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Linking increased airway hydration, ciliary beating, and mucociliary clearance through ENaC inhibition

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Abstract

The epithelial sodium channel (ENaC) is a key molecular target in the etiology of several diseases that are characterized by aberrant epithelial Na⁺ transport. For example, mutations involving gain or loss of ENaC function in the kidney underlie Liddle’s syndrome and type-1 pseudohypoaldosteronism, respectively, and cause suffering from severe abnormalities in blood pressure and whole body electrolyte homeostasis (23, 31). Similarly, in the airways of patients with cystic fibrosis (CF), ENaC is abnormally hyperactive, attributable in part to excessive proteolytic cleavage of ENaC in the absence of the CF gene product, the CF transmembrane conductance regulator (CFTR) (18). The disproportionate Na⁺ absorption contributes to a depletion of ASL and is believed to be responsible for mucus stasis and increased incidence of airway infections that lead to progressive decline in lung function and early mortality in patients with CF patients. ASL is also dehydrated in the airways of patients with chronic obstructive pulmonary disease (COPD) attributable to a tobacco smoke-induced decrease in CFTR-mediated anion secretion and a concomitant increase in mucus secretion, which together lead to mucus plugging, bacterial infection, and chronic neutrophilia (9, 32). The extracellular loops of ENaC are proteolytically cleaved by trypsin-like proteases and/or neutrophil elastase, leading to channel activation and increased Na⁺ absorption (6). COPD airways display an abundance of free neutrophil elastase that is predicted to hyperactivate ENaC, which will exacerbate mucus dehydration (6, 15).

Given the prominent role that ENaC plays in a number of diseases, it is not surprising that ENaC inhibitors (amiloride, benzamil, and related compounds) have been used therapeutically. For example, systemic administration of amiloride rapidly normalizes blood pressure and plasma electrolytes in patients with Liddle’s syndrome (21). Biological control of ENaC is complex and offers several opportunities for thera-
ENaC INHIBITION RESTORES ASL HEIGHT AND MCC

L23

Fig. 1. Compound A is a potent inhibitor of epithelial sodium channel (ENaC)-mediated short-circuit current (Isc). Top: representative Ussing chamber trace for Compound A vs. benzamil. Bottom: effective concentration (EC) curves for Compound A (EC50 0.177 nM, n = 8), P-552 (EC50 1.29 nM, n = 5), benzamil (EC50 21.9 nM, n = 13), and amiloride (EC50 0.454 µM, n = 2) in the Ussing chamber assay on normal human bronchial epithelial cells (HBEC) from healthy donors. Means ± SE.

Fig. 2. Retention of airway surface liquid (ASL) in HBECs. Airway surface hydration was determined by gravimetric methods. Vehicle (n = 30) and the short-acting ENaC inhibitor amiloride at 1 µM (n = 12) did not manage to restore the apically added volume from penetrating through the membranes, whereas Compound A at 1 µM (n = 12) significantly (**P < 0.001) retained 66% of the added volume at 24 h. Means ± SD.

peutic intervention. However, to date, no effective ENaC inhibitor exists for the treatment of chronic airway disease. Amiloride-like compounds are readily absorbed across epithelial barriers and thus distribute rapidly throughout the body, even if selectively applied to a specific tissue or fluid compartment such as the lung (16, 37). As a consequence, the therapeutic benefits of treating an ENaC defect in the airways are outweighed by unwanted actions of amiloride at other sites. As a case in point, chronic pulmonary application of amiloride significantly prolongs survival and greatly attenuates the pulmonary disease symptoms in a mouse model of chronic lung disease (airway-specific overexpression of the β-subunit of ENaC), but the rapid trafficking of amiloride from the airways into the systemic circulation causes excessive and undesirable renal excretion of fluid and Na+ (46) and hyperkalemia (2). The aim of the present study was to test the concept that ENaC inhibition would help rehydrate cigarette smoke (CS) exposed-airway cultures and thereby restore MCC. To achieve this goal, we developed a novel compound (Compound A), which has high affinity for ENaC and is poorly absorbed across airway epithelia. To further corroborate our studies, we also tested its actions in vivo in three separate, nondiseased species.

MATERIALS AND METHODS

Ussing Chamber Measurements

The in vitro potency of Compound A was tested in normal human bronchial epithelial (HBEC) cultures from healthy donors (MatTek, Ashland, MA) that were grown at an air-liquid interface (ALI) on Transwell permeable supports (0.4-µm polycarbonate membrane, 12-mm insert; Costar Corning, Corning, NY). Experiments were performed using modified Ussing chambers containing carboxygenated (95% O2-5% CO2) Krebs solution at 37°C. Cell layers were allowed to equilibrate before being voltage clamped at 0 mV and then briefly clamped at 10 mV to assess transepithelial electrical resistance. While measuring short-circuit current (Isc), we applied inhibitors cumulatively before benzamil (10 µM) was added on the final response plateau, to which calculated responses were standardized (100%). Compound A was tested compared with amiloride, benzamil, and P-552, a well characterized ENaC inhibitor developed by Parion Sciences (17), in a concentration range from 10 pM-30 µM.

Gravimetric Measurements of ASL Height

The efficacy and duration of Compound A, benzamil, and amiloride were observed by means of gravity in HBECs grown under ALI conditions (Donor 231849, cat. no. CC-2540; Lonza, Walkersville, MD) in a 24-well plate format using Transwell clear filters (0.4-µm polycarbonate membrane, 6.5-mm insert, Costar Corning). After 3–4 wk, HBEC ALI cultures were washed with PBS twice before the experiment was initiated. A sample (30 µl) of either vehicle or test compound (10 µM) was added apically, and inserts were weighed after removal of basolateral fluid (17). The weighing procedure was repeated at 4, 8, and 24 h.

XZ Confocal ASL Height Measurements and Ciliary Beating Frequency

Primary normal HBEC cultures were obtained by the UNC CF Center Tissue Core under protocols approved by the UNC Institutional Committee for the Protection of the Rights of Human Subjects as described, plated on 12-mm T-clear culture inserts, and studied 3–5 wk after seeding as described (45).

To measure ASL height, cultures were prewashed twice in PBS, and PBS (20 µl) containing 2 mg/ml rhodamine-dextran (10 kDa; Invitrogen, Carlsbad, CA) was added to cultures at the start of the experiment. To study absorption, the 20 µl PBS was left on HBEC surfaces (e.g., Fig. 3), whereas, under steady-state conditions, PBS was aspirated with a Pasteur pipette to bring ASL volume down to ≤10 µm (e.g., Figs. 8 and 9). Five predetermined points (one central and four 2 mm from the edge of the culture) were XZ scanned using a confocal microscope (Leica SP5; ×63/1.3 NA glycerol immersion lens) as described (45). Cultures were returned to the incubator between time points. For all studies, perfluorocarbon was added mucosally during imaging to prevent evaporation of the ASL.
To measure ciliary beat frequency (CBF), a digitized video was collected in 2.1-s segments using a Nikon TE2000 and a MegaPlus ES310 (Kodak) turbo video camera. CBF analysis was performed on digitized video using Sisson-Ammons Video Analysis software (42).

Cigarette Smoking of ALI Cultures

CS exposure to HBECs was conducted by using a smoke engine as previously described (8, 9). In brief, smoke from one Kentucky research cigarette (2R4F) was generated according to ISO standards (2 s/35-ml puff) and applied immediately to HBECs using a Borgwaldt smoke engine (LC1). One important difference to previous experiments was that, after mucosal addition of Compound A or vehicle (PBS) in 20 μl, excess PBS was aspirated from HBEC mucosal surfaces to set the ASL height at 7 μm before smoke exposure to induce a comparative situation for the two groups, and as such the increased baseline ASL height induced by Compound A was thus normalized.

In Vivo Efficacy Measurements

All studies in guinea pigs and rats were approved by the local ethical committee in Gothenburg. Animals were given free access to food and water during the conscious periods of the experimental protocols, and they were acclimatized for at least 1 wk in the animal facility with 12-h:12-h light/dark cycle at 21 ± 2°C and with 55 ± 15% relative humidity.

Tracheal potential difference. Male Dunkin Hartley guinea pigs (Charles River, Sulzfeld, Germany) were anaesthetized, and a breathing tube with an integrated electrode was inserted into the trachea to administer drugs and to measure tracheal potential difference (TPD) (AstraZeneca R&D, Mölndal, Sweden). The reference electrode was placed on the outside of the trachea. A catheter was introduced into the right carotid artery for blood pressure recordings, maintenance of anesthesia, and blood sampling. Online recordings of TPD were performed during a three-step dosing interval of each 30 min on 4–11 animals per treatment group and 8 control animals. The response by test compound on the TPD was measured per individual by taking the area under the curve (baseline predose) per dose compared with the full inhibition window for ENaC inhibition (50% in the present model) to produce a dose-response curve. The percentage evaluation...
Retention of colloids was in all cases evaluated 2 h after the administration of 99mTc in the conscious rat. Compound A at 100 µg/kg 4-h pre-MCC measurement (***, p<0.001) increased MCC vs. vehicle (1st bar) as demonstrated by the lower percentage of retention of 99mTc colloids. For the dose-response evaluation, Compound A was administered just before 99mTc. For the evaluation of duration, 100 µg/kg of Compound A was administered 2 and 4 h before the 99mTc administration (denoted as pre-99mTc). The retention of colloids was in all cases evaluated 2 h after the administration of 99mTc. Means ± SE, n = 3–39.

of the results was considered superior to presenting absolute data when comparing in vivo potency between compounds. Ninety minutes after the first dose, the animals were terminated, and the trachea and left lung lobes were removed and analyzed for content of test compound. To find out whether there were unwanted effects of the test compounds on sodium and potassium levels in the blood, blood electrolyte content at the end of the experiment was compared with baseline concentrations (data not shown).

Rat MCC. Female Wistar rats were randomized upon arrival. Compound A was run in four separate studies to build up both the dose-response relationship and the duration of effect where vehicles were included in each study. Data were pooled because there were no statistically significant differences between the vehicles in each study, giving vehicle (n = 39), 3 µg/kg (n = 4), 30 µg/kg (n = 6), 100 µg/kg (n = 13), 100 µg/kg 2-h pre-MCC measurement (n = 5), and 100 µg/kg 4-h pre-MCC measurement (n = 3). A human serum albumin-based-radiolabeled colloid particle (99mTc-albumin, Nanocoll, GE Healthcare, Piscataway, NJ) was used to estimate the distribution of delivered activity and the subsequent course of clearance from the airway by the mucociliary transportation by means of single-photon emission-computed tomography. In brief, the rats were anesthetized with isoflurane, and the test compound or vehicle was administered via passive inhalation in a nose-only exposure system. A novel closed exposure chamber was developed and used in combination with a vibrating mesh nebulizer (Aerogen, Galway, Ireland) to efficiently dose individual animals during ~2 min. Shortly after the drug/vehicle administration for the dose-response experiments, 99mTc-colloids (~50 MBq/rat) were dosed in a similar fashion. For duration of effect evaluation, the vehicle/compound was administered 2 or 4 h before the administration of 99mTc-colloids. The clearance of 99mTc from the lung (i.e., a measure of MCC) was determined by comparing lung levels of 99mTc immediately after the dosing of 99mTc and at a time point 2 h later. The rats were allowed to wake up in between imaging time points and again anesthetized with isoflurane shortly before the second measurement.

Sheep MCC. The Mount Sinai Medical Center (MSMC) Animal Research Committee approved all procedures used in this protocol. MSMC is fully accredited by the Association for Assessment of Laboratory Animal Care International.

Seven adult female sheep (37–54 kg) were used in the present study, once or twice with a washout of at least 1 wk between experiments. The animals were conscious and upright in a supportive cart and intubated for nebulized delivery of test compounds and radiolabeled 99mTc-Technetium-sulfur colloid (1). The test compound was delivered 4 h (Compound A at 0.3 and 3 mg/sheep, equivalent to 7.5 and 75 µg/kg) before measurements of MCC, whereas the corresponding vehicle was administered 1 h before measurements (3 ml 0.9% NaCl). MCC was measured as the retention of radiolabeled 99mTc every 5 min over a 1-h period and expressed as percentage of radioactivity present in the initial baseline image (100%). The vehicle data 1 h after dosing demonstrated normal MCC rate, and controls were not repeated at the 4-h predosing protocol for ethical reasons. Plasma samples were collected at nine time points over 24 h after compound administration for determination of plasma exposure of drug as well as sodium and potassium content in blood.

Renal electrolyte handling in the anesthetized rat. Twelve female Wistar rats were anesthetized by means of spontaneously breathing isoflurane (induction concentration of 5% followed by 2%). A catheter was placed in the carotid artery for blood sampling and measurement of mean arterial blood pressure and heart rate. Another catheter was placed in the vena jugularis for continuous infusion of test compound according to a three-step dose design, where each step had a duration of 30 min (bolus given over 6 min + infusion over 24 min, giving a total of: 0.02-0.2-2.0 µg/kg per min + 30) or vehicle (68 µl/kg per min during bolus and 17 µl/kg per min during infusion, giving a total of 1.6 ml/kg per h) plus a constant infusion of 0.9% NaCl (12 ml/kg per h) to secure urine production. The urinary bladder was catheterized for urine collection. The animals were left to stabilize for at least 30 min after surgery during constant basal infusions before any test compound or vehicle infusion was initiated. Urine was collected over 20-min periods, two at baseline (i.e., preexposure), followed by three 30-min exposures of increasing doses of test compound or vehicle, where urine was collected over the last 20 min for each dose. Additional urine was collected during the washout period (0–20, 20–40, and 40–60 min after cessation of the infusion.

Fig. 6. Compound A increases sheep MCC 4–5 h after dosing. Compound A was given at t = 0 h in 2 doses; 0.3 mg (red triangles) with no effect vs. vehicle (black squares) 4–5 h after dosing, whereas 3 mg (red circles) demonstrated clearly increased MCC vs. vehicle, given as percent retention of the 99mTc signal 60 min after colloid administration. Means ± SD, n = 2–3 (descriptive statistical analysis, only).
Blood samples were collected 5 min before the end of each urine collection period for electrolyte evaluation (iSTAT analyzer; Abbott Laboratories, Chicago, IL) and plasma concentration of drug (liquid chromatography-tandem mass spectrometry, LC-MSMS). Urine production, electrolytes (ABL700; Radiometer Medical ApS, Brønshøj, Denmark, for urine), as well as concentration of test compound (LC-MSMS) were evaluated.

Statistical Analyses

In vitro assays. All data are presented as means ± SE. Values of \( n \) refer to the number of cultures used in each group as appropriate. For normally distributed data, paired or unpaired Student’s \( t \)-tests were used. For not normally distributed data, Mann-Whitney \( U \) test or Wilcoxon matched-pairs test were used. For comparisons of multiple groups, ANOVA tests were used followed by Tukey-Kramer multiple-comparisons test (parametric) or ANOVA followed by Kruskal-Wallis Test (nonparametric). For experiments using primary HBECs, a minimum of four different donors supplied cultures for each experiment. For the Ussing chamber data, unconstrained concentration-response curves were fitted using an in-house Microsoft Excel add-in package by nonlinear, four-parameter, logistic-regression analysis. From these curves, pEC\(_{50}\) and Emax values were taken, from which mean values were calculated.

In vivo models. The sheep MCC evaluations were performed in only a few experiments, and adequate statistical analysis of the data was thus not possible (Student’s \( t \)-test between individual slopes for treatment group vs. control indicates significant differences, but only descriptive statistical analysis has been described). Rat MCC and guinea pig TPD were analyzed by means of one-way ANOVA followed by Dunnett’s post hoc test (GraphPad Prism v.5; GraphPad, San Diego, CA) and the rat electrolyte data analyzed by means of repeated-measures ANCOVA (SAS v.9). A difference of statistical

### Table 1. Average potassium and sodium blood levels from 2–3 sheep

<table>
<thead>
<tr>
<th>Time Point</th>
<th>Vehicle, ( n = 3 )</th>
<th>Compound A, 0.3 mg, ( n = 2 )</th>
<th>Compound A, 3 mg, ( n = 2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood K(^+), mM</td>
<td>Blood Na(^+), mM</td>
<td>Blood K(^+), mM</td>
</tr>
<tr>
<td></td>
<td>Mean  SD</td>
<td>Mean  SD</td>
<td>Mean  SD</td>
</tr>
<tr>
<td>Predose</td>
<td>4.4  0.7</td>
<td>152  10</td>
<td>4.7  0.0</td>
</tr>
<tr>
<td>Immediate</td>
<td>4.1  0.2</td>
<td>150  2.3</td>
<td>4.3  0.1</td>
</tr>
<tr>
<td>15 min</td>
<td>4.2  0.3</td>
<td>146  1.6</td>
<td>4.1  0.3</td>
</tr>
<tr>
<td>30 min</td>
<td>4.1  0.2</td>
<td>148  4.1</td>
<td>4.2  0.4</td>
</tr>
<tr>
<td>1 h</td>
<td>3.8  0.1</td>
<td>151  3.5</td>
<td>3.9  0.3</td>
</tr>
<tr>
<td>2 h</td>
<td>3.7  0.4</td>
<td>147  3.8</td>
<td>4.1  0.3</td>
</tr>
<tr>
<td>4 h</td>
<td>4.2  0.3</td>
<td>147  3.3</td>
<td>3.6  0.1</td>
</tr>
<tr>
<td>6 h</td>
<td>4.6  0.7</td>
<td>147  3.4</td>
<td>3.9  0.3</td>
</tr>
<tr>
<td>24 h</td>
<td>4.9  0.5</td>
<td>155  14.1</td>
<td>5.5  0.0</td>
</tr>
</tbody>
</table>

Sheep after having received vehicle (diurnal rhythm of blood electrolytes) or Compound A at 0.3 mg and 3 mg (corresponding to \( 7.5 \) and \( 75 \) \( \mu \)g/kg) by inhalation. The higher dose indicates increased blood potassium at 1 and 2 h after dosing vs. vehicle controls (explorative statistical analysis only).

In vitro assays. All data are presented as means ± SE. Values of \( n \) refer to the number of cultures used in each group as appropriate. For normally distributed data, paired or unpaired Student’s \( t \)-tests were used. For not normally distributed data, Mann-Whitney \( U \) test or Wilcoxon matched-pairs test were used. For comparisons of multiple groups, ANOVA tests were used followed by Tukey-Kramer multiple-comparisons test (parametric) or ANOVA followed by Kruskal-Wallis Test (nonparametric). For experiments using primary HBECs, a minimum of four different donors supplied cultures for each experiment. For the Ussing chamber data, unconstrained concentration-response curves were fitted using an in-house Microsoft Excel add-in package by nonlinear, four-parameter, logistic-regression analysis. From these curves, pEC\(_{50}\) and Emax values were taken, from which mean values were calculated.

In vivo models. The sheep MCC evaluations were performed in only a few experiments, and adequate statistical analysis of the data was thus not possible (Student’s \( t \)-test between individual slopes for treatment group vs. control indicates significant differences, but only descriptive statistical analysis has been described). Rat MCC and guinea pig TPD were analyzed by means of one-way ANOVA followed by Dunnett’s post hoc test (GraphPad Prism v.5; GraphPad, San Diego, CA) and the rat electrolyte data analyzed by means of repeated-measures ANCOVA (SAS v.9). A difference of statistical

![Fig. 7. Compound A affects renal electrolyte handling at urine concentrations in the nM range. Anaesthetized rats were given Compound A in a 3-step iv infusion dose design, where each step had a duration of 30 min (bolus for 6 min + infusion for 24 min, giving a total of: 0.02–0.2–2.0 µg/kg per min * 30) or vehicle in corresponding volumes. Urinary excretion of potassium (B) is significantly (*)\( P = 0.028 \) decreased as plasma and urine concentration of compound A approaches 10 nM (D), which significantly (\( P = 0.010 \)) increases blood potassium levels (C), whereas urinary excretion of sodium (A) is not significantly altered. Means ± SD, \( n = 6 \).](image-url)
Compound A demonstrated significant effects on renal potassium handling

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline 1</th>
<th>Dose 1</th>
<th>Dose 2</th>
<th>Dose 3</th>
<th>WO 0–20′</th>
<th>WO 20–40′</th>
<th>WO 40–60′</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood Na⁺, mM</td>
<td>Vehicle</td>
<td>137 ± 1</td>
<td>138 ± 2</td>
<td>138 ± 2</td>
<td>138 ± 2</td>
<td>138 ± 2</td>
<td>138 ± 1</td>
</tr>
<tr>
<td>Blood Na⁺, mM</td>
<td>Cmpd A</td>
<td>138 ± 1</td>
<td>139 ± 2</td>
<td>139 ± 2</td>
<td>138 ± 3</td>
<td>137 ± 2</td>
<td>137 ± 1</td>
</tr>
<tr>
<td>Blood K⁺, mM</td>
<td>Vehicle</td>
<td>4.2 ± 0.2</td>
<td>4.1 ± 0.3</td>
<td>4.1 ± 0.4</td>
<td>3.9 ± 0.2</td>
<td>3.9 ± 0.2</td>
<td>3.8 ± 0.2</td>
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<tr>
<td>Blood K⁺, mM</td>
<td>Cmpd A*</td>
<td>4.0 ± 0.3</td>
<td>3.9 ± 0.2</td>
<td>3.9 ± 0.4</td>
<td>3.9 ± 0.4</td>
<td>4.3 ± 0.3</td>
<td>4.3 ± 0.2</td>
</tr>
<tr>
<td>Blood Cl⁻, mM</td>
<td>Vehicle</td>
<td>102 ± 1</td>
<td>103 ± 0</td>
<td>104 ± 2</td>
<td>103 ± 2</td>
<td>104 ± 2</td>
<td>103 ± 2</td>
</tr>
<tr>
<td>Blood Cl⁻, mM</td>
<td>Cmpd A</td>
<td>103 ± 3</td>
<td>103 ± 3</td>
<td>104 ± 3</td>
<td>104 ± 5</td>
<td>105 ± 2</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>Plasma concentration, nM</td>
<td>Vehicle</td>
<td>0.7 ± 0.3</td>
<td>8.6 ± 2.1</td>
<td>0.6 ± 0.3</td>
<td>0.3</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>UNaV, μmol/20’</td>
<td>Vehicle</td>
<td>20 ± 14</td>
<td>20 ± 13</td>
<td>38 ± 50</td>
<td>54 ± 51</td>
<td>84 ± 36</td>
<td>93 ± 16</td>
</tr>
<tr>
<td>UNaV, μmol/20’</td>
<td>Cmpd A</td>
<td>27 ± 10</td>
<td>31 ± 26</td>
<td>53 ± 43</td>
<td>97 ± 68</td>
<td>180 ± 119</td>
<td>194 ± 131</td>
</tr>
<tr>
<td>UV, μmol/20’</td>
<td>Vehicle</td>
<td>27 ± 14</td>
<td>26 ± 17</td>
<td>24 ± 12</td>
<td>27 ± 22</td>
<td>36 ± 21</td>
<td>31 ± 12</td>
</tr>
<tr>
<td>UV, μmol/20’</td>
<td>Cmpd A</td>
<td>45 ± 15</td>
<td>35 ± 10</td>
<td>43 ± 20</td>
<td>34 ± 18</td>
<td>9.6 ± 9.3</td>
<td>3.0 ± 3.6</td>
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<tr>
<td>UCIV, μmol/20’</td>
<td>Vehicle</td>
<td>44 ± 28</td>
<td>46 ± 29</td>
<td>58 ± 37</td>
<td>75 ± 44</td>
<td>115 ± 27</td>
<td>115 ± 22</td>
</tr>
<tr>
<td>UCIV, μmol/20’</td>
<td>Cmpd A</td>
<td>36 ± 22</td>
<td>58 ± 27</td>
<td>83 ± 22</td>
<td>112 ± 37</td>
<td>154 ± 99</td>
<td>163 ± 119</td>
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<td>UV, μL/20’</td>
<td>Vehicle</td>
<td>125 ± 23</td>
<td>124 ± 23</td>
<td>167 ± 52</td>
<td>225 ± 45</td>
<td>370 ± 44</td>
<td>526 ± 115</td>
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<tr>
<td>UV, μL/20’</td>
<td>Cmpd A</td>
<td>176 ± 45</td>
<td>157 ± 50</td>
<td>228 ± 58</td>
<td>516 ± 416</td>
<td>607 ± 498</td>
<td>782 ± 653</td>
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<tr>
<td>Urine concentration, nM</td>
<td>Vehicle</td>
<td>6.5 ± 3.2</td>
<td>10.3 ± 7.9</td>
<td>11.6 ± 6.4</td>
<td>7.4 ± 6.8</td>
<td></td>
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</tbody>
</table>

Values are means ± SD, n = 6. Compound A (Cmpd A) demonstrated significant effects on renal potassium handling at plasma concentrations (Cp) approaching 10 nM in the anesthetized rat, i.e., below Cp after full efficacy on mucociliary clearance in sheep. The effect of Compound A on blood potassium was significantly increased and interacted with time (*P = 0.010) because of the decreased urinary excretion of potassium (†P = 0.028). UNaV, urinary sodium excretion; UKV, urinary potassium excretion; UCIV, urinary chloride excretion; UV, urine excretion; WO, washout.

significance is marked as *P < 0.05, **P < 0.01, or ***P < 0.001, unless the P values are given.

RESULTS

Compound A Has Significant In Vitro Potency Against ENaC

We generated a library of highly soluble compounds with long lung duration (t½ > 20 h in rats) and good potency against ENaC (μIC50 > 9) from which compound A was chosen. Compound A caused a rapid decrease in Isc, and the effects of Compound A and benzamil were not additive (Fig. 1), suggesting that they both targeted ENaC. All inhibitors tested in the Ussing chambers decreased the Isc to a similar degree as benzamil, and the rank order of potencies were Compound A > P-552 > benzamil > amiloride, with Compound A (IC50 = 0.177 nM, n = 8) demonstrating a potency shift of 3.5 log units vs. amiloride (IC50 = 0.454 μM, n = 2, benzamil IC50 = 21.9 nM, n = 13) and greater than sevenfold more potency than P-552 (IC50 = 1.29 nM, n = 5; Fig. 1C). Compound A demonstrated a great selectivity and secondary pharmacology profile with its main hits at α1A receptor (0.2 μM) and dopamine transporter (0.91 μM), whereas Nav1.5 (>33.3 μM), Nav1.2 (>33.3 μM), human ether-a-go-go (13% inhibition at 10 μM), M2 receptor (>100 μM), and β2 receptor (>100 μM) were essentially untouched (Millipore, Billerica, MA; binding assays of 124 and 154 targets, respectively; actives were followed up in functional assays).

Compound A Slows ASL Absorption

Despite having a submicromolar IC50 against ENaC in Ussing chambers, amiloride is essentially ineffective under thin film conditions because of its rapid removal from the ASL (37). To see whether Compound A was more effective, we tested its ability to slow ASL absorption. Because airway epithelial cultures can either absorb or secrete ASL (38), we used the absorptive mode by removing endogenous ASL and adding a bolus of test solution at 1 μM and t = 0 with the remaining volume assayed over time. Using a gravimetric method, we determined that airway hydration in HBEC cultures was significantly preserved with Compound A (66 ± 2%, n = 12) vs. vehicle (39 ± 8%, n = 30) or amiloride (28 ± 10%, n = 12) (Fig. 2).

XZ confocal microscopy revealed a similar pattern with the retention of ASL height being increased after Compound A that lasted over 6 h (Fig. 3, A and B). We then performed concentration responses for Compound A using data obtained 2 and 6 h after the volume challenge (Fig. 3, C and D), and potencies (IC50) were 0.018 μM and 0.052 μM, respectively.

Compound A Inhibits Guinea Pig TPD and Increases Rat and Sheep MCC Rates In Vivo

Because Compound A was efficacious in HBECs, we tested its ability to inhibit ENaC in vivo. The TPD has previously been shown to be a good measure of ENaC activity in anesthetized guinea pigs (10) and was dose dependently inhibited by all inhibitors tested (Fig. 4) with the rank order Compound A > P-552 > benzamil (ED50 being 4.7, 15, and 30 μg/kg, respectively). Inhibition of ENaC and increases in ASL hydration are predicted to increase MCC rates (36). Therefore, we investigated whether Compound A could affect rat MCC. As predicted by our in vitro data (Figs. 1–3), Compound A increased MCC, and a maximal effect was seen at 100 μg/kg (Fig. 5). However, when MCC was investigated 2 h after administration of Compound A, the effect was absent, thus demonstrating short duration in the rat (Fig. 5).

Compound A was also administered to sheep at two separate doses (0.3 and 3 mg per animal; 7.5 and 75 μg/kg, respectively), and MCC was measured 4–5 h after dosing (Fig. 6). Under these conditions, hardly any effect was seen with 0.3 mg (1.3-fold vehicle controls). However, close to full efficacy was demonstrated at 3 mg (2.7-fold vehicle control). Plasma concentrations of drug peaked 30–60 min after dosing at 24 and 15 μM, respectively, for the two animals receiving 3 mg. The higher dose was accompanied by a tendency for effects on blood potassium levels 1–2 h after dosing (Table 1), whereas the lower dose was devoid of effects on blood electrolytes.

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Effects on Rat Renal Electrolyte Handling During Continuous IV Infusion of Compound A

Compound A did not elicit any changes in renal electrolyte handling up to plasma concentrations of $0.7 \pm 0.3$ nM (equals dose 2). At this dose, there were no measurable levels of Compound A in urine. During application of dose 3, plasma concentrations of Compound A reached $8.6 \pm 2.1$ nM, and the urinary concentrations were $6.5 \pm 3.2$ nM. Under these conditions, potassium was no longer excreted into the urine ($P = 0.028$ for the interaction between treatment and time), and, shortly after cessation of drug infusion (washout), blood potassium levels increased relative to both previous levels and time-matched vehicle control. Values were significantly increased ($P = 0.010$) and interacted significantly with time ($P = 0.001$) (Fig. 7). Sodium concentrations in blood were not affected by Compound A, whereas there was a significant effect of Compound A on chloride blood concentrations ($P = 0.019$) (Table 2). Urine production was significantly increased with time for both vehicle and drug treatment ($P = 0.020$), with no interaction with drug and time on the excretion of sodium or chloride ions (Fig. 7). We also observed a hysteresis between plasma and urine concentration of Compound A and its effect on urinary potassium excretion and blood potassium levels, indicating the time delay of the system.

Compound A Increases ASL Rehydration and Restores Ciliary Beating in Cigarette-Smoke-Exposed HBECs

COPD is typically caused by chronic tobacco inhalation, and mucus dehydration is one of the symptoms of this disease (19, 27). Thus, as a first step toward testing whether Compound A can rehydrate ASL in airways of smokers/patients with COPD, we pretreated HBECs with vehicle or Compound A, amiloride, or benzamil and then exposed them to CS. It is important to note that, for these experiments, we removed excess solution from the mucosal surface (see MATERIALS AND METHODS) so as to start all treatments with an ASL of $\sim 7 \mu m$, which gives a

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Fig. 8. Compound A prevents cigarette smoke (CS)-induced ASL volume depletion. A: typical XZ confocal micrographs of ASL height (red) in the presence of either Compound A or vehicle (PBS) and following exposure to air (control) or 10 puffs of CS as indicated. Scale bar = 7 μm. B and C: mean ASL height over time when cultures were dosed with 1 μM Compound A or vehicle for 2 h followed by air or CS, respectively. All $n = 9–12$. Importantly, baseline ASL was set to $≈ 7 \mu m$ to induce a comparative situation between the 2 groups. D: heat maps of ciliary beat frequency (CBF) following air (control) or CS exposure in HBECs measured using the Sisson-Ammons Video Analysis system. E: mean CBF measured immediately before (0) or up to 3 h after exposure to air in the presence of vehicle or Compound A (1 μM). All $n = 6$. F: mean CBF measured immediately before (0) or up to 3 h after CS exposure with vehicle or Compound A (1 μM). air/vehicle; air/Compound A; CS/vehicle; CS/Compound A. Data shown as means ± SE. *$P < 0.05$ different ± air or CS; †$P < 0.05$ different ± Compound A.
balance between absorption and secretion, unlike how adding a bolus of test solution causes absorption to predominate (38). Under these conditions, CS causes a rapid decline in ASL height attributable to CS-mediated internalization of CFTR and/or a direct effect of acrolein on CFTR open probability (9, 29, 28). Compound A alone did not alter ASL height, as there was no excess fluid to absorb (Fig. 8, A and B). Following CS exposure, ASL height rapidly and significantly decreased in the vehicle-exposed group. Importantly, a 2.5-h preincubation with 1 μM Compound A completely prevented the CS-induced decrease in ASL height (Fig. 8, A and C). In contrast, amiloride and benzamil were less effective at blocking the CS-induced ASL diminution (Figs. 10 and 11). To examine the functional consequences of dehydration by CS, we measured HBEC CBF with or without Compound A pretreatment. In vehicle-treated cultures, CBF was significantly decreased, whereas Compound A completely prevented this (Fig. 8, D–F).

Because patients with COPD will typically use drugs such as Compound A after lung disease is manifest, rather than prophylactically, we next tested whether Compound A was capable of speeding up ASL rehydration after CS exposure. This was investigated by adding Compound A as a dry powder in perfluorocarbon 30 min after CS-induced ASL diminution vs. air controls (37). Whereas Compound A had no effect on ASL height when added after air exposure, we found that ASL height returned to normal levels significantly more quickly when Compound A was added compared with the control (Fig. 9, A and B). Again, amiloride and benzamil were less effective at inducing ASL rehydration (Figs. 10 and 11).

**DISCUSSION**

In the present study, we have demonstrated that ENaC inhibition increases airway hydration and prevents CS-induced ASL height depletion, and the concomitant decreased CBF in vitro. Furthermore, we found that the increased ASL height and airway hydration manifest as increased MCC in vivo. These data suggest that ENaC inhibition may be efficacious in the restoration of mucus hydration and mucus transport in chronic bronchitis.

An ENaC inhibitor alters the transmembrane potential, as the natural flux of the positively charged sodium ion is hindered from crossing the apical plasma membrane. Using Ussing chambers, we identified Compound A as a potent (IC\textsubscript{50} 0.177 nM) and selective inhibitor of ENaC (Fig. 1) and consequently used it as a tool compound through all models described to link in vitro and in vivo efficacy with the physicochemically same properties. Also, the effects of benzamil and Compound A on \(I_{sc}\) were not additive, suggesting that they both inhibited the same pathway. We also measured Com...
compound A activity in vivo in the TPD model in anaesthetized guinea pigs. The efficacy of inhibitors is commonly measured using these two experimental procedures, and compounds are then compared with regard to potency and efficacy. However, electrophysiological data do not always translate into changes in airway hydration and MCC; electrophysiological approaches usually isolate a single species of channel and override cellular driving forces (39). In contrast, ASL hydration needs not only the target channel, but also ionic driving forces and water permeability to be acting in concert. Thus the potency of an ENaC inhibitor is only one feature of its physiochemical properties, and the likelihood that it will be efficacious in airway hydration and clearing mucus is subject to additional factors, including the residence time of the drug in the airway lumen. Thus optimization of compounds by looking only at the potency can be misleading. However, using two independent methods of measuring ASL volume and height, we also found that Compound A was efficacious and had a good duration of action under thin film conditions (Figs. 2 and 3) with an IC₅₀ of 0.052 μM. The discrepancy between the in vitro potencies can be explained by the fact that the Ussing chamber assay is a target engagement read out in a closed system, whereas the ASL height measures are functional. Also, the mass of drug applied under thin film conditions is vastly lower than the mass used in the Ussing chambers attributable to the big difference in volume, and, under thin-film conditions, the epithelia are capable of sequestering or removing compounds (40). Although this phenomenon is sometimes overlooked in preclinical development, it represents the situation that is mostly seen in the lung and may explain why amiloride had little effect on MCC in humans (26).

In the present TPD study (modified from Ref. 10), we chose to look at the online responses of the compounds with the ability to follow the effect after an intratracheal bolus dose, giving a sense of the duration of effect of the compounds and ability to get both potency and efficacy in the same experiment. The ED₅₀ for Compound A in the guinea pig TPD model was ~5 μg/kg. Using MCC as the readout, full efficacy was observed in rats at 100 μg/kg and in sheep at 75 μg/kg. It was also noted that the duration of efficacy of Compound A was shorter in rats compared with sheep. Taken together, these data indicate that ENaC needs to be completely inhibited to get full enhancement of MCC in the healthy animal. Furthermore, complete inhibition alone is not sufficient to positively impact on mucus clearance rates, and any useful compound must stay in the ASL compartment for extended periods [see amiloride both preclinically and clinically (20) and Figs. 10 and 11]. Whether one needs to fully restore MCC to normal levels, or whether any partial increase in MCC [reduced for patients with both CF and severe COPD (30, 41)], needs to be explored in the appropriate patient group(s) once this class of compounds is in clinical development.

The ovine model of MCC is to date the most commonly used preclinical model to estimate efficacy and duration of drugs with different mechanisms of action on MCC (1). However, these sheep are healthy, and data may only be translatable to humans with normal MCC and normal airway homeostasis. How this model translates to the diseased state in COPD or CF remains to be demonstrated. To mimic the diseased status of CF or COPD, neutrophil elastase can be given to induce reduction of MCC in the sheep model via its inhibitory mechanism on ENaC (33). However, this is still an animal model rather than the disease per se and does not necessarily translate into the effect by ENaC inhibitors in COPD or CF. CF piglets have recently been developed that can be used in the preclinical and pharmacological evaluation of MCC (34), but the downside of this model is that the animals get very sick within 24 h postpartum because of its intestinal phenotype of CF, despite attempts to correct the gut phenotype.

It is well known that an apical ENaC inhibitor for the treatment of respiratory diseases needs to exhibit minimal exposure in the distal tubuli in the kidneys to avoid local ENaC inhibition and alterations in renal electrolyte handling (2, 25). Compound A demonstrates a tendency to increase blood potassium levels 1–2 h after a therapeutic dose in sheep and has an effect on the rat renal handling of potassium at nanomolar exposures in both plasma and urine, clearly indicating the unsuitied therapeutic properties of this drug. However, it is still useful as a tool compound because of its selectivity and efficacy. The fate of GS-9411 was similar; despite unmeasurable levels of the parent compound in urine, metabolites caused alterations in renal electrolyte handling, and investigation was stopped in phase 1 (25). Recent data from O’Riordon et al. (24) demonstrated no effect on electrolyte homeostasis by the follow-up compound, GS-5737, in a phase 1 safety and pharmacokinetic study at doses 10-fold of what produced a maximal
effect on MCC in healthy sheep, suggesting that safe ENaC antagonists can be developed.

The present study demonstrates that a potent and selective ENaC inhibitor with a long duration of action can indeed increase ASL height and airway hydration in a dose-dependent manner in an in vitro culture setting for more than 6 h (Figs. 2 and 3). In contrast, short-acting ENaC antagonists such as amiloride are largely without effect under thin film conditions despite being efficacious in Ussing chambers (Figs. 1, 10, and 11). With regard to the gravimetric methods, the change here largely reflects the meniscus, which because of hydrostatic interactions with the plastic cell culture insert wall, is absorbed much more slowly, as described by Harvey et al. (14). As demonstrated in the present study and previously (9), acute CS induces a rapid decrease in ASL height because of removal of CFTR from the epithelial surface. Pretreatment with the concentration of Compound A that produced a maximal response on ASL height (1 µM) prevented CS-induced ASL dehydration by keeping ENaC antagonized and thus preventing abnormal homeostasis of ASL volume. After CS exposure, ENaC continues to function (9), and our data suggest that ENaC drives the actual decline in ASL volume when CFTR is no longer present to balance absorption with secretion (Fig. 8). Our findings indicate that an ENaC inhibitor would indeed prevent the dehydration caused by smoking and thus has the potential to be efficacious in the treatment of smoke-induced chronic bronchitis. Furthermore, the ENaC inhibitor was able to restore ASL height within 30 min after addition of Compound A compared with the 4 h that ASL recovery would normally take after CS exposure (9). To see whether pretreatment with compound A and protection against the CS-induced ASL dehydration would have additional functional consequences, we measured the HBEC CBF after CS exposure with or without Compound A in the periciliary liquid. The CBF at an ASL height of 7–8 µm was ~5 Hz and decreased to ~<4 Hz following CS-induced ASL dehydration, indicating the necessity for appropriate ASL hydration in ciliary beating. However, in this study, we did not see differences of statistical significance.

In conclusion, Compound A demonstrated significant effects on tracheal potential difference as well as MCC in both rats and sheep, demonstrating a link between increased airway hydration, ciliary function, and MCC. These data support the hypothesis that ENaC inhibition may be efficacious in the restoration of mucus hydration and mucus transport in patients with chronic bronchitis. ENaC inhibition prevents CS-induced ASL dehydration and the decrease in CBF as demonstrated by the use of this potent, selective, and long-lasting ENaC inhibitor (Compound A). Furthermore, ASL height restoration after CS was also significantly more rapid when Compound A was given after the CS-induced ASL depletion. Our data suggest that ENaC inhibition may be efficacious in the restoration of mucus hydration and mucus transport in chronic bronchitis and support further study of ENaC inhibitors in this patient population.

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ENaC INHIBITION RESTORES ASL HEIGHT AND MCC


