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 investigated 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 using a bronchial epithelial cell line VA10. The results showed that azithromycin is superior to other macrolides in improving the respiratory epithelial barrier *in vitro*.

In addition to their actions on Gram-positive bacteria, macrolide antibiotics have been shown to have many additional effects, such as 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, leading 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.

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 or rhamnolipids derived from *Pseudomonas aeruginosa*, which is frequently implicated in cystic fibrosis. In addition, AZM and other macrolides, as cationic amphiphilic drugs, can bind phospholipids and inhibit their degradation, directly or indirectly contributing to the barrier-enhancing effects.

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). Increased TEER reflected a 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 gene expression of pathways involved in EMT were also explored.

Results

In vitro results showed that AZM reduced epithelial barrier failure, evidenced by increased transepithelial electrical resistance (TEER), reflecting decreased epithelial permeability. The 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 showed that viability was not significantly affected by any treatment.

To explain the improved barrier function observed in the bronchial epithelial cell culture after AZM treatment, the authors analyzed the genes for gap junctions, adherens junctions, desmosomes, and hemidesmosomes for all treatments at both time points (on days 14 and 21).

AZM increased the expression of occludin's largest isoform but decreased the expression of the other isoforms. Further, AZM decreased the levels of 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. Erythromycin, clarithromycin, and roxithromycin increased the mature form of DSG-1, and only solithromycin caused a significant increase in the precursor form of DSG-1. 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 increased TEER. Immunostaining for tight junction proteins showed a redistribution of claudin-4, occludin, ZO-1, JUP, and DSG-1 and their supra-apical location after AZM treatment. According to the authors, this protein redistribution probably contributes to the improvement of the respiratory barrier.

The effect of AZM treatment on phospholipid retention was most significant and was threefold that of the control. Erythromycin and clarithromycin also increased phospholipid retention. Transmission electron images of ALI cultures showed that only AZM treatment caused vesicle build-up, whereas erythromycin and clarithromycin did not show any effect.

Only AZM treatment positively affected gene categories related to the differentiation of keratinocytes and epidermis at both time points. On day 21, gene ontology (GO) enrichment analysis showed that AZM treatment positively enriched 242 significant gene sets for biological processes. These gene sets were not upregulated with the other tested macrolides. Roxithromycin treatment, which positively enriched the second-highest number of gene sets, only did so for 106 unique GO biological processes.

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 gene expression of pathways involved in EMT were also investigated. All macrolides downregulated gene sets involved in EMT. JAK-STAT, Notch, TGFβ, Hedgehog, and WNT signaling pathways were all affected negatively by the tested compounds but to varying degrees. At both time points (on days 14 and 21), TGFβ signaling, Notch signaling,

JAK-STAT signaling, Hedgehog signaling, and WNT signaling were negatively enriched only by AZM. 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 one-time point.

AZM also downregulated the gene expression of COL1A2, COL3A1, COL5A2, MMP2, and MMP9, suggesting its role in collagen remodeling. Since increased collagen turnover is a marker of stable COPD and is elevated during COPD exacerbations, these results could indicate 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 gene expression of pathways 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 distinct in terms of enhancement of the epithelial barrier, retention of phospholipids, vesicle build-up, and its effect on gene sets related to keratinocyte differentiation and establishment of the skin barrier.

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