European Journal of Pharmaceutics and Biopharmaceutics 85 (2013) 736–743

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

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

Research paper

One-month subchronic toxicity study of cell-penetrating peptides for insulin nasal delivery in rats

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ARTICLE INFO

Article history:
Received 20 June 2013
Accepted in revised form 13 September 2013
Available online 21 September 2013

Keywords:
Cell-penetrating peptide
Penetratin
Toxicity
Insulin
Nasal delivery
Long-term administration

ABSTRACT

Recently, cell-penetrating peptides (CPPs) based vehicles have been developed for the delivery of different payloads in animals. Our studies have shown that nasal absorption of insulin and other therapeutic peptides and proteins can be improved significantly by co-administration of the CPP penetratin. Successful development of suitable CPP-based delivery systems, however, will depend not only on the efficiency of CPPs to transport therapeutic agents across the biological barriers of the nasal cavity, but also on the risk of adverse effects such as toxicity and undesired immunogenicity, especially in chronic therapy. In this study, we investigated the bioavailability (BA) of insulin and the adverse effects on the nasal mucosa in rats following a long-term dosing regimen of L-penetratin and the novel penetratin analogue "PenetraMax." Following nasal delivery, a significantly higher BA for insulin (almost 100% relative to subcutaneous (s.c.) injections) was observed for PenetraMax in comparison with the parent penetratin peptide after chronic administrations in rats. Importantly, there was negligible biomarker leakage in nasal lavage fluid and the integrity of the nasal epithelium remained unaffected when PenetraMax was used in long-term multiple administrations. In addition, no significant difference in the release of inflammatory and immunogenicity mediators in plasma was observed after nasal administration of PenetraMax with or without insulin solution. In conclusion, PenetraMax, a novel CPP candidate, can open a new avenue in clinical trials for noninvasive nasal insulin delivery.

1. Introduction

Cell-penetrating peptides (CPPs) have proven their ability for intracellular delivery of various macromolecular therapies such as nucleic acids, proteins and peptides, as well as drug delivery carriers [1–5]. CPPs comprise a growing family of peptides that have opened a new avenue for noninvasive systemic delivery of a vast collection of biomolecules otherwise restricted in crossing the plasma membrane [6,7]. CPPs are short polybasic and/or amphipathic peptides (<40 amino acids) with a net positive charge derived from signal peptides, viral peptides or other sources and they have been used successfully to translocate covalently or non-covalently bound cargo across cell membranes [8–10]. Previously, we have shown that CPPs are capable of transcellular delivery of peptides and proteins across the intestinal and nasal epithelial membranes [11–16]. Recently, systemic delivery of proteins and peptides via the nasal route has received increased attention because of the great potential for noninvasive biodrug delivery. Sufficient bioavailability, however, has to date been a challenge to achieve [17–19]. We have previously demonstrated that penetratin, a peptide derived from the *Drosophila* Antennapedia Homeoprotein, to be amongst the most successful CPPs to overcome the permeability problems of peptides and proteins in the nasal cavity. Although the relative bioavailability of insulin can reach up to 50% after co-administration with penetratin, a relatively high dose of penetratin is required for this enhancement of nasal insulin absorption [13]. To address the efficiency of penetration, a screening study of various penetratin analogues suggested that the chain length, hydrophobicity, amphipathicity and basicity of the CPP contribute to their absorption-enhancing efficiency [20]. Penetratin structure modifications introduced in the "shuffle (R, K fix) 2" sequence were successful in overcoming the permeability barrier for peptides and proteins in the nasal cavity. These findings were further supported in an *in silico* study based on molecular orbital analysis with Self-Organizing Maps

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http://dx.doi.org/10.1016/j.ejpb.2013.09.014

(SOM) classification in which the structural and physiochemical factors mentioned above were associated with the intestinal insulin absorption-enhancing effect of CPPs. As predicted by SOM clustering, a novel penetratin analogue (Sample 6) had significantly greater capacity to interact with insulin and to enhance intestinal insulin absorption than that of the original penetratin sequence [21] (Now this optimised peptide Sample 6 was named as "PenetraMax.") Insufficient information, however, regarding long-term CPP toxicity restricts their application *in vivo* and preclinical development in general as a successful delivery system will depend equally on the efficiency of CPPs to transport therapeutic agents through the biological barriers as well as the risk of associated side effects such as toxicity and undesired immunogenicity [22,23]. Hence, it is of great interest to develop a novel highly effective nasal absorption promoter with negligible toxic effect on the nasal mucosa and low systemic toxicity, especially for chronic therapy.

In this study, we explored the potential of a novel CPP, PenetraMax, for nasal delivery of insulin in comparison with the parent penetratin peptide at varying concentrations in a one-month twice daily administration study in rats. In a clinical setting, absorption enhancers like CPPs would be used chronically and as such, their safety must be guaranteed for chronic administration. Thus, following the same dosing scheme as mentioned above, we also examined systemic toxicity by quantitatively assessing nasal biomarker release (lactate dehydrogenase (LDH), interleukin-1α (IL-1α), and tumour necrosis factor-α (TNF-α)) and systemic release of IL-1α and TNF-α. In addition, local effects on the nasal mucosa after 7 and 30 days repeated daily administrations were investigated by histopathological examination of the nasal epithelium.

2. Materials and methods

2.1. Materials

Recombinant human insulin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-penetratin and its analogue PenetraMax listed in Table 1 were synthesised by Sigma–Genosys, Life Science Division of Sigma–Aldrich Japan Co. (Hokkaido, Japan). All other chemicals were of analytical grade and are commercially available.

2.2. Preparation of the mixed insulin/CPP solutions

To prepare the insulin solution, specific amounts of recombinant human insulin were dissolved in 50 μL of 0.1 M HCl. The insulin-HCl solution was diluted to 1.4 mL with phosphate-buffered saline (PBS, pH 6.0) containing 0.001% methylcellulose, which prevents the adsorption of the insulin to the tube surface, and normalised with 50 μL of 0.1 M NaOH. Specific amounts of L-penetratin or PenetraMax were dissolved in PBS (pH 6.0) containing 0.001% methylcellulose. Aliquot of insulin solution was added to L-penetratin or PenetraMax solution, mixed gently, and adjusted to a specific concentration. Each insulin/CPP solution was clear after mixing.

Table 1
Molecular weight and amino acid sequences of L-penetratin and PenetraMax.

	Sequence	Mw
L-Penetratin	RQIKIWFQNRRMKWKK	2246.7
PenetraMax	KWFKIQMQIRRWKNKR	2246.7

F, phenylalanine; I, isoleucine; K, lysine; M, methionine; N, asparagine; Q, glutamine; R, arginine; W, tryptophan.

2.3. Nasal absorption study

This research was performed at Kobe Gakuin University and complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals. Male Sprague Dawley rats weighing 180–220 g were purchased from Japan SLC, Inc. (Shizouka, Japan). The animals were housed in temperature (23 ± 1 °C) and relative humidity (55 ± 5%) controlled rooms with free access to water and food during acclimatisation. Animals were fasted for 24 h before the experiments; however, they were allowed to drink water ad libitum.

Under an intraperitoneal (i.p.) injection of sodium pentobarbital (12.5 mg/kg; Somnopentyl; Kyoritsu Seiyaku Corp., Tokyo, Japan), insulin or insulin/CPP mixture was applied intranasally twice a day for 1, 7 or 30 days. Rats were restrained in a supine position during administration at an angle of 15° and total 10 μL of drug (5 μL/each nostril) was instilled at 1 cm depth in each nostril by micropipette with a narrow tip fitted. The procedure was performed gently and slowly and lasted 1–2 min allowing the rats to receive all of the prepared sample. Insulin doses were 1 IU/kg body weight and CPP concentrations were 0.5 and 2 mM.

Blood sampling to determine plasma insulin concentrations was performed on all groups after the first dose on days 1, 7 and 30 depending on the group. Following their anesthetization with an i.p. injection of sodium pentobarbital (50 mg/kg), the rats were restrained in a supine position on a thermostatically controlled board at 37 °C. Additional i.p. injections of sodium pentobarbital (12.5 mg/kg) were given every 1 h to maintain anaesthesia during the experiment for collection of blood samples. A 0.25 mL blood aliquot was taken from the jugular vein using 1 mL tuberculin heparinized syringes at 5, 10, 15, 30, 60, 120 and 180 min after dosing. Plasma was separated by centrifugation at 12,000 rpm (13,400*g*) for 1 min and stored at −80 °C until analysis. The plasma insulin concentration was determined using a human insulin ELISA kit (Mercodia AB, Uppsala, Sweden) and the absorbance at λ_{max} 450 nm was detected by using a microplate reader (POWERSCAN HT, DS Pharma Biomedical Co. Ltd., Osaka, Japan).

2.4. Pharmacokinetic analysis

The bioavailability of nasally administered insulin was calculated relative to a s.c. injection (1 IU/kg). Briefly, an insulin solution was prepared by dissolving an appropriate amount of insulin in PBS. The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were directly determined from plasma insulin concentration–time curves. The total area under the insulin concentration curve (AUC) for 0–3 h was estimated from the sum of successive trapezoids between each data point. The relative bioavailability (BA) of insulin was calculated relative to the s.c. injection as follows:

$$\mathrm{BA}\ (\%) = ([\mathrm{AUC}]/\mathrm{dose}) \times 100/([\mathrm{AUC}]_{\mathrm{s.c./dose_{s.c.}}})$$

2.5. Biomarker assay

Inflammatory blood biomarkers (IL-1α and TNF-α) for insulin solution (control), L-penetratin with and without insulin and PenetraMax with and without insulin were analysed on the first day of nasal administration for each group and at the end of each respective dosing regimen (after 1, 7 and 30 days of nasal administrations). Nasal lavage fluid was collected for analysis of LDH, IL-1α and TNF-α release by the nasal mucosal cells at the end of days 1, 7 and 30 respectively. Briefly, after blood sampling rats were killed with an i.p. overdose of pentobarbital. The nasal cavity was perfused with 10 mL PBS warmed to 37 °C at a flow rate 2 mL/