Expert Opinion

Tight junction modulator and drug delivery

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Recent progress in pharmaceutical technology based on genomic and proteomic research has provided many drug candidates, including not only chemicals but peptides, antibodies and nucleic acids. These candidates do not show pharmaceutical activity without their absorption into systemic flow and movement from the systemic flow into the target tissue. Epithelial and endothelial cell sheets play a pivotal role in the barrier between internal and external body and tissues. Tight junctions (TJs) between adjacent epithelial cells limit the movement of molecules through the intercellular space in epithelial and endothelial cell sheets. Thus, a promising strategy for drug delivery is the modulation of TJ components to allow molecules to pass through the TJ-based cellular barriers. In this review, we discuss recent progress in the development of TJ modulators and the possibility of absorption enhancers and drug-delivery systems based on TJ components.

Keywords: absorption enhancer, claudin, drug delivery, occludin, paracellular route, tight junction

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1. Introduction

Drug candidates, including chemicals, peptides, proteins, nucleic acids and their derivatives, can be efficiently identified by a combination of high-throughput technology and genome-based drug discovery. However, two steps are required for the clinical application of these drug candidates: movement of the molecules into the body and tissue through epithelial and endothelial cell sheets. These cell sheets regulate the movement of solutes between tissues within the body as well as between the outside and inside of the body.

Routes for passing of drug through the epithelial and endothelial cell sheets are classified into transcellular and paracellular routes (**Figure 1**). In the transcellular route, drugs are delivered by simple diffusion into the cell membranes and active transport via a receptor or transporter on the cell membrane [1,2]. Various transporters involved in the influx and efflux of peptides, organic anions and cations have been identified, and transcellular delivery systems using the transporters have been widely investigated [2-6]. Transporter-mediated drug delivery is tissue-specific and has low toxicity; however, the drugs must be modified for interaction with the transporter without loss of pharmaceutical activity. Thus, the transcellular route is not suitable for high-throughput production of drug candidates. The other route for drug delivery is the paracellular route. Tight junctions (TJs) seal the paracellular route and prevent the free movement of molecules in the paracellular space; therefore, a strategy for the paracellular delivery of drugs is the opening of TJs [7,8]. Compared with the transcellular route, the paracellular route has the advantages that drug modification is not needed and that one method can be applied for various drugs. Drug delivery systems through the paracellular route have been investigated as absorption enhancers since the 1980s. However, only sodium caprate is currently used as an absorption enhancer in pharmaceutical therapy.

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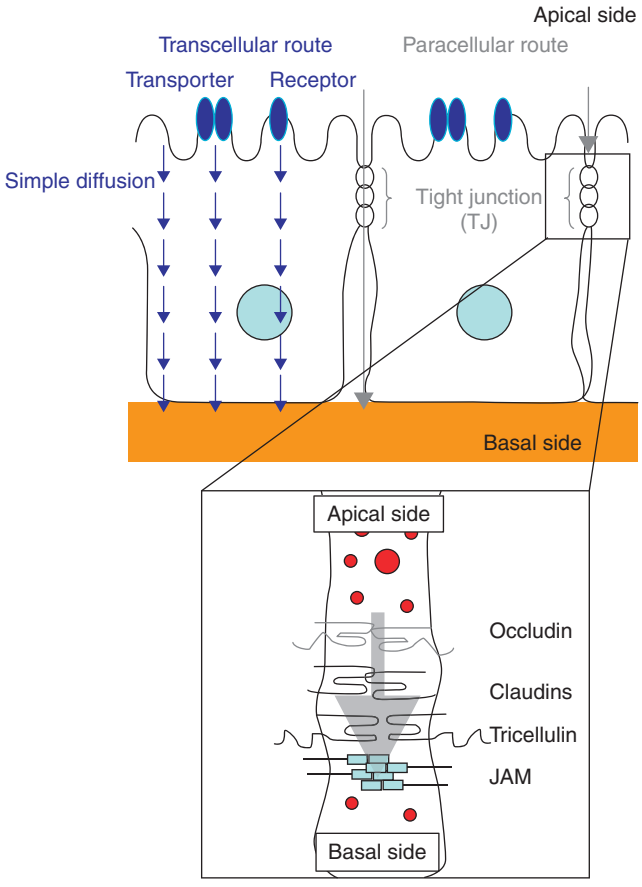

Figure 1. Schematic illustration of transport routes in epithelia.

It had been unclear how TJs regulated movement of solutes and what TJs were. In 1993, Furuse and colleagues determined that occludin, a protein with four transmembrane domains, is a component of TJs and that TJs consist of protein [9]. In 1998, Furuse and co-workers also identified another TJ protein, claudin-1 and -2 [10]. Claudins, a multigene family of at least 24 members, are key molecules of the TJ barrier [11]. Schematic biochemical machinery of TJs is shown in **Figure 1**, and modulation of the TJ components to allow drugs to pass through the paracellular route has been investigated as a novel strategy for drug delivery since the first report of TJ component-based drug delivery using an occludin peptide corresponding to part of the extracellular loop [12].

In this review, we examine recent topics in TJ-based drug delivery systems that use both approaches – TJ component/TJ modulator and TJ barrier – and discuss the future direction of such systems.

2. TJ components and TJ modulators

In the first section, we reviewed recent progress in TJ modulators over the past 2 years with respect to TJ components and modulators of TJ barrier.

2.1 Claudin

Claudin is a four-transmembrane TJ protein with a molecular mass of around 23 kDa, and comprises a family of at least 24 members [10]. Expression of each claudin member varies among cell types and tissues [13,14]. Claudins are thought to polymerize and form TJ strands in a homomeric and heteromeric manner, and the combination and mixing ratios of different claudin species determine the barrier properties of TJs, depending on the tissues [11]. For instance, deletion of claudin-1 causes dysfunction of the epidermal barrier [15], and deletion of claudin-5 causes dysfunction of the blood–brain barrier [16]. These findings indicate that a specific claudin modulator would be useful for tissue-specific drug delivery through the paracellular route. The C-terminal receptor binding region of *Clostridium perfringens* enterotoxin (C-CPE) is the only known modulator of claudin-4 [17]. Cells treated with C-CPE have decreased intracellular levels of claudin-4 as well as disrupted TJ barriers in epithelial cell sheets [17]. We previously found that the jejunal absorption-enhancing effect of C-CPE was 400-fold more potent than that of sodium caprate, the only clinically used absorption enhancer [18]. The development of other claudin modulators by using C-CPE as a prototype is a promising strategy. Deletion assays and site-directed mutagenesis assays indicate that the C-terminal 16 amino acids of C-CPE are involved in its modulation of claudin-4 and that Tyr residues at positions 306, 310 and 312 are critical for C-CPE activities [19,20]. Van Itallie and colleagues revealed that the structure of C-CPE is a nine-strand β sandwich and that the C-terminal 16–amino acid fragment is located in the loop region between the β8 and β9 strands, indicating that the claudin-4 binding site is on a large surface loop between strands β8 and β9 or on a domain containing these strands [21]. These findings indicate that peptides containing the loop structure formed by the β8 and β9 strands are likely to be novel claudin modulators. Considering the antigenicity of the claudin-4 modulator, smaller peptides are useful. Recently, the 12-mer peptide binders of claudin-4 were successfully identified using a random 12-mer peptide phage-display library [22]. The common claudin-binding motif <XX(Y/W)$(X)_{3\ or\ 4}$Y(Y/X)(L/I)XX> was also detected. The 12-mer peptide was bound to claudin with nanomolar affinity, but it did not modulate the claudin barrier. A 27-mer amino acid peptide corresponding to the extracellular loop region of claudin-1 modulated epithelial barrier through its interaction with claudin-3 [23]. Distinct species of claudins can interact within and between tight junctions [24]. Thus, a short peptide corresponding to the extracellular loop region of the heterotypically interacting claudin is also a candidate of claudin modulator.

2.2 Occludin

Occludin, a 65-kDa protein containing four transmembrane domains, was the first TJ-associated integral protein to be identified [9]. The initial strategy for TJ component-based

drug delivery was to use a synthetic peptide corresponding to the extracellular loop region of occludin *in vitro* [12]. The testes are rich in receptors for follicle-stimulating hormone. The effects of follicle-stimulating hormone-fused occludin peptide on the *in vivo* blood-testis barrier were investigated. The fusion protein modulated the blood–testis barrier, resulting in delivery of inulin into the testis [25]. Astrovirus infection causes diarrhea [26]. Moser and co-workers found that the astrovirus capsid disrupted occludin and increased the permeability of the TJ barrier without cytotoxicity in human intestinal cells [27]. A pro-inflammatory cytokine, IL-1β causes a functional opening of the intestinal TJ barrier without induction of apoptosis [28,29]. The IL-1β-induced enhancement of TJ permeability was mediated by downregulation of occludin through an increase in the myosin light chain kinase [29,30].

Thus, occludin peptides containing the ligand-targeting motifs and novel types of occludin modulators, such as the component capsid and the activator of myosin light chain kinase, may provide novel methods to deliver drugs into target tissues across endothelial cell sheets.

2.3 Ephrin

Ephrin-A2, a family of receptor tyrosine kinases, directly phosphorylates claudin-4 in epithelial cells, leading to the disruption of the epithelial barrier function [31]. Intravenous administration of ephrin-A2 ligand causes vascular permeability in the lungs, resulting in the leakage of albumin into the lungs of rats [32]. The ephrin-A2 ligand is altered in the disruption of the TJ barrier in the lungs of rats and in cultured lung vascular endothelial cells [32]. High levels of ephrin-A2 mRNA are also expressed in the intestine [33]. A modulator of the ephrin-A2 system will be a novel type of pulmonary and intestinal absorption enhancer.

2.4 Zonula occludens toxin

Zonula occludens toxin (Zot) is a 44.8-kDa envelope protein of *Vibrio cholera*, and zonulin is the intestinal Zot analogue that governs the permeability of intercellular TJs [34-36]. Zot and Zot derivatives are reversible TJ openers that enhance the delivery of drugs through the paracellular route without toxicity [35-40]. The Zots bind to a putative receptor on the apical surface of enterocytes, leading to protein kinase C-mediated polymerization of soluble G-actin and the subsequent loosening of TJs [38,41]. Zot enhanced the absorption of insulin in diabetic rats, and the bioavailability of oral insulin was sufficient to lower the serum glucose concentrations to an extent that was comparable to the parenteral injection of the hormone [35]. In 2001, an active fragment of Zot, ΔG with a molecular mass of 12 kDa, was identified [42]. In 2008, a hexapeptide derived from Zot, AT1002, was found to enhance absorption [43]. AT1002 increased permeability in human epithelial cell sheets without cytotoxicity and enhanced duodenal absorption of ciclosporin A.

2.5 Chitosan

Chitosan is derived from chitin, a polysaccharide found in the exoskeletons of insects, arachnids, and crustaceans. Chitosan is a nontoxic, biocompatible and mucoadhesive polymer that is a safe and efficient intestinal permeation enhancer for the absorption of drugs [44-46]. The chitosan-mediated activation of protein kinase Cα is followed by the redistribution of ZO-1 and an increase in TJ permeability, suggesting that the protein kinase Cα-dependent signal transduction pathway affects TJ integrity [47]. The oral administration of recently developed chitosan-coated nanoparticles containing insulin dramatically decreased blood glucose levels in diabetic rats [48].

2.6 HA, HAstV-1

Hemagglutinin (HA), a non-toxic component of the large 16S of the botulinum neurotoxin [49], and the human astrovirus serotype 1 (HAstV-1) capsid [27] may be a novel absorption enhancer via the paracellular route. The HA protein affected distribution of occludin, ZO-1, E-cadherin and β-catenin, and increased TJ permeability in human intestinal epithelial cells without cytotoxicity [49]. When HAstV-1 infected a Caco-2 cell monolayer from the apical side, the paracellular permeability was increased. UV-inactivated HAstV-1 also increased the permeability and disrupted occludin, indicating that the enhancement of the permeability was not dependent on viral replication [27]. Further analysis of the mode of action of these toxin- and virus-derived enhancers will lead to the development of novel intestinal absorption enhancers.

3. Physiological barriers modulated by TJ modulators

In the second section, we overviewed recent progress in TJ modulators with respect to the barrier separating different body compartments.

3.1 Blood–brain barrier

The blood–brain barrier, which comprises endothelial cell sheets with extremely tight junctions, limits the diffusion of hydrophilic molecules between the bloodstream and brain. Many pharmaceutical chemicals developed for the treatment of brain disorders cannot be applied in clinical therapy because they do not pass through the blood–brain barrier. Methods to open or reversibly regulate the blood–brain barrier have been investigated. Blood–brain barrier modulation based on the infection mechanisms of HIV has been proposed. Disruption of TJs occurs in the brains of HIV-infected patients [50-52], and tat protein, which is released from HIV-infected cells, decreases ZO-1 levels at the cell–cell borders in brain microvascular endothelial cells [53]. Tat treatment reduced expression of occludin, ZO-1, and ZO-2 in human brain microvascular endothelial cells via caveolin-1 and Ras signaling. Other HIV-1-derived proteins, gp120 and Nef,

Table 1. Candidates of absorption enhancer.

Target barrier	Candidates
Intestinal barrier	C-CPE
	AT1OO2
	Ephrin
	Chitosan and its derivatives
	Haemagglutinin
	HAstV-1 capsid
	Spermine
Blood-brain barrier	HIV-1 tat
	Sodium caprate
	Nitric oxide
Nasal barrier	AT1OO2
	Sperminated gelatin
	FDFWITP
Blood-testis barrier	C-type natriuretic peptide
	domain I of laminin β3

C-CPE: C-terminal of Clostridium perfringens enterotoxin; HAstv-1: Human astirovirus serotype 1; HIV: Human immunodeficiency virus.

can change the expression of TJ proteins *in vitro* [54]. Cocaine [55-56], sodium caprate [57] and nitric oxide [58] also modulate the blood–brain barrier.

3.2 Blood–testis barrier

Disruption of the blood–testis barrier affects spermatogenesis; thus, junctional proteins, such as occludin, ZO-1, and N-cadherin, could be the primary targets for testicular toxicants [59]. Monophthalates (mono-*n*-butyl phthalate and mono-2-ethylhexyl phthalate) were recently shown to disrupt the inter-Sertoli TJs in rat [60]. Phthalates are used as plasticizing and suspension agents in personal care products, plastics, paints, and pesticides. Monophthalates reduced the TJ barrier in Sertoli cells and induced the disappearance of ZO-1 and F-actin from around the cell periphery. The expression of occludin mRNA was also suppressed in a dose-dependent manner. C-type natriuretic peptide is a novel regulator of blood–testis barrier dynamics [61]. C-type natriuretic peptide regulates blood pressure, blood volume, fat metabolism, bone growth, and steroidogenesis in the testis and also reduces the expression of N-cadherin, occludin, and JAM-A [62,63]. Laminin fragments can also modulate the blood–testis barrier [64]. Treatment of primary Sertoli cells with domain I of laminin β3 caused a dose-dependent reduction in β1-integrin, occludin and ZO-1 and a decrease in the blood–testis barrier. Domain IV of laminin γ3 also reduced the expression of β1-integrin, occludin and JAM-A.

3.3 Epithelial barrier

Intranasal delivery is a convenient, reliable, rapid, and noninvasive delivery approach for low-molecular-weight compounds, and intranasal absorption enhancers have been developed for improvement of the nasal absorption of therapeutic macromolecules. AT1002, a polypeptide derived from Zot, enhanced not only intestinal absorption, but also nasal absorption of hydrophilic markers, PEG4000 and inulin [65]. Sperminated gelatin is a nasal absorption enhancer of insulin; when intranasally delivered, it decreases the plasma glucose level [66]. Aminated gelatin enhanced absorption of protein drugs through mucosal membranes with negligible mucosal damage [67].

Phage display technology is a powerful method for the selection of peptide ligands [68,69]. Recently, novel TJ modulators were isolated by using a phage-display library [70]. TJ-bound peptides were screened by using confluent monolayer cell sheets that were treated with a calcium chelator, EGTA. The polypeptide FDFWITP was isolated as a TJ binder. FDFWITP and its derivative peptides modulated TJ barriers without cytotoxicity, and these TJ-modulating activities were reversible. Thus, the phage-display system is a promising and powerful tool for developing TJ modulators.

4. Expert opinion

Many TJ-associated integral proteins, including occludin, claudin, tricellulin, ZO-1, ZO-2 and ZO-3, have been identified. These proteins play pivotal roles in the regulation of solute movement via the paracellular route, indicating that TJ modulators can be promising methods to deliver drugs. Studies of claudin-deficient mice initially indicated the possibility of TJ component-based drug delivery. Claudin-1-deficient mice lose their epidermal barrier function against a tracer with a molecular weight of around 600 Da, [15], indicating that claudin-1 modulators can enhance the transdermal absorption of drugs. The transdermal route is an easy, painless, and noninvasive method for drug administration, and the claudin-1 modulators have been the subject of pharmaceutical research. Claudin-5-deficient mice lose their blood–brain barrier [16], and small molecules (< 800 Da) selectively passed across the blood–brain barrier. The claudin-5 modulator will be a candidate for the pharmaceutical therapy of brain diseases. We found that the intestinal absorption-enhancing effects of a claudin-4 modulator were 400-fold more potent than those of a clinically used absorption enhancer [18]. Disruption of occludin or tricellulin increases TJ permeability [12,25,71]. These findings strongly indicate that modulation of TJ is a promising method for drug delivery. Because TJ proteins are poor in antigenicity, it is difficult to develop antibodies against the extracellular domain, resulting in a severe delay in the development of TJ modulators. At this point, there have been two breakthroughs in the development of TJ modulators. The first breakthrough is the determination of the structure of the only known claudin modulator, C-CPE [21]. The second breakthrough is the establishment of an efficient phage-display method to isolate a novel peptide to bind TJ components [22]. We believe that the

development of a claudin modulator by using C-CPE as a prototype will be successful, and that a peptide type of TJ modulator will be prepared in the near future. We are also optimistic about the production of a novel TJ modulator based on fragments of toxins, viruses and natural products. These fragments appear to use a novel mechanism to modulate the TJ barrier, and further analysis of this novel type of TJ modulator may lead to the next generation of TJ modulators (**Table 1**).

Very recently, Lee and colleagues proposed the lipid-protein hybrid model for TJ that the TJ proteins by themselves, and in combination with the lipids, serve, in addition, essential roles in barrier function, indicating that a lipid modulator can be a TJ modulator [72]. Glycosylated sphingosine, oxidized lipids and ether lipids were identified as TJ modulators, and the displacement of claudins and occludin from lipid raft was involved in the absorption-enhancing effect of sodium caprate [73,74]. Future investigation of the lipid-protein hybrid model for TJ may be the third breakthrough in the development of TJ modulators.

Declaration of interest

The authors state no conflict of interest and have received no payment in the preparation of this manuscript.

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