ELECTRONIC SUPPLEMENTARY MATERIAL

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SUPPRESSION OF CFTR PREMATURE TERMINATION CODONS AND RESCUE OF CFTR PROTEIN AND FUNCTION BY THE SYNTHETIC AMINOGLYCOSIDE NB54

SUPPLEMENTARY MATERIALS AND METHODS

Growth of stable cell lines expressing recombinant CFTR. CFTR-W1282X cDNA were stably transfected into HeLa and CFBE41o- cells using the TranzVector^{TM} (Tranzyme, Inc., Birmingham, AL) as described (1). Expression of the *CFTR* gene was coupled to the puromycin-N-acetyltransferase gene (*puro*) gene, allowing selection of a stable pool of cells expressing *CFTR* by growth with puromycin (4 mg/ml). Cells were otherwise maintained in DMEM supplemented with 10% FBS, along with penicillin and streptomycin, as previously described (2).

Procurement and Growth of Primary Airway Epithelial Cells. The UAB Institutional Review Board approved use of human cells and tissues. Primary human airway epithelial cells were derived from lung explants of CF subjects who provided written informed consent and were confirmed to harbor 2 severe CFTR mutations by methods described previously (3, 4). Briefly, tissues were debrided immediately after surgical resection, washed twice in Minimum Essential Media with 0.5 mg/ml DTT (Sigma-Aldrich, St. Louis, MO) and 25 U/ml DNAase I (Roche, Basel, Switzerland), and then placed in dissociation media containing MEM, 2.5 U/mL DNAse I, 100 µg/ml ceftazidime, 80 µg/mL tobramycin, 1.25 µg/mL amphotericin B, and 4.4 U/mL pronase (Sigma-Aldrich) for 24-36 hrs at 4 C. Loosened airway epithelial cells were then expanded in growth media containing BEGM (LONZA, Basel, Switzerland) supplemented with an additional 10 nM all trans-retinoic acid (Sigma) that was exchanged every 24 hrs. Following expansion, first or second passage cells were seeded on permeable supports for studies.

Once cells were 80-90% confluent, they were seeded on Snapwell 1.13 cm^2 permeable supports (1 x 10^6 cells/filter; Bayer, Pittsburgh, PN) or Costar 0.4 µm permeable supports (5 x 10^5 cells/filter; Bethesda, MD) after coating with NIH 3T3 fibroblast conditioned media, and grown in differentiating media containing DMEM/F12 (Invitrogen, Carlsbad, California), 2% Ultroser-G (Pall, New York, NY), 2 % Fetal Clone II (Hyclone, Logan, UT), 2.5 µg/ml Insulin (Sigma), 0.25 % Bovine Brain Extract (LONZA), 20nM Hydrocortisone (Sigma-Aldrich), 500 nM Triodothyronine (Sigma), 2.5 µg/ml Transferrin (Invitrogen), 250 nM Ethanolamine (Sigma-Aldrich), 1.5 µM Epinephrine (Sigma-Aldrich), 250 nM Phosphoethanolamine (Sigma-Aldrich), and 10 nM Retinoic acid (Sigma-Aldrich) until terminally differentiated, as previously described (4, 5).

SPQ studies of halide efflux in HeLa cells. HeLa cells stably transfected with a lentiviral system carrying a *CFTR-W1282X* cDNA under CMV promoter control were studied with the halide quenched dye 6-methoxy-N-(3-sulfopropyl)-quinolinium (SPQ, Molecular Probes Inc., Eugene, OR) as previously described (2, 6, 7). Briefly, cells were seeded at ~ 5 x 10^5 cells/coverslip and grown in DMEM supplemented with 10% FBS at 37℃ for 24-48 hours. The media was changed, and cells were treated for 24 hours with aminoglycoside compounds or vehicle (500 µg/ml) in media at 37℃ and 5% CO2. On the day of the study, cells were loaded with hypotonic SPQ (10 mM) for 10 min, and then placed in a NaI buffer to quench cellular fluorescence. The cells were then placed in a specially designed perfusion chamber and studied at room temperature. Fluorescence of individual cells was measured using a Zeiss inverted microscope (Thornwood,

NY) with excitation at 340 nm, emission at >410 nm using a PTI imaging system (Monmouth Junction, NJ) and a Hamamatsu camera (Bridgewater, NJ). Baseline fluorescence was measured in isotonic NaI buffer, followed by perfusion with isotonic dequench buffer (NaNO3 replaced NaI) to measure unregulated halide efflux, and then NaNO3 buffer with CFTR agonists (20 μM forskolin and 50 μM genistein to fully activate CFTR activity). At the end of each experiment, cells were returned to the NaI buffer for requench (1100 sec). Increase in fluorescence above the basal (NaI quenched) level is shown (% increase F > basal). The data are cumulative from multiple coverslips in each condition studied in a paired fashion. To account for toxicity seen with aminoglycosides and inadequate loading of SPQ, the top 50% of cells from each coverslip are reported in summary data obtained from each condition, as previously described (6, 8).

Functional screen in IB3 and CFNPE monolayers using SPQ fluorescence. Methods were similar to the SPQ experiments in HeLa cells, with only minor differences as noted as described online. The principle advantage of the technique is that it allows a greater number of identical conditions to be tested compared to conventional SPQ measurements using the perfusion apparatus. Cells were grown in a 96-well (full area) microtiter plates according to a microtiter plate map design. Cells were loaded with SPQ (2 mg/ml in DMEM) overnight (18 hrs) concurrent with administration of test drugs. SPQ fluorescence following addition of $NaNO_3$ buffer was read across the microtiter plate within a Turner Designs Multimode Plate Reader, and results normalized to an untreated control.

NaCl Ringer was used instead of NaI Ringer to quench SPQ fluorescence in the initial and recovery phase of the assay. For each assay, results were expressed as the change in fluorescence over basal, as previously published [23, 29, 30]. Cell toxicity of aminoglycosides was monitored using the CellTiterGLO luminescent cell viability assay (Promega, Madison, WI).

Voltage clamp studies in Ussing chambers. Inserts were mounted in Ussing chambers, and short-circuit current (I_{SC}) was measured under voltage clamp conditions using MC8 voltage clamps and P2300 Ussing chambers (Physiologic Instruments, San Diego, CA) as previously described (5). Monolayers were initially bathed on both sides with identical Ringers solutions containing (in mM) 115 NaCl, 25 $NaHCO_3$, 2.4 KH_2PO_4, 1.24 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 D-glucose (pH 7.4). Bath solutions were vigorously stirred and gassed with 95%O_2:5% CO_2. Short-circuit current measurements were obtained using an epithelial voltage clamp (Physiologic Instruments). A three mV pulse of one second duration was imposed every 3 seconds to monitor resistance, which was calculated using Ohm's law. Where indicated, the mucosal bathing solution was changed to a low Cl^- solution containing (in mM) 1.2 NaCl, 115 Na gluconate, and all other components as above. Amiloride (100 μM) was added to block residual ENaC current, followed by the CFTR agonists forskolin and genistein, as indicated (minimum five minutes observation at each concentration). $CFTR_{Inh}$-172 (10μM) was added to the mucosal bathing solution at the end of experiments to block CFTR-dependent I_{SC}. All chambers were maintained at

37℃ and vigorously gassed with 5% CO_2/95% O_2, and agonist stimulation was initiated within 30 min of placement into the chambers.

Mouse lines and treatment protocols. The *CFTR-G542X* mice used in this study contained the *Cftr*^{tm1Cam} knockout (9) and expressed a human *CFTR* transgene with the G542X premature stop mutation (10-12) (referred to as *Cftr-/-hCFTR*-G542X mice). Treatments consisted of subcutaneous injections of gentamicin or NB54 delivered in the hind limb once daily for 14 days. Treatment was initiated 16 days after birth; mice were weaned 23 days after birth. All mice were maintained on a liquid diet (Peptamen® Complete Elemental Diet, Nestlé) after weaning to prevent intestinal blockage. The animal protocols used in this work were reviewed and approved by the UAB Institutional Animal Care and Use Committee.

Short-circuit current measurements. Transepithelial I_{SC} measurements were carried out using MC8 voltage clamps, P2300 Ussing chambers, and P2303 sliders (Physiologic Instruments, San Diego, CA) under conditions previously described (10-12). Forskolin (10 μM) was added to both the mucosal and serosal solutions and the I_{SC} was continuously monitored for ≥ 10 min to ensure that a sustained response was obtained. Carbachol (100 μM) was then added to the serosal solution and incubation was continued for an additional 5 min. Glybenclamide (200 μM) was then added to both solutions to block the forskolin-activated CFTR short-circuit current.

Immunohistochemical staining of murine intestinal tissues. Immunofluorescence experiments were carried out as previously described (10-

12). The human CFTR-specific antiserum #4562 (rabbit) was raised against a *TrpE* fusion protein that included hCFTR NBD1 and a portion of the R domain (hCFTR amino acids 521–828).

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