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Discovery of tight junction modulators: significance for drug development and delivery

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Tight junction biology has many important applications, from improving knowledge of diseases characterized by the loss of epithelial or endothelial tissue barrier function to providing a mechanistic basis for improving non-invasive drug delivery. A variety of chemical and molecular biological tools have been developed, including high throughput cell-based screening of molecular libraries that facilitate discovery of novel peptides and lipids to modulate tight junction function safely and reversibly. Further development of novel tight junction modulating excipients necessitates consideration of physicochemical, toxicological, pharmaceutical and regulatory issues.

Tight junction physiology and therapeutic potential

Respiratory, reproductive, gastrointestinal, renal and epidermal epithelia function as a selective barrier between the outside environment and underlying tissue. Epithelial cells are polarized, both functionally and compositionally (membrane-bound lipids and proteins). This polarity is established partially by formation of specialized cell–cell junctions referred to as the Apical Junction Complex (AJC) [1]. The AJC is positioned at the apical side of the plasma membrane and is composed of two distinct junctional complexes—the tight junction (TJ) and the adherens junction (AJ). These junctions are distinct, as shown in the electron micrograph in Figure 1a, where the TJ is the most apical junction followed by the AJ. Desmosomes provide structural integrity and are dispersed along the basal–lateral junction. The AJ and the TJ are composed of transmembrane proteins that interact directly with the neighbouring cells, forming a selective barrier that regulates passage of molecules (reviewed in reference [2]). The TJ includes occludin, members of the claudin family and junctional adhesion molecule (JAM-A). The AJ of epithelial cells contains E-cadherin and members of the nectin family. Cytoplasmic scaffolding proteins, for example, zonula occludens (ZO-1), connect the TJs and AJs to the actin cytoskeleton and mediate signaling processes. Figure 1b shows a cross-section of immunofluorescent staining of junctional markers in differentiated normal human bronchial epithelial cells. TJs form a continuous belt of adhesions restricted to the apical membrane, as shown in Figure 1c, while E-cadherin is present along the entire basal–lateral membrane (Figure 1b) but does not provide barrier function. Immunostaining with specific antibodies against TJ proteins such as claudin 4 and ZO-1 results in a chicken wire-like staining pattern in sections parallel to the plane of the monolayers. TJs also form between adjacent endothelial cells in the vasculature. The 'tightness' of endothelial junctions varies, being very low in the spleen and endocrine glands and very high in the brain and cornea.

The AJ and TJ are dynamic structures, undergoing constitutive turnover and recycling. The cytosolic face of the AJC is composed of a large array of distinct proteins that direct the assembly of the AJC [3]. The assembly and disassembly of the AJC is thought to regulate epithelial morphogenesis and remodeling processes. Downregulation of expression of AJC proteins is not likely to be rapid enough to account for these dynamic processes while endocytosis of the AJC could provide a rapid and recycling mechanism of modulation. Cellular stress, such as pathogens, oxidative stress and cytokines can signal an increase in the endocytosis of the AJC. Studies have shown that endocytosis of AJ and TJ proteins can occur by clathrin or caveolae endocytic pathways or macropinocytosis (reviewed in reference [4]). Extracellular calcium is required for the formation of the AJC. Chelators, such as ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), are capable of disrupting intracellular junctions, decreasing transepithelial resistance and increasing permeability. Studies in intestinal epithelial cells suggest that calcium depletion stimulates endocytosis of the AJC via a

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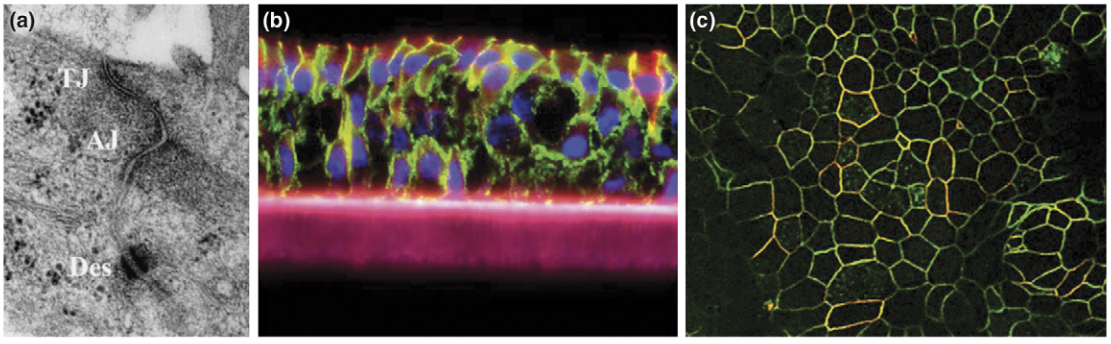

FIGURE 1

Epithelial tight junctions. **(a)** Transmission electron micrograph of the apical junctional complex in normal human bronchial epithelial cells. Tight junction (TJ), the adherens junction (AJ) and the desmosome (Des). **(b)** Cross-section of immunofluorescent staining of differentiated normal human bronchial epithelial cells for the junctional markers JAM-A (red) and E-Cadherin (green). DAPI (blue) marks the nuclei of each cell. **(c)** Confocal image of differentiated normal human bronchial epithelial cells, immunofluorescently labeled for ZO1 (red) and occludin (green). This image is looking down on the apical surface of the cell layer and shows the outlines of the tight junctions, which border all sides of the apical cells.

clathrin-dependent pathway [5]. Clathrin-mediated endocytosis can result in trafficking to an acidic endosome and eventually to the lysosome for degradation. Thus, targeting endocytosis of the AJC via a caveolar or macropinocytic pathway may be effective in modulating TJ dynamics. Suitably effective compounds may lead to an increase or decrease in TJ permeation, depending on the specific targeting mechanism. A better understanding of the signaling pathways that regulate TJ dynamics and recycling may reveal new approaches for TJ modulation.

Tight junction dysregulation in human disease

Various diseases involve pathological alteration of epithelial or endothelial barriers, including bacterial and viral infections, allergen response, immune hyper-responsiveness, inflammatory diseases, such as Crohn's disease (CD) and ulcerative colitis (UC), psoriasis, encephalitis, diabetes, multiple sclerosis and cancer [6,7]. In these diseases, the TJ barriers are disrupted resulting in an increase in permeability. Insight into the mechanisms of epithelial barrier modulation has come from studies of the effects of pro-inflammatory factors such as interferon (INF)-γ and tumour necrosis factor (TNF)-α. AJC internalization via macropinocytosis has been observed *in vitro* in intestinal epithelial cells treated with INF-γ. This effect was temporary, as removal of INF-γ resulted in recycling of the endosomal compartment back to the plasma membrane. Inhibition of myosin light chain phosphorylation, using inhibitors of Rho kinase (ROCK), blocked the INF-γ stimulated endocytosis of the AJC [8]. Clinical biopsies of inflamed regions of UC also reveal significant populations of TJ proteins in cytoplasmic vesicles and, in agreement with *in vitro* results, pharmacological inhibition of ROCK in CD patients prevented inflammatory responses [9]. No specific drugs have yet been developed that restore TJ integrity, although it has been shown that (1) it is possible to decrease TJ permeability by increasing the expression of certain TJ proteins, such as occludin [10] and (2) exposure of cell layers to epidermal growth factor decreases TJ permeability [11,12]. Identification of compounds that effectively decrease TJ permeability is a major future area for drug discovery and development.

Microorganisms have evolved mechanisms for breaching permeability barriers either by targeting junctions or cells, for example, viral and bacterial infections that alter the gastrointestinal (GI) barrier resulting in severe diarrhoea. Several microbial toxins target TJs [13] and represent various mechanisms that can be exploited for drug delivery. The second *Vibrio cholera* enterotoxin, ZOT [14], an analogue of human zonulin [15], was shown to increase intestinal muscosal permeability by reversibly altering tight junction structure [16]. The mechanism involves zonulin receptor binding and effects on protein kinase C dependent actin polymerization and altered regulation of the paracellular pathway [17].

Rotavirus causes severe secretory diarrhoea in infants and children. The virus infects columnar epithelial cells and inhibits the absorptive capacity of these cells, causing a net secretion of water and salts, which results in watery diarrhoea and severe dehydration. Rotavirus decreases transepithelial electrical resistance (TER) and increases paracellular permeability in Caco-2 monolayers [18]. Immunohistochemistry studies have shown that rotavirus infection decreases the levels of claudin-1, occludin and ZO-1 proteins in the perijunctional region. Rotaviruses bind to intestinal receptors that are not exposed to the intestinal lumen but are located beyond the TJ in the basolateral domain. The outer capsid protein VP4 and its proteolytic subfragment VP8 function to open the paracellular space sealed by the TJ [19]. VP8 also triggers the movement of some basolateral proteins towards the apical surface, suggesting that once the fence function constituted by the TJ is relaxed, basolateral proteins can diffuse in the plane of the membrane.

Experimental systems and methods

A variety of human epithelial cell lines and techniques can be used to study the mechanisms of drug mucosal absorption as previously described [20]. Primary human cells for drug absorption and formulation development studies are available from commercial sources, for example human-derived tracheal/bronchial epithelial cells cultured to form a pseudo-stratified columnar polarized epithelial cell layer that forms tight junctions similar to normal respiratory epithelium. Results using this *in vitro* system have a

good correlation with results from *in vivo* animal models and humans. These epithelial cells can be grown as a tissue layer in a 96-well format, suitable for high throughput analysis of TER, cytotoxicity, cell viability and drug permeation or in individual 24-well cell culture inserts, most suitable for immunocytochemistry and fluorescence microscopy. A variety of methods are used to evaluate the properties of cultured tissue/cells that measure the integrity of TJs, absorption properties of epithelial tissue barriers and potential toxicity associated with treatment conditions [20].

TER, a measure of TJ integrity (paracellular gate function), is calculated from the measured potential difference between the apical and basolateral sides of the cell layer, with passive ion flow being the dominant process reflecting changes in paracellular permeability. Resistance is expressed as both $\Omega\,cm^2$ and percent original TER value relative to a control:

$$\text{Normal resistance}(\Omega\,cm^2) = (\text{TER}_1 - \text{blank}) \times 0.12(\text{for 96-well format})$$

$$\text{Relative TER}\,(\%)\left(\frac{\text{TER}_1-\text{blank}}{\text{TER}_2-\text{blank}}\right) \times 100$$

A change in the properties of the TJ complex can have a major effect on TER with either minor or major affects on permeability of a peptide/protein drug, depending on the mechanism of the formulation or TJ modulator.

The functional restriction to paracellular transport through the TJ can be measured by the permeation (P_{app}) of a drug or hydrophilic marker, such as radiolabeled mannitol or a fluorescent-labeled macromolecule, across a cell/tissue layer. Sodium fluorescein (376 Da) and FITC-dextran (available in average MW from 4000 to 500 000 Da) are routinely used as models to evaluate size dependence of hydrophilic macromolecule transport. Permeability calculations for a test compound (Tc):

$$\text{Permeation}\% = \frac{C_b \times V_b}{C_a \times V_a} \times 100$$

$$\text{Apparent permeability}(P_{app})(\text{cm/s}) = \frac{V_b}{A \times C_a}\frac{C_b}{dt}$$

where C_b is the basolateral Tc concentration, C_a is the apical Tc concentration, V_b is the basolateral volume, V_a is the apical Volume, A is the filter surface area and dt is the elapsed time.

Immunofluorescence and confocal microscopy are used to evaluate the general morphological properties and integrity of monolayers used in TER and permeability studies and the distribution of cytoskeletal and TJ-associated proteins. Fluorescence markers are used to monitor changes in plasma membrane or TJ permeability, as well as to follow the distribution and localization of labeled formulation components and TJ modulating compounds.

Intramembrane lipid diffusion assays that allow visualization or measurement of lipid diffusion from one cell surface domain to the other are used to analyze the fence function of TJs (prevention of intermixing of lipids and integral membrane proteins in the outer leaflet of the plasma membrane). This is accomplished by labeling one side of the cell/tissue layer with a fluorescent lipid, such as BODIPYL-FL-C5-sphingomyelin, that does not interchange from inner and outer membrane leaflets.

Cytotoxicity assays are important for evaluating the potential toxicity of new TJ modulators. Two convenient assays that are adaptable to high throughput screening are (1) the MTT assay, which measures cell metabolic activity by spectrophotometric quantitation as an endpoint for cell proliferation and viability and (2) the lactate dehydrogenase (LDH) assay, which measures plasma membrane permeability based on release of LDH from damaged cells.

Screening peptide libraries for the discovery of tight junction modulators

Peptide library screening has great potential for the systematic identification of tight junction modulators (TJMs) for use as drugs to restore normal tight junction properties for disease states characterized by abnormal TJ permeability, or as excipients to enhance paracellular permeability for non-invasive drug delivery. Since its early development [21], phage display technology has been used widely for the screening of random combinatorial peptide libraries for drug discovery [22]. Phage display has been used to screen for peptides that modulate the activity of epithelial cell tight junctions [23]. Panning with a phage library that displays random 7-mers was performed using monolayers of human bronchial epithelial cells (16HBE14o$^-$) that were treated with the calcium chelator EGTA to increase accessibility to the junctional complex/paracellular space, followed by subtractive panning. Within 5 min of treatment with 1 mM EGTA, TER was reduced 75%. The effect of calcium chelation on tight junction integrity was reversible as demonstrated by recovery of TER over a 120 min period after EGTA removal. Microscopic examination of treated vs. untreated cells stained with FITC labeled anti-ZO1 or anti-claudin antibodies revealed that EGTA treatment created dispersed gaps between cells of an otherwise confluent monolayer and that the gaps were no longer observable 120 min after EGTA removal.

A novel peptide, FDFWITP, identified by phage display selection as a potential TJM, was synthesized in linear and cyclic forms with lysine residues added to improve solubility. The cyclic form of the peptide reduced TER in a concentration-dependent manner (80% reduction at 100 μM and 95% reduction at 500 μM) and was reversible within 2 h; the linear form only affected TER at the highest concentration. Interestingly, the constrained peptide did not increase permeation of the model small molecule, fluorescein. The highly selective activity of FDFWITP supports the hypothesis that ions and small molecules may be transported paracellularly across tight junctions by separate pathways.

Peptides displayed as unconstrained linear molecules can adopt numerous conformations, very few of which may represent stable structures. Panning with these unconstrained peptides against targets of interest usually leads to the isolation of peptides with low binding affinity. Herman *et al.* [24] developed a new peptide library technology based on a 20 amino acid Trp cage miniprotein, with multiple variable positions, displayed on bacteriophage T7. They demonstrated that the library could be used to identify peptides that bind to specific cell types and that may be suitable for the delivery of therapeutic compounds. The Trp cage is a hydrophobic cluster with Trp-25 buried in a central location, where it is shielded from solvent exposure. The residues that form the cage around Trp-25 include multiple proline residues (Pro-31, 36–38) that are oriented so that the proline rings are located on both faces of the indole ring of Trp

25, as well as a Phe side chain that completes the hydrophobic cluster [25,26]. The optimized Trp cage miniprotein TC5b (NLYIQWLKDGGPSSGRPPPS) has been extensively studied because it is an ultra fast, cooperatively folding system [27]. Within the optimized Trp cage sequence, there are positions where substitution does not compromise folding of the miniprotein. Some of these positions are solvent-exposed, making them ideal for display of random substitutions in a highly diverse library. Utility of the library was demonstrated by identification of specific binding ligands, including AAADPYAQWLQSMGPHSGRPPPR, which bound to human bronchial epithelial cells. A high complexity library based on the Trp cage miniprotein has demonstrated potential for identifying novel cell- and protein-binding peptides that could be used for the delivery of therapeutic molecules or as target-specific therapeutic agents.

Therapeutic development of novel tight junction modulators

Safe and effective non-invasive routes for delivery of macromolecular drugs [28,29] continue to be widely studied, including oral [30,31], transdermal [32,33], buccal [34], pulmonary [35,36] and intranasal (IN) [37,38] administration. For some peptide and protein drugs, marketing approval has been achieved for non-invasive delivery via pulmonary and IN administration, the latter being most established in terms of marketed products.

Tissue barriers associated with such non-invasive delivery pose a considerable challenge, particularly for macromolecules. This challenge can be met by employing permeation enhancers to facilitate paracellular transport [20,39,40]. Permeation enhancers can be selected empirically from among currently acceptable excipients for pharmaceutical use, or novel TJM excipients can be developed rationally.

For developing novel TJM excipients, the target profile for the pharmaceutical product(s) needs to be considered. The novel TJM excipient must be chemically stable and retain permeation enhancement under conditions relevant for therapeutic storage and use. Routine storage of drug products includes frozen, refrigerated and room temperature conditions (the latter providing maximum convenience for the patient). Dosage forms for therapeutic peptide and protein products include aqueous solutions, powders and lyophilized forms intended for reconstitution. It would be advantageous for a novel TJM excipient to be applicable to a range of these types of products and their storage conditions. In addition, the impact of the TJM excipient on the cost of goods should be evaluated. Typically, peptides and proteins have a higher cost of goods compared with low-molecular-weight compounds; this applies to both the active pharmaceutical ingredient (API) as well as the excipient(s). Thus, it is important to maximize potency of the TJM enhancer, particularly for a TJM peptide.

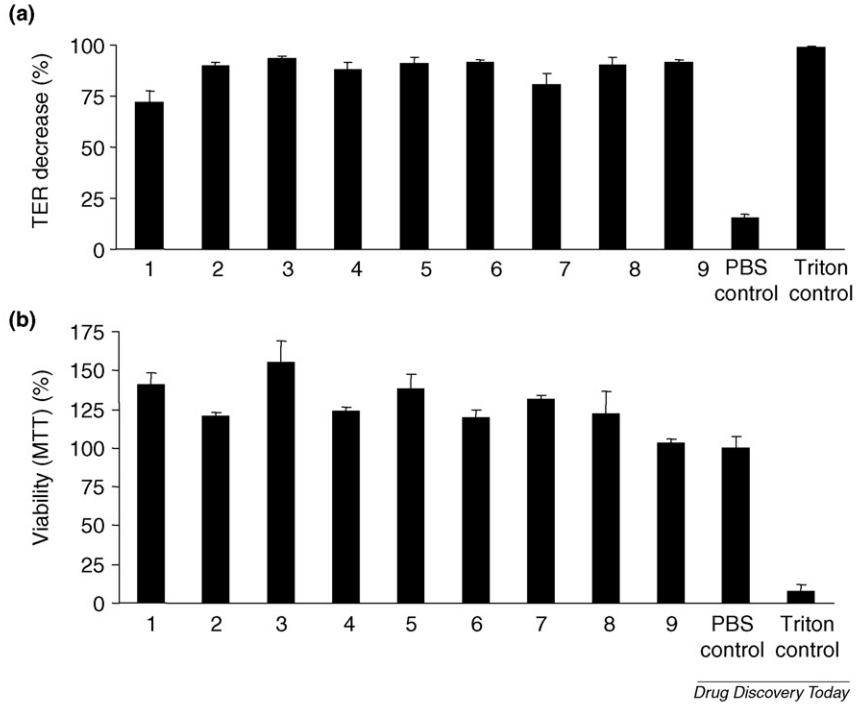

FIGURE 2

In vitro screening of tight junction modulating lipids. **(a)** % TER decrease. **(b)** % cell viability. Data for % TER decrease and % cell viability are after 1 h at 37 °C for (1) 1-palmitoyl-2-(5′-oxo-valeroyl)-sn-glycero-3-phosphocholine (POVPC), (2) 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC), (3) 1-*O*-hexadecyl-2-azelaoyl-sn-glycero-3-phosphocholine (C16–09:0), (4) glucosyl-sphingosine, (5) 1-*O*-octadecyl-2-*O*-methyl-glycerol-3-phosphocholine, (6) 1-palmitoyl-2-azelaoyl-sn-glycero-3-phosphocholine (16:0-09:0 (COOH)PC), (7) 1-palmitoyl-2-(9′-oxo-nonanoyl)-sn-glycero-3-phosphocholine (16:0-09:0 (ALDO)PC), (8) D-lactosyl-β-1′-D-erythro-sphingosine (lactosyl-(β)-sphingosine), (9) PN159, (PBS control) phosphate buffered saline (PBS) and (Triton control) 1% Triton X-100. Data from Chen *et al.* [45].

Novel excipients are typically not approved as stand-alone drugs, but rather are approved as a component of a specific drug product formulation. Development activities to support a novel TJM excipient include evaluation of pharmacological activity, level and duration of exposure, route of administration and whether or not the novel TJM excipient results in systemic (rather than local) exposure. It is always a good idea to consult with the appropriate drug review division before initiation of studies.

Recommended safety evaluations and toxicity data required to determine that a novel TJM is safe for use in a drug product formulation are found in the FDA Guidance for Industry: Nonclinical Studies for the Safety Evaluation of Pharmaceutical Excipients [41]. Additional relevant guidances are found in the ICH Safety Guidelines [42] and ICH Quality Guidelines [43]. Additional industry references, for example, reference [44], may also be informative.

Case study: tight junction modulating lipids

Chen *et al.* [45] reported on the identification of lipids that rapidly and reversibly alter TJ permeability in epithelial tissue. As tight junction proteins have been shown to associate with (and in some cases to affect) lipid raft structure, a screen was developed to identify lipids that alter tight junction properties. TER measurement was used to monitor TJ activity on bronchial/tracheal epithelial tissues using a microtiter format (Figure 2a). Toxicity was evaluated using measurements of both cell viability (MTT assay) and release of LDH from damaged cells (Figure 2b).

Among seven groups of lipids tested (sterols, sphingolipids, ceramides, glycosylated sphingosines, alkylglucosides, oxidized lipids and ether lipids), the latter four were identified as tight junction modulators. Individual lipids within these four separate classes showed up to 95% TER reduction at non-cytotoxic concentrations. Cells treated with alkylglucosides, however, showed very high cytotoxicity and low viability at concentrations of 0.2–0.4% (although enhanced transmucosal absorption has been reported [46,47]) compared with the other three lipid classes.

TER reduction was rapid, occurring within 1 min after treatment for the case of 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC). Reversibility of the TER reduction also was observed, with up to 90% recovery occurring within 1 h. Several of the identified lipids also provided enhanced permeation of relatively large molecules such as FITC-labeled dextran (3000 Da) and the therapeutic peptide, peptide YY 3–36 (PYY_{3-36}) (4050 Da).

Immunofluorescence staining of PGPC-treated cells with antibodies against ZO-1, occludin and claudin 4 was used to monitor tight junction structural integrity. These analyses showed no detectable changes in tight junction structural morphology, which is consistent with a non-destructive, submicroscopic alteration in tight junction function commensurate with the TER reduction and permeation enhancement. We note that as compounds become increasingly toxic, changes in TJ integrity become apparent as observed microscopically by immunofluorescence staining.

Case study: PN159, a novel tight junction modulating peptide

Peptides capable of modulating the function of epithelial tight junctions have been described [20,48]. A peptide sequence denoted PN159 was identified as a novel TJM peptide capable of reducing TER across a tissue barrier and increasing paracellular transport of 3 kDa MW dextran with low cytotoxicity and high retention of cell viability. The chemical stability of PN159 under therapeutically relevant storage conditions was established using a stability-indicating HPLC method. Aqueous solutions were stored at various temperature and pH conditions. PN159 was most chemically stable at low temperature and pH; for example, at 5 °C and pH 4.0 or pH 7.3 there was essentially complete recovery of TJM peptide after six months. Therefore, PN159 is chemically stable under storage conditions relevant to IN formulations.

Next, human epithelial tissue (previously shown to provide a good model for IN delivery [49,50]) was employed to examine the behaviour of PN159 *in vitro*. Solutions of a model low-molecular-weight drug (galantamine) in the absence and presence of 25, 50 and 100 μM PN159 were placed on the apical side of the tissue and cell viability, cytotoxicity, TER and drug permeation were monitored. PN159 provided a substantial reduction in TER (Figure 3a), with good cell viability and low cytotoxicity over the conditions studied (Figure 3b). This reduction in TER was consistent with TJ opening, resulting in a 2.4- to 3.4-fold increase in P_{app} for galantamine (Figure 4a).

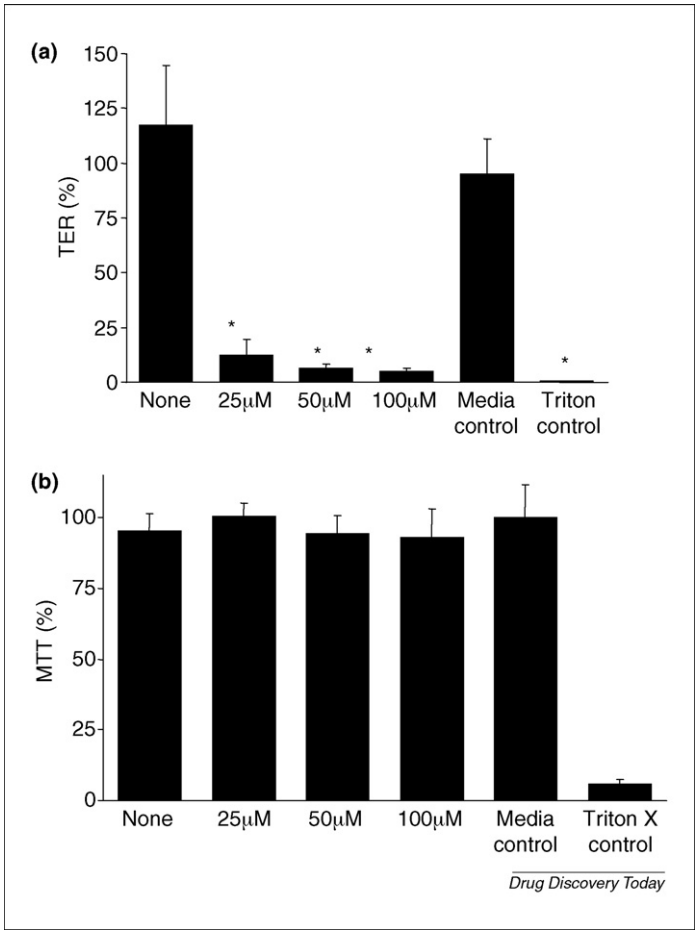

FIGURE 3

In vitro screening of tight junction modulating peptide PN159. **(a)** %TER. **(b)** %MTT. Measurements of transepithelial electrical resistance (TER) and toxicity are described in the text. Data from Chen *et al.* [48].

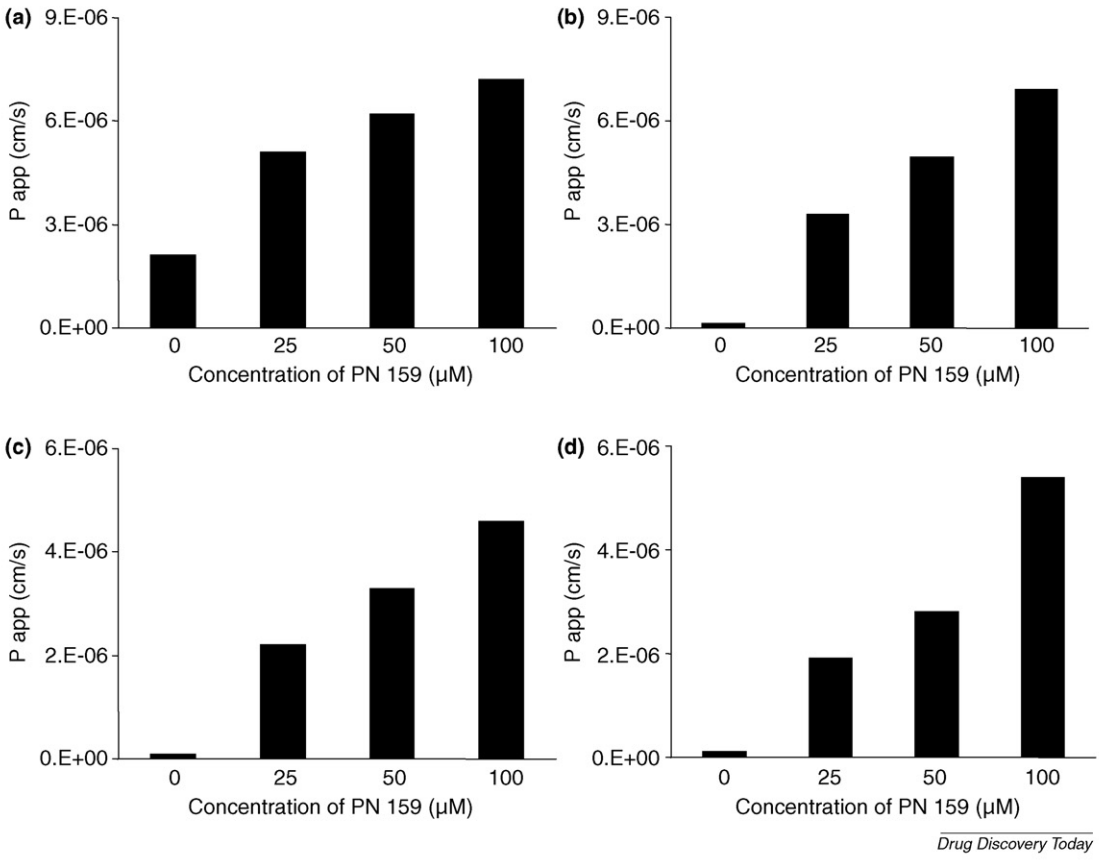

FIGURE 4

Effect of concentration of tight junction modulating peptide PN159 on *in vitro* P_{app}. **(a)** galantamine, **(b)** calcitonin, **(c)** human parathyroid hormone 1–34 (PTH_{1-34}) and **(d)** human peptide YY 3–36 (PYY_{3-36}). Measurements of apparent permeation (P_{app}) are described in the text. Data from Chen *et al.* [48].

Having established the utility of the TJM peptide PN159 for transmucosal formulations of a low-molecular-weight drug, it was important to determine whether the findings were applicable to larger molecules, for example, therapeutic peptides and proteins. For this purpose, salmon calcitonin was chosen as a model therapeutic peptide and tested in the *in vitro* tissue model in the absence and presence of 25, 50 and 100 μM PN159. In the absence of PN159, the P_{app} for calcitonin was 1×10^{-7} cm/s, about an order of magnitude lower than that for galantamine, presumably due to the difference in molecular weight. The data demonstrate a dramatic increase in calcitonin permeation in the presence of the PN159, with up to a 23- to 47-fold increase in P_{app}, compared with calcitonin alone (Figure 4b). It is interesting to note that the fold improvement in P_{app} in the presence of PN159 was more dramatic for salmon calcitonin compared with galantamine (Figure 4a). It is hypothesized that galantamine (a low-molecular-weight compound) has a relatively high P_{app} in the absence of permeation enhancers owing to its low molecular size, even relative to dimensions of the 'closed' TJ pores/channels. There is some, but expectedly limited, improvement in P_{app} when TJs were opened in the presence of PN159. Salmon calcitonin, with a relatively larger molecular size, has limited permeation when TJs are closed. Concomitantly, the presence of PN159 and the opening of TJs results in a dramatic increase in P_{app}.

In order to explore the generality of these findings, two other therapeutic peptides, human parathyroid hormone 1–34 (PTH_{1-34}) and human peptide YY 3–36 (PYY_{3-36}) were evaluated in the *in vitro* tissue model. In the absence of PN159, the P_{app} of these two peptides was consistent with that for calcitonin. In the case of PTH_{1-34}, the presence of PN159 afforded about a 3- to 5-fold increase in P_{app} (Figure 4c); when PYY_{3-36} was formulated in the presence of PN159, the P_{app} was increased about 17- to 45-fold (Figure 4d). These data demonstrate that PN159 has utility for enhancing transmucosal drug delivery for a variety of drug compounds, including various peptides.

Conclusions

The growing knowledge of TJ biology, the development of high throughput cell/tissue based assay systems and use of diverse molecular libraries have led to the identification of promising TJ modulating compounds that safely and reversibly open TJs to enhance tissue permeability and drug transport. New generations of peptide and lipid compounds identified as TJ modulators are amenable to optimization by structure–activity analysis and detailed mechanism studies. This will continue to improve bioavailability and extension of the molecular weight range of drugs that can be delivered effectively for chronic applications. The dynamic nature of tight junctions and the

involvement of specific endocytic pathways and intracellular signaling mechanisms that regulate TJ function offer new possibilities for the development of drugs with more specific mechanisms of action, including the ability to restore normal barrier properties to tight junctions that are dysregulated in disease.

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