Effects of antibiotics on bronchial epithelial differentiation

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14 ECTS thesis submitted in partial fulfillment of a Baccalaureus Scientarium degree in Molecular Biology

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Reykjavík, February 2015
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Abstract

Bronchial epithelial integrity plays a critical role in lung defense, as many pulmonary diseases arise when epithelial integrity is decreased. Tight junctions connect neighboring cells in epithelia and serve to separate the external compartments of the lung from the internal compartments, along with controlling the paracellular movement of water, ions and solutes. The epithelia also secretes antimicrobial peptides and activates pro-inflammatory pathways, making the epithelia crucial in lung defense. In this research, cells from the human airway epithelium derived basal cell line VA10 were treated with two distinct antibiotics, the macrolide azithromycin and the cephalosporin ceftriaxone to determine whether they can increase epithelial integrity, as determined by TER measurements. To further examine the effects of these antibiotics on the epithelia, a panel of tight junction proteins and other cell markers were observed with immunofluorescence. Both azithromycin and ceftriaxone treatment on early passage VA10 cells showed increased TER, when in contrast, azithromycin treatment on later passage VA10 cells did not show increased TER. The immunostaining of the proteins alone was not sufficient to conclude the effects of the antibiotics on epithelial integrity but gave insights into a possible difference in signal intensity when treated with variable concentrations of the antibiotics.
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<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
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<tr>
<td>ALI</td>
<td>Air-liquid interface</td>
</tr>
<tr>
<td>ASL</td>
<td>Air surface liquid</td>
</tr>
<tr>
<td>Azm</td>
<td>Azithromycin</td>
</tr>
<tr>
<td>BEGM</td>
<td>Bronchial epithelial growth medium</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane regulator</td>
</tr>
<tr>
<td>COPD</td>
<td>Chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-Diamidino-2-Phenylindole</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>DPB</td>
<td>Diffuse panbronchiolitis</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>IMF</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>JAM</td>
<td>Junctional adhesion molecule</td>
</tr>
<tr>
<td>MARVEL</td>
<td>MAL and related proteins for vesicle trafficking and membrane link</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>P/S</td>
<td>Penicillin/Streptomycin</td>
</tr>
<tr>
<td>SCRU</td>
<td>Stem cell research unit</td>
</tr>
<tr>
<td>TER</td>
<td>Transepithelial electrical resistance</td>
</tr>
<tr>
<td>ZO</td>
<td>Zonula occludens</td>
</tr>
</tbody>
</table>
Acknowledgements

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1. Introduction

1.1 Epithelial integrity

Healthy lungs are important for wellness as various defects in lungs can cause serious diseases, making a good lung defense crucial. Foreign organisms and chemical irritants can enter the body via inhalation, enabling contact with the airway epithelia which partly explains the importance of epithelial integrity (Parker & Prince 2011). The bronchial epithelial cells play a great role in lung defense, not only as a physical barrier but also in inflammatory processes (Asgrimsson et al. 2006). They are an important part of the innate immune system with numerous roles. The airway epithelia has surface, cytosolic and endosomal sensors that activate various pro-inflammatory signaling pathways as well as maintaining the balance of production and clearance of mucus and mucins in the respiratory tract. In addition to producing anti-microbial compounds, the epithelia also produces cytokines, chemokines, lipid mediators, peptides and reactive oxygen species that for instance regulate secretions and induce recruitment and activation of other leukocytes, such as T cells and neutrophils (Parker & Prince 2011) (Polito & Proud 1998). It is also a regulator of electrolyte content of the airway surface liquid (ASL), yet further evidence demonstrating the importance of the bronchial epithelium in lung defense (Asgrimsson et al. 2006). The airway epithelia makes up a complicated system, with many components that need to be correctly regulated in order to maintain epithelial integrity (Parker & Prince 2011).

1.1.1 Junctional complexes and other cell markers

Neighboring cells in the airway epithelia are connected by various junctional complexes that serve to separate the external and internal compartments of the lung, and control the paracellular movement of water, ions and solutes (Coyne et al. 2003). The tight junctions are an important part of the lung barrier and are just below the apical surface of the cells. They are categorized into three major groups: the claudin family, the tight junction-associated MARVEL protein family, which includes occludin, and the immunoglobulin-like proteins, which includes junctional adhesion molecule (JAM). Tight junctions are transmembrane proteins, meaning that they are made of both intracellular and extracellular components and connect to scaffold proteins within the cell that are capable of protein-protein interactions with other tight junctional components (Georas & Rezaee 2014) (Figure 1). Barrier properties
of the tight junctions differ among epithelia in different tissues regarding electrical resistance and ionic charge selectivity (Van Itallie & Anderson 2004).

![Diagram of tight junctions and adherence junctions in epithelial cells.](image)

**Figure 1. A schematic figure of tight junctions and adherence junctions in epithelial cells.** Tight junctions (occludins, claudins and JAMs) promote the epithelial integrity by regulating the movement of ions and solutes across the paracellular space. Zonula occludens link the tight junctions to the actin cytoskeleton (Neu et al. 2010).

At least 27 members of the claudin family have been identified. They are predicted to be tetraspanning molecules with two extracellular loops and intracellular N- and C-terminals. The claudin–claudin interactions in the tight junctions are formed with homo- and heterotypic aggregation between the extracellular loops. It is known that claudins contribute to paracellular selectivity, where some claudins increase paracellular permeability (“leaky” claudins) and others promote barrier integrity e.g. claudin 1(Georas & Rezaee 2014). Each claudin is thought to feature both different resistance and charge selectivity (Van Itallie & Anderson 2004).

Occludin is another tetraspanning tight junction molecule with two extracellular loops and intracellular N- and C-terminals that interact with scaffold molecules in the apical junctional complexes. Occludin has numerous functions, including regulating paracellular permeability. It has been implied to have a regulatory role in the apical junctional complexes because of its interaction with ZO-1 and the actin skeleton. There are also indications that occludin promotes the paracellular flux of macromolecules as well as acting like an adhesion molecule during tissue inflammation (Georas & Rezaee 2014).
Junctional adhesion molecules (JAM) are expressed in the basolateral surface of human airway epithelial cells, close to the tight junctional strands (Excoffon et al. 2008). JAM proteins consist of an extracellular domain, a transmembrane segment that spans the membrane once, and a short intracellular tail that can bind to several ligands. JAM-A is suggested to facilitate tight junction assembly, supported by the evidence that it associates with five PDZ proteins, including ZO-1 and can cause reorganization of the actin cytoskeleton. Another evidence is that its presence often correlates with decreased paracellular permeability as well as enhanced electrical resistance (Bazzoni 2003).

Direct interactions between claudins, occludins and JAMs have not been detected, meaning that scaffold proteins serve as their binding molecule. Zonula occludens proteins (ZO-1, ZO-2 and ZO-3) are important tight junctional scaffolding molecules that interact directly with claudins and occludins through their PDZ domains, linking them to the actin cytoskeleton and other signaling molecules. ZO-1 has been shown to be essential for clustering of claudins, barrier function, and strand formation. As mentioned above, ZO-1 is capable of interacting with JAMs and forming homodimers (Niessen 2007). Several studies have indicated that alterations in ZO-1 expression causes decreased barrier function in the airway epithelia (Georas & Rezaee 2014).

Other cell markers observed in this project were epithelial (E) and neural (N) cadherins, cytokeratins 5/6 and 17, LL-37, EGFR and p63. Cadherins are Ca\(^{2+}\) dependent glycoproteins that maintain the structural and functional integrity in tissues by serving as adherens junctional molecules, therefore providing strong cell-cell adhesion. Cadherins are integral membrane proteins that bind to cells at the extracellular domain and interact indirectly with the actin cytoskeleton at the cytoplasmic domain (Halbleib & Nelson 2006), (Takeichi 1988). Cytokeratins (CKs) are related to intermediate filaments and are thought to engage in preserving the structural integrity of cells with their intracellular network of filaments. Twenty cytokeratins have been identified but their expression pattern differs among epithelial cell type (Sundström B.E. & Stigbrand T.I. 1994). The cathelicidin-related antimicrobial peptide LL-37 is an important part of the ASL. ASL coats the luminal surface of the airway epithelial cells and contains various antimicrobial factors that kill inhaled bacteria. LL-37 disrupts the phospholipid membrane of bacteria, e.g. *Pseudomonas aeruginosa* at neutral pH (Abou et al. 2014). Epithelial growth factor receptor (EGFR) is a cell surface receptor that binds to members of the epidermal growth factor family, leading to cell proliferation (Anon 2015). *In vitro* studies have shown that a localized increase of EGFR is the result of both cigarette smoke exposure and damages in bronchial epithelium in bronchial
asthma (Puddicombe et al. 2000). The protein p63 plays an important role in regulating both epithelial proliferation and differentiation. It is expressed in the nucleus of certain subpopulations of basal cells in bronchial epithelia (Di Como et al. 2002).

1.1.2 Lung diseases

It is known that airway epithelial barrier dysfunction adversely affects lung function. Alterations in apical junctional complexes can cause increased epithelial permeability that leads to mucosal inflammation. Dysfunction of apical junction complexes can be the result of many causes, for example they can be degraded by proteases, disrupted or their expression can be silenced. Environmental exposures can also increase epithelial permeability, such as viruses, allergens, air pollution and smoke from cigarettes. Absorption in the airway maintains the surface layer for proper mucociliary clearance, making the airway epithelia slightly leaky. Altering selectivity of tight junctions in airway diseases is thought to affect both volume and composition of the airway surface fluid in a great manner (Van Itallie & Anderson 2004). Reduced barrier integrity is often the consequence of airway diseases, typical examples are cystic fibrosis and chronic obstructive pulmonary disease (COPD).

Cystic fibrosis is a genetic disease and is manifested by mutation in the cystic fibrosis transmembrane regulator (CFTR) protein in lung epithelia. CFTR regulates both chloride and sodium transport across the epithelia and may contribute to barrier function and tight junction assembly in the airway epithelia. In CFTR deficient cell lines, transepithelial electrical resistance (TER) has been shown to decrease and permeability to mannitol increase. Because of airway barrier dysfunction in cystic fibrosis patients, they are susceptible to paracellular invasion of pathogens, commonly *Pseudomonas aeruginosa*, causing both infection and inflammation as well as rupturing tight junctions (Georas & Rezaee 2014). The dysfunctional CFTR leads to reduced bicarbonate secretion through anion channels, resulting in decreased ASL pH. The abnormal acidic pH inhibits the activity of antimicrobial peptides in the ASL, including LL-37, therefore inhibiting bacterial killing in cystic fibrosis lungs (Abou et al. 2014).

COPD is a serious disease with extremely high morbidity. It is caused by environmental factors that, for instance, increase airway inflammation. The disease associated acute exacerbations are serious and result in a rapid failure in lung function (Kanoh & Rubin 2010). The inflammation caused by COPD, and asthma as well, can disrupt epithelial permeability by altering expression and function of airway epithelial junctional complexes
(Barnes et al. 2009). Dysfunction in airway epithelial integrity is correlated to many other pulmonary diseases such as diffuse panbronchiolitis (DPB), asthma and community acquired pneumonia.

1.2 Antibiotics

1.2.1 Macrolides

Macrolides are drugs containing a macrocyclic lactone ring of at least 12 elements. Fourteen- and 15- membered macrolides (e.g. azithromycin, clarithromycin and erythromycin) are commonly prescribed to patients with chronic inflammatory airway diseases. Interestingly, the major effects of macrolides are independent of their antimicrobial properties, but rather due to their ability to modulate inflammation, decrease mucus hypersecretion and alter expression of the mucin gene, among other things (Kanoh & Rubin 2010). Macrolides possess many diverse biological activities, such as suppressing pro-inflammatory cytokines and chemokines, influencing cell signaling, killing bacteria and stabilizing the airway epithelial cell membrane (Kanoh & Rubin 2010), the latter of which is the main focus of this project. Studies have shown that azithromycin is especially efficient in treating patients with the previously mentioned diseases. Results from in vitro studies have indicated that azithromycin affects tight junctional proteins in human airway epithelia, by altering their location in cells and by inducing their processing of smaller fragments (Asgrimsson et al. 2006).

1.2.2 Cephalosporins

Cephalosporins are a large family of β-lactam antimicrobial drugs. These antibiotics have a broad spectrum of activity and are generally categorized into generations; another benefit is their low level of toxicity. Their antimicrobial effects are due to interruption of the peptidoglycan synthesis in bacteria, therefore disrupting the bacterial cell wall. Third generation cephalosporins are very active against Gram negative bacteria, including Pseudomonas aeruginosa. The cephalosporin used in this project was ceftriaxone, a third generation compound. Along with second- and other third-generation cephalosporins, ceftriaxone is commonly used to treat patients with community-acquire pneumonia (Marshall & Blair 1999), acute exacerbations of both COPD (Hunter & King 2001) and chronic bronchitis (Wilson et al. 2003). It has also shown that combining β-lactam antimicrobtics with a macrolide is even more effective when treating patients with community-acquired...
pneumonia (Kanoh & Rubin 2010).

![Chemical structures of azithromycin and ceftriaxone](image)

**Figure 2. The chemical structure of the macrolide azithromycin (left) and the cephalosporin ceftriaxone (right)** (Wikipedia, The free encyclopedia 2015).

### 1.3 VA10 cell line

The cell line used for this project is the VA10 cell line, a basal epithelial cell line established from a human bronchial explant. It was made by transfecting primary bronchial epithelial cells with retroviral E6 and E7 oncogenes from human papilloma virus 16 (HPV). Immortalized bronchial epithelial cell lines are important when studying characteristics of the respiratory tract, such as function and structure. The VA10 cell line is an excellent candidate for such studies since it maintains an intact karyotype at low passages, generates both apical-basolateral polarity in three-dimensional culture and high TER when cultured on air-liquid interface culture or in suspended culture. The cells express phenotypic traits of basal cells, such as cytokeratins, integrin α6β4 and p63 as well as expressing functional tight junction proteins. Air-liquid interface culture is very beneficial when studying bronchial epithelial cells. VA10 cells cultured in such conditions are able to recapitulate the pseudostratified mucociliary phenotype observed in human lungs, where the basal surface of the cells are exposed to liquid and the apical surface is exposed to air, hence is an excellent model for studying the epithelial integrity (Figure 3) (Halldorsson et al. 2007).

![Air-liquid interface culture](image)

**Figure 3. Air-liquid interface culture.** The apical surface of bronchial epithelial cells is exposed to air and the basal surface is exposed to liquid (Hou et al. n.d.)
2. Aim of the project

The aim of the project was to compare a distinct class of antibiotic with the macrolide azithromycin which is known to increase epithelial integrity in VA10 cells. Furthermore, to determine whether increases in epithelial integrity as determined by TER measurements are also detectable using immunofluorescence staining of tight junctin proteins and other potentially relevant cell markers.
3. Materials and methods

3.1 Cell culture

All cell culture was performed in a sterile environment, where cells were only exposed to the air inside a laminar flow hood.

3.1.1 VA10 cell culture

The VA10 cell line with passage numbers, 30 and 19 was cultured in pre-warmed bronchial epithelial growth medium (BEGM) (Gibco) supplemented with 50 IU/ml penicillin and 50 μg/ml streptomycin (5% P/S) (Invitrogen) in polystyrene T25 flasks (Falcon) in a humidified environment at 37°C with 5% CO₂. Media was changed every 2-3 days and the cells split at a ratio 1:5 or 1:6 when confluency was reached. The p30 VA10 cells were in culture until p33, where they were then used for an ALI experiment, making the ALI culture a passage 34.

3.1.2 Air liquid interface (ALI) culture

Confluent VA10 cells monolayers were seeded onto collagen-coated Transwell filters (0.4 μm polyester) (Corning) of a 12-well plate. Collagen I (PureCol, Advanced Biomatrix) was diluted 1:44 in phosphate buffered saline (PBS) and 0.5 ml pipetted into each Transwell insert and incubated for at least 30 minutes at 37°C, aspirated and washed once with PBS. The monolayer VA10 cells were washed twice with 3 ml PBS and incubated for 10 minutes at 37°C after the second wash. One ml of trypsin-EDTA (0.25% trypsin, 1 mM EDTA in PBS) was added and incubated at 37°C for 3-5 minutes for dislodging of cells. The trypsin was neutralized with 3 ml PBS followed by centrifugation at 2000 rpm for 3 minutes to pellet the cells. The supernatant was aspirated and the pellet resuspended in 5 ml BEGM medium. At this point, the cells were p20 and p34. Cells were counted with a hemocytometer and 150,000 cells of the p34 were seeded into the upper chamber of Transwell filters, while due to limited cells, only 128,000 cells of the p20 cells were seeded. BEGM medium was added to both chambers for two days (0.5 ml to upper chamber and 1.5 ml to lower chamber), then the medium was replaced with Dulbecco’s modified eagle medium (DMEM/F-12 (1:1)) (Gibco) + 5% P/S supplemented with 10% fetal bovine serum (FBS). When there appeared to be a confluent and intact layer of cells on the filters, the medium from upper chamber was removed and cells were washed with PBS. The medium in the lower chamber was replaced.
with DMEM/F-12 + 5% P/S supplemented with 2% Ultroser G (Pall Corporation, Germany) and changed every two days followed with TER measurements and PBS wash.

3.1.3 TER measurement

TER describes the barrier integrity and is the electrical, ohmic resistance of the cell layer (Benson et al. 2013). The equipment used for TER measurements was a Millicell® ERS Voltohmter together with a probe that has two electrodes. Each electrode was inserted into each chamber, that is, to each side of the cell layer (Figure 4). In principle, a direct current (U) was applied to the two electrodes and the resulting current (I) was measured and the resistance (R) calculated according to Ohm’s law (R=U/I) (Benson et al. 2013). Prior to TER measurement 0.5 ml DMEM/F-12 medium was added into the upper chamber, followed with a 20 minute incubation at 37°C. The probe was sterilized prior to measurement by washing with 96% ethanol. TER measurements commenced the same day the media from upper chamber was removed for both the high passage and low passage cells, that is, ALI culture started.

![Figure 4. TER measurements of ALI culture. Two electrodes were inserted into each chamber, E1 into the Transwell cup and E2 into the underlying well (Benson et al. 2013).](image)

3.1.4 Antibiotics

Two antibiotics were used in this project, the macrolide azithromycin (Apótek, Landspítali) and the cephalosporin ceftriaxone (rocephalin) (Apótek, Landspítali). Passage 34 cells were treated with one concentration of azithromycin alongside a control; 40 μg/ml azithromycin or 0 μg/ml control. Cells in passage 20 were treated with variable concentrations against a control; 0 μg/ml control, 20 μg/ml, 100 μg/ml, 200 μg/ml ceftriaxone or 40 μg/ml azithromycin. The stocks were: 2 mg/ml azithromycin and 20 mg/ml ceftriaxone, both dissolved in water. The various dilutions were prepared using the stock solutions in
DMEM/F-12 medium as diluent and added to the lower chambers. The antibiotics were first added to the medium the same day TER measurements started (day 0).

### 3.2 Immunocytochemistry

#### 3.2.1 Methanol fixation

Cells from the ALI culture were fixed with methanol. This was done by washing the Transwells in chilled PBS and submerging them in wells filled with 100% methanol (-20°C) and letting them fix overnight at -20°C. The methanol was removed the following day and 100% acetone (-20°C) was added to each filter for 1 minute (protocol as given by http://www.zonapse.net/protocols/id6.html).

#### 3.2.2 Immunostaining

The methanol-fixed Transwell filters were excised from the cup and cut into four small slices. The individual filter slices were put in a 48 well plate and hydrated with IMF buffer (0.1% Triton X-100, 0.15 M NaCl 5 mM EDTA, 20 mM HEPES, pH 7.5) followed by two IMF washes. 100 μl of IMF buffer was added to each filter along with primary antibodies and incubated overnight at 4°C (see Table 1). Filters were then washed three times for 15 minutes each in IMF buffer and Alexa Fluor secondary antibodies were added to each filter in 100 μl of IMF buffer and incubated for two hours at room temperature (see Table 2). The filters were again washed four times for 15 minutes each in IMF buffer and stained with 0.1 μg/ml DAPI in PBS for 60 minutes at room temperature. The filters were again washed four times with PBS, once with water and fixed for 60 minutes in ice cold 95% ethanol at 4°C. At last, the filters were placed on a glass slide and a drop of antifade reagent (Fluoromount™ aqueous mounting medium) was added before they were covered with a glass coverslip. The filters were imaged using a Olympus FV1200 laser scanning confocal microscope.
Table 1. Primary antibodies and DNA stain for immunostaining of Transwell filters.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Isotype</th>
<th>Supplier</th>
<th>Catalogue number</th>
<th>Dilution</th>
<th>Initial conc. (μg/μl)</th>
<th>Final conc. (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claudin-1</td>
<td>rabbit</td>
<td>-</td>
<td>Invitrogen</td>
<td>519000</td>
<td>1:100</td>
<td>0.25</td>
<td>2.50</td>
</tr>
<tr>
<td>Claudin-4</td>
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<td>IgG1</td>
<td>Invitrogen</td>
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<tr>
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<td>IgG1</td>
<td>Invitrogen</td>
<td>331500</td>
<td>1:150</td>
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<td>3.35</td>
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<tr>
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<td>rabbit</td>
<td>-</td>
<td>Zymed</td>
<td>361700</td>
<td>1:25</td>
<td>0.25</td>
<td>1.0</td>
</tr>
<tr>
<td>ZO-1</td>
<td>rabbit</td>
<td>-</td>
<td>Abcam</td>
<td>Ab59720</td>
<td>1:100</td>
<td>0.10</td>
<td>1.0</td>
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<tr>
<td>E-cadherin</td>
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<td>BD biosciences</td>
<td>610181</td>
<td>1:100</td>
<td>0.25</td>
<td>2.50</td>
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<tr>
<td>N-cadherin</td>
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<td>LL-37</td>
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<td>-</td>
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<td>-</td>
<td>Cell signaling technology</td>
<td>D38B1</td>
<td>1:100</td>
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<td>N/A</td>
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<td>mouse</td>
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<td>Novocastra</td>
<td>NCL-L-p63</td>
<td>1:25</td>
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<td>N/A</td>
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Table 2. Secondary antibodies used for immunostaining of Transwell filters.

Initial concentration was 2 mg/ml for all antibodies and final concentration 2 μl/ml with the dilution of 1:1000.

<table>
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<tr>
<th>Secondary antibody</th>
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<tr>
<td>Alexa fluor® 488 goat anti-rabbit</td>
<td>Life technologies</td>
<td>A11008</td>
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<td>A11010</td>
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<tr>
<td>Alexa fluor® 488 goat anti-mouse IgG3 (γ3)</td>
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4. Results

4.1 TER measurements

4.1.1 VA10 ALI culture p34

VA10 ALI culture p34 were treated with or without 40 µg/ml azithromycin. Cells treated with 40 µg/ml azithromycin did not show higher TER than the control (Figure 5). This is in contrast to the study by Asgrimsson et al., where they showed that treatment with 40 µg/ml azithromycin increases TER significantly in VA10 ALI culture. These results implicate that the passage number used (34) was too high to give reliable TER measurements.

![Figure 5](image_url)

Figure 5. Results from TER measurements on VA10 ALI culture p34. Results showed that treatment with 40 µg/ml azithromycin did not give higher TER compared to the control.
4.1.2 VA10 ALI culture p20

VA10 ALI culture with lower passage number (p20) were treated with the macrolide azithromycin (40 µg/ml), the cephalosporin ceftriaxone (20, 100 or 200 µg/ml) or without antibiotics (control). Treatment with ceftriaxone increased TER in dose- and time- dependent manners, whereas the highest dose of ceftriaxone (200 µg/ml) gave a two-fold increase in TER. In contrast to the p34 experiment, treatment with 40 µg/ml azithromycin gave a three-fold increase in TER. TER was expected to decrease the first 4-5 days as a result of the cells encountering stress.

![Graph](image)

**Figure 6. Results from TER measurements on VA10 ALI culture p20.** Results showed that treatment with 40 µg/ml azithromycin gave a three-fold increase in TER and treatment with ceftriaxone increased TER in dose- and time- dependent manners, whereas the highest dose (200 µg/ml) gave a two-fold increase in TER.
4.2 Immunocytochemistry

4.2.1 VA10 ALI p34

Through the use of fluorescent immunostaining, the proteins of interest were able to be visualized for further study. All proteins were expressed in the cells as expected. The proteins were imaged with a 20x objective but some were also imaged with a 60x objective. The images presented here were achieved by collecting serial optical sections from the samples into one image, a projected image. The optical series gave additional insights and were therefore also examined when the images were interpreted. The following describes the representative figures 7-10, where VA10 cells were treated with either control or 40 µg/ml azithromycin. The signal for both claudin-1 and occludin (Figure 7) increases moderately in cells treated with 40 µg/ml azithromycin compared to the control. Additionally, cells treated with azithromycin appear larger in volume. JAM-A and claudin-4 (Figure 8) gave similar staining patterns, although the signal for claudin-4 does not seem to increase overall. The control for claudin-4 shows areas with moderate signal intensity and small circular cells, that is, more compact cells, but also areas with stronger signal and larger cells, whereas the azithromycin treatment appears to show that moderate signal and larger cells are dominant. The signal for JAM-A appears to increase slightly and larger circular, “pancake” cells (located at the apical side of the cell layers on the filter) are more frequent. Interestingly, the cells demonstrate a certain level of heterogeneity, where areas demonstrate different staining intensities, indicating the differentiation potential of the cells. Both LL-37 and CK-17 (Figure 9) have increased signal intensity when treated with azithromycin, especially CK-17. For those proteins, both larger and more frequent staining patterns can be detected, indicating larger, squamous-like cells. EGFR, p63, E-cadherin and N-cadherin did not demonstrate any change in signal intensities when compared to the control (Figure 10). The poor condition of the filter used for the cadherins hindered the interpretation of their images. Although there was no change in TER between the azithromycin treated cells and the control, it doesn’t seem to reflect a change in staining intensities in the immunostainings. The change in signal intensity for each protein compared to control is recapitulated in table 3.
Figure 7. Immunostaining of claudin-1 (A) and occludin (B) in control and 40 µg/ml azithromycin treated p34 cells. Cells were also stained with the nuclear stain DAPI (blue) and the images were taken with both a 20x objective and a 60x objective. The signal intensity for both claudin-1 and occludin increases moderately in azithromycin treated cells compared to the control, in addition to appear larger in volume.
Figure 8. Immunostaining of JAM-A (A) and claudin-4 (B) in control and 40 µg/ml azithromycin treated p34 cells. Cells were also stained with the nuclear stain DAPI (blue) and the images were taken with both a 20x objective and a 60x objective. The signal for JAM-A appears to increase slightly and larger circular “pancake” cells are more frequent. The control for claudin-4 shows areas with moderate signal intensity and small circular cells but also areas with stronger signal and larger cells, whereas the azithromycin treatment appears to show that moderate signal intensity and larger cells are dominant.
Figure 9. Immunostaining of LL-37 (A) and CK-17 (B) in control and 40 µg/ml azithromycin treated p34 cells. Cells were also stained with the nuclear stain DAPI (blue) and the images were taken with both a 20x objective and a 60x objective. Both LL-37 and CK-17 have increased signal intensity when treated with azithromycin, especially CK-17. For those proteins, both larger and more frequent intense staining patterns can be detected.
Figure 10. Immunostaining of EGFR, p63, E-cadherin and N-cadherin in control and 40 µg/ml azithromycin treated p34 cells. Cells were also stained with the nuclear stain DAPI (blue) and the images were taken with a 20x objective. EGFR, p63, E-cadherin and N-cadherin did not indicate any change in signal intensity compared to control, but the poor condition of the filter used for the cadherins hindered the interpretation of their images.
4.2.2 VA10 ALI p20

The imaging and interpretation of the VA10 ALI p20 samples was performed in the same way as for the p34 samples. The same panel of proteins was examined, additionally with ZO-1 and CK-5/6, but now also with ceftriaxone in three different concentrations. Interestingly, the results from p20 cells did not show the same results as seen in the p34 cells. Admittedly, this is not surprising given the different TER results. The following describes the representative figures 11-16, where VA10 cells were treated with control, azithromycin (40 µg/ml) or ceftriaxone (20, 100 or 200 µg/ml). Claudin-1 staining appears to show an increased signal when treated with 20 µg/ml ceftriaxone, and a moderate signal decrease when treated with 100 µg/ml while there is no apparent change for the highest dose and the azithromycin treated cells (Figure 11). The staining of occludin shows a more compact cell population and moderate increased signal intensity for both the highest dose of ceftriaxone and azithromycin treatment (Figure 11). This is in harmony with the TER measurements, that is, cells treated with 200 µg/ml ceftriaxone gave a two-fold increase in TER and cells treated with 40 µg/ml azithromycin gave a three-fold increase in TER, while the other doses did not show a difference in either TER measurements or in immunostaining. Treatment of ceftriaxone and azithromycin do not appear to change the signal intensity for claudin-4 and ZO-1, except the treatment with 20 µg/ml ceftriaxone which shows a possible increase in intensity and more compact cell population (Figure 12). None of the treatments indicate a change in signal intensity for E-cadherin and N-cadherin (Figure 13). All treatments appear to moderately decrease the signal intensity for CK-5/6 and cause the cells to be more compact, while in contrast, all treatments appear to increase the signal for LL-37 and diffuse mass of the protein can be detected, especially when treated with azithromycin (Figure 14). The intense spots in the CK-5/6 samples are most likely either precipitate from the secondary antibody or dead/abnormal cells at the apical side of the filter. Treatment with 100 and 200 µg/ml ceftriaxone and 40 µg/ml azithromycin appear to noticeably increase the signal intensity for both EGFR and p63, whereas staining of EGFR reveals a more compact cell population and staining of p63 seems to increase markedly compared to the control (Figure 15). The signal for JAM-A is weak and unclear, presumably due to poor quality of the antibody. Staining of JAM-A was only done on the control and samples treated with 200 µg/ml ceftriaxone and 40 µg/ml azithromycin where no difference was detected between the samples (Figure 16). The images of CK-17, taken with a 60x objective and presented here indicate that cells treated with 200 µg/ml ceftriaxone show decreased signal intensity and more compact cells are more frequent and evident compared to the control and other treatments, but when imaged with a 20x objective, no overall change in signal intensity is observed for CK-17 (Figure 16). Therefore, the area selected for imaging with a 60x objective is not a reflection of the whole filter. This is a concern that applies to all filters that were stained. The change in signal intensity for each protein compared to control is recapitulated in table 3.
Table 3. Estimation of signal intensity for each protein in each concentration compared to control for both VA10 ALI culture p20 and p34. The symbols ↑/↓ stand for slightly increased/decreased signal intensity while the symbols ↑↑/↓↓ stand for apparent increased/decreased intensity. The symbol – stands for no detection of altered signal intensity.

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<td>p20</td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone</td>
</tr>
<tr>
<td></td>
<td>20 µg/ml 100 µg/ml 200 µg/ml 40 µg/ml</td>
</tr>
<tr>
<td>Claudin-1</td>
<td>↑</td>
</tr>
<tr>
<td>Occludin</td>
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</tr>
<tr>
<td>JAM-A</td>
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<tr>
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<td>-</td>
</tr>
<tr>
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<td>↑</td>
</tr>
<tr>
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<td>-</td>
</tr>
<tr>
<td>EGFR</td>
<td>-</td>
</tr>
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<td>-</td>
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</tr>
<tr>
<td>N-cadherin</td>
<td>-</td>
</tr>
<tr>
<td>ZO-1</td>
<td>↑</td>
</tr>
<tr>
<td>CK-5/6</td>
<td>↓</td>
</tr>
</tbody>
</table>

↑↓: slightly increased/decreased signal intensity
↑↑/↓↓: apparent increased/decreased intensity
- : no detection of altered signal intensity
N/A: immunostaining not available
Figure 11. Immunostaining of claudin-1 (left column), occludin (center column) and merge with DAPI stain (right column) in p20 cells, taken with a 60x objective. Claudin-1 appears to show slight signal increase when treated with 20 µg/ml, and a slight signal decrease when treated with 100 µg/ml, no change seems to be for the other treatments. The staining of occludin shows more compact cell population and moderate increased signal intensity for both the highest dose of ceftriaxone and azithromycin treatment.
Figure 12. Immunostaining of claudin-4 (left column), ZO-1 (center column) and merge with DAPI stain (right column) in p20 cells, taken with a 60x objective. Treatment of ceftriaxone and azithromycin do not appear to change the signal intensity for claudin-4 and ZO-1, except treatment with 20 µg/ml ceftriaxone which shows a possible increase in intensity and more compact cell population.
Figure 13. Immunostaining of E-cadherin (left column), N-cadherin (center column) and merge with DAPI stain (right column) in p20 cells, taken with a 20x objective. None of the treatments demonstrate a change in signal intensity for E-cadherin and N-cadherin.
Figure 14. Immunostaining of CK-5/6 (left column), LL-37 (center column) and merge with DAPI stain (right column) in p20 cells, taken with a 60x objective. All treatments appear to slightly decrease the signal intensity for CK-5/6 and causing its configuration to seem smaller (the intense spots are assumed to be precipitate from the antibody or dead/abnormal cells). All treatments appear to slightly increase the signal for LL-37 and diffuse mass of the protein can be detected when treated with azithromycin.
Figure 15. Immunostaining of EGFR (left column), p63 (center column) and merge with DAPI stain (right column) in p20 cells, taken with a 60x objective. Treatment with 100 and 200 µg/ml ceftriaxone and 40 µg/ml azithromycin appear to noticeably increase the signal intensity for both EGFR and p63, whereas staining of EGFR reveals more compact cell population and staining of p63 seems to increase markedly compared to control.
Figure 16. Immunostaining of JAM-A (left column), CK-17 (center column) and merge with DAPI stain (right column) in p20 cells, taken with a 60x objective. Poor quality of JAM-A antibody resulted in a weak and unclear signal. Staining of JAM-A was only done on the control and samples treated with 200 µg/ml ceftriaxone and 40 µg/ml azithromycin whereas no difference was detected between the samples. Images of CK-17 indicate that compact cells are more frequently detected in cells treated with the antibiotics, especially with 200 µg/ml ceftriaxone compared to control. However, images of CK-17 with a 20x objective indicate no overall change in signal intensity for CK-17.
5. Discussions

In this study the effects of two distinct antibiotics, azithromycin and ceftriaxone on bronchial epithelial integrity were examined. Studies have shown that azithromycin contributes to higher TER and changes the processing of claudin-1, claudin-4, occludin and JAM-A, that is, changes their intracellular location and was demonstrated with immunostaining and Western blots (Asgrimsson et al. 2006).

Two passage numbers, p34 and p20 were studied in this experiment but only p20 showed higher TER when treated with azithromycin. Although p34 is not considered a high passage number, it likely has undergone clonal selection resulting in the loss of differentiation potential, hence a lack of TER. Cells with lower passage number (p20) showed promise for more reliable TER measurements and gave a three-fold increase in TER after treatment with 40 µg/ml azithromycin. This is consistent with results from Asgrimsson et al, where TER also increased three-fold with 40 µg/ml azithromycin treatment, although the measured TER values were higher overall. Azithromycin is a macrolide, derived from erythromycin. Erythromycin is effective when treating pulmonary diseases but has not been shown to affect processing of tight junctions (Asgrimsson et al. 2006). Azithromycin differs from erythromycin by its one methyl-substituted nitrogen atom in the lactone ring and one may suspect that its unique chemical structure could be the reason for its effect on tight junctions. The reason ceftriaxone, a cephalosporin was used in this experiment was to support the azithromycin argument. Both penicillin and erythromycin have been compared to azithromycin, with neither treatment resulting in an increase in TER or affecting proteins as demonstrated by Western blot (Asgrimsson et al. 2006). Therefore it was interesting to examine if this held true for other drugs used in the clinic, especially those used on lung patients. Ceftriaxone is not a macrolide but it is prescribed for patients with acute exacerbations of which *Pseudomonas* is often a cause. Since ceftriaxone is not a macrolide and its effects have not been shown to be independent of its antimicrobial properties, it was not expected to give results similar to azithromycin. Interestingly, ceftriaxone treatment caused TER to increase in dose- and time- dependent manners where the highest dose gave a two-fold increase. TER measurements were stopped on day 18, but if the culture was left to continue, it is tempting to speculate that TER would continue to increase, potentially resulting in the antibiotics producing similar TER measurements. It is worth noting that the relevant *in vitro* dose for azithromycin based on the clinical dose is 40 µg/ml, whereas the relevant dose of ceftriaxone for *in vitro* experiment like this one has not been adequately studied, although
the concentration has been determined to be around 100 µg/ml. Hence, treatment with 200 µg/ml ceftriaxone may be slightly toxic to cells, despite giving highest TER. Patients with community-acquired pneumonia treated with a combination of β-lactam and macrolide show lower mortality rates than patients treated only with β-lactam (Kanoh & Rubin 2010). Thus, the results presented here raise the question what would the effects of combining both antibiotics be on TER measurements?

In summary, the immunostaining of the proteins alone is not sufficient to conclude the effects of the antibiotics on epithelial integrity. The interpretation of the images is considered subjective since the results are not quantifiable, not to mention that the experiment was not repeated. Furthermore, the image is largely dependent on the area chosen for the imaging, as the filter tends to curl. In order to get significant results, gene expression of the proteins alongside Western blots would be useful. These methods would have given better insights into the study at hand, but were not performed due to time constraints of the project. The images gave some suggestions into the morphological status of the studied proteins and their intensity in staining. The projected images presented here were at times insufficient for interpretation so the optical series taken with the confocal microscopy revealed the cells appearance throughout the sample. The images of the tight junction molecules do not suggest stark changes in their intracellular location for either azithromycin or ceftriaxone, but the methods used in this project are different from the methods that Grimsson et al. used, namely the variable results may partly owe to the fact that their cells were cultured on glass slides. Comparison of the results between p34 and p20 shows inconsistency regarding half of the proteins studied (Claudin-1, JAM-A, CK-17, EGFR and p63). Although TER was not increased in p34, it does not necessarily indicate that the processing of the proteins could not be altered but the lack of TER in p34 experiment makes it less plausible. Overall, results from TER measurements do not seem to be in context with the results from immunostaining when each dose is examined, except for occludin that showed changes in both TER and in immunostaining when treated with highest dose of ceftriaxone and azithromycin, when compared to the control. The tight junctions were expected to show different processing in azithromycin cells, which was not observed in this research. E-cadherin however was not expected to show different processing, which is consistent with results from both p34 and p20 cells. Previous studies in the laboratory (SCRU) have indicated that LL-37 decreases in human airway epithelium derived basal cell lines when treated with 40 µg/ml azithromycin, demonstrated by gene expression (VA10 cell line) and immunostaining (BCi_NS1.1 cell line, (Walters et al. 2013)), (unpublished data), while results from this experiment indicate that LL-
37 increases. Cathelicidins are active participants in killing bacteria (Zanetti 2004), hence suggesting that treatment with antibiotics may decrease the levels of cathelicidins as the antibiotic is playing its role. The results presented here could be an anomaly, since the effects of the antibiotics were only determined by immunofluorescence and the research was not repeated, making interpretation of the results difficult. Furthermore, a diffuse mass appearance of the azithromycin treated cells could be due to LL-37 being secreted and a result of the fixation step. Previous studies with LL-37 and VA10 cells have shown that this is a typical appearance of cells without treatment (oral communication) so why this result appears after treatment could be potentially due to stress, since LL-37 is involved in the NF-kB signaling pathway that is associated with endoplasmic reticulum stress (Park et al. 2011). The effect of the antibiotics on the other proteins (EGFR, p63, CK-5/6, CK-17 and N-cadherin) has not previously been examined, therefore it is not known what results were to be expected for these proteins. The basal markers, EGFR and p63 are often linked together. Immunostaining on these proteins gave an interesting result with an increase in staining intensity when treated with azithromycin and ceftriaxone. There is not an obvious explanation but in order to make a conclusion, this would have to be followed up with Western blot or gene expression measurement.

Despite indications from the clinic claiming azithromycin to have beneficial effects on patients outside of its role as an antibiotic, there is relatively little evidence for the mechanism. This study has demonstrated some changes on the bronchial epithelium as a result of azithromycin treatment, but this was not limited to azithromycin as ceftriaxone treated resulted in similar changes. Further research on the effects of azithromycin on epithelial integrity is worth performing to reveal its properties and mechanism, along with researching different antibiotics, macrolides and non-macrolides for comparison. However, as mentioned above, immunofluorescence study alone is insufficient for these purposes.
6. References


Excoffon, K.J.D. a et al., 2008. Reovirus preferentially infects the basolateral surface and is released from the apical surface of polarized human respiratory epithelial cells. The Journal of infectious diseases, 197, pp.1189–1197.


