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Tight junction modulation and its relationship to drug delivery ☆

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Abstract

In order for therapeutic agents to exert their pharmacological effects, they have to cross the biological membranes into the systemic circulation and reach the site of action. Drugs cross the membranes by one of two pathways; paracellular or transcellular. Most drugs are transported transcellularly depending on their physiocochemical properties, however the paracellular route is usually the main route of absorption for hydrophilic drugs (proteins, peptides, etc.). The paracellular pathway is governed by the tight junctions (TJs). The modulation of the TJs by absorption enhancers for paracellular drug transport enhancement and hence drug delivery improvement has been hampered for so many years by lack of comprehensive understanding of the structure and function of the TJs. The TJs are a multiple unit structure composed of multiprotein complex that affiliates with the underlying apical actomyosin ring. TJ proteins identified include transmembrane proteins; occludin and claudin, and cytoplasmic plaque proteins; ZO-1, ZO-2, ZO-3, cingulin, and 7H6. Among the new absorption enhancers that evolved in the past few years is Zonula Occludens toxin, Zot. In vivo and in vitro studies have shown that Zot and its biologically active fragment ΔG could be effectively used to increase the transport/absorption of paracellular markers and low bioavailable drugs across the intestinal epithelium. Above all, the transient opening of the TJs by Zot suggests that it could be used as a novel approach for the safe drug delivery of therapeutic agents.

Keywords: Zonula Occludens toxin; Paracellular permeability; Blood borne barrier; Oral delivery; Zonulin

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Contents

1. Introduction

Alternatives to the parenteral route of drug delivery present a series of attractive advantages for the administration of therapeutic compounds via oral delivery. These advantages are particularly relevant for the treatment of pediatric patients and include the avoidance of pain and discomfort associated with injections and the elimination of possible infections caused by inappropriate use or reuse of needles. Moreover, oral formulations are less expensive to produce, because they do not need to be manufactured under sterile conditions.

In the past few years we have witnessed an explosion in research aimed at creating new drug delivery systems. This research has been fuelled by unprecedented challenges, such as the need to deliver new, more complex drugs (e.g., proteins, hormones, etc.) that are becoming available through recombinant DNA technology. Thus, considerable attention has been directed at finding ways to increase the oral bioavailability of these compounds. However, the intestinal absorption of these molecules is profoundly limited by their physicochemical characteristics.

The identification of therapeutic agents has been sometimes compromised by their biological behavior following administration. For drugs to be therapeutically effective, they have to possess favorable characteristics to cross the biological membranes into the systemic circulation and reach the site of action. Drugs cross the membranes via the transcellular or the paracellular routes (Fig. 1). The transcellular pathway involves the passage of the drug across the cells, while the paracellular pathway refers to the passage of drugs in between the adjacent cells. The major pathway for absorption or transport of a drug depends on its physicochemical characteristics as well as the membrane features. In general, lipophilic drugs cross the biological membrane transcellularly while hydrophilic drugs cross the membrane paracellularly. In order to ameliorate drug absorption via the transcellular pathway, the physicochemical features of the drug have to be manipulated (lipophilicity, pKa, conformation, H-bond characteristics, etc.) or the membrane characteristics have to be altered.

Agents used to increase the penetration or absorption of drugs are called absorption/penetration enhancers. In most cases, those that act via alteration of the characteristics of the membrane to be more permeable, tend to compromise cell viability. On the other hand, manipulation of the paracellular pathway could be used to increase the transport of hydrophilic drugs and modify the absorption route of the fraction absorbed paracellularly for other drugs. The manipulation of the paracellular route has only been explored recently because the structural features of the TJs governing the permeation via this route have been partially unraveled in the past few years. In this report, we will focus on the use of the paracellular pathway to increase the bioavailability of therapeutic agents. Novel absorption enhancers will be discussed with emphasis on Zonula Occludens toxin.

2. The paracellular route

Paracellular transport is the transport of drugs through the intercellular spaces. The paracellular

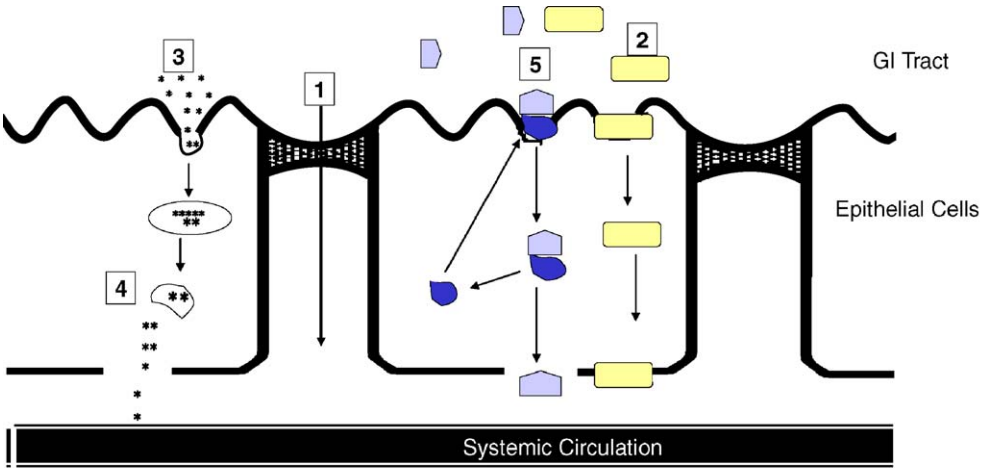

Fig. 1. A schematic representation of the paracellular transport (1) and the transcellular transport of drugs (,) or solutes (* *) across the epithelial cells of the GI tract into the systemic circulation. (2) transcellular passive diffusion, (3) transcellular endocytosis followed by exocytosis (4), (5) carrier-mediated transport processes between a specific carrier () and a drug ().

pathway is governed by TJs. TJs or Zonula Occludens constitute the major rate-limiting barrier towards the paracellular transport for permeation by ions and larger solutes [1]. The dimensions of the paracellular space lie between 10 and 30–50 Å, suggesting that solutes with a molecular radius exceeding 15 Å (~3.5 kDa) will be excluded from this uptake route [2]. TJs are dynamic structures, which normally regulate the trafficking of nutrients, medium sized compounds ($\leq$15 Å) and relatively large amounts of fluids between the intestinal lumen and the submucosa [3]. TJs serve two primary functions in epithelia and endothelia. They form a regulated barrier in the spaces between cells (the paracellular space), restricting the movement of molecules as small as ions across cell sheets and they act as a boundary within the plasma membrane itself, separating the compositionally unique apical and basolateral cell surface domains [4]. TJs play a role in the transduction of signals in one or more directions across cell membranes and in regulating links to the cytoskeleton of the cells [5].

3. Structural characteristics of tight junctions

The TJs are a multiple unit structure composed of multiprotein complex that affiliates with the underlying apical actomyosin ring. Among the TJ proteins identified so far are transmembrane proteins; occludin and claudin, and cytoplasmic plaque proteins; ZO-1, ZO-2, ZO-3, cingulin, and 7H6 [6]. In vitro, the electrical resistance is a measure of charge flow across the membrane and thus reflects the permeability of the paracellular shunt pathway [7] and the tightness of the tight junctions.

TJs assembly is the result of a complex cascade of biochemical events that ultimately lead to the formation of an organized network of TJs elements, the composition of which has been only partially characterized. Two candidates for the transmembrane protein strands, occludin and claudins, have recently been identified [8]. Several proteins have been identified in a cytoplasmic submembraneous plaque underlying membrane contacts, but their function remains to be established. Zonula Occludens-1 (ZO-1) and Zonula Occludens-2 (ZO-2) are the best characterized proteins within the cytoplasmic plaque of the TJs. Most immunoelectron microscopic studies have localized ZO-1 to precisely beneath membrane contacts[9]. Both ZO-1 and ZO-2 belong to the membrane-associated guanylate kinase (MAGUK) family of proteins [10]. They are bound to each other [11] and to 130 kDa protein (ZO-3). ZO-1 and ZO-2 each exists as a heterodimer [11] in a detergent-stable complex with ZO-3. ZO-1 is a phosphoprotein having a species-dependent relative mass between 210 and 225 kDa

[12,13]. ZO-1 binds directly to the COOH-terminal cytoplasmic tail of occluding [14]. ZO-2 is a 160 kDa phosphoprotein peripherally associated with the cytoplasmic surface of the TJ [11,15]. ZO-2 shares strong sequence homology with ZO-1 [15]. The expression of ZO-2 is restricted exclusively to TJs, whereas ZO-1 is also observed at some types of cadherin-based junctions, such as the intercalated discs of cardiac myocytes [16]. ZO-1 and ZO-2 are involved in creating the proper organization of proteins within the tight junctional plaque so that signaling events can be propagated [17]. ZO-3 (130 kDa, p130) shares strong sequence homology with both ZO-1 and ZO-2, and directly interacts with ZO-1 but not ZO-2 [18].

Several other peripheral membrane proteins have been localized to the TJs, including cingulin [19], 7H6 [20], rab13 [21], $G\alpha_{i-2}$ [22]and PKC [23]. Actin, although not exclusively localized to the TJs, was actually the first protein shown by morphological techniques to be associated with the junctional membrane [24]. The architecture of the actin cytoskeleton appears to be critical for TJ function. Most of the actin is positioned under the apical junctional complex where myosin II and several actin binding proteins, including α-catenin, vinculin and radixin have been identified [25]. These actin binding proteins, concentrated directly under the adherens junction, could serve as links to the plasma membrane [17]. Myosin movement along actin filaments is regulated by ATP and phosphorylation of the regulatory light chain by Ca^{2+}-calmodulin-activated myosin light chain kinase [26]. Increases in intracellular Ca^{2+} can affect phosphorylation of myosin regulatory light chain contraction of perijunctional actin and cause increased paracellular permeability [27].

Occludin is a transmembrane phosphoprotein of TJs of ~65 kDa [17]. It is expressed in the TJs of both epithelial and endothelial cells. The proposed folding topology places both NH_2 and COOH terminals within the cytoplasm, allowing the polypeptide to pass out and back inside twice within the NH_2 terminal half [17]. The two extracellular loops, 44 and 45 residues in length, lack consensus sites for glycosylation and although lacking obvious sequence homology with each other, share an extremely high hydrophobic residue and glycine content (25% tyrosine and 36% glycine in the first loop).The cytoplasmic domains are heavily charged, whereas the two extracellular loops contain a total of only one positive and three negative side chains [17]. Occludin has recently been shown to function as a cell–cell adhesion molecule and appears to participate in maintaining the intramembrane diffuson barrier [28]. Occludin is likely involved in establishing the seal at the sites of junctional strands [29].

Claudins interact with ZO-1, ZO-2 and ZO-3 and are able to polymerize and form TJ strands in the absence of their ZO binding region. Two additional cytoplasmic plaque proteins, cingulin (140 kDa phosphoprotein) and 7H6 antigen (155–175 kDa polypeptide) have been localized precisely to TJs using immuno-electron microscopic techniques. Two others are Symplekin (126.5 kDa) and ZA-1TJ. The novel protein, Symplekin [30] has been described to associate with TJs and with the nucleus. Similar to ZO-1, Symplekin is also expressed by cells that do not form TJs, where it appears to be only in the nucleus. ZO-1 also can be localized to the nucleus, but unlike Symplekin, only in growing but not in differentiated epithelial cells [31]. This dual localization for these TJs components suggests that TJs might also be involved in the regulation of gene expression, cell growth, and differentiation [32]. Another set of proteins has been localized only at the resolution of the light microscope to the apical junction complex and includes the small GTP-binding proteins Rab13 and Rab3B (25 kDa), the tyrosine kinase protooncogenes c-Src and c-Yes, and the Src substrate p120, which can bind cadherin/β-catenin complex [17]. These proteins are presumably involved in signal transduction and cell adhesion, although implications for regulation of the tight junction remain unclear. Beside Rab13, the other small GTP-binding proteins are known to regulate the cortical cytoskeleton; Rho regulates actin polymerization and focal adhesion formation [33]. In polarized epithelial cells, Rho also regulates TJ organization and permeability [34]. Other proteins, such as Rac and focal adhesion kinase (FAK), play a role in plasma membrane ruffling and focal adhesion formation [35].

4. Enhancing drug delivery via modulation of the TJs

Absorption enhancers are compounds capable of increasing the absorption of therapeutic agents and

hence improve therapeutic effectiveness. Many studies focused on evaluating absorption enhancers based on the extent of bioavailability enhancement achieved, the influence of formulation and physiological variables, toxicity associated with permeation enhancers and the mechanism of permeation enhancement [36]. Many studies focused on the investigation of absorption enhancers with compounds acting via one or more mechanisms, including influence on the thermodynamic activity of the drug in solution, alteration of the molecular structure of the cell membrane ranging from temporary membrane pore formation to complete membrane destruction, loosening of the TJs between epithelial cells, inhibition of the protease activity present in the mucosa, and alteration of the characteristics of the mucus in ways which reduce its diffusion barrier properties [37]. Ideally, the action of an absorption enhancer should be immediate and should coincide with the presence of the drug at the absorption site [36,38]. For a thorough evaluation of absorption enhancing compounds, it is important to study the extent of improved drug absorption and the absorption-enhancement–time profile as well as the toxicity of the enhancer [39]. In most cases, drug absorption enhancement is accompanied by mucosal damage induced by the enhancer [36,40]. Numerous classes of compounds with diverse chemical properties, including detergents, surfactants, bile salts, Ca^{2+} chelating agents, fatty acids, medium chain glycerides, acyl carnitine, alkanoyl cholines, *N*-acetylated α-amino acids, *N*-acetylated non-α-amino acids, chitosans, mucoadhesive polymers, and phospholipids have been reported to enhance the intestinal absorption of small drug molecules and large polypeptide drugs [36,41–50]. Many of these absorption enhancers act as detergents/surfactants to increase the transcelluar transport of drugs by disrupting the structure of the lipid bilayer and rendering the cell membrane more permeable and/or by increasing the solubility of insoluble drugs [51]. Others act as Ca^{2+} chelators and improve the paracellular transport of hydrophilic drugs by disrupting the TJs after the removal of extracellular Ca^{2+} from the medium or upregulation of intracellular Ca^{2+}. Ca^{2+} depletion induces global changes in the cells, including disruption of actin filaments, disruption of adherent junctions, and diminished cell adhesion [52]. In the case of surfactants, the potential lytic nature of these agents may cause exfoliation of the intestinal epithelium, irreversibly compromising its barrier functions [53]. Reports about some enhancers, including fatty acid sodium caprate and long chain acyl carnitines, have been shown to improve absorption without obvious harmful effects to the intestinal mucosa [53]. Sakai et al. tested the cytotoxicity of three absorption enhancers; sodium caprate, dipotassium glycyrrhizinate, and sodium deoxycholate, by the trypan blue exclusion test, the protein-release test, the neutral-red assay, the DNA-propidium iodide staining assay and the test for recovery of transepithelial electrical resistance (TEER) [54]. Among the three enhancers, dipotassium glycyrrhizinate was found not to be cytotoxic. They reported that sodium deoxycholate (0.1%w/v) and sodium caprate (0.5%w/v) were cytotoxic to the plasma membrane and nuclear membrane as indicated by the trypan blue exclusion, the protein release tests, and the DNA-propidium iodide staining test. Sodium glycolate (1%) has been shown to increase the absorption of insulin [55] and glucagons [56] from the nasal cavity. It induces slight cellular disruption in both in vitro and in vivo tests [37]. With respect to the mucus and mucous membranes, enhancers may act by alteration of the properties of the mucus layer, by opening the TJs between epithelial cells or by increasing membrane fluidity, either by creating disorders in the phospholipid domain in the membrane or by facilitating the leaching of proteins and lipids from the membrane [57]. Sodium lauryl sulfate is known to be capable of lysing biological membranes by a mechanism which appears to be a stepwise process involving both lipid solubilization and subsequent protein denaturation and solubilization [58,59]. Sodium lauryl sulfate was chosen as a positive control that promotes serious skin damage [60].

Based on these studies, it would appear that a transient opening of TJs would seem less damaging than a disruption of cell membrane structure [36]. Absorption enhancers include palmitoyl carnitine. It has been one of the most extensively studied and used absorption enhancers, which increases the intracellular concentration of Ca^{2+} ions however the exact mechanism of action is yet to be understood [48,53,61]. Some studies reported no

separation between toxic and effective concentrations [51]. Duizer et al. conducted absorption enhancement studies for palmitoyl carnitine with paracellular markers, mannitol and PEG4000, along with cytotoxicity studies (LDH, TEER reversibility). Their studies showed that palmitoyl carnitine expressed clear signs of cell damage at all effective (mannitol or PEG4000 transport enhancing) concentrations with LDH leakage and inability of the cells to recover full viability after exposure to the enhancer [62].

Several studies introduced sodium dodecyl sulfate, sodium caprate, and long-chain acylcarnitines to increase permeability through the paracellular pathways [53]. Sodium caprate (10–13 mM) increased the permeability of mannitol, PEGs, argovasopressin, and FITC-dextrans of 4000 and 20,000 molecular weight (MW) and compounds with MW < 1200 across Caco-2 cells [63]. Tomita et al. [64] and Lindmark et al. [49] proposed that the mechanism of paracellular transport enhancement by sodium caprate was via phospholipase C activation and upregulation of intracellular Ca^{2+}, leading to contraction of calmodulin dependent actin–myosin filaments and opening of TJs. Dodecylphosphocholine increases the permeability of hydrophilic compounds across Caco-2 cells by modulation of the TJs directly or through perturbation in the apical membrane and not by disruption of the cell membrane [51]. The enhancing effects by Quillaja saponin, dipotassium glycyrrhizinate, 18 beta-glycyrrhetinic acid, sodium caprate, and taurine were determined by changes in TEER and the amount of heparin disaccharide transported across Caco-2 cells. The results showed that these absorption enhancers can widen TJs and improve the transport of macromolecules and hydrophilic drugs [65].

An approach built on understanding the mechanisms by which TJ solute permeation is naturally regulated may provide insights into simple everyday problems, including improved bioavailability of orally administered drugs [1]. The discovery of an absorption enhancer that functionally and immunologically mimics an endogenous modulator of the TJs appears to be the ideal approach to use for the manipulation of TJs as a means to improve drug delivery. Among the recent absorption enhancers displaying this principle and exhibiting the safest and most effective promising results in enhancing drug delivery is Zonula Occludens toxin or Zot.

5. Discovery of Zonula Occludens toxin

Pathogenic *Vibrio cholerae* (*V. cholerae*) strains produce several endotoxins, e.g., cholera toxin (CT), Zonula Occludens toxin (Zot), and accessory cholera enterotoxin (Ace), as well as a haemolysin that may have enterotoxic effects [66]. Zot is a single polypeptide chain of 44.8 kDa, 399 amino acids (AA) in length, with a predicted pI of 8.5, of bacteriophage origin, present in toxigenic stains of *V. cholerae* [67] with the ability to reversibly alter intestinal epithelial TJs, allowing the passage of macromolecules through mucosal barriers [68]. It was first identified by Fasano et al. in the outer membrane of *V. cholerae* while searching for other factors responsible for the residual diarrhea observed in CT-*V. cholerae* vaccine candidates [69]. Zot possess multiple domains that allow a dual function as a morphogenetic phage protein and as an enterotoxin [70]. After cleavage at AA residue 287, a carboxyl terminal fragment of 12 kDa is excreted, that is probably responsible for the biological effect of the toxin [70]. The ~33 kDa N-terminal left after cleavage, is probably involved in CTXΦ[1] assembly [70] and remains associated to the bacterial membrane. Studies in Ussing chambers have shown that the activity of Zot is reversible, heat-labile, sensitive to protease digestion, and found in culture supernatant fractions containing molecules between 10 and 30 kDa in size [68]. To identify the Zot domain(s) directly involved in the protein permeating effect, several Zot gene deletion mutants were constructed and tested for their biological activity in Ussing chamber assay and their ability to bind to the target receptor on intestinal epithelial cell cultures [71]. With these studies, the Zot biologically active domain was localized toward the carboxyl terminus of the protein and coincided with the predicted cleavage product generated by *V. cholerae*. It was hypothesized that Zot

[1] CTXΦ is a filamentous bacteriophage that encodes cholera toxin and integrates site—specifically into the larger of the two *V. cholerae* chromosomes [97].

may mimic the effect of a functionally and immunologically related endogenous modulator of epithelial TJs [72]. The combination of affinity-purified anti-Zot antibodies and the Ussing chamber assay revealed an intestinal Zot analogue that was named Zonulin [73].

6. Insights into the Zonulin system

When Zonulin was studied in a nonhuman primate model, it reversibly opened intestinal TJs after engagement to the same receptor activated by Zot and therefore acts with the same effector mechanism described for the toxin [73]. Comparison of the amino termini of the secreted Zot fragment (AA 288–399) and its eukaryotic analogue, Zonulin, governing the permeability of intercellular TJ [71], revealed an 8-amino acid shared motif corresponding to 291–298 AA region, identified by the binding experiments as the putative binding domain. Zonulin has a molecular weight of 47 kDa, an N-terminal receptor binding motif that is structurally and functionally similar to the Zot binding motif [73], and a C-terminal domain probably involved in the rearrangement of the cytoskeletal elements functionally connected to the intercellular TJs [74]. Zonulin was detected in the human intestine, heart, and brain [69]. The physiological role of the Zonulin system remains to be established but it is likely that this system is involved in several functions, including TJ regulation during developmental, physiological, and pathological processes, including tissue morphogenesis, protection against microorganisms' colonization, the movement of fluid, macromolecules, and leucocytes between the bloodstream and the intestinal lumen and vice versa [74]. Dysregulation of this conceptual Zonulin model may contribute to disease states that involve disordered intercellular communication including developmental and intestinal disorders leading to autoimmune disease (celiac disease and type 1 diabetes), tissue inflammation, malignant transformation, and metastasis [75]. It was hypothesized that Zonulin has multiple functions with the adult form mainly in charge of the regulation of the paracellular permeability and its fetal counterpart possibly more involved in the regulation of molecules trafficking between body compartments during embryogenesis [73]. The fact that the interaction of bacteria with the intestinal mucosa induces Zonulin release, irrespective of their pathogenic traits or viability, can be interpreted as a bacteria independent mechanism of defense of the host that reacts to the abnormal presence of microorganisms on the surface of the small intestine. Following Zonulin induced opening of TJs, water is secreted into the intestinal lumen following hydrostatic pressure gradients [72] and bacteria are flushed out from the small intestine [74]. The increase in intraluminal Zonulin was found to be age related, to be detectable only in the small intestine (jejunum and ileum) but not in the colon, to correlate with an increase in intestinal permeability of the intestine, to precede the onset of diabetes by at least 3–4 weeks, to remain high in these diabetic prone rats, and to correlate with progression towards full-blown diabetes [76].

7. Identification of the Zot receptors

Zot binding to the surface of rabbit intestinal epithelium has been shown to vary along the different regions of the intestine [72]. Zot increases tissue permeability in the ileum but not in the colon where the presence of the colonic microflora and /or their bioproducts could be harmful if the mucosal barrier is compromised [68]. This binding distribution coincides with the regional effect of Zot on the intestinal permeability and with the preferential F-actin redistribution induced by Zot in the mature cells of the villi [72,77].

The identification of Zot receptor was initiated by binding studies using [125I] maltose binding protein (Mbp)-Zot in different epithelial cell lines, including IEC6 (rat, intestine), Caco-2 (human, intestine), T84 (human intestine), MDCK (canine, kidney), and BPA endothelial cells (bovine, pulmonary artery) [78]. The Zot receptor was purified by ligand-affinity chromatography [78]. Only IEC6 cells (derived from rat crypt cells) and Caco-2 cells (that resemble mature absorptive enteric cells of the villi), but not T84 (crypt-like cells) or MDCK, express receptors on their surface. Zot action is tissue specific, it is active on epithelial cells of the small intestine (jejunum and distal ileum) and on endothelial cells but not on

colonocytes or renal epithelial cells [77]. Moreover, Zot is active only on the mucosal side [77]. Zot showed differential sensitivity among the enterocytes with the mature cells of the tip of the villi being more sensitive than the less mature crypt cells [77].The number of Zot receptors may decrease along the villous crypt axis, and this explains why the effect of Zot on IEC6 cells requires longer exposure time compared to whole tissue [77]. Fluorescence staining of Zot binding to the intestinal epithelium is maximal on the surface of the mature absorptive enterocytes at the tip of the villi and completely disappears along the surface of the immature crypt cells, suggesting that the expression of Zot/Zonulin receptor is up-regulated during enterocyte differentiation [72]. It was proposed that the 66 kDa Zot/Zonulin putative receptor identified in intestinal cell lines is an intracellular modulator of the actin cytoskeleton and of the tight junctional complex, whose activation is triggered by the ligand engagement and subsequent internalization of the ligand/receptor complex [78]. Earlier studies discussed the partial characterization of the Zonulin receptor in the brain and revealed it is a 45 kDa glycoprotein containing multiple sialic acid residues with structural similarities to myeloid-related protein, a member of the calcium-binding protein family possibly linked to cytoskeletal rearrangements [79]. The functional significance of glycosylation of the Zonulin/Zot receptor remains unknown. However, the sialic acid residues may contribute to the stability (protection from proteolysis) or assist in translocation to the cell surface during synthesis [80]. Affinity column purification revealed another ~55 kDa Zot binding protein in the human brain plasma membrane preparation, which was identified as tubulin. Pretreatment with a specific protein kinase C inhibitor, CGP41251, completely abolished the Zot effects on both tissue permeability and actin polymerization [77]. Zot decreases the soluble G-actin pool with a reciprocal increase in the filamentous F-actin pool, thus exerting an effect on actin filaments (polymerization) [77]. Thus, Zot triggers a cascade of intracellular events that lead to a protein kinase C (PKC) α dependent polymerization of actin microfilaments strategically localized to regulate the paracellular pathway [77], and consequently leading to opening of the TJs at a toxin concentration as low as 1.1×10^{-13} M [72]. So far, the receptor was identified in the small intestine [72], brain [79], nasal epithelium [81], and possibly the heart [79] (since Zonulin was identified there) [82].

8. Zot as a TJ modulator for improved drug delivery

Extensive studies of the biological activity of Zot were triggered by its ability to allow for the oral administration of insulin, an agent exclusively administered subcutaneously to diabetic rats [83]. None of the animals treated with insulin and Zot experienced diarrhea, fever, or other systemic symptoms, and no structural changes were demonstrated in the small intestine on histological examination [83]. The observation that in rabbit small intestine, Zot does not affect Na^+-glucose coupled active transport, not cytotoxic, fails to completely abolish the transepithelial resistance, and induces a reversible increase of tissue permeability [68], made it a potential tool for studying intestinal TJ regulation [77]. This information is of valuable importance in cases of clinical conditions affecting the gastrointestinal system involving alteration in intestinal TJ function, including food allergies [84], malabsorption syndromes [85], and inflammatory bowel diseases [86]. In vitro experiments in the rabbit ileum demonstrated that Zot reversibly increased intestinal absorption of insulin (MW 5733 Da) by 72% and immunoglobulin G (140–160 kDa) by 52% in a time dependent manner [83]. In vivo [83,87] and in vitro [68,87] studies showed that the effect of Zot on tissue permeability occurs within 20 min of the addition of the protein to the intestinal mucosa and reaches a peak effect in 80 min. Zot was also tested in an in vivo primate model of diabetes mellitus. Insulin was intragastrically administered to diabetic monkeys either alone or in combination with increasing amounts of Zot. Measurements of blood insulin levels revealed that insulin bioavailability increased from 5.4% in controls to 10.7% and 18% when Zot 2 and 4 μg/kg were administered, respectively [88].

Karyekar et al. has recently reported that Zot increases the permeability of molecular weight markers (sucrose, inulin) and chemotherapeutic agents (paclitaxel and doxorubicin) across the bovine

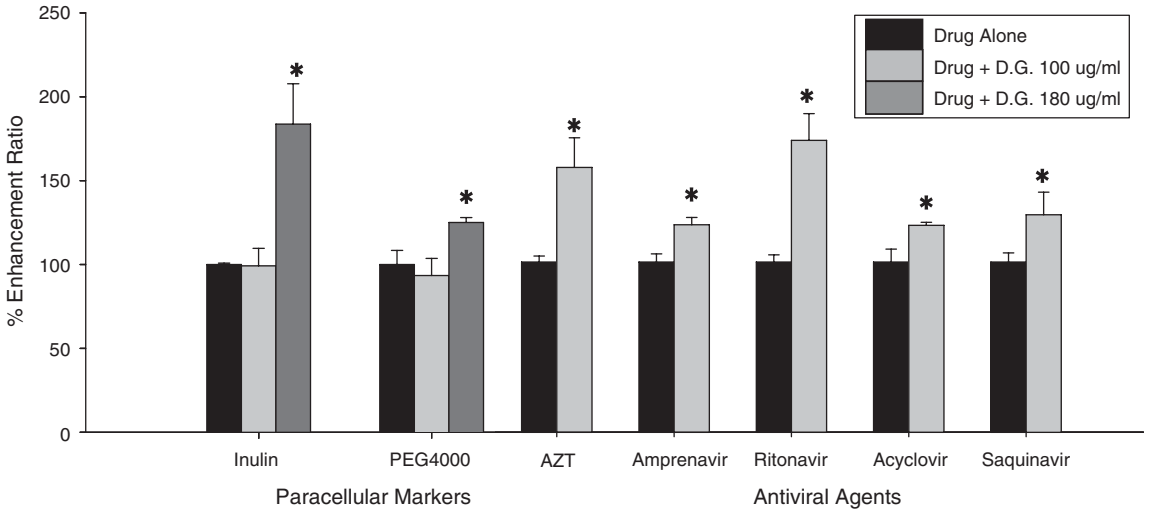

Fig. 2. The percent fold enhancement in the apparent permeability coefficient (Papp) of [^{14}C]Inulin, [^{14}C]PEG4000, [^{14}C]Zidovudine (AZT), [^{3}H]Amprenavir, [^{3}H]Ritonavir, [^{3}H]Acyclovir, and [^{3}H]Saquinavir in the presence of ΔG at 0, 100, and/or 180 μg/ml across Caco-2 cell monolayers ($N=3$). *Significant at $p<0.05$ compared to control. Data presented as mean (± SD).

brain microvessel endothelial cells in a reversible and concentration dependant manner and without affecting the transcellular pathway as indicated by the unaltered transport of propranolol in the presence of Zot [89]. A good correlation was established between the transport enhancement and the molecular size with r^2 of 0.875. Since many therapeutic agents including anticancer drugs have molecular weights ranging from 300 to 1000 Da, these studies introduced Zot as a potential absorption enhancer for the effective delivery of therapeutic agents to the central nervous system.

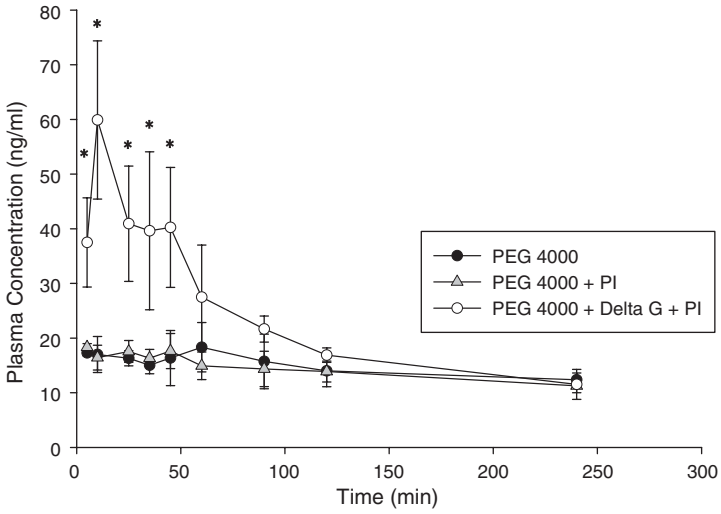

Fig. 3. Average Plasma concentration versus time profile for [^{14}C]PEG4000 alone and with ΔG (720 μg/kg) and/or PI administered ID to jugular vein cannulated Sprague Dawley rats. *Significant at $p<0.05$ compared to PEG4000. Data presented as Mean ± SD ($N=3$–5/group). Republished with permission from J Pharm Sci. © copyright, Vol 93(5), pp. 1310–9, 2004.

Moreover, studies have shown that Zot enhances the transport of drug candidates of varying molecular weights (mannitol, PEG4000, inulin) or low bioavailability (Doxorubicin, paclitaxel, acyclovir, cyclosporin A, anticonvulsant enaminones) up to 30 fold as seen with paclitaxel across Caco-2 cell monolayers, without modulating the transcellular transport [90,91]. In addition, the transport enhancing effect of Zot is reversible and non-toxic [68,91]. Recent studies have identified a smaller 12 kDa fragment of Zot, referred to as ΔG [71]. These studies focused on identifying the Zot domain(s) directly involved in the protein permeating effect. ΔG results in a cascade of intracellular events leading to actin cytoskeletal rearrangement, disengagement of junctional complex proteins, and finally opening of TJs.

ΔG displayed significant potential as a TJ modulator, which could be used to improve the bioavailability of drugs. In vitro studies showed that it is capable of significantly increasing the apparent permeability coefficients for a wide variety of therapeutic agents and markers across the Caco-2 cell model [92–94] (Fig. 2). In addition, ΔG improved the bioavailability of paracellular markers, mannitol, inulin, and PEG4000 (Fig. 3) after intraduodenal (ID) administration to rats in the presence of peptidase inhibitors (PI) [92,93]. The transport/absorption of different therapeutic agents exhibiting different physicochemical properties (lipophilicity/hydrophilicity, molecular weights, efflux properties, structural differences) and belonging to a wide range of drug classes was tested with ΔG. For example, when the anti-HIV protease inhibitors were administered orally to Sprague Dawley rats with ΔG, the rate and/or extent of drug absorption were significantly ameliorated, with the exception of drugs such as saquinavir, whose low oral bioavailability is mainly attributed to extensive first pass metabolism [95]. The in vivo studies with ΔG displayed up to 57 and 50 fold increases in drug bioavailability parameters; Cmax and AUC, respectively, as seen with cyclosporin A (Fig. 4) [95] after metabolic protection was provided.

Moreover, Zot is a good mucosal adjuvant, considering its ability to interfere with the suppression of specific cell mediated immunity, probably as a result of the increased dose and/or altered processing of antigen at the mucosal level [96]. To further characterize the role of Zot as an adjuvant, its ability

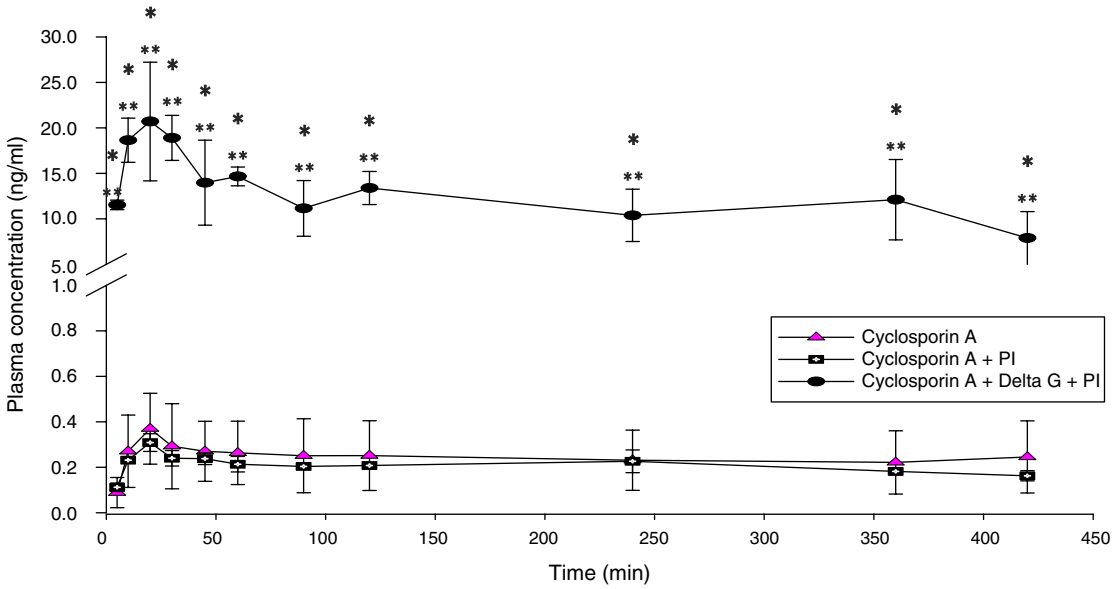

Fig. 4. Average Plasma concentration versus time profile for [^{3}H] cyclosporin A alone and with ΔG (720 μg/kg) and/or PI administered ID to jugular vein cannulated Sprague Dawley rats. *Significant at $p<0.05$ compared to cyclosporine A. **Significant at $p<0.05$ compared to cyclosporine A/ PI. Data presented as Mean ± SD ($N=3$–6/group). Republished with permission from J Pharmacol Exp Ther, Vol 312 (1), pp. 199–205, 2005.

to abrogate nasal tolerance to an unrelated protein as gliadin was examined [96]. When mice were given intranasally Zot with gliadin, the cytokine pattern showed reduced down-regulation of IL-2 and IFN-γ secretions, together with significantly lower suppression in T-cell proliferation. This suggested that the mechanism of adjuvanticity mediated by Zot preferentially leads to a functional activation of Th1-cell differentiation.

Recent studies have shown that Zot could be exploited to deliver soluble antigens (Ag) through the nasal mucosa for the induction of Ag-specific systemic and mucosal immune responses [81]. The coadministration of Zot and ovalbumin (Ova) was found to induce anti-Ova serum immunoglobulin G (IgG) titers that were approximately 40-fold higher than those induced by immunization with Ag alone. Zot also stimulated Ag-specific IgA titres in vaginal and intestinal mucosa.

9. Conclusion

The low bioavailability of drugs remains to be an active area of research. Novel approaches to improve drug delivery have been introduced lately. Among which, the modulation of TJs to improve paracellular drug transport appears to be a very appealing and attractive solution. Transient opening of TJs would be beneficial to the therapeutic effect because it avoids entry of metabolic waste as well as leakage of important proteins and nutrients. Zot was identified as a potential modulator of the TJs that initiates a cascade of intracellular events upon binding to its receptor leading to opening of the TJs. Extensive in vivo and in vitro studies have identified Zot receptors in the small intestine, the nasal epithelium, the heart and the brain endothelium. The discovery of Zot triggered studies to identify possible eukaryotic analogues. Comparison of the amino termini of the secreted Zot fragment (AA 288–399) and its eukaryotic analogue, Zonulin, governing the permeability of intercellular TJs [71], revealed an octapeptide amino acid shared motif corresponding to 291–298 AA region. The physiological role of the Zonulin system remains to be established but it is likely that this system is involved in several functions, including TJ regulation during developmental, physiological, and pathological processes, including tissue morphogenesis, protection against microorganisms' colonization, as well as the movement of fluid, macromolecules, and leucocytes between the blood stream and the intestinal lumen and vice versa [74]. In vitro and in vivo studies across the blood brain barrier have shown that Zot increases the transport and tissue accumulation of therapeutic agents when administered concurrently. In addition, Zot and its biologically active fragment ΔG displayed significant potential in enhancing the oral bioavailability of anticancer, immunosuppressant, and antiretroviral drugs in Caco-2 cells and Sprague Dawley rats. Moreover, toxicity studies have shown that Zot and ΔG do not compromise cell viability or cause membrane toxicity contrary to other absorption enhancers. Collectively, this report presents modulation of the TJs as a promising route for enhanced drug delivery approaches as evidenced by the novel modulator, Zot.

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