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Comparison of mucosal absorption-enhancing activity between a claudin-3/-4 binder and a broadly specific claudin binder

Koji Matsuhisa, Masuo Kondoh *, Hidehiko Suzuki, Kiyohito Yagi

Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka, Japan

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ABSTRACT

Intercellular spaces between adjacent mucosal epithelial cells are sealed by tight junctions (TJs) that prevent the free movement of solutes across the epithelium. Claudins (CLs), a family of 27 integral membrane proteins, are essential components for TJ seals. We previously used a CL-3/-4 binder, the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), to show that CL modulation is a promising method to enhance mucosal absorption. Recently, by using a C-CPE mutant library, we developed a CL binder (m19) with broad specificity to CL-1, -2, -4, and -5. Here, we compared the mucosal absorption-enhancing activity of C-CPE and m19. Both CL binders enhanced jejunal absorption of dextran with a molecular mass of 4000 and 150,000 Da and nasal absorption of dextran with a mass of 4000 Da but not 150,000 Da in rats. Although both binders showed similar nasal absorption-enhancing activity of dextran (4000 Da), m19 exhibited a more potent jejunal absorption-enhancing effect than that of C-CPE. These findings suggest that mucosal absorption-enhancing activity may be modified by modulating CL specificity.

1. Introduction

Recent drug discovery has shifted to the development of biologics, including nucleic acids, peptides, and proteins; these biologics represent over 30% of the new drugs worldwide. Most biologics are injected into patients because of their poor permeability that stems from their hydrophilic and degradable properties. Non-invasive drug delivery to the systemic circulation remains an important challenge.

Epithelium surrounds organisms and separates interior and outer bodies. Passage through epithelium is the first step in drug absorption. Routes for solute movement across epithelium are classified into transcellular and paracellular routes. Since the 1990s, various methods have been developed to deliver drugs via the transcellular route, including the use of simple diffusion and transporter- and receptor-mediated active transport. Some of these methods have been used for chemicals; however, the transcellular approaches have mainly been used for biologics [1,2]. Disrupting the intercellular seal has been the basic strategy for drug delivery via the paracellular route. Paracellular drug delivery has been studied for over 40 years, but its application to clinical use remains limited because of damage to the mucosal membrane [3,4].

Tight junctions (TJs), localized between adjacent epithelial cells, seal the intercellular space to prevent leakage of solutes across the epithelial cell sheets. Modulation of TJ-barriers has been a popular method to enhance epithelial absorption of drugs. However, the biochemical and functional structures of TJs had not been identified before 1998. Freeze-fracture replica electron microscopy analysis had shown that TJs form a series of continuous, anastomotic, and intramembranous particle strands [5], but in 1998, the first structural and functional component of TJs, claudin (CL), was identified [6]. CL is a tetra-transmembrane protein with a molecular mass of ~23 kDa; the CL family comprises 27 members [7,8]. Interestingly, the expression profiles and barrier-functions of the various CL family members differ among tissues. For instance, CL-1-deficient mice exhibit loss of the epidermal barrier, and CL-5-deficient mice show disruption of the blood–brain barrier [9,10]. The CLs are believed to form homo- and hetero-type strands on the lateral membrane of TJs [7,11], and the combination of CLs is thought to determine the properties of the TJ seals. Extensive research on CLs has provided insights into strategies for drug delivery via the paracellular route that involve modulating CLs.

Clostridium perfringens enterotoxin (CPE), a 35-kDa polypeptide, is a food poison in humans [12]. CPE binds via its C-terminal

Abbreviations: TJ, tight junction; CL, claudin; C-CPE, C-terminal fragment of *Clostridium perfringens* enterotoxin from 194 to 319 amino acids; CPE, *Clostridium perfringens* enterotoxin; FD-4, fluorescent labeled dextran with a molecular mass of 4000 Da; FD-150, fluorescent labeled dextran with a molecular mass of 150,000 Da; FBS, fetal bovine serum; BSA, bovine serum albumin; AUC, the area under the plasma concentration curve.

* Corresponding author. Address: Laboratory of Bio-Functional Molecular Chemistry, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan. Fax: +81 6 6879 8199.

E-mail address: masuo@phs.osaka-u.ac.jp (M. Kondoh).

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region, and a receptor of CPE is identical to CL-3/-4 [13–15]. Interestingly, the C-terminal receptor binding fragment of CPE reversibly modulates TJ barriers *in vitro*. We previously found that the C-terminal fragment of CPE, which corresponds to amino acids 184–319, is 400-fold more potent than the clinically used absorption enhancer sodium caprate in terms of its jejunal absorption-enhancing effect of dextran (4000 Da) and that this absorption-enhancing activity involves an interaction between the CPE fragment and CL-4 [16]. We also showed that a CL binder corresponding to amino acids 194–319 of CPE, called here C-CPE, enhances jejunal, pulmonary, and nasal absorption of a biologically active peptide [17]. Thus, we have established proof-of-concept for CL-targeted mucosal absorption of drugs by using CPE fragments. We also examined the functional domain map of C-CPE by using site-directed mutagenesis [18,19], and developed a C-CPE library containing randomly mutated functional residues, which we used as a CL binder screening system with a baculoviral display [20]. By using this system, we identified the broadly specific claudin binder m19 from the C-CPE mutant library [21]. Here, we compared the mucosal absorption-enhancing activity of C-CPE with that of the broadly specific claudin binder m19.

2. Materials and methods

2.1. Materials

Fluorescent-labeled dextrans with molecular masses of 4000 Da (FD-4) or 150,000 Da (FD-150) were purchased from Sigma–Aldrich (St. Louis, MO). A mouse fibroblast cell line (L cells) and mouse CL-1-, CL-2-, CL-4-, or CL-5-expressing L cells (CL-1/L cells, CL-2/L cells, CL-4/L cells, CL-5/L cells) were kindly provided by Dr. S. Tsukita (Kyoto University, Kyoto, Japan). Anti-His-tag antibody was obtained from Thermo Fisher Scientific Inc. (Waltham, MA). All reagents used were of research grade.

2.2. Fluorescence-activated cell sorting

The CL/L cells were maintained in modified Eagle's medium supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 atmosphere at 37 °C. The cells were resuspended in the culture medium. Cells (5.0×10^5 cells) were incubated with C-CPEs for 1 h at 4 °C. The cells were then washed with phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) twice, and then incubated with an anti C-CPE-fused tag (histidine) antibody. The cells were incubated with fluorescein-labeled secondary antibody, and C-CPE-bound cells were detected and analyzed with a flow cytometer and appropriate software (FACSCalibur and CellQuest, Becton Dickinson, New Jersey, USA).

2.3. Preparation of C-CPE

C-CPE and the broadly specific CL binder m19 were prepared as described previously [17,21]. Briefly, pET16b plasmids encoding C-CPE or m19 were transduced into *Escherichia coli* strain BL21 (DE3), and production of the recombinant proteins was induced by the addition of isopropyl-D-thiogalactopyranoside. The harvested cells were lysed in buffer A (10 mM Tris–HCl, pH 8.0, 400 mM NaCl, 5 mM $MgCl_2$, 0.1 mM phenylmethanesulfonyl fluoride, 1 mM 2-mercaptoethanol, and 10% glycerol). The lysates were applied to a HiTrap™ Chelating HP column (GE Healthcare, Buckinghamshire, UK), and the recombinant proteins were eluted with buffer A containing imidazole. The buffer was exchanged with PBS by using a PD-10 column (GE Healthcare), and the purified protein was stored at −80 °C until use. Purification of the recombinant proteins was confirmed by means of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), followed by staining with Coomassie Brilliant Blue. Protein was quantified with a BCA protein assay kit in which BSA served as the standard (Pierce Chemical, Rockford, IL).

2.4. In situ loop assay

Jejunal absorption of FD-4 or FD-150 was evaluated by using an *in situ* loop assay as described previously [16]. The experiments were performed according to the guidelines of the ethics committee of Osaka University. After 7-week-old Wister male rats were anesthetized with thiamylal sodium, 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. A mixture of FDs (2 mg) and CL binder (200 μg) was injected into the jejunal loop. Blood was collected from the jugular vein at the indicated time points. The plasma levels of FDs were measured with a fluorescence spectrophotometer (Tristar LBP941; Berthold Technologies, Bad Wildbad, Germany). The area under the plasma concentration curve (AUC) of the FDs from 0 to 4 h was calculated by using the trapezoidal method.

2.5. Nasal absorption assay

Nasal absorption of FDs was examined in 7-week-old Wister male rats. The experiments were performed according to the guidelines of the ethics committee of Osaka University. For the nasal absorption assay, a mixture of FDs (2 mg) and CL binder (50 μg) was intranasally injected into both sides of the nasal cavity. The total injection volume did not exceed 50 μl. Blood was collected at the indicated time points, and the plasma concentration of the FDs was measured with a fluorescence spectrophotometer, as described above.

2.6. Statistical analysis

Data were analyzed by using Dunnett's multiple comparison test; statistical significance was assigned at $p < 0.05$.

3. Results

Previously, we found that m19 binds to CL-1, -2, -4, and -5-expressing cells [21]. However, because approximately half of the CL molecule is embedded in the cell membrane, it is very hard to prepare recombinant CL proteins. Therefore, we first compared the affinities of C-CPE and m19 for CLs by using a flow cytometer and found that C-CPE bound to CL-4 whereas m19 bound to CL-1, -2, -4, and -5 (Fig. 1).

To investigate jejunal absorption of FD-4 and FD-150, we performed an *in situ* loop assay. C-CPE and m19 enhanced jejunal absorption of FD-4 (AUC from 0 to 4 h = 18.33 ± 3.12 μg h/ml and 31.91 ± 2.03 μg h/ml, respectively, Fig. 2A and B). C-CPE and m19 were more potent enhancers of jejunal absorption of FD-4 than of FD-150. Although C-CPE did not enhance jejunal absorption of FD-150, m19 did increase jejunal absorption of the larger dextran (Fig. 2C and D). C-CPE and m19 also increased nasal absorption of FD-4 (Fig. 3A and B); however, the absorption-enhancing effects were much weaker than those in the jejunum (AUC from 0 to 4 h: 1.52 ± 0.23 μg h/ml and 1.73 ± 0.16 μg h/ml, respectively). Neither C-CPE nor m19 enhanced nasal absorption of FD-150 (Fig. 3C and D). Thus, the absorption-enhancing activities of C-CPE and m19 differ.

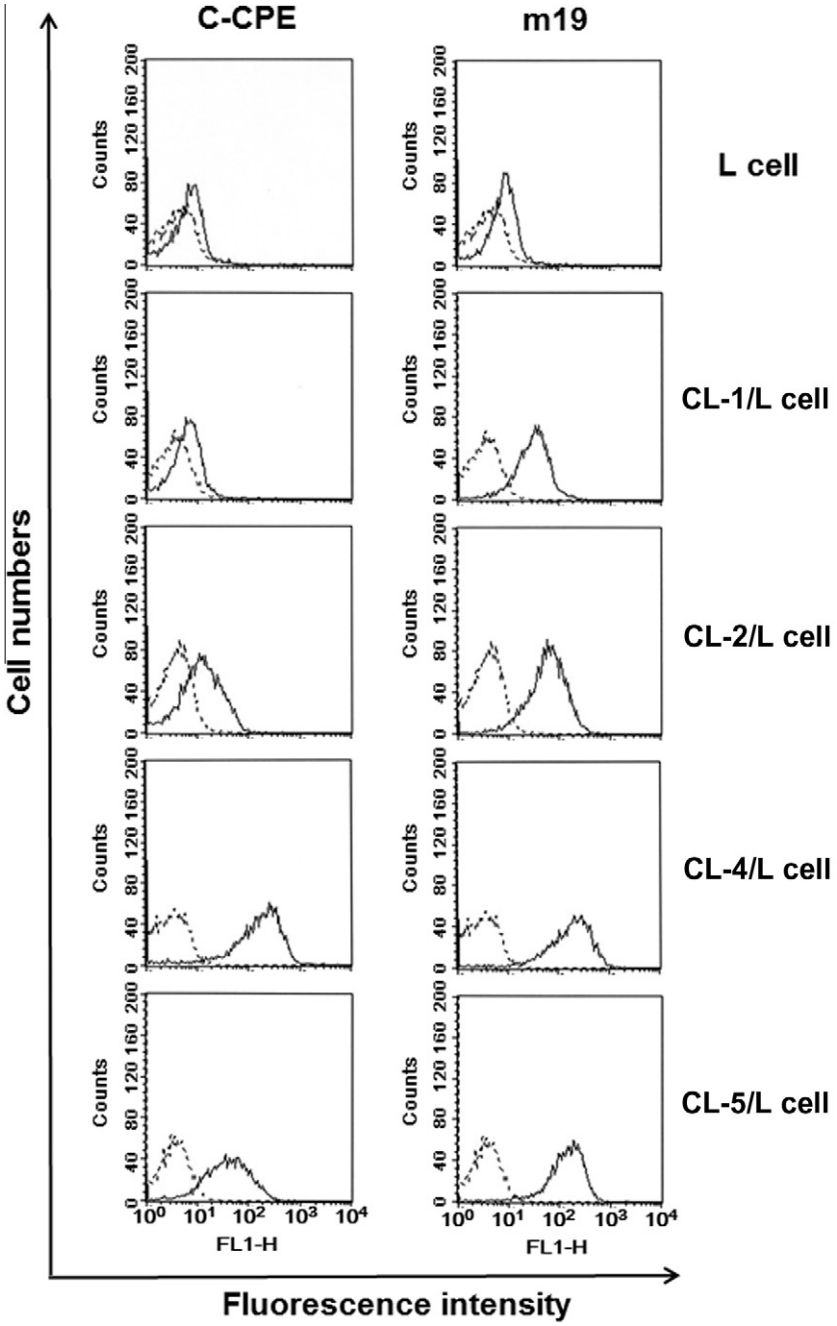

Fig. 1. Fluorescence-activated cell sorting analysis of the affinity of C-CPE and m19 for claudins. Cells were incubated with 10 μg/ml of C-CPE or m19 for 1 h at 4 °C. They were then washed twice with PBS before being incubated with a mouse anti-His-tag antibody. The cells were then incubated with fluorescent-labeled anti-mouse IgG. The cells were subjected to FACS analysis as described in the Section 2. The dotted histograms show cells treated with the anti-His-tag antibody and fluorescent-labeled IgG. The solid histograms show cells treated with C-CPE or m19, the anti-His-tag antibody, and fluorescent-labeled IgG.

4. Discussion

TJs contain several CLs, and the combination and mixing ratios of CL family members determine the barrier properties of the TJ seals [11,22]. Accordingly, a change in CL specificity may lead to a change in the absorption-enhancing properties of CL binders. Here, we compared the mucosal absorption-enhancing effects of a CL-3/-4 binder, C-CPE, with those of a CL-1/-2/-4/-5 binder, m19, and found that m19, the binder with the broader specificity, was a more potent jejunal absorption enhancer, but had similar nasal absorption-enhancing activity compared with C-CPE.

Heterogeneity of CL expression has been observed among tissues [23], including the gut, where heterogeneic CL subcellular localization has also been observed [7,24]. CL-8 is expressed in the ileum and colon, but not in the duodenum and jejunum. CL-4 expression is lower in the jejunum than in the colon. The C-terminal fragment of CPE, which corresponds to amino acids 184–319, enhances jejunal not colonic absorption of FD-4 [16]. Segment-specific expression of CLs has also been observed in kidney [7]. One possible explanation for the different mucosal absorption-enhancing effects of C-CPE and m19 in jejunal and nasal mucosa may be found in the tissue-specific heterogeneity of CL expression. CLs form homo- and hetero-type strands in TJs and the CL combination determines the properties of the TJ barriers [11,22]. CL-11 expression decreases TJ integrity in LLC-PK1 cells, but increases it in MDCK-II cells [25]. CL-4 expression, however, increases TJ integrity

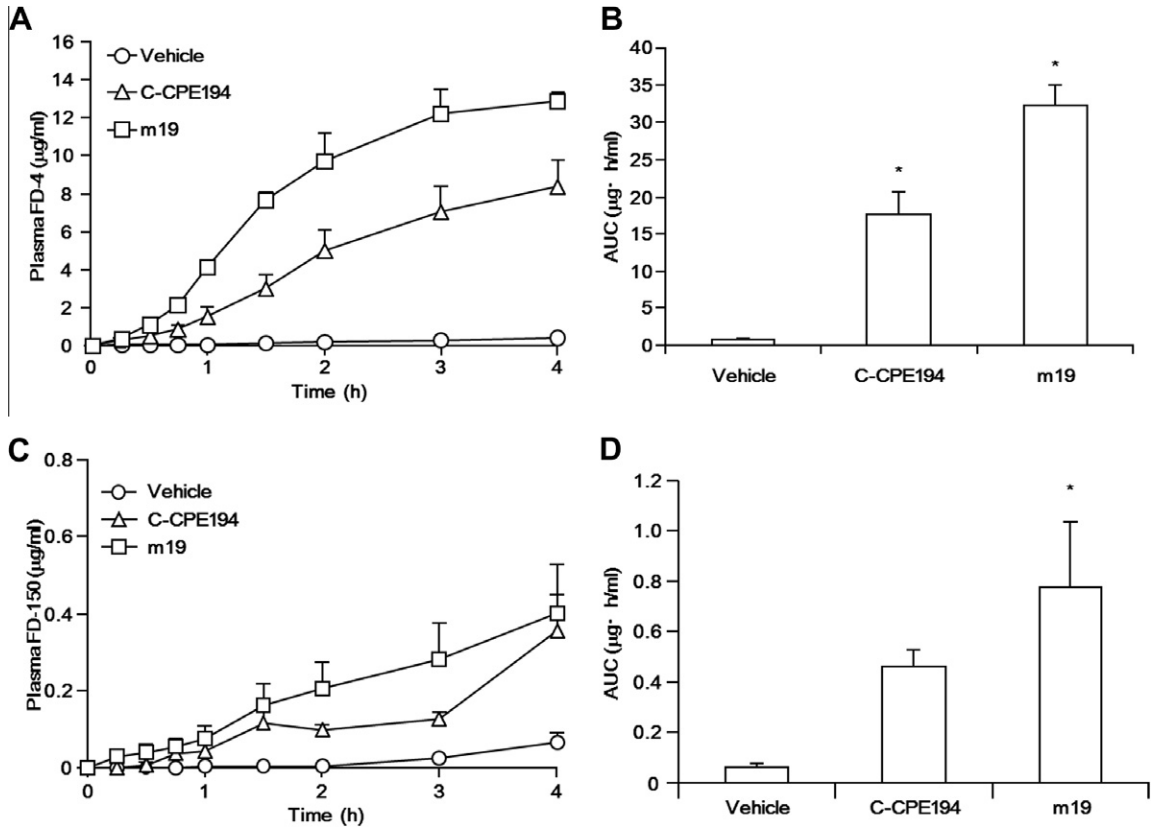

Fig. 2. Jejunal absorption-enhancing effect of C-CPE and m19. Rat jejunum was treated with a mixture of 2 mg of FD-4 (A and B) or FD-150 (C and D) and 0.2 mg of C-CPE or m19. Time-course changes of plasma FD levels were monitored (A and C), and the area under the concentration curve between 0 and 4 h (AUC) was calculated as described in the Section 2 (B and D). Data are means ± SE ($n = 4$). *Significantly different from the vehicle-treated group ($p < 0.05$).

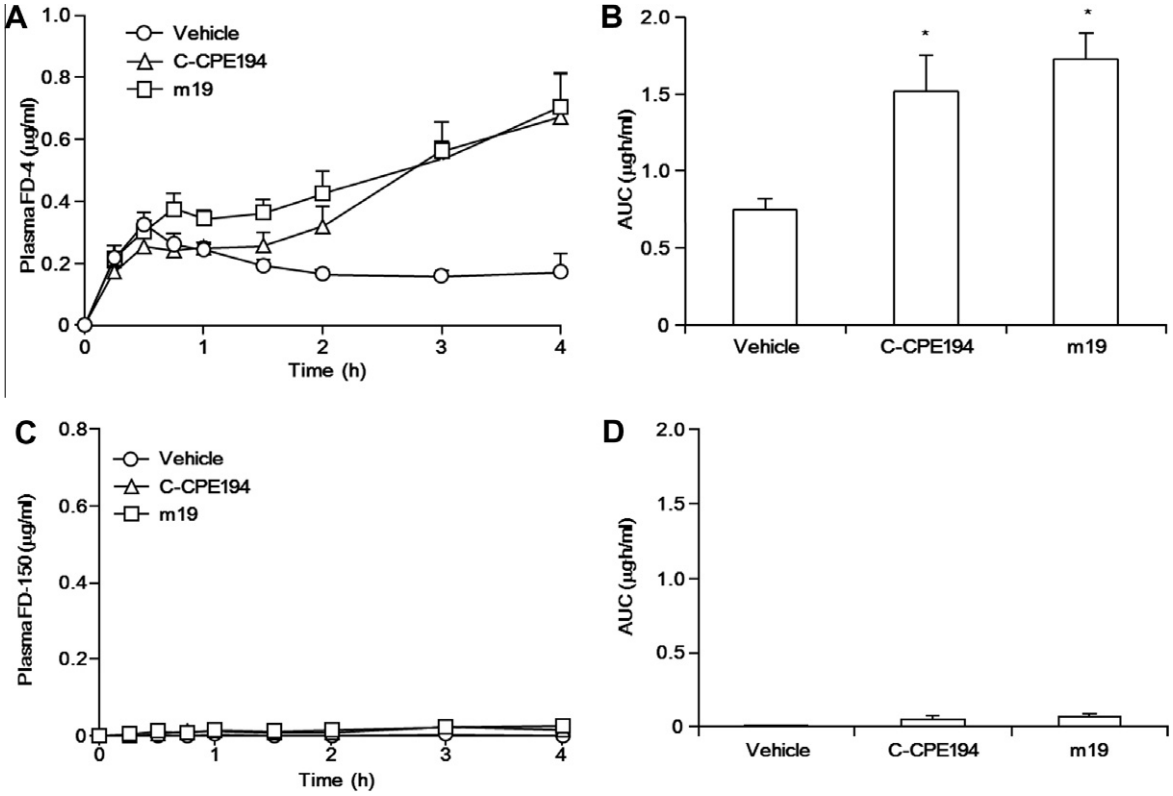

Fig. 3. Nasal absorption-enhancing effect of C-CPE and m19. A mixture of 2 mg of FD-4 (A and B) or FD-150 (C and D) and 0.05 mg of C-CPE or m19 was nasally administrated to rats. Time-course changes of plasma FD levels were monitored (A and C) and AUCs were calculated (B and D). Data are means ± SE ($n = 4$). *Significantly different from the vehicle-treated group ($p < 0.05$).

in both LLC-PK1 and MDCK-II cells. Thus, the properties of the TJ barriers, including the CL expression profiles, may differ between nasal and jejunal mucosa.

CPE binds to CL-3, -4, -6, -7, -8, and -14 [26]. Recently, Kimura et al. showed that CPE also interacts with CL-1 and -2 [27]. Although C-CPE is the receptor-binding domain of CPE, we have not assessed the interaction of C-CPE and m19 with CLs other than CL-1, -2, -4, and -5. A comprehensive understanding of the CL-specificity of C-CPE and m19 is important. However, it is difficult to prepare recombinant CL proteins, and CL-4 is the only CL recombinant protein that has been prepared to date [28]. Yet, we cannot overlook other potential differences in affinity for CLs between C-CPE and m19.

In summary, our findings indicate that mucosal absorption-enhancing activity may be modified by modulating the CL-specificity of CL binders.

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References

[1] S. Majumdar, S. Duvvuri, A.K. Mitra, Membrane transporter/receptor-targeted prodrug design: strategies for human and veterinary drug development, Adv. Drug Deliv. Rev. 56 (2004) 1437–1452.
[2] N. Mizuno, T. Niwa, Y. Yotsumoto, Y. Sugiyama, Impact of drug transporter studies on drug discovery and development, Pharmacol. Rev. 55 (2003) 425–461.
[3] M.A. Deli, Potential use of tight junction modulators to reversibly open membranous barriers and improve drug delivery, Biochim. Biophys. Acta 1788 (2009) 892–910.
[4] A. Yamamoto, T. Uchiyama, R. Nishikawa, T. Fujita, S. Muranishi, Effectiveness and toxicity screening of various absorption enhancers in the rat small intestine: effects of absorption enhancers on the intestinal absorption of phenol red and the release of protein and phospholipids from the intestinal membrane, J. Pharm. Pharmacol. 48 (1996) 1285–1289.
[5] L.A. Staehelin, Further observations on the fine structure of freeze-cleaved tight junctions, J. Cell Sci. 13 (1973) 763–786.
[6] M. Furuse, K. Fujita, T. Hiiragi, K. Fujimoto, S. Tsukita, Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin, J. Cell Biol. 141 (1998) 1539–1550.
[7] M. Furuse, S. Tsukita, Claudins in occluding junctions of humans and flies, Trends Cell Biol. 16 (2006) 181–188.
[8] K. Mineta, Y. Yamamoto, Y. Yamazaki, H. Tanaka, Y. Tada, K. Saito, A. Tamura, M. Igarashi, T. Endo, K. Takeuchi, S. Tsukita, Predicted expansion of the claudin multigene family, FEBS Lett. 585 (2011) 606–612.
[9] M. Furuse, M. Hata, K. Furuse, Y. Yoshida, A. Haratake, Y. Sugitani, T. Noda, A. Kubo, S. Tsukita, Claudin-based tight junctions are crucial for the mammalian epidermal barrier: a lesson from claudin-1-deficient mice, J. Cell Biol. 156 (2002) 1099–1111.
[10] T. Nitta, M. Hata, S. Gotoh, Y. Seo, H. Sasaki, N. Hashimoto, M. Furuse, S. Tsukita, Size-selective loosening of the blood–brain barrier in claudin-5-deficient mice, J. Cell Biol. 161 (2003) 653–660.
[11] M. Furuse, K. Furuse, H. Sasaki, S. Tsukita, Conversion of zonulae occludentes from tight to leaky strand type by introducing claudin-2 into Madin–Darby canine kidney I cells, J. Cell Biol. 153 (2001) 263–272.
[12] B.A. McClane, G. Chakrabarti, New insights into the cytotoxic mechanisms of *Clostridium perfringens* enterotoxin, Anaerobe 10 (2004) 107–114.
[13] P.C. Hanna, E.U. Wieckowski, T.A. Mietzner, B.A. McClane, Mapping of functional regions of *Clostridium perfringens* type A enterotoxin, Infect. Immun. 60 (1992) 2110–2114.
[14] J. Katahira, N. Inoue, Y. Horiguchi, M. Matsuda, N. Sugimoto, Molecular cloning and functional characterization of the receptor for *Clostridium perfringens* enterotoxin, J. Cell Biol. 136 (1997) 1239–1247.
[15] N. Sonoda, M. Furuse, H. Sasaki, S. Yonemura, J. Katahira, Y. Horiguchi, S. Tsukita, *Clostridium perfringens* enterotoxin fragment removes specific claudins from tight junction strands: evidence for direct involvement of claudins in tight junction barrier, J. Cell Biol. 147 (1999) 195–204.
[16] M. Kondoh, A. Masuyama, A. Takahashi, N. Asano, H. Mizuguchi, N. Koizumi, M. Fujii, T. Hayakawa, Y. Horiguchi, Y. Watanbe, A novel strategy for the enhancement of drug absorption using a claudin modulator, Mol. Pharmacol. 67 (2005) 749–756.
[17] H. Uchida, M. Kondoh, T. Hanada, A. Takahashi, T. Hamakubo, K. Yagi, A claudin-4 modulator enhances the mucosal absorption of a biologically active peptide, Biochem. Pharmacol. 79 (2010) 1437–1444.
[18] A. Takahashi, E. Komiya, H. Kakutani, T. Yoshida, M. Fujii, Y. Horiguchi, H. Mizuguchi, Y. Tsutsumi, S. Tsunoda, N. Koizumi, K. Isoda, K. Yagi, Y. Watanabe, M. Kondoh, Domain mapping of a claudin-4 modulator, the C-terminal region of C-terminal fragment of *Clostridium perfringens* enterotoxin, by site-directed mutagenesis, Biochem. Pharmacol. 75 (2008) 1639–1648.
[19] A. Takahashi, M. Kondoh, A. Masuyama, M. Fujii, H. Mizuguchi, Y. Horiguchi, Y. Watanabe, Role of C-terminal regions of the C-terminal fragment of *Clostridium perfringens* enterotoxin in its interaction with claudin-4, J. Control. Release 108 (2005) 56–62.
[20] H. Kakutani, A. Takahashi, M. Kondoh, Y. Saito, T. Yamaura, T. Sakihama, T. Hamakubo, K. Yagi, A novel screening system for claudin binder using baculoviral display, PLoS ONE 6 (2011) e16611.
[21] A. Takahashi, Y. Saito, M. Kondoh, K. Matsushita, S.M. Krug, H. Suzuki, H. Tsujino, X. Li, H. Aoyama, K. Matsuhisa, T. Uno, M. Fromm, T. Hamakubo, K. Yagi, Creation and biochemical analysis of a broad-specific claudin binder, Biomaterials 33 (2012) 3464–3474.
[22] M. Furuse, H. Sasaki, S. Tsukita, Manner of interaction of heterogeneous claudin species within and between tight junction strands, J. Cell Biol. 147 (1999) 891–903.
[23] K. Morita, M. Furuse, K. Fujimoto, S. Tsukita, Claudin multigene family encoding four-transmembrane domain protein components of tight junction strands, Proc. Natl. Acad. Sci. USA 96 (1999) 511–516.
[24] H. Chiba, M. Osanai, M. Murata, T. Kojima, N. Sawada, Transmembrane proteins of tight junctions, Biochim. Biophys. Acta 1778 (2008) 588–600.
[25] C.M. Van Itallie, A.S. Fanning, J.M. Anderson, Reversal of charge selectivity in cation or anion-selective epithelial lines by expression of different claudins, Am. J. Physiol. Renal Physiol. 285 (2003) F1078–1084.
[26] K. Fujita, J. Katahira, Y. Horiguchi, N. Sonoda, M. Furuse, S. Tsukita, *Clostridium perfringens* enterotoxin binds to the second extracellular loop of claudin-3, a tight junction integral membrane protein, FEBS Lett. 476 (2000) 258–261.
[27] J. Kimura, H. Abe, S. Kamitani, H. Toshima, A. Fukui, M. Miyake, Y. Kamata, Y. Sugita-Konishi, S. Yamamoto, Y. Horiguchi, *Clostridium perfringens* enterotoxin interacts with claudins via electrostatic attraction, J. Biol. Chem. 285 (2010) 401–408.
[28] L.L. Mitic, V.M. Unger, J.M. Anderson, Expression, solubilization, and biochemical characterization of the tight junction transmembrane protein claudin-4, Protein Sci. 12 (2003) 218–227.