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Efficiency of cell-penetrating peptides on the nasal and intestinal absorption of therapeutic peptides and proteins

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ABSTRACT

The purpose of our study was to investigate the potential of cell-penetrating peptides; penetratin as novel delivery vector, on the systemic absorption of therapeutic peptides and proteins across different mucosal administration sites. The absorption-enhancing feasibility of L- and D-penetratin (0.5 mM) was used for glucagon-like peptide-1 (GLP-1), and exendin-4 as novel antidiabetic therapy, in addition to interferon-β (IFN-β) as protein biotherapeutic model from nasal and intestinal route of administration was evaluated as first time in rats. Nasal route is the most feasible for the delivery of therapeutic peptides coadministered with penetratin whereas the intestinal route appears to be more restricted. The absolute bioavailability (BA (%)) values depend on the physichochemical characters of drugs, stereoisomer character of penetratin, and site of administration. Penetratin significantly increased the nasal more than intestinal absorption of GLP-1 and exendin-4, as the BA for nasal and intestinal administration of GLP-1 was 15.9% and 5%, and for exendin-4 were 7.7% and 1.8%, respectively. Moreover, the BA of IFN-β coadministered with penetratin was 11.1% and 0.17% for nasal and intestinal administration, respectively. From these findings, penetratin is a promising carrier for transmucosal delivery of therapeutic peptides and macromolecules as an alternative to conventional parenteral routes.

1. Introduction

As potential therapeutics, bioactive peptides and proteins offer high specificity and potency for treating several disease conditions (Kumar et al., 2006; Sato et al., 2006). Despite revolutionary progress in the large scale manufacturing of peptides and proteins, developing effective and convenient non-invasive delivery systems of this therapeutics remains a major challenge (Khafagy et al., 2007). Problems arise from the unfavorable physicochemical and biological properties of therapeutic peptides, proteins and hydrophilic macromolecules, which affect their absorption. So far, the parenteral route is the most common delivery system for such biopharmaceutical products (Mustata and Dinh, 2006). However, the oral delivery route, even if it is the most challenging for peptide and protein delivery regimens, remains the most convenient system as it is non-invasive, patient friendly and allows self-administration (Morishita and Peppas, 2006). The oral administration of biotherapeutic agents is notoriously difficult because of their poor permeability through the intestinal mucosa. This is associated with their high molecular weights, hydrophilicity and susceptibility to enzymatic degradation (Goldberg and Gomez-Orellana, 2003). Various pharmaceutical strategies that have been proposed to maximize peptide and protein bioavailability (BA) (Katsuma et al., 2006; Morishita et al., 2006; Werle and Takeuchi, 2008). However, acceptable BA has not attained yet.

Because of the complexity and poor success in the oral delivery of biotherapeutic peptides and proteins, the nasal route offers an interesting option to conventional parenteral routes of administration (Costantino et al., 2007). It offers numerous benefits as a non-invasive target issue for drug delivery. The large surface area of the nasal mucosa affords a rapid onset of therapeutic effects and there is no first-pass metabolism. These benefits maximize patient convenience, comfort and compliance (Türker et al., 2004). Nonetheless, as with any such surface the nasal mucosa poses a permeation barrier to macromolecular therapeutics such as peptides and proteins. There remain the problems of enzymatic degradation and rapid mucociliary clearance (Illum, 2003), which limit the ability of peptides and proteins to reach the general circulation in therapeutic quantities. Previously, several approaches are used to improve the absorption of peptides and proteins through the nasal mucosa, including using absorption enhancers (Pillion et al., 2002), carrier systems (Wang et al., 2006), and mucoadhesive polymers (Dyer et al., 2002). Although such methods have been successful in delivering biotherapeutic peptides, they have been hampered by limited biological efficiency at tolerable levels of safety.

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Thus, improving the membrane permeability of macromolecules should help achieve an acceptable BA of macromolecules (Morishita and Peppas, 2006; Goldberg and Gomez-Orellana, 2003). Nevertheless, highly cationic, naturally occurring low molecular weight peptide sequences enriched with basic amino acids or proline, and synthetic molecules such as model amphipathic peptides, can cross the lipid bilayer (Wagstaff and Jans, 2006). These cell-penetrating peptides (CPPs) efficiently translocate through the cell membrane without the need for a receptor, and can deliver a hydrophilic cargo (peptide or protein) into cells. This ability of these domains, together with their low toxicity, makes them promising potential delivery vectors (Lindsay, 2002; Tréhin and Merkle, 2004).

A previous study from our laboratory established that oligoarginine is talented for assisting the non-invasive absorption of biotherapeutic agents (Morishita et al., 2007). It produced high BA and showed no apparently undesirable effects on biological membranes, but required relatively high doses. Moreover, the absorption of INF-β was slightly improved by coadministration of oligoarginine. Therefore, the main goal of our present study is to evaluate the effects of penetratin as one of the most promising CPP to improve the BA of new therapeutic peptides, such as glucagon-like peptide-1 (GLP-1) and exendin-4 that offer a novel treatment option for patients with type 2 diabetes (Sadrzadeh et al., 2007). In addition, interferon-β (IFN-β), a model of high molecular weight protein has insufficient absorption through the mucosal route of administration. We also studied the stereoisomeric effects of L- and D-forms of penetratin and their abilities to enhance the BA of peptides and proteins drugs, and comparing the penetratin efficiency for enhancing biotherapeutics absorption through the nasal and intestinal routes, which were not examined before for *in vivo* absorption study and are used as first time.

2. Materials and methods

2.1. Materials

GLP-1 and exendin-4 were purchased from Sigma–Aldrich Co. (St Louis, MO, USA). Recombinant human IFN-β (0.6×107 IU/vial) was kindly supplied by Toray Industries, Inc. (Kanagawa, Japan). L- and D-penetratins (RQIKIWFQNRRMKWKK, 2247 Da, and purity >95% for each peptide) were synthesized by Sigma Genosys, Life Science Division of Sigma–Aldrich Japan Co. (Ishikari, Japan). All other chemicals were of analytical grade and available commercially.

2.2. Preparation of drug-penetratin solutions

Specific amounts of GLP-1 and exendin-4 equivalent to administered dose were dissolved in 1.5 ml phosphate buffered saline (PBS), pH 7.4, containing 0.001% methylcellulose to prevent adsorption of the CPP onto the tube surface. L- or D-penetratin (0.5 mM) were measured into polypropylene tubes and an appropriate aliquot of drug solution was added to the tubes and mixed gently to yield a clear solution. In the case of IFN-β, one vial of IFN-β was diluted on ice with 1 ml of PBS, pH 7.4, containing 0.05% Tween 20 to prevent the adsorption of IFN-β onto the tube surface, then 100 μl of IFN-β solution was diluted to prepare the IFN-β solution at 0.9×10^6 IU/ml in PBS. Specific amounts of L- or D-penetratin (0.5 mM) were measured into polypropylene tubes and an appropriate aliquot of the IFN-β solution was added to the tubes and mixed gently to yield a clear solution.

2.3. Nasal absorption study

This research was performed at Hoshi 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 Tokyo Laboratory Animals Science Co., Ltd. (Tokyo, Japan). Animals were housed in rooms controlled between 23 ± 1 °C and 55 ± 5% relative humidity and had free access to water and food during acclimatization. Animals were fasted for 24 h before the experiments.

The *in vivo* experiments were performed in rats as described (Hirai et al., 1981). Following anesthesia using an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), rats were restrained supine at an angle of 15° on a thermostatically controlled board at 37 °C and a small midline incision was made carefully in the neck. The trachea was then cannulated with a 7 cm long polyethylene tube with a diagonally cut end (2.08 mm outer diameter, o.d., 1.57 mm inner diameter, i.d.; Becton Dickinson, Franklin Lakes, NJ, USA), to maintain respiration. The esophagus was then cannulated with a blind-ended polyethylene tube (8 cm l.; 2.42 mm o.d.; 1.67 mm i.d.). This was moved rostrally to block the nasal cavity, to maintain solutions in the nasal cavity and eliminate oral absorption. The nasopalatine ducts were closed with medical cyanoacrylate polymer glue (Aron Alpha A® Super Glue, Daiichi Sankyo Co., Tokyo, Japan) to prevent drainage of the solution from the nasal cavity to the mouth. Rats were left on the board at 37 °C for a further 30 min to recover from the resulting surgery. After 30 min rest, each rat was then given 20 μl of the drug-penetratin mixture or drug solution alone (control) with a micropipette inserted directly 0.5 cm into each nostril. The doses were 0.1 mg/kg for GLP-1, 0.25 mg/kg for exendin-4, and 0.18×10^6 IU/kg for IFN-β. Additional i.p. injections of sodium pentobarbital (12.5 mg/kg) were used every 1 h to maintain anesthesia.

2.4. In situ intestinal loop absorption study

Rats, anesthetized as above, were restrained in a supine position on a thermostatically controlled board at 37 °C. The ileum was exposed following a small midline incision made carefully in the abdomen and its proximal-to-ileocecal junction segment (length 10 cm) was cannulated at both ends using polypropylene tubing (4 mm o.d., 2 mm i.d.; Saint-Gobain Norton Co. Ltd., Nagano, Japan). These were securely ligated to prevent fluid loss and carefully returned to their original location inside the peritoneal cavity. To wash out the intestinal contents, PBS at 37 °C was circulated through the cannula at 5.0 ml/min for 4 min using an infusion pump (KD Scientific Inc., Holliston, MA, USA). The cannulation tubing was removed and the segments were then tightly closed; about 1 ml of perfusion solution remained in the segments. Rats were left on the board at 37 °C for a further 30 min to recover from the surgery described above. After 30 min rest, 0.5 ml of drug-penetratin mixture or drug solution alone (control) was directly administered into the 6 cm ileal loop made from the 10 cm pretreated segment. The doses were 1.25 mg/kg for GLP-1, 1.25 mg/kg for exendin-4, and 2.25×10^6 IU/kg for IFN-β.

The BA of nasally and intestinally administered drugs was calculated relative to the intravenous (i.v.) route for administered drugs. Briefly, a drug solution was prepared by dissolving an appropriate amount of drug in PBS as described before for i.v. injections at 6.25×10^{-3} mg/kg for GLP-1, 12.5×10^{-3} mg/kg for exendin-4, and 3×10^3 IU/kg for IFN-β. To maintain the same physical conditions for the rats, the same surgery was performed on animals receiving i.v. injections of drugs as that performed on rats in the nasal and ileal loop absorption studies.

2.5. Analytical methods

During the nasal and *in situ* ileal loop experiments, a 0.25 ml blood aliquot was taken from the jugular vein using 1 ml tuberculin heparinized syringes before and 5, 10, 15, 30, 60, 120, 180

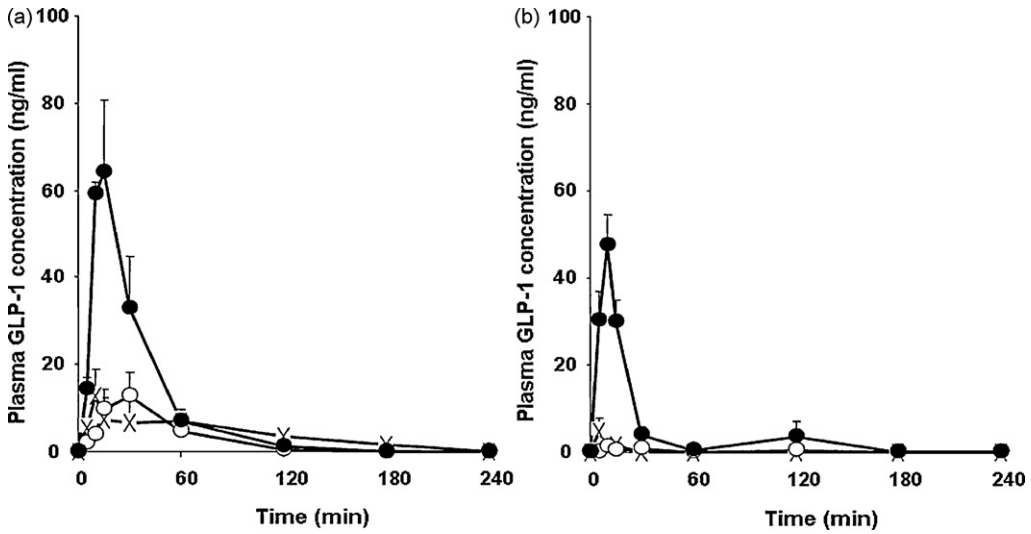

Fig. 1. Plasma GLP-1 concentration–time profiles following nasal (a) and intestinal (b) administration of GLP-1 (0.1 and 1.25 mg/kg, respectively) with L- or D-penetratin (0.5 mM). GLP-1 (×); L-penetratin (●); D-penetratin (○). Each data point represents the mean ± S.E.M. ($n = 3$).

and 240 min after dosing. Plasma was separated by centrifugation at 13,400 × *g* for 1 min and stored at −80 °C until analysis. Plasma concentrations of peptide drugs were determined using enzyme-linked immunoassays (ELISA). IFN-β and exendin-4 ELISA kits (Toray Industries, Inc.) and rat GLP-1 ELISA kit (Wako Pure Chemical Industries Ltd.) were used.

2.6. Data analysis

The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were directly determined from plasma drug concentration–time curves. The total area under the drug concentration curves (AUC) for 0–4 h was estimated from the sum of successive trapezoids between each data point ($[AUC]_{n.}$, $[AUC]_{situ}$, and $[AUC]_{i.v.}$ for nasal, *in situ*, and i.v. administrations, respectively). The absolute bioavailability (BA (%)) of drugs was calculated relative to the i.v. injections using the following equation.

$$BA(\%) = ([AUC]_{n.} \text{ or } [AUC]_{situ}/\text{dose}_{n.} \text{ or dose}_{situ}) \times 100/[AUC]_{i.v.}/\text{dose}_{i.v.}$$

2.7. Statistical analysis

Data are expressed as the mean ± standard error of the mean (S.E.M.). The significance of differences in mean values was evaluated using Student's unpaired *t* test. For multiple comparisons, a one-way layout analysis of variance (ANOVA) with Dunnett's test was applied. Differences were considered statically significant when *p* was less than 0.05.

3. Results

3.1. Effect of L- and D-penetratin on GLP-1 and exendin-4 absorption from the nasal cavity and ileal loop

The plasma profiles after nasal and intestinal delivery of GLP-1 and exendin-4 are shown in Figs. 1 and 2, respectively. Table 1 summarizes the pharmacokinetic parameters derived from the GLP-1 and exendin-4 concentration–time profiles following nasal and intestinal administration with L- or D-penetratin. For nasal delivery, L-penetratin significantly increased GLP-1 and exendin-

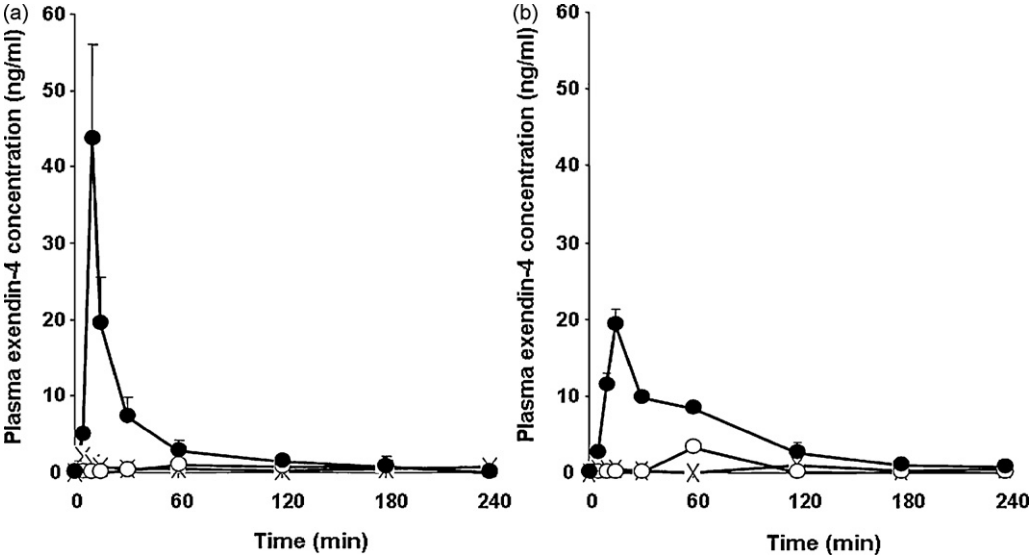

Fig. 2. Plasma exendin-4 concentration–time profiles following nasal (a) and intestinal (b) administration of exendin-4 (0.25 and 1.25 mg/kg, respectively) with L- or D-penetratin (0.5 mM). Exendin-4 (×); L-penetratin (●); D-penetratin (○). Each data point represents the mean ± S.E.M. ($n = 3$).

Table 1
Pharmacokinetic parameters following nasal and intestinal administration of drugs with D- or L-pentratin (0.5 mM).

	Nasal			Intestinal		
	C_{max}	T_{max}	AUC	C_{max}	T_{max} (min)	AUC
GLP-1 (control)	10.6 ± 3.5^{a}	45.0 ± 15.0	12.2 ± 3.3^{c}	4.2 ± 2.2^{a}	11.6 ± 5.3	0.8 ± 0.4^{c}
+D-penetratin	13.7 ± 4.6^{a}	23.3 ± 6.6	9.0 ± 3.2^{c}	2.1 ± 0.5^{a}	10.0 ± 0.0	0.9 ± 0.7^{c}
+L-penetratin	$72.4 \pm 11.5^{a*}$	$11.6 \pm 1.6^{*}$	$34.9 \pm 8.3^{c*}$	$47.0 \pm 16.8^{a*}$	13.3 ± 1.6	$11.0 \pm 3.4^{c*}$
Exendin-4 (control)	2.1 ± 1.1^{a}	5.0 ± 0.0	2.0 ± 1.2^{c}	0.6 ± 0.1^{a}	10.0 ± 2.8	0.9 ± 0.4^{c}
+D-penetratin	0.8 ± 0.5^{a}	65.0 ± 30.4	2.1 ± 1.3^{c}	$2.5 \pm 0.1^{a**}$	$60.0 \pm 10.0^{**}$	2.0 ± 1.2^{c}
+L-penetratin	$45.7 \pm 11.0^{a*}$	$11.6 \pm 1.6^{*}$	$15.1 \pm 2.3^{c**}$	$21.4 \pm 2.3^{a**}$	$30.0 \pm 1.5^{*}$	$17.9 \pm 1.0^{c**}$
IFN-β (control)	2.7 ± 0.5^{b}	180.0 ± 60.0	8.8 ± 2.3^{d}	0.8 ± 0.2^{b}	53.3 ± 33.8	0.0 ± 0.0^{d}
+D-penetratin	$18.9 \pm 6.0^{b*}$	84.0 ± 14.6	$80.1 \pm 32.6^{d*}$	$6.4 \pm 1.1^{b**}$	54.0 ± 6.0	$0.1 \pm 0.0^{d**}$
+L-penetratin	$15.0 \pm 4.0^{b*}$	60.0 ± 0.0	$44.7 \pm 8.0^{d*}$	0.6 ± 0.1^{b}	40.0 ± 10.0	0.0 ± 0.0^{d}

Each value represents the mean ± S.E.M. (n = 3–7). C_{max}, maximum concentration [a](ng/ml), or [b](IU/ml); T_{max}, time to reach C_{max}; AUC, area under the curve [c](ng h/ml), or [d](IU h/ml).
* $p < 0.05$, significant difference compared with the corresponding "control".
** $p < 0.01$, significant difference compared with the corresponding "control".

4 absorption at doses of 0.1 and 0.25 mg/kg, respectively, although D-penetratin showed no difference in enhancing ability compared with controls (Figs 1a and 2a). The same trend was found for the intestinal route; L-penetratin produced significantly higher permeability of GLP-1 (1.25 mg/kg) and exendin-4 (1.25 mg/kg) through the ileal membrane than did D-penetratin (Figs 1b and 2b). As observed for GLP-1 and exendin-4, the AUC for nasal administration was consistently higher than using the intestinal route (Table 1).

The systemic BAs of GLP-1 and exendin-4 coadministered with penetratin were significantly greater following nasal administration than with the intestinal route. Thus, the respective BA values for the nasal and intestinal administration of GLP-1 were 15.9% and 5%, whereas for the nasal and intestinal administration of exendin-4 they were 7.7% and 1.8% (Fig. 4).

3.2. *Effects of* L- *and* D-*penetratin on IFN-β absorption from the nasal cavity and ileal loop*

We next evaluated the effects of L- and D-penetratin (0.5 mM) on the extent of IFN-β absorption from the nasal cavity and ileal loop. Fig. 3 shows plasma IFN-β concentration–time profiles after administration from the nasal cavity and ileal loop in the presence or absence of 0.5 mM L- or D-penetratin. The pharmacokinetic parameters of IFN-β absorption after administration from these sites with or without L- or D-penetratin are summarized in Table 1. No apparent uptake was observed following nasal and intestinal administration of the IFN-β solution. On the contrary, when either L- or D-penetratin was coadministered with IFN-β (0.18×10^6 IU/kg) in the nasal cavity, we detected significantly higher effects on plasma IFN-β concentrations. However, the absorption-enhancing ability of D-penetratin was higher than that of L-penetratin (Fig. 3a). Fig. 4 shows that the relative BA of IFN-β was 6.2% and 11.1% when coadministered with L- and D-penetratin, respectively, through the nasal route compared with i.v. injection. Table 1 shows that L-penetratin did not stimulate the intestinal uptake of IFN-β (2.25×10^6 IU/kg) compared with the control. Conversely, D-penetratin improved the ileal absorption of IFN-β more significantly than did the L-form. The BA for intranasal administration of IFN-β with D-penetratin was approximately 65 times higher than for the intestinal route (Fig. 4).

4. Discussion

There has been much research on the non-invasive delivery of bioactive peptides and proteins, nevertheless these approaches have not yet been resulted an acceptable value of BA. For that, our

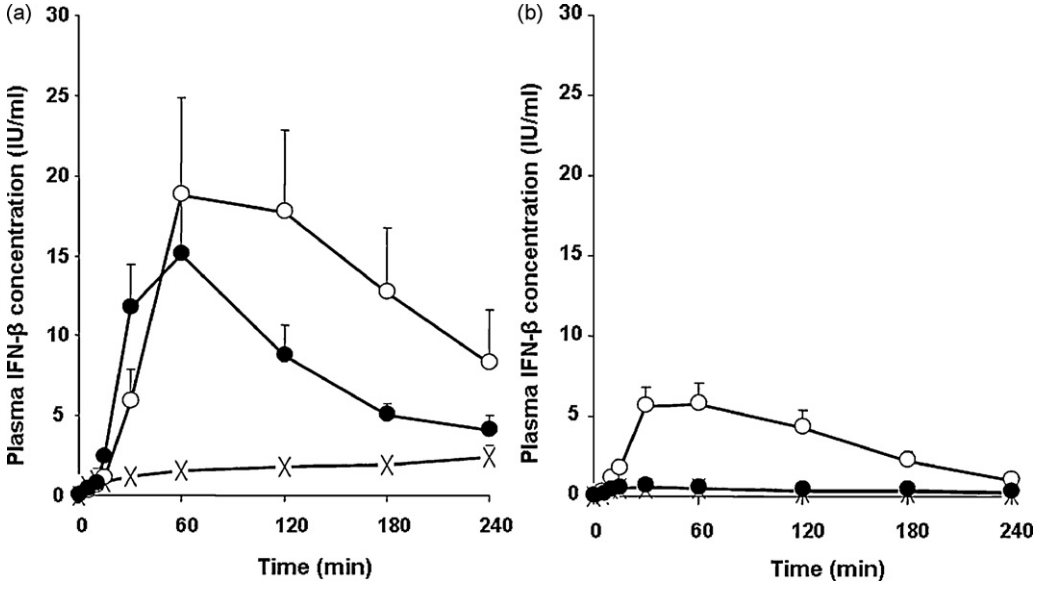

Fig. 3. Plasma IFN-β concentration versus time profiles following nasal (a) and intestinal (b) administration of IFN-β (0.18×10^6 and 2.25×10^6 IU/kg, respectively) with L- or D-penetratin (0.5 mM). IFN-β (×); L-penetratin (●); D-penetratin (○). Each data represents the mean ± S.E.M. (n = 3–5).

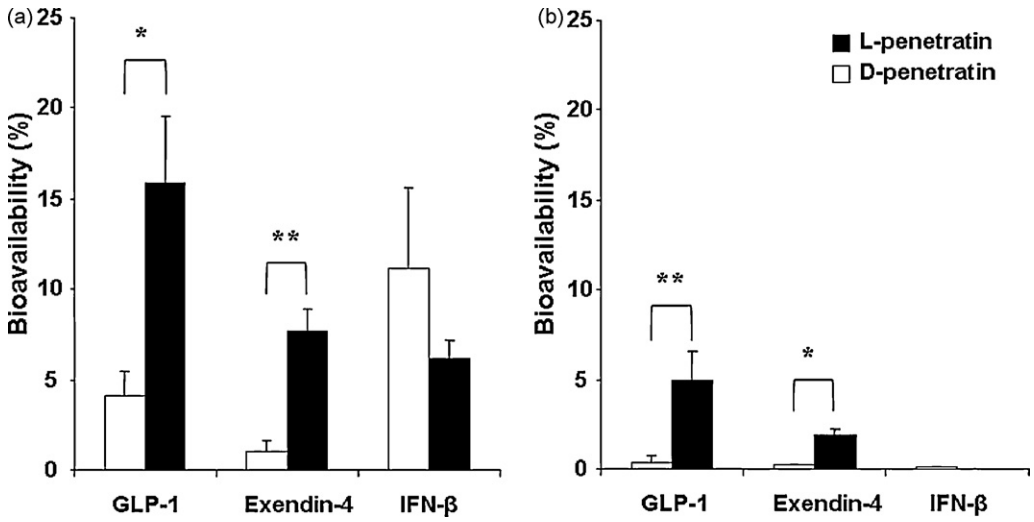

Fig. 4. Bioavailability of peptides and macromolecules after nasal (a) and intestinal (b) administration with L- or D-penetratin. Each value represents the mean ± S.E.M. ($n = 3–7$). $^{*}p < 0.05$, $^{**}p < 0.01$, significant difference.

study focused on improving the BA of therapeutic peptides using a non-parenteral route of administration, and aimed to overcome the challenges inherent in the development of clinical therapeutic delivery systems for biomolecules. In recent years, CPPs have been characterized for their high ability to translocate poorly permeable peptide through mucosal membranes efficiently without altering drug bioactivity (Schwarze et al., 1999).

Our strategy for achieving a significant BA of therapeutic peptides and proteins depends on the coadministration of biomolecules and CPPs as a physical mixture (Morishita et al., 2007). Previously, a conventional covalent link between a CPP and its cargo showed an insignificant effect on the pharmacological activity of therapeutic peptides and proteins compared with the drug itself (Kamei et al., 2008b). Our studies have shown that suitable CPPs can increase drug BA through biological membranes safely in a non-disruptive way (Morishita et al., 2007; Khafagy et al., 2009; Kamei et al., 2008a).

Penetratin is one of the most promising CPPs tested to date, characterized by a high content in basic amino acids (lysine and arginine) and by the presence of hydrophobic residues (most importantly tryptophan). It overcomes the plasma membrane barrier of mammalian cells through a macropinocytotic pathway and efficiently delivers molecular cargoes in a biologically active form, although, the mechanism by which CPP-cargo uptake across the cell membrane is still unclear (Jones et al., 2005; El Andaloussi et al., 2007). Coadministration of 0.5 mM of penetratin in a physical mixture with the administered peptides/proteins in this research, showed the remarkable enhancing efficiency without aggregation and irritation to the mucosa. We have also reported that the concentration of penetratin can be increased to 2 mM without induction of any toxicity (Khafagy et al., 2009). Therefore, the main goal of our present study is to evaluate the potentiality of penetratin as a bioenhancer of different clinically beneficial biodrugs providing a non-invasive tool for peptides delivery across mucosal membrane conserving adequate BA.

GLP-1 has a unique profile, considered highly desirable for an antidiabetic agent, particularly since the glucose dependency of its antihyperglycemic effects should minimize any risk of severe hypoglycemia. However, GLP-1 is still restrictedly administered by the parenteral route of administration (Deacon, 2004). As shown in Fig. 1, L-penetratin significantly enhanced nasal and intestinal GLP-1 absorption in rats, especially with nasal administration. In particular, the L-form enhanced intranasal GLP-1 absorption more than with *in situ* ileal loop administration. The highly positively charged residues of penetratin, providing electrostatic interaction with negatively charged peptides, might be associated with the absorption-enhancing efficiency of this CPP (Christiaens et al., 2004). Because GLP-1 has a low pI, a negative net charge at the physiological pH of the administration site has been proposed for its binding to positively charged penetratin, with a pI of 12.8 (Kamei et al., 2009). This property also allows penetratin to bind to electrostatic membrane constituents, such as anionic lipids and glycosaminoglycans at low micromolar concentrations, with potential consequences for endocytotic pathways (Poon and Gariépy, 2007).

According to these results, L- and D-penetratin have different efficiency on the nasal and intestinal GLP-1 absorption. Proteolytic stability is a critical requirement for the therapeutic application of CPPs, as it is essential that they can deliver their cargo effectively to the site of administration without being cleaved by proteases, which cause premature release of the cargo (Pujals et al., 2007). It is possible that differences in the sensitivity to enzymatic degradation between the L- and D-forms of peptides affect the enhancement of intestinal GLP-1 absorption by such CPPs (Kamei et al., 2008a). Peptides containing the L-forms of amino acids in the nasal cavity and intestinal lumen are more metabolically unstable than those containing the D-forms (Pappenheimer et al., 1994). In contrast, L-penetratin enhanced nasal GLP-1 absorption more strongly than the D-form. The formation of complexes via electrostatic interactions after mixing GLP-1 and penetratin is suspected to be degraded by peptidases of the nasal and ileal enzyme fluids (Khafagy et al., 2009; Kamei et al., 2008a). Clearly, a strong affinity between a CPP and its cargo is required to stabilize the resulting complex during transport and to achieve an optimum absorption. On the other hand, a sufficient affinity between a CPP and its cargo is also required to facilitate the release of the cargo after its cellular uptake, as potentially achieved through enzymatic digestion of the CPP or by competitive displacement of the cargo (Ziegler and Seelig, 2007). In addition, the internalization efficiency of penetratin is retained even when its sequence is partially modified, suggesting that fractionated penetratin might still show the ability to internalize a cargo drug (Christiaens et al., 2004).

The results of GLP-1 study show that coadministration of penetratin with GLP-1 significantly enhance the nasal BA in comparison with previous approach developed to provide transmucosal absorption as a possible alternative to injection treatment (Gutniak et al., 1997). The significant improvement of nasal GLP-1 absorption compared with the intestinal route might result from a deficiency in luminal proteases, minimal contact of peptides and proteins with

the resident proteases, or exposure to a smaller surface area. Hence, a smaller fraction of the total proteases is present when compared with the oral route; moreover, peptides and proteins are subjected to less dilution in the nasal route (Lee, 1988). This maximizes the concentration gradient for peptide and protein absorption, furthermore, the nasal mucosa is highly permeable than the oral route (Duchateau et al., 1986; Hayashi et al., 1985).

Exendin-4, a peptide share approximately 52% amino acid sequence resemblance to GLP-1 shows similar glucoregulatory activities to GLP1 (Göke et al., 1993). Although it has been approved only as parenteral dosage forms and has not established as non-invasive delivery system (Gallwitz, 2006). Fig. 2 and Table 1 show that L-penetratin markedly enhanced the nasal and intestinal absorptions of exendin-4, as with GLP-1 absorption. Thus, L-penetratin appears to act by electrostatic and/or hydrophobic interactions between its basic and/or hydrophobic residues and the negatively charged amino acids of exendin-4. Exendin-4 (Mw 4186, pI 4.5) has low pIs in physiological nasal and intestinal fluids. This might explain how the positively charged basic residues in L- or D-penetratins enhance their permeability across the mucosal membrane. There were rapid increases in plasma GLP-1 and exendin-4 concentrations to 64.2 and 43.6 ng/ml within 15 and 10 min, respectively, and these levels were maintained for at least 120 min, indicating the potential of intranasal coadministration with penetratin (Figs 1a and 2a). Compared with the intranasal route, the intestinal tract seems to have limited potential as a route for GLP-1 and exendin-4 coadministered with penetratin (Figs 1b and 2b) (Gedulin et al., 2008).

On the contrary, D-penetratin was more effective than the L-form in enhancing IFN-β absorption in the present study (Fig. 3), particularly through the nasal cavity (Fig. 3a and Table 1). The putative difference of cargo physicochemical parameters such as size and charge and penetratin's sensitivity to peptidases and proteases might explain this diversity. As can be anticipated from the structure of the reacting molecules, electrostatic force is not the only source of interactions between IFN-β and penetratin.

There are two possible explanations. First, penetratin is inherently more hydrophobic than oligoarginine. Second, it may also reflect the special hydrogen bond properties of the guanidinium group, which will contribute to a greater non-electrostatic free energy of binding by interacting with the membrane hydration layer (Gonçalves et al., 2005). IFN-β has a high Mw (22,000) and highly positive pI (9.6) and is therefore relatively positively charged in the nasal cavity and intestinal lumen. This might prevent intermolecular interactions with the hydrophilic oligoarginine. However, significant translocation under these conditions is observed for amphipathic CPPs such as penetratin. Penetratin is characterized by a high content of basic residues and by the presence of hydrophobic residues, most importantly tryptophan. We hypothesize that CPP with intrinsic hydrophobic properties, such as penetratin, which is more sensitive to hydrophobic cargoes. This illustrates the dynamic requirements for their association with biological membranes and for the subsequent escape of any cargo to the cell interior. Moreover, depending on the nature of the cargo and the vector, distinct pathways could be used leading to equivalent biological activity (Joliot and Prochiantz, 2008). The hydrophobic and the electrostatic contributions to the total free energy of binding are of the same order of magnitude for all amphipathic CPPs. In the case of penetratin, the hydrophobic effect is the main driving force for the partitioning of peptides into the cell membrane together with the formation of inter- or intramolecular hydrogen bonds as the peptide forms a secondary structure. This assumes that the two tryptophan residues of penetratin are important in penetrating into the membrane and in the interaction with a hydrophobic cargo (Persson et al., 2004).

As shown in Figs. 3 and 4, D-penetratin produced a higher BA for coadministered IFN-β than did L-penetratin, especially via the intranasal route. The formation of complexes via hydrophobic and/or H-bond interactions after mixing IFN-β and penetratin is suspected to affect the action of peptidases in the nasal and intestinal mucus fluid. The L-penetratin–IFN-β complex might be more readily degraded by enzymes because of the lower resistance of the L-form of the peptide to enzymatic degradation. In contrast, the high stability of the D-penetratin–IFN-β complex plays a critical factor in determining its effect on enhancing drug absorption. The relative BA demonstrated in Fig. 4 shows that penetratin has different enhancing efficiency for peptides. In the case of GLP-1, exendin-4, and IFN-β the difference of enzymatic activity and mucosal permeability of site of administration markedly affect the extent of absorbed drug.

5. Conclusions

The therapeutic application of penetratin regards to the site of administration depends on the physicochemical characters of biotherapeutic drugs, and the chirality of the constituent amino acids of penetratin isomers. Nasal delivery of therapeutic peptides and proteins coadministered with penetratin significantly improved the relative BA more than intestinal delivery. Penetratin markedly enhanced the absorption of peptides such as GLP-1 and exendin-4, in addition to protein as IFN-β. Particularly, penetratin significantly increase the BA of GLP-1 as an alternative treatment for type 2 diabetic patients, which may be feasible in the future for clinical application. These findings may be useful in establishing fundamental guidelines for the development of non-invasive delivery systems of many drugs, including therapeutic peptides and proteins, using penetratin as a carrier.

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