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Note

The role of intermolecular interactions with penetratin and its analogue on the enhancement of absorption of nasal therapeutic peptides

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ABSTRACT

We investigated the relationship between intermolecular binding and the ability of novel cell-penetrating peptides (CPPs) to enhance the nasal absorption of therapeutic peptides and proteins. The absorption-enhancing effect of a novel L-penetratin analogue, 'shuffle (R,K fix) 2' coadministered with different biotherapeutic peptides was evaluated after nasal administration in rats. Shuffle (R,K fix) 2 significantly increased the nasal absorption of insulin, glucagon-like-peptide-1 (GLP-1) and exendin-4, compared with the absorption seen with L-penetratin. Intermolecular binding was analyzed by surface plasmon resonance (SPR)-based binding assay. The binding characteristics implied that the higher the amount of CPP bound, the greater the nasal drug absorption. In addition, the calculated binding ratio between CPP and drug proved a critical aspect in enhancing the absorption of insulin and GLP-1. This difference in the enhancing effect of CPPs on nasal drug absorption is attributed to the degree of binding with the therapeutic macromolecule.

Cell-penetrating peptides (CPPs) have attracted much interest for their capacity to mediate the cellular uptake of poorly internalized conjugated bioactive macromolecules (Snyder and Dowdy, 2004; Deshayes et al., 2005). These CPPs include human immunodeficiency virus (HIV)-1Tat (48–60) (Vives et al., 1997; Nakase et al., 2008), oligoarginine (Mitchell et al., 2000; Futaki et al., 2001) and amphipathic peptides such as the *Drosophila* Antennapedia homeodomain (penetratin) (Derossi et al., 1994).

Our strategy using CPPs is promising for the intestinal and nasal absorption of therapeutic peptides and proteins because it provides higher bioavailability and shows no apparent undesirable effects on biological membranes (Morishita et al., 2007; Khafagy et al., 2009a,b). We have also shown that the non-invasive delivery of therapeutic peptides and proteins is improved significantly by coadministration of penetratin with these drugs (Khafagy et al., 2009a,b). However, more powerful vectors are required to maximize the bioavailability of biodrugs and to make them acceptable for clinical application. Therefore, we explored sequence modulation of the penetratin template design and obtained a potential candidate: shuffle (R,K fix) 2. This significantly enhanced the bioavailability of insulin through a nasal route compared with the parent molecule penetratin, without detectable epithelial membrane damage (amino acid sequences are shown in Table 1) (Khafagy et al., 2010). However, how penetratin and shuffle (R,K fix) 2 differ in their abilities to enhance drug permeation through cell membrane is unclear. Thus, the purpose of our study was to evaluate the relationship between the ability of shuffle (R,K fix) 2 to enhance nasal drug absorption and the intermolecular interactions involved compared with the parent molecule, penetratin.

Insulin–CPP solution was prepared by dissolving recombinant human insulin (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in 50 μL 0.1N HCl in polypropylene tubes, then diluted with 1.4 mL phosphate buffered saline (PBS), pH 7.4, and normalized with 50 μL 0.1N NaOH. Specific amounts of GLP-1 and exendin-4 (Sigma–Aldrich Co., St Louis, MO, USA) equivalent to a therapeutically administered dose were dissolved in 1.5 mL PBS. The peptide–drug solutions and 500 μM of L-penetratin or shuffle (R,K fix) 2 (were synthesized by Sigma Genosys, Life Science Division of Sigma–Aldrich Japan Co., Hokkaido, Japan; purity was >95% for each peptide) were mixed gently to adjust them to appropriate experimental concentrations as clear solution.

We examined the improvement of the nasal absorption of different biodrugs in rats coadministered with shuffle (R,K fix) 2 or L-penetratin. This research was performed at Hoshi University, and it 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 (Tokyo Laboratory Animals Science Co., Ltd., Tokyo, Japan). Nasal absorption study was performed in rats as described (Khafagy et al., 2009a). Each rat was given 20 μL of

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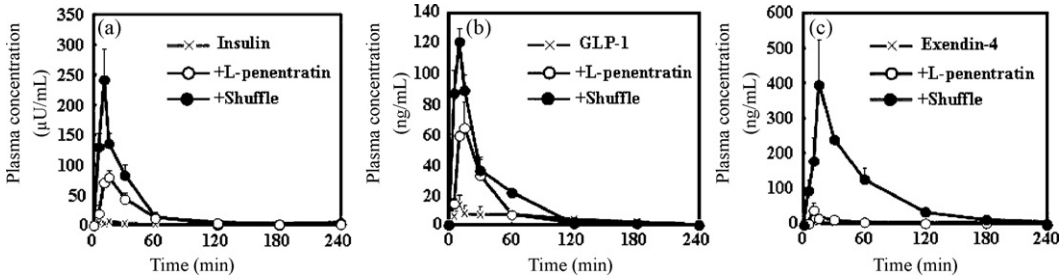

Fig. 1. Plasma peptide drugs concentration versus time profiles following nasal administration of (a) insulin (1 IU/kg); (b) GLP-1 (0.1 mg/kg); and (c) exendin-4 (0.25 mg/kg) with L-penetratin or shuffle (R,K fix) 2 (0.5 mM). Each data point represents the mean ± S.E.M. (n = 3–6).

Table 1
Molecular weights and amino sequences of L-penetratin and shuffle (R,K fix) 2.

CPP	Sequences	Mw
L-Penetratin	RQIKIWFQNRRMKWKK	2247
Shuffle (R,K fix) 2	RWFKIQMQIRRWKNKK	2247

F: phenylalanine, I: isoleucine, K: lysine, L: leucine, M: methionine, N: asparagine, Q: glutamine, R: arginine, and W: tryptophan.

the drug–CPP mixture or drug solution alone (control), using a micropipette inserted directly 0.5 cm into each nostril. The doses were 1 IU/kg body weight for insulin, 0.1 mg/kg for GLP-1 and 0.25 mg/kg for exendin-4. During the experiment, a 0.25 mL blood aliquot was taken from the jugular vein. Plasma concentrations of peptide drugs were determined by enzyme-linked immunoassay (ELISA). An insulin assay kit (Invitron Ltd., Monmouth, Wales, UK), rat GLP-1 ELISA kit (Wako Pure Chemical Industries Ltd., Osaka, Japan) and exendin-4 ELISA kits (Toray Industries, Inc., Fujisawa, Kanagawa, Japan) were used.

Fig. 1 shows a comparison of the efficiency of L-penetratin and shuffle (R,K fix) 2 on the nasal peptides absorption. Shuffle (R,K fix) 2 had a greater absorption-enhancing efficiency on the nasal absorption of insulin, GLP-1 and exendin-4 than did L-penetratin.

The binding characteristics between L-penetratin and shuffle (R,K fix) 2 and peptide drugs were analyzed by surface plasmon resonance (SPR)-based measurements to clarify the effects of physical CPP–peptide mixtures on the nasal absorption of these peptide drugs. The intermolecular interaction between peptide drugs and CPPs was analyzed by SPR (Biacore 2000, GE Healthcare Ltd., Buckinghamshire, UK). To measure the binding of L-penetratin or shuffle (R,K fix) 2 to peptides, either insulin, GLP-1, or exendin-4 was immobilized at the carboxymethyl dextran (CM5) sensor chips surface (GE Healthcare Ltd., Buckinghamshire, UK) using amine coupling (Kamei et al., 2009). For binding measurements, different concentrations of L-penetratin (1–200 μM) or shuffle (R,K fix) 2 (1–20 μM) were injected for 5 min followed by an additional 5 min dissociation phase.

The absorption-enhancing effect of the oligoarginine–drug physical mixture corresponds with the intermolecular binding between the drug and the oligoarginine (Kamei et al., 2009). As shown in Fig. 2a–c, the binding sensorgram response increased proportionately to the L-penetratin concentration (1–200 μM) injected into peptide-immobilized flow cells at pH 7.4. However the sensorgram responses for shuffle (R,K fix) 2 had higher values with the lower concentrations (1–20 μM) used for L-penetratin (Fig. 2d–f). This suggests that the binding affinities between shuffle (R,K fix) 2 and insulin, GLP-1 or exendin-4 are much greater than for L-penetratin.

To determine the binding characteristics between peptide drugs and CPPs, the equilibrium binding of each cycle was calculated using BIAevaluation software, and then the dissociation constant (KD) and the maximum amount (R_{max}) were calculated using equilibrium amounts based on fitting using MULTI software followed by Scatchard analysis. The immobilized molecule is referred to as the 'ligand' and the injected molecule is referred to as the 'analyte'. The maximum binding capacity in the absorption experimental conditions (B_{max}) was calculated using the following equation:

$$B_{max} = [L]_t \cdot R_{max}/L_i \quad (1)$$

where $[L]_t$ is the total ligand concentration at absorption experimental condition and L_i is the amount of immobilized ligand. The bound ($[A]_b$) and unbound ($[A]_f$) analyte concentrations were cal-

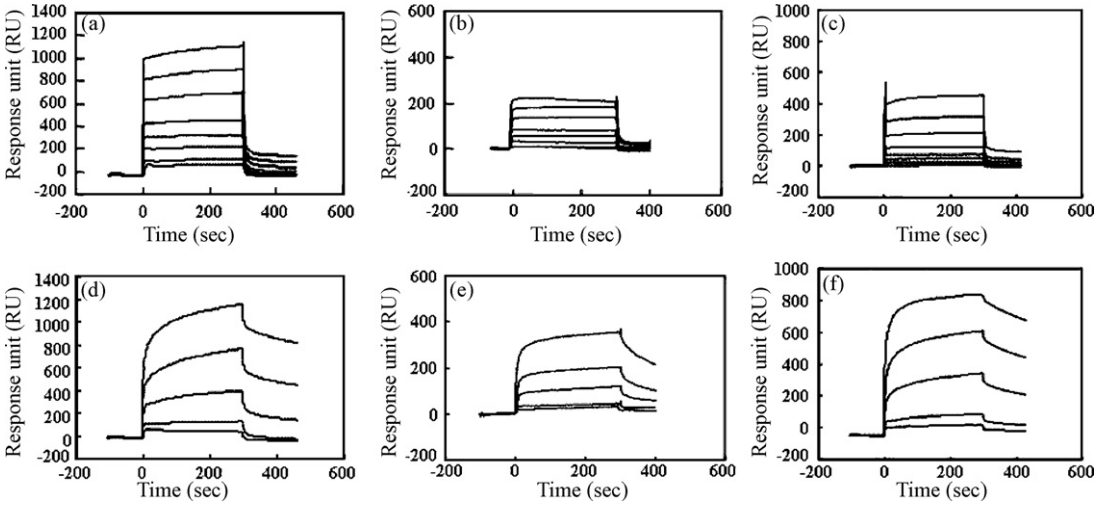

Fig. 2. Binding sensorgrams after the injection of various concentrations of L-penetratin (1–200 μM; a–c) or shuffle (R,K fix) 2 (1–20 μM; d–f) into a peptide-immobilized flow cell at pH 7.4. Sensorgrams are shown for insulin (a and d), GLP-1 (b and e) and exendin-4 (c and f).

Table 2
Binding parameters between L-penetratin and immobilized peptide drugs derived from the SPR study.

	Insulin		GLP-1	Exendin-4	
	High	Low		High	Low
Binding parameters					
Immobilized peptide amount (pmol/mm^2)	0.221	0.221	0.078	0.132	0.132
KD (μM)	2.793	74.583	42.6	14.622	520.952
R_{max} (pmol/mm^2)	118.06×10^{-3}	500.07×10^{-3}	89.02×10^{-3}	52.75×10^{-3}	43.19×10^{-2}
Binding ratio (L-penetratin/peptide)	535.82×10^{-3}	226.94×10^{-2}	113.61×10^{-2}	39.98×10^{-2}	32.73×10^{-1}
Nasal absorption experimental condition					
Applied L-penetratin (μM)	500		500	500	
Applied peptide (μM)	33.1		151.6	298.5	
B_{max} (μM)	17.7	75.1	172	119.3	977.5
Unbound L-penetratin (μM)	418.6		346.6	160.43	
Bound L-penetratin (μM)	81.4		153.3	339.5	
Bound L-penetratin ratio (%)	16.2		30.6	67.9	

KD (μM) and R_{max} (pmol/mm^2) values were calculated from the Scatchard analysis.
Binding ratio (L-penetratin/peptide) was calculated by dividing R_{max} by immobilized peptide amount.
The parameters at the nasal absorption experimental condition were calculated using Eqs. (1)–(3) described in the text.

culated using the following equations:

$$[A]_t = [A]_f + B_{max} \cdot [A]_f/(KD + [A]_f) \quad (2)$$

$$[A]_b = [A]_t - [A]_f \quad (3)$$

where $[A]_t$ is the total analyte concentration. The bound analyte concentration at absorption experimental condition $[A]_b$ was used as the index of binding affinity between peptide drugs and CPPs.

Table 2 shows the calculated binding parameters between L-penetratin and insulin, GLP-1 and exendin-4 in the nasal absorption experiments demonstrated that the bound concentrations were 16.2%, 30.6% and 67.9%, respectively. On the other hand, Table 3 shows higher bound concentration of shuffle (R,K fix) 2 with insulin, GLP-1 and exendin-4 (33.2%, 91.9% and 98.2%, respectively). These demonstrate a significant increase of the nasally absorbed peptides coadministered with shuffle (R,K fix) 2 under the experimental conditions. The difference in the binding characteristics between shuffle (R,K fix) 2 and L-penetratin may be attributed to the modification of amphiphilic residue (tryptophan) positions and fixing the basic (Arg and Lys) residue positions (Table 1). This possibly allows a high degree of conformational flexibility of the interacting moieties (Christiaens et al., 2002; Polyansky et al., 2009).

The relationship between the concentration of L-penetratin or shuffle (R,K fix) 2 that bound to the peptide drug in nasal absorption experiments, and the absorption-enhancing efficiency of CPPs was evaluated. The amount of L-penetratin or shuffle (R,K fix) 2 that bound to insulin was concentration dependent but tended to saturate at approximately 300 μM for both CPPs (Fig. 3a and d). Table 4 shows the calculated ratio between bound concentrations of CPPs added to peptide drugs, using the binding characteristics between CPPs and immobilized peptides in Tables 2 and 3. The binding ratios between L-penetratin and insulin and GLP-1 were approximately 2.5:1 and 1:1, respectively. However, higher binding ratios between shuffle (R,K fix) 2 and insulin and GLP-1 (5:1 and 3:1, respectively). This explain the significantly higher absorption of nasal administration of insulin and GLP-1 coadministered

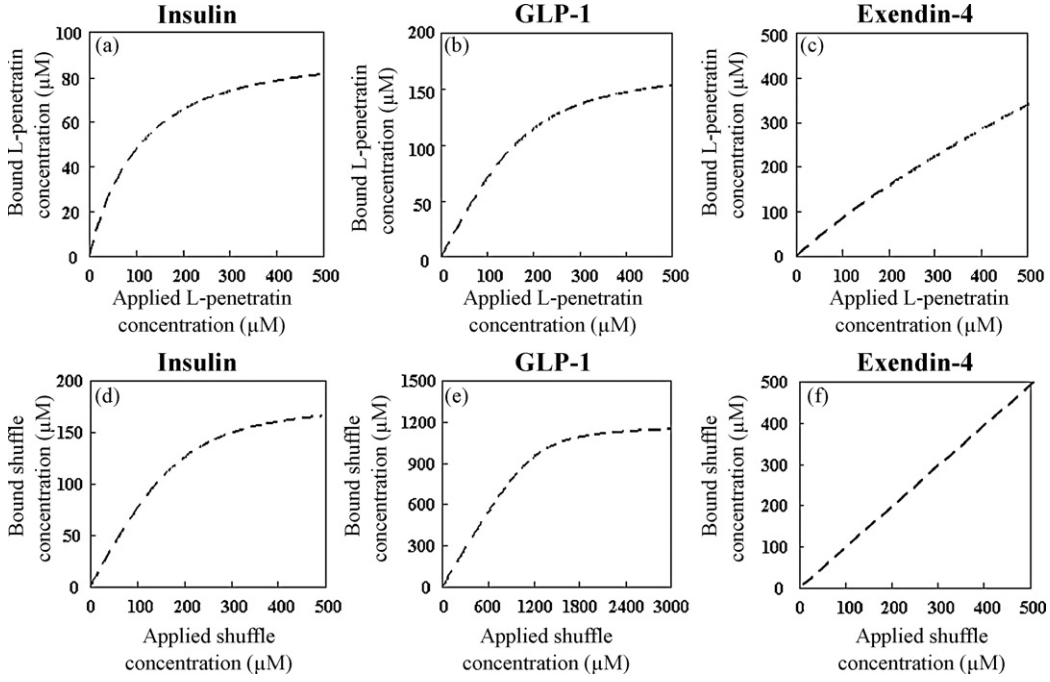

Fig. 3. Relationship between the total concentrations of L-penetratin (a–c) and shuffle (R,K fix) 2 (d–f) added to insulin (33.1 μM; a and d), GLP-1 (151.6 μM; b and e) or exendin-4 (298.5 μM; c and f). The L-penetratin or shuffle (R,K fix) 2 concentrations bound to peptide drugs were calculated using the parameters listed in Tables 2 and 3 and Eqs. (1)–(3) as described in the text.

Table 3
Binding parameters between shuffle (R,K fix) 2 and immobilized peptide drugs derived from the SPR study.

	Insulin	GLP-1	Exendin-4
Binding parameters			
Immobilized peptide amount (pmol/mm^2)	0.221	0.078	0.132
KD (μM)	33.9	64.3	21.4
R_{max} (pmol/mm^2)	1.217	0.614	0.756
Binding ratio (shuffle (R,K fix) 2/peptide)	5.52	7.383	5.73
Nasal absorption experimental condition			
Applied shuffle (R,K fix) 2 (μM)	500	500	500
Applied peptide (μM)	33.11	151.6	298.57
B_{max} (μM)	182.9	1188.3	1711.1
Unbound shuffle (R,K fix) 2 (μM)	333.8	40.5	8.6
Bound shuffle (R,K fix) 2 (μM)	166.1	459.4	491.3
Bound shuffle (R,K fix) 2 ratio (%)	33.2	91.9	98.2

KD (μM) and R_{max} (pmol/mm^2) values were calculated from the Scatchard analysis. Binding ratio (shuffle (R,K fix) 2/peptide) was calculated by dividing R_{max} by immobilized peptide amount.
The parameters at the nasal absorption experimental condition were calculated using Eqs. (1)–(3) described in the text.

Table 4
The binding ratio between CPP and peptide drugs at nasal absorption conditions.

CPP	Insulin	GLP-1	Exendin-4
L-Penetratin	2.5	1.01	1.13
Shuffle (R,K fix) 2	5.16	3.01	1.64

with shuffle (R,K fix) 2. Thus, the binding ratio between a drug and a CPP obtained in binding experiments may be an index to clarify the enhancing efficacy of nasal peptide absorption. Conversely, in the case of exendin-4, approximately the same binding ratios were observed with L-penetratin and shuffle (R,K fix) 2, although, shuffle (R,K fix) 2 significantly increased the absorption of exendin-4 compared with L-penetratin. The binding between a drug and a CPP involves electrostatic and hydrophobic interactions and hydrogen bonding, and these depend on the type of CPP. Furthermore, not only the binding efficiency between a drug and CPP but also other factors such as the internalization efficiency of the CPP itself may affect absorption enhancement (Kamei et al., 2009; Khafagy et al., 2009a, 2010).

In conclusion, we determined that the significant enhancing effect of coadministered L-penetratin or shuffle (R,K fix) 2 on the nasal absorption of biodrugs is related to the intermolecular binding between a drug and a CPP. In addition, the binding ratio between L-penetratin or shuffle (R,K fix) 2 and peptide drugs is critical for the enhancement of nasal insulin and GLP-1 absorption.

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