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Applicability and Limitations of Cell-Penetrating Peptides in Noncovalent Mucosal Drug or Carrier Delivery Systems

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ABSTRACT

Our recent studies show that cell-penetrating peptides (CPPs) have potential to improve the intestinal absorption of peptide and protein drugs safely and effectively when used in the noncovalent drug–CPP approach. To clarify the applicability and limitations of this strategy, the present study examined the effects of cargo size on the absorption-stimulatory effect of CPPs. Different sizes of hydrophilic macromolecular dextran (4.4, 10, and 70 kDa) and polystyrene-based nanoparticles (20, 100, and 200 nm) were chosen as the model cargos in this study. In an *in situ* rat intestinal absorption study, CPPs (octaarginine, Tat, penetratin, and PenetraMax) increased the intestinal absorption of dextran, and the efficiency varied according to the molecular size of dextran. Among the CPPs, D-penetratin showed an enhancing effect even when coadministered with the largest dextran (70 kDa). By contrast, an *in vitro* study of Caco-2 cell uptake showed that the ability of CPPs to deliver nanoparticles into epithelial cells was dependent on their particle size and that the relatively poor internalization of 200-nm nanoparticles could be facilitated by coincubation with CPPs. These findings suggest that the intrinsic uptake properties of macromolecules and particulate cargos determine the effectiveness of their intestinal mucosal delivery using the noncovalent CPP method.

Introduction

The cell-penetrating peptides (CPPs) are potential delivery vectors to transport protein- and nucleic acid–based biopharmaceuticals safely into cells.[1-4] In addition to increasing the delivery of macromolecular substances, CPPs can also boost the internalization of nano-sized particulate systems such as liposomes and polymer micelles into living cells by surface modification.[5,6] We have recently studied the ability of CPPs to improve the mucosal absorption of peptide and protein drugs *via* the oral and nasal routes of administration. We have demonstrated that typical CPPs such as oligoarginine and penetratin can effectively deliver these drugs from the site of administration to the systemic circulation.[7-11] Drugs that can be delivered effectively using CPPs include insulin, glucagon-like peptide-1, exendin-4, and interferon beta.[12,13] The *in vitro* study confirmed that the epithelial cellular uptake of insulin was significantly increased by coincubation with CPPs and that the increased drug uptake contributed to the net epithelial permeation and absorption to blood.[14] It is important that an effective enhancing effect of CPPs on the absorption of drugs be achieved by using the noncovalent approach based on the physical mixture of drug and CPP.[11,12,15] Thus, CPPs could be considered potential enhancers to improve the oral bioavailability of various biopharmaceuticals. However, the limitations on their capability to deliver cargos in a noncovalent manner remain unknown.

We have previously clarified that in our noncovalent strategy, the intermolecular interaction between a macromolecular drug and CPP is essential for increasing the epithelial cellular uptake of drugs.[12,15] CPPs exert their absorption-enhancing ability without chemical conjugation to the cargo, although some type of molecular interaction, such as electrostatic binding, is required. In other words, only drugs or delivery carriers that noncovalently bind to CPPs can be delivered to the systemic circulation. One of the limitations of a noncovalent delivery strategy using CPPs is that such intermolecular interactions are strongly influenced by the physicochemical characteristics of the drugs, delivery carriers, and CPPs. In the case of the covalent strategy for CPPs, it has been reported

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that a 116 kDa β-galactosidase[16] or 100 nm liposomes[17] can be successfully internalized into cells.

To our knowledge, such limitations on the application of CPPs in noncovalent delivery systems have not been examined in terms of the effects of molecular size and various delivery carriers. Therefore, the aim of this study was to clarify the molecular size-dependent changes in the absorption-boosting effect of CPPs. The intestinal absorption of dextran of different average molecular size was used as the hydrophilic macromolecular model compound in the presence of CPPs in rats. In addition, to examine whether our noncovalent approach using CPPs is useful in the mucosal delivery of carriers, we examined the effect of CPPs on the uptake of nanoparticles in an intestinal epithelial model using Caco-2 cells. Understanding the limitation to use the functional peptides as a mucosal delivery systems is essential for determining their effectiveness, applicability, and feasibility in drug delivery.

Materials and Methods

Materials

Fluorescein isothiocyanate–labeled dextrans with average molecular weights of 4.4, 10, and 70 kDa (FD-4, FD-10, and FD-70, respectively) were purchased from Sigma-Aldrich Corp. (St. Louis, MO). FluoSpheres carboxylate–modified microspheres with average diameters of 20, 100, and 200 nm, and RIPA buffer were purchased from Thermo Fisher Scientific Inc. (Waltham, MA). The CPPs listed in Table 1 were synthesized by Sigma-Genosys, Life Science Division of Sigma-Aldrich Japan Co. (Hokkaido, Japan). Human colon adenocarcinoma–derived Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD) at passage 18. Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose, nonessential amino acids, antibiotics mixture (10,000 U/mL penicillin, 10 mg/mL streptomycin, and 29.2 mg/mL L-glutamine in 10 mM citric acid–buffered saline), 0.05% trypsin–ethylenediaminetetraacetic acid, and Hank's balanced salt solution (HBSS) were purchased from Gibco Laboratories (Lenexa, KS). Fetal bovine serum was purchased from Biowest (Nuaille, France). All other chemicals were of analytical grade and are commercially available.

Preparation of the CPP and Dextran Solutions and Nanoparticle Suspension

Specific amounts of CPPs (R8, Tat, penetratin, and PenetraMax, as listed in Table 1) were dissolved in phosphate-buffered saline (PBS, pH 7.4) or HBSS containing 0.001% methylcellulose, which prevents adsorption of CPPs to the tube wall. For the animal study, a specific amount of dextran (FD-4, FD-10, and FD-70) was dissolved in PBS (pH 7.4) with 0.001% methylcellulose and then mixed with CPP solution in PBS just before administration to rats. For the cell study, dextran was dissolved in HBSS with 0.001% methylcellulose to prepare a 7.0 mg/mL of stock solution, and the supplied nanoparticle suspensions with 2% solids (20, 100, and 200 nm diameter) were diluted 20 times using HBSS with 0.001% methylcellulose to prepare a 0.1% stock suspension. The nanoparticle suspension and CPP in HBSS solution were added separately to the cell to final concentrations of 0.01% and 60 μM, respectively.

Table 1
Amino Acid Sequences of the CPPs Used in This Study

Peptides	Sequences[a,b]
L-R8	RRRRRRRR
D-R8	rrrrrrrr
L-Tat	GRKKRRQRRRPPQ
D-Tat	grkkrrqrrrppq
L-penetratin	RQIKIWFQNRRMKWKK
D-penetratin	rqikiwfqnrrmkwkk
L-PenetraMax	KWFKIQMQIRRWKNKR
D-PenetraMax	kwfkiqmqirrwknkr

[a] F: phenylalanine; G: glycine, I: isoleucine, K: lysine, M: methionine, N: asparagine, P: proline, Q: glutamine, R: arginine, W: tryptophan.
[b] Uppercase and lowercase letters indicate the L- and D-forms of the amino acids, respectively.

In Situ *Closed-Loop 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 of that institution. Male Sprague-Dawley rats weighing 180–220 g were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed under controlled temperature (23 ± 1°C) and relative humidity (55 ± 5%) and given free access to water and food during acclimatization. The animals were fasted for 24 h before the experiments. For the experiments, the rat was anesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg; Somnopentyl, Kyoritsu Seiyaku Corp., Tokyo, Japan) and restrained in a supine position on a thermostatically controlled board at 37°C. An additional i.p. injection of sodium pentobarbital (12.5 mg/kg) was given every 1 h to maintain the anesthesia.

A small midline incision was made carefully in the abdomen, the ileum was exposed, and the proximal-to-ileocecal junction segments (length = 10 cm) were cannulated at both ends with polypropylene tubing. The tubing was ligated securely to prevent fluid loss, and the segments were carefully returned to their original location inside the peritoneal cavity. To wash out the intestinal contents, PBS warmed to 37°C was circulated through the cannula at 5.0 mL/min for 4 min using an infusion pump (KD Scientific Inc., Holliston, MA). The rat was left on the board at 37°C for a further 30 min to recover from the physiological stress of the surgery. After 30 min of rest, the cannulation tubing was removed, the segments were closed tightly, and 0.5 mL of the dextran-CPP solution or control dextran solution was administered directly into the 6-cm ileal loop made from the original 10-cm segment. The dose of dextran (FD-4, FD-10, and FD-70) was 10 mg/kg (4 mg/mL). The dose of CPPs was 1.25 μmol/kg body weight (500 μM). The pH of the mixed dextran-CPP solutions was adjusted to 7.4. During the experiments, a 0.25-mL sample of blood was taken from the jugular vein before dosing and again at 5, 10, 15, 30, 60, 120, and 180 min after dosing. A tuberculin syringe (1 mL, Terumo Corp., Tokyo, Japan) was heparinized in the usual way by coating the syringe wall with aspirated heparin and then expelling all the heparin by depressing the plunger to the needle hub. The plasma was separated by centrifugation at 13,400 × *g* for 1 min. The plasma concentration of dextran was measured with a microplate fluorometer (Synergy HT, BioTek Instruments Inc., Winooski, VT) at excitation and emission wavelengths of 485 and 528 nm, respectively. The area under the plasma concentration–time curve for 0-4 h was estimated from the sum of the successive trapezoids between each data point.

Cell Culture

Caco-2 cells were cultured in 75 cm^2 culture dishes (Becton Dickinson, Franklin Lakes, NJ) with 10 mL of culture medium comprising Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum, 0.1 mM MEM–nonessential amino acids, 2 mM glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. The seeding density for culture was 8.0 × 10^5 cells/dish. The cells were maintained in an incubator at 37°C, 95% relative humidity, and 5% CO_2.