BBB and confirmed that it could be reduced at physiological conditions in the brain and release its active form. These findings indicate that for those who need to release a free drug in the brain and maintain activity, a disulfide bond might be the most efficient linkage across the BBB.

1. Introduction

In 1997, two endogenous μ-opioid receptor agonists, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2, EM-1) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2, EM-2) were reported by (Zadina et al., 1997). The agonists exhibited high affinity and selectivity for the μ-opioid receptor. Both EM-1 and EM-2 induce potent antinociceptive effects in various pain models without some of the undesirable side effects of morphine (Carrigan et al., 2000; Czapla et al., 2000; Sakurada et al., 2001; Wilson et al., 2000). Despite the potential advantageous properties, endomorphins and their potential analogs are only active when they are injected directly into the brain. When systemically administered, they tend to lose effectiveness because they are unable to be transported effectively into the brain to exert their biological effects. The major obstacle to targeting the brain for therapy is the existence of the blood–brain barrier (BBB). The BBB is primarily formed of brain capillary endothelial cells with tight junction structures. This structure protects the brain against unwanted substances, including pathogenic organisms and therapeutic molecules, entering into the central nervous system (CNS) from blood circulation. Therefore, the BBB is the most important physical and enzymatic barrier for most neuropeptides, including EMs, when administered systemically.

To overcome the BBB and improve uptake of a drug in the brain, various strategies have been developed, including invasive and non-invasive methods. The principal defect of the invasive strategies is that they are highly traumatic with low therapeutic efficiency and substantial side effects. In contrast, the non-invasive methods provide significant advantages because of their maintaining of the integrity of the BBB and increasing the permeability, stability, bioavailability, and/or receptor affinity of peptides. The following non-invasive approaches have been applied: chemical modification of the peptide itself, conjugation of the peptide to shuttle compounds and encapsulation of the peptide in a

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substance (Brasnjevic et al., 2009; Malakoutikhah et al., 2011; Witt et al., 2001).

Among these strategies, much attention has been given to the use of peptide-vector-mediated delivery systems, such as the cell-penetrating peptides (CPPs). CPPs are a group of small naturally derived peptides with the ability to translocate through cellular membranes and these peptides have been researched in recent decades (Bechara and Sagan, 2013; Fonseca et al., 2009; Vives et al., 2008). CPPs have been widely reported as a novel technology for enhancing the delivery of therapeutic drugs, e.g., small molecules, peptides, proteins, liposomes and nanoparticles, across the BBB (Herve et al., 2008; Scherrmann and Temsamani, 2005; Zou et al., 2013). Most articles focus on the selection of CPPs that achieve efficient transport across the BBB and the expansion range of the transport molecule. However, very few examples of the various types of conjugation between biologically active cargo and CPPs have been published, and in many cases, the conjugation of CPPs does not allow sufficient cargo delivery through the BBB and into the CNS for biological activity. It is common to include at least two types of linkages, including labile linkages and stable linkages. The labile linkages are usually ester and disulfide conjugates, and thiol-maleimide is often used as a stable linkage. We concentrate here on the impact of different linkages on BBB permeability.

In this report, we explored a SynB3 (RRLSYSRRRF) peptide derived from the antimicrobial peptide protegrin 1 (Harwig et al., 1995) as a CPP carrier, which is the most efficient CPPs in terms of delivering different moleculars across the BBB (Rousselle et al., 2000, 2002, 2003; Temsamani et al., 2005). We coupled EM-1 with SynB3 via three covalent linkages, including amide, maleimide and disulfide linkages, and measured the brain uptake and pharmacological effects. First, we compared the antinociceptive activities of three conjugates using a tail-flick test after i.v. administration and assessed metabolic stability in mouse brain homogenate and serum. Then, using the near-infrared (NIR) fluorescent dye Cy7, the tissue penetration of the fluorescent chimeric peptide was measured *in vivo* and *in vitro* by a whole mouse imaging system and fluorescent microscopy. The present data indicated that the chimeric peptide with a disulfide linkage had the maximal analgesic effect and was delivered much more efficiently into the brain among the three linkages. Thus, finally, we moved toward *in vivo* and *in vitro* proof of the cleavability of the disulfide linkage through *in vivo* antinociception behavior and *in vitro* MS analysis.

2. Materials and methods

2.1. Materials and animals

All reagents and chemicals, unless otherwise stated, were obtained from Sigma–Aldrich (Dorset, U.K.) or J&K Scientific Ltd., Commercial Fmoc-protected amino acid, *O*-(1H-benzotriazole-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), 1-hydroxybenzotriazole (HOBT) and *N*,*N*-diisopropylethylamine (DIEA) were obtained from GL Biochem (Shanghai) Ltd. (China). MBHA resin (0.45 mmol·g^{-1}) were obtained from Tianjin Nankai Hengcheng S&T Co., Ltd. (China). The Cy7 peptide was obtained from Fanbo Biochemicals Ltd. (China). All reagents and chemicals were used without further purification.

Male Kunming mice (Animal Center of Medical College of Lanzhou University, Gansu, China) were employed for analgesia studies (18–22 g) and metabolic stability analyses. The SPF level Kunming mice (18–22 g, Animal Center of Medical College of Lanzhou University, Gansu, China) were used for optical imaging scans. All animals were cared for, and experiments were performed in accordance with the principles and guidelines of the Ethics Committee of Lanzhou University.

2.2. Peptide synthesis

The peptides were synthesized on MBHA resin using the standard Fmoc-chemistry-based strategy. All crude peptides were purified by reversed-phase high-performance liquid chromatography (RP-HPLC) and were then characterized by electrospray ionization mass spectrometry (ESI-MS).

2.3. Synthesis of conjugates

The synthesis of the amide linkage procedure was performed using the same instrument as described above in Section 2.2. The other linkages, including maleimide (Ede et al., 1994; Song et al., 2009) and disulfide (Carlsson et al., 1978; Xie et al., 2005), used methods described in previous papers.

2.4. Assessment of antinociception

The antinociceptive responses were assessed in mice with the tail-flick test. Nociception was evoked by immersing the mouse's tail in hot water ($50 \pm 0.2^\circ$ C) and measuring the latency to withdrawal. Before treatment, the latency to tail-flick [control latency (CL)] was determined, and no response within 5 s was excluded. The tail-flick responses were measured at different times after i.v. injection of drugs. The latency to tail-flick was defined as the test latency (TL). The maximum cut-off time was set at a latency of 10 s, and 0.9% saline was used as control (n = 6–8 per group). The antinociceptive response was expressed as a percentage of the maximal possible effect (%MPE), which was calculated using the following equation: %MPE = 100 × (TL–CL)/(10-CL).

2.5. Metabolic stability studies

Enzymatic degradation studies of EM-1 and its chimeric peptide were performed in mouse brain homogenate and mouse serum. Fresh blood (male Kunming mice) was collected, and 100% serum was obtained after centrifugation (20,000 × g by 20 min at 4 °C) as described previously (Liu et al., 2006). The supernatant was separated and stored at −80 °C until required. The 15% mouse brain homogenate was prepared following a previously reported protocol (Gillespie et al., 1992; Mallareddy et al., 2011). Briefly, mouse brains were isolated, pooled, homogenized with 50 mM Tris–HCl (pH 7.4), and stored at −80 °C until used. The protein content of the suspension was determined using a BCA protein assay kit (Thermo, Rockford, IL, U.S.). A final protein concentration of 2.3 mg/mL in 50 mM Tris buffer, pH 7.4, was used for all incubations.

The stability of peptides was determined by RP-HPLC analysis. Approximately 10 μL of the three different chimeric peptide stock solutions were added to 190 μL of brain homogenate or serum. Incubations were conducted at 37 °C, and an amount of 20 μL of the aliquots was withdrawn from the mixture at 0, 10, 15, 30, 60 and 90 min in triplicate. The enzyme activity was quenched at the required time by placing the tube on ice and acidifying with 90 μL of a 0.5% acetic acid solution. Approximately 90 μL of acetonitrile was added immediately for precipitated proteins. The samples were then centrifuged at 13,000 × g for 15 min at 4 °C. Then, 50 μL of the supernatant was taken and analyzed by RP-HPLC. The degradation rate constants (k) were obtained by least squares linear regression analysis of logarithmic peak area [(ln (A_t/A_0)], where A_t is the amount of the remaining peptide and A_0 is the initial amount of peptide *versus* time courses with at least five time