Non-Antibiotic Macrolides Prevent Human Neutrophil Elastase-Induced Mucus Stasis and Airway Surface Liquid Volume Depletion

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Abstract

Mucus clearance is an important component of the lung’s innate defense system. A failure of this system brought on by mucus dehydration is common to both cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD). Mucus clearance rates are regulated by the volume of airway surface liquid (ASL) and by ciliary beat frequency (CBF). Chronic treatment with macrolide antibiotics is known to be beneficial to both CF and COPD patients. However, chronic macrolide usage may induce bacterial resistance. We have developed a novel macrolide, 2'-desoxy-9-(S)-erythromycylamine (GS-459755), which has significantly diminished antibiotic activity against *Staphlococcus aureus*, *Streptococcus pneumonia*, *Moraxella catarrhalis* and *Haemophilus influenzae*. Since neutrophilia frequently occurs in chronic lung disease and human neutrophil elastase (HNE) induces mucus stasis by activating the epithelial sodium channel (ENaC), we tested the ability of GS-459755 to protect against HNE-induced mucus stasis. GS-459755 had no effect on HNE activity. However, GS-459755 pretreatment protected against HNE-induced ASL volume depletion in human bronchial epithelial cells (HBECs). The effect of GS-459755 on ASL volume was dose-dependent (IC$_{50}$ ~3.9 μM) and comparable to the antibacterial macrolide azithromycin (IC$_{50}$ ~2.4 μM). Macrolides had no significant effect on CBF, or the transepithelial water permeability. However, the amiloride-sensitive transepithelial voltage, a marker of ENaC activity, was diminished by macrolide pretreatment. We conclude that GS-459755 may limit HNE-induced activation of ENaC and may be useful for the treatment of mucus dehydration in CF and COPD without inducing bacterial resistance.
**Introduction**

Mucus clearance operates continuously to facilitate the removal of inhaled pathogens and particles (27). The airway surface liquid (ASL) lines the lung’s mucosal surfaces and acts as a lubricant to maintain mucus clearance (38). The ASL is composed of two layers: a mucus layer, which traps inhaled particles and an underlying periciliary layer (PCL), which keeps mucus at an optimum distance from the epithelia to affect mucus clearance (17, 38). The height of the PCL is approximately the length of outstretched cilia (7 µm) while the mucus layer height varies considerably (2-70 µm) (39). In normal airways, CFTR mediates anion secretion in both the superficial epithelia and in submucosal glands to drive liquid secretion and maintain ASL at its optimal height for efficient mucus transport with absorption of excess liquid across the epithelia being controlled by the epithelial Na⁺ channel (ENaC) (4). CFTR is a cAMP-regulated Cl⁻ channel and as such, its activity is strongly influenced by adenosine, which is formed extracellularly and stimulates purinergic receptors to raise cAMP (12). ENaC has a reciprocal relationship with CFTR and is active in the absence of cAMP. However, the extracellular loops of ENaC must be proteolytically cleaved by intracellular furin-type convertases or extracellular serine proteases in order for the channel to become active and to conduct Na⁺ (25). In addition to cleavage by furin and serine proteases, ENaC can also be cleaved and activated by human neutrophil elastase (HNE) (7). Thus, it is likely that ENaC is abnormally activated in conditions of chronic neutrophilia (i.e. in CF and COPD lungs) due to increased mucosal HNE levels, leading to enhanced Na⁺ absorption which may be contributory to the observed mucus dehydration/mucus stasis (5).

COPD is the fourth leading cause of death in the United States with over 1 million people affected (28). COPD is a syndrome which encompasses both emphysema and chronic bronchitis
(CB) that is most often caused by cigarette smoke (CS) (16) that is characterized by mucus accumulation, a robust immune response and in later stages of the disease, chronic neutrophilia (24). More recently it has emerged that altered ion channel activity also occurs, which is predicted to contribute to mucus dehydration and deficient mucus clearance (8, 11). Macrolide antibiotics typically used in the clinic are characterized by the presence of a macrocyclic lactone ring containing 14 or 15 atoms to which one or two sugars are attached via glycosidic bonds (34). As antibiotics, they inhibit bacterial protein synthesis by binding to the bacterial ribosomal 50S subunit which interferes with transpeptidation/translocation (21). They are often used to treat gram positive bacteria such as *Streptococcus pneumonia* and some gram negative bacteria including *Haemophilus influenzae* (21). In addition to being antibiotic, macrolides also have immunomodulatory and anti-inflammatory properties. For example, they have successfully been used to treat diffuse panbronchiolitis, COPD and CF, likely by also acting in an immunomodulatory role (14, 29, 31, 41). More specifically, macrolides have been shown to affect NFkB and ERK MAPK activation, decrease IL-8 secretion and decrease MUC5AC expression with efficacy in the micromolar range (22). Since chronic antibiotic usage can induce bacterial resistance, it has been proposed that a non-antibacterial macrolide would be therapeutically beneficial in treating chronic airway disease without inducing bacterial resistance. Accordingly, we designed a novel macrolide, GS-459755, which has negligible antibacterial ability. Since HNE-induced mucus stasis is thought to be contributory to chronic lung disease, we tested the ability of clinically established macrolides (erythromycylamine and azithromycin) vs. GS-459755 to prevent HNE-induced mucus stasis and ASL volume depletion in vivo and in vitro.
Experimental Procedures

Sheep tracheal mucus clearance velocity (TMV) measurements. TMV was evaluated as described (37). See also, online supplemental methods.

GS-459755 Chemistry. The synthesis steps are included in the online supplement and in Fig. S1.

MIC<sub>90</sub> determination. Cation adjusted Mueller-Hinton broth (DIFCO Lot #7158099) was used for all testing. The media was supplemented with 3% Lysed horse blood (Hemostat Lot#H03218) for testing S. pneumoniae or made up as Haemophilus Test Medium (HTM) for testing H. influenzae. Minimum inhibitory concentrations (MICs) for each compound were determined by the broth microdilution method recommended by the CLSI (M7-A8, 2009). A total of 50 aerobic isolates representing 4 species were tested. These isolates included clinical isolates as well as strains from the American Type Culture Collection (ATCC). Twelve to thirteen isolates of each species were tested. The species included within this study included Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae and Moraxella catarrhalis. ATCC quality control strains were tested on a daily basis and were included in the data analysis: S. aureus ATCC 29213, S. aureus ATCC 25923, S. pneumoniae ATCC 49619, H. influenzae ATCC 49247, H. influenzae ATCC 49766 and M. catarrhalis ATCC 49143.

Human neutrophil elastase activity assays. HNE activity was measured by determining the change in 405 nm absorbance of N-methoxysuccinyl-Ala-Ala-Pro-Val p-nitroanilide) in a plate reader (PerkinElmer EnVision) over time as an indication of cleavage.

Human bronchial epithelial cell (HBEC) culture. Primary human bronchial epithelial cultures (HBECs) were obtained by the UNC CF Center Tissue Core under protocols approved
by the UNC Institutional Committee for the Protection of the Rights of Human Subjects as described, plated on 12 mm T-clear culture inserts and studied 3-5 weeks after seeding (33).

**Measurements of ciliary beat frequency (CBF).** Digitized video was collected in 2.1 second segments using a Nikon TE2000 and a MegaPlus ES310 (Kodak) turbo video camera. CBF analysis was performed on digitized video using Sisson-Ammons Video Analysis (SAVA) software (46).

**HBEC bioelectric and confocal ASL height measurements.** Transepithelial electric potential ($V_t$) was measured with voltage sensing microelectrodes as described (44). To measure the height of the ASL, PBS (20 µl) containing 2 mg/ml rhodamine-dextran (10 kDa; Invitrogen, USA) was added to cultures at the start of the experiment. In some cases, after addition of PBS, all available fluid was aspirated with a Pasteur pipette to bring ASL volume down to minimal levels (35). Five predetermined points (one central, four 2 mm from the edge of the culture) were XZ scanned using a confocal microscope (Leica SP5; glycerol 63X immersion lens) as described (35). Cultures were returned to the incubator between time points. For all studies, perfluorocarbon (PFC) was added mucosally during imaging to prevent evaporation of the ASL.

**Measurement of transepithelial water flow.** HBECs were bilaterally loaded with 3 µM calcein-AM (Invitrogen, USA) for 30 min at 37°C (30). Calcein-loaded HBECs were observed by XZ confocal microscopy. Transepithelial osmotic water flow was initiated with the mucosal addition of 200 µL of hyperosmotic solution (Ringer plus 75 mM NaCl) and cell height and serosal bath intensity were tracked over time.

**Surface Biotinylation.** Apical membrane proteins were biotinylated as previously described (42). Polarised HBECs were washed three times with PBS supplemented with 1 mM
MgCl2 and 1 mM CaCl2 (PBS++). Sulfo-NHS-biotin (0.5 mg/ml) in borate buffer (85 mM NaCl, 4 mM KCl, 15 mM Na2B4O7, pH 9) for was applied onto the apical membrane and incubated for 30 min with gentle agitation. PBS++ supplemented with 10% (v/v) FBS was added to the basolateral bath to prevent biotinylation of basolateral proteins. Cells were lysed in lysis buffer (0.4% sodium deoxycholate, 1% NP-40, 50 mM EGTA, 10 mM Tris-Cl, pH 7.4 and Protease inhibitor) and protein concentration was determined by BCA assay. 300 μg of total protein were incubated overnight with 100 μl of neutravidin agarose beads at 4°C with agitation. Biotinylated proteins bound to beads were washed three times with lysis buffer and eluated in 30 μl of Laemmli buffer supplemented with 10% (v/v) β-mercaptoethanol by first incubating at room temperature for 10 minutes, followed by heating at 95°C for another 10 minutes.

**Western Blotting.** Biotinylated proteins were fractionated on 4–12% Bis–Tris gel (Invitrogen) alongside pre-stained protein standards (Santa Cruz, CA) and transferred onto PVDF Membrane. Membranes were incubated in PBS-T containing 5% (w v⁻¹) nonfat milk powder for 1 h at room temperature before incubation with primary antibodies; anti-γENaC (Gilead) (diluted 1:1000) or Anti-GAPDH (Abcam, Cambridge, UK) (1:5000), followed by incubation with species-specific HRP-conjugated secondary antisera (GE Heathcare). Immunostained proteins were visualized with ECL select chemiluminescent substrate (GE Healthcare). Densitometric quantification was performed using ImageJ (NIH).

**Statistical analyses.** HNE cleavage data was fitted to a four parameter inhibition curve using GraphPad Prism. Differences between means were analyzed by Students T-tests or Mann-Whitney U tests as appropriate. In some cases, data were analyzed with ANOVA followed by the Tukey test where the variances were homogeneously distributed. In the case of non-homogeneity of variance, significance of difference between means was determined by
either ANOVA followed by Dunn’s Multiple Comparison Test, the Mann-Whitney U test, or the Wilcoxon Signed Rank Test as appropriate. All values are expressed as mean ± SEM and $p < 0.05$ is considered statistically significant.
Results

Macrolides prevent HNE-induced mucus stasis in vivo.

It has previously been reported that chronic macrolide usage ameliorated COPD symptoms and it is possible that these beneficial effects were independent of antibiotic properties (14). Accordingly, we tested whether the antibacterial macrolide erythromycylamine altered mucus clearance in vivo by measuring ovine TMV. HNE alone significantly reduced TMV as compared to baseline (Fig. 1A). Pretreatment with aerosolized erythromycylamine (2 mg/kg) prevented the HNE-induced decrease in mucus clearance (Fig. 1A). This effect was not due to stimulatory actions of the drug as erythromycylamine treatment alone had no effect on TMV. Erythromycylamine not only prevented the HNE-induced reduction in TMV, but was seen to reverse the effect. Treatment with 2 mg/kg erythromycylamine 4h after HNE challenge resulted in a restoration of TMV to pre-challenge values (Fig. 1B).

Erythromycylamine is minimally absorbed after oral administration (10). However, the structurally related antibiotic macrolide azithromycin can be administered orally and has been shown to prevent exacerbations in COPD patients when dosed daily by mouth (2). When instilled into the lung of sheep via a nebulizer, azithromycin prevented the HNE-induced decrease in TMV (n=2; data not shown). Furthermore, orally administered azithromycin also prevented the HNE-induced decrease of TMV in sheep, suggesting that this action is preserved in structurally related macrolides (Fig. 1C).

Since chronic antibiotic use can lead to bacterial resistance, we hypothesized that a non-antibiotic macrolide with the characteristic lactone ring and the C3,C5-carbohydrate structure preserved would be a useful therapeutic agent for the treatment of chronic mucus stasis in the
lung but would not induce bacterial resistance. We designed a novel macrolide, GS-459755, which preserved the basic macrolide structure but significantly reduced antibiotic activity (Fig. S1 and Table 1). Aerosol pre-treatment with 2 mg/kg GS-459755 1 h before HNE prevented the HNE-induced decrease in TMV in a similar fashion to that seen with erythromycylamine (Supplemental Figure S2A). In contrast, 0.2 mg/kg GS-459755 was ineffective in blocking the HNE-induced effects. When given 4h after the HNE challenge, 2 mg/kg GS-459755 reversed the HNE-induced reduction in TMV (Supplemental Figure S2B). Both pre-treatment and post-treatment results with GS-459755 were of similar magnitude as that seen with erythromycylamine (Fig. 1 vs. S2).

**Macrolides do not alter HNE activity.**

We next tested whether macrolides including GS-459755 could alter HNE activity in vitro. The potent and specific HNE inhibitor ONO-5046 (23) significantly inhibited the ability of HNE to cleave a specific peptide (Fig. 2). In contrast, neither erythromycylamine, nor azithromycin, nor GS-459755 had any significant effect on HNE activity (Fig. 2).

**Macrolides prevent HNE-induced ASL volume depletion without affecting CBF.**

A change in TMV in response to HNE could be due to altered ciliary beating. To test for this, we measured the ability of HNE ± macrolides to affect HBEC CBF. Using the SAVA system (see Methods), we measured a basal CBF of ~5 Hz. Consistent with previously published data (3), addition of HNE had no effect on CBF, and 1.5 h preincubation with either azithromycin or GS-459755 in the presence of HNE had no additional effect on CBF (Fig. 3). Together, these data suggest that the protection from HNE-induced slowing of TMV seen in the presence of macrolides was not due to a change in CBF.
Since mucus clearance rates are also dependent on ASL hydration (43), we next looked at the ability of HNE and macrolides to alter ASL volume homeostasis. We washed HBEC apical surfaces 3 times with PBS and then loaded the apical surface with 20 μl PBS containing a fluorescent dextran. Under these conditions, normal HBECs adjust ion transport activity to render a steady-state ASL height of ~7 μm (Fig. 4A, B) (9, 15). We found that 100 nM HNE significantly diminished ASL height to 5.6 ± 0.1 μm (Fig. 4A, B). Addition of either azithromycin or GS-459755 in the absence of HNE had no effect on ASL height at 2 h (Control, 8 ± 1.0 μm; azithromycin, 7.9 ± 1.1 μm; GS-459755, 7.3 ± 1.0 μm). In contrast, pretreatment for 90 min with either macrolide prevented HNE-induced ASL height depletion (Fig. 4A, B). We next performed dose responses for both compounds, whilst keeping HNE levels constant at 100 nM. As can be seen in Fig. 4C, the effects of both macrolides were dose-dependent with IC50s of 2.4 ± 0.2 μM for azithromycin and 3.9 ± 0.3 μM for GS-459755.

Unlike the rescue effect seen in vivo (Fig. 1B), azithromycin failed to restore ASL height in vitro when added 1 h after HNE had already depleted ASL volume (Fig. 4D). These data suggest that macrolides preserve existing fluid on airway surfaces, rather than generate new secretions. To differentiate between the two possibilities, we pretreated HBECs with bumetanide, an inhibitor of NKCC-type co-transporters, which inhibits endogenous Cl− secretion and leads to a collapse of the ASL volume (44). Bumetanide caused ASL volume to decrease by ~50%, and neither azithromycin pretreatment nor HNE exposure caused an additional effect on ASL height (Fig. 5A). Importantly, combined addition of azithromycin and bumetanide followed by HNE prevented ASL volume depletion (Fig. 5A). For the experiments shown in Figs. 4A-D and Fig. 5A, HBECs were treated with 20 μl of PBS apically at t=0. To confirm that this increase in ASL height was due to reduced absorption rather than the initiation of
bumetanide-independent secretion, we measured ASL height after addition of azithromycin followed by HNE with minimal apical volume. To achieve this condition, we added 20 μl of test solution containing the fluorescent rhodamine-dextran ± HNE and aspirated excess solution, which yielded a starting height of ~5 μm as previously described (35). Following this protocol, no additional increase in ASL height was observed (Fig. 5B), suggesting that the beneficial effects of the macrolides were due to preservation of existing fluid rather than induction of secretion. Next, to confirm that macrolides had no effect on Cl⁻ secretion, we measured adenosine and UTP-induced ASL secretion. Both compounds robustly increased ASL height, likely through P2Y₂/Ca²⁺-activated Cl⁻ channels and A2B/cAMP/CFTR respectively (Fig. 5 C, D) (12). However, neither mode of secretion was affected by azithromycin pre-treatment (Fig. 5 C, D).

**Macrolides do not affect the transcellular water permeability.**

Airway epithelia have a relatively high water permeability which enables isosmotic absorption and secretion (13, 30). Simultaneous measurements of epithelial cell volume and transepithelial water flow revealed that when faced with a mucosal hypertonic challenge, the columnar epithelial cells shrank as they lost water across their apical membranes (Fig. 6A, B), and the serosal compartment became concentrated as water moved transepithelially into the mucosal compartment (Fig. 6C). HNE exposure (30 min) had no effect on the rate or magnitude of cell shrinkage (Fig. 6A, B) and exposure to azithromycin either with or without HNE had no additional effect on this parameter. Surprisingly, HNE alone caused a significant increase in transepithelial water movement compared to control cultures (Fig. 6C). However, azithromycin had no additional effect on this property (Fig. 6C), suggesting that the macrolides do not alter ASL height and mucus transport by affecting water permeability. To see whether the increase in
transepithelial water movement was due to altered resistance of the epithelia, we measured the transepithelial electrical resistance (Rt) before and after HNE exposure using Ussing chambers. There was no difference in Rt pre- or post HNE exposure (Fig. 6D), suggesting that the increased water flux is not due to an increase in leakiness of the epithelial tight junctions.

**Azithromycin prevents the HNE-induced increase in transepithelial voltage.**

Since macrolides affected ASL height without exerting any additional influence on water flux, it is likely that these changes were entirely due to altered ion transport. Accordingly, we measured the transepithelial voltage (Vt) as a direct indicator of ion transport. Vt significantly increased by ~5 mV after HNE addition (Fig. 7A). Consistent with the lack of effect of macrolides alone on TMV or ASL volume regulation (Figs. 2 and 6), azithromycin alone had no effect on Vt (Fig. 7). However, as would be predicted by the ASL measurements, azithromycin abolished the HNE-induced increase in Vt (Fig. 7A). To interrogate this change further, we measured the bumetanide- and amiloride-sensitive components of Vt as markers of Cl⁻ secretion and Na⁺ absorption, respectively. There was no significant difference in the bumetanide-sensitive Vt between vehicle and azithromycin-exposed cultures (Fig. 7B). In contrast, the amiloride-sensitive Vt was significantly reduced after azithromycin and HNE exposure (Fig. 7B). This is consistent with the hypothesis that macrolides protect against HNE-induced activation of ENaC.

We then repeated this protocol with GS-459755-treated cultures. Again, the transepithelial Vt increased with HNE. This increase was abolished by GS-459755 pretreatment (Fig. 7C), which was also due to a change in the amiloride-sensitive Vt but not to a change in the bumetanide-sensitive Vt (Fig. 7D).
Azithromycin limits activation of ENaC by HNE.

To confirm that the effects of azithromycin/HNE on amiloride-sensitive Vt were due to effects on ENaC, we co-expressed αβγENaC in Xenopus laevis oocytes and looked for the change in current following HNE exposure (Fig. S3). HNE robustly activated a current (Fig. S3) that was absent in H2O-injected controls (data not shown). Consistent with the electrophysiological data from HBECs, azithromycin pre-treatment significantly prevented the activation of ENaC by HNE (Fig. S3).

HNE has previously been shown to induce activation by cleaving γENaC (1). Therefore, to test whether the effects of HNE on amiloride-sensitive Vt seen in Fig. 7 were due to changes in γENaC cleavage, we surface biotinylated HBECs and probed for cleavage of γENaC in the presence of azithromycin. Under basal, thin film conditions, where ENaC is silent or near-silent, cleaved γENaC was essentially undetectable in the plasma membrane (Fig. 8A, B). However, a 5 min treatment with 100 nM HNE significantly increased the cleaved form of γENaC. Again, consistent with the electrophysiological data from HBECs (Fig. 7) and Xenopus oocytes, pretreatment with HNE prevented cleavage of γENaC (Fig. 8A, B).
Discussion

The direct effects of cigarette smoke on proteases and the immune system due to oxidative stress have been well described (19). However, there is now substantial evidence demonstrating that cigarette smoke also induces the rapid removal of CFTR from the plasma membrane and diminishes CFTR gene expression (8, 11, 18, 40). This data is consistent with mucus dehydration and the decrease in mucus clearance seen in COPD patients (11). In both diseases, after mucus clearance fails and chronic neutrophilia sets in, increased levels of HNE are detected in the airway lumen (26, 32). The role of ENaC in the development of CF lung disease is controversial (20). While CFTR has been shown to be directly affected by cigarette smoke (8, 11, 18, 40), to date, no direct effects of cigarette smoke on ENaC have been reported. However, the γ subunit of ENaC is proteolytically cleaved by HNE (15) and by bacterial proteases (6), suggesting that ENaC may be abnormally active in COPD patients. Furthermore, HNE slows mucus clearance and induces ASL volume depletion (Figs 1, 4). Regardless of its role in COPD pathogenesis, COPD mucus is dehydrated (11, 36) and inhibition of ENaC is an attractive target to help rehydrate COPD airways, even if this dehydration is initiated solely by a lack of CFTR.

We tested whether the HNE-induced decrease in mucus clearance could be prevented by macrolide exposure. As an initial proof of concept, we dosed sheep with the inhaled antibiotic erythromycinylamine (Fig. 1). This pretreatment prevented the HNE-induced reduction in TMV. Erythromycinylamine was also efficacious when added after HNE. Since erythromycinylamine has therapeutic potential, we developed a novel macrolide-based molecule with significantly diminished antimicrobial activity against several common pathogens, which we termed GS-459755 (Fig. S1). Our findings indicated that the efficacy of GS-459755 in the sheep TMV
model was comparable to erythromycylamine and azithromycin (Figures 1 and S2), suggesting that it was independent of the anti-bacterial properties.

Neither HNE nor macrolides had any effect on CBF (Fig. 3). However, mucus clearance rates are also strongly affected by ASL hydration levels, and the more dilute the mucus, the faster it is cleared (43). HNE significantly reduced ASL height as compared to the vehicle control (Fig. 4), which is consistent with the reduction in TMV seen in vivo. Azithromycin and GS-459755 prevented HNE-induced ASL height depletion, although neither compound was efficacious when added alone. Both macrolides tested displayed a dose-dependent effect with IC₅₀s in the micromolar range, suggesting that they had similar mechanisms of action (Fig. 4).

One important difference was noted between the in vivo TMV experiments and the in vitro ASL height experiments: When azithromycin was added after HNE had already depleted ASL volume, no rescue effect was noted, unlike in vivo where macrolides were capable of restoring TMV when added after HNE (Fig. 5), suggesting that macrolides did not induce ASL secretion. To investigate this discrepancy further, we probed the HNE/macrolide response with bumetanide, an inhibitor of basolateral NKCC transporters, which blocks transepithelial Cl⁻ secretion (44, 47). Consistent with our previous findings, bumetanide caused ASL height to collapse to ~4 μm and HNE had no additional effect. This height reflects the height of bent-over cilia and is typically the lowest ASL height that can be measured in a fully ciliated culture (44).

Interestingly, our data suggest that macrolides reduce HNE-stimulated absorption, rather than induce secretion. Given that distal secretions constantly move up the airways, such a protective measure would be capable of preserving mucus transport in vivo. This hypothesis was further tested by measuring the effects of adenosine and UTP to induce ASL secretion in the presence and absence of azithromycin (Fig. 5). In this case, no difference in the magnitude of ASL
secretion was detected, further indicating that macrolides predominantly affect absorption. Thus, the results could be explained by macrolides preserving existing secretions in the lung.

ASL volume homeostasis is dependent on ion transport (Na⁺ absorption and Cl⁻ secretion) and also on transepithelial water permeability. For example, if airway epithelia were impermeant to H₂O there would be no net change in ASL height, even if there was a large accompanying ion flux. Accordingly, we also measured cell height and serosal bath intensity as an indicator of transapical and transepithelial water flux respectively to determine whether HNE and macrolides also affected net water movement. There was no difference in cell shrinkage under any of the conditions tested, suggesting that macrolides do not affect transapical water permeability (Fig. 6). Surprisingly, HNE significantly increased the concentration of rhodamine-dextran in the serosal bath. Since HNE had no effect on rates of cell shrinkage, we conclude that HNE increased the water permeability of the paracellular pathway. Under normal conditions, the majority of water flow in leaky epithelia is thought to be transcellular (45). In contrast, our data suggest that HNE induced additional paracellular water flow. We measured Rᵣ in the presence and absence of HNE and macrolides and found no direct effects on Rᵣ, suggesting that HNE is not contributing to a non-specific degradation of the tight junctions. Azithromycin had no effect on water flux, suggesting that altered barrier properties do not contribute to the protective effects of macrolides against HNE-induced ASL height depletion.

Since macrolides had no discernible effect on water permeability, we next measured the transepithelial voltage and looked for cleavage of apical membrane γENaC, as indicators of ion transport under thin film conditions with an intact ASL, since this most accurately reprises the in vivo situation. Under these conditions, Cl⁻ secretion is partially active, due to endogenous adenosine and ATP which are present in the ASL and stimulate CFTR and CaCC, respectively.
ENaC can be partially active due to the presence of endogenous extracellular proteases but is largely inactive due to the accumulation of SPLUNC1 (15). HNE exposure increased $V_t$ in control cultures and induced cleavage of $\gamma$ENaC (Fig. 7 and 8). However, the effect of HNE was abolished by macrolide pretreatment, suggesting that macrolides do indeed prevent HNE from cleaving $\gamma$ENaC to preserve ASL volume and mucus clearance. These results were reprised in Xenopus oocytes, suggesting that the effects of macrolides are not specific to airway epithelia.

Our data suggest that macrolides prevent the cleavage of $\gamma$ENaC by HNE directly inhibiting HNE proteolytic activity. The effect may be due to steric hindrance of the HNE cleavage site on the $\gamma$ subunit of ENaC by the macrolides. However, a direct interaction between either azithromycin or GS-459755 and $\gamma$ENaC remains to be tested.

In conclusion, we have developed a novel macrolide, GS-459755, that has significantly reduced antibacterial activity whilst retaining other key attributes of the macrolide family. Like the antibiotic macrolides erythromycylamine and azithromycin, this compound can protect against HNE-induced mucus stasis and ASL volume depletion, possibly by protecting bronchial epithelial cells from proteolytic activation of ENaC. Based on this data, we propose that such non-antibacterial macrolides may be suitable for chronic treatment of CF and COPD patients without inducing bacterial resistance.
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Disclosures

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Author Contributions:

RT measured ASL height, water permeability and Vt and co-wrote the paper. CD performed CBF and ASL height measurements. ALG performed ASL height experiments and analyzed/interpreted the data. CDT designed and performed surface biotinylation/Western blot analysis of ENaC and analyzed data. MJS performed resistance measurements, measured ENaC currents in Xenopus Oocytes and wrote appropriate sections. JRS, TCC, and WMA designed and executed sheep TMV studies and wrote appropriate sections. AY performed chemical synthesis. JL performed HNE inhibition assay. GP led macrolide chemistry development. WRB designed GS-459755. CDW had advisory function and interpreted data. SW led the project and co-wrote the paper.
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**Figure Legends**

Fig. 1. Macrolides prevent the HNE-induced reduction of ovine tracheal mucus velocity (TMV).

(A) Pre-treatment with aerosolized erythromycylamine (2 mg/kg) 1 h prior to HNE (1190 mU).

■, Vehicle/baseline TMV. ●, Erythromycylamine alone. ▼, HNE alone. ▲, Erythromycylamine followed by HNE. (B) Treatment with aerosolized erythromycylamine 4 h after HNE increases TMV. ●, HNE. ▲, erythromycylamine 4 h after HNE. (C) 4 days of pre-treatment with orally administered azithromycin significantly limits the HNE-induced reduction in TMV. ●, HNE alone. ■, HNE after azithromycin treatment. * = p < 0.05 vs HNE alone.

Fig. 2. Macrolides do not inhibit HNE activity. The ability of 8.8 nM HNE to cleave N-methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide was measured over time and in the presence of the macrolides erythromycylamine (●), azithromycin (■) and GS-459755 (◆) vs. the HNE-specific inhibitor ONO-5046 (▲). All n=4.

Fig. 3. Ciliary beat frequency is not affected by macrolide exposure. Mean CBF was measured by high speed video microscopy in HBECs under basal conditions or following 1.5 h preincubation with vehicle, 100 µM azithromycin or 100 µM GS-459755 (open bars) ± 100 nM HNE (closed bars). All n=5.
Fig. 4. Macrolides prevent HNE-induced ASL absorption. (A) XZ-Confocal images of airway surface liquid (ASL) (red) in HBECs pre-incubated bilaterally with 100 μM azithromycin, GS-459755 or vehicle (PBS) for 1.5 h prior to a 2 h mucosal exposure to 20 μl PBS containing 1 mg/ml rhodamine-dextran ± 100 nM HNE. B) Mean data taken from A. All n=6. (C) Dose response curves for azithromycin (open squares) and GS-459755 (closed circles) in the presence of 100 nM HNE. All n=4-7. (D) Mean sequential ASL height with HNE added 2 h before azithromycin. N=6. * = p < 0.05 compared to vehicle. † = p < 0.05 different ± macrolide or ± bumetanide as appropriate.

Fig. 5. Macrolides Do Not Stimulate ASL Secretion. (A) Mean ASL height taken after 1.5 h bilateral pre-incubation with 100 μM azithromycin or vehicle followed by 2 h mucosal exposure to 20 μl PBS ± 100 nM HNE. Where noted, HBECs were incubated over the entire period with 100 μM serosal bumetanide. (B) Mean ASL height with time in HBECs pretreated for 90 min with bilateral 100 μM azithromycin and 100 μM serosal bumetanide followed by 100 nM mucosal HNE. ●, 0 μl PBS added at t=0; □, 20 μl PBS added at t=0. Both n=6. (C) Mean ASL height measured before (control) and at timed intervals following exposure to 100 μM adenosine (ADO) added as a dry powder suspended in perfluorocarbon. (D) Mean ASL height measured before (control) and at timed intervals following exposure to 100 μM UTP (ADO) added as a dry powder suspended in perfluorocarbon. For C and D, open bars denote vehicle; closed bars denote 90 min pretreatment with bilateral 100 μM azithromycin. * = p < 0.05 compared to vehicle. † = p < 0.05 different ± macrolide or ± bumetanide as appropriate.
Fig. 6. Macrolides do not affect transapical or transepithelial water permeability. (A) XZ-Confocal images of calcein-AM stained HBECs (pseudocolored red) pre-incubated bilaterally with 100 μM azithromycin (Azithro) or vehicle (PBS) for 1.5 h. HBECs were then exposed mucosally to 20 μl PBS ± 100 nM HNE for 1 h (see Experimental Procedures). (B) Mean change in cell height before (0) and after exposure to mucosal hypertonicity, as measured by XZT scanning. All n=6. (C) Mean change in serosal fluorescence before (0) and after exposure to mucosal hypertonicity, as measured by XZT scanning. □, vehicle; Δ, vehicle/HNE; ●, azithromycin; ▼, azithromycin/HNE. All n=6. * = p < 0.05 different ± HNE. (D) Normalized transepithelial electrical resistance in HBECs before (open bars) and after (closed bars) addition of HNE (100 nM). Both n=11.

Fig. 7. Macrolides inhibit the HNE-induced increase in amiloride-sensitive transepithelial voltage (Vt). (A) and (C) Mean Vt across HBECs with vehicle or bilateral azithromycin (100 μM) (Azithro) or GS-459755 respectively (open bars) and following 10 min exposure to HNE (100 nM; closed bars). (B) Change in Vt in NL HBECs bilaterally exposed to vehicle (open bars) vs azithromycin (100 μM; gray bars; all n=12). (D) Change in Vt in NL HBECs bilaterally exposed to vehicle (open bars) vs. GS-459755 (100 μM; gray bars; all n=10). For B and D, after vehicle or macrolide, cultures were then exposed to HNE (100 nM) followed by sequential addition of 100 μM serosal bumetanide (100 μM) then mucosal amiloride (100 μM) in the presence of bumetanide. * = p < 0.05 different ± HNE. † = p < 0.05 different ± macrolide.
Fig. 8. Azithromycin prevents cleavage of apical membrane γENaC in HBECs. HBECs were pretreated with vehicle or 10 μM azithromycin for 1 h followed by a brief (5 min) treatment with 100 nM HNE. A, Representative western blot of biotinylated γENaC probed with an antibody that recognizes cleaved γENaC. B, bargaph of mean integrated densitometry taken from A. All n=6. * = p < 0.05 different ± HNE. † = p < 0.05 different ± macrolide.
Table 1. GS-459455 has up to 1000-fold less antibacterial activity than azithromycin. 

MIC₉₀s were determined for *S. aureus* (ATCC 29213 and 25923), *S. pneumoniae* (ATCC 49619) *M. catarrhalis* (ATCC 49143) and *H. influenzae* (ATCC 49247 and 49766).

<table>
<thead>
<tr>
<th>Macrolide</th>
<th>Structure</th>
<th>MIC₉₀ (µg/mL) (N = 12-13)</th>
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<tr>
<td></td>
<td></td>
<td>*S. aureus</td>
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<tr>
<td>GS-459755</td>
<td>2'-Desoxy-9-(S)-erythromycylamine</td>
<td>&gt; 128</td>
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<tr>
<td>Azithromycin</td>
<td>9-Deoxy-9a-aza-9a-methyl-9a-homoerythromycin A</td>
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