Modulating tight junctions on airway epithelial cells to enhance paracellular transport of antifibrotic drugs

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Introduction

The lungs provide a unique absorptive surface for drug delivery and the inhalation route allows for the deposition of high drug concentrations while minimizing systemic effects. However, some pharmacologically active compounds cannot be administered through the inhalation because of inadequate bioavailability for sub epithelia targeting, and this may limit the usefulness of these compounds. This lack of uptake across the epithelia can be caused by many reasons including; poor aqueous solubility, degradation of active compound with the local enzymes and poor membrane permeability [1]. Poor membrane permeation can occur due to the large-molecular weight of a compound, as is the case with proteins and other macromolecules, or an insufficient partition coefficient that allows uptake or transport into biological membranes, as with many hydrophilic, low-molecular weight compounds. Airway epithelial cells are a major obstacle for drug delivery to the lung parenchyma. Several transport routes exist in airway epithelial cells, which potentially can be exploited for enhancing drug permeability. Compared to the transcellular pathways via passive diffusion, transporters, adsorptive and receptor-mediated transports, the paracellular flux is very limited. The Epithelial barrier forms tight junctions that restrict or completely block the free passage of hydrophilic molecules [2]. Therefore, a promising strategy for drug delivery is the modulation of the tight junctions to allow molecules to pass through this cellular barrier where sub epithelium targeting is required. The paracellular route has more advantages compared to the transcellular route including; 1) drug modification is not needed and 2) various drugs could benefit by the same tight junction modulator. Selected compounds that are capable of modulating tight junctions and thereby increase paracellular drug transport have been investigated for intestinal drug absorption [3] but limited studies have been performed to modulate airway tight junctions [4]. These modifiers include hypertonic saline [5], sodium caprate (sodium salt of medium chain fatty acid), and oleic acid (fatty acid). It has been shown that hypertonic solution of NaCl increases the permeability of the tight junctions of the airway epithelium through an unknown mechanism [5]. It has been claimed that the opening of the tight junctions is simply due to mechanical stress caused by shrinking of the cells, but in a study by Hogman et al. using lanthanum ions transported after modulating the tight junctions with hypertonic saline, they demonstrated that this mechanism is unlikely to be the major effect [5]. They have suggested that hypertonic NaCl can effect on tight junctions by affecting the intracellular concentration of calcium ions and/or cAMP, or that it affects the structure of the cytoskeleton. They have also shown that this effect is reversible in a mouse model. Sodium caprate has been also shown to dilate tight junctions reversibly, increase the permeability of fluorescein Na and decrease transepithelial electrical resistance (TEER) via phospholipase C activation and upregulation of intracellular Ca^{2+}, which can lead to contraction of actin–myosin filaments attached to the intracellular domain of tight junctions [6]. Oleic acid has an effect on membrane fluidity and paracellular permeability of dermal, gastrointestinal, alveolar and blood–brain barriers [7]. The exact target for oleic acid is not known yet, but membrane microdomains or lipid rafts could be involved. It has been demonstrated that it has a reversible effect on tight junction dilation in an experiment *in vivo*. To the authors' knowledge, only Na caprate is currently used as an absorption enhancer in pharmaceutical applications [8].
In this study, the effect of already stablished tight junction modulators for the oral route including; hypertonic NaCl, Na caprate, and oleic acid on an antifibrotic hydrophilic drug PXS25 has been investigated on airway epithelial cells.

Materials and methods

Materials

All chemical reagents were purchased from Sigma-Aldrich and used as supplied, unless otherwise stated. The Calu-3 sub-bronchial epithelial cell line was purchased from American Type Culture Collection. Cell culture materials were obtained from Invitrogen Australia and PXS25 was provided by Pharmaxis Ltd.

Methods

Calu-3 cells (passage 35–45) were grown in Dulbecco's Modified Eagle's medium: F-12 containing 10% (v/v) foetal calf serum (Invitrogen, Sydney, NSW, Australia), 1% (v/v) non-essential amino acid solution and 1% (v/v) L-glutamine solution (Invitrogen). Cells were maintained in a humidified 95% air 5% CO_2 atmosphere at 37°C and were subcultured according to American Type Culture Collection recommendations. Cells were cultured on Transwell cell culture inserts using air-liquid interface (ALI) method and seeded at a density of 1.65×10^5 cells/insert [9]. The medium was replaced three times a week and any apical surface liquid or mucus removed.
Calu-3 cells were treated for 1 hr with each modulator (oleic acid 100 µM, Na caprate 1 mM and NaCl 3%) and three wells were assigned for each experiment. Transepithelial electrical resistance measured before and after treatment using an epithelial voltohmmeter (EVOM, World Precision Instruments, USA) and corrected by subtracting the blank inserts and multiplied by the area of the transwell inserts.
Concurrently, epithelial permeability studies using fluorescein sodium (flu-Na), a marker for paracellular transport, were performed to evaluate barrier formation and tight junction functionality. Briefly, 250 µL of 2.5 mg/mL flu-Na solution were added on the apical chamber, while 600 uL of Hanks Balance Salt Solution (HBSS, Gibco Invitrogen, USA) were added into the basolateral chamber. At pre-determined time points up to 4 hours, 100 µL samples were taken from the basolateral chamber and subsequently replaced with fresh buffer to maintain sink conditions. Samples were placed in a black, 96 well plate and fluorescence reading was subsequently measured using Spectromax plate reader with excitation and emission wavelengths settings of 485 and 520 nm, respectively. The apparent permeability coefficient (Papp) of flu-Na across the epithelial cells was calculated using this equation: $Papp = (V/AC_0)(dC/dt)$, where V is the volume of solution in the basolateral chamber, A is the surface area of the Transwell membrane (cm^2), C_0 is the initial concentration in the apical chamber (µg/mL) and dC/dt is the flux (rate of change in cumulative mass transport) of flu-Na.
The cytotoxicity of each modulator on Calu-3 cells was investigated using a MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay from Promega). Cells were seeded in 96 well-plate at the density of 5×10^4 cell per well and incubated overnight at 37 ºC. Then each modulator (100 µl) was added to the seeded cells and incubated for 24 hours, followed by the addition of 20 of MTS reagents and incubation for 2 hours. The optical absorbance was determined at 490 nm with Spectromax micro-plate reader. Results were expressed as the IC_{50} (50% inhibitory concentration) of the modulators.
PXS25, an hydrophilic compound with limited transcellular transport, was applied on the Calu-3 previously treated with tight junction modulators to observe the effect of these modulators on its bioavailability. Briefly, 100 µL of 100 µM PXS25 solution were added on the apical chamber of the Traswell, while 600 uL of Hanks Balance Salt Solution (HBSS, Gibco Invitrogen, USA) were added into the basolateral chamber. At pre-determined time points up to 4 hours, 100 µL samples were taken from the basolateral chamber and subsequently replaced with fresh buffer to maintain sink conditions. Then samples were analysed with a LC-MS/MS spectrometer validated method.
LC-MS/MS analysis was performed using an Agilent triple quadrupole mass spectrometer. Separation was performed using an Oasis HLB (30 mg, 1 mL, Waters, Milford MA, USA) solid phase extraction method. The analysis was conducted using c18 (Agilent) column with gradient of two mobile phases; A, methanol/water/formic acid (5/94.9/0.1 v/v/v); B, methanol/water/formic acid (94.9/5/0.1 v/v/v) at a flow rate of 0.4 mL/min. Samples were analysed in triplicate in the negative ion mode.

Results

The cytotoxic concentration of oleic acid and Na caprate on Calu-3 cells was determined by calculating their IC_{50}. NaCl has been previously tested and found to be safe [10]. Oleic acid presented an IC_{50} of 120 µM, Na caprate 24 mM respectively. Calu-3 cells were only treated for 1 hour with the tight junction modulators while the IC_{50} was measured after 24 hours exposure. Therefore all three modulators were considered safe at the concentrations studied.
After 12 days in air-liquid interface culture, Calu-3 cells were treated with modulators for 1 hour and TEER measured before and after the modulators treatment. Before treatment the Calu-3 cells presented a TEER of $749 \pm 78\ \Omega\ cm^2$ while after one hour treatment the TEER measurement dropped significantly (Table 1).
Linear flux was observed with all of modulators during the 4 hours transport experiment. All modulators increased the permeability of flu-Na from cell membrane (Figure 1A). As expected, there was an inverse correlation between the apparent permeability (Papp) of passively transported flu-Na across the Calu-3 cell monolayers and measured TEER after treatments (Figure 1B). Treatments caused lower TEER values and higher Papp for flu-Na. This demonstrated that treating Calu-3 monolayer with these modulators, affected the tight junction's integrity and made them more permeable.

Similar findings were observed with PXS25 transport. All treatments caused an increase in PXS25 transport. For example treating cells with NaCl increased the PXS25 concentration in the basal chamber from 12.70 ± 0.94 to 22.31 ± 0.88 ng/ml (Figure 2).

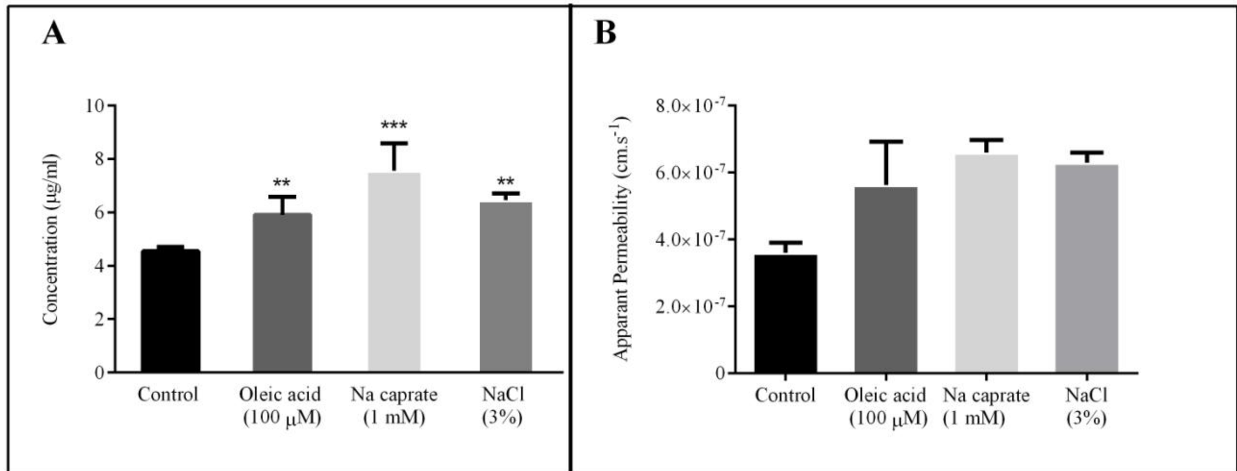

Figure 1- A, Transport of flu-Na across Calu-3 cells after tight junction modulators treatment (Oleic acid, Na caprate and NaCl) for 1 hour. **B**, Apparent permeability of flu-Na measured within 4 hours of transport after treatments. ***, P ≤ 0.01, ***P ≤ 0.001 significantly different from non-treated monolayer (n=3, Mean ±* SD)

Table 1- Transcellular electric resistance measurement before and after treating calu3 cells with tight junction modulators for one hour.

	Before treatment (Ω cm^2)	After treatment (Ω cm^2)
Control (HBSS)	749 ± 78	762 ± 103
NaCl (3%)	612 ± 12	109 ± 27*
Na caprate (1 mM)	673 ± 41	64 ± 3*
Oleic acid (100 μM)	808 ± 67	102 ± 10*

* *P ≤ 0.05,TEER was significantly different from non-treated monolayer (n=3, Mean* ± SD)

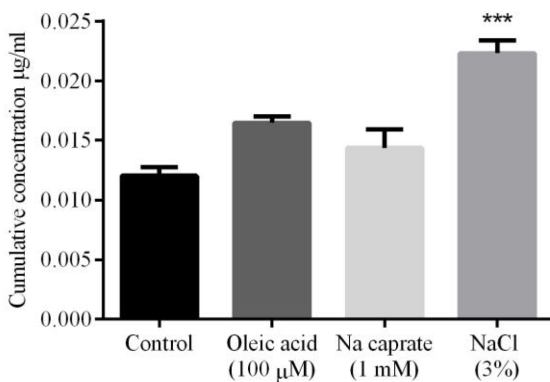

Figure 2- Transport of PXS25 across Calu-3 cells after 1 hr treatment with the tight junction modulators (n= 3, mean ± SD). **** P ≤ 0.001, significantly different from non-treated monolayer*

Discussion and conclusions

Opening of tight junctions could enhance paracellular permeability of biological barriers for a variety of drugs, including hydrophilic compounds, biopharmaceuticals, like peptides, proteins, nucleic acids, and viral vectors, without the need to modify drugs. This concept has already been proven clinically for several tight junction modulators on intestinal epithelial cells [8]. In this study we have shown that modulators, like Na caprate, oleic acid, or NaCl could also be used to improve paracellular transport in Calu-3 lung epithelia cells and consequently used to enhance sub-epithelial uptake of hydrophilic drugs delivered to the lung.

References

1 . Saaber D, Wollenhaupt S, Baumann K, Reichl S: Recent progress in tight junction modulation for improving bioavailability, Expert Opin Drug Dis, 2014, 9: pp.367-381.
2 . Mitic L L, van Itallie C M, Anderson J M: Molecular Physiology and Pathophysiology of Tight Junctions - I. Tight junction structure and function: lessons from mutant animals and proteins, Am J Physiol-Gastr L, 2000, 279: pp.G250-G254.
3 . Matsuhisa K, Kondoh M, Takahashi A, Yagi K: Tight junction modulator and drug delivery, Expert Opin Drug Del, 2009, 6: pp.509-515.
4 . Everett R S, Vanhook M K, Barozzi N, Toth I, Johnson L G: Specific modulation of airway epithelial tight junctions by apical application of an occludin peptide, Mol Pharmacol, 2006, 69: pp.492-500.
5 . Nilsson H, Dragomir A, Ahlander A, Johannesson M, Roomans G M: Effects of hyperosmotic stress on cultured airway epithelial cells, Cell Tissue Res, 2007, 330: pp.257-269.
6 . Coyne C B, Ribeiro C M P, Boucher R C, Johnson L G: Acute mechanism of medium chain fatty acid-induced enhancement of airway epithelial permeability, J Pharmacol Exp Ther, 2003, 305: pp.440-450.
7 . Sztriha L, Betz A L: Oleic-Acid Reversibly Opens the Blood-Brain-Barrier, Brain Res, 1991, 550: pp.257-262.
8 . Aungst B J: Absorption Enhancers: Applications and Advances, Aaps J, 2012, 14: pp.10-18.
9 . Haghi M, Young P M, Traini D, Jaiswal R, Gong J, Bebawy M: Time- and passage-dependent characteristics of a Calu-3 respiratory epithelial cell model, Drug development and industrial pharmacy, 2010, 36: pp.1207-1214.
10 . Hogman M, Mork A C, Roomans G M: Hypertonic saline increases tight junction permeability in airway epithelium, Eur Respir J, 2002, 20: pp.1444-1448.