Nonantibiotic macrolides prevent human neutrophil elastase-induced mucus stasis and airway surface liquid volume depletion

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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 epithelium to affect mucus clearance (17, 38). The height of the PCL is approximately the length of outstretched cilia (7 μm) whereas 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) because of increased mucosal HNE levels, leading to enhanced Na+ absorption that 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 that encompasses both emphysema and chronic bronchitis (CB) that is most often caused by cigarette smoke (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 pneumoniae* 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 NF-κB and ERK MAPK activation, decrease IL-8 secretion, and decrease MUC5AC expression with efficacy in the microsomal range (22). Since chronic antibiotic usage can induce bacterial resistance, it has been proposed that a nonantibacterial macrolide would be therapeutically beneficial in treating chronic airway disease without inducing bacterial resistance.
Accordingly, we designed a novel macrolide, 2′-desoxy-9-(S)-erythromycin (GS-459755), that 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 (erythromycin and azithromycin) vs. GS-459755 to prevent HNE-induced mucus stasis and ASL volume depletion in vivo and in vitro.

EXPERIMENTAL PROCEDURES

Sheep TMV measurements. Tracheal mucus clearance velocity (TMV) was evaluated as described (37). In brief, sheep were nasally intubated with an endotracheal tube 7.5 cm in diameter (Mallinckrodt Medical, St. Louis, MO) after topical anesthesia of the nasal passages with 2% lidocaine solution. The cuff of the tube was placed just below the vocal cords (verified by fluoroscopy) to allow for maximal exposure of the tracheal surface area. To minimize possible impairment of TMV caused by endotracheal tube cuff inflation, the cuff was deflated throughout the study except during drug and/or HNE delivery. To alleviate the effects of prolonged intubation, inspired air was warmed and humidified (Bennett humidifier; Puritan-Bennett, Le- nexa, KS). TMV was measured by a roentgenographic technique. Five to 10 radiopaque Teflon/bismuth trioxide disks, 1 mm in diameter, 0.8-mm thick, and 1.8 mg in weight, were insufflated onto the trachea. A modified suction catheter connected to a source of continuous compressed air at a flow of 3–4 l/min was used to introduce the particles via the endotracheal tube. The catheter remained within the endotracheal tube during sufflation of Teflon particles, so that no contact with the tracheal surface was made. The cephalad-axial velocities of the individual disks were recorded on videotape from a portable image intensifier unit. Individual disk velocities were calculated by measuring the distance traveled by each disk during a 1-min observation period. For each run, the mean values of all individual disk velocities were calculated. A collar containing radiopaque reference markers of known length was worn by the sheep and was used as a standard to correct for magnification effects inherent in the fluoroscopy unit.

Airway treatments/challenges were performed by using the nebulizer-dosimeter system previously described (38). Decreases in TMV were induced by aerosol administration of HNE solution. Macrolide solutions were administered by nebulization either prophylactically (1 h before HNE challenge) or therapeutically (4 h after HNE delivery). To alleviate the effects of prolonged intubation, inspired air was warmed and humidified (Bennett humidifier; Puritan-Bennett, Le- nexa, KS). TMV was measured by a roentgenographic technique. Five to 10 radiopaque Teflon/bismuth trioxide disks, 1 mm in diameter, 0.8-mm thick, and 1.8 mg in weight, were insufflated onto the trachea. A modified suction catheter connected to a source of continuous compressed air at a flow of 3–4 l/min was used to introduce the particles via the endotracheal tube. The catheter remained within the endotracheal tube during sufflation of Teflon particles, so that no contact with the tracheal surface was made. The cephalad-axial velocities of the individual disks were recorded on videotape from a portable image intensifier unit. Individual disk velocities were calculated by measuring the distance traveled by each disk during a 1-min observation period. For each run, the mean values of all individual disk velocities were calculated. A collar containing radiopaque reference markers of known length was worn by the sheep and was used as a standard to correct for magnification effects inherent in the fluoroscopy unit.

Oocyte studies. Xenopus laevis oocytes were prepared and injected as described (12a). Injected oocytes were kept in modified Barth’s solution [CaCl2, 0.82 MgSO4, and 15 HEPES, adjusted to pH 7.35 with Tris] at 18°C. Oocytes were studied 24 h after RNA injection by the two-electrode voltage-clamp technique as previously described (12a). Oocytes were pretreated with 10 mmol/L pH 9.2 Tris and soaked in 10 mmol/L pH 9.2 Tris. The solutions were administered by nebulization either prophylactically (1 h before HNE challenge) or therapeutically (4 h after HNE challenge). The TMV response to the different experimental paradigms was measured over 8 h and changes were reported as percentage of TMV at baseline as in previous studies (38). Studies involving sheep were performed with the approval and oversight of the Institutional Animal Care and Use Committees at the Mount Sinai Medical Center.

Chemistry. The synthesis is shown in Fig. 1. A solution of 9-amino-9-deoxy erythromycin (compound 1, erythromycinlamine; 10 g, 14 mmol), N-(benzyloxy carbonyl) succinimide (3.65 g, 14.6 mmol) in dichloromethane (DCM; 200 ml) was stirred at 21°C for 18 h. Solvents were concentrated under reduced pressure and the residue was redissolved in ethyl acetate. The solution was washed with saturated NaHCO3 solution, dried (MgSO4), and concentrated to afford compound 2 (11.6 g, 98%). ES/MS was calculated for C43H42N2O14 868.5, found m/z = 869.5 (M+H+).

A solution of compound 2, DCM (100 ml), and triethylamine (TEA; 1.3 g, 13 mmol) was cooled in an ice-water bath while acetic anhydride (1.97 g, 19.3 mmol) was added in one portion. The bath was removed and the reaction was stirred for 5.5 h. NaH2PO4 (0.5 M aqueous solution) was added and the organic layer was separated, dried (MgSO4), and concentrated to afford compound 3 (11.5 g, 95%). ES/MS was calculated: for C45H44N2O15 910.5, found m/z = 911.5 (M+H+).

A solution of compound 3 and 4-dimethylaminopyridine (DMAP; 4.6 g, 37 mmol) in DCM (100 ml) was cooled to −40°C. Benzyl chloroformate (CbzCl; 4.3 g, 25 mmol) was added at a rate to maintain the reaction temperature below −40°C. The reaction mixture was then stirred at −20°C for 3 days. After this time the reaction was cooled to −40°C and DMAP (4.6 g, 38 mmol) and CbzCl (4.3 g, 25 mmol) was added. The reaction mixture was then stirred at −20°C for an additional 48 h. The reaction mixture was added to a cold NaOH solution (2 N, 100 ml). The layers were separated and the organic layer was washed with water (3 × 50 ml) and dried (MgSO4). The organic layer was concentrated under reduced pressure and the residue was purified by column chromatography (silica gel, DCM-acetonitrile (ACN), 0 to 100%) to yield compound 4 (9.1 g, 69%). ES/MS was calculated: for C53H82N2O15 1,044.6, found m/z = 1045.6 (M+H+).

Compound 4 (5 g, 4.8 mmol) in methanol (50 ml) was heated to 50°C for 36 h. The solvents were removed under reduced pressure and the residue was purified by column chromatography (silica gel, DCM-ACN, 0 to 100%) to afford compound 5 (4.0 g, 83%). ES/MS was calculated: for C55H84N2O17 1,086.6 (M+H+).

To compound 5 (3 g, 3.0 mmol) was added 1,1-thiocarbonyldimidazole. The system was evacuated and replaced with N2 (g). Anhydrous ACN (3 ml) was added and the reaction was stirred at 48°C for 36 h. The solution was added to anhydrous toluene (100 ml) at 90°C. To this was added tributyltin hydride (3 g, 10 mmol) and azobisisobutyrimidazole (0.5 g, 3 mmol). After 1 h, the reaction was concentrated under reduced pressure. To this was added ACN (50 ml) which was extracted with hexanes (3 × 50 ml). The ACN layer was concentrated and purified on preparative HPLC (H2O-ACN, 5–95% ACN) to yield compound 6 (2.5 g, 85%). ES/MS was calculated: for C57H88N2O18 1,128.7, found m/z = 1129.6 (M+H+).

A solution of compound 6 (1.8 g, 1.83 mmol) in methanol (15 ml) was purged with N2 (g), and charged with palladium/charcoal (5%, 200 mg). The mixture was subjected to hydrogenation conditions. After 4 h, the reaction was filtered through celite and concentrated under reduced pressure to afford compound 7, which we termed GS-459755 (1.24 g, 95%). ES/MS was calculated: for C53H70N2O15 718.5, found m/z = 719.5 (M+H+). The oral bioavailability of GS-459755 was low (less than 1% in the rat, data not shown).

MIC90 determination. Cation-adjusted Mueller-Hinton broth (DIFCO lot no. 7158099) was used for all testing. The medium was supplemented with 3% lysed horse blood (Hemostat lot no. H03218) for testing S. pneumoniae or made up as Haemophilus test medium for testing H. influenzae. Minimum inhibitory concentrations (MICs) for each compound were determined by the broth microdilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) (M7-A8, 2009). A total of 50 aerobic isolates representing four 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, S. pneumoniae, H. 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.

**HBEC culture.** Primary human bronchial epithelial cultures (HBECs) were obtained by the University of North Carolina (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 wk after seeding (33).

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

**HBEC bioelectric and confocal ASL height measurements.** Trans-epithelial electric potential (Vt) 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) 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 by using a confocal microscope (Leica SP5; glycerol ×63 immersion lens) as described (35). Cultures were returned to the incubator between time points. For all studies, perfluorocarbon 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) 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). Polarized HBECs were washed three times with PBS supplemented with 1 mM MgCl2 and 1 mM CaCl2 (PBSx3). Sulfos-NHS-biotin (0.5 mg/ml) in borate buffer (85 mM NaCl, 4 mM KCl, 15 mM Na2B4O7, pH 9) was applied onto the apical membrane and incubated for 30 min with gentle agitation. PBS supplemented with 10% (vol/vol) 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

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Fig. 1. Synthesis of GS-459755, a nonantibiotic macrolide. 9-Amino-9-deoxy erythromycin (compound 1; erythromycylamine) was converted into 2'-desoxy-9-(S)-erythromycylamine (compound 7; GS-459755) by a 6-step process (see EXPERIMENTAL PROCEDURES).
three times with lysis buffer and eluted in 30 μl of Laemmli buffer supplemented with 10% (vol/vol) β-mercaptoethanol by first incubating at room temperature for 10 min, followed by heating at 95°C for another 10 min.

Western blotting. Biotinylated proteins were fractionated on 4–12% Bis-Tris gel (Invitrogen) alongside prestoned protein standards (Santa Cruz, CA) and transferred onto PVDF membrane. Membranes were incubated in PBS-Tween containing 5% (wt/vol) nonfat milk powder for 1 h at room temperature before incubation with primary antibodies, anti-γENaC (Gilead) (diluted 1:1,000), or anti-GAPDH (Abcam, Cambridge, UK) (1:5,000), followed by incubation with species-specific HRP-conjugated secondary antisera (GE Healthcare). Immunostained proteins were visualized with ECL select chemiluminescent substrate (GE Healthcare). Densitometric quantification was performed with ImageJ (NIH).

Statistical analyses. HNE cleavage data was fitted to a four parameter inhibition curve by use of GraphPad Prism. Differences between means were analyzed by Student’s t-tests or Mann-Whitney U-tests as appropriate. In some cases, data were analyzed with ANOVA followed by the Tukey’s test where the variances were homogeneously distributed. In the case of nonhomogeneity 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 means ± SE, 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 compared with baseline (Fig. 2A). Pretreatment with aerosolized erythromycylamine (2 mg/kg) prevented the HNE-induced decrease in mucus clearance (Fig. 2A). This effect was not due to stimulatory actions of the drug since erythromycylamine treatment alone had no effect on TMV. Erythromycylamine not only prevented the HNE-induced reduction in TMV, but it was seen to reverse the effect.

Treatment with 2 mg/kg erythromycylamine 4 h after HNE challenge resulted in a restoration of TMV to prechallenge values (Fig. 2B).

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. 2C).

Since chronic antibiotic use can lead to bacterial resistance, we hypothesized that a nonantibiotic 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, that preserved the basic macrolide structure but significantly reduced antibiotic activity (Fig. 1 and Table 1). Aerosol pretreatment 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 (Fig. 3A). In contrast, 0.2 mg/kg GS-459755 was ineffective in blocking the HNE-induced effects. When given 4 h after the HNE challenge, 2 mg/kg GS-459755 reversed the HNE-induced reduction in TMV (Fig. 3B). Both pretreatment and posttreatment results with GS-459755 were of similar magnitude as that seen with erythromycylamine (Fig. 2 vs. Fig. 3).

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. 4). In contrast, neither erythromycylamine nor azithromycin nor GS-459755 had any significant effect on HNE activity (Fig. 4).

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

Fig. 2. Macrolides prevent the human neutrophil elastase (HNE)-induced reduction of ovine tracheal mucus velocity (TMV). A: pretreatment with aerosolized erythromycylamine (2 mg/kg) 1 h prior to HNE (1,190 mU). ■, Vehicle/baseline TMV; ●, erythromycylamine alone; ▲, HNE alone; ▼, erythromycylamine followed by HNE. B: treatment with aerosolized erythromycylamine (Erythro) 4 h after HNE increases TMV. ●, HNE; ▲, erythromycylamine 4 h after HNE. C: 4 days of pretreatment with orally administered azithromycin significantly limits the HNE-induced reduction in TMV. ●, HNE alone; ■, HNE after azithromycin treatment. *P < 0.05 vs. HNE alone.
measured the ability of HNE ± macrolides to affect HBEC CBF. Using the SAVA system (see EXPERIMENTAL PROCEDURES), 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. 5). 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 three 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. 6, A and B) (9, 15). We found that 100 nM HNE significantly diminished ASL height to 5.6 ± 0.1 μm (Fig. 6, A and 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. 6, A and B). We next performed dose responses for both compounds, while keeping HNE levels constant at 100 nM. As can be seen in Fig. 6C, the effects of both macrolides were dose dependent with IC50 values 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. 2B), azithromycin failed to restore ASL height in vitro when added 1 h after HNE had already depleted ASL volume (Fig. 6D). These data suggest that macrolides preserve existing fluid on airway surfaces, rather than generating new secretions. To differentiate between the two possibilities, we pretreated HBECs with bumetanide, an inhibitor of NKCC-type cotransporters, 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. 7A). Importantly, combined addition of azithromycin and bumetanide followed by HNE prevented ASL volume depletion (Fig. 7A). For the experiments shown in Fig. 6, A–D and Fig. 7A, 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. 7B), 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. 7, C and D) (12). However, neither mode of secretion was affected by azithromycin pretreatment (Fig. 7, C and D).

Macrolides do not affect the transcellular water permeability. Airway epithelia have a relatively high water permeability that 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. 8, A and B), and the serosal compartment became concentrated as water

Table 1. GS-459455 has ≤1,000-fold less antibacterial activity than azithromycin

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<tr>
<th>Macrolide</th>
<th>Structure</th>
<th>MIC90 (μg/ml) (N = 12–13)</th>
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<tr>
<td>GS-459755</td>
<td>2′-Desoxy-9-(S)-erythromycyclamine</td>
<td>&gt;128</td>
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<tr>
<td>Azithromycin</td>
<td>9-Deoxy-9a-aza-9a-methyl-9a-homoerythromycin A</td>
<td>2</td>
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MIC90 values were determined for Staphylococcus aureus (ATCC 29213 and 25923), Streptococcus pneumoniae (ATCC 49619), Moraxella catarrhalis (ATCC 49143), and Haemophilus influenzae (ATCC 49247 and 49766).
moved transepithelially into the mucosal compartment (Fig. 8C). HNE exposure (30 min) had no effect on the rate or magnitude of cell shrinkage (Fig. 8, A and 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 with control cultures (Fig. 8C). However, azithromycin had no additional effect on this property (Fig. 8C), 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. 8D), 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.

**Fig. 4.** 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 erythromycin (●), azithromycin (■), and GS-459755 (●) vs. the HNE-specific inhibitor ONO-5046 (▲). All n = 4.

**Fig. 5.** Ciliary beat frequency (CBF) is not affected by macrolide exposure. Mean CBF was measured by high-speed video microscopy in human bronchial epithelial cells (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 (solid bars). All n = 5.

Accordingly, we measured the \( V_t \) as a direct indicator of ion transport. \( V_t \) significantly increased by ~5 mV after HNE addition (Fig. 9A). Consistent with the lack of effect of macrolides alone on TMV or ASL volume regulation (Figs. 4 and 8), azithromycin alone had no effect on \( V_t \) (Fig. 9). However, as would be predicted by the ASL measurements, azithromycin abolished the HNE-induced increase in \( V_t \) (Fig. 9A). To interrogate this change further, we measured the bumetanide- and amiloride-sensitive components of \( V_t \) as markers of \( Cl^- \) secretion and \( Na^+ \) absorption, respectively. There was no significant difference in the bumetanide-sensitive \( V_t \) between vehicle- and azithromycin-exposed cultures (Fig. 9B). In contrast, the amiloride-sensitive \( V_t \) was significantly reduced after azithromycin and HNE exposure (Fig. 9B). 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 \( V_t \) increased with HNE. This increase was abolished by GS-459755 pretreatment (Fig. 9C), which was also due to a change in the amiloride-sensitive \( V_t \) but not to a change in the bumetanide-sensitive \( V_t \) (Fig. 9D).

**Azithromycin limits activation of ENaC by HNE.** To confirm that the effects of azithromycin/HNE on amiloride-sensitive \( V_t \) were due to effects on ENaC, we coexpressed αβγENaC in *Xenopus laevis* oocytes and looked for the change in current following HNE exposure (Fig. 10). HNE robustly activated a current (Fig. 10) that was absent in H2O-injected controls (data not shown). Consistent with the electrophysiological data from HBECs, azithromycin pretreatment significantly prevented the activation of ENaC by HNE (Fig. 10).
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. 9 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. 11, A and 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. 9) and Xenopus oocytes, pretreatment with azithromycin prevented cleavage of γENaC (Fig. 11, A and 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). Whereas 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. 2 and 6). 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 erythromycylamine (Fig. 2). This pretreatment prevented the HNE-induced reduction in TMV. Erythromycylamine was also efficacious when added after HNE. Since erythromycylamine 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. 1). Our findings indicated that the efficacy of GS-459755 in the sheep TMV model was comparable to erythromycylamine and azithromycin (Figs. 2 and 3), suggesting that it was independent of the antibacterial properties.

Neither HNE nor macrolides had any effect on CBF (Fig. 5). 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 compared with the vehicle control (Fig. 6), 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.

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**Fig. 7.** Macrolides do not stimulate ASL secretion. A: mean ASL height taken after 1.5 h bilateral preincubation with 100 μM azithromycin (Azith) 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 azithromycin. Under basal, thin-film conditions, where ENaC is silent and probed for cleavage of ENaC. Again, consistent with the electrophysiological data from HBECs (Fig. 9) and Xenopus oocytes, pretreatment with azithromycin prevented cleavage of γENaC (Fig. 11, A and B).

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Both macrolides tested displayed a dose-dependent effect with IC$_{50}$ values in the micromolar range, suggesting that they had similar mechanisms of action (Fig. 6). 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. 7), 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$^{-}$/H$^{+}$ secretion (44, 47). Consistent with our previous findings, bumetanide caused ASL height to collapse to $\sim$4 $\mu$m 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. 7). 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 H$_2$O 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. 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 $R_t$ in the presence and absence of HNE and macrolides and found no direct effects on $R_t$, suggesting that HNE is not contributing to a nonspecific 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 reproxes the in vivo situation. Under these conditions, Cl$^{-}$ secretion is partially

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**Fig. 8.** Macrolides do not affect transapical or transepithelial water permeability. A: XZ confocal images of calcein-AM stained HBECs (pseudocolored red) preincubated bilaterally with 100 $\mu$M azithromycin (Azithro) or vehicle (PBS) for 1.5 h. HBECs were then exposed mucosally to 20 $\mu$l PBS $\pm$ 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 XZ scanning. All n = 6. C: mean change in serosal fluorescence before (0) and after exposure to mucosal hypertonicity, as measured by XZ scanning. $\square$, vehicle; $\triangle$, vehicle/HNE; $\bullet$, azithromycin; $\blacktriangle$, azithromycin/HNE. All n = 6. *P < 0.05 different $\pm$ HNE. D: normalized transepithelial electrical resistance in HBECs before (open bar) and after (solid bar) addition of HNE (100 nM). Both n = 11.
active, owing to endogenous adenosine and ATP that are present in the ASL and stimulate CFTR and CaCC, respectively. ENaC can be partially active owing 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 $\beta$-ENaC (Figs. 9 and 11). However, the effect of HNE was abolished by macrolide pretreatment, suggesting that macrolides do indeed prevent HNE from cleaving $\beta$-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 $\beta$-subunit of ENaC by the macrolides. However, a direct interaction between azithromycin or GS-459755 and $\beta$-ENaC remains to be tested.

In conclusion, we have developed a novel macrolide, GS-459755, that has significantly reduced antibacterial activity while retaining other key attributes of the macrolide family. Like the antibiotic macrolides erythromycin 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. On the basis of this data, we propose that such nonantibacterial macrolides may be suitable for chronic treatment of CF and COPD patients without inducing bacterial resistance.

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NONANTIBIOTIC MACROLIDES AND MUCUS CLEARANCE

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AUTHOR CONTRIBUTIONS

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