Azithromycin is in vitro superior to other macrolides in the respiratory epithelial barrier enhancement, phospholipid retention, vesicle build-up, and its effect on gene sets related to keratinocyte differentiation | 1

Antibacterial medications called macrolides are frequently used to treat respiratory diseases. Erythromycin was the first macrolide to be discovered in 1952. Later chemical modifications of erythromycin led to macrolides with longer half-lives, better tissue distribution, and similar or better antibacterial activity. The respiratory epithelium is an important barrier against external agents, and its failure allows agents to infiltrate the sub-epithelial stroma and trigger an inflammatory response. In this study, the authors from Iceland used a bronchial epithelial cell line, VA10, to investigate the non-antibacterial effects of macrolides, erythromycin, clarithromycin, roxithromycin, azithromycin (AZM), solithromycin, and an aminoglycoside, tobramycin, on the differentiation of bronchial epithelial cells and the integrity of the respiratory barrier. The results showed that azithromycin is superior to other macrolides in improving the respiratory epithelial barrier *in vitro*.

Besides their actions on Gram-positive bacteria, macrolide antibiotics have many additional effects, like immunomodulatory effects, lipid remodeling, epidermal differentiation, inhibition of mucus secretion, and barrier enhancement. (Kricker, Jennifer A. *et al.* Nonantimicrobial Actions of Macrolides: Overview and Perspectives for Future Development. Pharmacological Reviews, Volume 73, Issue 4, 1404-33. https://pharmrev.aspetjournals.org/article/S0031-6997%2824%2900694-X/fulltext)

AZM has been shown to improve lung function in both chronic obstructive pulmonary disease (COPD) and cystic fibrosis. COPD is characterized by an epithelial-to-mesenchymal transition (EMT) phenotype, which leads to barrier failure, dysfunctional mucociliary clearance, and easier paracellular access of infectious agents to the underlying submucosa. AZM has been reported to suppress EMT mediated by the transforming growth factor (TGF)-β1. Also, AZM and other macrolides, as cationic amphiphilic drugs, can bind to phospholipids and inhibit their degradation, directly or indirectly contributing to the barrier-enhancing effects.

The same research group has previously found that AZM improves the bronchial epithelial barrier *in vitro* and prevents barrier failure in bronchial epithelial cells treated with culture medium derived from *Pseudomonas aeruginosa*, frequently implicated in cystic fibrosis.



About the study

The authors compared the effects of the macrolides erythromycin, clarithromycin, roxithromycin, azithromycin, solithromycin, and the aminoglycoside tobramycin, at an equimolar concentration of 35 µM, on the bronchial epithelial cell line VA10 cultured under air-liquid interface (ALI) conditions for three weeks. The effects of antibiotics on culture differentiation, cell-cell junctions, and epithelium were evaluated by RNA sequencing, barrier integrity assays, and immunostaining on days 14 and 21. The epithelial barrier failure was evidenced by transepithelial electrical resistance (TEER), because increased TEER reflects decreased epithelial permeability. The ability of macrolides to induce phospholipid retention was investigated in a VA10 monolayer cell culture treated with macrolides for three days.

Since COPD is characterized by dysfunctional mucociliary clearance and an epithelial-to-mesenchymal transition (EMT) phenotype leading to barrier failure, the effects of macrolides on the expression of genes involved in EMT pathways were also explored.

Results

AZM reduced epithelial barrier failure, evidenced by increased transepithelial electrical resistance (TEER). AZM effects on epithelial barrier enhancement were also evidenced by decreased paracellular flux and increased thickness of the epithelial cell layer. Erythromycin and clarithromycin also significantly increased TEER, whereas roxithromycin and tobramycin did not affect TEER but moderately increased epithelial cell layer thickness.

The viability assay demonstrated that viability was not significantly affected by any treatment.

Analysis of the gap junctions, adherens junctions, desmosomes, and hemidesmosomes for all treatments on days 14 and 21 showed that AZM increased the expression of the largest isoform of occludin but decreased the expression of the other isoforms. AZM also decreased

the precursor and mature forms of the protein DSG-1, which belongs to the E-cadherin superfamily and is one of the most important desmosomal cadherins. AZM also downregulated CAV-1 at the RNA and protein levels. Since CAV-1 interacts with tight junctions and affects their expression and localization, this effect of AZM could also contribute to an increase in TEER. Immunostaining for tight junction proteins showed a redistribution of claudin-4, occludin, ZO-1, JUP, and DSG-1 after AZM treatment. According to the authors, this protein redistribution probably contributes to the improvement of the respiratory barrier.

Erythromycin, clarithromycin, and roxithromycin increased the mature form of the protein DSG-1, and only solithromycin caused a significant increase in the precursor form of DSG-1.

The effect of AZM treatment on phospholipid retention was the most significant (threefold that of the control). Erythromycin and clarithromycin also increased phospholipid retention.

Only AZM treatment positively affected genes related to keratinocyte and epidermis differentiation at both time points. On day 21, AZM treatment positively enriched 242 significant gene sets that were not upregulated with the other tested macrolides. Roxithromycin treatment positively enriched the second-highest number of gene sets (106 gene sets).

All macrolides downregulated gene sets involved in epithelial-to-mesenchymal transition phenotype, which characterizes COPD. Tested compounds negatively affected JAK-STAT, Notch, TGFβ, Hedgehog, and WNT signaling pathways, but to varying degrees. At both time points (on days 14 and 21), only AZM negatively enriched TGFβ, Notch, JAK-STAT, Hedgehog, and WNT signaling. Clarithromycin treatment had a similar effect, whereas roxithromycin, solithromycin, and tobramycin did not affect JAK-STAT signaling. Erythromycin negatively enriched TGFβ, JAK-STAT, and WNT signaling at a single time point.

AZM also downregulated the gene expression for COL1A2, COL3A1, COL5A2, MMP2, and MMP9. This suggests a role of AZM in collagen remodeling, and one of the many avenues by which AZM asserts its effects in COPD.

Conclusion

This *in vitro* study investigated the effect of macrolides on bronchial epithelial cells and the integrity of the respiratory barrier. All macrolides (erythromycin, clarithromycin, roxithromycin, AZM, solithromycin, and an aminoglycoside, tobramycin affected the expression of genes involved in the epithelial-to-mesenchymal transition, metabolism, and immunomodulation. Treatment with AZM, clarithromycin, and erythromycin increased TEER and induced phospholipid retention. AZM treatment was specific in terms of the epithelial barrier enhancement, phospholipid retention, vesicle build-up, and its effect on gene sets related to keratinocyte differentiation and establishment of the skin barrier.

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