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Progress in absorption enhancers based on tight junction

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Absorption enhancers have been investigated since the 1960s, in order to assist the transfer of drugs across the paracellular space in the intestinal epithelium. However, few absorption enhancers are presently used clinically, due to the difficulty of developing enhancers with high specificity and low toxicity. Using high-throughput genomic techniques, new drug candidates such as, non-Lipinski molecules, peptides, antibodies and nucleic acids, are being discovered, so the need for oral drug delivery strategies using absorption enhancers is gaining importance. The key to addressing this issue is to understand the molecular mechanism of the paracellular route in epithelial cell sheets. Towards this end, basic research in cell biology has revealed the components that regulate the paracellular route, and how the transport of substances is regulated. Based on these findings, novel strategies for enhancing drug absorption have been proposed. In this article, the authors first survey the development of absorption enhancers, then outline recent progress in the cell biology of tight junctions, and finally discuss novel approaches for absorption enhancers based on these advances.

Keywords: absorption, claudin, occludin, tight junction

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1. Introduction

Recent progress in genomic research has dramatically impacted drug discovery. Target proteins for diseases are easily identified from genomic information, so target-based drug design is now possible. Moreover, high-throughput technologies, such as combinatorial chemistry synthesis and high-throughput screening systems, have accelerated genomic drug discovery. So far, not only 'Lipinski' and 'non-Lipinski' chemical compounds, but also peptides, antibodies and nucleic acids and their derivatives, are promising candidates as pharmaceutical agents. Antibodies, nucleic acids and nucleic acid derivatives may exhibit high potency in personalised medicine since they can readily recognise individual differences in proteins and genes. However, non-Lipinski compounds are poorly absorbed when orally administered, as they are often hydrophilic and/or biodegradable. Indeed, high-throughput screening using Caco-2 cells is conducted in order to predict intestinal absorption, but non-Lipinski compounds often precipitate during screening. The development of drug delivery systems has, therefore, been unable to keep up with the production of lead compounds with pharmaceutical activity. At present, the development of oral drug delivery systems applicable for the high-throughput production of drug candidates is a pivotal issue in the pharmaceutical sciences.

Oral routes are preferred for the administration of therapeutic compounds because needle-borne infections and the pain associated with injections are avoided. The intestinal epithelium has the largest surface area (> 200 m^2) in the body for the absorption of drugs. The plasma membrane of epithelial cells acts as a major barrier between the inside and the outside of the body, and the free movement of substances

from the lumen to the bloodstream is restricted by the epithelial cell sheets. In most cases, orally administered drugs are absorbed across the epithelial cell sheets in the intestine, followed by movement of drugs from the lumen to the systemic fluid. However, the intestinal absorption of drugs is limited by their physicochemical properties. There are three transepithelial pathways for passing molecules from the intestinal lumen to the bloodstream: transcellular carrier-mediated transport, transcellular passive transport and paracellular transport. The uptake of hydrophobic molecules usually occurs by passive transport, as the molecules enter the epithelial cells by simple diffusion through the apical cell membrane. In contrast, hydrophilic molecules are almost impermeable to the epithelial cell sheets. Some hydrophilic molecules, including sugars and amino acids, are absorbed by active transport systems, such as transporters and receptors on the cell membrane [1-3]. In physiological conditions, selective transport systems in the intestine are critical for preventing infection and inflammation caused by foreign substances in the intestinal tract. Therefore, paracellular routes in the intestine are strictly regulated and usually prevent the influx of foreign substances [3].

As mentioned above, the development of an efficient system for delivering compounds across intestinal epithelial cell sheets is vital for the oral delivery of drugs produced by genomic drug discovery. Consequently, many approaches have been investigated. These approaches can be classified into two major categories: the active transcellular route and the paracellular route (Figure 1). Epithelial cells express various membrane nutrient transporters and receptors for the intestinal absorption of nutrients, allowing ligand-mimetic compounds to also be absorbed. For example, an approach using vitamin B_{12} receptors has been reported [4-6], where the administration of vitamin B_{12}-conjugated peptides lead to enhanced absorption of peptides [4]. Additionally, approaches using transporters have been widely investigated because various transporters are also expressed in the brush-border membrane of intestinal epithelial cells and are involved in the efficient absorption of nutrients or endogenous compounds. For example, PepT1 is a transporter of dipeptides and tripeptides, and the expression level of PepT1 in the small intestine is the major factor determining the intestinal absorption of peptidomimetic compounds, such as β-lactam antibiotics, angiotensin-converting enzyme inhibitors, and the dipeptide-like anticancer drug bestatin [7-12]. PepT2, another peptide transporter, is responsible for the renal reabsorption of small peptides, as well as β-lactam antibiotics and other peptidomimetic drugs [13,14]. Other influx transporters expressed in the intestine, such as ASBT, OATP-B, OATP-D, OATP-E and OATP-3, help improve the intestinal absorption of drugs [15-20]. However, transporters that support efflux into the lumen exist in the intestinal membrane, and the excretion of their substrates decreases net absorption in the intestine [21-24]. The inhibition of intestinal efflux transporters leads to improved oral bioavailability of a coadministered drug [25], and the combined use of influx transporters and the inhibition of efflux transporters can effectively control the transcellular intestinal absorption of drugs. As the recognition of compounds by receptors and transporters is critical for oral delivery via the transcellular route, the structure–activity relationship of compounds must be considered. Moreover, the release of drugs on the basal side in the intestine needs to be taken into account.

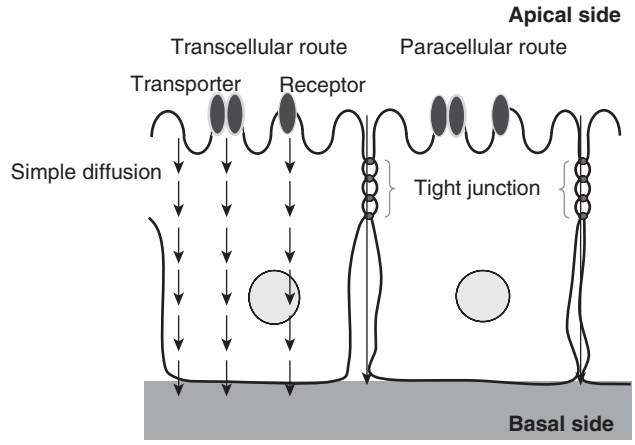

Figure 1. Scheme showing transport routes within epithelia.

Another route for oral drug delivery is the paracellular route. Adjacent epithelial cells tightly contact each other in the intestine to form epithelial cell sheets; these sheets prevent the free movement of substances between the apical and basal side of the intestinal epithelium. A key structure for regulating the intercellular transport of substances is tight junctions (TJs). As TJs seal the intercellular space, the delivery of drugs via the paracellular route must modulate the barrier function of TJs, allowing drugs to move through open pores in TJs. Open TJs may allow the movement of many types of drugs, without the need for chemical modification of the compounds. Therefore, an absorption enhancer that opens TJs may be useful for genomic high-throughput drug discovery. Indeed, there have been attempts to develop TJ openers as absorption enhancers. These attempts can be roughly classified as calcium chelators and surfactants. However, both these approaches cause unacceptable side effects, such as exfoliation of the intestinal epithelium and irreversible disruption of TJ barrier function [26,27]; sodium caprate is presently the only intestinal absorption enhancer used for pharmaceutical therapy in Japan and Sweden. Clearly, strategies for drug delivery using the paracellular route require further development.

In this review, the authors describe the background to the cell biology of TJ and the history of absorption enhancers. Based on these discussions, the authors raise issues that need to be addressed in the development of absorption enhancers, and provide a perspective of the future of absorption enhancers.

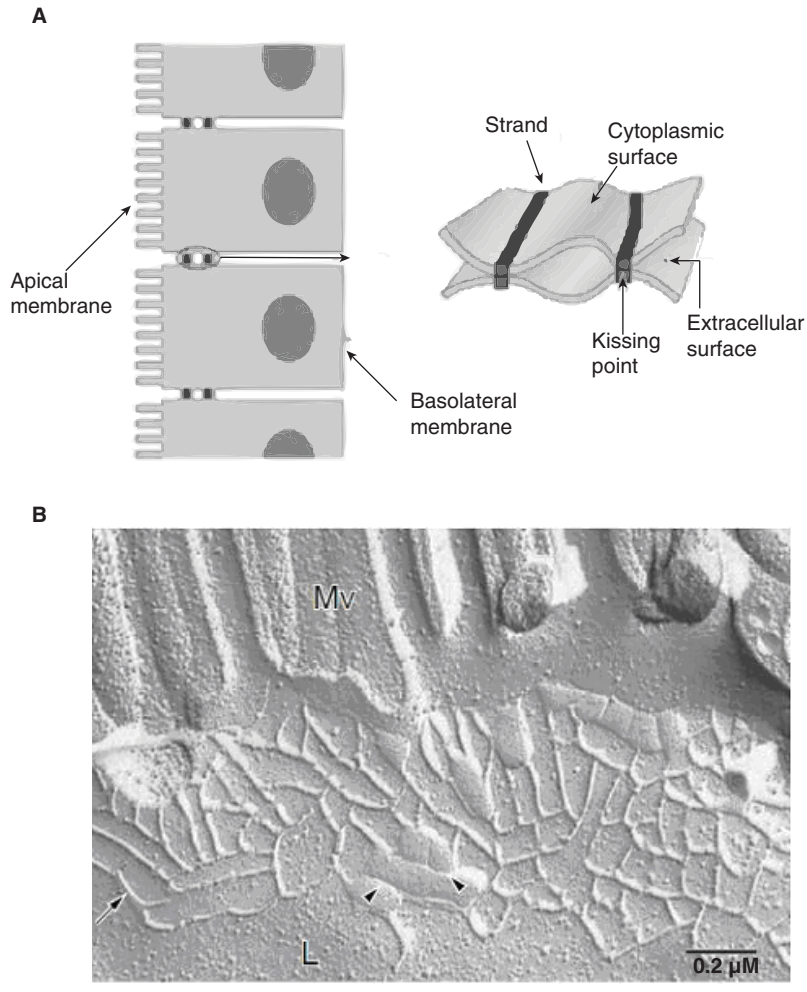

Figure 2. Structure of tight junctions. A. Schematic drawing. Tight junctions (circled) are located at the apical part of lateral membranes. TJs comprise an elaborate network of paired strands, which form the so-called 'kissing points' that eliminate the extracellular space. TJ strands also function as a fence between apical and basolateral membrane domains. **B.** A freeze-fracture replica image of glutaraldehyde-fixed mouse intestinal epithelial cells. Between apical microvilli (Mv) and the lateral membrane (L), TJs appear as a set of continuous, anastomosing strands in the P-face (arrows), with complementary grooves in the E-face (arrowheads).
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TJ: Tight junction.

2. Tight junctions

Over 40 years ago, Farquhar and Palade discovered the junctional complex between adjacent epithelial cells [28]. This complex is located in the apical compartment of the lateral membrane and consists of three components: TJs, adherens junctions and desmosomes [28]. Adjacent cell membranes are 15 – 20 nm apart at adherens junctions and desmosomes, but are in contact at TJs. The intercellular spaces, which are completely absent at TJs, are called 'kissing points'. In simple epithelial cell sheets, adherens junctions and desmosomes play a role in the mechanical linkage between adjacent cells, whereas TJs are critical for intercellular sealing [29,30]. Thus, understanding the barrier function of TJs is necessary for developing absorption enhancers for drugs delivered via the paracellular route. For some time, the biochemical and functional properties of TJs were unclear because they are located in the lateral membrane between adjacent cells. However, from 1993, details of the molecular components of TJ cell – cell adhesion were determined by Tsukita and his colleagues. In this section, the authors review the present understanding of TJs.

Freeze-fracture replica electron microscopy analysis has revealed that TJs appear as a series of continuous, anastomotic and intramembranous particle strands or fibrils (Figure 2), but what precisely TJs are has long been a topic of discussion. Two models have been proposed: the protein model and the lipid model. In the protein model, TJ strands are composed of

integral membrane proteins polymerising linearly within the lipid layer of the cell membrane, whereas in the lipid model, inverted cylindrical micelles constitute TJ strands [31].

In 1993, Tsukita and colleagues discovered the first component of TJ strands, occludin [32]. Occludin is a transmembrane protein with four transmembrane domains and a molecular mass of ~ 60 kDa. Immuno-replica electron microscopy analysis has revealed that occludin is incorporated into TJ strands. However, formation of TJ strands without occludin has been observed in endothelial cells of non-neuronal tissue and in Sertoli cells [33,34]. Moreover, it has been demonstrated that TJ strands form despite genetic disruption of occludin [35]. Thus, data on occludin suggests that another molecule is involved in the formation of TJ strands.

In 1998, Furuse *et al.* identified two novel components of TJ strands, claudin-1 and -2 [36]. These proteins, each with a molecular mass of ~ 23 kDa and consisting of four transmembrane domains, were localised in TJ strands [37-39], and the exogenous expression of claudin led to formation of TJ strands in claudin-absent fibroblast cells [40,41]. Thus, claudin is critical for the formation of TJ strands. Claudin is a family of > 24 members; interestingly, the expression profiles and barrier function of each member varies with tissue type [42-45]. For example, claudin-1 and -5 are widely expressed in a variety of tissues, and the expression of claudin-6 is observed before birth [36,46]. Gene-knockout analyses have revealed the biological function of claudin. Claudin-1- and claudin-5-deficient mice lose the epidermal barrier and blood–brain barrier, respectively [47,48], whereas the barrier function boundary between the blood and testis was eliminated in claudin-11 knockout mice [38,49]. Thus, each claudin family member is responsible for a barrier function of TJs. Most cell types express more than two members of the claudin family, and each TJ strand contains several types of claudin. Each claudin strand between adjacent cell membranes pairs in a heterotypic and homotypic manner [43,50,51]. Therefore, it has been proposed that the complexity of TJ strands is controlled by the combination and mixing ratio of > 24 members of the claudin family. Indeed, the exogenous expression of claudin-11 or -15 has been shown to result in an elevated barrier function of TJs in MDCK II cells, but not in LLC-PK1 cells [52,53]. It has also been demonstrated that claudin-3 forms paired strands with claudin-1 and -2, but claudin-1 does not form paired strands with claudin-2 [50]. Considering the complexity of intra-tissue relationships in an organism, it is reasonable that the properties of the TJ barrier would be determined by a combination of claudin family members.

The occludin and claudin results mentioned above are relevant to bicellular junctions between adjacent cells. Tricellular points exist in epithelial cell sheets at the intersection between three cells and are composed of three pairs of TJ strands associated tightly and laterally [54]. Thus, the central point in a tricellular junction is a pore without TJ strands, and sealing this central point is essential for the barrier function and fence function of the epithelium. In 2005, Ikenouchi *et al.* identified a key molecule, tricellulin, for barrier function at tricellular contacts of epithelial cells [55]. Tricellulin is a tetra-transmembrane protein with a molecular weight of ~ 64 kDa, and is localised at tricellular junctions. The C-terminal region in tricellulin was similar to the C-terminal region in occludin. Knockdown of tricellulin results in dysfunction of epithelial barrier function, accompanied by abnormal organisation of TJs.

As reviewed in this section, our knowledge of the molecular architecture of TJs has accumulated. Presently, the main focus of research in TJs is to understand the regulatory mechanisms of the barrier function of occludin, claudin, and tricellulin. Recently, Umeda *et al.* found that the major PDZ-domain-containing cytoplasmic proteins in TJs, ZO-1 and -2, control the polymerisation of claudins in TJ strands [56]. ZO-1 and -2 comprise the plaque structures of TJs together with cingulin, the Par-3/Par-6/aPKC complex, ZONAB, and GEF-H1/Lfc [56,57] (Figure 3). Further research clarifying the regulation of TJ barrier function by these proteins is underway.

3. The history of absorption enhancers and tight junctions

Surfactants and chelators were used to enhance the absorption of drugs in the 1960s [58,59]; since then, bile salts and fatty acids have also been found to enhance the absorption of drugs [26,60]. Research into the mechanisms behind these absorption enhancers reveals that some enhance the absorption of drugs through the paracellular route. Depletion of extracellular calcium by ethylenediaminetetraacetic acid (EDTA) activates protein kinase C and modulates junctional integrity, resulting in expansion of the paracellular route [27,61]. Sodium caprate is the absorption enhancer presently used in clinical therapy. The proposed model by which sodium caprate enhances the absorption of drugs is that phospholipase C activation-induced contraction of calmodulin-dependent actin-myosin filaments opens TJs [60,62]. A nitric oxide donor, N-ethyl-2-(1-ethyl-hydroxy-2-nitroso-hydrazino)-ethanamine, increases the permeability of drugs via a paracellular route [63]. Deoxycholate and glycocholate are believed to enhance intestinal permeation via the paracellular route [64-67]. Palmitoylcarnitine enhances intestinal absorption with a change in distribution of the TJ protein [68,69]. Several mucoadhesive polymers, such as chitosan, its derivatives and polyacrylic acid derivatives, have been used as absorption-enhancers [60,70-75]. Chitosan derivatives induce a redistribution of F-actin and ZO-1, resulting in an opening of TJs [75,76]. Although these agents enhance drug absorption, most cause unacceptable side effects, such as the irreversible opening of paracellular routes and cell membrane damage. In particular, the concomitant influx of toxic substances with the drug through open TJs cannot occur if this approach is to be clinically useful. What do these endeavours to develop absorption enhancers

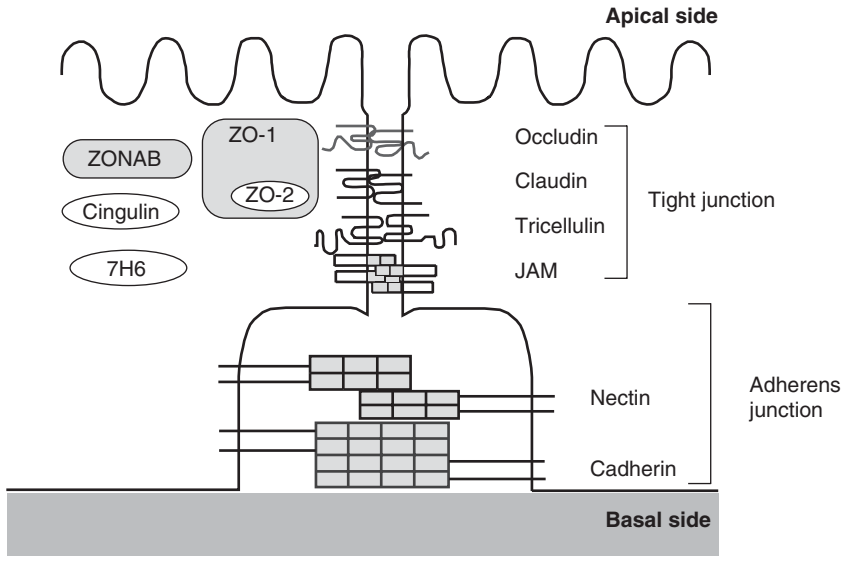

Figure 3. The composition of tight junction and adherens junction.

signify? Some researchers believe that the prospect for drug delivery via the paracellular route is very poor, and others think that attention should be focused on the transcellular route for the oral administration of absorption-enhancement drugs.

As mentioned above, absorption enhancers were developed before 1998, followed by a gradual increased understanding of the paracellular route/TJ mechanism. When these enhancers were developed, the components of TJs, a key architecture in the paracellular route, had not yet been identified (Tables 1 and 2). Thus, these absorption enhancers were developed independently of TJ-components, and can, therefore, be considered as first-generation enhancers.

4. Tight junction component-based absorption enhancers

As cell biology of TJ progressed, TJ-components-based approaches for drug delivery through the paracellular route were gradually developed. In this section, the authors review the development of novel second-generation enhancers.

The first target molecule for molecular-based oral drug delivery was occludin. Occludin has four-transmembrane domains and two extracellular loops. Occludin seals TJs through interactions between extracellular occludin loops in the lateral membranes of adjacent cells on lateral membrane [30,32]. The putative approach for modulating TJs using occludin is the downregulation of occludin protein levels and the prevention of interactions between adjacent occludin molecules. Indeed, Wong and Gumbiner showed that a synthetic peptide corresponding to the putative extracellular domain of occludin modulated TJ permeability [77]. A correlation between the perturbation of TJ permeability and a decrease in occludin levels was observed, while other components of TJs, such as ZO-1, ZO-2 and cingulin, were not affected when cells were treated with the occludin peptide [77]. Lancaz-Vieira *et al.* found that 9 or 10 amino acids, homologous to segments of the extracellular loop of occludin, impaired junction resealing [78]. Tavelin *et al.* showed that another short (14 amino acids) peptide corresponding to the extracellular loop region of occludin could be a prototype of a new class of TJ modulators [79]. Although the molecular mechanisms of these occludin peptides have never been fully clarified, the turnover of occludin, but not its synthesis, was affected by treating cells with the occludin peptide [77,79]. Taken together, the peptides may act as antagonists of the occludin extracellular loop and perturb interactions between adjacent occludin molecules in the lateral membrane; the resulting increase in occludin turnover could result in the modulation of TJs.

Claudin is believed to be a key molecule for TJ functions, such as barrier function and selective ion transport [42,80]. As with occludin, claudin is also a four-transmembrane domain protein with two extracellular domains. However, there have been no reports of the modulation of TJs using synthetic peptides corresponding to the extracellular region of claudin, although a study using an enterotoxin peptide has been reported. *Clostridium perfringens* enterotoxin (CPE) is a 35-kDa polypeptide that causes food poisoning in humans. CPE has two functional domains: an N-terminal cytotoxic domain and a C-terminal receptor-binding domain [81,82]. The receptor for CPE was identified by Katahira *et al.* in 1997 [83]. In 1999, Morita *et al.* found that claudin-4 is identical to the receptor for CPE [37]. Fujita *et al.* found that CPE interacts with claudin via the second claudin extracellular loop domain [84]. Interestingly, Sonoda *et al.* showed that treatment of cells with the C-terminal region of CPE (C-CPE) resulted

Table 1. Absorption enhancers.

Absorption enhancer	Target molecule
EDTA	Ca^{2+} (1980)
Oleic acid	Cell membrane (1980)
NO	Unknown (1998)
Sodium caprate	Phospholipase C (1980)

EDTA: Ethylenediaminetetraacetic acid; NO: Nitric oxide.
The years in brackets represent the year when each compound was first used as an absorption enhancer.

Table 2. Progress in cell biology of TJ.

Years	Events
1993	Identification of occludin
1998	Identification of claudin
1999 onwards	Clarification of TJ barrier function of claudin
2005	Identification of tricellulin

TJ: Tight junction.

in a decrease in TJ integrity, accompanied by a decrease in claudin-4 levels [85]. The authors of the present paper had previously discovered that the ability of C-CPE to enhance jejunal absorption was 400-fold more than that of sodium caprate, a clinically used absorption enhancer [86] (Figure 4). Thus, claudin is a novel target molecule for drug delivery. In particular, as CPE is an enterotoxin in humans, and as C-CPE is a fragment of CPE corresponding to the claudin-4 receptor region in the jejunum, C-CPE should be stable in the jejunum. C-CPE may, therefore, be a useful prototype for developing claudin targeting absorption enhancers.

Claudin consists of > 24 members, with each family member having different expression profiles and barrier functions [41,42,51,87]. The expression profiles and barrier functions of claudin in the gastrointestinal tract are not fully understood, but future research may provide information on claudin family members that could be used to develop effective oral drug delivery systems. To this end, RNA interference is a promising method for modulating claudin function. Indeed, knockdown of claudin by RNA interference revealed dysfunction of claudin in cells [88], so further development of knockdown of claudin in the gastrointestinal tract will likely provide new strategies for oral drug delivery.

Another unique approach has been developed by Fasano *et al*. They have investigated the application of Zonula Occludens toxin (Zot) for oral drug delivery. Zot is a toxin produced by the pathogen *Vibrio cholera*, and is a single 44.8-kDa polypeptide chain capable of altering intestinal epithelial TJ [89,90]. Zot activates protein kinase C-dependent polymerisation of actin filaments, followed by opening of TJs [91]. For example, Zot increases oral absorption of insulin [92]. A 12-kDa C-terminal Zot fragment encompassing the functional domain modulates TJs [93]. This Zot fragment significantly increases the oral absorption of ciclosporin A (acyclovir), a compound which, by itself, has low oral absorption [94]. A protein of ~ 66 kDa from intestinal cells was identified as a Zot receptor; it was suggested that this receptor is localised on the surface of the mature absorptive enterocyte at the tip of the villi and is absent from the surface of immature crypt cells [91,95].

As reviewed above, the theoretical development of methods to enhance absorption have been explored, and molecular-based methods developed in the future should focus on a new concept, namely a TJ-based strategy.

5. Expert opinion

Most biochemical components of TJs were identified a decade ago. Occludin peptide, Zot, a fragment of Zot and C-CPE decrease the integrity of TJs in intestinal cell models, and the knockdown of occludin or claudin also reduces TJ barrier function in intestinal cell models [76,79,85,93,96,97]. Intestinal absorption enhancement in animal models is observed following treatment with CPE fragment or Zot [86,92]. Thus, TJ component-based oral drug delivery systems, a novel type of absorption enhancer, are beginning to be developed.

Taking into consideration the history of research in TJ, the oral absorption enhancers can be classifed into two categories: first-generation and second-generation strategies for enhancing absorption. The borderline separating these generations is determined by whether the strategy is based on a component of the TJ barrier (Table 2). First-generation strategies for devising absorption enhancers, such as chelators and surfactants, were primarily developed in the 1960s [58,59]. These chelators and surfactants often induce severe side effects, such as exfoliation of the intestinal epithelium and the release of protein and phospholipids from the intestinal membrane [26,98]. These first-generation enhancers open TJs, allowing the influx of drugs into the systemic fluid through the mucosal epithelium. Therefore, substances besides the desired drugs can enter into the systemic fluid. The intestinal epithelium is a barrier preventing the entry of pathogenic microorganisms and xenobiotics, so the influx of toxic substances induced by the opening of TJs may cause severe side effects, such as inflammation and unexpected infectious diseases. Sodium caprate is the only first-generation enhancer used for pharmaceutical therapy in Japan, Denmark and Sweden. Some researchers believe that the limitations described above show that drug delivery through the paracellular route is not ideal, and that strategies should focus on delivery systems utilising transcellular routes. Is this true? Should we focus on transcellular routes using simple diffusion and transporter systems?

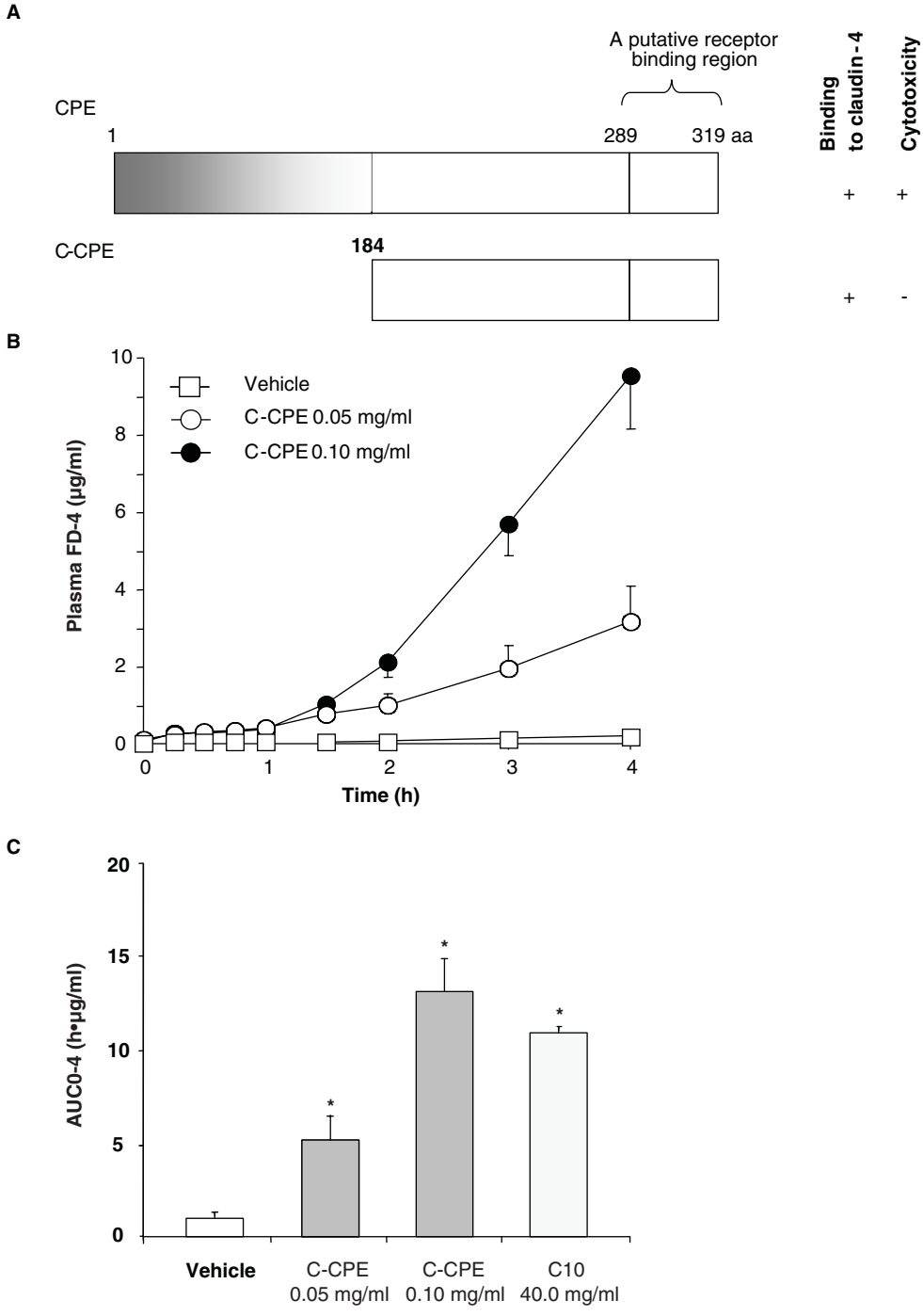

Figure 4. C-CPE is a novel absorption enhancer. A. Diagram of CPE and C-CPE structures. C-CPE is the C-terminal fragment of CPE [83]. C-CPE binds to claudin-4 and decreases TJ barrier function, as indicated by a decrease in TER [85,97]. **B.** and **C.** The effect of C-CPE on jejunal absorption in rats. Rat jejunum was treated with FD-4 (10 mg/ml) in the presence of vehicle C-CPE or C10. The FD-4 levels in plasma collected from the jugular vein were determined (B), and the AUC0-4 h was calculated (C). Data are means ± SE (n = 4).

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*Significant difference from the vehicle-treated group ($p < 0.05$).

AUCO-4: Area under the plasma concentration-time curve from 0 to 4 h; C10: Sodium caprate; C-CPE: C-terminal region of *Clostridium perfringens* enterotoxin; CPE: *Clostridium perfringens* enterotoxin; FD-4: Fluorescein isothiocyanate-dextran with a molecular weight of 4000; TJ: Tight junction.

It is important to note that basic concepts leading to the development of the first enhancers emerged before 1993, when the first component of TJs, occludin, was identified. The first functional component of the TJ barrier, claudin, was found in 1998 [32] (Table 2). Claudin is the only molecule known to form a variety of epithelial barriers with different properties, as the combination and mixing ratios of over 24 members of the claudin family determine the barrier properties of individual TJ strands [50,51]. Indeed, expression of claudin-11 increases and decreases the TJ barrier in MDCK II and LLC-PK1 cells, respectively [52]. Thus, recent progress in biology of TJ reveals that a component of the TJ determines TJ properties, and we, therefore, predict that second-generation enhancers will be based on molecules critical for TJ barrier function.

However, as the opening of TJs by such enhancers leads to the influx of drugs into the systemic fluid, it is probable that the principal side effect of the first-generation enhancers, the influx of substances other than the drug of interest, will also inevitably be a side effect with the second-generation enhancers. However, several lines of evidence from research with claudin indicate that a strategy based on this compound may circumvent the most serious side effects in drug delivery via the paracellular route. Findings using claudin-deficient mice showed that the barrier function of claudin is selective towards compounds depending on their molecular size [42,47,48]. Overexpression of claudin also revealed that claudin plays a role in selective barrier function based on the charge of the compounds [52,99-101]. Thus, at the least, size- and charge-dependent influx is expected (Figure 5A). A point that still needs to be proven regarding TJs is how the passage of substances through the paracellular route is regulated. In 1973, freeze-fracture replica electron microscope analysis revealed that TJs constitute a set of continuous intramembranous particle strands [102]. In 2003, elegant experiments performed by the Tsukita lab revealed the dynamic behavior of TJ strands [103]. This was observed using fluorescein-labelled, claudin-expressing cells in real time, and found that TJ strands were occasionally broken and annealed, and associated with each other in an end-to-side and side-to-side manner. Tsukita's group proposed a model in which local breaking and annealing of paired TJ strands enables solutes to pass across TJs, while maintaining the structural integrity of the TJs [42] (Figure 5B). This model suggests that the intestinal absorption of substances could be regulated by member-specific modulation of claudin.

Towards this end, our group has already found that a claudin modulator, C-CPE, appears to be a prototype novel enhancer that enhances jejunal absorption of dextran with molecular masses of up to 10 kDa [86]. C-CPE is a C-terminal fragment of CPE, the receptor binding region of CPE, and, therefore, the binding specificity of C-CPE to claudin family members, such as claudin-3, -4, -6, -7, -8 and -14, may be identical to that of CPE [84]. Previous reports showed that the expression profile of claudin family members differed among positions and among cell types in the gut of animal models [104,105]. Therefore, we are attempting to modulate the claudin-specificity of C-CPE and to regulate permeable substance-specificity for the application of claudin modulators to pharmaceutical therapy. To this end, we have performed functional domain mapping of C-CPE and have identified several residues responsible for the C-CPE-mediated modulation of claudin [96,106-108]. We are presently screening new claudin modulators by a phage display system, using C-CPE as a prototype [109].

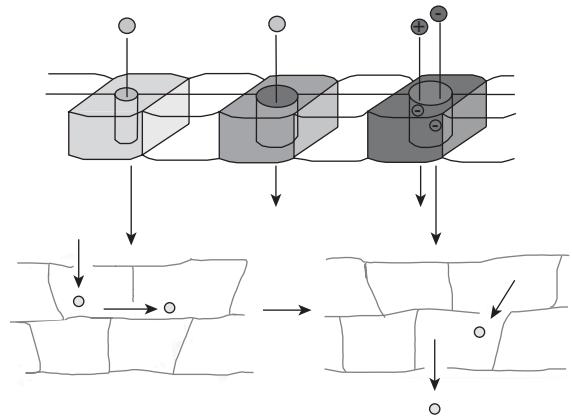

Figure 5. Proposed mechanisms for paracellular transport of solutes across TJs. A. Aqueous pores for small molecules such as inorganic ions within claudin-based TJ strands. Depending on the diameters of their aqueous pores, TJ strands can exhibit size selectivity (left and centre). When crucial sites within the pores of TJs are, for example, negatively charged owing to acidic amino acid residues in the extracellular loops of the claudins, cations can pass through the pores more easily than anions, thereby generating charge selectivity (right). **B.** The dynamic reorganisation of TJ strands. Local breaking and annealing of paired TJ strands (lines) enables solutes (circles) to pass across TJs, while maintaining the structural integrity of the TJs.
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TJ: Tight junction.

Blocking antibodies for claudin are useful for modulation of barrier-function of claudin, but all available antibodies for claudin recognise the intracellular C-terminal domain of the protein. Preparation of antibodies against claudin and antagonists of claudin is important. In addition, given the various combinations of claudin family members in TJ strands, knockdown of claudins responsible for intestinal permeability using RNA interference is also an attractive strategy.

Progress in cell biology of TJ has resulted in potent new strategies for designing absorption enhancers, and second-generation enhancers have been established. It is true that these second-generation enhancers have the potential to overcome side effects observed with the first-generation enhancers, but it is still unclear whether we can strictly regulate the paracellular transport of substances and drugs by controlling the claudin system, whether we can quickly

and reversibly modulate the paracellular route and how we should formulate drugs containing the enhancers. Therefore, it is clear that many further breakthroughs are required in order to establish pharmaceutical therapies using enhancers. We strongly believe that progress in the biology of TJs will provide these breakthroughs, and that TJ-based delivery systems will be used in pharmaceutical therapy in the future.

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