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Trends in drug delivery through tissue barriers containing tight junctions

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Abbreviations: TJ, tight junctions; ABC, ATP binding cassette; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol tetraacetic acid; ZO, zonula occludens protein; TAMP, tight junction-associated marvel protein; marvel, MAL and related proteins for vesicle trafficking and membrane link; TER, transepithelial electrical resistance; ECD, extracellular domains; C10, sodium caprate; n.a., not accessible; FD, fluorescein isothiocyanate dextran; CPE, *Clostridium perfringens* enterotoxin; cCPE, C-terminal fragment *Clostridium perfringens* enterotoxin; siRNA, small interfering ribonucleic acid

A limitation in the uptake of many drugs is the restricted permeation through tissue barriers. There are two general ways to cross barriers formed by cell layers: by transcytosis or by diffusion through the intercellular space. In the latter, tight junctions (TJs) play the decisive role in the regulation of the barrier permeability. Thus, transient modulation of TJs is a potent strategy to improve drug delivery. There have been extensive studies on surfactant-like absorption enhancers. One of the most effective enhancers found is sodium caprate. However, this modulates TJs in an unspecific fashion. A novel approach would be the specific modulation of TJ-associated marvel proteins and claudins, which are the main structural components of the TJs. Recent studies have identified synthetic peptidomimetics and RNA interference techniques to downregulate the expression of targeted TJ proteins. This review summarizes current progress and discusses the impact on TJs' barrier function.

Introduction

Tissue barriers. To guarantee specific organ function, the body must maintain compartments with a special environment and gradients for various kinds of solutes and ions. To sustain these gradients, organs have layers of cells which form barriers between the environment and the organ tissue. Most of these are epithelial cells such as the brush border cells in the intestine or the cells in the lining of the kidney tubules. Others are the endothelial cells which line blood vessels, forming the blood-brain barrier or the blood-retina barrier, for instance. Other examples are the Sertoli cells forming the blood-testis barrier or Schwann cells which electrically seal the myelin sheaths of neurons. The common function of these barrier-forming cells is to regulate the transport of water, ions, nutrients and other solutes, in order to maintain the organ-specific environment in the steady-state. In addition, noxious agents and metabolic end products are removed from the organ tissue by means of efflux transporters. However, these functions may also impair the uptake of many drugs.

Barrier-forming cells are polarized. They possess an apical region facing the outer lumen and a basolateral plasma membrane region facing the organ side. Proteins (i.e., transport proteins, channels and receptors) within the apical plasma membrane cannot move to the basolateral side and *vice versa*. The protein patterns in these membrane compartments are different and they fulfil different functions, i.e., in the directed transport of molecules. This so-called fence function is due to a protein complex, the tight junctions (TJs), which are organized in strand networks. TJ-strands span the plasma membrane of adhering cells, like a belt at the interface between the apical and basolateral sites. TJs also establish a barrier in the extracellular space between neighboring cells by interactions between their TJ-strand networks. In consequence, these cells together with their TJs form a barrier which regulates transcellular flux indirectly and paracellular flux directly. Transcellular permeability is limited by the lipid surface of the cell and the outwards or inwards directed transporter activity (i.e., ABC transporters or Glut-1).[1] In general, only small lipophilic molecules are passively absorbed across barriers by the transcellular pathway if they are not a substrate of any of the transporters. Paracellular permeability can differ greatly between the diverse tissue barriers, depending on the composition of the expressed TJ proteins and on their expression level. Thus, TJs regulate paracellular passage of small hydrophilic substances, ions and water. However, large hydrophilic substances need an active permeation process, e.g., carrier-mediated transport.[2-5]

To improve the delivery of pharmaceutical agents through tissue barriers, drug enhancing methods have been developed. The largest body of information on a drug enhancer is available for caprate, as this is the best investigated agent for this purpose and has been used in clinical studies. Therefore, the main part of this review concerns caprate, as this is the standard approach in the field.

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Tight junctions and claudins. TJ-complexes mainly consist of the transmembrane proteins occludin, tricellulin, marvelD3, as well as the claudin protein family and various scaffolding proteins, such as zonula occludens proteins 1–3 (ZO-1 to 3).[6-9] Occludin was the first transmembrane TJ-protein discovered.[10] This is specifically located in TJs and plays a regulatory role. Nonetheless, since occludin-knockout mice are viable and fail to display defective barrier function, its physiological importance is still unclear. It may well be relevant to regulation under pathological circumstances, as it has multiple phosphorylation sites.[11,12] In addition, occludin can be partially replaced by the other tight junction-associated marvel protein(TAMP) family members, tricellulin and/or marvelD3.[6] Tricellulin has recently been shown to be mainly localized at tricellular contacts.[13] Knockdown of tricellulin expression by RNA interference technology leads to impaired TJ organization, a decrease in transcellular electrical resistance (TER) and a size-selective increase in permeability.[14] Thus, tricellulin plays an important role in sealing tricellular contacts in epithelial barriers. However, a more essential TJ-protein family has been identified—the claudins.[15] The claudin family is currently thought to consist of up to ~27 members.[16] Claudins have a dominant influence on the properties of the TJs. They are expressed in a tissue-dependent combination, which results in tissue-specific barrier characteristics, since some claudins lead to a leakier barrier and others to a tighter barrier. Thus, the functional backbone of the TJ is formed by the claudins.[5]

Claudins and TAMPs consist of two extracellular domains (ECDs) and four transmembrane domains, whereas the C- and N-terminal domains are located in the cytoplasm. Many of the claudins can bind via the C-terminus to the PDZ domain of the scaffolding protein ZO-1, which links them to the actin cytoskeleton. ZO-1 and -2 are essential for the spatial organization, but not for the formation of the claudin-based TJ-strands.[17] To form these strands, the claudins can interact in different ways, either *trans* (interaction between claudins in two neighboring cells) and *cis* (established between claudins along the same membrane).[18,19] There are also homo- and heterophilic interactions between claudins and TAMPs.[20,21]

Although some claudins have very similar sequences, their expression can lead to quite different TJ characteristics. For example, claudin-2 is known to be involved in paracellular pore formation for cations, whereas claudin-5 seals the paracellular cleft for small molecules.[22,23] Therefore, detailed information is necessary about the structural differences between claudins, especially their ECDs. In this context, some studies point to the importance of both ECDs for proper tightening of the paracellular cleft,[24] ion selectivity[25-27] and the involvement in strand formation.[18,28] In summary, claudins, particularly their ECDs, are potent targets for manipulating paracellular permeability.

Substances to Modulate Tight Junctions

In general, many organs are protected by a barrier to avoid contact with unwanted or even toxic substances. Regrettably, this means that drugs are also less able to reach their destination. This is true for the blood-brain barrier, which prevents the uptake of almost all large molecules and more than 98% of small molecule drugs into the brain.[29] Consequently, only small (< 500 Da) lipophilic drugs with fewer than 8–10 hydrogen bond to solvent water are believed to cross the blood-brain barrier.[30] Most designed drugs are only poorly absorbed, due to their size or hydrophilicity. To face this problem of low bioavailability, two major strategies are being pursued in pharmacological research. One is to modify and couple drugs so that they can cross a barrier. The other strategy is to use an absorption enhancer which increases barrier leakiness.

The first strategy is implemented, for example, by employing prodrugs. These are initially inactive compounds, which are modified to raise transcellular permeability, either by increased lipophilicity or by coupling to a ligand, which allow the drug to use a transporter.[31] After uptake, those drugs are activated, e.g., by enzymatic conversion. Other compounds achieve carrier-mediated uptake after modification, e.g., nanoparticles conjugated to aminosubstituted vitamin B_{12} derivatives.[32]

Whereas the first strategy attempts to exploit transcellular uptake, the second strategy focuses on the paracellular pathway. For transient modification of the TJs, promising candidates have been described in the recent years: small molecules,[33-35] peptides[36] and small interfering ribonucleic acid (siRNA).[37] These are specifically directed against TJ molecules, especially against claudins, in contrast to indirect TJ modulation by the calcium chelators ethylenediamine tetraacetic acid/ethylene glycol tetraacetic acid (EDTA/EGTA)[38] or the clinically used hyperosmolar mannitol.[39] It has been demonstrated that hyperosmolar saline solution transiently enhances analgesic drug delivery through the perineural barrier in rats.[40]

Since the claudins make the dominant contribution to the function of the TJs, specific targeting of the claudins is an obvious and promising approach. Additionally, the tissue-specific expression of the claudins allows tissue-barrier specific modulation, which should lead to fewer side effects.

Surfactant-like agents. Various agents, such as surface active agents (surfactants), lipids and polymers, have been tested for their efficiency in improving the systemic availability of drugs.[33] So far only capric acid ($C_{10}H_{20}O_2$) has achieved clinical relevance.

Sodium caprate (C_{10}), the sodium salt of capric acid, also known as sodium decanoate, has been approved (in Japan and Sweden) as an absorption enhancer in a rectal suppository for the antibiotic ampicillin, with the trade name Doktacillin®.[41] Lindmark and colleagues showed that 50 mg/person C_{10} significantly increased ampicillin concentration in the serum of adult humans.[42] This absorption enhancement of C_{10} was also clinically demonstrated for oral applications.[42] Since capric acid is present in dairy products, e.g., milk and plant oils (i.e., coconut oil), C_{10} is accepted by the US American Food and Drug Administration for use in food (Sec. 172.863 Salts of fatty acids. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=172&showFR=1; 2013).

C_{10} is an amphiphilic substance, with a hydrophilic "head" (carboxylate ion) and a hydrophobic "tail" (aliphatic C_{10} chain) and has a critical micelle concentration of about 50 mM. At this

Table 1. Increased drug uptake by caprate in vivo

Drug	Caprate concentration	Species	Administration	Literature
berberine	100 mg/kg	rat	oral	45
berberine	50 mg/kg	rat	oral	46
oleanolic acid	100 mg/kg	rat	oral	43
cefotaxime	0.25% w/v	rat	oral	105
ropivacaine	20 mM in 10 ml saline	sheep	epidural	52
cyclosporine A	0.25% w/v	rat	oral	50
oligonucleotides	25–100 mg/kg	pig	enteral	48

concentration, C_{10} may improve the solubility of hydrophobic substances and acts like a detergent.[43] C_{10}-micelles may enhance in vivo absorption via transcellular transport.[44] On the other hand, it is well established that C_{10} widens TJs, thus serving to open the barriers.[34] Several in vivo studies have shown that this enhances drug uptake (**Table 1**).

The drug berberine is normally poorly absorbed after oral administration. Two studies have found that its bioavailability is enhanced when it is orally administered together with C_{10}. The intestinal mucosa is not damaged.[45,46] The in vitro enhancement ratios were 2.08, 1.49 and 3.49 in the rat duodenum, jejunum and ileum, respectively (C_{10}: 0.2% w/v).[46] C_{10} (100 mg/kg) also increases the permeability for nanoparticles across the intestinal epithelium of rats[47] and for chemically modified antisense oligonucleotides (C_{10}: 25, 50 and 100 mg/kg) in pigs[48] after enteral application. After intraduodenal administration, the uptake of nucleotide prodrugs into the liver was enhanced (C_{10}: 86 mg/kg).[49] Intracarotid injections of C_{10} lead to a transient, reversible and molecular weight-dependent opening of the blood-brain barrier in the rat, beginning 5 min after injection.[50,51] After epidural injection of C_{10} with the anesthetic ropivacaine, the maximal intrathecal concentration of ropivacaine was elevated.[52] This effect is transient and reversible and limited to the arachnoid barrier.

Cell culture experiments have been performed to characterize the detailed mode of action of C_{10}. These studies have mostly used cells from the colon[53,54] or kidney,[55,56] but also freshly isolated brain capillaries[55] and excised rat intestinal mucosa.[57] In these experiments TER decreased and/or the permeability of molecules with a molecular weight between 182 and 10,000 Da increased after incubation with C_{10} (**Table 2**).

The decrease in TER depends on the C_{10} concentration, the incubation time and the cell line used. 7.5–10 mM C_{10} seems to be the optimal concentration for MDCK and HT-29/B6 cells. Lower concentrations are less effective and higher concentrations do not decrease TER further. The maximum effect of C_{10} on TER is already reached within 5–30 min (50% of control value) and lasts for up to 300 min. After removal of C_{10}, the TER returns to control values if the initial C_{10} concentration is below 30 mM.[56] In Caco-2 cells, concentrations of 25 mM to 50 mM C_{10} are necessary to increase permeability.[53] In all studies, C_{10} caused much higher permeation ratios for small molecules (up to 1 kDa) than for large molecules (4 to 20 kDa).[53,54,57] This biphasic permeability corresponds to the size-dependent permeation of molecules through Caco-2 or human intestinal epithelial (T84) monolayers, as shown by Watson and colleagues. A permeation study was performed with 24 polyethylene glycols of different molecular weight (238 to 1250 Da; ~3.5 to 7.39 Å). Permeation decreased markedly between 238 and 326 Da, but remained at a constant low value with the larger oligomers. After C_{10} treatment, the permeation profile was still biphasic, but was enhanced 6-fold over the whole size range. EGTA treatment also caused increased permeability, but no size selectivity was observed.[58] However, molecules >20 kDa did not permeate after C_{10} treatment.[54]

Table 2. Increased drug uptake by caprate in vitro

Cell type	TER	Permeability (mol. weight)	Literature
Caco-2	↓	↑ mannitol (182 Da) ↑ polyethylene glycol (900 Da) ↑ decapeptide (~1 kDa)	53
HT-29/B6	↓	↑ fluorescein (330 Da) ↑ FD4, FD10 (4, 10 kDa) → FD20 (20 kDa)	54
MDCK-I	↓	↑ FD4 (4 kDa)	56
MDCK-II	n.a.	↑ lucifer yellow (457 Da)	55
excised rat intestinal mucosa		↑ sodium fluorescein (376 Da) ↑ FD4 (4 kDa)	57

Caco, human colorectal adenocarcinoma cell line; HT, human colon carcinoma cell line; MDCK, Madin-Darby canine kidney cells line; ↓ decreasing; ↑ increasing; → no change; n.a. not accessible; FD, fluorescein isothiocyanate-dextran.

Thus, C_{10} increases the permeability of cell monolayers in a biphasic, size-selective manner. This observation points toward modulation of the paracellular, rather than the transcellular pathway after C_{10} treatment.[54]

The mode of action of C_{10} was illustrated by Maher et al.[44] In their model, C_{10} at concentrations up to 50 mM induces contraction of the actomyosin perijunctional ring, dilating the paracellular space.[34] This effect is mediated by an increase in the intracellular Ca^{2+} level, caused by phospholipase C activation and the subsequent cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG).[59] Intracellular Ca^{2+} forms complexes with calmodulin (CaM) in response to IP3, which in turn activate CaM-dependent protein kinases and the myosin light chain kinase.[60] Phosphorylation of the regulatory light chain of myosin

II induces the contraction of the actomyosin perijunctional ring.[61] Contraction of the actin myosin ring complex is also induced by another signaling pathway, mediated by DAG and protein kinase C.[62] The TJ complex linked by ZO-1 to the actin cytoskeleton is then redistributed from the TJs to the cytoplasm.[34,60,62-64]

However, recent reports have shown that this model cannot be completely true as a general mode of action. In HT-29/B6 cells, for example, no morphological change in the perijunctional actomyosin ring could be observed after incubation with 10 mM C_{10}.[54] Furthermore, the effect of C_{10} could not be blocked with inhibitors of the actin-myosin interaction, phospholipase C, Rho-kinase or by Ca^{2+} chelators.[54] The redistribution of the TJ-complex is under discussion as well, since several studies have reported that the membrane localization of ZO-1 is unchanged (30 mM C_{10};[65] 7.5 mM C_{10}[55]). However, claudin-1, claudin-4, junctional adhesion molecule and β-catenin in human airway epithelial cells and claudin-5 in brain endothelial cells were reduced after C_{10} treatment.[55,65] In MDCK cells, claudins-4, -5 and occludin were displaced from lipid rafts to more fluid membrane domains, whereas claudins-1, -2 and -3 were unchanged after C_{10} treatment.[56] In this context, it should be noted that another group found that both occludin and claudin-1 were partially removed from TJs after C_{10} treatment in skin epithelial cells.[66] Slightly different observations have been made with HT-29/B6 cells. While no changes could be observed in claudins-1 to -5, -8, occludin and tricellulin in the total lysate or membrane fraction, there was a selective and reversible decrease in tricellulin and claudin-5 at tricellular and bicellular TJs, respectively.[54] The reported differences in the effect of C_{10} on TJ-protein localization could be related to the kind of cell line and their diverse TJ-protein expression patterns. However, claudins and occludin are connected via ZO-1 to the actin cytoskeleton and no changes in ZO-1 localization upon C_{10} treatment were observed in HEK-293 (human embryonic kidney 293), MDCK, bEND5 (mouse brain endothelioma) and mouse brain capillaries.[55] The conclusion from these findings would be that there is more specific modulation of TJ-proteins, rather than just the modulation of the actin-myosin ring complex alone. Interestingly, the decreases in TER and claudin-5 in the membrane are reached within 5 min, suggesting that these events are directly linked.[55,56] Since claudin-5 tightens the TJ against molecules with a molecular weight less than 800 Da, this may explain why low molecular weight molecules permeate much more rapidly than higher molecular weight proteins.[23]

But how is the permeability of macromolecules enhanced by C_{10}? This issue was also addressed by Watson and colleagues, who showed that the unspecific absorption enhancer EGTA increased the permeability of 10 kDa fluorescein isothiocyanate dextran (FD10) 21-fold, whereas C_{10} only results in a 1.9-fold increase.[58] This might be explained if C_{10} acts on tricellulin. In this scenario, the decrease in tricellulin allows macromolecules up to a certain size (10 kDa) to pass via the paracellular pathway, since it is known that tricellulin acts as a barrier to macromolecules.[54,67] As tricellular contacts are rarer than bicellular contacts and the possibilities of macromolecular permeation are correspondingly rare, this could explain why low molecular weight molecules are more permeable. However, the exact mechanism behind the modulation of specific membrane proteins is still unclear.

If C_{10} is to be used as a drug enhancer, its cytotoxic effects must be studied. 50 mM C_{10} is at least harmful in Caco-2 cells.[53] Caco-2 cell cultures are apparently more sensitive to the cytotoxic effects of C_{10} than the intestine in vivo, since there is no evidences of mucosal irritation or damage after exposure to twice the concentration used in the cell culture experiments.[53] Perhaps this could be explained by the dilution effect in vivo, by the protective intestinal mucus layer or by the high regeneration of mucosa cell.[41] Furthermore, while the t_{max} (time point at which the maximal concentration is reached) of a drug or marker administered with C_{10} in vivo is between 10 to 60 min,[43,48,57] the in vitro effect lasts at least 300 min.[56] After removal of C_{10}, it takes 120 min until the TER is back to control values.[54] This may contribute to the stronger cytotoxic effects.

The mode of administration of C_{10} and its effective concentration also influence cytotoxicity. Possibilities include reincubation with C_{10} or coadministration in a liquid or solid form, e.g., tablets by spray freeze-drying.[43,57,68] Because the uptake of C_{10} in vivo is quite fast (t_{max} ~7 min,[48]) after injection, the drug is injected before and after the application of C_{10}.[52] C_{10} was coadministered with two different size markers combined in tablet or in liquid form. It was found that the solid form enhanced uptake much better than the liquid form. Additionally, the smaller marker is taken up to a greater extent than the larger marker.[57] An in situ drug delivery study showed that co-administration of C_{10} and FD4 achieved the optimal effect compared with pre-incubation with C_{10}.[68] In summary, C_{10} enhances paracellular drug uptake for molecules with a molecular weight less than 20 kDa. No difference between hydrophobic and hydrophilic drug enhancement was reported. However, most of the drugs investigated tended to be hydrophilic.

In addition to C_{10}, tartaric acid, sodium taurodeoxycholate, sodium dodecyl sulfate (SDS) or p-t-octyl phenol polyoxyethylene-9.5 (Triton X-100) were tested as penetration enhancers with surface activity.[69] They were tested at the rat jejunum and have potential as enhancing systems for oral delivery of poorly absorbed hydrophilic compounds such as protein or peptide drugs. These approaches have not been further developed.

A further group of surfactant-like drug enhancers are short chain alkylglycerols.[70] Intra-arterial injection leads to concentration-dependent enrichment of co-applied cytostatic drugs and antibiotics in the brain.[71] Even the penetration of large molecules like albumin could be significantly increased.[72] Opening of the blood-brain barrier in vivo is reversible and lasts from a few minutes to about one hour, depending on the concentration used.[71,73] In vitro and in vivo analysis showed that alkylglycerols are nontoxic.[74] In freshly isolated brain capillaries, the small molecule fluorescein diffused into the capillary lumen after addition of alkylglycerols through the paracellular cleft, indicating opening of the paracellular diffusion barrier.[72] However, the exact mode of action remains unknown.

Peptides. Specific targeting of TJ proteins could also be achieved by using peptides which are thought to bind to

extracellular parts of TJ transmembrane proteins. Occludin has been the first target for barrier modulation, using an 18 amino acid peptide derived from the ECD2. After calcium depletion of T84 cells, the ECD2 peptide prevents reformation of TJs and binds to claudin-1, occludin and the junctional adhesion molecule-A.[36] Peptides derived from the N-terminal half of the first ECD of human occludin increased the permeability of airway epithelia.[75] Moreover, a 22-amino acid peptide of the ECD1 corresponding to the residues 209–230 of rat occludin was injected into the testis of adult rats (1.5–10 mg/testis) and was able to open the blood-testis barrier.[76] This peptide fused to the follicle stimulating hormone (FSH) specifically opened the blood-testis barrier after intraperitoneal injection (40 μg/adult rat), without disturbing the epithelia of other organs.[77]

A similar effect was shown in T84 cell monolayers for a peptide related to the amino acids 53–80 of the ECD1 of mouse claudin-1, whereas claudin-1_146–160 and claudin-1_31–54 did not increase TER or FD3 permeability. This peptide increased paracellular gastric permeability for sucrose 1 day after oral administration in rats (0.1 mg/kg body weight).[78] A similar peptide was used to improve the delivery of pharmaceutical agents through the perineural barrier. Beginning at 48 h after perineural injection of 400 μM peptide, there was an improved effect of opioid receptor agonists (e.g., DAMGO) or sodium channel blockers (tetrodotoxin) on raised the mechanical nociceptive threshold.[79] This demonstrated that the perineural barrier was opened by an induction mechanism carried by the peptide.

Another ligand for a subgroup of claudins is the *Clostridium perfringens* enterotoxin (CPE), a common source of many symptoms of *Clostridum perfringens*-related food borne diseases. Among other claudins (6, 8 and 14), the main binding partners for CPE are the ECDs2 of claudin-3 and claudin-4.[80] CPE could be separated into a cytotoxic N-terminal and a claudin binding C-terminal part at the very end of CPE (290–319).[81] The larger C-terminal fragment (amino acid 194–319, cCPE) is capable of modulating TJs and increases the permeability of CPE sensitive claudin expressing epithelial cells (3.5 μg/ml, 18 h).[82] In rats, it was demonstrated that 1 μg/μl cCPE enhanced the nasal mucosal absorption of dextran ≥4 kDa and jejunal absorption and also for 150 kDa dextrans one hour after administration.[83] Since claudin-4 is highly expressed in human epithelial ovarian carcinomas, but not in normal ovary tissue, cCPE was tested as drug enhancer for cancer treatment. After application of 5 μg/ml cCPE for 24 h, the claudin-4 expression level decreased and claudin-4 was removed from TJs in cell culture, without showing any cytotoxicity (0.01–15 μg/ml, 0–48 h).[84] In a human epithelial ovarian carcinoma xenograft model, Gao and coworkers also found suppression of tumor growth by 59% compared with the vehicle, when the mice were treated with taxol (20 mg/kg) combined with cCPE (0.1 mg/kg) twice a week for a period of four weeks.

A toxicological study in mice using intravenous administration (5 mg/kg) or nasal cCPE administration (2 mg/kg), did not increase biochemical markers of liver and kidney injury but, after six administrations once a week, the cCPE-specific serum immunoglobulin G rose.[85]

Specific targeting of claudins and occludin is an innovative and promising approach modulating TJ expressing cells directly and avoiding effects of the drug enhancer on other cell types. Peptides directed against certain claudins interfere with claudin-interactions only in TJs containing the target claudins of the peptide. Therefore, the effect is tissue barrier specific. Since peptide-treated barriers open slowly, but stay open for a period of hours, long-term treatments with antibiotics or chemotherapy are conceivable.

A more rapidly acting but less specific peptide is a 6-mer, FCIGRL, called AT1002. This short, synthetic peptide corresponds to the amino acids 288–293 of the zonula occludens toxin from *Vibrio cholera*.[86] Both the toxin and the peptide reversibly open TJs and increase paracellular permeability in intestinal cells by binding to proteinase activating receptor (PAR) 2 and, subsequently, to actin polymerization mediated by protein kinase Cα activation.[87] In male Sprague-Dawley rats, intestinal uptake of cyclosporin A was increased by co-treatment with 10–40 mg/kg AT1002 (1.5 to 2.5-fold, 0–120 min after application).[88] Nasal administration of the low bioavailability agent ritonavir (Abbott Laboratories), an antiretroviral drug to treat HIV infections, was increased 2.55-fold up to 240 min with AT1002 when co-administered with the bioadhesive polymer carrageenan.[89] One problem with the use of AT1002 is the instability of the peptide in neutral to basic pH conditions. It has been recently shown that the stability of AT1002 could be increased up to 6 h in 5% dextrose solution.[90,91] Another approach to stabilize AT1002 is the systematic exchange of amino acids, especially, the cysteine at position two.[92]

siRNA and antibodies. An even more specific new approach to modulate tissue barrier functions is the administration of siRNA found to knockdown the expression of TJ proteins in barrier-forming epithelial[93,94] and endothelial cells.[95] This affects ZO-1,[96] occludin[97] or claudins.[98]

By selectively decreasing claudin-5 at the TJs, the chance of drugs reaching their target in the brain increases, as claudin-5 tightens the blood-brain barrier for small molecules.[23] Campbell et al. injected siRNA into mice specifically to modulate claudin-5 expression in blood vessels in the brain.[99] 20 μg siRNA per mouse suppresses the expression of a protein by degrading its mRNA. Claudin-5 selective siRNA reduced claudin-5 protein content and size-selective opening (up to 1 kDa) of the blood-brain barrier between 24 and 72 h post injection. In a mouse model of traumatic brain injury, they also showed that water efflux to the blood from the brain—caused by edema—is increased.[99] siRNA in complex with in vivo-jetPei™, an in vivo delivery agent, is now being used in a phase I clinical trial (www.polyplus-transfection.com/transfection-reagents/5_in-vivo delivery/1_dna_sirna_delivery/dna-sirna-delivery-in-vivo-jetpei/).

Selective targetting of claudins is also the goal of several therapeutic anti-claudin antibodies since some types of tumors, such as breast, pancreatic or ovarian cancers, exhibit increased expression of claudins-3 and -4.[100,101] Therefore, a monoclonal antibody, KM3907, with in vivo antitumor activity (10 mg/kg) was developed. KM3907 recognized claudins-3 and -4, but not claudins-5, -6 and -9.[102] Another monoclonal antibody against claudin-4 (KM3934) has also shown promising antitumor

efficacy in preclinical models.[103] A monoclonal anti-claudin-1 antibody, which binds to the first ECD of claudin-1, was proven to efficiently inhibit infection of primary human hepatocytes with all major genotypes of hepatitis C virus. Fofana and coworkers preincubated cell cultures in vitro with 10 μg/ml for one hour without observing cytoxic effects (up to 1000 μg/ml) or effects on TJ integrity.[104]

This demonstrates that the development of antibodies against ECDs of TJ proteins is possible. The design of non-cytotoxic antibodies targeting single TJ components could raise new options for the development of drug enhancers. Since TJ protein binding proteins and peptides take a short time to bind to their target, but several hours to show an effect on permeability, these antibodies should be analyzed for their potential as drug enhancers.

Conclusion

Different drug enhancing approaches, with very different properties, are available in experimental and clinical investigations. Hyperosmolar solutions have been used since the 1970s. These drastic procedures immediately and transiently disrupt the TJs. As yet, mannitol is the only compound with any clinical relevance. Nevertheless, hyperosmolar solutions give limited reproducibility, strong side-effects are common and a molar dosage is used.

Milder approaches have since been developed, based on surface active agents. They also show rather unspecific modes of action. However, there is evidence that some TJ proteins can be selectively targeted. The activity appears directly after administration and lasts for a short time. Millimolar concentrations are effective.

During the last decade, specific peptide ligands of ECDs of TJ proteins have been designed. They affect single TJ proteins, allowing selective targeting of specific barriers, depending on the expression pattern of the TJ proteins. Micromolar amounts of the peptides were found to be effective. However, the transient action has a lag phase of several hours and lasts for many hours.

The most advanced approach is to administer siRNA for downregulation of selected TJ proteins. This method is highly specific. Submicromolar dosage is required; drug delivery is improved after one day and lasts for a second day.

In summary, unspecific approaches need higher dosages, have a shorter duration of action and may cause more side effects. Specific procedures require lower doses, may cause fewer side effects and longer lasting effects. Longer opening times are disadvantageous. Consequently, one should prefer immediately acting agents, such as caprate, for the time necessary to deliver the drug, i.e., opening for some hours. On the other hand, procedures selectively targeting single TJ proteins are advantageous and, specific for defined tissue barriers. Moreover, fewer secondary effects would be expected. However, even more specific approaches, e.g., siRNA, have a longer duration of action which, in turn, may raise the risk of side effects.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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