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Research Article

Pulmonology

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# Repurposing tromethamine as inhaled therapy to treat CF airway disease

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In cystic fibrosis (CF), loss of CF transmembrane conductance regulator (CFTR) anion channel activity causes airway surface liquid (ASL) pH to become acidic, which impairs airway host defenses. One potential therapeutic approach is to correct the acidic pH in CF airways by aerosolizing  $\text{HCO}_3^-$  and/or nonbicarbonate pH buffers. Here, we show that raising ASL pH with inhaled  $\text{HCO}_3^-$  increased pH. However, the effect was transient, and pH returned to baseline values within 30 minutes. Tromethamine (Tham) is a buffer with a long serum half-life used as an i.v. formulation to treat metabolic acidosis. We found that Tham aerosols increased ASL pH in vivo for at least 2 hours and enhanced bacterial killing. Inhaled hypertonic saline (7% NaCl) is delivered to people with CF in an attempt to promote mucus clearance. Because an increased ionic strength inhibits ASL antimicrobial factors, we added Tham to hypertonic saline and applied it to CF sputum. We found that Tham alone and in combination with hypertonic saline increased pH and enhanced bacterial killing. These findings suggest that aerosolizing the  $\text{HCO}_3^-$ -independent buffer Tham, either alone or in combination with hypertonic saline, might be of therapeutic benefit in CF airway disease.

## Introduction

Airways evolved with a thin layer of fluid, the airway surface liquid (ASL), that is rich in host defense mechanisms and strategically situated at the interface with the environment (1–9). Appropriate ASL volume, pH, and ionic composition are critical for optimal airway host defense (7, 10–19). In cystic fibrosis (CF), dysfunction of an anion channel, CF transmembrane conductance regulator (CFTR), inhibits 2 important airway host defenses: antimicrobial factors and mucociliary transport (20, 21). Loss of CFTR-mediated  $\text{HCO}_3^-$  secretion acidifies ASL pH and inhibits antimicrobial factors. Loss of  $\text{Cl}^-$  and  $\text{HCO}_3^-$  secretion also alters the viscoelastic properties of mucus and reduces mucociliary transport. With these and potentially other host defense impairments, the airways fail to eradicate and dispose of invading microorganisms. As a result, recurrent airway infections and inflammation cause a progressive decline in lung function. Despite advances in antibiotic therapy and mucus clearing maneuvers, airway disease continues to shorten the lives of people with CF (22, 23).

In newborn CF piglets, acidic ASL pH inhibits antimicrobial factors. Instillation of  $\text{HCO}_3^-$  onto the airway surface of CF piglet trachea raised ASL pH and enhanced bacterial killing (20). Conversely, acidifying the ASL of non-CF piglets by increasing  $\text{CO}_2$  tension inhibited bacterial killing. Small changes in ASL pH resulted in a large defect in bacterial killing due to the inhibitory effect of acidic pH on both individual antimicrobial factors and on their synergistic interactions (24).

Another important arm of the airway host defense is mucociliary transport. Mucociliary transport was impaired in vivo in CF piglets. In freshly excised trachea, impaired mucus detachment from CF submucosal glands disrupted mucociliary transport, although it remains uncertain whether defective  $\text{HCO}_3^-$  secretion, liquid secretion, or a combination are responsible. Additional studies revealed that an acidic pH increased ASL viscosity (25).

One potential therapeutic approach to raise ASL pH is to aerosolize pH buffers. Indeed, a clinical trial of aerosolized  $\text{HCO}_3^-$  in people with CF is ongoing (ClinicalTrials.gov). Tromethamine (Tham) or

**Conflict of interest:** The University of Iowa Research Foundation has submitted patent applications for CF pigs and has licensed materials and technologies to Exemplar Genetics. M.J. Welsh holds equity in Intrexon Corp., which owns Exemplar Genetics.

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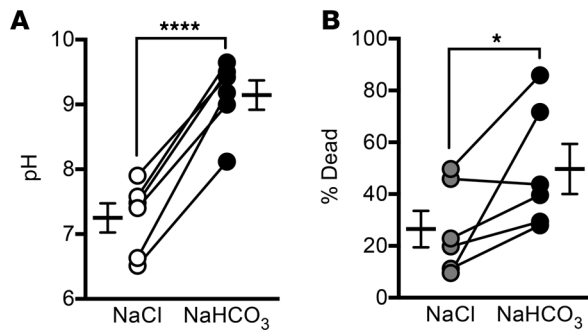
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**Figure 1. Cystic fibrosis (CF) sputum antimicrobial activity in the presence of NaHCO<sub>3</sub>.** (A) CF sputum pH and (B) CF sputum antimicrobial activity, in the presence of 200 mM NaCl or 200 mM NaHCO<sub>3</sub> (1:1 v/v) at 5% CO<sub>2</sub>. Data are mean ± SEM; some error bars are hidden by symbols. Each data point indicates sputum from a different donor  $n = 6$ . \* $P < 0.05$ , \*\*\*\* $P < 0.001$  from a Wilcoxon signed-rank test.

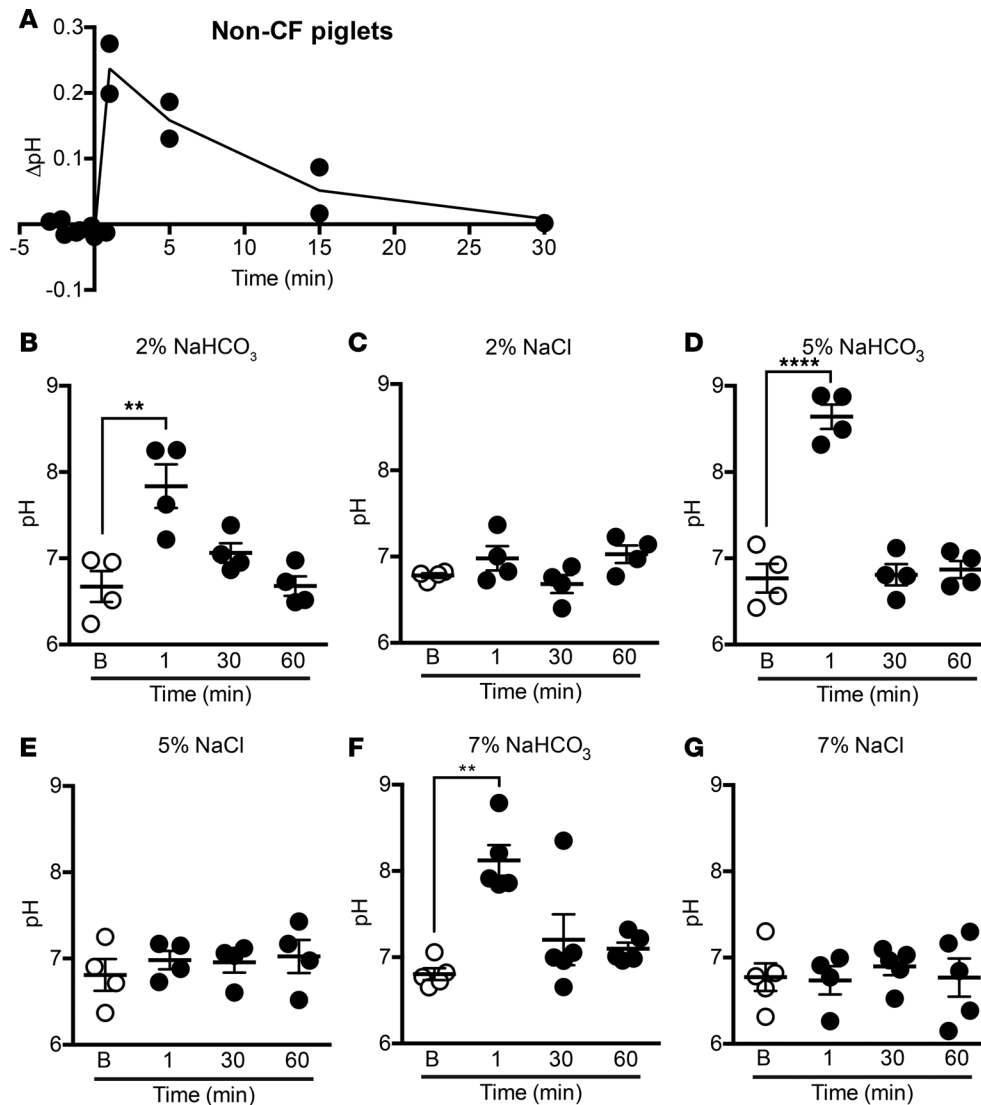
tris[hydroxymethyl]aminomethane acetate) is an FDA-approved buffer in clinical use, used to reverse metabolic acidosis (26–28). In contrast to the short-term effect of i.v. NaHCO<sub>3</sub>, Tham alkalizes serum with an effect that persists for 16–48 hours (28). Tham is also used as an excipient for inhaled preparations of prostacyclin (29) and nasal preparations of ketorolac (30). Because Tham has a long half-life and prolonged buffering capacity in serum, we hypothesized that inhaled Tham would increase ASL pH for a longer duration than HCO<sub>3</sub><sup>-</sup> and would enhance ASL bacterial killing. We tested this hypothesis in both pigs and humans with CF.

## Results

*NaHCO<sub>3</sub> increases the pH of CF sputum and enhances its antibacterial activity.* To investigate the effect of increasing CF sputum pH on bacterial killing, we collected sputa from individuals with CF. We mixed each sputum sample with an equal volume of either NaHCO<sub>3</sub> or NaCl. We measured pH in a humidified chamber at fixed 5% CO<sub>2</sub> using a planar opto-electrode. We also measured bacterial killing. Compared with an equimolar concentration of NaCl, NaHCO<sub>3</sub> increased sputum pH (Figure 1A). We interrogated the bacterial killing properties of CF sputum by examining the viability of *Staphylococcus aureus* (*S. aureus*), one of the first bacteria to infect CF lungs (23, 31, 32). We attached *S. aureus* to small gold grids and probed the antibacterial properties of the sputum-buffer mixture (20). We recovered the grids 15 minutes later and examined *S. aureus* viability. NaHCO<sub>3</sub> increased the CF sputum ability to kill *S. aureus* in comparison with an equimolar concentration of NaCl (Figure 1B). These data indicate that adding NaHCO<sub>3</sub> raises CF sputum pH and enhances its ability to rapidly kill bacteria.

*NaHCO<sub>3</sub> transiently increases nasal pH.* Nebulized NaHCO<sub>3</sub> is used safely in patients suffering from acute chlorine gas inhalational injury (33–35). In that clinical setting, the goal is an immediate neutralizing effect from NaHCO<sub>3</sub>. However, in CF airways, a longer effect may be required. To test the effect of NaHCO<sub>3</sub>, we aerosolized 250 μl of 200 mM NaHCO<sub>3</sub> onto the surgically exposed trachea of non-CF piglets and measured ASL pH. NaHCO<sub>3</sub> alkalized ASL pH, but the effect was short-lived and returned to baseline within 30 minutes (Figure 2A). In addition, we aerosolized 250 μl of 2% (238 mM) NaHCO<sub>3</sub> into the nose of healthy human volunteer subjects. Similar to our findings on the porcine tracheal surface, NaHCO<sub>3</sub> increased ASL pH, but pH returned to baseline within 60 minutes (Figure 2, B and C). Increasing the concentration of NaHCO<sub>3</sub> to 5% or 7% had no additional effect on the duration of the rise in nasal pH (Figure 2, D–G). Thus, these data suggest that the effect of NaHCO<sub>3</sub> on ASL pH is transient. Possible explanations include transepithelial HCO<sub>3</sub><sup>-</sup> absorption and/or a rapid shift of HCO<sub>3</sub><sup>-</sup> to CO<sub>2</sub> and H<sub>2</sub>O.

*HCO<sub>3</sub><sup>-</sup> is not required for a pH-related increase in ASL antimicrobial activity.* The transient effect of HCO<sub>3</sub><sup>-</sup> on ASL pH prompted us to find buffers with a longer duration. ASL antimicrobial activity is pH regulated, yet it remains uncertain whether HCO<sub>3</sub><sup>-</sup> regulates antimicrobial activity independently of its effect on pH. If HCO<sub>3</sub><sup>-</sup> is not required, then any buffer that increases ASL pH may have a therapeutic potential in CF airways. To investigate whether or not, at a constant pH, HCO<sub>3</sub><sup>-</sup> enhances cationic peptide activity, we tested the antimicrobial properties of human β-Defensin-3 (hBD-3; an antimicrobial peptide that is inhibited by ionic strength and Ca<sup>2+</sup>) (36) and LL-37 (an antimicrobial peptide that is not inhibited by ionic strength or Ca<sup>2+</sup>) (37) at different concentrations of HCO<sub>3</sub><sup>-</sup> and a constant pH of 7.5. We found that increasing HCO<sub>3</sub><sup>-</sup> concentration in the absence of Ca<sup>2+</sup> did not increase hBD-3 and LL-37 activity; in fact, it slightly inhibited their activity (Figure 3, A and B). In the presence of Ca<sup>2+</sup>, we found that increasing HCO<sub>3</sub><sup>-</sup> inhibited both hBD-3 and LL-37 antimicrobial activity (Figure 3, C and D). These data suggest that, under a constant

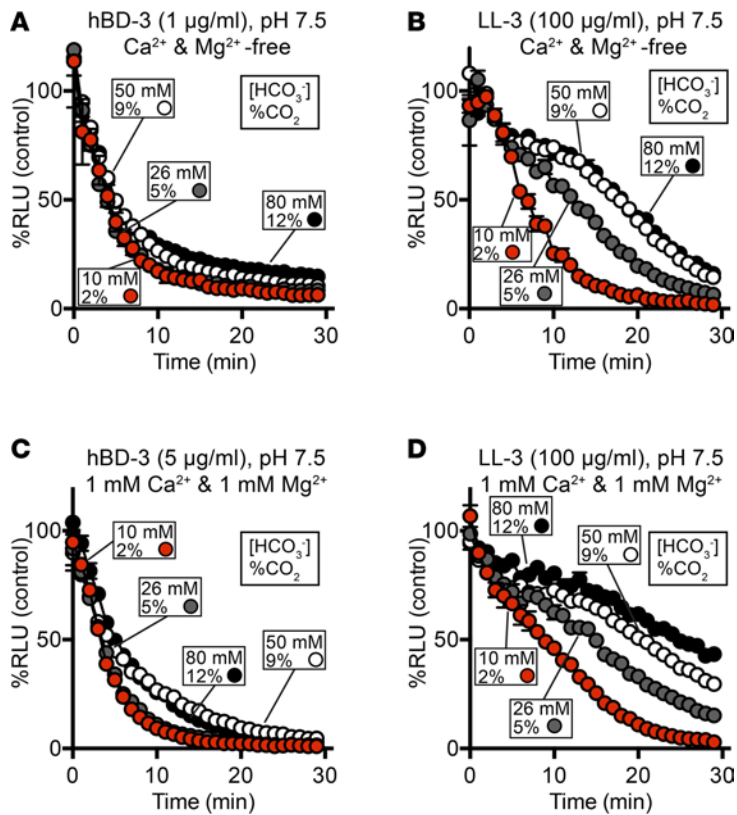


**Figure 2. Effect of NaHCO<sub>3</sub> on airway surface liquid (ASL) pH.** (A) Data represent change in tracheal ASL pH of WT newborn piglets after instillation of 250 µl 200 mM NaHCO<sub>3</sub>. Each data point indicates measurements from a different animal,  $n = 2$ . (B–G) Data represent human nasal pH at baseline and after instillation of 250 µl (B) 2% NaHCO<sub>3</sub>, (C) 2% NaCl, (D) 5% NaHCO<sub>3</sub>, (E) 5% NaCl, (F) 7% NaHCO<sub>3</sub>, and (G) 7% NaCl. Data are mean ± SEM; some error bars are hidden by symbols.  $n = 4–5$ , 1-way ANOVA, \*\* $P < 0.01$ , \*\*\*\* $P < 0.001$ .

alkaline pH, increasing HCO<sub>3</sub><sup>-</sup> concentrations does not enhance hBD-3 and LL-37 antimicrobial activity. On the contrary, it inhibits it to a small degree. These data, together with our prior finding that increasing pH enhances ASL antimicrobials (20, 24), suggest that any buffer that can increase pH might be used therapeutically to alkalinize ASL in CF.

**Tham buffers in the ASL physiological pH range.** One possible candidate is Tham (tris[hydroxymethyl]aminomethane), an amine compound with pKa of 8.07 at 25°C. Tham has a long half-life when given via i.v. and controls pH in the physiological range (27). We measured the buffering capacity of 0.3 M Tham and 0.3 M NaHCO<sub>3</sub> (equilibrated with 5% CO<sub>2</sub>). We titrated both buffers with 0.1 M HCl and calculated the buffering capacity ( $\beta$ ), the acid equivalents needed to change the pH of a buffer by 1 pH unit. By plotting the acid titration curve of both NaHCO<sub>3</sub> and Tham (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/jci.insight.87535DS1) and the first derivative (Supplemental Figure 1, C and D) against pH, we found that NaHCO<sub>3</sub> had a significant buffering capacity at a pH range from 6–7 (Supplemental Figure 1C), whereas Tham had a buffering capacity at a slightly broader pH range from 6.5–8.5 (Supplemental Figure 1D). Moreover, Tham has an additional buffering range at lower pH values (pH 3–6). These data suggest that Tham has an optimal buffering capacity that is within the pH range of both CF and non-CF ASL and a wider range than HCO<sub>3</sub><sup>-</sup>/CO<sub>2</sub> (38–40).

**Tham enhances hBD-3 and LL-37 antimicrobial activity.** An alkaline pH increases the activity of individual ASL antimicrobial peptides and their synergistic interactions (24). Therefore, we tested the effect of Tham on hBD-3 and LL-37 antimicrobial activity. We used a bioluminescence-based assay in which



**Figure 3. Antimicrobial activity of cathelicidin (LL-37) or human  $\beta$ -Defensin-3 (hBD-3) in the presence of varying concentrations of NaHCO<sub>3</sub>.** Data represent relative luminescence (RLU) of *S. aureus* (Xen-29) as a percentage of control (no added antimicrobial and same buffer conditions) at isohydric pH (same pH of 7.5), and varying concentrations of NaHCO<sub>3</sub>/CO<sub>2</sub> (red circles, NaHCO<sub>3</sub> 10 mM/CO<sub>2</sub> 2%; gray circles, NaHCO<sub>3</sub> 26 mM/CO<sub>2</sub> 5%; open circles, NaHCO<sub>3</sub> 50 mM/CO<sub>2</sub> 9%; closed circles, NaHCO<sub>3</sub> 80 mM/CO<sub>2</sub> 12%) in the presence (C and D) and in the absence (A and B) of Ca<sup>2+</sup> and Mg<sup>2+</sup>. Graphs depict (A) 1  $\mu$ g/ml hBD-3 and (B) 100  $\mu$ g/ml LL-37 in the absence of Ca<sup>2+</sup> and Mg<sup>2+</sup>. Graphs depict (C) 5  $\mu$ g/ml hBD-3 and (D) 100  $\mu$ g/ml LL-37 in the presence of 1 mM Ca<sup>2+</sup> and 1 mM Mg<sup>2+</sup>. Data are mean  $\pm$  SEM; some error bars are hidden by symbols. Results are from a single experiment in triplicate. Each experiment was repeated at least 3 times with similar results.

*S. aureus* Xen-29 was modified to express luminescence genes (41). Compared with an iso-osmolar nonionic control (pH = 7.02), Tham (pH = 8.65) enhanced antimicrobial activity of both hBD-3 (Figure 4A) and LL-37 (Figure 4B). These data suggest that increasing pH independently of HCO<sub>3</sub><sup>-</sup> concentration enhances hBD-3 and LL-37 antimicrobial activity.

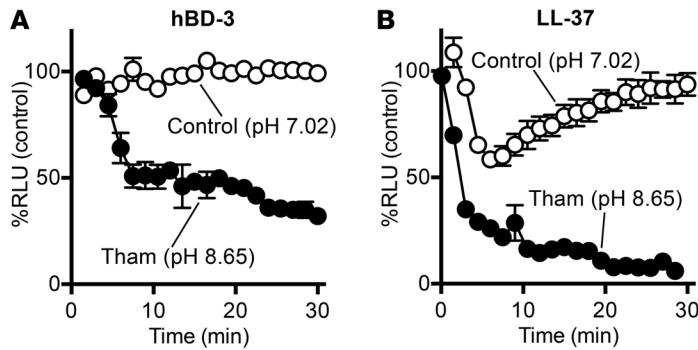
*Tham increases the pH of tracheal ASL in CF piglets and enhances bacterial killing.* To investigate whether Tham produces a prolonged increase in tracheal ASL pH, we aerosolized Tham onto the airways of non-CF piglets. Tham raised pH immediately after administration (Figure 5A). While the

effect of HCO<sub>3</sub><sup>-</sup> on ASL returned to baseline by 30 minutes (Figure 2A), the effect of Tham was still higher than baseline at 60 minutes (Figure 5A). We also tested whether aerosolized Tham rescues the bacterial killing defect in CF piglets. We exposed the mucosal surface of airways in newborn CF piglets via a small tracheal widow and aerosolized Tham. We measured ASL pH and bacterial killing 15 minutes after administration of Tham or NaCl as a control. Tham increased pH (Figure 5B) and enhanced bacterial killing compared with NaCl (Figure 5C). The increase in bacterial killing was more pronounced compared with what we found in CF sputum, perhaps because the antimicrobial factors in CF sputum may have been inactivated by proteases, thereby limiting the killing capacity of sputum when compared with ASL (42, 43). Thus, these data suggest that Tham increases ASL pH in a sustained manner and it corrects the bacterial killing defect seen in CF pigs.

*Tham increases human nasal pH, and the effect is prolonged.* To examine whether Tham could also provide a long-tasting buffering effect in human airways, we tested the effect of acute Tham administration on nasal pH. Aerosolized Tham alkalinized the nasal pH of healthy human subjects, with the effect lasting at least 2 hours (Figure 6A). Administration of an iso-osmolar solution of xylitol, a nonionic sugar, had no effect on nasal pH (Figure 6B). These findings are similar to the effect of Tham on tracheal ASL pH in pigs. We also investigated the effect of Tham on nasal pH in individuals with CF during one of their clinic visits. In the clinic setting, we were limited to pH measurements obtained 30 minutes after drug administration. Aerosolization of 250  $\mu$ l Tham increased nasal pH for 30 minutes, the total duration of the experiment (Figure 6C). Disruption of epithelial integrity in the nasal epithelia could lead to serum leakage and increase pH to serum levels. The high transepithelial nasal voltage (Vt) seen in CF patients requires an intact epithelia (44, 45). We found that Tham had no effect on nasal Vt (Figure 6D). Thus, Tham appears to produce no disruption of the epithelial barrier integrity, and increases in pH are likely secondary to a direct buffering effect on ASL.

*Tham in combination with hypertonic saline (7% NaCl) increases CF sputum pH and enhances bacterial killing.* Hypertonic saline is often used in individuals with advanced CF airway disease to accelerate mucociliary transport (46, 47). However, we and others reported that salt inhibits individual antimicrobial peptides and their synergistic interactions (48–52). Because Tham may be useful as a primary or adjunct inhalational therapy, we asked whether Tham would increase pH in the presence of hypertonic saline and whether a





**Figure 4. Effect of tromethamine (Tham) on cathelicidin (LL-37) or human  $\beta$ -Defensin-3 (hBD-3) antimicrobial activity.** Data represent relative luminescence (RLU) of *S. aureus* (Xen-29) as a percentage of control (no added antimicrobial and same buffer conditions) at an ionic strength of 125 mM (1% Tryptic Soy Broth, 100 mM NaCl) in the presence of 10 mM xylitol (nonionic control, open circles, pH 7.02) or 10 mM tromethamine (closed circles, pH 8.65) and (A) 1  $\mu$ g/ml hBD-3 or (B) 10  $\mu$ g/ml LL-37. Data are mean  $\pm$  SEM; some error bars are hidden by symbols. Results are from a single experiment in triplicate. Each experiment was repeated at least 3 times with similar results.

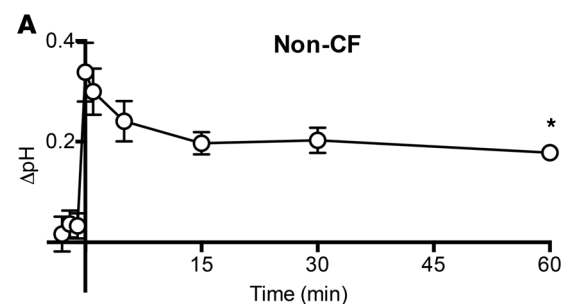
mixture of Tham and hypertonic saline would enhance bacterial killing. We collected sputa from CF subjects and mixed it in a 1:1 (w/v) dilution of Tham (300 mM), hypertonic saline (1,197 mM, 7% NaCl), or a combination of Tham (300 mM) and hypertonic saline (1,197 mM, 7% NaCl). Compared with hypertonic saline or isotonic saline (in Figure 1), Tham alone or in combination with hypertonic saline raised the pH of CF sputum (Figure 7A). Similar to the effect of  $\text{HCO}_3^-$  on CF sputum (Figure 1), Tham alone or in combination with hypertonic saline, increased the ability of CF sputum to kill *S. aureus* compared with hypertonic saline alone (Figure 7B). Although there was a tendency for lower bacterial killing with Tham and hypertonic saline in combination, in comparison with Tham alone, the difference was not statistically significant. Thus, Tham alone or in combination with hypertonic saline is effective at increasing ASL pH and reversing the bacterial killing defect in CF sputum.

## Discussion

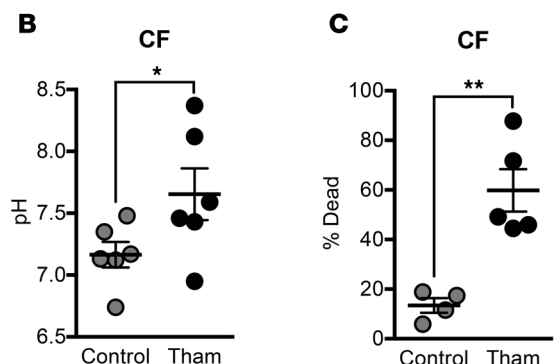
Loss of the CFTR anion channel reduces ASL pH. Our results suggest that both  $\text{HCO}_3^-$  and Tham increase ASL pH in pigs and humans with CF. However, Tham increased ASL pH for a longer duration than  $\text{HCO}_3^-$ . Tham also enhanced ASL bacterial killing in pig airways and human CF sputum. Thus, increasing ASL pH with Tham might enhance host defense and thereby benefit people with CF.

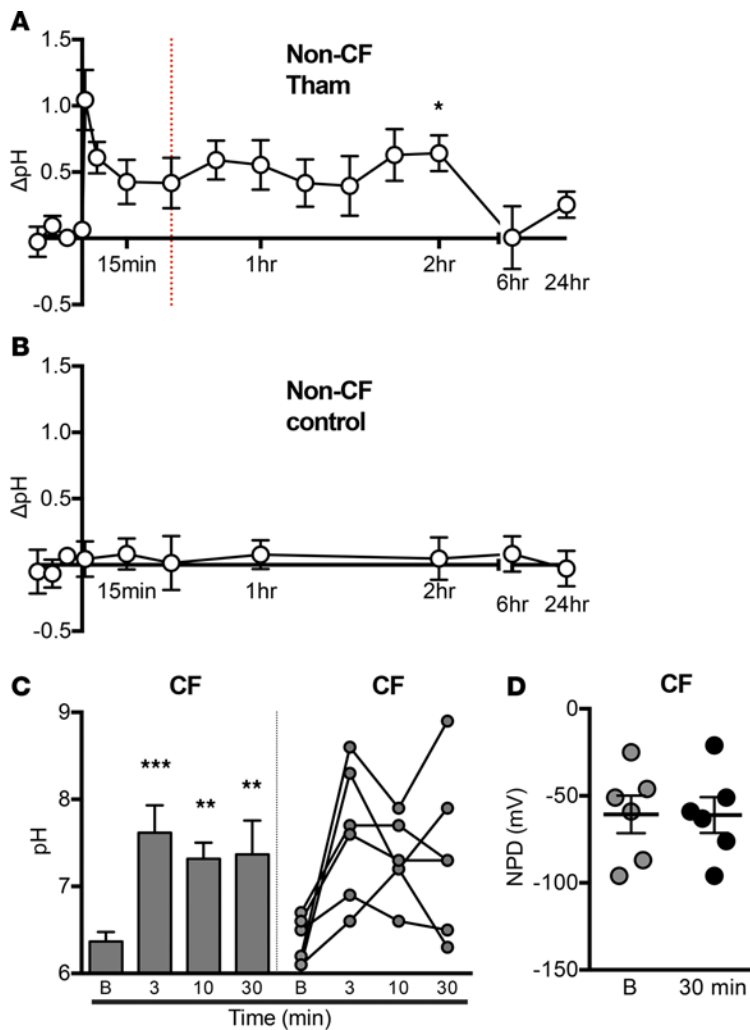
The surface of the airways lies in proximity with the environment and is constantly exposed to microorganisms. The liquid covering the airways contains a plethora of cationic antimicrobial factors that form the first line of defense against invading bacteria. In previous work, we showed that an abnormally acidic ASL inhibits the activity of individual antimicrobial factors and their combined synergistic interaction (24). Yet, whether the contribution of pH-mediated antimicrobial factor inhibition is  $\text{HCO}_3^-$  dependent has been uncertain. Here, we show that, both in the presence and absence of  $\text{Ca}^{2+}$ ,  $\text{HCO}_3^-$  did not increase cationic peptide bacterial killing when pH was constant. On the contrary, there was a small inhibition. This result suggested that pH buffers other than  $\text{HCO}_3^-$ , such as Tham, might increase ASL antimicrobial activity.

In this study, we identified an FDA-approved i.v. buffer that might be repurposed as



**Figure 5. Effect of tromethamine (Tham) on tracheal airway surface liquid (ASL) pH of newborn piglets and ASL bacterial killing.** (A) Data represent change in tracheal ASL pH of WT newborn piglets after instillation of 250  $\mu$ l 0.3 M tromethamine. Data are mean  $\pm$  SEM,  $n = 3$ , Wilcoxon signed-rank test,  $*P < 0.05$ . (B) ASL pH in exposed tracheal window of CF untreated piglets (gray circles) and 15 minutes after tromethamine treatment (closed circles). Data are mean  $\pm$  SEM; some error bars are hidden by symbols.  $n = 6$ , Wilcoxon signed-rank test,  $*P < 0.05$ . (C) ASL bacterial killing in exposed tracheal window of CF untreated piglets (gray circles,  $n = 4$ ) and 15 minutes after tromethamine treatment (closed circles,  $n = 4-5$ ). Data are mean  $\pm$  SEM, Wilcoxon-Mann-Whitney test,  $**P < 0.01$ .





**Figure 6. Effect of tromethamine (Tham) on nasal pH measurements.** Data represent nasal pH of healthy volunteers after instillation of 250  $\mu$ l (A) 0.3 M tromethamine (red dashed line represent the duration of the effect of HCO<sub>3</sub><sup>-</sup>) or (B) 0.3 M xylitol (nonionic control). Data are mean  $\pm$  SEM; some error bars are hidden by symbols,  $n = 5$ , Wilcoxon signed-rank test, \* $P < 0.05$ . (C) Nasal pH of subjects with cystic fibrosis (CF) at baseline and after instillation of 250  $\mu$ l tromethamine. Bar graph on the left represents the mean pH  $\pm$  SEM. The graph on the right shows pH data from individual subjects over time. Data are mean  $\pm$  SEM; some error bars are hidden by symbols,  $n = 6$ , 1-way ANOVA with Holm-Sidak's Multiple Comparison Test, \*\* $P < 0.01$ , \*\*\* $P < 0.005$ . (D) Transepithelial voltage (Vt) in mV of CF subjects at baseline (B, gray circles) and 30 minutes (closed circles) after exposure to 250  $\mu$ l 0.3 M tromethamine.  $n = 6$ , Wilcoxon signed-rank test.

an aerosol to alkalinize the ASL of CF airways (53). Tham is a biologically inert weak amine base with an elimination half-life of 16–48 hours after i.v. administration (28). In addition to i.v. usage, Tham is included as an expedient in many topical, injectable, and/or inhalational FDA-approved drugs. Both iloprost inhalation solution (Ventavis) and nasal Ketorolac Tham (SPRIX) are formulated with Tham with a safe toxicological profile. Similar to its effect on pH in the serum, in the airways, Tham may alkalinize the ASL by buffering protons. In the serum of patients with acidosis, Tham is protonated. Because protonated Tham is cell impermeable, cellular absorption is reduced, and as a result, the effect on serum pH is sustained (28). A similar mechanism may be responsible for the prolonged effect in the airways.

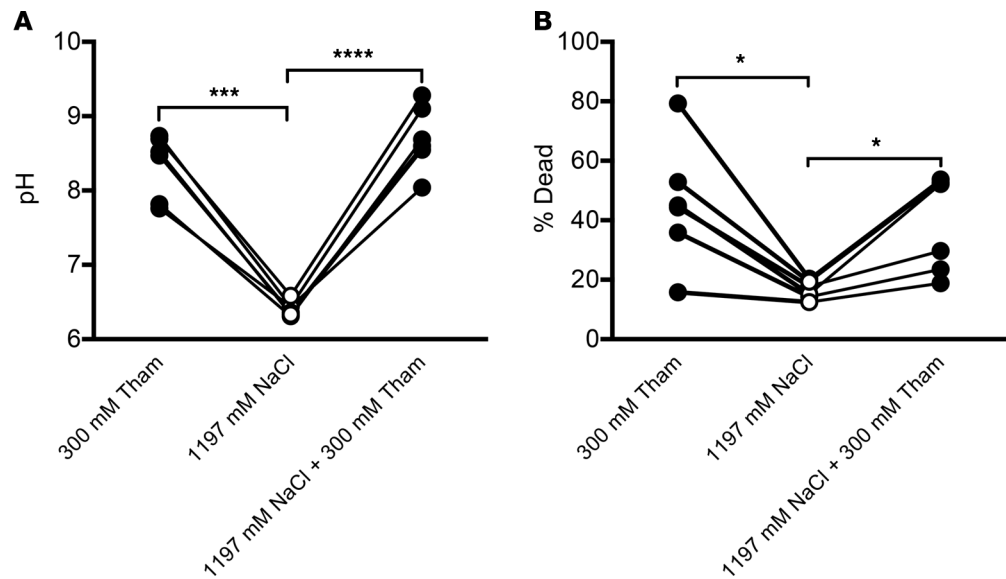
Loss of CFTR reduces airway epithelial HCO<sub>3</sub><sup>-</sup> secretion, diminishes ASL pH, and interferes with at least 2 important host defenses: antibacterial activity and mucociliary transport. Each defect may independently contribute to lung disease in CF (10). Treating people with CF

with antibiotics improves their clinical status, without correcting the mucus abnormalities. Accordingly, inhaled Tham might be beneficial, even if it only corrects the antimicrobial defect. While increasing ASL pH will change the viscoelastic properties of CF mucus, it is unclear whether such changes in pH and viscosity would enhance MCT.

This study has several advantages: (i) we studied 2 species, humans and pigs; (ii) we examined the effect of Tham on host defense both in vivo and in vitro; (iii) we tested pristine uninfected airways, as well as sputum from people with advanced CF airway disease; and (iv) we assayed ASL pH and a host defense defect associated with CF airway disease.

This study has also limitations. The experiments were not designed to measure the half-life of Tham in ASL. Moreover, we studied an important pathogen in early CF airway disease, *S. aureus*. However, *P. aeruginosa* is the predominant pathogen in advanced CF airway disease. Therefore, it remains to be determined whether inhaled Tham will also restore killing of *P. aeruginosa*. Lastly, our studies examined a transient effect of Tham and raised the question of whether intermittent correction of the ASL pH is sufficient to reverse the bacterial killing host defense defect in CF airways. Should therapy be targeted toward early (infant) or late (adult) CF airway disease? Additional longitudinal studies in pigs and humans will be designed to answer these questions.

Hypertonic saline increases mucociliary transport and decreases the frequency of CF exacerbations (46, 54–58). We and others have previously shown that salt inhibits ASL cationic peptides (52, 59). Our data show that Tham increases sputum pH and improves bacterial killing even in the presence of hypertonic saline. We speculate that perhaps a combination of Tham and hypertonic saline might be of therapeutic benefit in CF airways.



**Figure 7. Antimicrobial activity of sputum from subjects with cystic fibrosis (CF).** Data represent pH and antimicrobial activity of CF sputum mixed at 1:1 (v/v) with 300 mM tromethamine (Tham), 1,197 mM NaCl, or a combination of 1,197 mM NaCl/300 mM Tham. **(A)** CF sputum pH. **(B)** CF sputum antimicrobial activity. Each data point indicates sputum from a different donor.  $n = 6$ , 1-way ANOVA with Holm-Sidak's Multiple Comparison Test,  $*P < 0.05$ ,  $***P < 0.005$ ,  $****P < 0.001$ .

## Methods

**CF sputum preparation.** We recruited adults diagnosed with CF and attending the University of Iowa Hospital and Clinics CF clinic. Patients were asked to swallow saliva and to expectorate all secretions during routine clinic visits. Sputum plugs were visually identified and separated from saliva, weighed, and stored at  $-70^{\circ}\text{C}$  in small, tightly sealed containers to minimize water loss. Sputum was mixed in a 1:1 v/v with either 200 mM NaCl, 200 mM  $\text{NaHCO}_3$ , 1,197 mM NaCl, 300 mM Tham, or 1,197 mM NaCl/300 mM Tham. To guarantee adequate mixing of sputum with the different solutions, the mixture was homogenized (Sonic Dismembrator Model 100, Fisher Scientific) for 30 seconds. All experiments were conducted in a humidified chamber with constant 5%  $\text{CO}_2$ .

**pH measurements.** To measure nasal pH, we used a Sandhill ZepHr PHNS-P (Sandhill Scientific) Mobidium pH probe with an internal reference electrode as previously described (40). Prior to each study, the pH probe was calibrated in buffer solutions of pH 6, 7 and 8 (VWR).  $V_t$  was recorded with an Oakton pH6+ meter (Cole-Palmer) and corrected to temperature. The probe was positioned at 6 cm from the most caudal aspect of the columella. The probe remained in position until the readings were stable. All measurements were taken from the right nostril and by the same operator. Subjects on intranasal medications (steroids or anticholinergics) or with history of nasal or sinus surgery were excluded from the study. Adverse events, including nasal congestion or epistaxis, were collected but none reported.

To measure pH in sputum, we used a needle-type fiber optic pH meter (World Precision Instruments) (20). The pH meter was calibrated before each set of measurements using standard pH buffer solutions. All measurements were obtained in a humidified chamber at  $37^{\circ}\text{C}$  and constant 5%  $\text{CO}_2$  to mimic physiologic conditions.

To measure pH in pigs in vivo, we used noninvasive dual lifetime referencing to interrogate a  $3 \times 3$  mm planar optode (pH sensitive foil, PreSens GmbH) applied directly to the tracheal surface of anesthetized pigs (20). pH-dependent polarized light phase shift was measured using a single-channel pH meter (pH-1 mini; PreSens GmbH). The tip of the fiber optic pH meter was kept at the same constant distance from the tracheal surface in all samples and confirmed by recording the amplitude. Calibration before each set of measurements was done by measuring phase shift from flat filters soaked in standard pH buffers. All measurements were obtained in a humidified chamber at  $37^{\circ}\text{C}$  and constant 5%  $\text{CO}_2$  to mimic physiologic conditions.

**Nasal  $V_t$  measurements.** Nasal  $V_t$  measurements were obtained in subjects with CF after Tham administration according to the description by Solomon et al. (60) with Electronic Data Capture (ADInstruments), KCI



calomel electrodes (Thermo Fischer Scientific Inc.), and 3% agar nasal catheter and reference bridges (61).

*Methods for measuring buffering capacity of  $\text{HCO}_3^-$  and Tham.* An automated endpoint titration was carried out using Titralab 856 workstation (Radiometer Analytical). We measured pH using calomel combined pH electrode (pHC4000, Radiometer Analytical). HCl (100 mM) was delivered into 5 ml of buffer at a rate of 0.2 ml/min. Titration was carried out to endpoint of pH 3. The volume of HCl delivered was recorded and used to calculate the acid equivalents needed to raise pH by 1 unit or buffering capacity.

*Methods for administering  $\text{HCO}_3^-$  or Tham.* To alter tracheal pH in pigs using  $\text{HCO}_3^-$  or Tham, we aerosolized the solutions to the exposed tracheal surface as previously described (20). Pigs were initially sedated with Ketamine (Ketaject, Phoenix; 20 mg/kg, i.m. injection) and Xylazine (AnaSed, Lloyd; 2 mg/kg, i.m. injection) and anesthetized using Propofol (Diprivan, Fresenius Kabi; 2 mg/kg, i.v. injection). The trachea was surgically exposed and accessed anteriorly. A small anterior window through the tracheal rings accessed a tracheal window to interventions and pH measurements. All studies were obtained in a 100% humidified chamber at 37°C and constant 5%  $\text{CO}_2$  to mimic physiologic conditions.

To change nasal pH in human nostrils, we administered Tham intranasally using a 250  $\mu\text{l}$  preloaded Accuspray syringe (BD Biosciences) (40).

*Bacterial killing assays.* To interrogate individual bacteria killing in CF sputum or in pigs, we used bacteria-coated grid assays (20, 24). Gold grids (200 mesh, Ted Pella) were functionalized using a series of intervention: 100 mM 11-mercaptoundecanoic acid (MUA in 100% alcohol, Sigma-Aldrich) for 60 minutes at room temperature; 1:1 mixture of 100 mM N-ethyl-N-(3-diethylaminopropyl) carbodiimide (EDC); 100 mM N-hydroxysuccinimide (NHS) for 30 minutes at room temperature; and 0.1 mg/ml Neutravidin (Sigma-Aldrich) at 37°C for 1 hour.

We used 2 different strains of *S. aureus* (SH1000, a human strain for CF sputum killing studies, and SA43, a porcine strain for pig studies). Bacteria were grown to mid-log phase, incubated with 0.2 mg/ml N-hydroxy-sulphosuccinimide-biotin (sulpho-NHS-biotin) for 60 minutes at room temperature, quenched with 100 mM glycine to bind excess free biotin, and allowed to attach to Neutravidin-coated grids for 15 minutes prior to the experiment.

After bacteria-coated grids were exposed to pig trachea for 5 minutes or CF sputum for 15 minutes, they were immediately rinsed in phosphate buffered saline and stained with SYTO9/propidium iodide (Live/Dead BacLight Bacterial viability assay, Invitrogen), mounted on slides, and imaged on a laser-scanning confocal microscope (Olympus FV1000). Live (green) and dead (red) bacteria were automatically counted using Image-based Tool for Counting Nuclei (ITCN 1.6, UCSB Center for Bio-Image Informatics) an ImageJ (NIH) plugin.

*Antimicrobial factors and luminescence antibacterial assay.* ASL antimicrobial factors included recombinant human  $\beta$ -defensin-3 hBD-3 (Peptotech) and human LL-37 (AnaSpec). Cationic peptides were dissolved in acidified water (0.01% acetic acid) that contained 0.1% BSA.

We used *S. aureus* Xen-29 (Caliper LifeSciences Bioware). *S. aureus* Xen-29 was derived from *S. aureus* 12600, a pleural fluid isolate, which is also designated as NCTC8532. *S. aureus* Xen-29 possesses a stable copy of the modified *Photobacterium luminescens luxABCDE* operon at a single integration site on the bacterial chromosome. For maintenance of luminescence, the bacteria were grown in Tryptic Soy Broth (TSB) in the presence of kanamycin (10  $\mu\text{g}/\text{ml}$ ).

Antimicrobial peptide activity was tested in a buffer composed of 1% TSB (casein peptone 17 g/l, soya peptone 3 g/l, NaCl 5 g/l,  $\text{K}_2\text{HPO}_4$  2.5 g/l, glucose 2.5 g/l) and supplemented with 10 mM potassium phosphate buffer with pH adjusted by varying the ratio of monobasic to dibasic phosphate and 100 mM NaCl to achieve an ionic strength of 125 mM.

Bacteria were grown overnight at 37°C in medium described above, diluted 1:100, and grown to exponential phase. Bacteria were harvested by centrifugation and suspended in the 1% TSB buffer. Bacteria ( $5 \times 10^4$  CFU) were incubated with antimicrobial factors in 96-well plates (Optiplat; Packard Instruments) in a total volume of 120  $\mu\text{l}$ . Luminescence was measured with a luminometer (Spectra Max L, Molecular Devices) and reported as relative light units (RLU). A previous study determined that reductions in luminescence have an excellent correlation with a decrease in CFU (59). All experiments included control bacteria that did not receive antimicrobials but were incubated in buffer of identical ionic strength and pH. Data are shown as relative luminescence as a percentage of control (RLU % control).

*Preparation of isohydric (same pH) solutions.* To test antimicrobial peptide activity under the same pH conditions and increasing concentrations of  $\text{HCO}_3^-$ , we used 4 solutions all adjusted to the same pH of 7.5

and the same ionic strength (~161 mM in the presence of CaCl<sub>2</sub> or MgSO<sub>4</sub> and ~154 mM in the absence of CaCl<sub>2</sub> or MgSO<sub>4</sub>) and supplemented with 1% TSB. To test the effect of increasing HCO<sub>3</sub><sup>-</sup> concentrations at the same pH in the absence of calcium or magnesium, we used the same 4 solutions without the addition of CaCl<sub>2</sub> or MgSO<sub>4</sub>:

10 mM NaHCO<sub>3</sub>: NaCl 140 mM, KCl 3 mM, CaCl<sub>2</sub> 1 mM, **NaHCO<sub>3</sub> 10 mM**, NaH<sub>2</sub>PO<sub>4</sub> 1.25 mM, MgSO<sub>4</sub> 1 mM, D-glucose 10 mM, **CO<sub>2</sub> 2%**.

26 mM NaHCO<sub>3</sub>: NaCl 124 mM, KCl 3 mM, CaCl<sub>2</sub> 1 mM, **NaHCO<sub>3</sub> 26 mM**, NaH<sub>2</sub>PO<sub>4</sub> 1.25 mM, MgSO<sub>4</sub> 1 mM, D-glucose 10 mM, **CO<sub>2</sub> 5%**.

50 mM NaHCO<sub>3</sub>: NaCl 100 mM, KCl 3 mM, CaCl<sub>2</sub> 1 mM, **NaHCO<sub>3</sub> 50 mM**, NaH<sub>2</sub>PO<sub>4</sub> 1.25 mM, MgSO<sub>4</sub> 1 mM, D-glucose 10 mM, **CO<sub>2</sub> 9%**.

80 mM NaHCO<sub>3</sub>: NaCl 70 mM, KCl 3 mM, CaCl<sub>2</sub> 1 mM, **NaHCO<sub>3</sub> 80 mM**, NaH<sub>2</sub>PO<sub>4</sub> 1.25 mM, MgSO<sub>4</sub> 1 mM, D-glucose 10 mM, **CO<sub>2</sub> 12%**.

**Animals.** We studied both female and male newborn pigs with targeted disruption of the *CFTR* gene *CFTR*<sup>-/-</sup>, generated from mating *CFTR*<sup>+/-</sup> pigs. We also studied the WT littermates. We obtained all pigs from Exemplar Genetics.

**Statistics.** Data are presented as points from individual humans or animals or sputum samples obtained from individual donors, with mean ± SEM indicated by bars. For statistical analysis, we used a Wilcoxon signed-rank test or a Wilcoxon Mann Whitney test to compare 2 groups and a 1-way ANOVA for multiple comparisons. In Figure 7, A and B, and Figure 6C, we used Holm-Sidak's Multiple Comparison Test for pairwise differences in pH or bacterial killing. Differences were considered statistically significant at *P* < 0.05.

**Study approval.** The University of Iowa Animal Care and Use Committee approved all animal studies. The University of Iowa IRB approved all human studies and collection of sputum samples. A written informed consent was received from participants prior to inclusion in the study.

## Author contributions

MHAA, MJW, DAS, and JZ designed studies. MHAA, JLL, NDG, KAS, JAR, and PJT conducted experiments and acquired data. MHAA, DAS, MJW, and JZ analyzed data. MHAA, DAS, PST, MJW, and JZ wrote the manuscript.

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