Journal of Cystic Fibrosis xxx (xxxx) xxx

Contents lists available at ScienceDirect

Journal of Cystic Fibrosis

journal homepage: www.elsevier.com/locate/jcf

Original Article

Amphotericin B induces epithelial voltage responses in people with cystic fibrosis

Rajeev S. Chorghade[a], Bo Ram Kim[b], Janice L. Launspach[b], Philip H. Karp[b], Michael J. Welsh[b,c], Martin D. Burke[a,d,e,f,g,*]

[a] Department of Chemistry, University of Illinois at Urbana-Champaign, Urbana, IL 61801, USA
[b] Department of Internal Medicine and HHMI, Pappajohn Biomedical Institute, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA
[c] Department of Molecular Physiology and Biophysics, Roy J. and Lucille A. Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA
[d] Department of Biochemistry, University of Illinois at Urbana-Champaign, Urbana IL, USA
[e] Arnold and Mabel Beckman Institute, University of Illinois at Urbana-Champaign, Urbana IL, USA
[f] Carle Illinois College of Medicine, University of Illinois at Urbana-Champaign, Champaign, IL 61820, USA
[g] Carl R. Woese Institute for Genomic Biology, University of Illinois at Urbana-Champaign, Urbana IL, USA

ARTICLE INFO

Article history:
Received 15 July 2020
Revised 19 September 2020
Accepted 25 November 2020
Available online xxx

ABSTRACT

Background: Approximately 10% of people with cystic fibrosis (CF) have mutations that result in little to no CFTR production and thus cannot benefit from CFTR modulators. We previously found that Amphotericin B (AmB), a small molecule that forms anion channels, restored HCO_3^- secretion and increased host defenses in primary cultures of CF airway epithelia. Further, AmB increased ASL pH in *CFTR*-null pigs, suggesting an alternative CFTR-independent approach to achieve gain-of-function. However, it remains unclear whether this approach can be effective in people.

Methods: To determine whether AmB can impact physiology in people with CF, we first tested whether Fungizone, a clinically approved AmB formulation, could cause electrophysiological effects consistent with anion secretion in primary cultures of CF airway epithelia. We then evaluated the capacity of AmB to change nasal potential difference (NPD), a key clinical biomarker, in people with CF not on CFTR modulators.

Results: AmB increased transepithelial Cl^- current and hyperpolarized calculated transepithelial voltage in primary cultures of CF airway epithelia from people with two nonsense mutations. In eight people with CF not on CFTR modulators, intranasal Fungizone treatment caused a statistically significant change in NPD. This change was similar in direction and magnitude to the effect of ivacaftor in people with a *G551D* mutation.

Conclusions: Our results provide the first evidence that AmB can impact a clinical biomarker in people with CF. These results encourage additional clinical studies in people with CF to determine whether small molecule anion channels can provide benefit.

1. Introduction

Cystic fibrosis (CF) is caused by loss-of-function mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) anion channel [1]. Loss of CFTR-mediated HCO_3^- and Cl^- secretion to the airway surface liquid (ASL) compromises airway host defenses in people with CF and contributes to the chronic infection and inflammation that characterize CF airway disease [2,3]. Small molecule CFTR modulators such as ivacaftor, tezacaftor, and elexacaftor, which partially increase the activity of many CFTR mutants, have achieved significant clinical impact in people with CF [4–6]. However, approximately 10% of people with CF have nonsense mutations and/or splice defects that result in little to no CFTR production, or they cannot tolerate CFTR modulators, and thus cannot benefit [7,8].

We recently reported that the anion channel-forming small molecule natural product, amphotericin B (AmB), increased ASL height, increased transepithelial HCO_3^- secretion, and increased ASL pH in cultured CF airway epithelia [9]. Though AmB channels

* Corresponding author.
E-mail address: mdburke@illinois.edu (M.D. Burke).

https://doi.org/10.1016/j.jcf.2020.11.018
1569-1993/

are only modestly anion selective, this imperfect surrogate for the CFTR protein also increased ASL pH, decreased ASL viscosity, and increased ASL antibacterial activity in genetically diverse primary cultures of CF airway epithelia. Further, AmB increased ASL pH in *CFTR*-null pigs *in vivo*. These results suggest that CFTR-independent functional replacement with a small molecule surrogate may hold promise for addressing CF airway disease caused by nonsense mutations. Moreover, AmB is an already clinically approved drug that has been safely aerosolized to the lungs to treat or prevent intrapulmonary fungal infections in more than 1,600 people [10,11] and safely administered via intranasal perfusion in many previous studies [12–17].

The preclinical development and successful clinical translation of CFTR modulators provides a roadmap for the development of new CFTR therapeutics. Ivacaftor, the first clinically approved CFTR modulator, was initially tested via electrophysiological measurements in Ussing chambers. Specifically, ivacaftor increased transepithelial current and hyperpolarized transepithelial voltage in primary cultures of airway epithelia from people with G551D-CFTR mutations [18]. These electrophysiological studies suggested the potential for ivacaftor to change nasal potential difference (NPD), a key clinical biomarker widely used to evaluate experimental therapies in people with CF [19]. In an initial study in adults with CF caused by at least one G551D-CFTR mutation, ivacaftor changed NPD relative to placebo [20]. In subsequent clinical studies, ivacaftor also improved lung function as evaluated by metrics such as forced expiratory volume in one second (FEV_1), and improved health-related quality of life [4,21–23].

Here we report a similar series of electrophysiology studies in Ussing chambers with primary cultures of airway epithelia from people with CF caused by nonsense mutations. Collectively, these studies showed that AmB increased transepithelial Cl^- current, hyperpolarized calculated transepithelial voltage, and increased Cl^- secretion in CF airway epithelia. A subsequent clinical study in 8 people with CF not on modulators further showed that intranasal AmB caused a statistically significant change in NPD.

2. Materials and methods

2.1. Cell line growth conditions

CuFi-1 (ΔF508/ΔF508) cells (Welsh Laboratory, University of Iowa) were first grown from cryostock on Thermo Scientific Bio-Lite Cell Culture Treated 75 cm^2 flasks, seeded at 1×10^3 cells per cm^2 [24]. Prior to seeding, these flasks were coated with 4 mL of 60 µg/mL human placental collagen type IV (Sigma Aldrich 234154) for a minimum of 18 h at room temperature, rinsed twice with PBS, and dried. The cells were cultured with 12 mL Bronchial Epithelial Cell Growth Medium (BEGM) BulletKit (Lonza CC-3170), which includes the basal medium and eight SingleQuots of supplements (bovine pituitary extract (BPE), 2 mL; hydrocortisone, 0.5 mL; hEGF, 0.5 mL; triiodothyronine, 0.5 mL). The gentamycin-AmB aliquot was discarded and the medium was instead supplemented with 50 µg/mL penicillin-streptomycin (Corning Cellgro 30–001-CI), 50 µg/mL gentamycin (Sigma Aldrich G1397), and 2 µg/mL fluconazole (Sigma Aldrich F8929). The original CF transplant donor was genotyped by Integrated Genetics. The cell line was secondarily confirmed by the ATCC repository to have the correct genotype. MycoAlert Mycoplasma detection kit (Lonza LT07–418) was used to detect any RNA transcripts common to a broad spectrum of mycoplasma. The cell line was confirmed to be mycoplasma-free.

Cells were grown to 90% confluence at 37 °C in 5% CO_2, changing medium every two days, and then trypsinized with 4 mL of 0.25% trypsin containing 1 mM EDTA (Gibco 25200-056). Trypsin was inactivated with 10 mL HEPES-buffered saline solution (Lonza CC-5024) with 1% bovine calf serum. Cells were spun down in an Eppendorf Centrifuge 5430R at 1500 rpm for 5 min and resuspended in BEGM medium for passaging. For culturing on membrane supports for differentiation, cells were resuspended after centrifugation in Ultroser G medium comprised of 1:1 DMEM:Ham's F-12 (Corning 10–092-CV) supplemented with 2% v/v Ultroser G (Crescent Chemical 67042). The membrane supports used were Corning Costar 0.4 µm 24 well plate Transwell polycarbonate 0.33 cm^2 membrane inserts (Corning 3413). These membranes were coated with collagen in the same manner as the flasks detailed above. Each Transwell insert was seeded with 115,000 cells. These membranes were allowed to mature at an air-liquid interface for a minimum of 14 days to reach full differentiation, with the Ultroser G medium changed every other day. After maturation, medium was changed every seven days. For covariate control, membranes used in experiments were as close in age and maturation as possible.

2.2. Primary cultures of airway epithelia

Airway epithelial cells were obtained from human trachea and bronchi of CF specimens obtained from the Iowa Donor Network, either as post-mortem specimens or from tissue deemed not fit for transplant. All samples were deidentified in the Cell Culture Core Repository and patient identification information was not provided to the researchers running experiments. Patients were not recruited. Studies were approved by the University of Iowa Institutional Review Board. We have complied with all relevant ethical regulations and informed consent was obtained from all participants. After protease enzymatic digestion, cells were seeded onto collagen-coated semi-permeable membranes (0.33 cm^2, Corning 3413 polycarbonate) and grown at an air-liquid interface using previously described methods [25]. Primary W1282X/W1282X nasal epithelial cells were obtained from the Cystic Fibrosis Foundation. Airway epithelial cell cultures were analyzed after they had differentiated and at least 14 days after seeding.

2.3. Studies of $H^{13}CO_3^-$ and Cl^- efflux from POPC liposomes

Palmitoyl oleoyl phosphatidylcholine (POPC) was obtained as a 25 mg/mL solution in $CHCl_3$ from Avanti Polar Lipids (850457C). The solution was stored at −20 °C under an atmosphere of dry argon and used within 3 months. Cholesterol (Sigma Aldrich C8667) was purified by recrystallization from ethanol. $NaH^{13}CO_3$ was obtained as a white solid from Sigma Aldrich (372382).

Prior to preparing a lipid film, the lipid solution was warmed to ambient temperature to prevent condensation from contaminating the solution and degrading the lipid film. 42 mg of solid cholesterol was added to a 20 mL scintillation vial (Fisher Scientific), followed by 14 mL of the POPC solution. The solvent was removed with a gentle stream of nitrogen, and the resulting lipid film was stored under high vacuum for a minimum of twelve hours prior to use. For $H^{13}CO_3^-$ efflux experiments, the film was rehydrated with 2 mL of 250 mM $NaH^{13}CO_3$, 40 mM HEPES buffer, pH 7.5 (D_2O) and vortexed vigorously for approximately 3 min to form a suspension of multilamellar vesicles (MLVs). For Cl^- efflux experiments, the film was rehydrated with 2 mL of 250 mM NaCl, 40 mM HEPES buffer, pH 7.5. To obtain a sufficient quantity of large unilamellar vesicles (LUVs), at least two independent lipid film preparations were pooled together for the subsequent formation of LUVs. The lipid suspension was then subjected to 15 freeze-thaw cycles, where the suspension was alternatingly allowed to freeze in a liquid nitrogen bath, followed by thawing in a 50 °C water bath. The resulting lipid suspension was pulled into a Hamilton (Reno, NV) 1 mL gastight syringe and the syringe was placed in an Avanti Polar Lipids Mini-Extruder (610000). The lipid solution

was then passed through a 5.00 μm pore size Whatman Nuclepore hydrophilic polycarbonate filter (VWR 28158-067) 35 times, the newly formed large unilamellar vesicle (LUV) suspension being collected in the syringe that did not contain the original suspension of MLVs to prevent the carryover of MLVs into the LUV solution. To obtain a sufficient quantity of LUVs, at least four independent 1 mL preparations were pooled together for the dialysis and subsequent efflux experiments. The newly formed LUVs were dialyzed using Thermo Scientific Slide-A-Lyzer G2 dialysis cassettes (3 mL, 3500 MWCO, 87723). The LUV suspension was dialyzed 3 times against 600 mL of 62.5 mM $MgSO_4$, 40 mM HEPES buffer, pH 7.3. The first two dialyses were two hours long, while the final dialysis was performed overnight. Determination of total phosphorus was performed as described previously [9].

For $H^{13}CO_3^-$ efflux experiments, the pooled LUV suspension was then diluted to 70 mM lipid with 62.5 mM $MgSO_4$ in 40 mM HEPES buffer pH 7.3 (D_2O), and 0.025% (w/v) ^{13}C D-glucose (1–^{13}C) (Sigma Aldrich 297046) was added as an internal standard. For each experiment, 5 µL of DMSO or sterile water vehicle, AmB in DMSO (70 µM final concentration, 100X stock solution), or Fungizone in sterile water (70 µM AmB final concentration, 100X stock solution) was added to 500 μL of the liposome suspension with mixing for 1 hour. The liposome suspension was then immediately transferred to an oven dried New Era (Vineland, NJ) 5 mm NMR sample tube for analysis via NMR ^{13}C NMR spectra were acquired on a Bruker Avance III HD 500 MHz NMR spectrometer equipped with a 5 mm BBFO CryoProbe. The ^{13}C frequency was set to 125.83 MHz, and spectral width was 31,512 Hz. The instrument was locked on D_2O. Experimental conditions were: acquisition time, 0.93 s; 30° pulse width, 3.3 μs; relaxation delay, 0.2 s; number of scans, 256; temperature 23 °C. To effect complete ion release, 40 μL of a 10% (v/v) solution of triton X-100 (Sigma Aldrich X100) was added to the liposome suspension before data acquisition. After lysis of the liposome suspension, the integration of the signal corresponding to extravesicular HCO_3^- relative to the integration of the ^{13}C glucose standard was normalized to correspond to 100% efflux. For each experimental run with AmB addition, the signal corresponding to extravesicular HCO_3^- after 1 hour was integrated relative to the ^{13}C internal standard for each free induction decay (FID).

For Cl^- efflux experiments, the pooled LUV suspension was diluted to 70 mM lipid with 62.5 mM $MgSO_4$ in 40 mM HEPES buffer pH 7.3. Cl^- measurements were obtained using a Thermo Scientific Orion Star A211 Benchtop pH Meter (12–645–519) equipped with an Orion combination Cl^- electrode (Thermo Fisher Scientific 9617BNWP). Measurements were made on 4 mL suspensions that were magnetically stirred in 20 mL vials incubated at 23 °C. For each experiment, a baseline reading was established for at least one minute prior to addition of AmB, Fungizone, DMSO, or sterile water vehicle. After establishing a stable baseline reading, 40 µL of DMSO vehicle, AmB (70 µM final concentration, 100X stock in DMSO), sterile water vehicle, or Fungizone (corresponding to 70 µM AmB final concentration, 100X stock in sterile water) was added. The concentration of extravesicular Cl^- in each sample was measured every 10 s for 10 min after addition. At the conclusion of each experiment, 100 μL of a 10% (v/v) solution of triton X-100 (Sigma Aldrich X100) was added to the liposome suspension to effect complete ion release.

The efflux data from each run was normalized to the percent of total ion release from 0 to 100%. The scaling factor S was calculated for each experiment using the following relationship:

$$\left[\frac{[\mathrm{Ion}]_{final}}{[\mathrm{Ion}]_{initial}} - 1\right] \cdot S = 100$$

Each data point was then multiplied by S before plotting as a percentage of total $H^{13}CO_3^-$ or Cl^- efflux.

2.4. $H^{14}CO_3^-$ transport across CuFi-1 epithelial monolayers

Fully differentiated CuFi-1 (ΔF508/ΔF508) epithelia (0.33 cm^2) were cultured as described above. All experiments were run less than 2 months post seeding. ^{14}C-labeled sodium bicarbonate was obtained as a sterile 35.7 mM aqueous solution pH 9.5 (MP Biomedicals 0117441H). Fresh Ultroser G media was added to the basolateral membrane immediately prior to experimentation. A suspension of vehicle, AmB, or Fungizone in perfluorocarbon-72 (Fisher Scientific Acros 123792500) was prepared and vortexed vigorously for 30 s immediately prior to addition to ensure homogenization. The apical membrane was treated with 20 μL of vehicle, AmB, or Fungizone, and the cultured epithelia were incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. At the end of the treatment period, 5 μL of a 1.4 mM $H^{14}CO_3^-$stock solution in Ultroser G media was added to the basolateral media. The cultured epithelia were then incubated at 37 °C in 5% CO_2 for 10 min. After 10 min, the apical membrane of the cultured epithelia was immediately washed with 200 μL of PBS. The ASL wash was diluted in scintillation cocktail (Perkin Elmer 6013199) and analyzed via liquid scintillation counting. The measured basolateral-to-apical $H^{14}CO_3^-$secretion was then normalized to the vehicle-treated controls.

2.5. Ussing chamber analysis of CuFi-1 epithelia

Fully differentiated CuFi-1 (ΔF508/ΔF508) epithelia (0.33 cm^2) were cultured as described above. All experiments were run less than 2 months post seeding. Amiloride hydrochloride hydrate was obtained as a solid from Sigma Aldrich (A7410). Fungizone (Amphotericin B for Injection, USP, NDC 39822-10555, X-Gen Pharmaceuticals) was obtained as a lyophilized solid from McKesson (1670876). The indicated mass of Fungizone (250 µg/mL) refers to the total mass of Fungizone material, not the mass of amphotericin B alone.

Differentiated cultures of CuFi-1 epithelia were mounted in modified Ussing chambers (Physiologic Instruments EM-CSYS-6). The epithelial cultures were bathed on the basolateral membrane with a Cl^--containing Ringer's solution (mM): 135 NaCl, 5 HEPES, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose (pH 7.4) at 37 °C, gassed for at least 10 min with compressed air. The epithelial cultures were bathed on the apical membrane with a low Cl^- solution containing (mM): 135 sodium gluconate, 5 HEPES, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose (pH 7.4) at 37 °C, gassed for at least 10 min with compressed air. The pH of both solutions was adjusted to pH 7.4 using saturated tromethamine in double distilled water (ddH_2O). For experiments with zero Na^+ in the apical solution, the epithelial cultures were bathed on the apical membrane with a low Cl^- solution containing (mM): 135 N-methyl-D-glucamine (NMDG), 135 mM gluconic acid, 5 HEPES, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose (pH 7.4) at 37 °C, gassed for at least 10 min with compressed air. For experiments with zero basolateral Cl^-, the epithelial cultures were bathed on the basolateral membrane with a zero Cl^- solution containing (mM): 135 sodium gluconate, 5 HEPES, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 calcium gluconate, 1.2 magnesium gluconate, 5 glucose (pH 7.4) at 37 °C, gassed for at least 10 min with compressed air.

Bath solutions were stirred by bubbling continuously with compressed air. All experiments were run in voltage clamp mode, in which transepithelial voltage (Vt) was clamped to 0 mV. Transepithelial current (It) and conductance (Gt) were measured with an epithelial voltage clamp (Physiologic Instruments VCC-MC8). Data was collected using the Acquire and Analyze program (Physiologic Instruments). Measurements of transepithelial current and conductance were normalized per cm^2 of epithelial surface area.

For experiments with Fungizone or low Cl^- vehicle, each epithelial culture was bathed with 5 mL Cl^--containing Ringer's solution on the basolateral membrane and 3.33 mL low Cl^- solution on the apical membrane. After establishing a baseline reading for 10 min, 3.33 µL amiloride in DMSO (100 µM final concentration, 1000X stock solution) was added to the apical solution. After ~15 min of amiloride treatment, 1.67 mL of Fungizone dissolved in low Cl^- solution (250 µg/mL or 2.05 µg/mL final concentration, 3X stock solution + 100 µM amiloride) or low Cl^- vehicle (containing 100 µM amiloride) was added to the apical solution slowly via syringe to avoid introducing air bubbles, and transepithelial current and conductance were recorded for at least one hour after addition.

For experiments with basolateral addition of Fungizone or vehicle, each epithelial culture was bathed with 3.33 mL Cl^--containing Ringer's solution on the basolateral membrane and 5 mL low Cl^- solution on the apical membrane. After establishing a baseline reading for 10 min, 5 µL amiloride in DMSO (100 µM final concentration, 1000X stock solution) was added to the apical solution. After ~15 min of amiloride treatment, 1.67 mL of Fungizone dissolved in Cl^--containing solution (250 µg/mL final concentration, 3X stock solution) or vehicle was added to the basolateral solution slowly via syringe to avoid introducing air bubbles, and transepithelial current and conductance were recorded for at least one hour after addition.

For experiments with basolateral addition of Cl^- or gluconate after AmB or vehicle addition, each epithelial culture was bathed with 3 mL zero Cl^- solution on the basolateral membrane and 3.33 mL low Cl^- solution on the apical membrane. After establishing a baseline reading for 10 min, 3.33 µL amiloride in DMSO (100 µM final concentration, 1000X stock solution) was added to the apical solution. After ~15 min of amiloride treatment, 1.67 mL of Fungizone dissolved in low Cl^- solution (250 µg/mL final concentration, 3X stock solution + 100 µM amiloride) or low Cl^- vehicle (containing 100 µM amiloride) was added to the apical solution slowly via syringe to avoid introducing air bubbles, and transepithelial current and conductance were recorded for approximately 30 min. After 30 min, 3 mL of either Cl^--containing Ringer's solution or zero Cl^- solution was added to the basolateral solution slowly via syringe, and transepithelial current and conductance were recorded for at least 30 min after addition.

2.6. Ussing chamber analysis of primary cultures of CF airway epithelia

Fully differentiated primary cultures of CF bronchus and nasal epithelia (0.33 cm^2) were used for these experiments. Amiloride hydrochloride hydrate was obtained as a solid from Sigma Aldrich (A7410). Ivacaftor was obtained as a solid from Selleck Chemicals (S1144). Forskolin was obtained as a solid from Sigma Aldrich (F3917). Differentiated cultures of airway epithelia were mounted in modified Ussing chambers (Physiologic Instruments EM-CSYS-8). The epithelia were bathed on the basolateral membrane with a Cl^--containing Ringer's solution containing (mM) 135 NaCl, 5 HEPES, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose (pH 7.4) at 37 °C, gassed for at least 10 min with compressed air. The epithelial cultures were bathed on the apical membrane with a low Cl^- solution containing (mM) 135 sodium gluconate, 5 HEPES, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 5 glucose (pH 7.4) at 37 °C, gassed for at least 10 min with compressed air. The pH of both solutions was adjusted to pH 7.4 using saturated tromethamine in double distilled water (ddH_2O). Bath solutions were stirred by bubbling continuously with compressed air. All experiments were run in voltage clamp mode, in which transepithelial voltage (Vt) was clamped to 0 mV. Transepithelial current (It) and conductance (Gt) were measured with an epithelial voltage clamp (Physiologic Instruments VCC-MC8). Data was collected using the Acquire and Analyze program (Physiologic Instruments). Measurements of transepithelial current and conductance were normalized per cm^2 of epithelial surface area.

For experiments involving apical addition of ivacaftor, each primary culture of CF (ΔF508/G551D) airway epithelia was bathed with 5 mL Cl^--containing Ringer's solution on the basolateral membrane and 5 mL low Cl^- solution on the apical membrane. After establishing a baseline reading for 10 min, 5 µL amiloride in DMSO (100 µM final concentration, 1000X stock solution) was added to the apical solution. After at least 15 min of amiloride treatment, 10 µL of DMSO vehicle or 5 µL of ivacaftor in DMSO (10 µM final concentration, 1000X stock solution) and 5 µL of forskolin in DMSO (10 µM final concentration, 1000X stock solution) was added to the apical solution, and transepithelial current and conductance were recorded for at least one hour after addition.

For experiments involving apical AmB addition, each epithelial culture was bathed with 5 mL Cl^--containing Ringer's solution on the basolateral membrane and 3.33 mL low Cl^- solution on the apical membrane. After establishing a baseline reading for 10 min, 3.33 µL amiloride in DMSO (100 µM final concentration, 1000X stock solution) was added to the apical solution. After at least 15 min of amiloride treatment, 1.67 mL of Fungizone dissolved in low Cl^- solution (250 µg/mL final concentration, 3X stock + 100 µM amiloride) or low Cl^- vehicle (containing 100 µM amiloride) was added to the apical solution slowly via syringe to avoid introducing air bubbles, and transepithelial current and conductance were recorded for at least one hour after addition.

2.7. NPD measurements in people with CF

NPD experiments were performed according to the protocols approved by the University of Iowa Institutional Review Board (IRB ID# 199207428). Written informed consent was received from all participants prior to inclusion in the study. Eligible subjects had a confirmed diagnosis of cystic fibrosis. At screening, subjects had to be ≥18 years of age.

NPD measurements were performed using standardized protocols from the Cystic Fibrosis Foundation Therapeutics, Inc. Therapeutic Development Network (CFFT-TDN). NPD measurements were recorded every 1 second using LabChart software. The prespecified NPD endpoint was the change in transepithelial electrical potential after intranasal perfusion with vehicle or AmB treatment following an initial perfusion with zero Cl^- solution for each person.

For NPD measurements, bridges connected to electrodes were placed under the inferior turbinate of the nasal mucosa and in the subcutaneous space of the forearm while the nasal mucosa was bathed in a sequential series of perfusion solutions:

1 Cl^--containing Ringer's solution
2 Cl^--containing Ringer's solution containing 100 µM amiloride
3 Zero Cl^- solution containing 100 µM amiloride
4 Zero Cl^- solution containing 100 µM amiloride (vehicle) OR Zero Cl^- solution containing 100 µM amiloride + 250 µg/mL Fungizone (122 µM AmB)

NPD measurements were quantified as an average NPD measurement during the final 30 s of the initial intranasal perfusion with zero Cl^- solution and as an average NPD measurement during the final 30 s of intranasal perfusion with vehicle or Fungizone.

2.8. ASL AmB concentration determination in cultures of CuFi-1 epithelia

Fully differentiated CuFi-1 (ΔF508/ΔF508) epithelia (0.33 cm^2) were cultured as described above. All experiments were run less than 2 months post seeding. AmBisome (Amphotericin B Liposome for Injection, USP, NDC 0469-3051-30, Astellas Pharmaceuticals US, Inc.) was obtained as a lyophilized solid from McKesson (529618). The indicated mass of AmBisome (1 mg/mL) refers to the total mass of AmBisome material, not the mass of amphotericin B alone.

Fresh Ultroser G media was added to the basolateral membrane immediately prior to experimentation. A suspension of vehicle or 1 mg/mL AmBisome in perfluorocarbon-72 (Fisher Scientific Acros 123792500) was prepared and vortexed vigorously for 30 s immediately prior to addition to ensure homogenization. The apical membrane was treated with 20 μL of vehicle or AmBisome, and the cultured epithelia were incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. In preparation for ASL collection, 0.1 µL capacity microcapillary tubes (Drummond Scientific NC1453214) were placed into 200 µL pipette tips (Denville Scientific P1122).

At the end of the treatment period, microcapillary tubes were gently touched around the edge of the apical membrane of each epithelial culture insert until completely filled with ASL via capillary action. After collecting 0.1 µL ASL, a p200 pipette was used to push the entire sample into 39.9 µL molecular biology grade water (Corning 46-000-CM) in a 300 µL capacity HPLC insert (Agilent 5182-0549). AmB concentrations were then quantitively analyzed using an Agilent 1260 series Infinity II analytical HPLCMS equipped with an Agilent 6530 LC/Q-TOF. A Zorbax Eclipse C_{18} reverse phase column (Agilent, 4.6 × 150 mm, 5 µm) was used. HPLC experimental conditions were: Injection volume: 25 µL; Flow rate = 1.2 mL/minute; Eluent: MeCN: 25 mM NH_4OAc in H_2O, 1:19 → 19:1 over 8 min. UV detection at 406 nm was used. For MS analysis, AmB was analyzed using Dual Agilent Jet Stream Electrospray Ionization (Dual AJS ESI) in positive mode. Experimental MS conditions were: Gas temperature: 325 °C; Fragmentor voltage: 175 V, Skimmer voltage: 65 V. To quantify the final AmB concentration in the ASL of CuFi-1 epithelia, the area under the curve for the peak corresponding to AmB was integrated and compared to a standard curve containing known AmB concentrations in molecular biology grade water.

2.9. Statistics

No data were excluded. All data indicate mean ± SEM. Statistical analysis represents *P* values obtained from analysis of variance (ANOVA) with Tukey's multiple comparisons test or unpaired Student's *t*-test where indicated. ns, not significant. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. On the basis of pilot experiments, we chose sample sizes that adequately power each experiment to detect a difference in outcomes between groups. Epithelial samples were manually assigned at random into control and treatment groups for each experiment. The investigators were not blinded to allocation during experiments and outcome assessment.

3. Results

3.1. Fungizone forms anion-permeable channels

Fungizone is an FDA-approved, water-soluble formulation consisting of AmB, sodium deoxycholate, and Na_3PO_4. In our prior studies, we did not evaluate the ion channel forming capacity of Fungizone or determine whether this clinical formulation could cause anion secretion in CF airway epithelia similar to laboratory grade AmB [9].

Both laboratory grade AmB and Fungizone caused HCO_3^- (**Fig. S1A**) and Cl^- efflux (**Fig. S1B**) from 10% cholesterol/POPC liposomes. Further, Fungizone increased transepithelial $H^{14}CO_3^-$ secretion (**Fig. S1C**) to levels similar to that of laboratory grade AmB in cultures of CuFi-1 (ΔF508/ΔF508) airway epithelia [24]. These results suggest that despite the presence of sodium deoxycholate and Na_3PO_4 in the formulation and the modified aqueous solubility, the Fungizone clinical formulation forms transmembrane ion channels and increases anion secretion in CF airway epithelia.

3.2. AmB causes electrophysiological changes in CF airway epithelia

We next asked whether 250 µg/mL Fungizone (122 µM AmB), a concentration that has been safely delivered intranasally to people with fungal infections, can cause electrophysiological changes consistent with increased anion secretion in cultures of CF airway epithelia. Cultures of CuFi-1 (ΔF508/ΔF508) airway epithelia were mounted in modified Ussing chambers, and the transepithelial voltage was clamped to 0 mV. The epithelial cultures were bathed with a Ringer's solution (135 mM NaCl) on the basolateral membrane and a low Cl^- solution (4.8 mM Cl^-) on the apical membrane. This was intended to mimic an NPD experiment, where perfusion with a zero Cl^- solution establishes a strong electrochemical gradient favoring anion secretion. After establishing a baseline reading for approximately 10 min, 100 µM amiloride was added to the apical solution for 10 min to inhibit the epithelial Na^+ channel (ENaC). Next, vehicle or Fungizone (250 µg/mL) was added to the apical solution, and transepithelial current and conductance were measured for at least 30 min. Changes in transepithelial current, conductance, and calculated transepithelial voltage were quantified 10 min after each intervention. Fungizone caused statistically significant increases in transepithelial current (Fig. 1A) and transepithelial conductance (Fig. 1B), and hyperpolarized calculated transepithelial voltage (Fig. 1C) relative to vehicle treatment. Conversely, addition of Fungizone to the basolateral solution decreased transepithelial current, suggesting that the increased transepithelial current observed previously is specific to apically localized AmB channels. We speculate that the decreased transepithelial current observed with basolateral AmB addition is due to AmB-mediated permeabilization of the basolateral membrane to cations.

3.3. AmB-mediated electrophysiological changes depend upon basolateral Cl^-

Importantly, unlike CFTR, AmB channels are only modestly anion selective and have significant permeability to cations in addition to anions [9]. Thus, we reasoned that the increased transepithelial current observed could be due to AmB-mediated Na^+ absorption and/or AmB-mediated Cl^- secretion at the apical membrane of CF airway epithelia. We therefore aimed to determine whether Cl^- or Na^+ were responsible for the AmB-mediated transepithelial current. When Na^+ was removed from the apical solution and replaced with N-methyl-D-glucamine (NMDG), apical Fungizone increased transepithelial current, consistent with AmB-mediated Cl^- secretion (Fig. 1D). The magnitude of this effect was reduced relative to experiments with Na^+ present (Fig. 1A), suggesting that AmB-mediated Na^+ absorption might also contribute to the transepithelial current when Na^+ is present in the apical solution. To determine whether the sustained current in the absence of Na^+ is due to AmB-mediated Cl^- secretion, we repeated the same experiment with a zero Cl^- solution on the basolateral membrane (Fig. 1D). With zero basolateral Cl^-, Fungizone

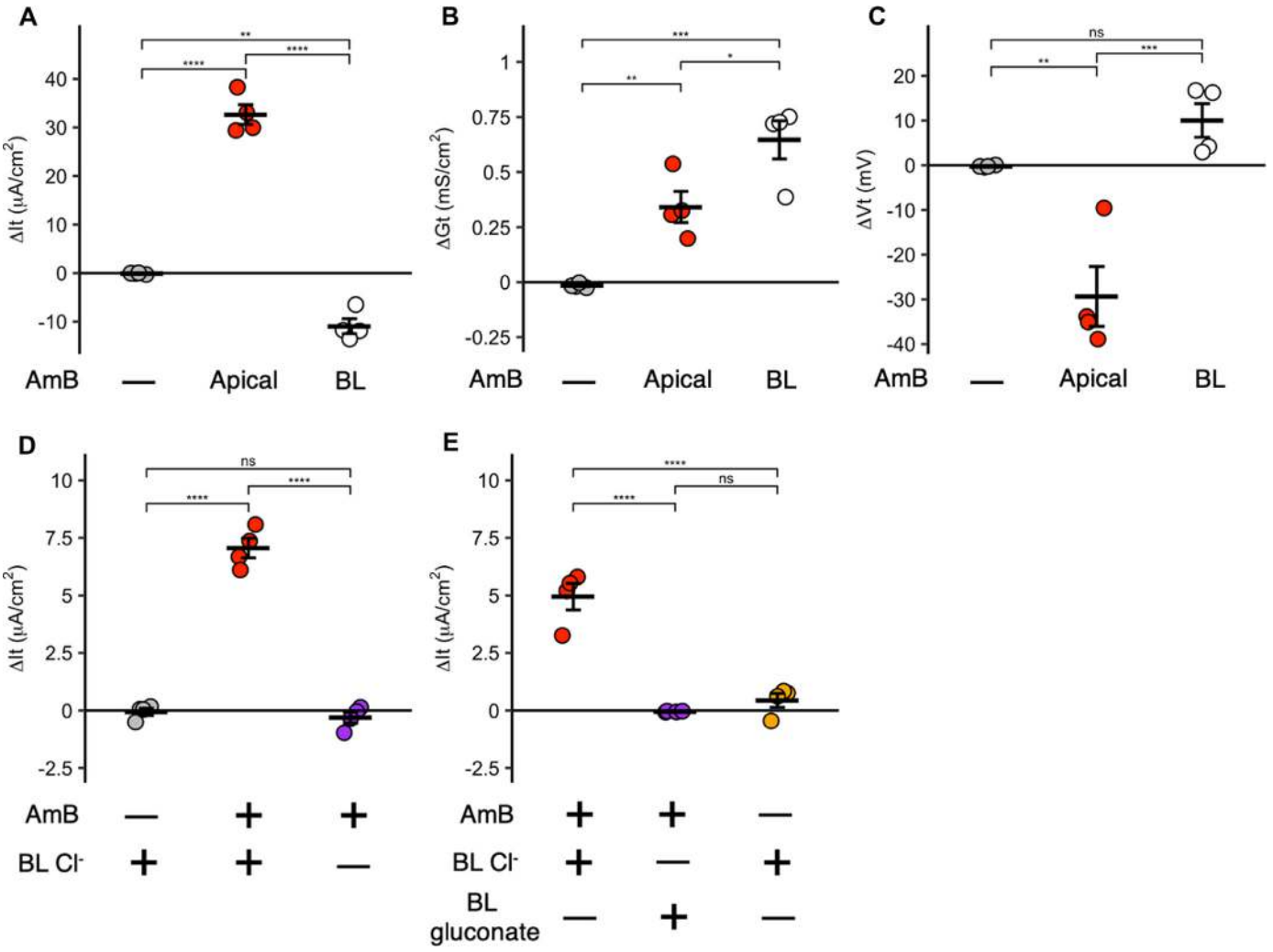

Fig. 1. Apical Fungizone addition increased transepithelial current and conductance and hyperpolarized calculated transepithelial voltage in cultures of CuFi-1 (ΔF508/ΔF508) airway epithelia. In an Ussing chamber assay, apical addition of 250 μg/mL Fungizone increased transepithelial current (A), increased transepithelial conductance (B), and hyperpolarized calculated transepithelial voltage (C), while basolateral (BL) Fungizone decreased transepithelial current ($n = 4$). When Na^+ was replaced with NMDG in the apical solution, 250 μg/mL Fungizone increased transepithelial current (D), consistent with AmB-mediated Cl^- secretion. The magnitude of the apical AmB-mediated increase in transepithelial current in the presence of basolateral Cl^- was reduced with zero Na^+ in the apical solution (****$P < 0.0001$ by analysis of variance (ANOVA) with Tukey's multiple comparison test). Removing Cl^- from the basolateral solution abolished the sustained AmB-mediated current ($n = 4$). (E) 30 min after Fungizone treatment in the presence of zero basolateral Cl^-, basolateral addition of 67.5 mM Cl^- increased transepithelial current, while basolateral gluconate addition did not ($n = 4$). Bars and whiskers indicate mean ± SEM. ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$ by analysis of variance (ANOVA) with Tukey's multiple comparison test.

caused only a transient increase in transepithelial current which rapidly returned to baseline levels, suggesting that the sustained AmB-mediated current depends upon the presence of basolateral Cl^-.

To further determine whether the AmB-mediated increase in current is at least in part attributable to Cl^- secretion, after adding AmB with a zero Cl^- basolateral solution (135 mM Na^+ gluconate), we added an equal volume of buffer containing 135 mM NaCl to the basolateral solution. With this addition, the basolateral concentrations of Na^+, K^+, Mg^{2+}, Ca^{2+}, and ionic strength all remained the same, but the concentration of basolateral Cl^- increased. The addition of basolateral Cl^- increased transepithelial current, consistent with AmB-mediated Cl^- secretion (Fig. 1E). As controls, addition of basolateral gluconate or addition of basolateral Cl^- in the absence of apical AmB caused minimal changes in transepithelial current. Collectively, these results suggest that the Fungizone-mediated increase in transepithelial current is at least partially due to basolateral-to-apical Cl^- secretion.

3.4. AmB causes electrophysiological changes similar to those caused by ivacaftor in primary cultures of CF airway epithelia

Primary cultures of CF airway epithelia represent a more physiologically relevant model system relative to epithelial cultures grown with a cell line. We thus next aimed to determine whether AmB could cause electrophysiological changes similar to those of ivacaftor in primary cultures of CF airway epithelia. We studied primary cultures of CF (ΔF508/G551D) bronchus epithelia using a low Cl^- (135 mM Na^+ gluconate, 4.8 mM Cl^-) apical solution similar to what is used in an NPD experiment. Consistent with what was observed in previous studies, apical addition of 10 μM ivacaftor/10 μM forskolin increased transepithelial current and conductance (Fig. 2A) and hyperpolarized calculated transepithelial voltage (Fig. 2B). Similarly, apical addition of 250 μg/mL Fungizone increased transepithelial current and conductance (Fig. 2C) and hyperpolarized calculated transepithelial voltage (Fig. 2D) in primary cultures of CF airway epithelia from the same donor. These results suggest that AmB causes electrophysiological effects similar

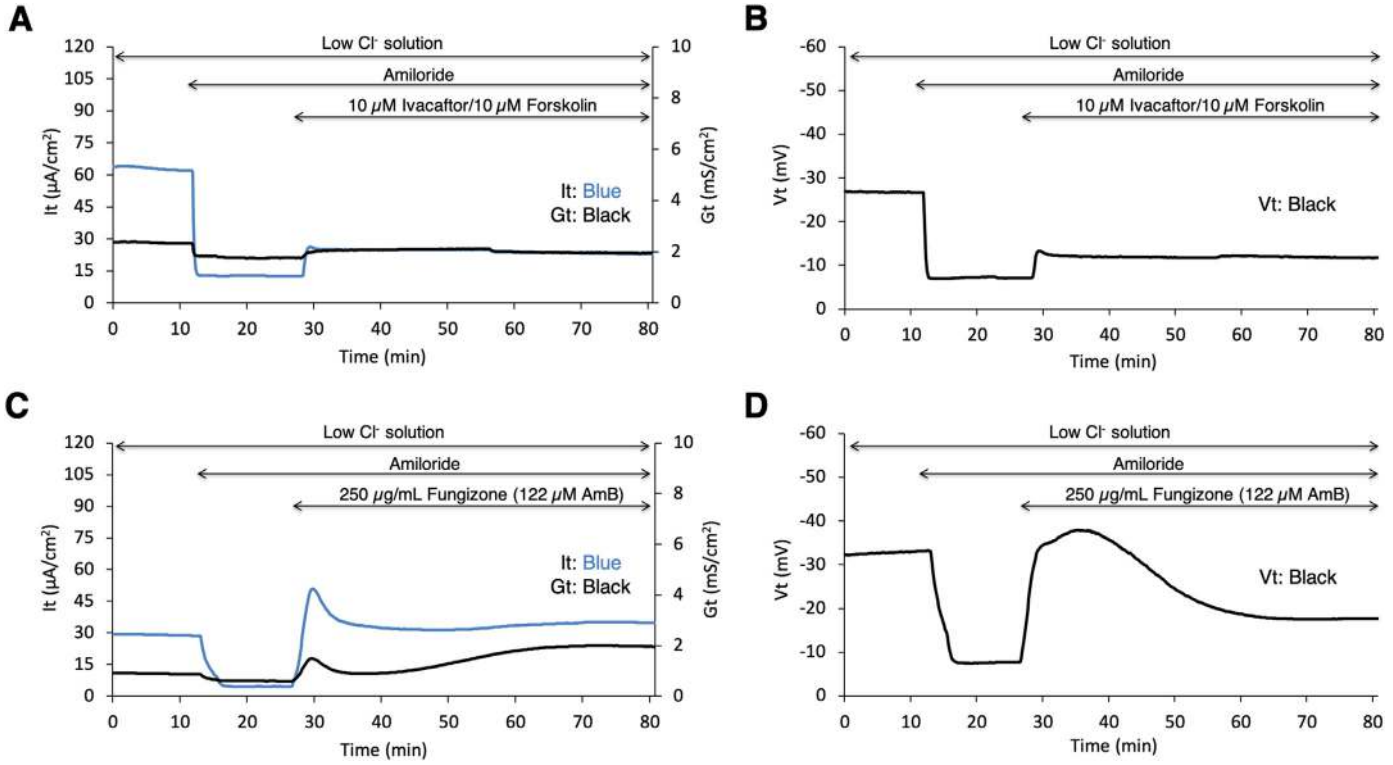

Fig. 2. Fungizone caused electrophysiological changes in primary cultures of CF (ΔF508/G551D) airway epithelia similar to those caused by ivacaftor. Addition of 10 μM ivacaftor/10 μM forskolin increased transepithelial current and conductance (A) and hyperpolarized calculated transepithelial voltage (B) in primary cultures of CF (ΔF508/G551D) bronchus epithelia ($n = 2$). Apical addition of 250 μg/mL Fungizone (122 μM AmB) similarly increased transepithelial current and conductance (C) and hyperpolarized calculated transepithelial voltage (D, $n = 2$).

to those caused by ivacaftor in primary cultures of CF airway epithelia.

3.5. AmB causes electrophysiological changes in primary cultures of CF airway epithelia from people who cannot benefit from CFTR modulators

We then asked whether Fungizone could cause similar electrophysiological effects in genetically diverse primary cultures of CF bronchus epithelia from people with at least one nonsense mutation, including epithelial cultures from a person with two nonsense mutations not addressable with CFTR modulators (W1282X/CFTR-del2,3). Fungizone statistically significantly increased transepithelial current (Fig. 3A) and conductance (Fig. 3B) and hyperpolarized calculated transepithelial voltage (Fig. 3C) in primary cultures of CF bronchus epithelia. To determine whether AmB can permeabilize and cause electrophysiological changes in nasal epithelia from people who cannot benefit from CFTR modulators, we obtained primary cultures of human nasal epithelia from three donors homozygous for the severe W1282X-CFTR nonsense mutation through the Cystic Fibrosis Foundation (CFF) RARE initiative. 250 μg/mL Fungizone (122 μM AmB) increased transepithelial current (Fig. 3D) and conductance (Fig. 3E) and hyperpolarized calculated transepithelial voltage (Fig. 3F) in primary cultures of W1282X/W1282X human nasal epithelia from all three donors tested. Collectively, these results suggested that AmB may change NPD in people with CF caused by a range of CFTR mutations, including people who cannot benefit from CFTR modulators.

3.6. AmB changes nasal potential difference in people with CF

With these promising preclinical results, we proceeded to test the possibility that AmB could generate a conductance consistent with anion secretion in people with CF. We thus tested the ability of AmB to change NPD in the presence of a Cl^- concentration gradient using a modified clinical protocol (**Fig. S2A**). None of our eight participants were taking CFTR modulators. The study included one person with two mutations not addressable with CFTR modulators (R553X/N1303K), one person with two probable mutations not addressable with CFTR modulators (M470 variant/unknown), five people with a single ΔF508 mutation and one nonsense mutation, and one person homozygous for the ΔF508 mutation who was unable to tolerate CFTR modulator treatment. In each participant, we treated one nostril with vehicle and the other nostril with Fungizone (AmB). Specifically, in the vehicle nostril, after an initial 4 min perfusion with zero Cl^- solution, we perfused with zero Cl^- solution for 4 min ("Vehicle"). In the other nostril ("Amphotericin B"), after the initial 4 min perfusion with zero Cl^- solution, we perfused for 4 min with 250 μg/mL Fungizone in zero Cl^- solution. We then quantified the NPD reading over the final 30 s of each perfusion. To analyze the change in NPD for each treatment, we subtracted the average NPD over the final 30 s of the perfusion with vehicle or AmB from the average NPD over the final 30 s of the initial zero Cl^- solution perfusion.

AmB caused NPD to become more negative in seven out of the eight people with CF (Fig. 4A, **Fig. S2B-C**). The average change in NPD relative to vehicle was -2.9 ± 1.1 mV ($p = 0.044$). This change is similar in direction and magnitude to the NPD change in response to administration of a zero Cl^- solution containing isoproterenol (−3.7 mV) caused by 150 mg ivacaftor treatment for 14 days in people with a G551D-CFTR mutation [20]. The change in NPD caused by AmB was also similar to the effect (approximately −4 mV) caused by the same dose of ivacaftor in a subsequent study which evaluated the change in NPD in response to administration of a zero Cl^- solution containing

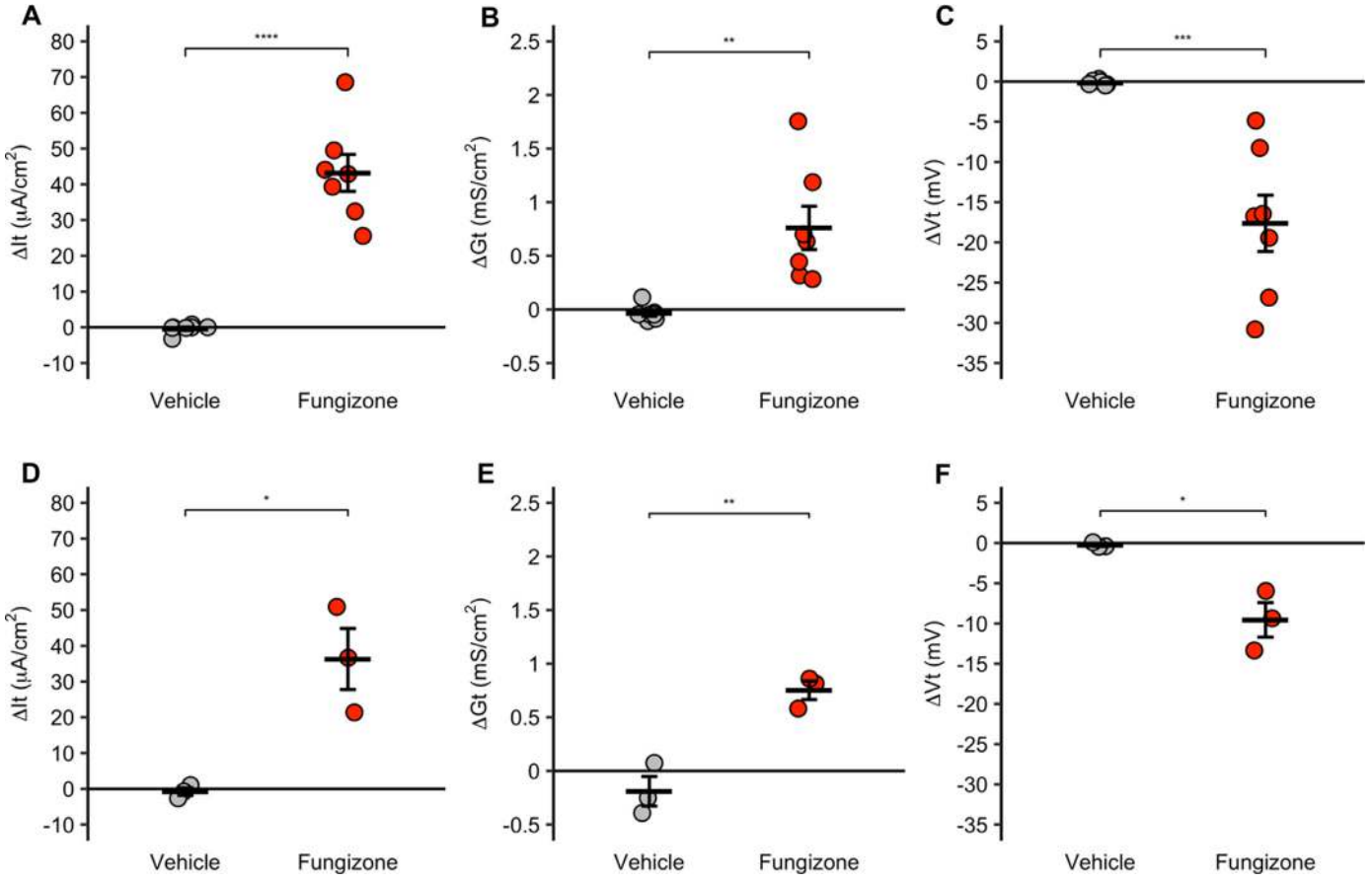

Fig. 3. Fungizone caused electrophysiological changes in genetically diverse primary cultures of CF bronchus and nasal epithelia. 250 μg/mL Fungizone (122 μM AmB) increased transepithelial current (A) and conductance (B) and hyperpolarized calculated transepithelial voltage (C) in genetically diverse primary cultures of CF bronchus epithelia with at least one nonsense mutation (W1282X/CFTR-del2,3; ΔF508/L1254X; ΔF508/dele2,3; ΔF508/R553X; ΔF508/2184-delA; ΔF508/G542X; ΔF508/2622–1G→A). Each data point in A-C indicates the average measurements from 1 to 3 epithelia for seven human donors (two epithelia were measured for most donors). Fungizone increased transepithelial current (D), increased transepithelial conductance (E), and hyperpolarized calculated transepithelial voltage (F) in primary cultures of CF nasal epithelia from 3 people with two W1282X-CFTR mutations. Each data point in D-F indicates the average measurements from two epithelia for each human donor. Bars and whiskers indicate mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$ by unpaired Student's *t*-test.

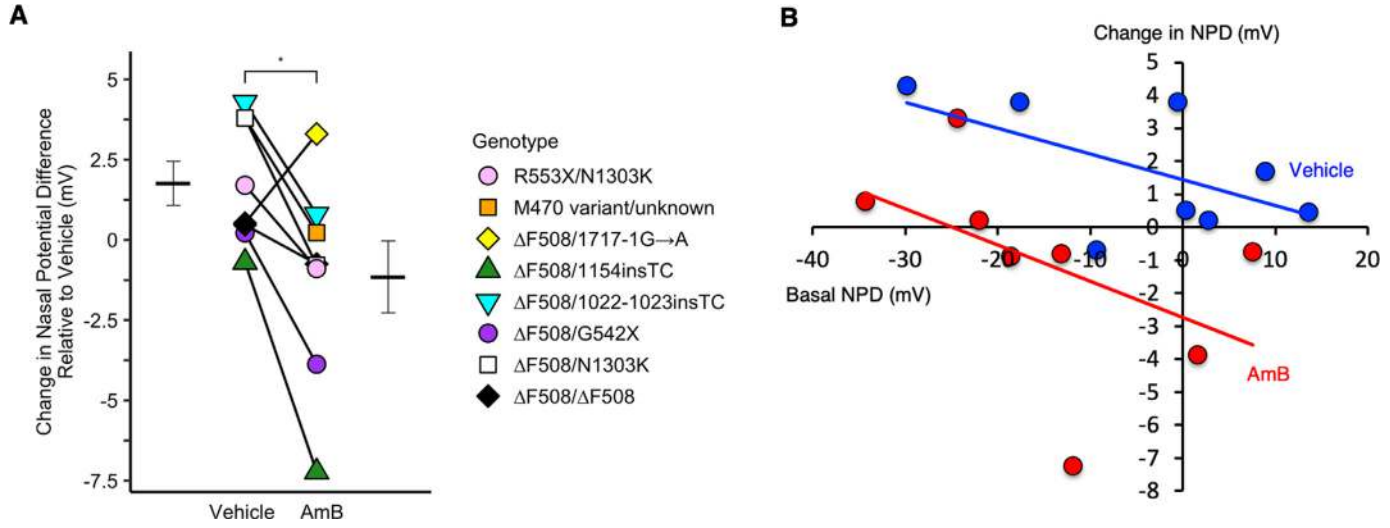

Fig. 4. AmB changed nasal potential difference in people with CF. (A) AmB caused a statistically significant change in NPD in people with CF not on modulators ($n = 8$). (B) Plot of basal voltage compared to change in voltage with either vehicle or AmB treatment ($n = 8$). Bars and whiskers indicate mean ± SEM. $^{*}P = 0.044$ by unpaired Student's *t*-test.

isoproterenol in the most polarized single nostril at each visit [26].

Further, we plotted the basal voltage during the initial zero Cl^- perfusion relative to the change in voltage caused by either zero Cl^- vehicle or AmB (Fig. 4B). The change in NPD caused by AmB was greater than vehicle independent of the basal voltage, increasing confidence that AmB is responsible for the observed changes.

3.7. AmB forms channels in cultured CF airway epithelia at concentrations which have been safely achieved in the airways

With the goal of further evaluating the therapeutic potential of this pharmacological approach, we next aimed to determine whether AmB could increase anion secretion and ASL pH in cultured CF airway epithelia at concentrations which have been safely delivered to the airways to treat and prevent intrapulmonary fungal infections. In a 2009 study, AmB was delivered to the airways via inhalation, and AmB concentrations in the upper and lower airways were quantified via bronchoalveolar lavage [27]. Notably, AmB concentrations of at least 3 μg/mL (3.25 μM) were retained in the proximal and distal airways at least 14 days after a single administration. Aerosolized AmB has also been shown to be an effective antifungal therapy against *Aspergillus* infections (MIC = 1 μM for most strains), providing strong evidence that the effective concentrations achieved and sustained in the airways are at least 1 μM.

2.05 μg/mL Fungizone (1 μM AmB) increased transepithelial current (**Fig. S3A**) and conductance (**Fig. S3B**) and hyperpolarized calculated transepithelial voltage (**Fig. S3C**) in cultures of CuFi-1 epithelia. Further, using the microcapillary tube-based assay we previously developed to collect 0.1 μL ASL from epithelial cultures maintained at an air-liquid interface [9], the ASL AmB concentration which increased ASL pH in cultures of CuFi-1 epithelia was determined. 1 mg/mL AmBisome (37.7 μM AmB), which previously increased ASL pH in cultures of CuFi-1 epithelia and in $CFTR^{-/-}$ pigs *in vivo* [9], was added to the apical membrane of cultures of CuFi-1 epithelia for 48 h as a suspension in perfluorocarbon-72 vehicle, and the ASL AmB concentration was quantified via HPLC/MS analysis and comparison with a standard curve of known AmB concentrations (**Fig. S3D-F**). Based on this analysis, the lowest apical AmB concentration previously tested in CuFi-1 epithelia, which increased ASL pH by 0.25 pH units relative to vehicle, was calculated to be 0.71 μM AmB [9]. This effect was sustained with calculated ASL AmB concentrations up to 285 μM [9]. Collectively, these results suggest that AmB can increase anion secretion and increase ASL pH in cultured CF airway epithelia at concentrations which have been safely achieved and sustained in the airways.

4. Discussion

Loss of CFTR-mediated anion secretion to the ASL impairs respiratory host defenses and leads to CF airway disease. We previously found that AmB, a clinically approved small molecule natural product, formed anion-permeable channels and increased host defenses in primary cultures of CF airway epithelia [9]. In this study, AmB caused electrophysiological changes in primary cultures of CF airway epithelia consistent with Cl^- secretion. Further, AmB changed NPD in people with CF not treated with CFTR modulators. Collectively, these results suggest that AmB generated apical membrane anion channels in people with CF. Moreover, AmB caused changes in the electrophysiological properties of CF airway epithelia *in vivo* that are similar to those caused by CFTR modulators. These results provide the first clinical evidence that small molecule channels can impact human physiology.

This study has advantages. (*i*) We evaluated AmB-mediated electrophysiological effects in differentiated primary cultures of CF airway epithelia *in vitro* as well as nasal epithelia in people with CF *in vivo*. (*ii*) By quantifying NPD measurements as the final 30 s of perfusion for each intervention, our NPD measurement methodology was not biased by the interpreter.

Our study also has limitations. (*i*) Ussing chamber studies in which cultures of CF airway epithelia are bathed with an excess of apical solution and intranasal perfusion with a zero Cl^- solution may not accurately represent native ASL conditions in the lower airways of people with CF. Though apical AmB addition caused electrophysiological effects that are also consistent with Na^+ absorption in cultures of CF airway epithelia, we previously found that ASL concentrations of Na^+, K^+, Mg^{2+}, and Ca^{2+} were unchanged with AmB treatment for 48 h relative to vehicle-treated controls [9].

(*ii*) In this study we did not characterize the effects of sustained apical AmB addition on epithelial membrane integrity or epithelial toxicity. Consistent with the observed increase in transepithelial conductance in our Ussing chamber assay, 122 μM AmB decreased transepithelial resistance in cultures of CF airway epithelia. However, transepithelial resistance did not decrease below 500 Ω^*cm^2 after 1 hour of AmB treatment, suggesting that AmB did not disrupt the integrity of the epithelial membrane. We did not expose the epithelia to this concentration of AmB for longer than 1 hour, but we previously found that apical addition of 50 μM AmB to cultures of CuFi-1 epithelia for at least 28 days did not decrease transepithelial electrical resistance (TEER) or cause toxicity as assessed by a lactate dehydrogenase (LDH) assay relative to vehicle-treated controls [9]. Collectively, these results suggest that high concentrations of AmB at the apical membrane do not disrupt epithelial membrane integrity or cause epithelial toxicity with sustained treatment.

(*iii*) We did not evaluate the effects of agents which modify epithelial anion channels on the AmB-mediated electrophysiological effects. Continuous, non-regulated small molecule-mediated anion secretion in airway epithelia could potentially disrupt overall ion homeostasis and/or cause other deleterious effects. However, we previously found that, similar to the function of CFTR, inhibition of the basolateral Na^+/K^+ ATPase with ouabain abolished AmB-mediated HCO_3^- secretion, and inhibition of the basolateral Na^+-K^+−2Cl^- cotransporter (NKCC) with bumetanide abolished the AmB-mediated increase in ASL height in CF airway epithelia [9]. These results suggest that like CFTR, the function of AmB channels can be modulated by physiological conditions and regulated by the activity of the endogenous protein network which drives epithelial anion secretion.

(*iv*) Finally, though these results suggest that AmB can impact physiology, we have not yet determined whether this pharmacological approach can improve lung function in people with CF. A key requirement for efficacy via this strategy will be safely delivering a sufficient quantity of AmB directly to the airways of people with CF for sustained periods. In 31 studies, nebulized AmB has been extensively used to treat and prevent pulmonary fungal infections in more than 1,600 people, including use in people with neutropenia, allergic bronchopulmonary aspergillosis (ABPA), *Aspergillus* empyema, *Aspergillus*-related ulcerative tracheobronchitis, candidal anastomotic infection, bronchopulmonary candidiasis, endobronchial histoplasmosis, pulmonary zygomycosis, and as a prophylaxis following lung transplantation [10,11]. In some studies, inhaled AmB doses up to 100 mg/day were well tolerated for at least four days [11], and nebulized AmB as a prophylaxis following lung transplantation was safely continued for life in 412 people for up to five years [28].

The broad use of inhaled AmB as an effective antifungal therapy against *Aspergillus* infections as well as a study which determined that AmB concentrations of at least 3.25 μM were retained in the airways at least 14 days following a single administration

suggest that AmB concentrations of at least 1 μM have been safely achieved and sustained in the airways following inhalation [27]. In this study, we found that 1 μM AmB caused electrophysiological effects consistent with anion secretion in cultures of CuFi-1 epithelia, and we calculated that ASL AmB concentrations less than 1 μM similarly increased ASL pH in cultures of CuFi-1 epithelia maintained at an air-liquid interface. Further, we previously found that AmB increased transepithelial HCO_3^- secretion for at least seven days after a single apical addition in cultures of CuFi-1 epithelia [9]. These results further suggest the prospect of this pharmacological approach to benefit people with CF.

In addition to the extensive use of inhaled AmB to treat and prevent intrapulmonary fungal infections and as a prophylaxis following lung transplantation, preliminary case reports suggest that AmB has been safely delivered to the airways to address ABPA in 17 people with CF who had not received a lung transplant [29–33].

Several important steps are needed to enable future clinical studies of efficacy in people with CF. These include detailed toxicology, pharmacokinetic, and pharmacodynamic studies with inhaled AmB in animals, such as CF pigs [34,35]. These studies will enable determination of the safety and tolerability of inhaled AmB when administered chronically, and quantification of airway AmB concentrations following inhalation will be useful towards determining the appropriate dose range and optimal dosing frequency for translation to people with CF.

Alternative CFTR-independent pharmacological strategies which could in theory similarly benefit people with CF who cannot receive treatment with CFTR modulators include the use of ENaC inhibitors to increase epithelial fluid secretion and improve mucociliary clearance (MCC) [36]. However, this strategy has yet to achieve significant clinical benefit for people with CF. ENaC inhibitors such as amiloride and benzamil have suffered from limitations such as rapid clearance and thus short half-lives in the airways, while inhalation of GS-9411 caused hyperkalemia in a Phase I study which halted further clinical development [36]. In addition to small molecules which directly inhibit ENaC, camostat, an ENaC-activating protease inhibitor which increased MCC in guinea pigs and sheep, caused adverse effects and tolerability issues in a Phase II clinical trial [36]. This approach may also be limited due to the inability to restore Cl^- and HCO_3^- secretion, which play essential roles in mucus expansion and ASL antibacterial activity [34,37].

Small molecule potentiators of non-CFTR epithelial anion channels such as TMEM16A have the theoretical potential to similarly increase epithelial anion secretion independent of CFTR function [38]. Inhaled denufosol, a P2Y2 agonist which increases intracellular [Ca^{2+}] and thereby activates TMEM16A, failed to improve lung function in people with CF as assessed by FEV_1 in a Phase III clinical trial. The more recently developed small molecule TMEM16A potentiator ETX001 increased ASL height in primary cultures of CF airway epithelia and increased MCC in sheep treated with $CFTR_{inh\text{-}172}$ [38]. However, it has not been determined whether this approach can benefit people with CF. Notably, the physiological role of TMEM16A in CF pathophysiology remains unclear. In addition to increasing Cl^- secretion, activation of TMEM16A may increase inflammatory mediators and lead to excessive mucus secretion, thus impairing pulmonary function. These effects may limit the potential of this approach to benefit people with CF [39,40].

Unlike these approaches, small molecule channels have the potential to increase Cl^- and HCO_3^- secretion in people with CF independent of CFTR and thereby restore ASL physiology without disrupting overall ion homeostasis or causing non-specific deleterious effects. Further, AmB is an already FDA-approved drug. Given the encouraging safety profile of AmB when sustained in the airways at or above concentrations which form channels in CF airway epithelia, the findings presented in this work support additional studies to test whether inhaled AmB can improve pulmonary function and benefit people with CF caused by nonsense mutations.

Importantly, aerosolized delivery of small molecule channels directly to the airways will not address CF disease in other organs, such as distal intestinal obstruction syndrome (DIOS) in the gastrointestinal (GI) tract. This work encourages additional studies to determine whether AmB can increase anion secretion in cultures of CF intestinal epithelia and benefit people with DIOS.

Authors' contribution

R.S.C., B.R.K., J.L.L., M.J.W., and M.D.B. designed experiments and interpreted data. R.S.C., M.J.W., and M.D.B. wrote the manuscript. R.S.C. performed HCO_3^- and Cl^- efflux experiments from POPC liposomes, cultured CuFi-1 epithelia, measured HCO_3^- secretion in cultures of CuFi-1 epithelia, and measured ASL AmB concentrations in cultures of CuFi-1 epithelia. P.H.K. cultured primary epithelia. R.S.C. performed Ussing chamber experiments in cultures of CuFi-1 epithelia and primary cultures of CF airway epithelia. B.R.K. and J.L.L. performed NPD measurements in people with CF.

Declaration of Competing Interest

R.S.C., M.J.W., and M.D.B. are inventors on patent applications PCT/US15/58806, PCT/US18/55435, PCT/US2017/26806, and/or PCT/US20/18/055435, submitted by UIUC, which cover use of AmB to treat CF. cystetic Medicines, a company for which M.J.W. and M.D.B. are founders and consultants, has licensed these patent applications.

Acknowledgements

This work was supported by the National Institutes of Health (5R35GM118185 to M.D.B. and HL091842 to M.J.W.), and by Emily's Entourage. M.J.W. is an HHMI Investigator.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2020.11.018.

References

[1] Stoltz DA, Meyerholz DK, Welsh MJ. Origins of cystic fibrosis lung disease. N Engl J Med 2015;372(4) 351-62.
[2] Ratjen F, Bell SC, Rowe SM, Goss CH, Quittner AL, Bush A. Cystic fibrosis. Nat Rev Dis Primers 2015;1:15010.
[3] Cutting GR. Cystic fibrosis genetics: from molecular understanding to clinical application. Nat Rev Genet 2015;16(1):45–56.
[4] Ramsey BW, Davies J, McElvaney NG, Tullis E, Bell SC, Drevinek P, et al. A CFTR potentiator in patients with cystic fibrosis and the G551D mutation. N Engl J Med 2011;365(18) 1663-72.
[5] Rowe SM, Daines C, Ringshausen FC, Kerem E, Wilson J, Tullis E, et al. Tezacaftor-ivacaftor in residual-function heterozygotes with cystic fibrosis. N Engl J Med 2017;377(21) 2024-35.
[6] Keating D, Marigowda G, Burr L, Daines C, Mall MA, McKone EF, et al. VX-445-Tezacaftor-Ivacaftor in Patients with Cystic Fibrosis and One or Two Phe508del Alleles. N Engl J Med 2018;379(17) 1612-20.
[7] Quon BS, Rowe SM. New and emerging targeted therapies for cystic fibrosis. BMJ 2016;352:i859.
[8] Veit G, Avramescu RG, Chiang AN, Houck SA, Cai Z, Peters KW, et al. From CFTR biology toward combinatorial pharmacotherapy: expanded classification of cystic fibrosis mutations. Mol Biol Cell 2016;27(3):424–33.
[9] Muraglia KA, Chorghade RS, Kim BR, Tang XX, Shah VS, Grillo AS, et al. Small–molecule ion channels increase host defences in cystic fibrosis airway epithelia. Nature 2019;567(7748) 405-8.
[10] Xia D, Sun WK, Tan MM, Zhang M, Ding Y, Liu ZC, et al. Aerosolized amphotericin B as a prophylaxis for invasive pulmonary aspergillosis: a meta analysis. Int J Infect Dis 2015;30:78–84.

[11] Kuiper L, Ruijgrok EJ. A review on the clinical use of inhaled amphotericin B. J Aerosol Med Pulm Drug Deliv 2009;22(3) 213-7.
[12] Ebbens FA, Georgalas C, Luiten S, van Drunen CM, Badia L, Scadding GK, et al. The effect of topical amphotericin B on inflammatory markers in patients with chronic rhinosinusitis: a multicenter randomized controlled study. Laryngoscope 2009;119(2) 401-8.
[13] Weschta M, Rimek D, Formanek M, Podbielski A, Riechelmann H. Effect of nasal antifungal therapy on nasal cell activation markers in chronic rhinosinusitis. Arch Otolaryngol Head Neck Surg 2006;132(7):743–7.
[14] Ebbens FA, Scadding GK, Badia L, Hellings PW, Jorissen M, Mullol J, et al. Amphotericin B nasal lavages: not a solution for patients with chronic rhinosinusitis. J Allergy Clin Immunol 2006;118(5) 1149-56.
[15] Ponikau JU, Sherris DA, Weaver A, Kita H. Treatment of chronic rhinosinusitis with intranasal amphotericin B: a randomized, placebo-controlled, double-blind pilot trial. J Allergy Clin Immunol 2005;115(1) 125-31.
[16] Weschta M, Rimek D, Formanek M, Polzehl D, Podbielski A, Riechelmann H. Topical antifungal treatment of chronic rhinosinusitis with nasal polyps: a randomized, double-blind clinical trial. J Allergy Clin Immunol 2004;113(6) 1122-8.
[17] Ponikau JU, Sherris DA, Kita H, Kern EB. Intranasal antifungal treatment in 51 patients with chronic rhinosinusitis. J Allergy Clin Immunol 2002;110(6) 862-6.
[18] Van Goor F, Hadida S, Grootenhuis PD, Burton B, Cao D, Neuberger T, et al. Rescue of CF airway epithelial cell function *in vitro* by a CFTR potentiator. VX-770. Proc Natl Acad Sci USA 2009;106(44) 18825-30.
[19] Rowe SM, Clancy JP, Wilschanski M. Nasal potential difference measurements to assess CFTR ion channel activity. Methods Mol Biol 2011;741: 69–86.
[20] Accurso FJ, Rowe SM, Clancy JP, Boyle MP, Dunitz JM, Durie PR, et al. Effect of VX-770 in persons with cystic fibrosis and the G551D-CFTR mutation. N Engl J Med 2010;363(21):1991–2003.
[21] Chang EH, Tang XX, Shah VS, Launspach JL, Ernst SE, Hilkin B, et al. Medical reversal of chronic sinusitis in a cystic fibrosis patient with ivacaftor. Int Forum Allergy Rhinol 2015;5(2) 178-81.
[22] Adam RJ, Hisert KB, Dodd JD, Grogan B, Launspach JL, Barnes JK, et al. Acute administration of ivacaftor to people with cystic fibrosis and a G551D-CFTR mutation reveals smooth muscle abnormalities. JCI Insight 2016;1(4):e86183.
[23] Hisert KB, Heltshe SL, Pope C, Jorth P, Wu X, Edwards RM, et al. Restoring cystic fibrosis transmembrane conductance regulator function reduces airway bacteria and inflammation in people with cystic fibrosis and chronic lung infections. Am J Respir Crit Care Med 2017;195(12) 1617-28.
[24] Zabner J, Karp P, Seiler M, Phillips SL, Mitchell CJ, Saavedra M, et al. Development of cystic fibrosis and noncystic fibrosis airway cell lines. Am J Physiol Lung Cell Mol Physiol 2003;284(5) L844-54.
[25] Karp PH, Moninger TO, Weber SP, Nesselhauf TS, Launspach JL, Zabner J, et al. An *in vitro* model of differentiated human airway epithelia. Methods for establishing primary cultures. Methods Mol Biol. 2002;188 115-37.
[26] Rowe SM, Liu B, Hill A, Hathorne H, Cohen M, Beamer JR, et al. Optimizing nasal potential difference analysis for CFTR modulator development: assessment of ivacaftor in CF subjects with the G551D-CFTR mutation. PLoS ONE 2013;8(7):e66955.
[27] Monforte V, Ussetti P, Lopez R, Gavalda J, Bravo C, de Pablo A, et al. Nebulized liposomal amphotericin B prophylaxis for Aspergillus infection in lung transplantation: pharmacokinetics and safety. J Heart Lung Transplant 2009;28(2) 170-5.
[28] Peghin M, Monforte V, Martin-Gomez MT, Ruiz-Camps I, Berastegui C, Saez B, et al. 10 years of prophylaxis with nebulized liposomal amphotericin B and the changing epidemiology of Aspergillus spp. infection in lung transplantation. Transpl Int 2016;29(1):51–62.
[29] Casciaro R, Naselli A, Cresta F, Ros M, Castagnola E, Minicucci L. Role of nebulized amphotericin B in the management of allergic bronchopulmonary aspergillosis in cystic fibrosis: case report and review of literature. J Chemother 2015;27(5) 307-11.
[30] Hayes D Jr, Murphy BS, Lynch JE, Feola DJ. Aerosolized amphotericin for the treatment of allergic bronchopulmonary aspergillosis. Pediatr Pulmonol 2010;45(11) 1145-8.
[31] Tiddens HA, Pfaff SJ, Zanden Tvd, Ruijgrok EJ. Weekly nebulization of liposomal amphotericine B for the treatment of prednisone dependent ABPA. Pediatr Pulmonol 2003.
[32] Laoudi Y, Paolini JB, Grimfed A, Just J. Nebulised corticosteroid and amphotericin B: an alternative treatment for ABPA? Eur Respir J 2008;31(4) 908-9.
[33] Proesmans M, Vermeulen F, Vreys M, Boeck KD. Use of nebulized amphotericin B in the treatment of allergic bronchopulmonary aspergillosis in cystic fibrosis. Int J Pediatr 2010:2010.
[34] Pezzulo AA, Tang XX, Hoegger MJ, Abou Alaiwa MH, Ramachandran S, Moninger TO, et al. Reduced airway surface pH impairs bacterial killing in the porcine cystic fibrosis lung. Nature 2012;487(7405) 109-13.
[35] Cooney AL, Abou Alaiwa MH, Shah VS, Bouzek DC, Stroik MR, Powers LS, et al. Lentiviral-mediated phenotypic correction of cystic fibrosis pigs. JCI Insight 2016;1(14).
[36] Shei RJ, Peabody JE, Kaza N, Rowe SM. The epithelial sodium channel (ENaC) as a therapeutic target for cystic fibrosis. Curr Opin Pharmacol 2018;43 152-65.
[37] Hoegger MJ, Fischer AJ, McMenimen JD, Ostedgaard LS, Tucker AJ, Awadalla MA, et al. Impaired mucus detachment disrupts mucociliary transport in a piglet model of cystic fibrosis. Science 2014;345(6198) 818-22.
[38] Danahay HL, Lilley S, Fox R, Charlton H, Sabater J, Button B, et al. TMEM16A potentiation: a novel therapeutic approach for the treatment of cystic fibrosis. Am J Respir Crit Care Med 2020;201(8) 946-54.
[39] Cabrita I, Benedetto R, Schreiber R, Kunzelmann K. Niclosamide repurposed for the treatment of inflammatory airway disease. JCI Insight 2019;4(15).
[40] Kunzelmann K, Ousingsawat J, Cabrita I, Dousova T, Bahr A, Janda M, et al. TMEM16A in cystic fibrosis: activating or inhibiting? Front Pharmacol 2019;10:3.