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Pharmacological Properties of 552-02, a Novel Epithelial Sodium Channel Blocker with Potential Clinical Efficacy for Cystic Fibrosis Lung Disease

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Running title: A Novel ENaC Blocker for CF Lung Disease

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The abbreviations used are: ANOVA, analysis of variance; ANCOVA, analysis of covariance; ASL, airway surface liquid; ALI, air-liquid interface; CBE, canine bronchial epithelia; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DMSO, dimethylsulphoxide; ENaC, epithelial sodium channel(s); ETT, endotracheal tube; HBE, human bronchial epithelia; HTBE, human tracheobronchial epithelia; HS, Hypertonic saline; I_{sc}, short-circuit current; KRB, Krebs-Ringer bicarbonate; LCMS, liquid chromatography mass spectrometry; K_{off}, Off rate constant; MC, mucociliary clearance; NHE, sodium-proton exchanger; P2Y2, purinergic receptor; R_t, transepithelial resistance;.

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Abstract

Amiloride improves mucociliary clearance (MC) by blocking airway epithelial sodium channels (ENaC) and expanding airway surface liquid (ASL). However, amiloride's low potency and rapid absorption by airway epithelia translated into a short duration of efficacy as an aerosolized therapy for cystic fibrosis (CF) patients. To improve ENaC blocker CF pharmacotherapy, a more potent and durable ENaC blocker tailored for aerosol delivery was synthesized. Parion 552-02 was tested for potency and reversibility of ENaC block, epithelial absorption and biotransformation, selectivity, durability of ASL expansion under isotonic and hypertonic conditions in canine and human CF bronchial epithelial cells, and drug dissociation on ENaC in *Xenopus* oocytes. Short-circuit current assessed compound potency and reversibility, patch-clamp recordings of ENaC current assessed drug off-rate (K_{off}), a gravimetric method and confocal microscopy measured mucosal water retention and ASL height, and drug absorption and biotransformation were assessed using LCMS. Amiloride and 552-02 were tested *in vivo* for MC activity in sheep immediately, and 4-6 hours after aerosol dosing. Compared to amiloride, compound 552-02 was 60 -100 fold more potent, 2-5 fold less reversible, slower at crossing the epithelium, and exhibited a 170 fold slower K_{off}. 552-02 exhibited greater ASL expansion over 8 h *in vitro,* and was more effective than amiloride at increasing MC immediately and 4-6 h post dosing. When combining hypertonic saline and 552-02, a synergistic effect on ASL expansion was measured in canine or CF bronchial epithelia. In summary, the preclinical data support the clinical utility of 552-02 +/-hypertonic saline for CF lung disease.

Introduction

The pulmonary disease in patients with cystic fibrosis (CF) reflects genetic mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that produce defective epithelial ion transport. The CF airway epithelial ion-transport abnormalities lead to a well described pathophysiological cascade that adversely affects the lung's innate defense mechanism. Impaired anion secretion by the CFTR channels and absence of CFTR inhibition on the epithelial sodium channel (ENaC) are major contributors towards the pathogenesis of CF (Boucher et al., 1988;Quinton, 1989;Rowe et al., 2005;Welsh, 1990). The CF ion-transport abnormalities lead to a decrease in hydration of the ASL (Matsui et al., 1998a). The loss of ASL volume/height degrades cilia function and causes mucus adhesion, which ultimately produces the chronic inflammation and infection that leads to a significant decline in pulmonary function (Boucher, 2007;Livraghi and Randell, 2007;Tarran et al., 2005).

Aerosol pharmacotherapies specifically targeting the ion transport defect in CF patients have been tested. These therapies include hypertonic saline (HS) (Donaldson et al., 2006;Elkins et al., 2006), amiloride (Pons et al., 2000), INS37217 (Deterding et al., 2005) and MOLI1901 (Grasemann et al., 2007). Clinical studies demonstrated that aerosolized HS (6 – 7% NaCl solution) in CF adults was safe, enhanced MC, decreased the number of pulmonary exacerbations, and improved pulmonary function when administered 2 to 4 times a day (Donaldson et al., 2006;Elkins et al., 2006). Recently, aerosolized HS (7% NaCl) was found to be safe and well tolerated in CF infants, supporting a preventative treatment strategy (Subbarao et al., 2007). The mechanism of action of aerosolized HS therapy reflects the direct addition of sodium and chloride ions to the airway surface, generating an osmotic gradient that expands the ASL and increases MC (Boucher, 2007;Donaldson et al., 2006;Hirsh, 2002;Tarran et al., 2007).

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The multi-dose regimen (2-4 times a day) in these studies was necessary to offset the rapid rate of salt and water absorption from the ASL in CF patients and produce the sustained increase in MC required for an overall improvement in pulmonary function.

Amiloride was designed as an orally-active potassium-sparing diuretic, but when administered as an aerosol to CF patients, the transient block of ENaC resulted in an increase in ASL volume and a short-acting enhancement of MC in CF patients (App et al., 1990;Hirsh et al., 2004;Kohler et al., 1986). To potentially improve the durability of HS in CF patients, a combination therapy of amiloride and HS was tested. Interestingly, the expected synergistic effect (combination > either therapy alone) was not found (Donaldson et al., 2006). The proposed mechanism for the amiloride block of the HS-induced increase in MC reflected amiloride's action (inhibition) on the aquaporin channels in the respiratory epithelium (Donaldson et al., 2006). This amiloride-sensitive aquaporin channel hypothesis was challenged by Levin 2006 (Levin et al., 2006) who reported amiloride had no effect on AQ-3, 4 and 5 in AQP-transfected Fisher rat thyroid cells, and in CF and normal bronchial epithelia cells. Regardless of the amiloride aquaporin controversy, previous reports are consistent with lack of amiloride selectivity for ENaC. For example, it has been reported that amiloride inhibits the sodium-proton exchanger (NHE) at higher concentrations (Kleyman and Cragoe, Jr., 1988;Repishti et al., 2001) and to a lesser extent the sodium-calcium exchanger (Antolini et al., 1993;Kleyman and Cragoe, Jr., 1988).

To improve ENaC blocker CF therapy, a novel chemical entity should more efficiently block ENaC, be more selective, maintain an increase in MC better than amiloride, and when combined with hypertonic saline (HS), outperform itself or HS alone. To design and synthesize an ENaC blocker with the aforementioned attributes, we modified the pyrazinoylguanadine structure of amiloride (Hirsh et al., 2006). A focused library of novel ENaC blocker compounds were tested for potency (IC_{50}) and for drug recovery

(reversibility of short-circuit current), identifying Parion compound '552-02' as a potential lead compound for CF lung disease (Hirsh et al., 2006).

In this study, we tested the hypotheses that a novel ENaC blocker, 552-02, is more efficacious and more selective on ENaC, promoting and maintaining an expansion in the ASL and increasing and maintaining elevated MC as an aerosolized therapy compared to amiloride. Further studies were performed to investigate whether compound 552-02 when used in combination with HS acted synergistically, expanding ASL volume to values greater than measured with 552-02 or HS alone.

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Methods

Cell Culture. Canine bronchial epithelial tissue used for primary culture was provided by Marshall Bio Resources (North Rose, NY) from animals undergoing scheduled sacrifice approved by the Veterinary staff to ensure the humane care and treatment of experimental animals. Human bronchial epithelial cells were provided by the Tissue Culture Core of the Cystic Fibrosis Center at UNC-CH under the auspices of protocols approved by the Institutional Committee on the Protection of the Rights of Human Subjects. Human bronchial tissue was harvested from excess donor lung tissue at the time of lung transplantation from a portion of the main stem or lobar bronchus. Protocols for primary human or canine bronchial epithelial culture are similar to previous described methods (Hirsh et al., 2004). Canine or human bronchi were incubated in MEM medium containing 0.1% protease (Sigma Type XIV) and 50 μg/mL DNase at 4 °C for a minimum of 24 h. Fetal bovine serum (10 %) was added to the medium and the epithelial layer scraped and rinsed to improve cell yield. Cells were then centrifuged for 5 minutes at 500 x g. Re-suspended cells were seeded at a density of 0.25 - 0.4 x 10^6 /cm^2 on 0.4 μm porous collagen coated (human placenta type VI Sigma) Snapwell™ or Transwell® (Corning Costar Corp., Cambridge, MA) membranes (1.13 and 4.7 cm^2) and maintained at an air-liquid interface in hormonally defined medium supplemented with penicillin and streptomyocin (Matsui et al., 1998b).

CBE and HBE Short-Circuit Current and Reversibility Measurements. Bronchial epithelial monolayers from 6 -14 day old cultures grown on permeable membrane supports were mounted in modified Ussing chambers (Physiologic Instruments Inc., San Diego, CA). All experiments were performed in Krebs-Ringer bicarbonate solution (KRB), at pH 7.4 containing: 140 mM Na^+, 120 mM Cl^-, 5.2 mM K^+, 1.2 mM Ca^{2+}, 1.2 mM

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Mg^{2+}, 2.4 mM HPO_4^{2+}, 0.4 mM $H_2PO_4^-$, 25 mM HCO_3^- and 5 mM glucose. The epithelium was bathed on both sides with warmed (37°C) KRB circulated by gas lift with 95% O_2 / 5% CO_2, maintaining the pH at 7.4. The transepithelial voltage was clamped to 0 mV, except for 0.2-s pulses (+5 mV) every 20 s to calculate transepithelial resistance (R_t). The short-circuit current (I_{sc}) and R_t were digitized and recorded on a computer. Data were acquired and analyzed using Acquire and Analysis (V. 1.2) software (Physiological Instruments). The 50% inhibition of I_{sc} concentration (IC_{50}) was calculated from apical drug additions ranging from 10^{-11} to 10^{-4} M (~ half log increments) and analyzed using nonlinear regression (Prism V.3, Graphpad software Inc) using the equation:

$$Y=Bottom + (Top-Bottom)/(1+10^{((LogEC50-X)*HillSlope)}) \quad \text{(Eqn.1)}$$

where X is the logarithm of concentration; Y is the response; Y starts at bottom of the curve and increases with a sigmoid shape (identical to the "four parameter logistic equation".) Stocks of ENaC blocker were dissolved in DMSO at a concentration of ~ 10 mM and stored at < - 10° C until use.

Following a full concentration-effect study, the percent recovery of I_{sc} from apical sodium channel blocker exposure was measured 3 min after completion of three mucosal bath replacements with KRB. The percent recovery was calculated as recovered current after the third wash divided by predrug I_{sc} x 100.

Drug off-rate kinetic measurements in *Xenopus* Oocytes. Oocytes were harvested from adult frogs with approved animal use protocols (IACUC, UNC-Chapel Hill). The oocytes follicular cell layer was removed enzymatically with collagenase and hyalurinidase (4 mg/mL Type 2; 1h, 23 ºC) in modified Barth's solution (MBS) containing (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 20 N-

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(2-hydroxy)piperazine-N'-2-ethanesulfonic acid (Hepes), pH 7.5 with NaOH. Following, oocytes were washed in 5 volume-equivalents of enzyme-free MBS solution containing 10 mM EGTA and 1% (w/v) bovine serum albumin to quench residual enzyme activity. Subsequently, oocytes were shrunken in hypertonic MBS solution (supplemented with 230 mM sucrose) for 0.5 - 1h and mature oocytes (stage V-VI) with clear separation of their plasma and vitelline cell membranes were visually selected and stored overnight in isotonic MBS solution. The selected oocytes were injected the next day with cRNAs of rat $\alpha\beta\gamma$ENaC subunits (0.3 ng each subunit in 50 nL/oocyte; Drummond, Nanojet) prepared from cDNA constructs (a gift from Dr. Bernard Rossier, Univ. Lausanne, Switzerland). Oocytes were used for patch-clamp analysis of drug off-rate kinetics 16 - 48h after injection of ENaC cRNAs. Patch-clamp measurements of ENaC-mediated currents were made only after the oocyte vitelline membrane was removed (forceps, Roboz #5) in hypertonic MBS solution (15-min). The patch-clamp bath solution contained (in mM): 110 Li-aspartate, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 Hepes, titrated to pH 7.35 with LiOH. The patch pipette was filled with solution containing (in mM): 75 Tris-aspartate, 17 NaCl, 3 MgATP, 0.2 Na_2GTP, 0.1 $CaCl_2$, 1 EGTA, 10 Hepes titrated to pH 7.35 with NaOH. Patch-clamp measurements of ENaC-mediate currents were recorded from outside-out macropatches using an EPC-7 patch-clamp amplifier (List, Darmstadt, Germany). Current was digitized at 1 kHz (16-bit, ITC, Instrutech, Long Island, NY) after low-pass filtering (0.1 kHz; -3 dB, Bessel) and acquired with PC running HEKA-PULSE acquisition software (Bruxton Corp, Seattle, WA). Patch-pipettes (borosilicate Warner Instruments, Hamden, CT) were fabricated from thin-walled glass using a 3-stage pull routine (DMZ Universal Puller, Zeitz Instruments, Germany). Pipette resistance with the indicated patch solutions was 7.6 ± 0.2 mΩ (mean ± SEM, n = 24). Currents were recorded at 0-mV, membrane potential (V_m), with flowing bath conditions. Drug washout

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was achieved with a Fast-step solution exchanger (Warner Instruments, Hamden CT). Exchange of bath solution at the patch was ~70-ms, (Caldwell et al., 2005). The V_m was not adjusted for an ~2-mV diffusion potential between pipette and bath solutions as previously reported (Caldwell et al., 2004). A Ag:AgCl electrode connected to the bath via a 3% agar bridge containing 1M KCl served as the ground electrode. Experiments were performed at ~23 °C.

Model fitting: Following drug washout from the bath, the time-dependent membrane current (I_{mem}) in outside-out macropatches was fitted with a single exponential kinetic model for analysis of drug off-rates:

$$I_{mem} = I_{ss} - I_{wash} e^{-t/\tau\,wash} \qquad \text{(Eqn. 2)}$$

Where I_{wash} is the time-dependent drug-sensitive Na^+-current and I_{ss} is the steady-state current achieved following drug washout. The time course of drug washout is described with time constant, τ_{wash}. I_{mem} was fitted beginning ~100 ms after switching to drug-free patch superfusion solution. Fit parameters were first adjusted manually and then automatically for convergence by minimization of least-squares using the Levenburg-Marquardt algorithm (Table Curve 2D).

The drug off-rate constant k_{off} (s^{-1}) was calculated as the inverse of the fitted time constant:

$$\tau_{wash} = 1/k_{off} \qquad \text{(Eqn. 3)}$$

The fractional current recovery (frct rcvry) after drug washout was calculated from measured steady-state current before (I_{ss} pre-drug) and 10 – 120-s after drug exposure (I_{ss} post-drug) as shown:

frct rcvry = I_{ss} post-drug/ I_{ss} pre-drug (Eqn. 4)

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Sodium Channel Absorption and Biotransformation by Human Tracheobronchial Epithelial Cells (HTBE). The rate of serosal appearance of amiloride and 552-02 (both 100 μM) by HTBE (MatTek Corp., MA) was measured after administering ~ 200 μL/cm^2 drug to the apical surface. The rate of serosal appearance of drug was measured using cultures grown at an ALI for no less than 24 days on 0.4 μm, 30 mm, PTFE Millicells (Millipore Corp., Billerica, MA) using hormonally defined media (MatTek Corp., MA). The average R_t prior to the start of the experiment was 422 Ωcm^2. Samples (250 μL) were collected from the serosal compartment over 240 min and applied to a 4.6 mm x 150 mm I.D, dC18, 5 μm Atlantis column (Waters Corp., Milford, MA) maintained at 40°C. Elution of sodium channel blockers was achieved with a 12-min, mobile phase consisting of a 0.02% trifloroacetic acid pH = 3.0: 0.02% trifloroacetic acid acetonitrile, linear gradient (95/5 – 56/64); at a flow rate of 1.5 mL/min. The column was monitored by an on-line fluorescence detector 474 (λ = 362 nm, ex; 412 nm, em) and a mass spectrometer micromass ZQ (Waters Corp., Milford, MA). Concentrations are determined by measuring intrinsic fluorescence of each compound using HPLC. Quantitative analysis employs a 7 point standard curve generated from authentic reference standard materials of known concentration and purity.

Sodium-Proton Exchange Assay. A Chinese hamster lung fibroblast cell line (PS120), lacking all endogenous Na^+/H^+ exchangers, was stably transfected using Lipofectin (GIBCOBRL) with human NHE1 or NHE2 or NHE3. Cells were grown in medium (DMEM) supplemented with 25 mM $NaHCO_3$, 10 mM HEPES, pH 7.4, 50IU/ml penicillin, 50 mg/ml streptomycin, 10% fetal bovine serum (FBS), and 800 mg/ml G418 in a 5% CO_2-95% O2 incubator at 37°C, and grown on glass coverslips until they reached 50–70% confluency. The cells were loaded with the acetoxymethyl ester 29,79-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF-AM; 5 mM) in Na1 medium (in mM: 130

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NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 25 glucose, and 20 HEPES, pH 7.4) for 20 min at 22°C and then washed with TMA1 medium (in mM: 130 tetramethylammonium chloride, 5 KCl, 2 $CaCl_2$, 1 $MgSO_4$, 1 NaH_2PO_4, 25 glucose, and 20 HEPES, pH 7.4) to remove the extracellular dye. The coverslip was mounted at an angle of 60° in a 100 μL fluorometer cuvette designed for perfusion and maintained at 37°C. The cells were pulsed with 40 mM NH_4Cl in TMA1 medium for 3 min, with or without 100 μM amiloride or 552-02 followed by TMA1 medium, which resulted in the acidification of the cells. Na1 medium was then added, which induced alkalinization of the cells. The Na^+/H^+ exchange rates (H^+ efflux) were calculated, as the product of Na^+-dependent change in pHi and the buffering capacity at each pHi and were analyzed with the use of a nonlinear regression data analysis program (Origin V 6.0) and fitted using a general allosteric model described by the Hill equation:

$$V = V_{max} * [H^+]^n / (k^n + [H^+]^n) \quad \text{(Eqn. 5)}$$

where *[H+]* is proton concentration calculated from $pH = -\log[H^+]$, *V* is velocity, *K* is affinity constant, and *n* is apparent Hill coefficient, and V_{max} is the maximum velocity.

Animal Preparation for MC Studies. All procedures were approved by the Mount Sinai Animal Research Committee to ensure the humane care and treatment of experimental animals. The methods were similar to (Hirsh et al., 2004). Briefly, Adult ewes (approx. 40 kg) were restrained in an upright position. The animals' heads were immobilized and the nasal passages anesthetized with 2% lidocaine. The animals were nasally intubated with a 7.5 mm internal diameter ETT. The cuff of the ETT was placed just below the vocal cords and its position was verified by bronchoscopic visualization. After intubation, the animals were allowed to equilibrate for approximately 20 minutes prior to initiating MC measurements.

MC Measurement. Test articles, either 4 mL of H_2O (vehicle), 4 mL Amiloride (3 mM) or 4 mL 552-02 (3 mM), were aerosolized using the Pari LC JetPlus nebulizer to free-breathing sheep. The nebulizer had a flow rate of 8 L/min and the time to deliver the nebulized test article solution was 10 – 15 min. Depending on the interval of pharmacologic action being tested (immediate or long-acting), the radio-label ^{99m}Technicium-sulfur colloid (^{99m}T SC) (3.1 mg/mL; containing approximately 20 mCi) was administered immediately after test article (T=0 format) or 4 hours after test article (T=4 format). The radio-label was aerosolozied using a Raindrop Nebulizer that produced a droplet with a mass median aerodynamic diameter of 3.6 µm. The nebulizer was connected to a dosimetry system consisting of a solenoid valve and a source of compressed air (20 pounds per square inch). The output of the nebulizer was directed into a plastic T connector, one end of which was connected to the ETT, the other was connected to a respirator. The system was activated for one second at the onset of the respirator's inspiratory cycle. The respirator was set at a tidal volume of 300 mL, with an inspiratory to expiratory ratio of 1:1, and a rate of 20 breaths/min. Radiolabeled aerosol was administered for ~ 5 minutes. The sheep were then extubated, and data capture by gamma camera was initiated. This first time point established the baseline deposition image and was assigned the value of 0% clearance. Total radioactive counts in the lung were collected every 5 minutes during the 1-hour observation periods and every 15 min thereafter. A washout period of at least 7 days (half life of ^{99m}TC = 6 h) separated studies for each animal.

Canine Airway Surface Liquid Drug Durability Assay. Using a gravimetric procedure, mucosal surface liquid mass was measured on canine bronchial cells that had been grown at ALI culture on 12 mm PTFE transwells (Corning) for a minimum of 6 days. At the start of the experiment, each transwell insert was removed from the

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transwell plate (lower compartment), blotted dry, weighed, and 50 µL of vehicle (0.1% DMSO), ENaC blocker (10 µM), HS (1.5%), or 1.5% HS with 552-02 (10 µM) (pre and post HS), was applied to the mucosal surface and the change in mass recorded. After weighing, the cells were immediately returned to a transwell plate (lower chamber, 500 µL, KRB, pH 7.4) and placed in a 37 º C, 5% CO_2 incubator. Experiments were performed in KRB at 37 º C. To reduce artifact due to an apical carbohydrate osmotic gradient formed during volume loss, glucose was not included in the apical KRB. The mass of mucosal surface liquid was monitored serially over 8 hours. The mass of surface liquid was converted to volume in µL. Data are reported as % initial volume (100 % = 50 µL).

The Effect of 552-02 on Human CF Bronchial Epithelial ASL Height Measurements Under Static and Sheer Stress Conditions. Primary human CF bronchiolar epithelia were plated on 12 mm T-clear inserts coated with human placental collagen and grown at an ALI. For all experiments cells were allowed to differentiate for a minimum of 3 weeks and were highly ciliated. Cultures that had an R_t < 200 Ωcm^2 were not included in this study. To study the effect of sheer-stress, a mechanical device imposing *in vivo*-like forces directly to the respiratory epithelium (phasic motion (0.5 dynes/cm2) was applied 30 min prior to measuring ASL height and for 4 hours after the initial measurement. To apply sheer stress to the cultures, the culture plates housing the cells were phasically rotating in the incubator to deliver a phasic shear-stress of 0.5 dynes/cm^2 (Tarran et al., 2005). The ASL was labeled by adding 10 µL of FITC-dextran (2 mg/mL) in Hanks Balanced Salt Solution (HBSS) for 1 minute, following which the culture was placed in an incubator at 37ºC to re-equilibrate for 1 hour. To prevent ASL dehydration, 100 µL of perflorocarbon (PFC, Fluorinert FC-77 from 3M) was added to the apical surface.

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For fluorescent microscopy, cultures were removed from the incubator and placed on a glass coverslip with a basolateral volume of 200 µL HBSS containing 10 mM glucose and 10 mM HEPES, pH 7.4. The ASL height was measured by an X-Z scanning confocal microscope (Leica Microsystems). Based on previous observations, basal ASL volumes were estimated to be between 1-10 µL, for all experiments, we assumed the resting ASL volume to be 5 µL. Both compound 552-02 and/or 0.2 mg NaCl were added to the apical membrane as dry powder in PFC to yield approximate concentrations of 10 µM for 552-02 and 4 % hypertonic saline.

To minimize variability, the ASL height measurements were made in five separate locations. The measurements were averaged and the standard deviation calculatedThe average ASL height for each image was determined using Image J software version 1.36b (Rasband, W.S., U. S. National Institutes of Health, Bethesda, MD).

Materials. Racemic compound 552-02 methanesulfonate was synthesized by Albany Molecular Research Inc. (Albany, NY) (Hirsh et al., 2006), cell culture media, bovine serum albumin, fetal bovine serum, bovine pituitary extract, epidermal growth factor, penicillin, retenoic acid, DNase, human placenta collagen VI, streptomyocin, amiloride (Midamor®), and lidocaine were purchased from Sigma-Aldrich (St. Louis, MO). The ^{99m}Tc-SC was purchased from Mallinckrodt Medical Inc. (St. Louis, MO). Salts and solvents were of analytical or HPLC grade and purchased from VWR International.

Statistical Analysis. All values are depicted as the mean $\pm$ S.D unless otherwise specified. Data from *in vitro* assays (potency, reversibility comparisons of k_{off} and frct rcvry were tested for significance (*indicates a P <0.05) using a paired t-test GraphPad Prism (V 4.0) and SigmaStat (v 2.03). Amiloride off-rate analysis was performed before or after exposure to 552-02. A one-way ANOVA followed by a Dunnet's post hoc analysis was used to test for significance for Na^+/H^+ exchanger activity. A two-way ANOVA with repeated measures followed by a bonferroni post hoc analysis was used to

test for significance for surface liquid volume ASL height with more than two treatments groups. Ovine MC versus time data (0-1 and 4 -6 hour) was fitted using a linear regression model and the slopes of each line were compared using an ANCOVA, for the immediate MC activity. For the durable MC format (T=4), due to unequal time collection from 5-6 h in some of the animals, significance difference between amiloride and 552-02 was determined at specific time points using a paired t tests. Non-linear, linear regression, and statistical analysis were performed using the program GraphPad Prism (V 4.0).

Results

ENaC Blocker Potency, Maximal Efficacy, and Reversibility. Potency, maximal efficacy, and reversibility of the novel sodium channel blocker 552-02 on ENaC in CBE and HBE were measured and compared to the prototypic ENaC blocker, amiloride (Figure 1 and Table1). The differences in the aforementioned properties of the two ENaC blockers were determined by recording the change in I_{sc} in response to increasing concentrations of drugs (10^{-11} to 10^{-4} M) in the apical bath and after apical wash. Active sodium transport was the dominant component of I_{sc} in CBE and HBE (Figures 2 A and B, respectively), as evidenced by the observation that the maximal effective concentrations of selected blockers decreased the I_{sc} (> 96 %) from the baseline value. The shape of the concentration-effect curves for amiloride and 552-02 were sigmoidal and similar to a drug-receptor binding complex curve exhibiting saturable binding. The calculated IC_{50} and % I_{sc} recovery (reversibility of drug to binding site complex) from maximal block after a full concentration-effect response of 552-02 and amiloride are shown in Table 1.

Compound 552-02 was two orders of magnitude more potent (100 fold) than amiloride in blocking epithelial sodium channel generated I_{sc} in CBE and approximately 60 fold more potent in normal HBE. The significant difference in IC_{50} between the two ENaC blockers suggest that 552-02 is accessing additional auxiliary binding sites in the channel pore, which would provide for a more stable drug-channel interaction (Hirsh et al., 2006).

To calculate the reversibility of I_{sc} after full-block, the apical compartment was washed three times and the I_{sc} recorded for 15 min thereafter. Compound 552-02 was less reversible compared to amiloride for CBE (19 vs. 90 % recovery, respectively), and 38 vs 90 % of basal I_{sc} recovered in HBE (Table 1). If a more vigorous wash-out were

applied (more volume exchanges or longer duration), the I_{sc} from 552-02 would fully recover to values which are similar to what was measured after washout from amiloride induced block (Table 1).

Using this protocol, no significant decay in basal current was noted over the duration of exposure or recovery intervals for control (untreated) tissues.

Off-Rate Kinetics and I_{Na} Recovery. To assess if the slow recovery of I_{sc} following 552-02 washout could be explained by slow dissociation of the drug from ENaC, patch clamp recordings from *Xenopus* oocytes injected with cRNAs of rat αβγ-ENaC subunits were utilized to measure kinetics of drug dissociation from the channel. Figure 3 (panel A) depicts a current record showing baseline current and drug-induced inhibition of I_{Na}. Bath superfusion of 552-02 (1 μM) reversibly inhibited I_{Na}. Rapid switching to drug-free patch superfusion solution resulted in a slow (τ_{wash} = 26.8 s), near-complete recovery of the 552-02-inhibited current as shown (Figure 3 A). Subsequently, a saturating amiloride concentration (10 μM) was applied and inhibited I_{Na} to the same extent as 552-02 (Figure 3 A). Amiloride washout (τ_{wash} = 236 ms; inset) resulted in a rapid and complete recovery of I_{Na}. In Figure 3 panel B, summary data for multiple experiments revealed an approximate 170 fold slower off rate for 552-02 than amiloride. The off-rate constant (k_{off}) for 552-02 was 0.039 $\pm$ 0.007 s^{-1}, whereas the k_{off} for amiloride was 6.57 $\pm$ 0.98 s^{-1}, reflecting a more stable 552-02-channel complex. Summary data for the fractional I_{Na} recovery after drug washout is shown in Figure 3 panel C. Like amiloride, 552-02 is a reversible blocker.

Drug absorption by HTBE. The retention of ENaC blockers in the ASL is a property that contributes to the durability of a pharmacological effect. The rate of serosal appearance of amiloride or 552-02 using HTBE cultures was measured after

administering 100 μM of test article to the apical surface. Compound 552-02 was absorbed by the HTBE at a significantly ($P<0.05$) slower rate compared to amiloride (Figure 4). This finding was also confirmed using a starting concentration of 10 μM of test article on CBE (data not shown). Furthermore, no significant (< 5 %) biotransformation of either amiloride or 552-02 by HTBE cultures was detected on the mucosal or serosal side with LCMS. The slower rate of 552-02 absorption was consistent over the duration of the study.

Na^+/H^+ exchange activity. To test the effect of amiloride or 552-02 on the NHE, human NHE 1, 2 and 3 activity was monitored over a wide pH range. Amiloride (100 μM) significantly blocked NHE 1 and 2 activity as represented by an ~ 90 % decrease in the V_{max} compared to control, whereas 552-02 (100 μM) only decreased NHE 1 V_{max} by 27% and had no affect on NHE 2 activity (Figure 5 A and B). 552-02 (100 uM) produced a decrease (27 %) on NHE3 V_{max} compared to control, whereas amiloride had no effect.

Ovine MC. MC was expressed as the percent of inhaled radiolabel ^{99}Tc-SC cleared from a central region of the right lung over time. To test the immediate action of ENaC blockers on MC, the radiolabel was administered immediately following vehicle or drug aerosolization, and the clearance of radiolabel was monitored for 60 min thereafter (the T=0 format Figure 6, panel A). The clearance versus time data for test articles (vehicle, amiloride, and 552-02) were fitted using a linear regression analysis and the slope of the fitted line for each test article calculated. Aerosol dosing of vehicle ($H_2O_{sterile}$; 4 mL), or equimolar concentrations (3 mM ; 4 mL) of amiloride, or 552-02 all were associated with positive slope values: 12.8, 26.0, and 43.3 respectively (Figure 6A). The difference between the 3 slopes were analyzed using ANCOVA and were found to be significantly different ($P<0.0001$). Amiloride approximately doubled the slope (26.0) as compared to vehicle control ($P<0.05$), similar to previous reported data (converted to retention) (Hirsh

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et al., 2004). Compound 552-02 showed the greatest MC efficacy (a slope of 43.3 reflecting a > 40% particle clearance) and was significantly ($P<0.05$) better at enhancing MC than amiloride (Figure 6A).

To test the durability of drug activity on enhancing MC, test articles were first administered by aerosol and four hours later the radiolabel ^{99}Tc-SC was administered (T=4 format). MC was monitored for 2 hours thereafter, yielding a total duration of action test of 6 hours. MC measured 4 hours after vehicle administration was very slow (Figure 6B), with a slope equal to 1.8, that was significantly different than t=0 vehicle slope. This observation suggested that the vehicle was active on MC immediately after dosing. Furthermore, the MC after amiloride delivery was not significantly different than vehicle (Figure 6B). Compound 552-02, however, produced a persistent acceleration of MC when measured 4 hours post delivery (slope = 12.3), which was significantly different compared to either vehicle or amiloride ($P<0.05$).

Durability of 552-02 activity with or without hypertonic saline on surface liquid retention in CBE. To test the *in vitro* durability of ENaC blockers on ASL volume, CBE cells were used and the change in surface liquid volume over 8 h was measured (gravimetrically). A 50 μL volume was administered to the surface that consisted of KRB (control with 0.1% DMSO) with or without 552-02 (10 μM) or amiloride (10 μM). The CBE absorbed > 80 % of the delivered amount of buffer over 8 h (Figure 7A). Amiloride (10 μM) did not significantly slow the rate of absorption compared to vehicle (Figure 7A). In contrast, 552-02 (10 μM) significantly inhibited the rate of absorption, with approximately 75% of the initial volume retained on the CBE surface at 8h (Figure 7A).

Using the same protocol, administration of 1.5 % HS produced an immediate 'secretion' (20% increase in surface liquid volume) which persisted for 2 h, followed by rapid volume loss (rate of fluid loss -4.2 $\pm$ 0.7 μL/cm^2/h) (Figure 7B). In contrast,

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administration of equivolume of buffer (vehicle) resulted in a monotonic absorption of fluid (-3.1 μL/cm^2/h), 552-02 (10 μM) significantly decreased absorption of isotonic buffer (-1.3 μL/cm^2/h). Importantly, combining 552-02 (10 μM) and 1.5% HS, produced an immediate and sustained increase in surface liquid volume. The net fluid movement for the combination over the entire experimental interval was secretory (+0.7 $\pm$ 1.0 μL/cm^2/h), which was significantly different compared to 1.5% HS (-4.2 μL/cm^2/h) and 552-02 alone (-1.3 μL/cm^2/h) (Figure 7B).

To test whether 552-02 nonselectively blocks aquaporin channels as reported for amiloride (Donaldson et al., 2006), 552-02 (10 μM) was added before or after administration of 1.5 % HS. Both 552-02 (10 μM) prior to 1.5 % HS or post 1.5 % HS produced an ~ 35% increase in surface liquid volume at 8 h compared to the initial starting volume 50 μL (Figure 7C). This increase was significantly different from control (< 15%) ($P<0.0001$), was 70% greater than 10μM 552-02 alone (Figure. 7B), and was 80% greater than HS alone (Figure 7B). These data suggest that, unlike amiloride, 552-02 does not inhibit water flow.

Effect of sheer stress with and without 552-02 on ASL height in CF HBE. To test whether 552-02 only blocks liquid absorption, or whether it can also induce liquid secretion, we measured the effects of compound 552-02 on ASL height (volume) under thin-film conditions (Tarran and Boucher, 2002). Cultures utilized for these experiments were maintained under standard static conditions or under phasic-motion conditions that possibly more closely mimic the *in vivo* condition (Tarran et al., 2005). CF bronchial epithelial cell ASL height was measured using confocal microscopy in the X-Z plane. Images of the ASL height (green in color) for cultures maintained under static or under phasic-motion (sheer stress) conditions, with or without 10 μM 552-02, are shown in Figure 8 panel A. Under static conditions, ~ 10 μM 552-02 maintained ASL height at 5

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μm but did not increase ASL height compared to control after 4 hours (Figure 8 panel B). In CF cultures maintained under phasic motion conditions without drug, ASL height was significantly greater ($P<0.05$) when compared to cells under static conditions. Importantly, the ASL height in CF cultures maintained under phasic-motion treated with 552-02 was significantly increased compared to control cultures under phasic-motion, consistent with ASL secretion induced by 552-02.

Effect of hypertonic saline with or without 552-02 (pre and post addition) on ASL height in CF HBE. To test if hypertonic saline and compound 552-02 act synergistically on CF bronchial cell ASL height (volume), and to determine if compound 552-02 selectively blocks ENaC and not aquaporin channels in CF airway epithelia, 552-02 (~10 μM) was added to the apical surface pre and post apical administration of ~ 0.2 mg NaCl as a mimic of HS (Figure 9). The change in ASL height was measured in the X-Z plane using confocal microscopy for 240 min. Representative confocal images of ASL height for each treatment are shown in Figure 9 A. Figure 9 B displays summary data for these experiments. ASL height remained constant without salt addition over 4 hours. Adding 0.2 mg of NaCl to the surface of the epithelia caused an immediate eight fold increase in ASL height compared to control. However, the significant ASL expansion decreased steadily to a value not significantly different from control by 4 h. To determine if 552-02 enhanced the osmotic effect of HS, we tested the effects of 0.2 mg NaCl in combination with 552-02 (~10 μM) administered pre or post NaCl. In both cases, an immediate increase in ASL height was produced using the combination therapy, with a slower decline in the loss of ASL height over the time-course (Figure 9B). The greatest effect was observed with 552-02 delivered first, followed by NaCl (11 fold increase in ASL height after 10 min compared to control.) The overall time course experiments indicated that the predose 552-02/NaCl combination had the most significant effect, not only

causing the largest increase but also in maintaining the expansion of ASL. The observation that 552 pretreatment was most effective in adding to the osmotic action of HS again demonstrates that 552-02 did not non-specifically block osmotically-driven water flow like amiloride.

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Discussion

Emerging evidence suggests that CF lung pathophysiology is linked to dehydration of airway surfaces caused by epithelial ion transport defects (Baconnais et al., 2005;Boucher, 2007;Donaldson et al., 2007). One pharmacologic approach to rehydrate airway surfaces is aerosol ENaC blocker therapy. The ENaC blocker standard for CF pharmacotherapy has been amiloride, an orally active potassium-sparing diuretic. Although exerting short-term improvements in MC or lung function (App et al., 1990;Knowles et al., 1990;Kohler et al., 1986), it has not consistently improved overall pulmonary function when used with current complex medical regimens (Burrows et al., 2006;Graham et al., 1993;Pons et al., 2000). A review of ENaC blocker CF pharmacotherapy suggested a lack of respiratory benefits with amiloride (Burrows et al., 2006). Importantly, amiloride pre-administered prior to HS aerosol therapy was not effective (Donaldson et al., 2006).

Compound 552-02 was specifically designed for aerosol delivery to the pulmonary system as a more selective, potent, long-acting ENaC blocker to promote an expansion in ASL volume, with or without hypertonic saline. Compound 552-02; *N*-(3,5-diamino-6-chloropyrazine-2-carbonyl)-*N'*-4-[4-(2,3-dihydroxypropoxy)phenyl]butyl-guanidine methanesulfonate (Figure 1B), was selected from a series of novel ENaC blockers to produce a selective block on airway epithelial sodium channels with a potency up to two orders of magnitude greater than amiloride (Hirsh et al., 2006). To overcome the *in vivo* shortcomings in CF using amiloride (Hirsh et al., 2004;Hofmann et al., 1997), a selection criteria for ENaC blockers was formed: greater intrinsic-activity at ENaC (lower IC_{50}), less reversibility (much slower K_{off}), a decrease in drug absorption, maintained expansion of ASL, and compatibility with HS therapy.

In this study, we demonstrated that 552-02 blocked the majority (> 95%) of I_{sc} with a calculated IC_{50} of ~ 7 nM (Table 1). The increase in potency of 552-02 compared to amiloride provides greater efficacy, a critical consideration for drugs delivered via aerosol. The higher

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IC_{50} value reported in this study for amiloride is within the calculated standard deviation for amiloride for human airway epithelium compared to past studies (Hirsh et al., 2004). The greater potency of 552-02 is likely a function of more efficient binding of the molecule associated in the pre-M2 region (Kashlan et al., 2005;Kellenberger et al., 2003;Schild et al., 1997) to possibly three auxiliary binding sites in the vicinity of the proposed site for amiloride (Hirsh et al., 2006). The lower % recovery after washing (Table 1) and more specifically, the 170 fold slower K_{off} for 552-02 measured in patch clamp studies (Figure 3), are congruent with a tighter 552-02/channel complex compared to amiloride.

An important ENaC blocker criterion is slower transepithelial drug absorption. In this study using human airway epithelia, penetration of 552-02 into the serosal compartment was significantly slower than amiloride (Figure 4), consistent with a slower transepithelial absorption.

To test if 552-02 is more selective for ENaC in contrast to amiloride, Na+/H+ exchanger activity was measured in the presence and absence of 552-02 or amiloride. Although 552-02 contains the amiloride structure, 552-02 diminished the effect on NHE 1 V_{max} by ~ 27%, and was inactive on NHE 2 activity compared to 90 % by amiloride. Also, 552-02 decreased the V_{max} by 27 % on NHE 3, whereas amiloride had no effect. The reduced pharmacological activity of 552-02 on the NHEs and the predicted maximum human plasma concentration of 552-02 from aerosol delivery of ~ 3.5 nM (Doran et al. 2006) predict a minimal block of 552-02 on NHE activity.

To investigate whether more potent, less reversible, and less permeant ENaC blockers are more effective in accelerating MC than amiloride, a whole animal sheep model was employed to compare the effects of equimolar concentrations (3 mM) of amiloride or 552-02. An increase in MC in this assay reflects an increase in ASL volume (Mentz et al., 1986) and was used to test the assumption that ENaC blockers increase ASL volume under physiologic, phasic respiratory motion conditions. Compound 552-02 significantly outperformed amiloride when assayed for acute effects (Figure 6A), with clearance rates closely resembling values obtained in human

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patients with genetically complete absence of ENaC function (pseudohypoaldosteronism) (Kerem et al., 1999).

To test whether 552-02 was more durable than amiloride *in vivo*, MC was measured 4 to 6 h post drug delivery. No differences were measured in MC over this interval between control and amiloride; however, an acceleration of MC was maintained in animals exposed to 552-02, with clearance rates significantly greater than amiloride over the 4 – 6 hour interval (Figure 6B). These *in vivo* data strongly support the hypothesis that by blocking epithelial sodium channels with potent, less reversible, less permeant compounds, a sustained increase in MC can be obtained. Studies in CF patients will be required to assess what duration of effect on MC is required for clinical benefit.

To test whether beneficial effects are achievable when combining HS and 552-02, ASL volume retention and ASL height measurements were performed in CBE (Figure 7), and CF bronchial airway epithelia (Figure 9). After adding Krebs buffer to CBE, the buffer was rapidly absorbed by the epithelium (Figure 7A). Comparing effects of equimolar concentrations of ENaC blockers on the retention of surface liquid by CBE, 552-02 was far superior at maintaining surface volume compared to amiloride. Compared to control, equivolume HS (1.5%) additions to CBE surfaces produced an initial ASL volume expansion (osmotically driven secretion) during the first two hours (figure 7B). However, once the surface liquid became isotonic, fluid absorption dominated (-4.2 $\mu L/cm^2/h$) (Figure 7B). Also, when HS was combined with 552-02, pre or post HS treatment, virtually all the buffer and osmotically driven secreted liquid was retained on CBE surfaces for the duration of the assay (8 h). These data support the hypothesis that a greater more durable MC effect might be measured using a combination therapy consisting of 552-02 and HS.

To investigate whether ENaC blockers alone, and particularly 552-02, could not only conserve ASL volume on airway surfaces, but also induce ASL volume secretion measurement of ASL volume under thin-film conditions were performed on CF bronchial epithelia. Previous

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studies using confocal imaging indicated that normal ASL volume homeostasis in cell cultures under static conditions is maintained in part by the accumulation of adenosine in the ASL (Lazarowski et al., 2004). CF airway epithelia cannot maintain ASL height consistent with normal epithelial cultures under static conditions, due to the absence of CFTR. In contrast, phasic motion-induced shear-stress produced sufficient ATP onto the surface of the epithelia to raise the ASL ATP concentration that activates the purinergic receptor (P2Y2), activating a calcium activated chloride channel and partially inhibiting ENaC, to produce volume secretion. Consistent with these reports, CF cultures under static conditions could not maintain adequate ASL height (5 μm) for MC. Furthermore, the addition of 552-02 to the ASL of CF cultures under static conditions did not increase ASL height (Figure 8). However, ASL height in CF cultures under phasic motion conditions was significantly raised, consistent with the actions of elevated ATP concentrations in the phasic motion cultures. As predicted, the addition of 552-02 to the CF culture under phasic motion conditions produced a larger anion induced ASL volume secretion than observed with no 552-02 treatment (Figure 8). These data suggest that ENaC block under physiologic conditions not only conserves ASL volume, but also induces ASL volume secretion. Similar interactions are predicted for normal epithelia under static conditions, where ENaC block should increase both adenosine and ATP stimulated chloride and volume secretion.

To improve the clinical efficacy of amiloride, a pilot study testing the combination therapy (aerosolized amiloride followed by HS) in CF patients measuring pulmonary function was performed. Unexpectedly, amiloride with HS was less effective than HS alone (Donaldson et al., 2006). The lack of synergism between HS and amiloride was explained by an apparent non-selective action of amiloride on epithelial aquaporin water channels (Donaldson et al., 2006). Levin et al. (Levin et al., 2006) challenged the data, reporting that amiloride had no affect on AQ-3, 4 and 5 in AQP-transfected cells, and in CF and normal bronchial epithelial cells. Regardless of the amiloride aquaporin controversy, we investigated whether ENaC blocker 552-

02 exhibited any activity on ASL volume expansion when delivered pre or post HS administration. Both in CBE and in CF bronchial epithelia, irrespective of the sequence, 552-02 administration with HS was significantly superior with respect to ASL volume expansion than HS treatment alone (Figure 7B and C and Figure 9). Thus, the combined data from CBE and CF bronchial epithelial argue strongly that 552-02 does not promote a block of transepithelial osmotically-driven water flux, and indeed the sequence of 552-02 followed by HS may be the preferred arm for clinical trials.

In summary, compound 552-02 is a novel potent, more selective, less epithelial permeant, more durable, epithelial sodium channel blocker than amiloride. When administered as an aerosol 552-02 produced a sustained increase in MC *in vivo* for periods greater than 5 hours. Furthermore, when used as a combination therapy with HS *in vitro*, compound 552-02 produced a sustained increase in ASL volume that was greater than 552-02 or HS alone. The use of 552-02 aerosol therapy alone, or in combination with HS, could be clinically beneficial by hydrating airway surfaces and, hence, restoring the efficacy of the primary innate defense mechanism to the CF lung.

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Footnotes

a) A portion of this work was presented in abstract form: (2006) *Ped. Pulmonol.*, Suppl 29, No. 256.

b) Dr. Andrew J. Hirsh, Parion Sciences Inc., 2525 Meridian Parkway, Suite 260, Durham, NC 27713

c) Senior authors M. Ross Johnson, and Richard C. Boucher contributed equally

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Legends for Figures

Figure 1. Structures of the potassium-sparing diuretic "amiloride" (Midamor®) **a**) and a novel epithelial sodium channel blocker 552-02 **b**) in their unprotonated forms.

Figure 2. Representative I_{sc} concentration-effect relationship (approximately 10 pM – 60 μM) for amiloride (•) and 552-02 (□) in canine (panel **A**) and human (panel **B**) bronchial epithelial cells. The cells were equilibrated for 10 to 20 min in KRB prior to mucosal compound administration to allow for a steady basal I_{sc}. The concentration-effect data plotted in panels **A** and **B** are representative of the average I_{sc} measured approximately 1 min post administration to the mucosal bath for each concentration, averaged over 60 seconds and analyzed using nonlinear regression (see method).

Figure 3. Drug dissociation kinetics and reversibility of I_{Na} inhibition with 552-02 compared with amiloride. In panel **A**, current recording from an excised outside-out macropatch configured from a *Xenopus* oocyte injected with cRNAs of rat αβγ-ENaC subunits (see methods). Inward current (-6.6 pA) decreased (-3.8 pA) within 100 ms of 552-02 superfusion (1 μM, bath) as shown. Time course of I_{Na} recovery after drug removal from bath was used to assess kinetics of dissociation from ENaC. 552-02 dissociation from the channel resulted in slow but near complete recovery of the inward I_{Na} (time constant, τ_{wash} = 26.8 s, red curve) to -6.2 pA. Subsequent amiloride superfusion (amil 10 μM, bath) also inhibited I_{Na} (-3.8 pA) with a rapid and complete recovery after drug washout (*inset;* amiloride washout, τ_{wash} = 236 ms, vertical and horizontal scale bars are 2 pA and 200 ms, respectively). In panel **B,** comparison of 552-02 and amiloride dissociation kinetics. The slower dissociation of 552-02 from ENaC resulted from an ~170-fold reduced off-rate constant (k_{off} = 0.039 ± 0.007 s^{-1}) compared to amiloride (k_{off} = 6.57 ± 0.98 s^{-1}; **P = 0.001, paired t-test, mean ± sem, n = 6), reflecting a relatively more stable ENaC-552-02 inhibitor complex. Note* ordinate is log-scale. In Panel **C,** 552-02 inhibition of I_{Na} was reversible. I_{Na} that recovered after 552-02 washout was nearly complete

(552-02 frct rcvry = 0.975 ± 0.004 vs. amil frct rcvry = 1.068 ± 0.016; **P = 0.003, mean ± sem, n = 6). Current was recorded at 0-mV, membrane potential (t = 23 °C), with Li^+ as charge carrier.

Figure 4. Absorption of amiloride or 552-02 by HTBE cells. The rate of serosal appearance of amiloride or 552-02 was calculated after 2h equilibration. Amiloride or 552-02 (100 µM), were added to the mucosal surface at the start of the experiment serosal samples were collected for up to 4 hours (amiloride n=10, 552-02 n=46). ** indicates significance (P<0.001) from amiloride.

Figure 5. Effect of ENaC blockers on sodium/proton exchange activity in PS120 NHE1, 2 and 3 transfected cells. The activity Na+/H+ exchange ($n \geq 3$) was reported in µM/s and was measured over a pH range of 0.05 to 0.75. All data were fit using nonlinear regression displaying the parameters in the corresponding color scheme black (■) control, red (•) 552-02, blue (▲) amiloride for NHE1 panel **A**, NHE2 panel **B**, and NHE 3 panel **C**. The calculated V_{max} values were used to assess significance from control. Results are reported as the mean ± SEM for V_{max}. The '*****' indicates significance (P<0.05) from vehicle.

Figure 6. Effect of aerosolized vehicle $H_2O_{(sterile)}$ (4 mL), amiloride (3 mM; 4 mL), or 552-02 (3 mM; 4 mL), on *in vivo* ovine MC. The MC data are reported as the rate of ^{99}TC-SC cleared from the central region of the lung immediately after dose administration 0-1 (Panel **A**), and to test for drug durability the clearance from 4 to 6 h after dosing was measured (Panel **B)**. Clearance data was fit using linear regression analysis and the calculated slope=ΔY/ΔX of each line is reported next to each data set. The '*****' indicates significance (P<0.05) from vehicle, and † indicates significance (P<0.05) from amiloride for panel **A**. The '*****' indicates significance (P<0.05) from vehicle and amiloride for panel **B**. Note, only 5 animals from each group were reported for the 5 to 6 h data set (panel **B**). 552-02 was significantly different (P<0.05) from vehicle starting at 0.25 h and for the duration of the assay.

Figure 7. Durability and selectivity of 552-02 (10 µM) with and without hypertonic saline on

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apical surface liquid retention by canine bronchial epithelial cells. Fifty μL of modified KRB with or without ENaC blocker (amiloride or 552-02; 10 μM) were added to the apical surface of the transwell. In panel **A** the percent of surface liquid remaining after 8 hours of treatment (vehicle, 552-02 or amiloride) was measured. The mass of surface liquid retained was measured and converted to microliters. † indicates a significant difference ($P<0.001$) from amiloride and control. In panel **B** a time-course measuring the percent of surface liquid gained or lost after an initial 50 μL was added to the apical surface using modified KRB and 0.1% DMSO (control □), 10 μM 552-02 in modified KRB (♦),1.5% hypertonic saline (HS; ∇), or 10 μM 552-02 in 1.5% hypertonic saline (●). The values on top of each data set represent the rate of loss (-) or gain (+) in surface liquid from 2 to 8 h in $\mu L/cm^2/h$. To test whether 552-02 blocks aquaporin channels in our culture system panel **C** represents the percent of surface liquid remaining on CBE after 8 h of control (modified KRB and DMSO), 10 μM 552-02 then HS or HS then 10 μM 552-02. The † indicates a significant difference ($P<0.001$) from amiloride and control.

Figure 8. Effect of 552-02 under static or sheer stress conditions on CF bronchial cell ASL height. Representative images of CF bronchial ASL labeled with a fluorescent dye (green color) under static and shear stress conditions with or without 10 μM 552-02 (Panel **A**.) A composite graph showing the effect of static and shear stress conditions on CF bronchial cell ASL height with and without 552-02 (panel **B**.) Solid bars represent cells under static conditions hatched bars represent cells under shear stress conditions(n=3). * indicate significance ($P<0.0001$) from control cells under static conditions; † indicates significance of 552-02 treated cells ($P<0.003$) from control cells under shear stress conditions. ASL height was measured in the X-Z plane

Figure 9. Effect of 552-02 with or without HS on CF bronchial cell ASL height. Representative images of ASL height from control, 0.25 mg NaCl, or 0.25 mg NaCl pre and post administration of approximately 10 μM 552-02 (P5) on CF bronchial cells are shown in panel **A**. In panel **B** a graph summarizing the effects of 552-02 with and without NaCl, including pre and post

administration on CF cell ASL height (n=3). * indicate significance (P<0.05) from HS; † indicates significance (P<0.05) from control. ASL height was measured in the X-Z plane.

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Table 1.

Intrinsic Blocking Activity and Recovery of Epithelial Sodium Channel Blockers on Sodium-Dependent I_{sc} Using Primary Canine and Human Bronchial Epithelial Cells

	CBE		HBE	
Compound	**IC_{50} (nM)**	**% Recovery**	**IC_{50} (nM)**	**% Recovery**
Amiloride[a]	781±331 (40)	90.0±27.5 (39)	389±188 (22)	89.5±10.7 (4)
552-02	7.54±2.71 (461)	18.6±10.4 (426)	6.40±5.93 (18)	38.2±22.0 (9)

All values are the mean ± S.D; number in parenthesis represents the number of observations; [a] Commercially available epithelial sodium channel blockers. The IC_{50} value was calculated fitted to a nonlinear regression sigmoidal concentration-effect curve with no weighting.

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Figure 1.

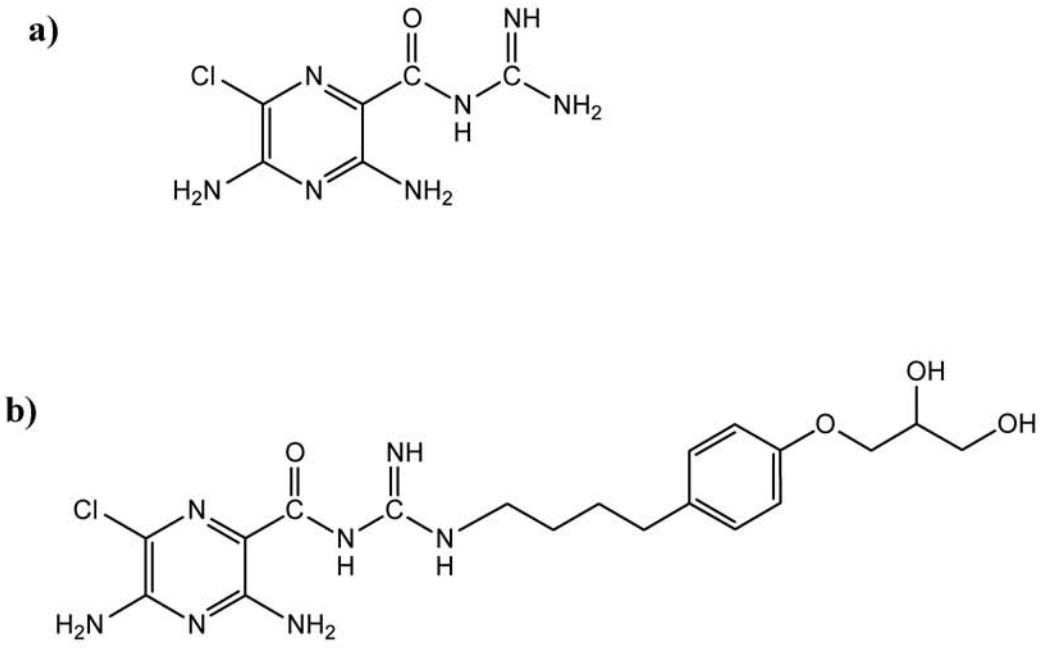

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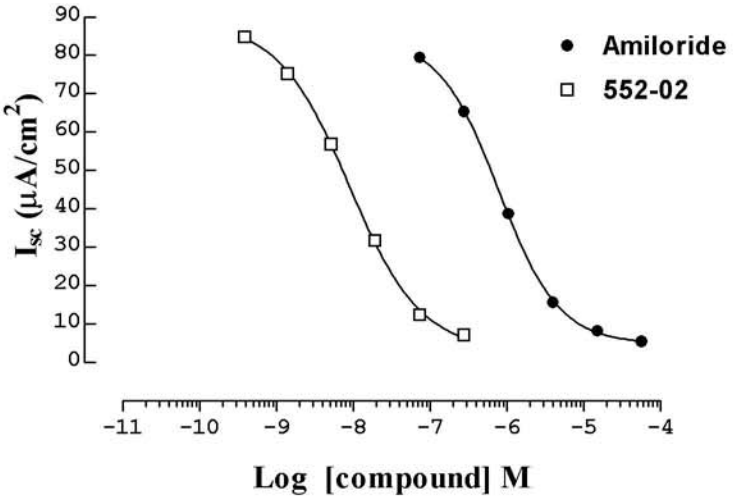
Figure 2.

A.



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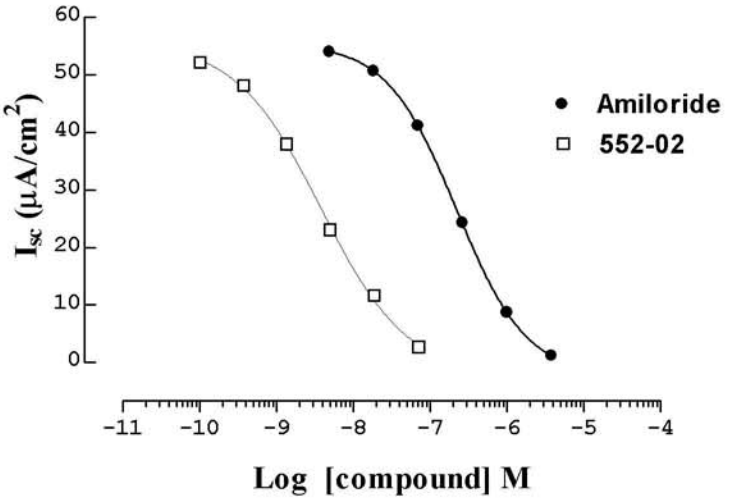

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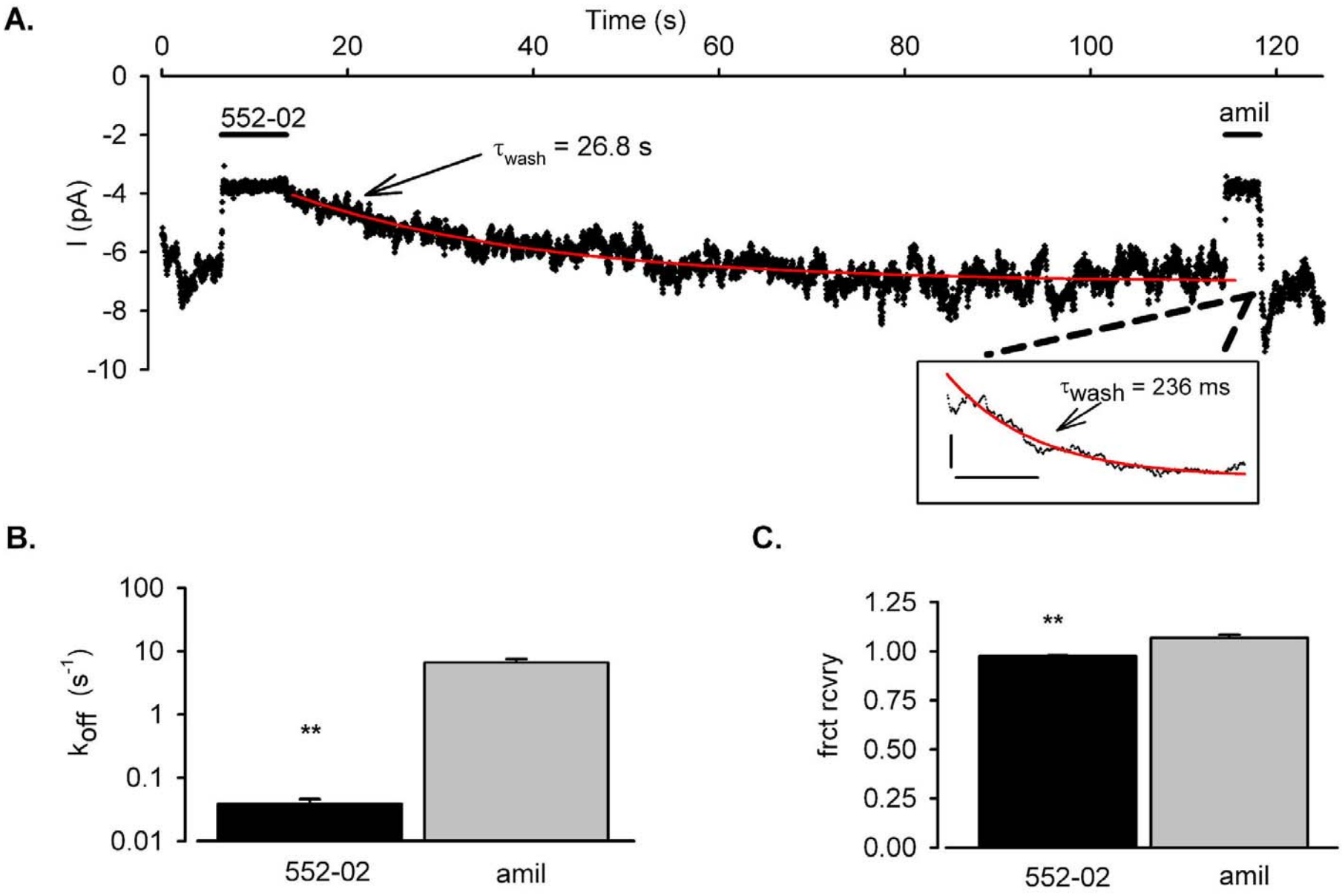

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Figure 4.

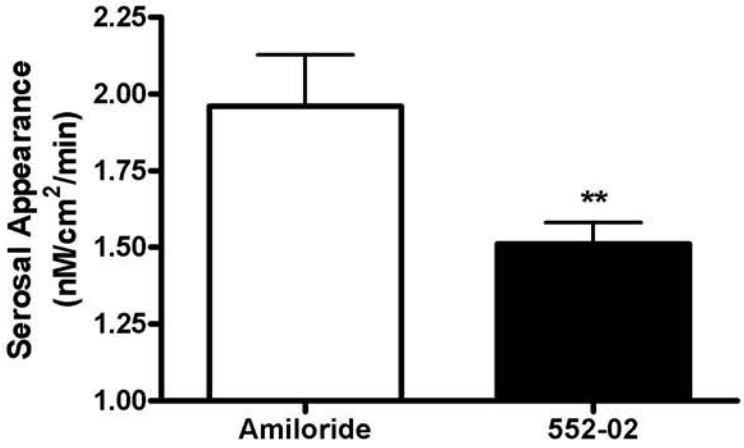

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Figure 5

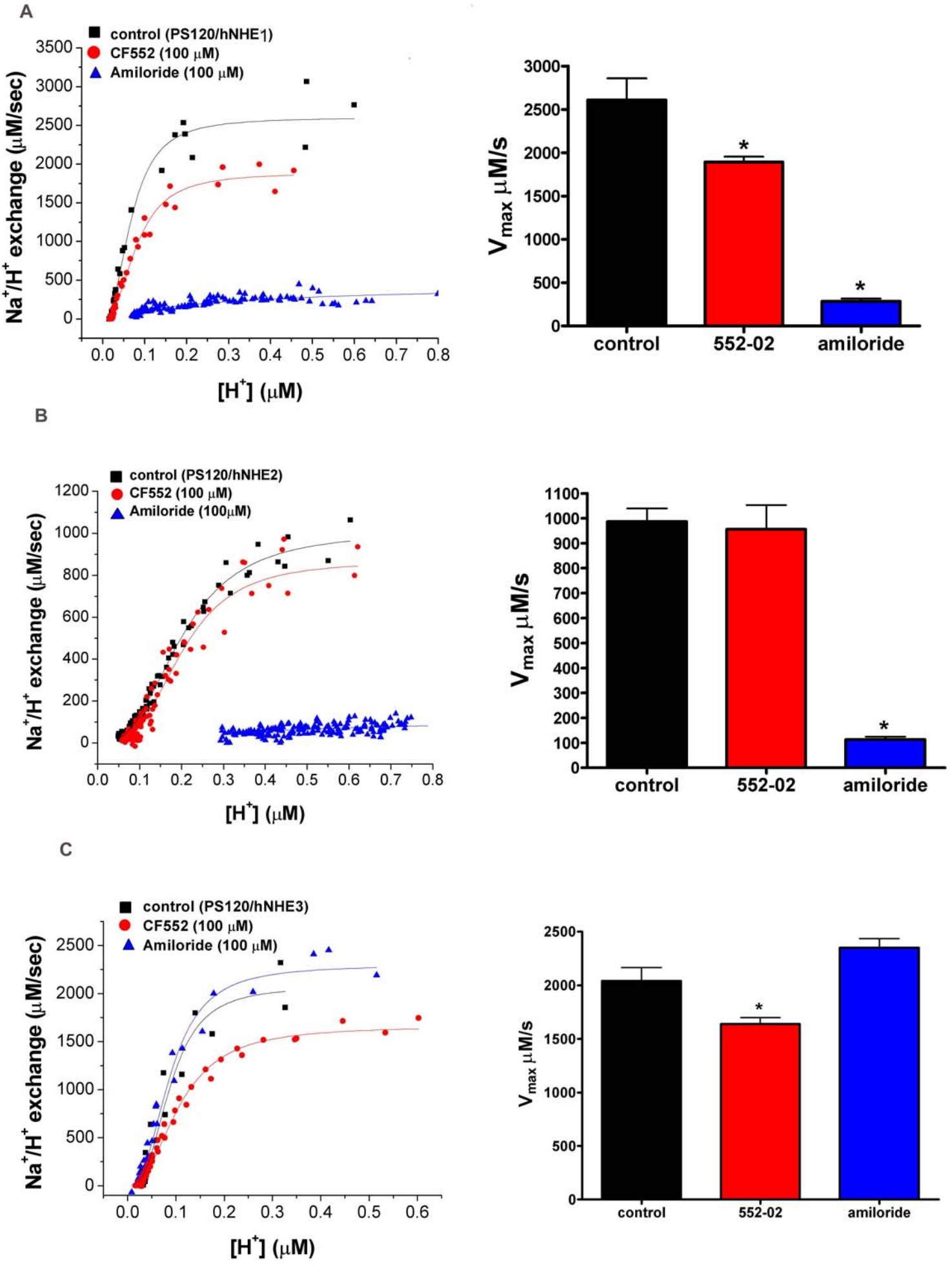

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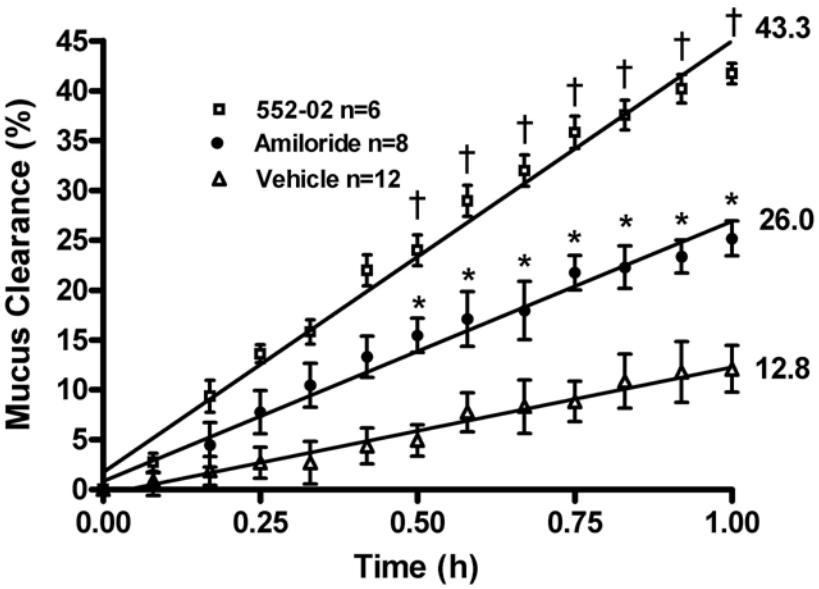
Figure 6.

A.



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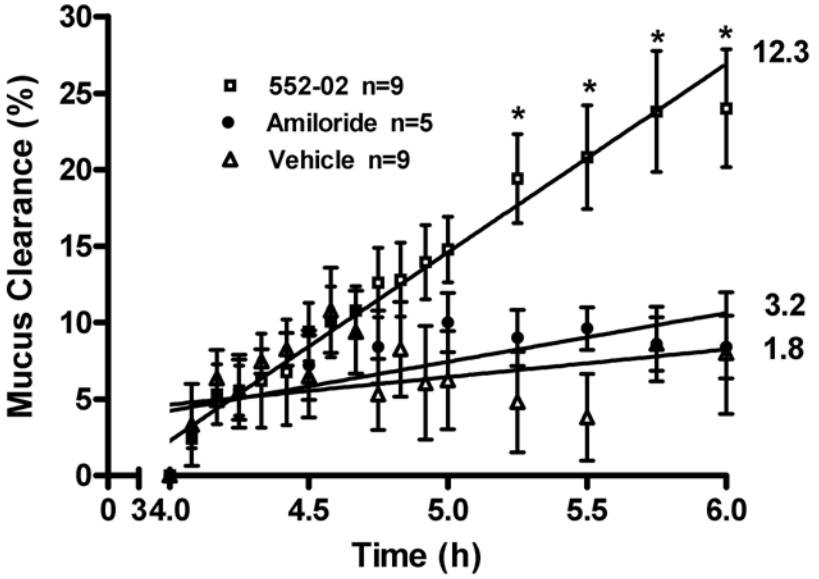

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Figure 7.

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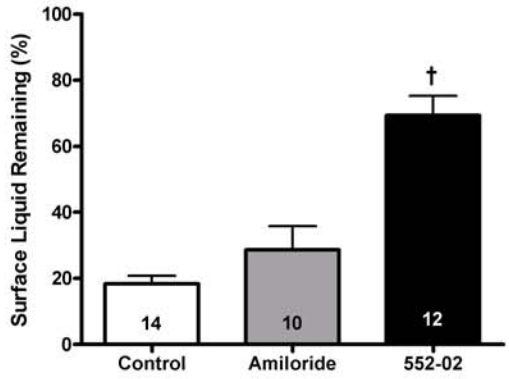

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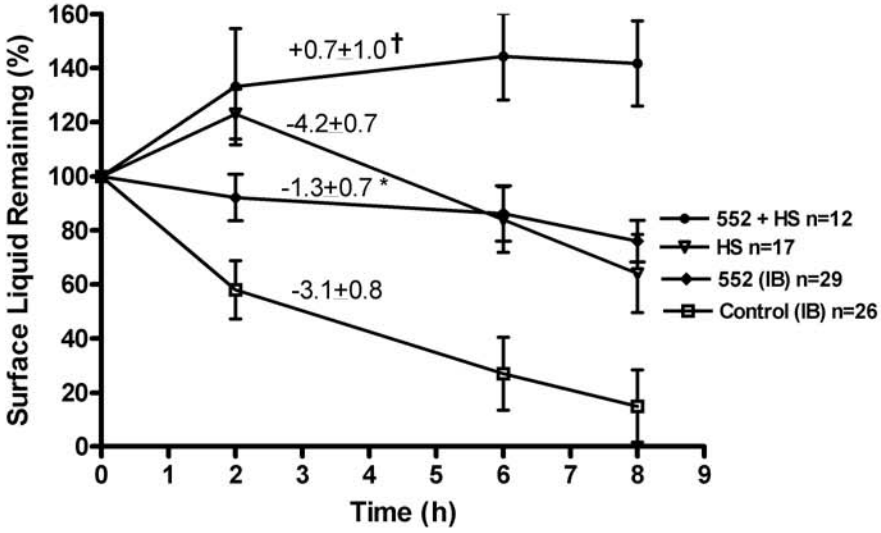

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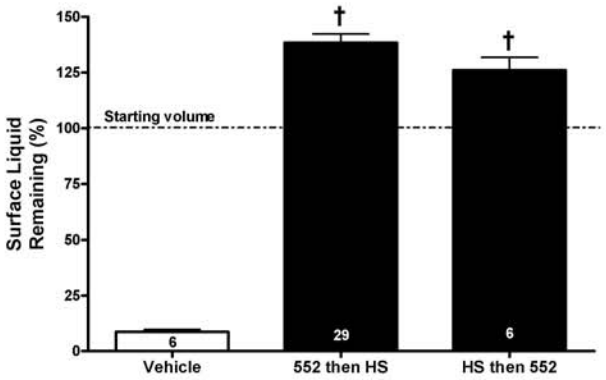

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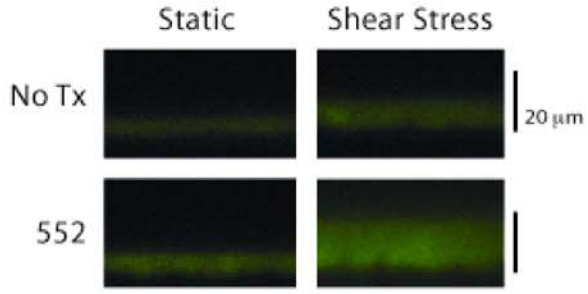
Figure 8.

A.



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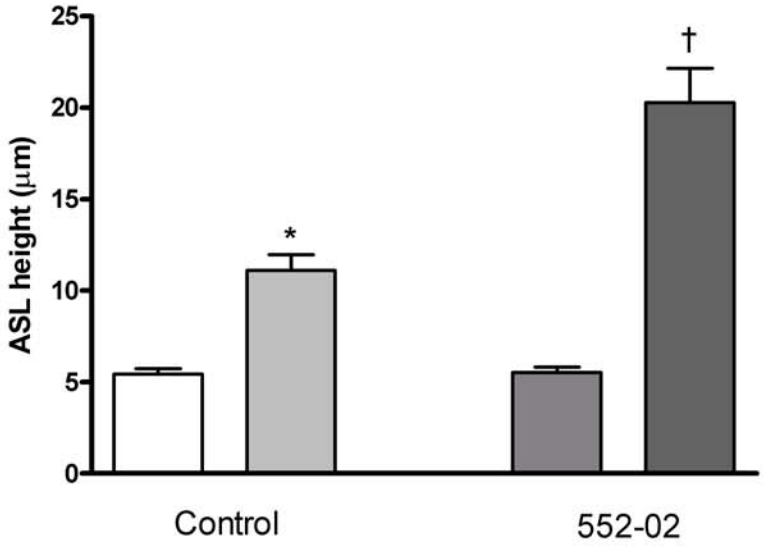

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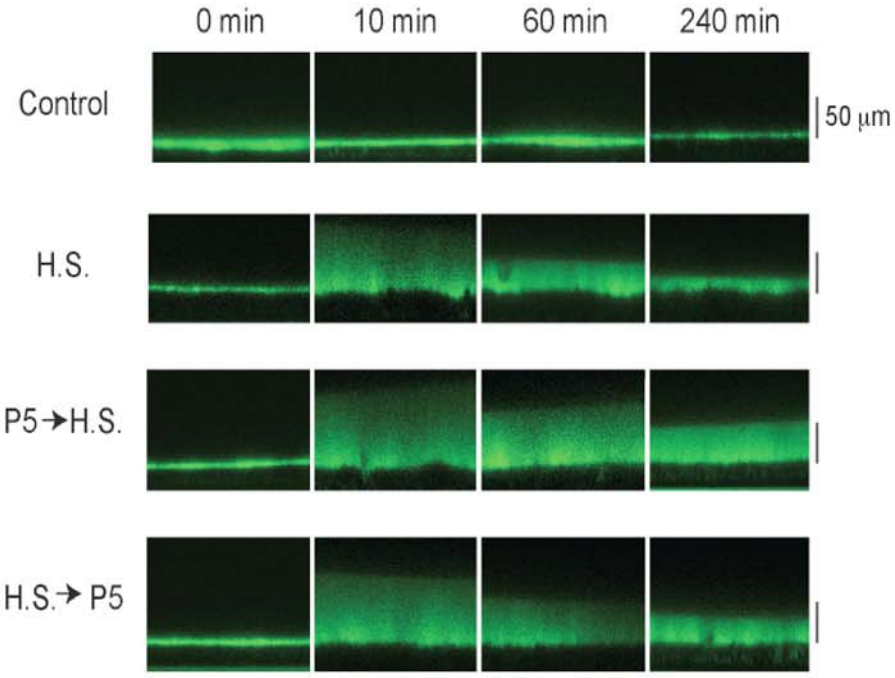
Figure 9.

A.



B.