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A claudin-4 modulator enhances the mucosal absorption of a biologically active peptide

Hiroshi Uchida [a,*], Masuo Kondoh [b,**], Takeshi Hanada [a], Azusa Takahashi [b], Takao Hamakubo [c], Kiyohito Yagi [b]

[a] Department of Biopharmaceuticals Research, Biopharma Center, Asubio Pharma Co., Ltd., Gunma, Japan
[b] Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan
[c] Department of Molecular Biology and Medicine, Research Center for Advanced Science and Technology, The University of Tokyo, Tokyo, Japan

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ABSTRACT

Biologics, such as peptides, proteins and nucleic acids, are emerging pharmaceuticals. Passage across the epithelium is the first step in the absorption of biologics. Tight junctions (TJ) function as seals between adjacent epithelial cells, preventing free movement of solutes across the epithelium. We previously found that modulation of a key TJ component, claudin-4, is a potent method to enhance jejunal absorption when we used dextran as a model drug and the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE) as a claudin-4 modulator. Here, we investigated whether the claudin-4 modulator enhances jejunal, nasal and pulmonary absorption of a biologics human parathyroid hormone derivative, hPTH(1-34). The claudin-4 modulator enhanced nasal but not jejunal and pulmonary absorption of hPTH(1-34). C-CPE is hydrophobic with low solubility of less than 0.3 mg/ml, but deletion of 10 amino acids at the N-terminal of C-CPE increased its solubility by 30-fold. Moreover, the N-terminal truncated C-CPE bound to claudin-4, modulated the TJ-barrier and enhanced jejunal absorption of dextran. The N-terminal-truncated C-CPE also enhanced jejunal and pulmonary absorption of hPTH(1-34). This report is the first to indicate that a claudin-4 modulator may be a promising enhancer of the jejunal, pulmonary and nasal absorption of a peptide drug.

1. Introduction

Recent progress in genomic and proteomic technologies has yielded some new biologics, such as peptides, proteins and nucleic acids, as pharmaceutical candidates. Passage across the mucosal epithelium of the intestine, nose and lung is the first step in drug absorption. However, most biologics are hydrophilic molecules that are poorly absorbed by the mucosa. Although injection is a compelling route for the administration of biologics, a transmucosal delivery system would be an ideal administration route for biologics because it is noninvasive and therefore would provide a higher quality of life to patients. However, it is difficult to develop a transmucosal delivery system since the epithelium plays a pivotal role in the barrier separating the inside of the body from the outside environment.

Tight junctions (TJ) exist between adjacent epithelial cells and seal the paracellular space, preventing free movement of solutes [1]. To facilitate drug absorption, modulators of the epithelial barrier have been investigated since 1960s [2,3]. Many TJ modulators, such as fatty acids, bile salts, a polysaccharide and a toxin fragment, have been developed [4–6]. However, the biochemical structures of TJs remained uncharacterized until 1998, and a drug absorption enhancer based on TJ-components has never been fully developed [7]. Occludin, a 65-kDa tetra-transmembrane protein, was the first TJ-structural component to be identified [8]. Claudin, a 23-kDa integral membrane protein bearing tetra-transmembrane domains, is the functional component of the TJ-barrier [9,10]. The claudin family consists of 24

Abbreviations: TJ, tight junction; C-CPE, the C-terminal fragment of *Clostridium perfringens* enterotoxin; C-CPE184, C-terminal fragment of *Clostridium perfringens* enterotoxin from 184 to 319 amino acids; hPTH, human parathyroid hormone; CPE, *Clostridium perfringens* enterotoxin; DDM, n-Dodecyl-β-D-maltoside; EDC, N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide; NHS, N-hydroxysuccinimide; C-CPE194, C-terminal fragment of *Clostridium perfringens* enterotoxin from 194 to 319 amino acids; C-CPE205, C-terminal fragment of *Clostridium perfringens* enterotoxin from 205 to 319 amino acids; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; BV, budded baculovirus; TBS, Tris-buffered saline; ELISA, enzyme-linked immunosorbent assay; SPR, surface plasmon resonance; TEER, transepithelial electric resistance; FD-4, fluorescein isothiocyanate-dextran with a molecular weight of 4 kDa; AUC, the area under the plasma concentration; BA, bioavailability.

* Corresponding author at: Department of Biopharmaceuticals Research, Biopharma Center, Asubio Pharma Co., Ltd., Gunma 370-0503, Japan. Tel.: +81 276 86 7359; fax: +81 276 86 5787.
** Corresponding author at: Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan. Tel.: +81 6 6879 8196; fax: +81 6 6879 8199.
E-mail addresses: uchida.hiroshi.b3@asubio.co.jp (H. Uchida), masuo@phs.osaka-u.ac.jp (M. Kondoh).

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members with different barrier functions among tissues. For example, claudin-1 and -5 function in the epidermal barrier and blood-brain barrier, respectively [11,12]. Claudin is heterogeneously expressed in epithelial cells of the gut, lung and nose [13–15]. The extracellular loop domains of claudin between adjacent cells are estimated to be involved in the paracellular tightness of the cleft between cells, resulting in prevention of solute movement by the formation of TJs [16,17]. Therefore, a molecule that can bind to the extracellular loop domain of claudin may be a novel type of mucosal-absorption enhancer.

The 35-kDa *Clostridium perfringens* enterotoxin (CPE) causes food poisoning in humans [18]. The receptor for CPE is claudin-4 [10]. The C-terminal fragment of CPE (amino acids 184–319; C-CPE184) is the receptor-binding region of CPE. Treatment of cells with C-CPE184 disrupted the TJ-barrier through its interaction with the second extracellular loop of claudin-4, indicating that C-CPE184 is a claudin-4 modulator [10,19,20]. We previously found that C-CPE184 enhanced the jejunal absorption of dextran (molecular mass, 4 kDa) over 400-fold compared with a clinically used absorption enhancer, sodium caprate, and that deletion of the claudin-4-binding region in C-CPE attenuated the absorption-enhancing effect of C-CPE [21]. Claudin-4 is also expressed in nasal and pulmonary epithelial cells [14,15,22]. Thus, a claudin-4 modulator may be a promising candidate for the enhancement of not only jejunal absorption but also pulmonary and nasal absorption of biologics; however, the ability of a claudin modulator to enhance the mucosal absorption of biologics has not yet been investigated.

In the present study, we investigated whether claudin-4 modulation enhanced jejunal, nasal and pulmonary absorption of a peptide drug, human parathyroid hormone derivative (hPTH(1-34)) using C-CPE184 and its derivatives. We found that a claudin-4 modulator was a novel mucosal-absorption enhancer of a peptide drug.

2. Materials and methods

2.1. Materials

Human parathyroid hormone derivative (hPTH(1-34)) was prepared as described previously [23]. n-Dodecyl-β-D-maltoside (DDM) was purchased from Dojindo Laboratories (Kumamoto, Japan). Anti-claudin antibodies and anti-his-tag antibody were obtained from Invitrogen (Carlsbad, CA) and EMD Chemicals Inc. (Darmstadt, Germany), respectively. CM5 sensor chips, amine-coupling reagents (N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), and ethanolamine–HCl) and HBS-EP+ (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P20) were obtained from GE Healthcare (Buckinghamshire, UK). All reagents used were of research grade.

2.2. Preparation of N-terminal-truncated C-CPE derivatives

We prepared expression vectors of N-terminal region-truncated C-CPE184 (amino acids 184–319). These vectors expressed amino acids 194–319 (C-CPE194), 205–319 (C-CPE205), 212–319 (C-CPE212), 219–319 (C-CPE219) and 224–319 (C-CPE224). Insert fragments of each C-CPE mutant were amplified from C-CPE184 cDNA (kindly provided by Dr. Y. Horiguchi, Osaka University) by polymerase chain reaction with forward primers (5′-ATGCTCGAGGATATAGAAAAAGAAATCCTT-3′ for C-CPE194, 5′-ATGCTCGAGGCTACAGAAAGATTAAATTTAACTG-3′ for C-CPE205, 5′-ATGGGGGAGGCTACAGAAAGATTAAATTTAACTG-3′ for C-CPE212, 5′-ATGCTCGAGGCTGAAGCCCAAGATTAAATTTAACTG-3′ for C-CPE219, 5′-ATGCTCGATCTATGAAGATTAAATTTAACTG-3′ for C-CPE224) and a common reverse primer (5′-TTTGCTAGCTAAGATTCTATATTTTTGTCC-3′). The resultant C-CPE fragments were cloned into the pET16b vector. The plasmids were transduced into *E. coli* BL21 (DE3), and protein expression was stimulated by the addition of isopropyl-1-thio-β-D-galactoside. The cell lysates were applied to HisTrap™ HP (GE Healthcare), and C-CPEs were eluted with imidazole. The solvent was exchanged with phosphate-buffered saline (PBS) by gel filtration, and the purified proteins were stored at −80 °C until use. Purification of the proteins was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by staining with Coomassie Brilliant Blue. C-CPEs were quantified by using a BCA protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL) with bovine serum albumin as a standard.

2.3. Preparation of claudin-displaying budded baculovirus (BV)

Claudin-displaying BV was prepared as described previously [24]. Briefly, mouse claudin-1 and -4 cDNA fragments were cloned into the baculoviral transfer vector pFastBac1 (Invitrogen). Recombinant baculoviruses were generated by using the Bac-to-Bac system according to the manufacturer's instructions (Invitrogen). Sf9 cells were cultured in Grace's Insect medium (Invitrogen) containing 10% FBS at 27 °C and infected with the recombinant baculovirus. Seventy-two hours after infection, the BV fraction was isolated from the culture supernatant of the infected Sf9 cells by centrifugation at 40,000 × *g* for 25 min. The pellets of the BV fraction were suspended in Tris-buffered saline (TBS) containing protease inhibitor cocktail (Sigma–Aldrich, St. Louis, MI) and then stored at 4 °C. The expression of claudins in the BV fraction was confirmed by SDS-PAGE and immunoblot with antibodies against claudins.

2.4. Enzyme-linked immunosorbent assay (ELISA) with claudin-displaying BV

The claudin-displaying BV were diluted with TBS and adsorbed to the wells of 96-well immunoplates (Nunc, Roskilde, Denmark) overnight at 4 °C. The wells were washed with PBS and blocked with TBS containing 1.6% BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan) for 2 h at room temperature. C-CPE or C-CPE derivatives were added to the wells and incubated for an additional 2 h at room temperature. The wells were washed with PBS and incubated with anti-his-tag antibody for 2 h at room temperature. The immuno-reactive proteins were detected by a horseradish peroxidase-labeled secondary antibody with 3,3′,5,5′-tetramethylbenzine as a substrate. The reaction was terminated by the addition of 0.5 M H_2SO_4, and the immuno-reactive proteins were measured at 450 nm.

2.5. Preparation of recombinant claudin-4 protein

Recombinant claudin-4 protein was prepared by using Sf9 cells infected with recombinant baculovirus, as previously reported [25,26]. Briefly, the C-terminal his-tagged claudin-4 cDNA fragment was cloned into pFastBac1, and recombinant baculovirus was generated by using the Bac-to-Bac baculovirus expression system. Sf9 cells were infected with the recombinant baculovirus. After 52–56 h of infection, the cells were harvested by centrifugation. The cells were washed with PBS and were resuspended in 10 mM Hepes, pH 7.4, 120 mM NaCl with protease inhibitor tablets (Complete Mini, EDTA-free, Roche Applied Science (Indianapolis, IN)), 1 mM phenylmethylsulfonyl fluoride and 20 units/ml DNase I. The cells were lysed by the addition of 2% of DDM and were then centrifuged. The resultant supernatant was applied to HisTrap™ HP, and claudin-4 was eluted with imidazole. The solvent for claudin-4 was exchanged to PBS containing 0.2% DDM by gel

filtration with a HiTrap Desalting column (GE Healthcare). Purification of claudin-4 was confirmed by SDS-PAGE followed by staining with Coomassie Brilliant Blue.

2.6. Surface plasmon resonance (SPR) analysis

SPR measurements were performed with a Biacore T100 instrument (GE Healthcare). Amine-coupling chemistry was used to immobilize claudin-4 at 25 °C on a CM5 sensor chip surface docked in a Biacore T100 and equilibrated with HBS-EP+. The carboxymethyl surface of the CM5 chip was activated for 2 min with a 1:1 ratio of 0.4 M EDC and 0.1 M NHS at a flow rate of 10 μl/min. Claudin-4 was diluted to 2.5 μg/ml in 10 mM MES buffer (pH 6.5) and injected for 2 min over the surface at a flow rate of 10 μl/min. Excess activated groups were blocked by a 5-min injection of 1 M ethanolamine (pH 8.5) at a flow rate of 10 μl/min. Approximately 1000 RU of claudin-4 was immobilized by using this protocol. Single-cycle kinetics experiments were performed at 25 °C with a flow rate of 30 μl/min [27]. C-CPE or its derivatives were serially diluted (1.25, 2.5, 5, 10 and 20 nM) in running buffer (HBS-EP+). Within a single binding cycle, samples of C-CPE or its derivatives were injected sequentially in order of increasing concentration over both the ligand and the reference surfaces. The reference surface, an unmodified flowcell, was used to correct for systematic noise and instrumental drift. Also, prior to the binding cycle for C-CPE or its derivatives, buffer was injected. These "blank" responses were used as a double-reference for the binding data [28]. The sensorgrams were globally fitted by using a 1:1 binding model to determine k_a, k_d and K_D values with the Biacore T100 Evaluation Software version 2.0.1.

2.7. Transepithelial electric resistance (TEER) assay

Caco-2 cells were seeded onto BD BioCoat™ Fibrillar Collagen Cell culture inserts (BD Biosciences, San Jose, CA) at a density of 1×10^5 cells/insert and cultured for 5 days. TJ barriers were formed by a 3-day culture in Entero-STIM™ (BD Biosciences) medium for cellular differentiation. C-CPE or its derivatives were added to the apical side of the chamber. After 18 h of incubation, the TEER values were measured with a Millicell-ERS epithelial volt-ohmmeter (Millipore, Billerica, MA). The percentage changes of TEER values were calculated by the ratio to TEER value in 100 μg/ml of C-CPE184. EC50 values, at which the TEER ratio is 50%, were calculated by using the four-parameter logistic function of DeltaSoft version 3 (BioMetallics, Princeton, NJ) from dose–response curves of the TEER ratio.

2.8. In situ loop assay

Jejunal absorption of hPTH(1-34) or fluorescein isothiocyanate-dextran with a molecular mass of 4 kDa (FD-4) was evaluated by using an in situ loop assay as described previously [21]. The experiments were performed according to the guidelines of the ethics committee of Osaka University or Asubio Pharma Co. Ltd. After 7-week-old Wister male rats were anesthetized with pentobarbital, a midline abdominal incision was made, and the jejunum was washed with PBS. A 5-cm long jejunal loop was prepared by closing both ends with sutures. hPTH(1-34) (100 μg) was co-administered with C-CPEs into the loop or administered 4 h after the administration of C-CPEs. Blood was collected from the femoral artery by using a cannulated polyethylene tube at the indicated time points. EDTA (1 mg/ml) was immediately added to the blood sample, and the plasma was recovered by centrifugation. To avoid degradation of hPTH(1-34), aprotinin (500 IU/ml) was immediately added to the plasma, and the plasma was stored at −80 °C until use. The plasma hPTH(1-34) was quantified by radioimmunoassay (RIA) with anti-hPTH antibody. Anti-hPTH antibody was added to the plasma and then incubated with [^{125}I-Tyr34] hPTH(1-34) (15,000–20,000 cpm/100 μl) for 24 h. The anti-rabbit IgG goat antibody was added, and anti-hPTH antibody bound to the anti-rabbit IgG goat antibody was separated by centrifugation. The radioactivity in the sediment was counted with a gamma counter (PerkinElmer Inc., Waltham, MA). The area under the plasma concentration time curve (AUC) from 0 to 120 min after administration was calculated by the trapezoidal method. Relative bioavailability (BA) was calculated with the following equation: BA (%) = (AUC (ng·min/ml)/dose (μg/kg))/(AUC (iv)(ng·min/ml)/dose (iv) (μg/kg)). AUC (iv) indicates the $AUC_{0-120\ min}$ of intravenously administered hPTH(1-34) (10 μg/kg), and the AUC value is 208.6 ± 52.7 ng·min/ml.

Rats were anesthetized with thiamylal sodium, and a jejunal loop was made, as described above. A mixture of FD-4 (2 mg) and C-CPEs was co-administered into the jejunal loop. Blood was collected from the jugular vein at the indicated time points. The plasma levels of FD-4 were measured with a fluorescence spectrophotometer (Fluoroskan Ascent FL; ThermoElectron Corporation, Waltham, MA). The AUC of FD-4 from 0 to 6 h ($AUC_{0-6\ h}$) was calculated by the trapezoidal method.

2.9. Nasal and pulmonary absorption assay

Nasal and pulmonary absorption of hPTH(1-34) was examined in 7-week-old Sprague–Dawley male rats. The experiments were performed according to the guidelines of the ethics committee of Asubio Pharma Co. Ltd. For the nasal absorption assay, 200 μg of hPTH(1-34) was intranasally administered to both sides of the nasal cavity 0 or 4 h after nasal administration of C-CPEs. The total injection volume did not exceed 20 μl. For the pulmonary absorption assay, a polyethylene tube (PE-240, Clay Adams, Becton Dickinson & Co., Sparks, MD) was inserted into the trachea of each rat. A MicroSprayer (Penn-Century, Inc., Philadelphia, PA) was used to perform pulmonary injections of C-CPEs; then, after 0 or 4 h, 150 μg of hPTH(1-34) was administered with the MicroSprayer. Blood was collected at the indicated time points, and the plasma concentration of hPTH(1-34) was measured by RIA, as described above. AUC and BA (%) values were calculated, as described above.

2.10. Statistical analysis

Data were analyzed by using analysis of variance (ANOVA) followed by Dunnett's multiple comparison test, and statistical significance was assigned at $p < 0.05$.

3. Results

3.1. Effects of C-CPE on jejunal, nasal and pulmonary absorption of a peptide drug

We previously found that a claudin-4 modulator, C-CPE184, is a novel type of absorption enhancer by using dextran as a model drug [21]. In the present study, we investigated whether the claudin-4 modulator enhances jejunal, nasal and pulmonary absorption of a peptide drug, hPTH(1-34). When hPTH(1-34) was administered with C-CPE184, C-CPE184 enhanced nasal absorption of hPTH(1-34) by 2.5-fold as compared to the vehicle-treated group. However, C-CPE184 did not enhance jejunal and pulmonary absorption of hPTH(1-34) (Fig. 1 and Table 1). Next, we examined whether pre-treatment of mucosa with C-CPE184 enhanced absorption of hPTH(1-34). When hPTH(1-34) was administered after 4 h of treatment with C-CPE184, the jejunal, nasal and pulmonary absorption of hPTH(1-34) was significantly

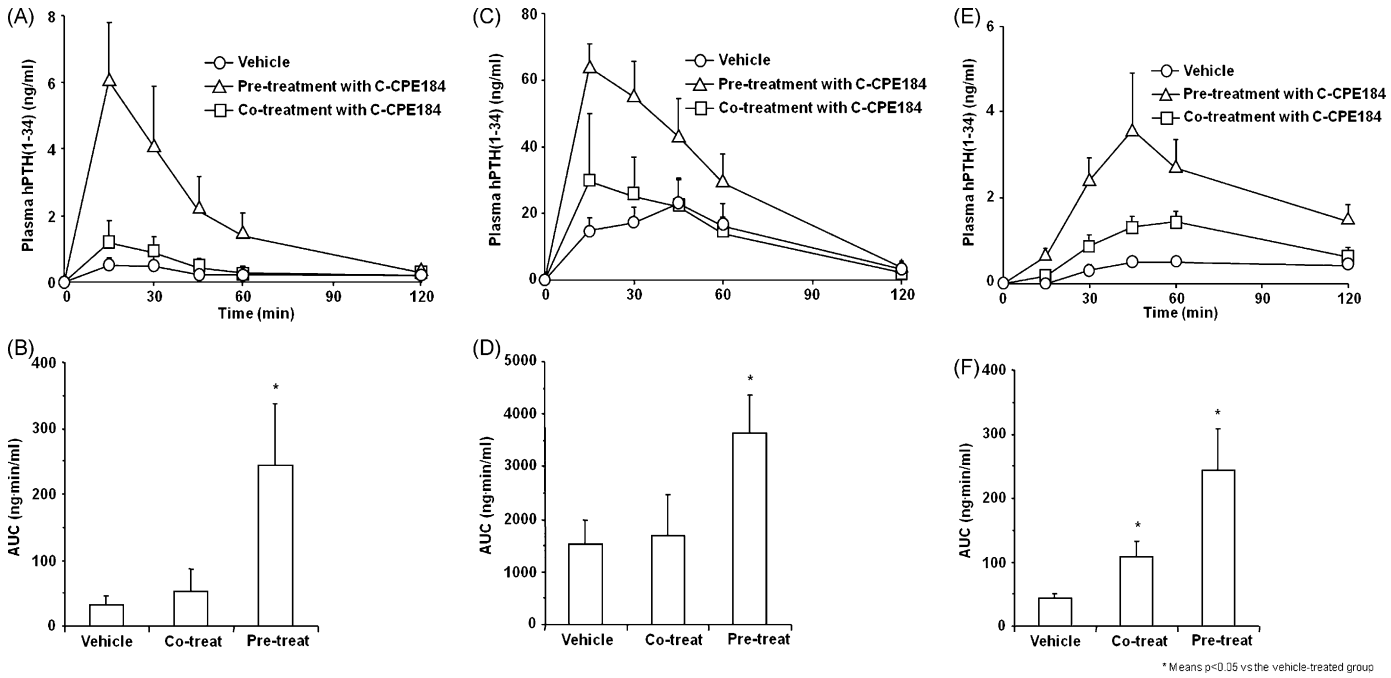

Fig. 1. Effect of C-CPE184 on mucosal absorption of hPTH(1-34) in rats. (A, B) Jejunal absorption of hPTH(1-34). Jejunum was co-treated with hPTH(1-34) (100 μg) and C-CPE184 (20 μg), or jejunum was treated with hPTH(1-34) 4 h after treatment with C-CPE184. (C, D) Pulmonary absorption of hPTH(1-34). hPTH(1-34) (150 μg) was pulmonary administered with C-CPE184 (5 μg) or 4 h after administration of C-CPE184. (E, F) Nasal absorption of hPTH(1-34). hPTH(1-34) (200 μg) was nasally administered with C-CPE184 (2 μg) or 4 h after administration of C-CPE184. Plasma hPTH(1-34) levels were measured at the indicated periods. Time-course changes in plasma hPTH(1-34) levels (A, C, E) and AUC from 0 to 120 min (B, D, F) were calculated. Data are mean ± SE (n = 3-6). Co-treat indicates co-treatment with both hPTH and C-CPE184, and Pre-treat indicates treatment with hPTH 4 h after C-CPE184-treatment. *Significantly different from the vehicle-treated group ($p < 0.05$).

Table 1
Parameters of mucosal absorption of hPTH(1-34) in C-CPE184-treated rats.

Treatments	Jejunum		Nasal		Pulmonary	
	Cmax (ng/ml)	BA (%)[a]	Cmax (ng ml)	BA (%)	Cmax (ng ml)	BA (%)
Vehicle	0.9 ± 0.3	0.4 ± 0.2	0.7 ± 0.1	0.3 ± 0.0	27.8 ± 6.5	14.6 ± 4.4
Co-treat	1.2 ± 0.6	0.6 ± 0.4	1.5 ± 0.2*	0.8 ± 0.2*	35.2 ± 18.9	14.9 ± 6.5
Pre-treat	6.0 ± 1.8*	2.7 ± 0.9*	3.6 ± 1.3	1.4 ± 0.4*	67.6 ± 7.S**	34.5 ± 6.6*

[a] BA (%) = (AUC/Dose)/(AUC iv/Dose iv).
Data are means ± SE.
*$p < 0.05$, **$p < 0.01$, as compared to vehicle-treated group.

increased 7.5-, 5.6- and 2.4-fold compared to the vehicle-treated group (Fig. 1 and Table 1).

3.2. Preparation of N-terminal-truncated C-CPE184-319 derivatives

The solubility of C-CPE184 is less than 0.3 mg/ml in PBS due to its hydrophobicity (Table 2). An increase in solubility without loss of claudin-4-modulating activity might improve the mucosal-absorption-enhancing activity of C-CPE184. Van Itallie et al. showed that the removal of the 10 N-terminal amino acids from C-CPE184 to yield C-CPE194 results in high solubility (10 mg/ml)

Table 2
Solubility of C-CPE184 and the N-terminal-truncated mutants.

Derivatives	Molecular size (kDa)	Solubility[a] (mg/ml)
C-CPE184	18.2	<0.3
C-CPE194	17.3	>10
C-CPE205	16.1	>4
C-CPE212	15.4	Insol.[b]
C-CPE219	14.7	Insol.
C-CPE224	14.2	Insol.

[a] Solvent is PBS.
[b] Insol., insoluble.

[26]. Although C-CPE194 is a claudin-4 binder, whether C-CPE194 modulates the TJ-barrier remains unclear. C-CPE194 contains nine β-sheets and one α-helix, and its 16 C-terminal amino acids are believed to comprise the claudin-4-binding region (Fig. 2A) [26,29]. Based on this information, we prepared five different N-terminal-truncated C-CPE184 derivatives: C-CPE194, which lacks the 10 N-terminal amino acids; C-CPE205, which is truncated prior to the β1-sheet; C-CPE212, which is truncated after the β1-sheet; C-CPE219, which is truncated after the α-helix; and C-CPE224, which is truncated before the β2-sheet (Fig. 2A). The C-CPEs were expressed in *E. coli* (Fig. 2B). The solubility of C-CPE194 (>10 mg/ml) and C-CPE205 (>4 mg/ml) in PBS was greater than that of C-CPE184 (<0.3 mg/ml) (Table 2). However, C-CPE212, C-CPE219 and C-CPE224 formed solid inclusion bodies in *E. coli*, and these inclusion bodies could not be dissolved without 2 M urea. Therefore, further experiments were performed with C-CPE184, C-CPE194 and C-CPE205.

3.3. Characterization of C-CPE194 and C-CPE205

To study the interaction between the C-CPEs and claudin-4, we performed ELISA with claudin-4-displaying BV, as described previously [24]. When C-CPEs were added to claudin-4-displaying

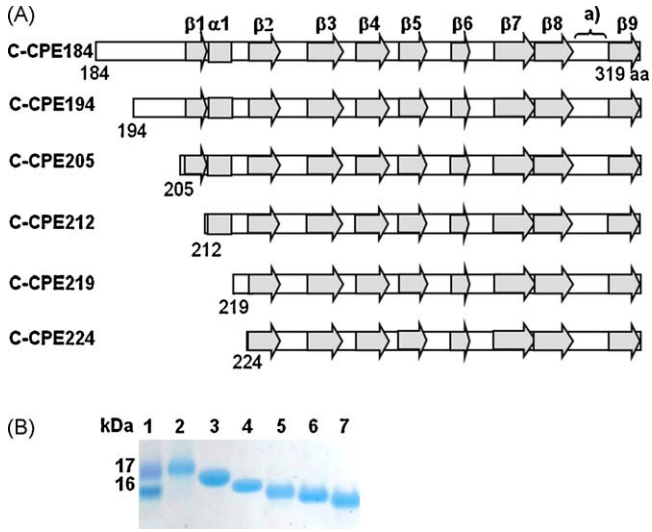

Fig. 2. Preparation of C-CPEs. (A) Schematic structure of C-CPEs. Van Itallie et al. determined the 3-dimensional structure of C-CPE194 containing nine β-sheets and one α-helix [26]. Based on the structural information, we designed five N-terminal truncated C-CPE184 derivatives. (B) CBB staining. C-CPEs were prepared and then purified by affinity chromatography. Lane 1, a maker for molecular weight; lane 2, C-CPE184; lane 3, C-CPE194; lane 4, C-CPE205; lane 5, C-CPE212; lane 6, C-CPE219; lane 7, C-CPE224.

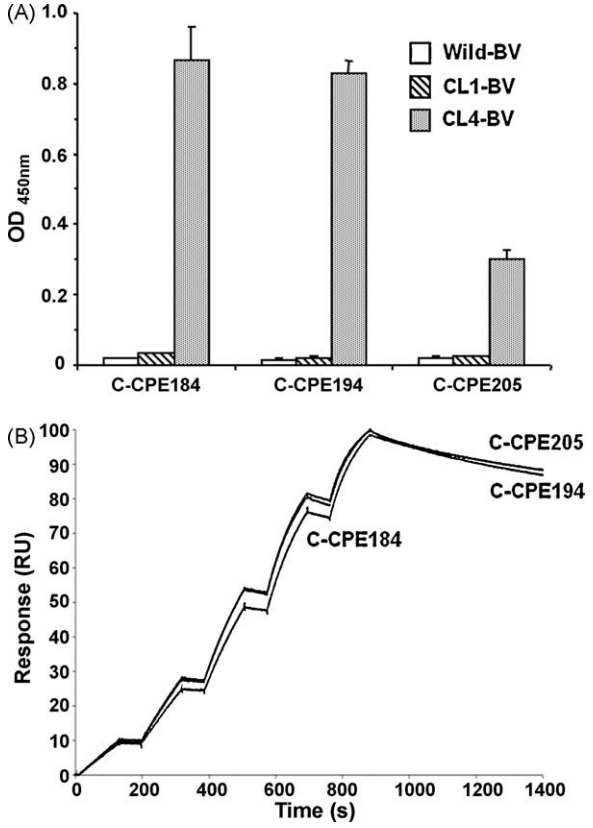

Fig. 3. Interaction of C-CPEs with claudin. (A) ELISA. The immunoplate was coated with wild-type BV (Wild-BV), claudin-1-displaying BV (CL1-BV) or claudin-4-displaying BV (CL4-BV), and then C-CPEs were added to the well. C-CPEs bound to BVs were detected by the addition of anti-his tag antibody and a labeled secondary antibody. Data are means ± SD (n = 3). (B) SPR assay. Claudin-4 was immobilized on a CM5 sensor chip by the amine-coupling method. C-CPEs were injected sequentially at concentrations of 1.25, 2.5, 5, 10 and 20 nM. The association phase was monitored for 120 s at a flow rate of 10 μl/min, and the dissociation phase was followed for 600 s at the same flow rate. The maximum values of response (Rmax) for all curves were compensated to 100 RU.

Table 3
Binding kinetics of C-CPEs to claudin-4.

Derivatives	k_a (l/Ms)	k_d (l/s)	K_D
C-CPE184	5.96×10^5	2.55×10^{-4}	429 pM
C-CPE194	7.13×10^5	3.24×10^{-4}	455 pM
C-CPE205	7.67×10^5	2.87×10^{-4}	374 pM

BV-adsorbed immunoplates, C-CPE194 and C-CPE205 bound to claudin-4-displaying BV but not mock BV or claudin-1-displaying BV (Fig. 3A). We performed SPR analysis to compare the affinities of C-CPEs to claudin-4. Claudin-4 proteins were fixed on the sensor chip, and C-CPEs were injected. Then, we measured the interaction between claudin-4 and C-CPEs. As shown in Fig. 3B and Table 3, C-CPE184, C-CPE194 and C-CPE205 had almost the same affinity to claudin-4 with K_D values of 429, 455 and 374 pM, respectively. The association and dissociation rates of C-CPE194 and C-CPE205 were also similar to those of C-CPE184. C-CPE184, C-CPE194 and C-CPE205 showed similar TJ-modulating activities in Caco-2 monolayer cells; their EC50 values were 0.49, 0.57 and 0.51 μg/ml, respectively (Fig. 4A and Table 4). We performed in situ loop assays to examine the jejunal absorption of FD-4 by C-CPEs. C-CPE194 and C-CPE205 enhanced the jejunal absorption of FD-4 similar to C-CPE184 at 0.2 mg/ml (Fig. 4B–D). Treatment with C-CPE194 or C-CPE205 at 1.0 mg/ml yielded a greater and earlier absorption of FD-4 than treatment at 0.2 mg/ml (Fig. 4B, C). We could not test 1.0 mg/ml of C-CPE184 due to its low solubility.

3.4. Jejunal and pulmonary absorption of hPTH(1-34) by co-treatment with C-CPE194

C-CPE194 enhanced the jejunal absorption of FD-4 to a similar extent as C-CPE184 and C-CPE205; C-CPE194 was also 30- and 3-fold more soluble than C-CPE184 and C-CPE205, respectively. C-CPE194 enhanced the jejunal absorption of hPTH(1-34) at 0.2 and 4.0 mg/ml (Fig. 5A). The AUC values were increased 11.0- and 18.4-fold as compared to the vehicle-treated group (Fig. 5B), and the Cmax and BA of the jejunal absorption of hPTH(1-34) were also increased by C-CPE194 (Table 5). Additionally, the pulmonary absorption of hPTH(1-34) was enhanced by C-CPE194 (AUC = 3080.0 ± 1994.3 ng·min/ml in vechicle-treated group, AUC = 13,397.7 ± 5830.1 ng·min/ml in C-CPE194 (0.8 mg/ml)-treated group) (Fig. 5C, D). The Cmax and BA of hPTH(1-34) were also increased by C-CPE194 (Table 5).

4. Discussion

Biologics are generally hydrophilic and poorly absorbed by the mucosa; therefore, many biologics are administered via injection. The development of a delivery system to allow biologics to pass across the epithelial barrier in mucosa is a pivotal issue for pharmaceutical therapy with biologics, since mucosal administration is needle-free, non-invasive, convenient and comfortable for patients [30,31]. We previously found that C-CPE184 enhanced jejunal absorption of dextran with a molecular mass of <10 kDa through its modulation of the claudin-4 barrier [21]. In the present study, we investigated the effect of a claudin-4 modulator on the mucosal absorption of a biologic, hPTH(1-34), and we found that a claudin-4 modulator is also a potent jejunal, nasal and pulmonary absorption enhancer of this biologic.

CPE is a 35-kDa polypeptide consisting of 319 amino acids [32]. The functional domain of CPE is divided into an N-terminal toxic domain and a C-terminal receptor-binding domain [33]. The receptor-binding fragments of CPE correspond to amino acids 169–319, 171–319, 184–319, 194–319 and 290–319 [20,26,33–35]. Among these fragments, only C-CPE184 and C-CPE194 have been

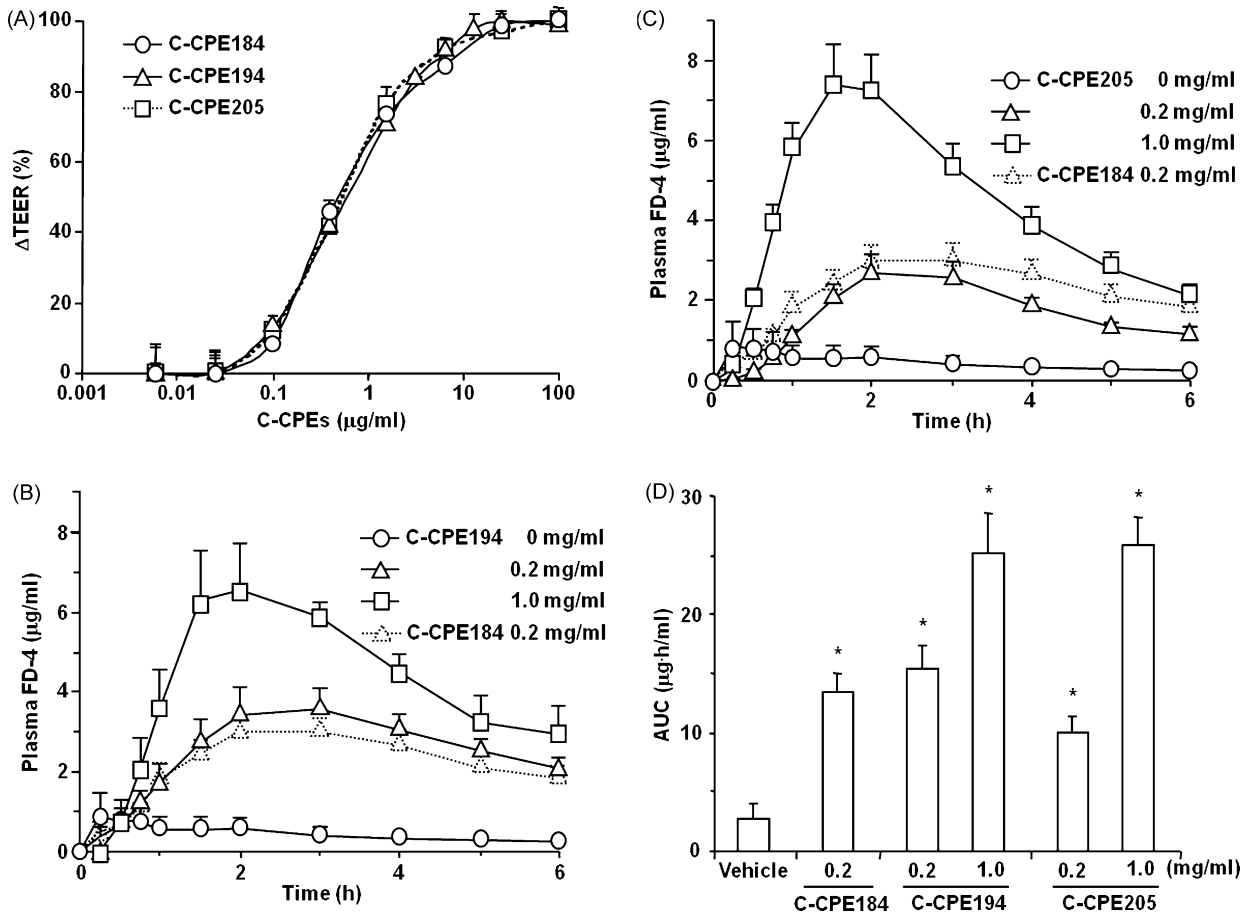

Fig. 4. Modulation of the TJ-barrier by C-CPEs. (A) Caco-2 cells were seeded on a BioCoat™. When TJ-barriers were developed and the cell sheets reached a plateau in their TEER value, C-CPEs were added to the wells from the basal side at the indicated concentration. After 18 h, the TEER was measured. The ΔTEER was calculated as the ratio to reduced TEER values at 0 and 100 μg/ml of C-CPE184 as 0% and 100%, respectively. Data are mean ± SD (n = 3). (B–D) Jejunal absorption of FD-4. Jejunum were treated with FD-4 and C-CPEs at the indicated concentration. Time-course changes in plasma FD-4 levels (B, C) and AUC from 0 to 6 h (D). Data are means ± SE (n = 4–9). *Significantly different from the vehicle-treated group ($p < 0.05$).

proven to bind to claudin-4 [10,26], and a mucosal-absorption-enhancing effect was proven only for C-CPE184 [21]. The claudin-4 modulator C-CPE184 is a 400-fold more potent jejunal absorption enhancer of dextran as compared to a clinically used absorption enhancer, sodium caprate [21]. However, the low solubility of C-CPE184 (<0.3 mg/ml in PBS) has limited its applicability. This low solubility may result in the slow onset of TJ opening due to limiting the access of C-CPE184 to claudin. Last year, Van Itallie et al. made a breakthrough by truncating the N-terminal of C-CPE184 by 10 amino acids to yield C-CPE194 [26]. They found that C-CPE194 has affinity to claudin-4 and high solubility (>10 mg/ml); moreover, they determined the 3-dimensional structure of C-CPE194, which contains nine β-sheets and one α-helix, and they suggested that the intervening surface loop spanning region 304–312 (located between the β8 and β9 sheets) may be a claudin-binding domain. Based on the structural data for C-CPE194, we prepared five N-terminal-truncated C-CPE184 derivatives: C-CPE194, C-CPE205, C-CPE212 (without the β1 sheet), C-CPE219 (without the β1 sheet and α helix), and C-CPE224 (without the β1 sheet and α helix). C-CPEs lacking the β1 sheet are soluble in PBS containing 2 M Urea but insoluble in PBS. C-CPE184, C-CPE194 and C-CPE205 have almost the same kinetics parameters for binding to claudin-4 and the same TJ-barrier modulating activity (Table 3, Fig. 4A). Thus, the β1 sheet appears to be critical for maintaining the structure of C-CPE, and the N-terminal region corresponding to amino acids 184–204 may not be involved in claudin-4 binding or TJ-barrier modulation.

Table 4
TJ-modulating activities of C-CPEs in Caco-2 cells.

Derivatives	EC50 values[a]
C-CPE184	0.49 μg/ml
C-CPE194	0.57 μg/ml
C-CPE205	0.51 μg/ml

[a] The concentration of C-CPEs at which a 50% decrease in TEER value was observed in Fig. 4A.

Biologics must escape degradation by mucosal enzymes to be absorbed by the mucosa. C-CPE184 (0.2 mg/ml) did not enhance jejunal or pulmonary absorption of hPTH(1-34). However, when hPTH(1-34) was administered 4 h after treatment with C-CPE184, jejunal, pulmonary and nasal absorption was enhanced. Thus, hPTH(1-34) may be degraded in the jejunal and pulmonary mucosa before the enhancement of its absorption by co-administered C-CPE184. Indeed, another claudin-4 modulator, C-CPE194, which is 30-fold more soluble than C-CPE184, significantly enhanced the jejunal and pulmonary absorption of hPTH(1-34). These findings indicate that modulation of claudin-4 may be a potent strategy for mucosal-absorption enhancement of biologics.

Meanwhile, a critical issue in the clinical application of the claudin-4 modulator as a mucosal-absorption enhancer is its safety. Problems with the safety of a claudin-4 modulator include the safety of a claudin-4 modulator in itself and the safety of the modulation of claudin-4, i.e., entry of unwanted substances by the

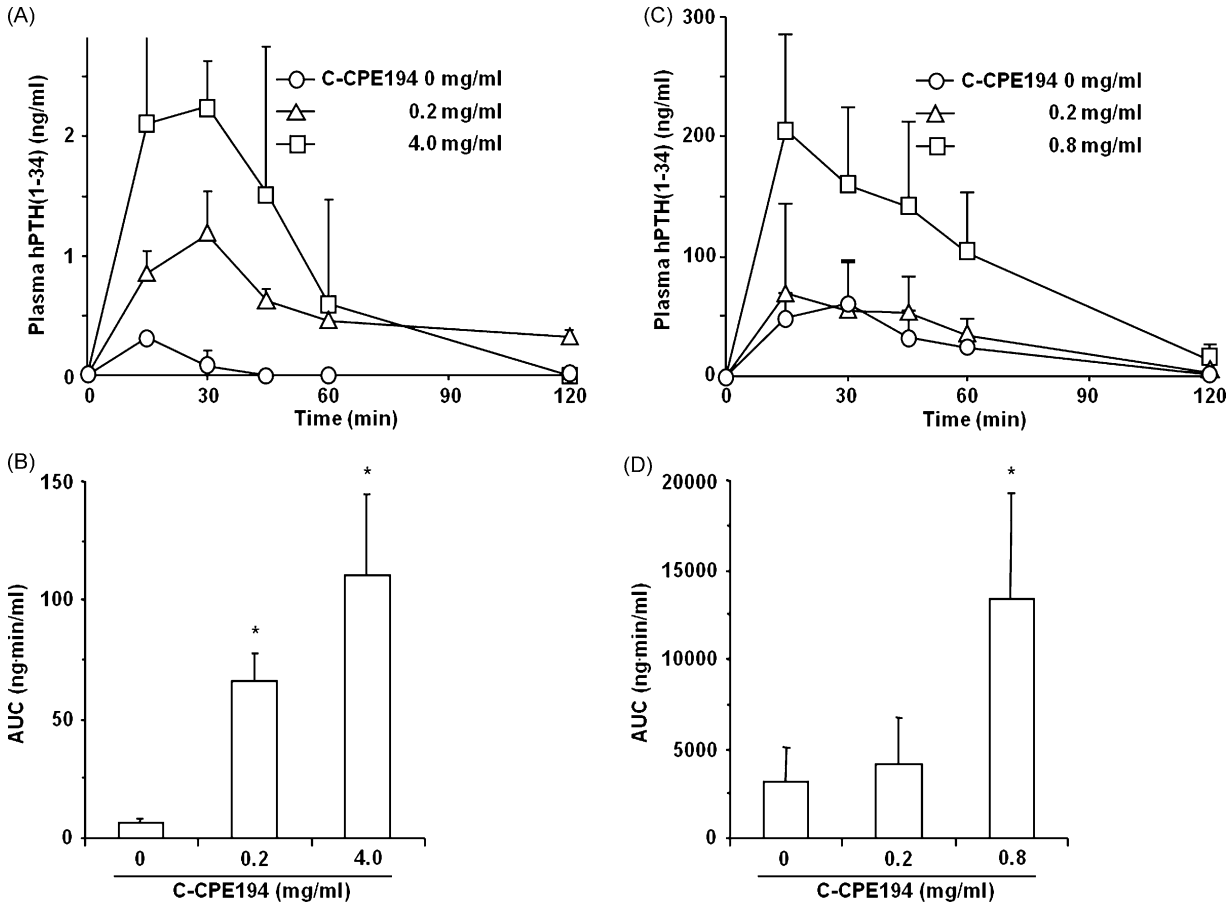

Fig. 5. Mucosal absorption of hPTH(1-34) by a claudin-4 modulator. (A, B) Jejunal absorption of hPTH(1-34). Rat jejunum was treated with hPTH(1-34) (100 μg) and C-CPE194 at the indicated doses. Time-course changes in plasma hPTH(1-34) (A) and AUC from 0 to 120 min (B) were analyzed. (C, D) Pulmonary absorption of hPTH(1-34). hPTH(1-34) (150 μg) and C-CPE194 at the indicated doses were pulmonary administered, and time-course changes in plasma hPTH(1-34) concentration (C) and AUC from 0 to 120 min (D) were analyzed. Data are mean ± SE ($n = 3$). *Significantly different from the vehicle-treated group ($p < 0.05$).

opening of TJs. As mentioned above, C-CPEs are the only claudin-4 modulator. C-CPEs are polypeptide fragments of CPE consisting of more than 120 amino acids, and C-CPEs themselves may have antigenicity. Claudin-4 modulator significantly enhanced jejunal absorption of dextran with a molecular mass of less than 10 kDa [21], and C-CPEs (>14.2 kDa) may not be absorbed by the modulation of claudin-4. Moreover, *C. perfringens* are indigenous bacterium, and immunological tolerance may be induced. Thus, the antigenicity of C-CPE might be partly negligible. Because absorption enhancers would be used as an additive in drugs, they would be repeatedly administered. To avoid the risk of antigenicity, the development of a chemical compound-type or a 30–40 mer peptide-type of claudin modulator is needed. The determination of the 3-dimensional structure of claudin is also important for the theoretical development of promising claudin modulators. Ling et al. prepared a 12-mer peptide-type claudin-4 binder which did not modulate TJs [36], and Van Itallie et al. determined the structure of C-CPE [26]. A peptide-type claudin modulator will be developed in the near future.

The other safety issue is the possible influx of unwanted substances that could be caused by the opening of TJs. C-CPE has demonstrated no damages to mucosal epithelial tissue in rat intestine [21]. Treatment of cells with C-CPE decreased the level of intracellular claudin-4 proteins paralleled by a disruption of the TJ-barrier [10]. Claudin contains the clathrin-sorting signal in its C-terminal intracellular domain, and claudin was often internalized [37,38]. Taken together, these results indicate that C-CPEs may disrupt the TJ-barrier, allowing the movement of solutes through the paracellular route. Do claudin modulators reversibly modulate the TJ-barrier and specifically regulate the movement of solutes? Disruption of the TJ-barrier by C-CPE is reversible, and the TJ-barrier gradually recovered after the removal of C-CPE [10]. The

Table 5
Parameters of mucosal absorption of hPTH(l-34) in C-CPE 194-treated rats.

C-CPE194 (mg/ml)	Jejunum		C-CPE194 (mg/ml)	Pulmonary	
	Cmax (ng/ml)	BA (%)[a]		Cmax (ng/ml)	BA (%)
0	0.3 ± 0.0	0.1 ± 0.0	0	62.3 ± 32.8	26.5 ± 17.4
0.2	1.2 ± 0.4*	0.8 ± 0.2**	0.2	78.7 ± 66.1	34.1 ± 21.6
4.0	2.8 ± 0.2*	1.3 ± 0.3**	0.8	205.2 ± 79.4*	100.6 ± 39.3*

[a] BA (%) = (AUC/Dose)/(AUC iv/Dose iv).
Data are mean ± SE.
*$p < 0.05$, **$p < 0.01$ as compared to the vehicle-treated group.

quick recovery of TJ-barriers will need to be facilitated. One approach is the development of a quickly reversible claudin modulator. Another approach is the development of a claudin inducer for the combination of a claudin modulator and inducer. Another approach is the reduction of unwanted transport using the properties of claudins. Claudin comprises a multigene family consisting of 24 members. Claudin forms paired TJ strands by polymerization in a homomeric and heteromeric manner, and the claudin strands interact in a homotypic and heterotypic manner between adjacent cells [39,40]. TJ-barrier properties are believed to be determined by the combination and mixing ratios of claudin species [41]. Interestingly, the diversity of claudin may contribute to the regulation of specific solute movement through the paracellular route [17]. The expression profiles of claudin in mucosal epithelium exhibit heterogeneity [13–15,42]. The development of claudin modulators with solute and tissue specificity will reduce the non-specific influx of solutes caused by the modulation of TJs.

In summary, we found that claudin-4 modulator enhanced the jejunal, pulmonary and nasal absorption of a peptide drug. This report is the first to indicate that a claudin-4 modulator may be a mucosal-absorption enhancer of biologics.

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