Expert Opinion

Advances in nasal drug delivery through tight junction technology

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New approaches for enhancing intranasal drug delivery based on recent discoveries on the molecular biology of tight junctions (TJ) are significantly improving the bioavailability of 'non-Lipinsky' small molecules, and peptide, protein and oligonucleotide drugs. As knowledge of the structure and function of the TJ has developed, so has the ability to identify mechanism-based TJ modulators using high-throughput molecular biology-based screening methods. The present review focuses on recent developments on the TJ protein complex as a lipid raft-like membrane microdomain, the emerging role of unique endocytic pathways in regulating TJ dynamics, and the utility of techniques such as RNA interference and phage display to study TJ components and identify novel peptides and related molecules that can modulate their function. Experimental and statistical methodologies used for the identification of new classes of TJ modulators are described, which are capable of reversibly opening TJ barriers with broad potential to significantly improve intranasal and, eventually, oral drug delivery. The development of an advanced intranasal formulation for the obesity therapeutic PYY_{3-36}, the endogenous Y2 receptor agonist is also reviewed.

Keywords: calcium, claudin, design of experiments, E-cadherin, endocytosis, epithelium, formulation development, intranasal drug delivery, junctional adhesion molecule, lipid rafts, Lipinsky's rule of five, obesity, occludin, paracellular transport, permeation assays, phage display, PYY_{3-36}, RNA interference, tight junction, transepithelial electrical resistance

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1. Introduction

The nasal mucosa is an increasingly attractive site for the systemic administration of drugs [1-3] using a formulation (i.e., a solution of components designed to stabilise, solubilise and enhance drug permeation) and a spray device. It has a relatively high surface area, good vascularity and responds to reversible modification using permeation enhancing formulations for safe and effective drug transport. In particular, it offers an improved delivery route for non-Lipinski small molecules and true macromolecules [4,5], and can avoid problems associated with oral and intravenous drug delivery. Effective delivery has been a major impediment to the development of the full potential of non-Lipinski small molecules, and of protein, peptide and oligonucleotide drugs.

Intranasal delivery has several advantages, including:

- delivery of a wide range of therapeutics (small and large molecules)
- a rapid onset (time of maximum blood concentration [T_{max}]) of adequate blood levels (maximum concentration [C_{max}])
- no first pass hepatic metabolism or mechanistically based gastrointestinal toxicity from high mesenteric drug concentrations
- more flexible dosing schedules and control of drug effects
- greater patient comfort and convenience
- no pulmonary toxicity
- less drug degradation

- the potential for direct delivery to the brain through the olfactory region [6-8]

Over the past two decades, intranasal delivery technology has progressed from the use of simple formulations suitable for small molecule drugs to the inclusion of permeation enhancers with excellent safety profiles to facilitate delivery of high-molecular-weight compounds. Advances in tight junction (TJ) biology now make possible the development of mechanism-based tight junction modulators (TJMs) that in the near future may replace complex formulations with single molecule compounds possessing greater ability to enhance systemic drug delivery [9].

TJs are structures that form a barrier between adjacent epithelial cells with a narrow band just beneath the apical surface, and are found in all tissues of the body. They perform two vital functions: as a barrier or gate to the movement of molecules between cells in the paracellular space, and as a fence to prevent diffusion of integral membrane proteins between the apical and basolateral surfaces, thus preserving, for example, the special functions of receptor-mediated endocytosis at the apical surface and exocytosis at the basolateral surface. High-molecular-weight drugs need to pass through TJ barriers in order to become systemically available and to move to their sites of action. As part of the body's normal activity, TJs selectively modulate paracellular permeability by opening and closing in response to various signals inside and outside of cells, including responding to cytokines, immune cells, nutrients, calcium depletion and lipid-modifying agents. TJs consist of a variety of integral membrane and peripheral, or associated, proteins, which are anchored in the membranes of two adjacent cells and interact across the paracellular space by noncovalent forces. In the cytoplasm, TJ membrane proteins interact with scaffold proteins to connect them with the cellular cytoskeleton and various signal transduction and transcriptional pathways involved in the regulation of TJ function. Dysregulation of TJ function occurs in a variety of diseases [10,11], particularly inflammation, cancer and CNS pathologies where normal tissue permeability and cell adhesion interactions are altered [12-14].

2. Transmembrane proteins of the junctional complex

Epithelial cells characteristic of the nasal mucosa, like other tissues, are joined together by TJs. These are schematically represented in **Figure 1**. The closely associated adherens junction (AJ; not shown) is found on the basolateral side, but is not circumferentially continuous, like the TJ, and therefore does not contribute significantly to the epithelial barrier properties. The cell membranes of adjacent cells are intimately connected by proteins of the junctional complexes, to an extent that one can measure a significant transepithelial electrical resistance (TEER). Freeze fracture electron microscopy (but not thin sections) visualises TJs as a network of strands that appear as rows of 10-nm particles within the plane of the plasma membranes of neighbouring cells [15,16]. These strands have been predicted to contain pores that dynamically open and close [17].

Three major types of integral membrane proteins comprise TJs: occludin, claudins and junctional adhesion molecules (JAM) [18-20]. These junctional proteins are involved in cell–cell adhesion and are dynamically regulated. A group of cytoplasmic scaffolding proteins, including a family of zonula occludens (ZO) proteins, connect TJs and AJs to the cytoskeleton and mediate intracellular signalling (**Figure 1**). A number of additional cytosolic and nuclear proteins interact directly or indirectly with TJ scaffolding proteins and are also involved in regulating diverse functions including paracellular permeability, cell polarity, gene transcription, tumour suppression and cell proliferation.

Occludin is a 65-kDa tetraspan integral membrane protein with two extracellular loops separated by a short cytoplasmic loop, with both N- and C-terminal regions located in the cytoplasm. Occludin is phosphorylated at serine/threonine residues in the C-terminal region and appears to be preferentially expressed in TJs, whereas the nonphosphorylated form may reside along the basolateral membrane [21]. Tyrosine phosphorylation of occludin is associated with recovery of cells from ATP depletion or during calcium repletion, in parallel with the establishment of TEER [22].

Claudins are a family of 24 isoforms of a tetraspan membrane protein ~ 23 kDa in size with two extracellular loops, short cytoplasmic N- and C-termini, and no sequence homology to occludin. The C-termini of most claudins end with tyrosine and valine residues comprising a site that mediates an interaction with the plaque proteins ZO-1, -2 and -3 via PDZ binding domains [23]. Epithelial and endothelial TJs of various tissues are distinguished by different claudin isotypes, which define the physiological characteristics of tissue barriers [24]. Claudins may be the single most important component of the TJ because they alone can form TJ strands [25]. Claudins play a key role in regulating ion flux as major components of paracellular channels, where individual claudins function as ion-specific pores [26-28].

JAM-1, more recently designated JAM-A, is a 43-kDa glycosylated protein belonging to the IgG superfamily [29,30]. It is characterised by two extracellular V-type Ig domains, a single transmembrane domain and a short intracellular C-terminus, which binds the PDZ domain of ZO-1. JAM-1 forms homophilic contacts between the first V-type Ig domains of apposing JAM-1 molecules. JAM-1 facilitates the migration of leukocytes through the paracellular pathway of endothelial cell layers [31,32] and constitutes the receptor for coxsackie and adenoviruses, and reovirus [33]. Although JAM-1 is localised at the TJ, it may not be an integral component, but rather is indirectly tethered to claudins via ZO-1. Other known PDZ partners include Afadin/AF6, CASK/Lin2, MUPP1, and Par3 [29]. The use of monoclonal antibodies and calcium switch experiments combined with phage display analysis indicates

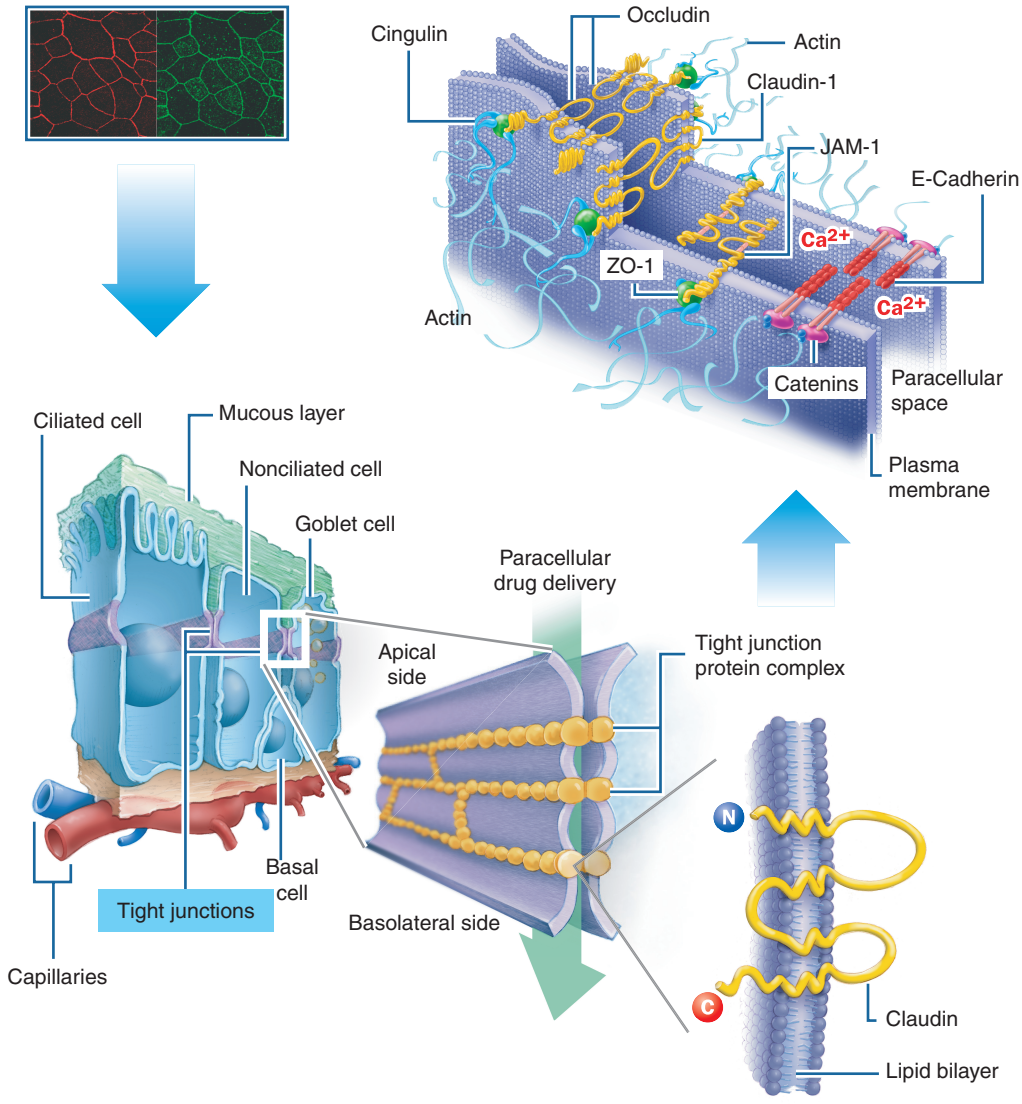

Figure 1. Cellular and molecular anatomy of tight junctions. The lower part of the diagram represents tissue structure showing several cell types (left), with a progressively more molecular representation of the tight junction, a claudin molecule in the plasma membrane (right) and the structure of several important tight junction proteins and their connections to scaffolding proteins and actin (upper right); JAM-1 and E-cadherin are located on the basolaterol side of occludin and claudin. The microscopic picture (upper left) shows the 'chicken wire'-like appearance of tight junctions looking down from the apical side of the tissue as visualised by specific tight junction antibody stains against claudin 4 and ZO-1.
JAM: Junctional adhesion molecule; ZO: Zonula occluden.

that a region located within the N-terminal Ig-like loop (residues 111 – 123), which includes residues within the putative homodimer interface as determined by X-ray crystallography, is critical for JAM-mediated regulation of TJs and leukocyte transmigration [34].

E-cadherin is a 120-kDa transmembrane protein, which is a constituent of the AJ. It is important for initiating and maintaining cell–cell contacts and is required for the formation and maintenance of TJs. It is a dimer that mediates calcium-dependent cell adhesion by binding to actin through catenin accessory proteins. Modulation of E-cadherin function affects tumour invasiveness, metastasis and inflammatory processes [35,36].

In polarised epithelial cells, cell–cell adhesion forms specialised membrane structures consisting of claudin-based TJs and E-cadherin-based AJs aligned from the apical to the basal side of the lateral membrane. Nectin is a calcium-independent immunoglobulin-like cell–cell adhesion molecule, which localises at AJs and is associated with E-cadherin through their respective cytoplasmic tail-binding proteins, afadin and catenins. The nectin afadin system may play an important role in the organisation of TJs as well as AJs in

epithelial cells [37]. In addition, PAR-6, protein kinase C-α (PKC-α) and PAR-3 are cell polarity proteins that also cooperate in the establishment of cell–cell adhesion-induced epithelial junction complex [38].

3. Molecular structure and function

3.1 Tight junction proteins in lipid raft-like membrane microdomains

Recent work indicates that TJ proteins may be localised in membrane microdomains, regions of the plasma membrane endocytic compartment, which are detergent-insoluble rafts highly enriched in cholesterol and sphingolipids compared with the rest of the plasma membrane [39]. Detergent insolubility may result from tight packing of the acyl chains of lipids in the liquid ordered phase that enhances lipid interactions and excludes detergents. Hyperphosphorylated occludin and ZO-1 appear to be localised with caveolin in detergent insoluble membrane microdomains in T84 epithelial cells [40]. Disassembly of TJs by either chelation of extracellular calcium [41], or *Clostridium difficile* toxins [42] results in their displacement from membrane microdomains, by a caveolin-mediated mechanism. It was further demonstrated that major pools of hyperphosphorylated occludin and ZO-1 are found in raft-like membrane microdomains with characteristics of detergent-insoluble glycolipid rafts. TJ disassembly by calcium chelation resulted in displacement of TJ proteins from the raft-like compartment.

3.2 Intracellular signalling and the role of calcium

Intercellular junctions not only have adhesive functions that provide barriers to paracellular transport of molecules and cells, and a fence function preserving cellular polarity, but also contain important components involved in signal transduction pathways, which regulate epithelial proliferation and differentiation [41,43]. Signals are transmitted from the cell interior to TJs and regulate assembly and function. Calcium-dependent E-cadherin-mediated cell–cell adhesion functions as the initial signal that triggers the assembly of all intercellular junctions. Several different signalling pathways and proteins are involved including protein kinase A (PKA), monomeric and heterotrimeric G proteins and PKC isotypes. The Rho-family GTPases may be of particular importance in the regulation of paracellular permeability by mediating changes in the actin-cytoskeleton through changes in the activities of myosin light chain phosphastase and kinase and modulation of occludin phosphorylation. TJs have been linked to Ras, known to mediate growth factor and extracellular matrix signalling with major influences on cell–cell adhesion. The Raf pathway, for example, is activated by Ras and regulates cell cycle entry.

Calcium is important for maintaining cell–cell junctions in epithelial and endothelial cells [44]. Reduced extracellular calcium decreases TEER and increases paracellular transport of fluorescein isothiocyanate (FITC)-labelled dextran 4000 and small proteins; restoration of calcium reverses these effects. Low calcium may disrupt the AJ by removing calcium from binding sites on E-cadherin extracellular domains, which stabilise the dimeric structure important for cell adhesion. TJs may also be affected by the loss of membrane association of ZO-1, -2 and occludin. Klingler *et al.* [45] have shown that when calcium is removed in the presence of PKA inhibitors, barrier properties are preserved, suggesting a role for PKA in modulating calcium-dependent TJ and AJ function. The effect of low calcium on TJ can be overcome by diacylglycerol-mediated activation of PKC [46], implicating PKC phosphorylation in calcium-modulation of TJ function. Intracellular calcium is also important for TJ integrity. In contrast to extracellular calcium chelation that does not alter ZO-1/actin interactions, lowering intracellular calcium affects ZO-1/actin binding and changes the subcellular localisation of occludin [47].

3.3 Role of endocytosis in junctional dynamics

Recent studies suggest that the junctional complex consisting of AJ and TJ is a surprisingly dynamic structure that can be rapidly disassembled and reconstituted in response to specific extracellular stimuli [48]. These studies further indicate that regulation of TJ proteins may involve an endocytic process. Endocytosis is a cellular process by which integral membrane proteins can be internalised and directed to specific cellular compartments. Discrete regions of the membrane invaginate into the cytoplasm as vesicles in an energy-dependent process requiring dynamin GTPase [49,50].

Matsuda *et al.* [51] have demonstrated this dynamic behaviour of TJs, particularly claudins, in cultured epithelial cells expressing claudin-3 fused at its N-terminus to green fluorescent protein (GFP), allowing visualisation of its behaviour by immunofluorescence microscopy. Tightly opposed membranes of TJs were observed to be endocytosed together into one of the adjoining cells without disruption of the paracellular claudin homodimer structures, yielding one cell with claudin-containing vesicles and the adjacent cell losing its TJ claudins. During internalisation, claudin was segregated from other TJ components (occludin, JAM and ZO-1) to generate claudin-enriched vesicles.

E-cadherin is constitutively internalised in confluent madin darby canine kidney (MDCK) cells and recycled back to the cell surface by exocytosis [52]. This process is increased in pre-confluent cells as well as after depletion of extracellular calcium, which is required for the stabilisation of E-cadherin-mediated cell–cell interactions. PKC was found to regulate endocytosis and recycling of E-cadherin [53]. Treatment of cells with phorbol esters increased the rate of endocytosis of E-cadherin, as well as decreasing its recycling back to the surface.

Kartenbeck *et al.* [54] found that when calcium ions were depleted from culture medium, the opposed membranes of AJs were endocytosed into their own cytoplasm. Recently, Ivanov *et al.* [55] used a model of calcium depletion to define the pathway of internalisation of AJ and TJ proteins

(E-cadherin, p120 and β-catenins, occludin, JAM-1, claudins 1 and 4 and ZO-1) in T84 epithelial cells. Using protease protection assays, TJ components lost sensitivity to degradation under conditions of calcium depletion and redistributed into a subapical intracellular compartment where condensed patches of E-cadherin, β-catenin, JAM-1 and ZO-1 were observed under the apical F-actin. Characterisation of the early endocytic pathway involved in this internalisation ruled out caveolae/lipid rafts and macropinocytosis, but showed that endocytosis of TJ and AJ after calcium depletion occurred via a clathrin-mediated pathway. At later stages of calcium depletion there was a loss of localisation of TJ and AJ proteins with early endosomes, an increased sensitivity to methyl-β cyclodextrin (MβCD) suggesting a later involvement of cholesterol-dependent events, and colocalisation with syntaxin-4 (TJ and AJ proteins) and Na^+K^+ATPase (AJ proteins only). Internalised junctional proteins were also shown to recycle back to the apical region of the lateral membrane.

Although clathrin-mediated endocytosis of E-cadherin occurs in MDCK cells [52], clathrin may not mediate junctional endocytosis in stratified epithelia. Establishment of cadherin-dependent cell–cell contacts in human epidermal keratinocytes is known to be regulated by the Rac1 small GTP-binding protein. Akhtar and Hotchin [56] utilised GFP-tagged Rac1 expression vectors to examine the subcellular distribution of Rac1 and its effects on E-cadherin-mediated cell–cell adhesion. Expression of GFP-L61Rac1 inhibited the uptake of transferrin-biotin, suggesting that endocytosis of E-cadherin was a clathrin-independent mechanism. This was supported by the observation that caveolin, rather than clathrin, localised around these structures. Furthermore, an inhibitory form of dynamin, known to inhibit internalisation of caveolae, inhibited formation of these cadherin vesicles.

4. Factors that influence intranasal drug absorption

Nasal drug permeability is affected by a variety of structural, biochemical, and physiological properties of the nasal mucosa, as well as the physicochemical characteristics of the drug and the formulation [2]. Depending on the lipophilicity of the compound, drugs may permeate nasal tissue through the transcellular route (characteristic of hydrophobic small-molecule drugs) or the paracellular route through TJs (common for more hydrophilic and/or polypeptide compounds in the presence of permeation enhancers). Small-molecule drugs, as well as the individual components of a formulation (i.e., those ingredients that solubilise, stabilise and enhance permeation of the drug) may be metabolised in the nasal mucosa, whereas peptides and proteins may be subject to protease degradation [57,58].

Mucus is released by goblet cells of the upper respiratory part of the nasal cavity and provides a protective, viscous layer ~ 5 μm thick, which bathes the cilia. Mucocilliary clearance (MCC) refers to the action of the cilia in transport of mucus along with dissolved substances (e.g., allergens, bacteria, viruses, toxins) toward the nasopharynx for eventual discharge into the gastrointestinal tract. For the MCC to be effective, the cilia must beat in a coordinated fashion, an energy-dependent process involving microtubule mechanics [59]. Calcium ion appears to strongly affect ciliary motility. Increased calcium influx increases beat frequency and the reduction of extracellular calcium results in the loss of ciliary beating, whereas repletion of calcium restores beating. Calcium channel blockers, such as verapamil, reduce ciliary beat frequency [60].

4.1 Permeation enhancers

There are many groups of excipients used in formulation development [61,62]. Permeation enhancers that have been evaluated in the past for use in nasal formulations include surfactants, bile salts, fusidates, chelators, fatty acid salts, phospholipids, glycyrrhetinic acid derivatives, cyclodextrins, alkyl glycosides and chitosan. For many compounds, including surfactants and bile salts, there appears to be a direct correlation between absorption promotion and local toxicity involving cellular damage [63]. For some compounds, altered TJ function may be involved.

Francis *et al.* [64] have shown that MβCD, a cholesterol solubilising agent, reversibly alters TJ barrier function by stimulating cholesterol efflux. MβCD (20 mM) in both apical and basolateral media reduced cholesterol levels by 70 – 80% with no significant effect on cell viability. Recovery of cholesterol content to initial values was nearly complete 22 h after the removal of MβCD. A decrease in immunostaining for occludin and ZO-1 at TJs indicated that changes in cytoskeletal organisation during long incubations with MβCD had physically disrupted the TJ network. The observed changes in paracellular permeability during cholesterol efflux may be related to increased levels of lipid-derived second messengers, some of which may trigger changes in the phosphorylation status of TJ proteins.

Dodecylphosphocholine (DDPC) can improve the paracellular permeability of hydrophilic compounds across Caco-2 cell monolayers by modulating TJs [65,66]. The alkyl chain as well as the zwitterionic head group of DDPC are required for its activity. DDPC appears to act by modulating the permeability of TJs by a mechanism that results in decreased TEER, increased permeability of paracellular markers (e.g., mannitol) with no change in the permeability of the transcellular marker testosterone, and redistribution of the TJ-associated protein ZO-1. The effect of DDPC on Caco-2 cells (e.g., decrease in TEER) is reversible, and is not caused by gross cytotoxicity.

Tomita *et al.* [67] studied the mechanism of paracellular permeability by EDTA, sodium caprate (C10) and decanoylcarnitine (DC). The results suggested that EDTA activates PKC by depletion of extracellular calcium via chelation resulting in expansion of the paracellular route, C10 increases the intracellular calcium level by an interaction with the cell membrane independent of cell polarity resulting in contraction with actin microfilament, and DC interacts specifically with the apical membrane to increase the intracellular calcium level.

Chitosan is a de-acetylated D-glucosamine polymer derived from chitin, a component of the exoskeletons of crustaceans (shrimp, crab and other shellfish), and has been tested as an excipient for transepithelial drug delivery systems. It has been shown to disrupt intercellular TJs, thus increasing permeability by translocation of TJ proteins from the membrane to the cytoskeleton [68]. Immunofluorescent localisation of ZO-1 revealed loss of membrane-associated ZO-1 and occludin from the cytosolic and membrane fractions into the cytoskeletal fraction.

Ohtake *et al.* [69,70] evaluated the ability of poly-L-arginine (poly-L-Arg) to increase the paracellular permeability of hydrophilic macromolecules across rabbit nasal epithelium. Apical poly-L-Arg appeared to increase predominantly the paracellular transport via disorganisation of TJ and AJ proteins. TJ disassembly involved both serine/threonine phosphorylation of ZO-1 by PKC activation, and tyrosine dephosphorylation of occludin, and their subsequent internalisation.

Leroy *et al.* [71] have demonstrated that alkylphospholipids can alter the E-cadherin complex and the TJ. Incorporation of alkylphospholipids into the plasma membrane lipid bilayer causes a rapid and reversible decrease in TEER concomitant with increased paracellular permeability. Unlike MβCD, alkylphospholipids do not specifically displace lipids from raft-like membrane domains, but do change the detergent-solubility of ZO-1 and occludin.

Ahsan *et al.* [72] evaluated the effects of permeability enhancers, tetradecylmaltoside (TDM) and MβCD, on insulin movement across human bronchial epithelial cell monolayers (16HBE14o$^-$). Both agents decreased TEER and increased manitol permeability when applied to the apical side of cell monolayers, but only TDM was effective in enhancing permeability of insulin to the basolateral side, which was also accompanied by some degradation.

5. Experimental models and methods for studying intranasal drug delivery

The main barriers opposing nasal drug delivery are the mucus layer lining the nasal cavity, the mucociliary clearance mechanism, and the mucosal TJs. Nasal absorption studies can be performed with a variety of animals [73-76], mounted tissue strips [77], and tissue/cell culture models. Tissue and cell culture models have the advantage of being able to use human primary cells of nasal origin in a high-throughput monolayer format; they provide direct access to both the apical and basolateral tissue surfaces for evaluation of drug transport pathways and permeation mechanisms. Cultured tissue monolayers have longer viability compared with isolated tissue strips, provide more sensitive evaluation of inhibitory compounds and toxicity effects, are amenable to mechanistic analysis of transport and metabolism under conditions of genetic manipulation of the cell population and minimise time-consuming, expensive animal studies. Of particular importance is the need to correlate results of cell/tissue culture models with the behaviour of formulations and individual excipients in appropriate animal models, which are ultimately required for regulatory approval and safety/efficacy studies in humans.

5.1 Tissue and cell culture

A variety of human epithelial cell lines have been used to study the mechanisms of drug mucosal absorption, including the colon carcinoma cell line model (Caco-2) [78] and recently a bronchial epithelial cell line 16HBE14o$^-$ [79,80]. These models retain their cell shape, thickness and physiological properties, such as TEER and permeability, and the ability to grow in confluent monolayers of polarised epithelial cells. The RPMI 2650 cell line originated from human nasal septum tumour tissue [81], but represents the undifferentiated state compared with 16HBE14o$^-$ (differentiated). Primary human nasal epithelial cells have been used successfully to study nasal absorption [82], but are very difficult to culture.

An established source of primary human cells for drug absorption and formulation development studies is the Epi-Airway™ System (MatTek Inc., MA, USA) [201], which consists of normal, human-derived tracheal/bronchial epithelial cells cultured to form a pseudostratified columnar epithelial cell layer, a highly differentiated model, which forms TJs similar to the respiratory epithelium found in the nasal cavity. Transmission electron microscopy shows numerous microvilli and cilia on the apical surface of the cultures and confirms the presence of TJs. In addition, mucus is secreted from the apical surface of these tissue cultures.

A variety of methods are used to evaluate the properties of cultured tissue/cells, which measure the integrity of TJs, absorption properties of epithelial tissue barriers and potential toxicity associated with treatment conditions [83,84].

5.2 Transepithelial electrical resistance

TEER is a measure of the integrity of TJs (paracellular gate function) and is observed to increase as TJs form when cells grow to confluence. It is calculated from the measured potential difference between the apical and basolateral sides of the cell monolayer and is inversely related to ion flow through the TJs and the cell membrane of the monolayer. TEER is a measure of instantaneous ion flow through the paracellular pathway. The contribution of ion pumps, ion transporters and ion channels are measured separately by other methods. The relative contribution of these pathways varies according to the type of epithelium, although ion flow in tissue monolayers is the dominant process reflecting changes in paracellular permeability. TEER and permeability may not be inversely correlated in a simple way (unpublished results). A change in the properties of the TJ complex can have a major effect on TEER with either minor or major affects on permeability of a peptide/protein drug, depending on the mechanism of the permeation enhancer or TJ modulator that is used in a formulation.

5.3 Permeation assays

Permeation across a cell monolayer of a drug or hydrophilic marker such as radiolabelled mannitol or a fluorescent-labelled macromolecule is a measure of the functional restriction to paracellular transport through the TJ and its structural integrity. Sodium fluorescein (molecular weight 376 Da) and FITC-dextran (available in approximate molecular weight fractions of 4000 – 500,000 Da) are routinely used as model compounds to estimate the size dependence of hydrophilic macromolecule transport [85]. The permeability coefficient, like TEER, is an indicator of monolayer integrity [86,87].

5.4 Immunofluorescence and confocal microscopy

The general morphological properties and integrity of monolayers used in TEER and permeability studies and the distribution of cytoskeletal and TJ-associated proteins can be characterised by fluorescence microscopy [84]. Fluorescence markers are used to monitor changes in plasma membrane or TJ permeability, as well as to follow the distribution and localisation of labelled formulation components and TJ modulating compounds. As shown in **Figure 1** (top), 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. Live cell imaging is preferable whenever possible, as fixation procedures can give misleading or artifactual results.

5.5 Intramembrane lipid diffusion assays

Analysis of the fence function of TJs (prevention of the intermixing of lipids and integral membrane proteins in the outer leaflet of the plasma membrane) can be achieved using methods that allow visualisation or measurement of lipid diffusion from one cell surface domain to the other [84]. This is done by labelling one side of the monolayer with a fluorescent lipid, such as BODIPYL-FL-C5-sphingomyelin, which does not 'flip-flop' between the inner and outer membrane leaflets. For example, the apical domains of insert-grown MatTek cells labelled with BODIPYL-FL-C5-sphingomyelin–bovine serum albumin complexes, washed and mounted on microscope slides in the presence or absence of test compound (or EDTA control), are observed by confocal microscopy and the diffusion of labelled lipid from the apical to basolateral cell surface is evaluated.

5.6 Cytotoxicity assays

The development of nasal delivery formulations and the identification and optimisation of new TJ modulators requires that potential toxicity effects are identified and monitored early in the process along with drug transport activity parameters, such as permeation. Two convenient assays, which are adaptable to high-throughput screening are: the dimethylthiazol diphenyl terazolium bromide (MTT) assay, which measures cell metabolic activity by spectrophotometric quantitation as an end point for cell proliferation and viability [88]; and the lactate dehydrogenase (LDH) assay, which measures plasma membrane permeability based on the release of LDH from damaged cells [89].

6. Identification of tight junction modulators

With the advances in TJ biology, cell-based models and assay systems for the screening and characterisation of compounds that modulate TJ function, it is now possible to develop mechanism-based TJMs with improved drug delivery properties. In principle, TJMs could operate through one of several mechanisms; for example, downregulation of TJ protein expression, physical inhibition of TJ interactions, modification of intercellular signalling, or through membrane-mediated changes, such as alteration of endocytic pathways. Examples of TJ modulation that result in increased paracellular permeation include the action of synthetic peptides derived from the extracellular domains of occludin [90] and a C-terminal fragment of *Clostidium perfringens* enterotoxin as a modulator of claudin 4 [91].

A number of experimental approaches can be used to identify those TJ targets most suitable for pharmacological manipulation to enhance paracellular drug delivery:

- gene expression profiling can be used to determine which TJ genes are expressed in a given tissue or cell line, and in particular, which TJ proteins are specifically involved in the formation of TJs
- RNA interference (RNAi) technology can be used to perform expression knock down studies and thus help to evaluate which TJ proteins are important to TJ function, and may be modified to enhance drug delivery
- gene cloning and expression can be used to produce pharmaceutical quantities of the most important TJ proteins as high-throughput screening reagents for screening and excipient formulation development
- phage display technology can be used to generate peptide libraries for selecting peptides that bind and alter the function of TJ components
- high-throughput tissue culture assays are important for identifying compounds that reversibly open TJs and increase drug permeation

6.1 Gene expression profiling and the identification of tight junction targets using RNA interference

Gene expression can be evaluated by a variety of techniques including high-density microarray hybridisation [92] and high-throughput quantitative multiplex polymerase chain reaction (PCR)-based methods for mRNA analysis [93]. Functional analysis of individual genes can be studied using RNAi technology. Small interfering RNAs (siRNAs) are double-stranded RNA molecules 20 – 22 nucleotides in length, which are able to silence a single gene in a sequence-specific manner by inducing specific degradation of the target mRNA via a sequence-guided RNA silencing complex (RISC) [94]. By

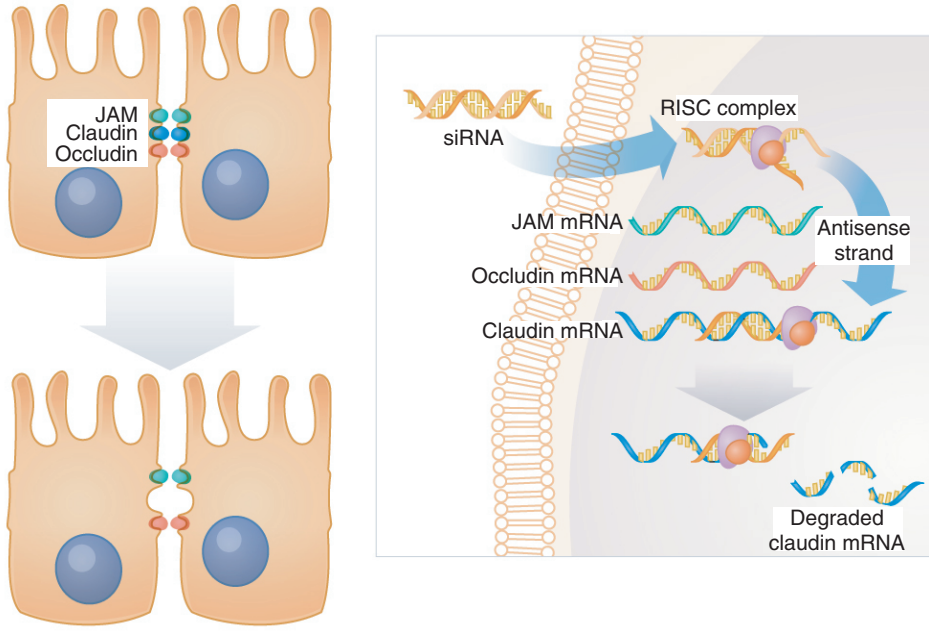

Figure 2A. The application of RNAi technology in TJ function analysis. siRNA against the mRNA of specific TJ proteins (e.g., claudin) are transfected into cells, resulting in target mRNA degradation and subsequent loss of protein from the TJ.
JAM: Junctional adhesion molecule; RISC: RNA silencing complex; RNAi: RNA interference; siRNA: Small interfering RNA; TJ: Tight junction.

introducing siRNA into epithelial cells or monolayers targeting the mRNA of a given TJ protein, the importance of individual TJ proteins on paracellular gate, fence and transport properties can be evaluated (**Figure 2A**).

Dutzar *et al.* [95,96] analysed the differential expression and function of TJ genes in normal (differentiated) and undifferentiated respiratory epithelial cells. Claudins 1, 3, 4, 7, 9, 12 and 20 showed high levels of RNA expression, whereas claudins 2, 5, 6, 8, 10, 11 and 14 – 19 were low or undetectable. Claudins 1, 12 and 20 were expressed in both differentiated and undifferentiated tissue. Claudins 3 and 4 were expressed only in differentiated tissues. The effects of siRNA knockdown of claudins 1, 3, 4, 9, 12, 20, JAM-1 and occludin were evaluated in functional assays for TJ formation and permeability. Knockdown of claudin 4 inhibited TJ formation and resulted in a significant decrease in TEER and an increase in dextran-4000 permeability. Lesser effects were observed for occludin and JAM-1 knockdowns, whereas inhibiting claudin 12 expression had little effect. Combination knockdowns between claudin 4 and occludin or JAM-1 showed synergistic effects.

6.2 Expression, purification and characterisation of tight junction proteins

So far, only the crystal structure of human JAM-1 extracellular domain has been solved [97], whereas structural information on other TJ components is largely unknown. In order to study the specific protein–protein interactions between and among claudins expressed in epithelial cells and to obtain quantities of TJ proteins for structural studies, phage display targets and development of antibody reagents, Chen *et al.* [98] carried out expression and purification studies of TJ proteins in the baculovirus system using poly-histidine as an N-terminal tag to facilitate purification. Nine TJ proteins including claudins 1, 3, 4, 5, 7, 9, 12, occludin and JAM-1 have been produced, most to > 95% purity, thus allowing protein–protein interaction studies, structural analysis, and excipient screening for TJ modulators.

6.3 Phage display and the identification of peptides that bind to and modulate tight junction components

Phage display is a powerful method for the selection of peptide ligands with affinity for selected targets [99]. This method utilises the ability of bacteriophage (phage) to express random sequence libraries of peptides, ranging from 7 to > 20 residues in length, which are displayed on the exterior of the phage where they are accessible for screening against target molecules. Libraries displaying billions of diverse peptides can be generated by cloning randomised oligonucleotides into the phage. Ligands that bind to the target molecule (e.g., a purified TJ protein or epithelial cell monolayer) are identified by a process (panning), in which a library is placed in contact with the target; phage that display nonbinding peptides are removed by washing, and bound phage are recovered by elution from the target and amplified (**Figure 2B**). The panning process is repeated for

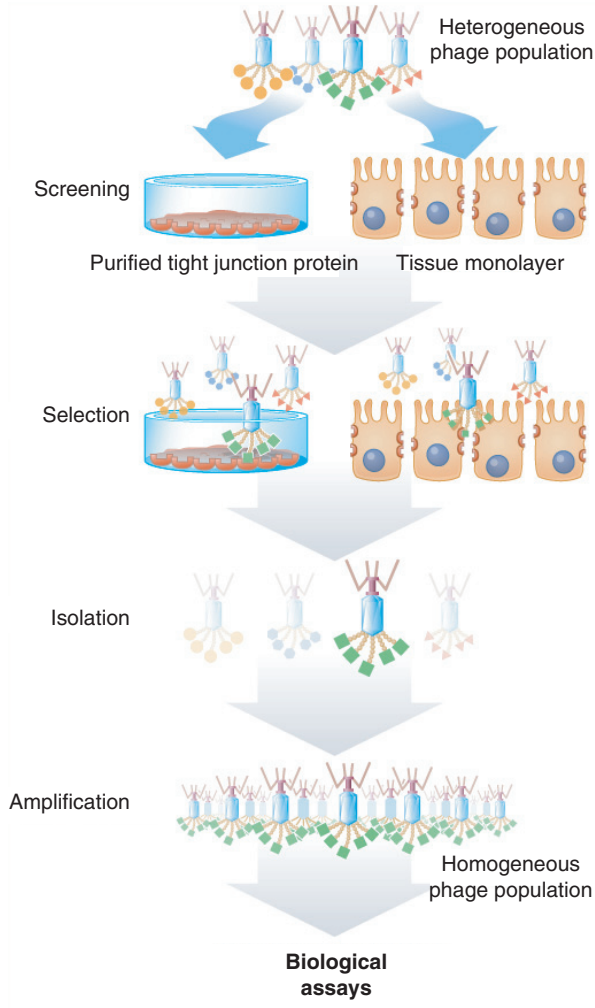

Figure 2B. The application of phage-display technology in the identification of tight junction modulator peptides. Peptide libraries expressed on bacteriophage are used to select for peptides that bind specifically to tight junction proteins as described in the text.

several rounds to enrich for phage displaying peptides that have binding affinity for the target. After panning, the amino acid sequences of the peptide ligands are then determined indirectly by DNA sequencing. Peptides derived from the biopanning process are then synthesised by standard solid-phase Merrifield synthesis techniques and further testing is performed.

Phage display was used to search for peptides that bind to and modulate TJs of epithelial cells. Panning was performed [99] using monolayers derived from human bronchial epithelial cells (16HBE14o$^-$) and a commercially available phage library displaying peptides consisting of seven random amino acids (New England Biolabs, MA, USA). In order to make the TJs more accessible during panning, a calcium switch step was performed, involving the treatment of cell monolayers possessing well-established TJs with EGTA. Subtractive panning against untreated cell monolayers was also performed to remove phage displaying peptides that bind to areas of the cells other than the TJ complex. This approach has led to the identification of TJ binding/modulating ligands.

For example, the ability of a peptide, identified by phage display, to open TJs was tested with monolayers of epithelial cells (MDCK) by monitoring TEER [100]. The peptide was synthesised in linear and cyclic versions with lysine residues (and a glycine linker) added to improve solubility. As shown in **Figure 3**, the cyclic form of the peptide (PN78) reduced TEER in a dose-dependent manner and was reversible within 2 h. The linear form (PN80) had a relatively small effect at the highest concentration tested. A scrambled cyclic sequence (PN76) and the control peptide Lys-Lys-Lys-Lys-Lys-Lys-Lys-Gly-Gly (data not shown) had no effect on TEER. These results demonstrate that the cyclic form of peptide PN78 can affect TJ integrity in a reversible process and is a potential candidate TJM. Finally, the fact that different peptides, although having similar potencies for reducing TEER, can differ significantly in the extent of permeation of different size molecules was noted (unpublished observations).

6.4 Identification of peptides that modulate epithelial tight junctions

In addition to phage display, various peptide synthesis and screening paradigms can be used to identify potential TJ modulators. Prieve *et al.* [101] used three different cell lines: MDCK (kidney epithelial cells), Caco-2 (intestinal epithelial cells), 16HBE14o$^-$ (respiratory epithelial cells), and primary respiratory epithelial tissue (EpiAirway), as model systems to screen a custom library of peptides designed based on a wide range of structural and physical–chemical properties; for example, variation of helicity and hydropathy. Compounds were characterised by their effects on TEER, the passage of different molecular weight FITC-dextrans through tissue monolayers, and cytotoxicity assays. Transcellular and paracellular transport pathways were differentiated by observing the behaviour and localisation of fluorescent-labelled compounds by microscopy. A small subset of peptides was found to reduce TEER in the concentration range 2 – 500 µM. TEER changes were observed as early as 15 min after treatment followed by rapid TEER recovery. Peptide effects on TJ structure were characterised by fluorescence microscopy analysis of immunostaining patterns of specific TJ components and electron microscopy. **Figure 4A** shows results for one promising class of small peptides comparing its ability to affect TEER and tissue monolayer permeability. The peptide designated PN159, shows a dose-dependent reduction in TEER with a rapid onset and quick recovery of TEER on removal. A specific structural modification (PNC-10) resulted in loss of activity. PN159 also demonstrated an ~ 25-fold increase in permeation of dextran-4000 compared with a PBS control formulation (**Figure 4B**) and low activities in the MTT and LDH toxicity assays (data not shown). This class of compounds and others

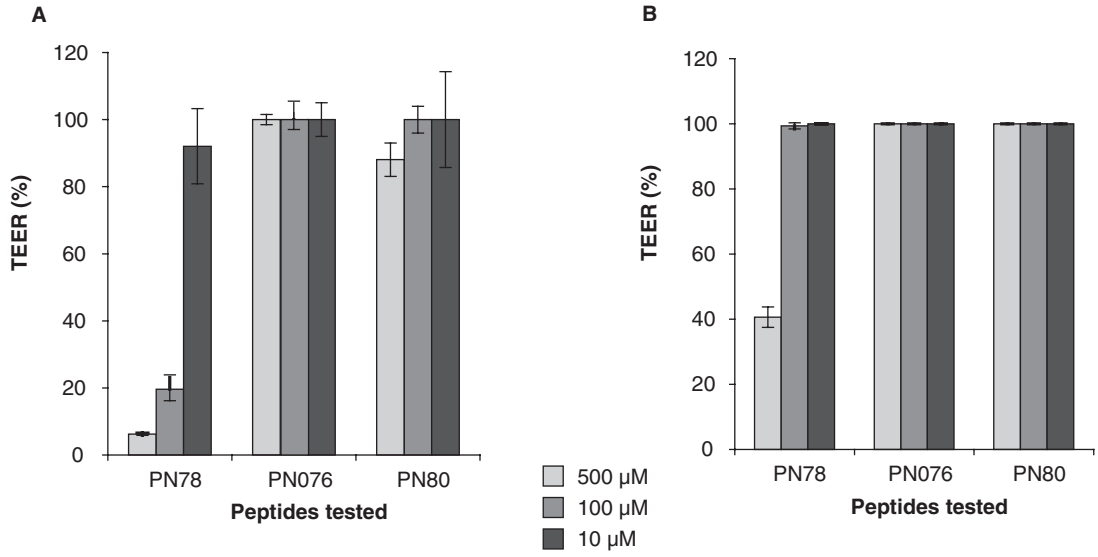

Figure 3. Effect of phage display-derived peptides on TEER. MDCK cell monolayers were treated with each peptide at three concentrations (500, 100 and 10 µM) for 30 min and then the peptide was removed. TEER was measured at 15 min and 2 h after peptide removal. Error bars indicate the standard deviation. **A.** TEER at 15 min after treatment. **B.** TEER at 2 h after treatment.
MDCK: Madin darby canine kidney; TEER: Transepithelial electrical resistance.

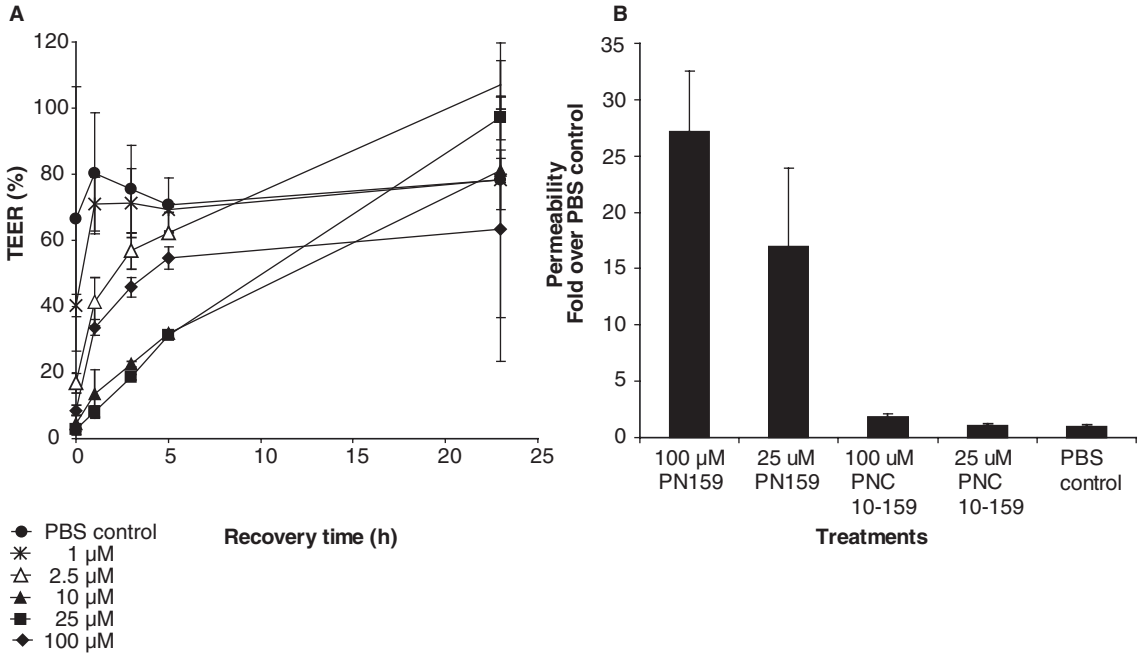

Figure 4. Peptide tight junction modulators on EpiAirway inserts. **A.** Reversible effects on TEER after 15 min of treatment with PN159 TJM peptide (100, 25, 10, 2.5 and 1 µM) or PBS control. **B.** TJM peptides affect permeability of Dextran 4400.
TEER: Transepithelial electrical resistance; TJM: Tight junction modulator.

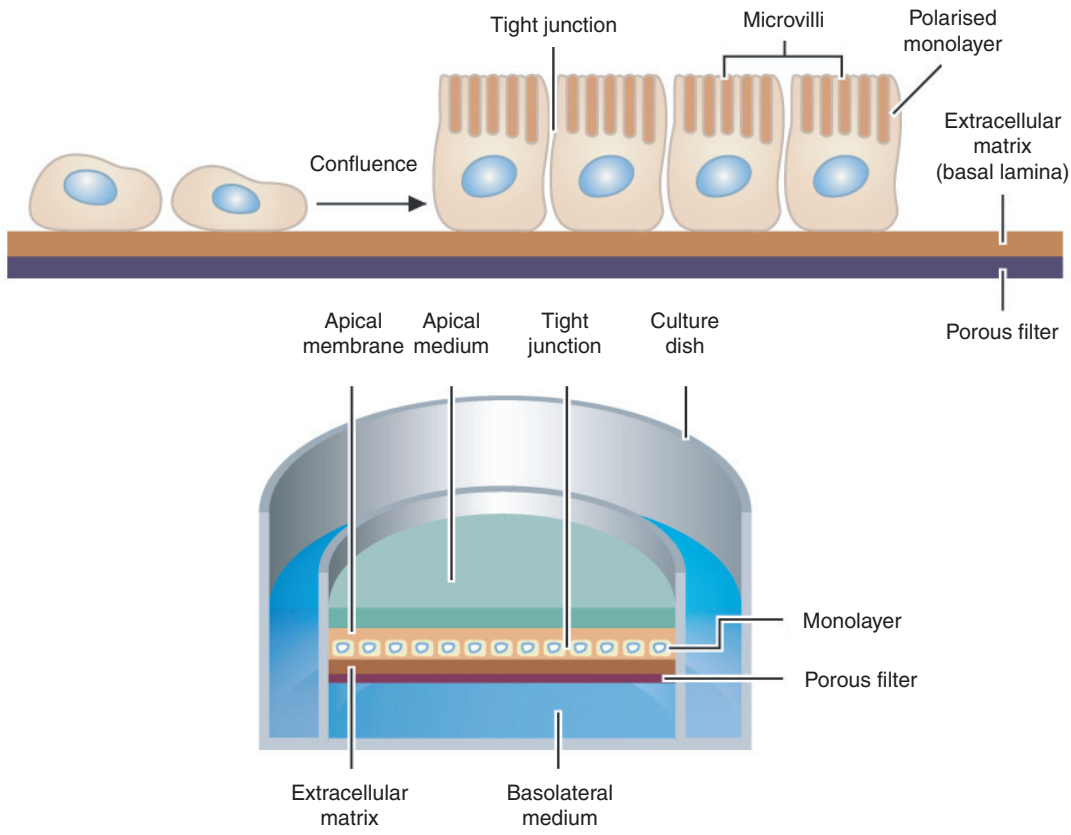

Figure 5. Formulation screening using epithelial cell monolayers. Cultures were grown on individual inserts. Testing of multiple formulations per plate were performed in microtitre well formats. Cells were grown to confluence in nutrient-containing media on inserts at 37°C in a CO_2 incubator. Formulations or individual test compounds were applied to the apical tissue surface.

identified by the various molecular biology approaches described herein have the potential for development as a new generation of potent and safe TJ modulators for macromolecule drug delivery with broad applications to nasal and gastrointestinal routes of administration.

7. Nasal drug delivery formulation for clinical development of the obesity therapeutic PYY_{3-36}

The recent advances in TJ biology, tissue culture models and high-throughput functional assays have led to better formulations for clinical development. A recent example of this success is the development and clinical testing of a formulation for the delivery of the investigational drug PYY_{3-36} [4,102], which is being co-developed by Nastech Pharmaceutical Company, Inc. and Merck & Co., Inc. for the treatment of obesity. Peptide YY is a naturally occurring hormone, produced by specialised endocrine cells (L cells) in the gut after a meal, which physiologically inhibits food intake by modulating appetite circuits in the brain specifically involving the Y2 receptor. Its derivative, PYY_{3-36}, is a promising therapeutic candidate for the treatment of obesity [103]. Nasal delivery offers major advantages over injection for long-term therapy. The essential features necessary for clinical development of a formulation for chronic peptide drug delivery include long-term drug stability, efficient delivery across the nasal mucosa resulting in adequate bioavailability, no irritation or long-term toxicity and preservative effectiveness for extended multiple dosing from a single vial.

The formulation developed for PYY_{3-36} clinical development was designed from components (i.e., individual excipients) that were optimised using a 'design of experiments' approach in which statistical models were used to define the relationship between formulation variables (inputs) and performance (outputs) [104,105]. To accomplish this, the primary human epithelial tissue model (EpiAirway) was used to evaluate the extent of TJ opening by measuring decreases in TEER and its recovery, whereas drug permeability was determined from measurements of drug appearance in the basal media (**Figure 5**). Cell viability and cytotoxicity were monitored by MTT and LDH assays, respectively. Data from a representative screening

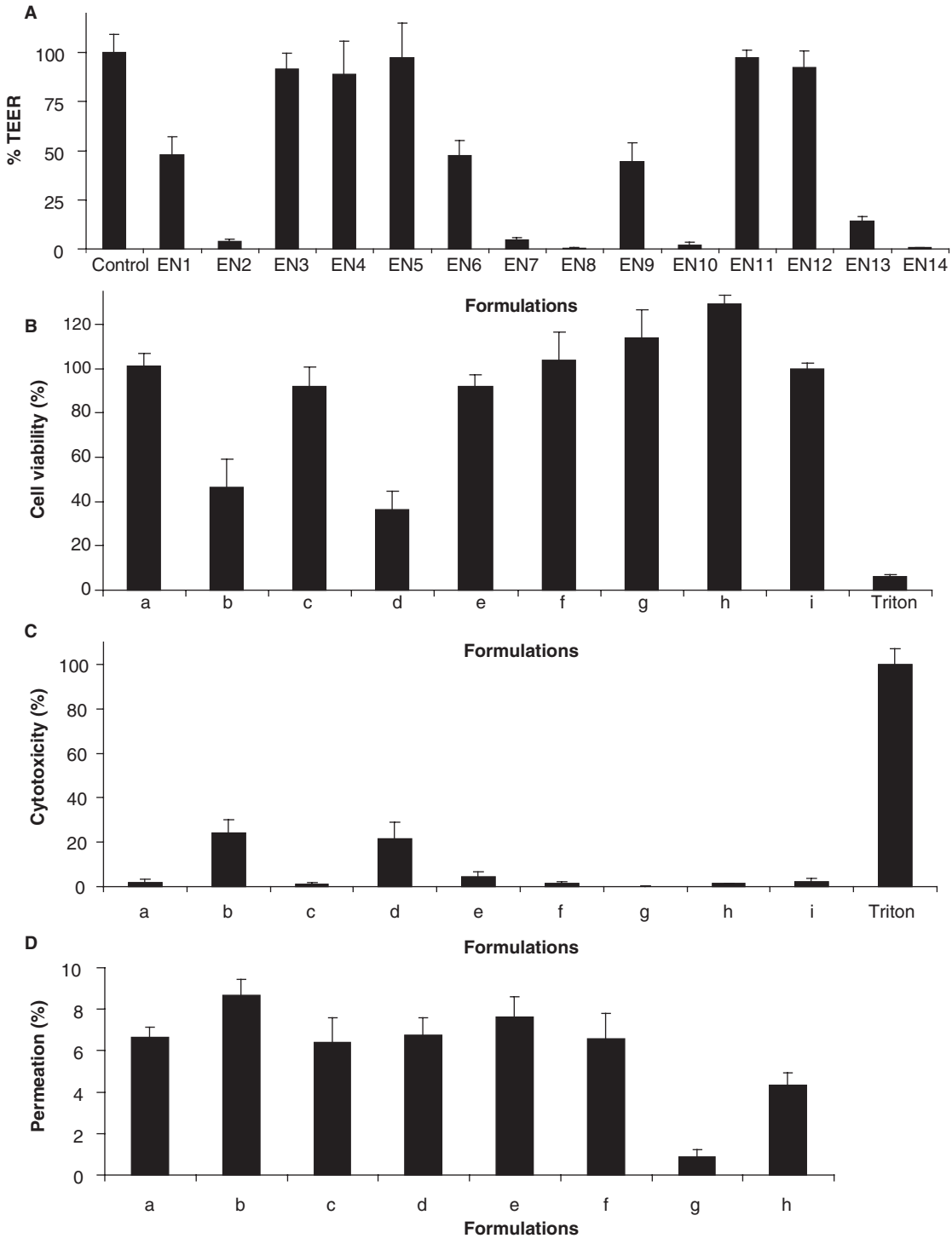

Figure 6. Formulation optimisation parameters. A formulation for nasal delivery of the obesity therapeutic candidate, PYY_{3-36}, was developed by optimising seven individual components (excipients) of the formulation (buffer, salts, permeation enhancers and so on) with respect to several parameters (described in the text). **A.** Percentage TEER was measured relative to a buffer and NaCl control. **B.** Percentage cell viability was measured using the MTT assay (Section 5.6) relative to a solution of Triton X-100 as a positive control. **C.** Percentage cytotoxicity was measured using the LDH assay (Section 5.6) relative to Triton X-100 as a positive control. **D.** Drug permeation was measured using a permeability assay (Section 5.3) in which permeation of the drug in a formulation is measured relative to the drug in a buffer NaCl solution (% permeation).

EN: Permeation enhancer; LDH: Lactate dehydrogenase; MTT: Dimethylthiazol diphenyl terazolium bromide; TEER: Transepithelial electrical resistance.

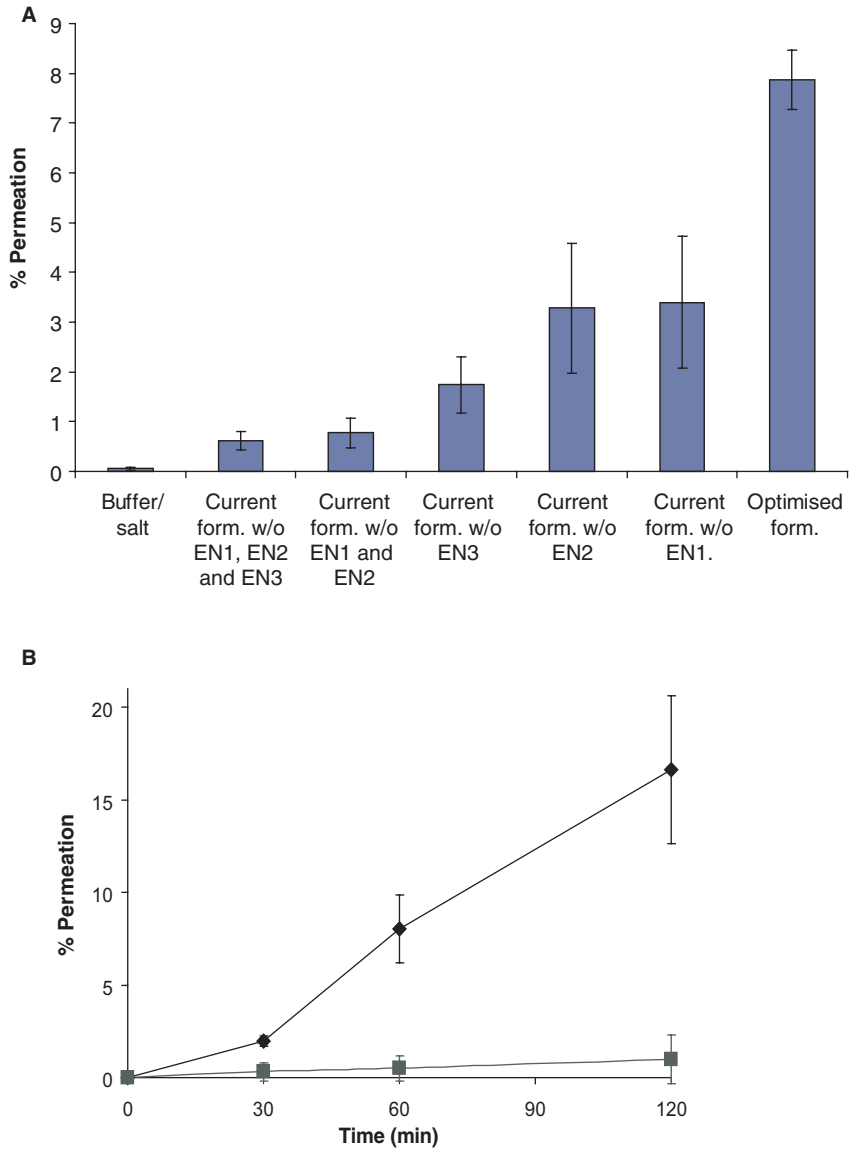

Figure 7. Optimised formulation of PYY_{3-36}. A. The contribution of individual permeation enhancers and other excipients in the optimised formulation in drug permeation assays using the MatTek tissue model system (Section 5.1). **B.** The percentage drug permeation (relative to a buffered salt solution) as a function of time for the optimised formulation.
w/o: Without.

experiment are depicted in **Figure 6**. **Figure 6A** shows the TEER results for a subset of permeation enhancers. There was a substantial reduction in TEER for several enhancers tested, indicating TJ opening. The decrease in TEER has been shown to correlate with the permeation of drug across the cell layer. For a set of representative formulations (a – i) evaluated in the toxicity assays (**Figures 6B** and **6C**), two of the candidate formulations (b and d) exhibited a reduced cell viability by the MTT assay (**Figure 6B**) and increased cell cytotoxicity by the LDH assay (**Figure 6C**), demonstrating the ability of the *in vitro* cell model to differentiate formulations with varying degrees of toxicity. **Figure 6D** shows the corresponding results of these formulations in drug permeation assays.

In total, > 200 different formulations were evaluated. Multivariate analysis was performed to determine the effect each formulation component had on seven output variables (drug permeability, osmolality, stability at refrigerated and accelerated conditions, TEER, MTT and LDH assays) [102]. This consisted of an initial analysis of each formulation component for some level of correlation with output parameters ($p < 0.1$). With the subset identified, either a linear regression or stepwise logistic selection model was used. The results (not shown) indicated that one excipient correlated to osmolality and toxicity, two correlated to PYY_{3-36} permeation, three affected drug stability and five impacted paracellular resistance. Based on these analyses, an optimised PYY_{3-36} formulation (i.e., maximally effective combination of components)

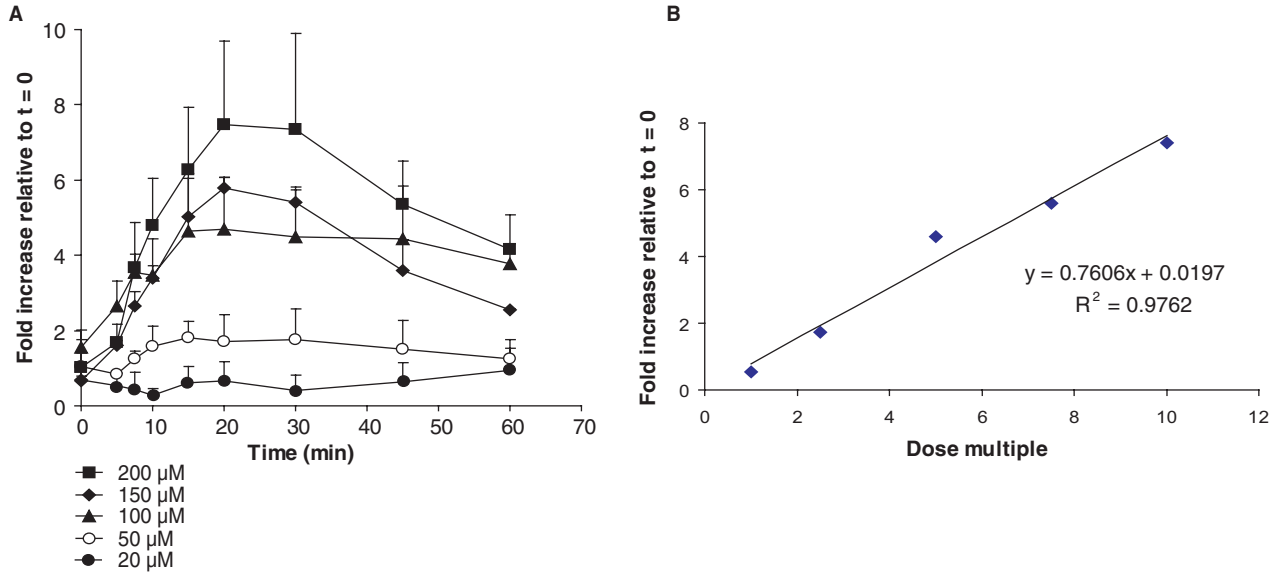

Figure 8. Clinical evaluation of a nasal formulation for PYY_{3-36}. Data from a Phase I human clinical trial. **A.** Serum levels following escalating intranasal administration of the indicated doses. **B.** Demonstration of dose linearity.

was selected for further evaluation. This optimised formulation contained two stabilisers, two permeation enhancers, one chelating agent, and one preservative in a buffer at pH 5. **Figure 7A** shows the contribution of several (but not all) individual excipients to drug permeability (singly or together) for the optimised formulation and the percentage permeation as a function of time (**Figure 7B**), using the EpiAirway *in vitro* tissue model system. This approach to formulation development resulted in a > 100-fold increase in tissue layer permeability of PYY_{3-36} compared with a simple saline formulation, and showed no significant cellular toxicity.

Finally, it was determined that treatment of EpiAirway tissue monolayers with optimised formulation results in opening TJs to form a paracellular channel with a hydrodynamic diameter < 10 nm, as defined by permeability studies in which 70-kD FITC-dextran passed between cells, whereas 500-kD-FITC-dextran was excluded (S-C Chen, unpublished observations). Under these conditions TJ morphology appeared unaltered by confocal microscopic evaluation of tissue sections immunostained against claudin 4 and ZO-1. The BODIPY-FL-C5-Sphingomyelin (BFCS) assay showed that BFCS was largely restricted to the apical side of the membrane, following formulation treatment, indicating that the TJ 'fence' function remained intact. These results suggest that this formulation does not open TJs to an extent that would permit large protein antigens, viruses, or cells to pass through; nor does it interfere with the polarised distribution of membrane proteins.

PYY_{3-36} nasal delivery using the optimised formulation was evaluated by extensive preclinical animal testing and demonstrated a good safety margin for nasal, cardiovascular and systemic toxicity for PYY_{3-36} including:

- no observable nasal toxicity in rats, rabbits and dogs, and no microscopic or gross pathologic findings (at 12x the expected human dose)
- a pharmacokinetic profile in rats and rabbits characterised by a linear response in C_{max} and total AUC with ~ 20% absolute bioavailability (i.e., compared with intravenous dosing)
- no drug-related findings observed so far in chronic systemic toxicity studies in two species

The PYY_{3-36} nasal spray development programme has now completed three Phase I clinical studies. Results of a Phase IA dose-ranging study [106] showed that nasal administration resulted in effective transfer of PYY_{3-36} into the bloodstream and that there was a linear relationship between dose administered and plasma levels of PYY_{3-36} (**Figure 8**). Treatment was well tolerated, with drug-related side effects that were generally mild and resolved without treatment. The excellent *in vitro* toxicity profile has thus far been realised *in vivo*: the PYY_{3-36} intranasal formulation has shown good nasal tolerability, with a cumulative experience of nearly 1000 administrations so far.

8. Expert opinion

The key objectives of modern nasal drug delivery research are to apply the growing knowledge of TJ molecular biology to the development of *in vitro* and *in vivo* assay systems and models for the identification and optimisation of advanced formulations and mechanism-based compounds that reversibly alter the properties of TJ components in a way that enhances tissue permeability and drug transport. This has led to improved bioavailability and extension of the molecular

weight range of drugs that can be effectively and safely delivered for chronic applications, effectively breaking the tyranny of Lipinski's rule of five in drug design research. The dynamic nature of TJs and the involvement of specific endocytic pathways and intracellular signalling mechanisms that regulate TJ function, offer new possibilities for the development of compounds with more specific mechanisms of action. A new generation of peptide-related compounds has been identified as TJMs, which are amenable to optimisation by structure–activity analysis and detailed mechanism studies. From comparative *in vitro* toxicity studies with a preclinically validated formulation used for PYY_{3-36} human Phase I clinical trials, the early indication is that these new compounds will be safe for chronic administration. The knowledge gained from nasal epithelial TJ biology and TJM development will be directly applicable to developing formulations of macromolecular drugs for oral delivery. Finally, as TJ pathophysiology is an important component of cancer, a key initiation step in inflammation and a route for invasion in many infectious diseases, it is hoped that this drug delivery directed research may provide insights into the development of entirely new therapeutic drug classes for these important conditions.

Acknowledgements

PDF files of meeting abstracts cited herein with full experimental data and results can be found on the Nastech website [202]. The authors thank their colleagues at Nastech for their reading of the manuscript and their contributions to the research discussed in this review: E Bell, G Brandt, R Costantino, L Chen, S-C Chen, K Cui, B Dutzar, R Herman, M Houston, M Kleppe, K Makienko, M Prieve, T Sileno and R Witkowska.

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