EUROPEAN RESPIRATORY *journal*

FLAGSHIP SCIENTIFIC JOURNAL OF ERS

Early View

Original research article

A novel thiol-saccharide mucolytic for the treatment of muco-obstructive lung diseases

Annalisa Addante, Wilfred Raymond, Irina Gitlin, Annabelle Charbit, Xavier Orain, Aaron Wolfe Scheffler, Aditi Kuppe, Julia Duerr, Maria Daniltchenko, Marika Drescher, Simon Y. Graeber, Anne-Marie Healy, Stefan Oscarson, John V. Fahy, Marcus A. Mall

Please cite this article as: Addante A, Raymond W, Gitlin I, *et al*. A novel thiol-saccharide mucolytic for the treatment of muco-obstructive lung diseases. *Eur Respir J* 2023; in press (https://doi.org/10.1183/13993003.02022-2022).

A novel thiol-saccharide mucolytic for the treatment of muco-obstructive lung diseases

Annalisa Addante[1,2], Wilfred Raymond[3], Irina Gitlin[3], Annabelle Charbit[3], Xavier Orain[3], Aaron Wolfe Scheffler[4], Aditi Kuppe[1,2], Julia Duerr[1,2], Maria Daniltchenko[1], Marika Drescher[1], Simon Y. Graeber[1,2,5], Anne-Marie Healy[6], Stefan Oscarson[7], John V. Fahy[3,8]* and Marcus A. Mall[1,2,5]*

[1]Department of Pediatric Respiratory Medicine, Immunology and Critical Care Medicine, Charité - Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

[2]German Centre for Lung Research (DZL), associated partner, Berlin, Germany

[3]Cardiovascular Research Institute, University of California San Francisco, San Francisco, CA, United States

[4]Department of Epidemiology and Biostatistics, University of California San Francisco, San Francisco, CA, United States

[5]Berlin Institute of Health (BIH) at Charité – Universitätsmedizin Berlin, Berlin, Germany

[6]School of Pharmacy and Pharmaceutical Sciences, Trinity College Dublin, Dublin, Ireland

[7]Centre for Synthesis and Chemical Biology, University College Dublin, Belfield, Dublin 4, Ireland

[8]Division of Pulmonary and Critical Care Medicine, University of California San Francisco, San Francisco, CA, United States

* J.V.F. and M.A.M. contributed equally as senior authors.

Corresponding author: Marcus A. Mall, M.D., Department of Pediatric Respiratory Medicine, Immunology and Critical Care Medicine, Charité - Universitätsmedizin Berlin, Augustenburger Platz 1, 13353 Berlin, Germany; Phone: +49 30 450 566 182, Fax: +49 30 450 566 931, Email: Marcus.Mall@charite.de

Take home message: MUC-031, a novel thiol-saccharide mucolytic drug, is potent and fast acting in rheology-based sputum assays and improves mucus obstruction, airway inflammation and survival in a mouse model of muco-obstructive lung disease.

Word count: 4442

Abstract

Background: Mucin disulfide cross-links mediate pathologic mucus formation in muco-obstructive lung diseases. MUC-031, a novel thiol-modified carbohydrate compound, cleaves disulfides to cause mucolysis. The aim of this study was to determine the mucolytic and therapeutic effects of MUC-031 in sputum from patients with cystic fibrosis (CF) and mice with muco-obstructive lung disease (βENaC-Tg mice).

Methods: We compared the mucolytic efficacy of MUC-031 and existing mucolytics (N-acetyl cysteine [NAC] and rhDNase) using rheology to measure the elastic modulus (G') of CF sputum, and we tested effects of MUC-031 on airway mucus plugging, inflammation and survival in βENaC-Tg mice to determine its mucolytic efficacy *in vivo*.

Results: In CF sputum, compared to the effects of rhDNase and NAC, MUC-031 caused a larger decrease in sputum G', was faster in decreasing sputum G' by 50%, and caused mucolysis of a larger proportion of sputum samples within 15 minutes of drug addition. Compared to vehicle control, three treatments with MUC-031 in one day in adult βENaC-Tg mice decreased airway mucus content (16.8±3.2 vs. 7.5±1.2 nl/mm^2, P<0.01) and bronchoalveolar lavage cells (73,833±6,930 vs. 47,679±7,736 cells/ml, P<0.05). Twice daily treatment with MUC-031 for two weeks also caused decreases in these outcomes in adult and neonatal βENaC-Tg mice and reduced mortality from 37% in vehicle-treated to 21% in MUC-031 treated in βENaC-Tg neonates (P<0.05).

Conclusion: MUC-031 is a potent and fast acting mucolytic that decreases airway mucus plugging, lessens airway inflammation, and improves survival in βENaC-Tg mice. These data provide rationale for human trials of MUC-031 in muco-obstructive lung diseases.

Word count: 253

Keywords: mucus plugs; mucolytic; cystic fibrosis; MUC-031; N-acetylcysteine

Introduction

Pathologic mucus occludes airways to decrease airflow and cause airway infection and inflammation in multiple chronic muco-obstructive lung diseases including cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) [1, 2]. Important mechanisms of pathologic mucus formation include increases in the concentration of mucins [3, 4] and non-mucin polymers (e.g. DNA), and increases in mucin disulfide crosslinks caused by oxidant acids generated by the activity of peroxidases secreted by neutrophils in chronic airway inflammation [5].

Thiol drugs have mucolytic effects because they cleave disulfide bridges between mucins through a chemical process of thiol exchange [6]. They can also scavenge reactive oxygen species to have anti-oxidant effects [7]. Relatively few thiol drugs have been formulated for inhaled delivery, the preferred route of administration to achieve mucolysis. The most widely used inhaled thiol drug is N-acetylcysteine (NAC), a drug whose clinical efficacy is limited by a side effect of bronchoconstriction. Mechanisms of NAC-induced bronchoconstriction include its hyperosmolar formulation and potential for sulfite generation when cysteine is catabolized by aminotransferases [8]. We previously reported that carbohydrates can be functionalized with a thiol group to generate thiol-saccharide compounds with potency advantages over NAC [5]. Additionally, because aminotransferases do not act on carbohydrates, thiol-saccharides will not generate sulfite catabolites.

Airway mucus plugging is increasingly recognized as an important cause of airflow obstruction in a spectrum of chronic lung diseases beyond CF. For example, identification and scoring of mucus plugs in computed tomography (CT) lung scans from patients with COPD and asthma revealed large subgroups of patients with high mucus plug scores that associated strongly with measures of airflow obstruction [9, 10]. There are few mucolytic drug options for these patients. Although recombinant human deoxyribonuclease I (rhDNase) and hypertonic saline (HS) are effective in patients with CF [11-13], they have not shown efficacy in COPD or bronchiectasis

[14-17], and there is a high unmet need for a well-tolerated and effective mucolytic for COPD and other muco-obstructive lung diseases .

MUC-031 is a novel thiol-saccharide mucolytic developed as part of an NHLBI-funded translational program project grant [18]. The efficacy of mucolytic drugs like MUC-031 can be tested *in vitro* using a cone and plate rheometer to quantify the elastic and viscous properties of sputum before and after drug treatment, and *in vivo* in animal models of muco-obstructive lung disease. Mice overexpressing the β-subunit of the epithelial sodium channel in their airways (βENaC-Tg) develop key features of muco-obstructive lung disease including airway mucus plugging, airway inflammation, and early mortality [19-21]. Here we set out to use rheology to determine if MUC-031 is a more potent mucolytic than rhDNase and NAC. We also tested if MUC-031 decreases airway mucus plugging and inflammation and improves survival in βENaC-Tg mice.

Methods

Key details about experimental methods are provided below and additional details are in the online supplement.

Synthesis of MUC-031

We have proposed thiol-modified carbohydrates ("thiol-saccharides") as novel thiol-based mucolytics because of their efficacy and favorable physico-chemical properties for inhaled delivery [5]. Carbohydrate scaffolds are natural and non-toxic and their polar nature and high aqueous solubility leads to ease of penetration into glycosylated mucin gels. In addition, the abundance of hydroxyl groups and chiral centers on carbohydrate scaffolds allows many possibilities for the introduction of a thiol group and subsequent structure-activity relationship studies. For example, we have shown that introduction of a thiol group in the 6-position of galactose generates a thiol saccharide (methyl 6-thio-6-deoxy-α-D-galactopyranoside) with better mucolytic activity than N-acetyl cysteine [5]. Methyl 6-thio-6-deoxy-α-D-galactopyranoside and MUC-031 are part of a library of approximately 30 thiol-sacccharides synthesized by the Centre for Synthesis and Chemical Biology in University College Dublin (UCD) using synthetic chemistry approaches similar to that previously described for methyl 6-thio-6-deoxy-α-D-galactopyranoside [5]. Additional representative structures have been disclosed in the patent literature [22, 23]. MUC-031 was chosen as the thiol-saccharide for further clinical development by the translational program project grant investigators for reasons related to its mucolytic potency and solution stability. Once selected as the lead, large scale synthesis of MUC-031 was done at Cascade Chemistry, Inc. (Eugene, OR, USA).

Study participants

Induced sputum was collected from healthy controls using a 12 minute induction protocol previously described [24]. Spontaneously expectorated sputum samples were collected from adult patients with CF according to protocols and informed consent procedures approved by

the Committee on Human Research at the University of California, San Francisco (UCSF). Demographics and clinical characteristics of study participants are shown in table 1.

Sputum rheology

The elastic (G') and viscous (G'') moduli of sputum were measured using a cone and plate rheometer (AR-2000 and DHR-2 devices, TA Instruments, New Castle, DE, USA) [5]. Detailed methods are provided in the online supplement. Briefly, aliquots of sputum were interrogated in a strain-controlled mode by oscillating the cone geometry at 5% strain and measuring the torque. After completion of baseline measurements, the geometry was raised and test compound solution was added and mixed with the sputum. Timed oscillation measurements were taken repeatedly at 2-minute intervals for at least 30 minutes (figures 1a, 1b). To accommodate baseline variability in sputum G' and G", a varying-coefficient model (VCM) was estimated via a generalized additive modeling framework (GAM). Each sample tested is treated independently by GAM, including separate aliquots from the same donor. The model generates a smooth term βj as function of time that is referred to as normalized G' or normalized G". In initial experiments we noticed that the addition of PBS to CF sputum decreased the G' and G" values. Sputum samples in which the mucolytic effects of MUC-031 and NAC were compared were treated with protease inhibitor cocktail (Halt™, ThermoFisher Scientific, Waltham, MA, USA) and EDTA, but sputum samples in which the mucolytic effects of MUC-031 and rhDNase were compared were not treated with protease inhibitors because these inhibitors could decrease the activity of Mg^{2+}/Ca^{2+}-dependent rhDNase.

Animal studies

All animal studies were approved by the animal welfare authority responsible for the Charité – Universitätsmedizin Berlin (Landesamt für Gesundheit und Soziales Berlin, Berlin, Germany). Treatment studies were performed in βENaC-Tg mice and wild-type littermates on the C57BL/6N strain background [19, 25]. MUC-031 (131 mg/mL) or vehicle alone were applied by intratracheal instillation (adult mice) or intranasal instillation (neonatal mice) in a volume of

1 µl/g body weight. To determine acute and chronic treatment effects of MUC-031 in established muco-obstructive lung disease, adult βENaC-Tg and wild-type mice were treated either three times in one day or twice a day for two weeks. To determine effects of preventive treatment, neonatal βENaC-Tg and wild-type mice were treated from the first day of life two times a day for two weeks.

BAL cell counts and cytokines measurements

Bronchoalveolar lavage (BAL) was obtained and cell counts were determined as previously described [19]. KC, TNF-α and IL-13 were measured using commercially available cytometric bead array (CBA) kits (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

Histology and airway morphometry

Left lungs were sectioned transversally at the level of the proximal intrapulmonary main axial airway. Our previous studies in βENaC-Tg mice showed that this airway region consistently exhibits mucus obstruction and that response to therapy with mucolytic agents in this airway region correlates with responses in distal airways [20, 26, 27]. Airway mucus content was assessed by determining the volume of Alcian blue-periodic acid Schiff (AB-PAS) positive material per surface area (nl/mm^2) of the airway, as previously described [20, 28].

Mucin agarose gel electrophoresis

Mucin Western blotting was performed as previously described [29, 30]. For Western blots of human sputum we used a mouse monoclonal antibody against human MUC5B (sc-393952, Santa Cruz, Dallas, TX, USA) and a mouse monoclonal antibody against human MUC5AC (MA5-12178, Invitrogen, Waltham, MA) and for Western blots of BAL supernatants from mice we used mouse monoclonal antibody against murine MUC5B (sc21768, Santa Cruz, Dallas, TX, USA). Details on the immunoblotting procedures are provided in the online supplement.

Statistical analysis

Analysis of rheology data: a varying-coefficient model was estimated via a generalized additive modeling framework, as described above and in the online supplement. Timepoints for which treatment conditions led to halving of the baseline elastic or viscous moduli of CF sputum samples were used to generate cumulative event curves and analyzed for significant difference with GraphPad Prism 9.2 (GraphPad Software, San Diego, CA, USA).

Analysis of data from mouse studies: data were analyzed with GraphPad Prism 8.2.0 (GraphPad Software, San Diego, CA, USA) and are reported as mean ± SEM. Statistical analyses were performed using one-way ANOVA, two-way ANOVA, Kruskal-Wallis test and Kaplan-Meier survival analysis as appropriate, and $P < 0.05$ was accepted to indicate statistical significance.

Results

Cross-linking of mucin polymers in CF sputum is revealed by marked increases in elastic modulus

Consistent with prior reports from us and others [5, 31], we found that the G' of sputum in health is higher than its G" across a broad range of frequencies (supplementary figure S2a). This G' dominance and plateau, as well as the identical dependence of G' and G" on frequency (G' and G" are parallel lines), are hallmarks of a cross-linked gel. In CF sputum, the predominant abnormality is a large increase in elastic response indicative of a densely cross-linked gel (supplementary figure S2a), and the G′ and G″ at a frequency of 1.0 Hz is markedly higher in CF than in health (supplementary figure S2b). Based on these data, we focused our analysis of the mucolytic effects of MUC-031 on its effects on sputum G'.

Pretreatment with a protease inhibitor cocktail inhibits PBS effects on the G' of CF sputum

We found that addition of PBS decreases the G' of CF sputum (supplementary figure S1), and based on our prior work [32], we hypothesized that this PBS effect is due to sputum protease activity. Although pretreatment of sputum with EDTA (an inhibitor of metalloproteinases [MMPs]) does not inhibit this PBS effect on sputum G', pretreatment with Halt (which inhibits serine proteases, cysteine proteases, aspartic acid proteases and aminopeptidases), combined with EDTA significantly decreased the PBS effect (supplementary figure S1). These data suggest that proteases liberated from the mucin matrix following addition of PBS may explain its G' lowering effects and that neutrophil serine proteases (neutrophil elastase, cathepsin G and proteinase 3) or cysteine proteases (cathepsin S and L) are the most likely protease mediators of this effect.

MUC-031 is a more potent and faster acting mucolytic than rhDNase and NAC

To compare rhDNase and MUC-031, we tested rhDNase at 5 µg/mL and 20 µg/mL based on drug levels reported in sputum from treated patients with CF [33]. We found that MUC-031 (5

mM) caused a larger decrease in G' in CF sputum than rhDNase (figure 1c) and that the mucolytic effects of 5 µg/mL and 20 µg/mL of rhDNase were similar (supplementary figure S4). To compare the speed of onset of mucolysis for rhDNase and MUC-031, we used two analysis approaches. First, we compared the time taken for sputum G' to decrease by at least 50% and found that the time was shorter for MUC-031 than for rhDNase (7.5 vs 11.9 minutes, figure 1c). Second, we defined mucolysis as a 50% decline from baseline in G' and we compared the proportion of CF sputum samples that underwent mucolysis within 15 minutes of addition of drug. We found that the percentage of MUC-031-treated sputum samples that underwent mucolysis by 15 minutes was much larger for MUC-031 than for rhDNase (87% vs 47%, $P < 0.05$, figure 1d).

To compare MUC-031 and NAC, we tested both drugs at 2.5 mM because of the robust effects of MUC-031 seen at 5 mM and because we reasoned that the addition of Halt and EDTA to inhibit autolysis by proteases would increase signal to noise ratio. Compared to NAC, MUC-031 caused a larger decrease in G' than NAC (figure 1e). In addition, MUC-031 had much faster speed of onset, as evidenced by a shorter time for the G' to decrease by 50% (12.5 minutes for MUC-031 and ~30 minutes for NAC, figure 1e) and a much larger proportion of sputum samples that underwent mucolysis by 15 minutes (69% for MUC-031 and 25% for NAC, $P < 0.01$, figure 1f).

To explore dose response effects for MUC-031 at high vs. low levels of protease activity, we compared the effects of different drug concentrations in CF sputa pretreated with EDTA alone or in CF sputa pretreated with EDTA plus Halt. In CF sputa pretreated with EDTA, we found a dose response effect for the 2.5 mM and 5 mM MUC-031 concentrations whereas in CF sputa pre-treated with Halt plus EDTA we found a dose response effect for the 0.5 mM and 2.5 mM MUC-031. In these Halt plus EDTA experiments, the effect of MUC-031 at 0.5 mM was equivalent to the effect of NAC at 2.5 mM (supplementary figure S3).

We also compared the effects of rhDNase, MUC-031, and NAC and PBS control on the viscous modulus (G") of CF sputum. The effects of the drugs on G" were much smaller than the effects seen for G' (supplementary figure S5 and S6).

MUC-031 cleaves MUC5B and MUC5AC in CF sputum

To determine if MUC-031 cleaves the major gel forming mucins in the airway (MUC5B and MUC5AC), we compared the effects of rhDNase and increasing concentrations (0.1 mM to 10 mM) of MUC-031 and DTT on the size of MUC5B and MUC5AC in Western blots of CF sputum. While rhDNase had no effect on high-molecular-weight intensity of MUC5B or MUC5AC, we found a dose-dependent reduction of multimer intensity for both mucins by MUC-031 (figure 2).

MUC-031 decreases airway mucus plugging and airway inflammation in adult βENaC-Tg mice

To investigate the mucolytic efficacy of MUC-031 *in vivo*, we first studied the effects of MUC-031 treatment in adult βENaC-Tg mice and wild-type littermate controls in single day (acute) and then 14-day (chronic) treatment protocols. For the acute treatment protocol, MUC-031 or vehicle alone was administered by intratracheal instillation to βENaC-Tg and wild-type mice three times in one day after which lung tissue and BAL was analyzed for airway mucus obstruction and inflammation outcomes. Consistent with results from previous studies [20, 26], vehicle-treated adult βENaC-Tg mice showed airway mucus plugging and airway inflammation that was not evident in vehicle-treated wild-type mice (figure 3 and supplementary figure S7a). Specific findings in BAL in the βENaC-Tg mice included increases in total cell number, macrophages (enlarged morphologically), neutrophils, and eosinophils. Specific findings in lung tissue were increases in total and intraluminal mucus volume density. Compared to vehicle-treated βENaC-Tg mice, MUC-031 treated βENaC-Tg mice showed significant decreases in total and intraluminal mucus volume density in the airways, whereas intraepithelial mucus volume density (a measure of goblet cell metaplasia) did not change

(figure 3a-d). MUC-031 treated βENaC-Tg mice also showed significant decreases in BAL total cell counts with a trend to decrease BAL macrophage and neutrophil numbers (macrophage size decreased significantly) (figure 3e and supplementary figure S7a).

For the chronic treatment protocol, MUC-031 or vehicle alone was administered by intratracheal instillation twice daily for 14 days to adult βENaC-Tg mice and wild-type littermate controls. No clinical signs or symptoms suggestive of toxicity or adverse events and no deaths were observed in adult wild-type or βENaC-Tg mice during chronic treatment with MUC-031. Compared to vehicle-treated βENaC-Tg mice, MUC-031 treated mice showed significant decreases in total and intraluminal mucus volume density in the airways without any significant change in intraepithelial mucus volume density (figure 4a-d). In addition, MUC-031 treated mice showed significant decreases in BAL total cell counts, macrophages, macrophage size, and a trend towards a decrease in neutrophils (figure 4e and supplementary figure S7b). Furthermore, MUC-031 treated mice showed a significant decrease in BAL levels of TNF-α, a slight increase of IL-13 without any effect on KC levels (figure 4f). Treatment of adult wild-type mice with MUC-031 twice daily for 14 days was associated with an increase in intraepithelial mucus volume density in the airways and an elevated expression of *Muc5ac* transcripts (figure 4d and supplementary figure S8a). Further, chronic treatment of adult wild-type mice with MUC-031 did not significantly change BAL cells or TNF-α levels, but was associated with a slight increase of IL-13 and there was a trend for an increase in KC levels (figure 4f).

MUC-031 improves survival in neonatal βENaC-Tg mice in context of decreased airway mucus plugging and inflammation

βENaC-Tg mice do not have airway mucus plugging or airway inflammation at birth but develop these pathologies shortly after birth where they contribute to increased mortality [20, 26]. A notable feature of neonatal βENaC-Tg mice compared to adult βENaC-Tg mice is that they show features of airway type 2 inflammation [20, 26, 34-36]. We investigated if preventive treatment of newborn βENaC-Tg mice with MUC-031 improves survival by preventing

development of airway mucus plugging and airway inflammation. Neonatal βENaC-Tg and wild-type mice were treated with intranasal instillation of MUC-031 or vehicle control twice daily for 14 days from the first day of life. Similar to the chronic treatment in adult mice, no clinical signs suggestive of toxicity were observed in neonatal mice of either genotype, and no deaths were observed in wild-type mice during preventive treatment with MUC-031. Compared to vehicle-treated βENaC-Tg mice, preventive treatment with MUC-031 in βENaC-Tg mice significantly improved survival (figure 5a). This survival benefit was likely conferred because preventive MUC-031 treatment significantly decreased airway mucus plugging, as evidenced by decreases in the total and intraluminal mucus volume density in the airways of surviving βENaC-Tg mice (figure 5b-d). In addition, preventive treatment with MUC-031 in βENaC-Tg mice significantly decreased intraepithelial mucus volume density reflecting goblet cell metaplasia (figure 5e). Furthermore, Western blotting of mucins in BAL showed partial depolymerization of MUC5B and a decrease in MUC5B concentration (figure 5f).

In exploring the effects of preventive treatment with MUC-031 in βENaC-Tg mice on lung inflammation, we found that MUC-031 significantly decreased BAL total cell counts, macrophages, eosinophils and macrophage size (figure 6a, b, and supplementary figure S7). MUC-031 also decreased BAL IL-13 levels but, in contrast to findings in adult βENaC-Tg mice, increased BAL TNF-α levels (figure 6b).

Discussion

Airway mucus plugging is an important cause of airflow obstruction and nidus for inflammation and infection in patients with a spectrum of muco-obstructive lung diseases including CF and COPD, and there are few mucolytic drug options for these patients. Here we show that a novel thiol-saccharide compound (MUC-031) is a more potent mucolytic drug than rhDNase and NAC and that its administration to βENaC-Tg mice - a model of muco-obstructive lung disease - causes beneficial effects on lung health that provide rationale for clinical trials in humans.

Confirming prior reports [5, 32, 37], we show here that the biophysical signature of healthy airway mucus is that its elastic modulus (G') is less than 1 Pascal (Pa) across a broad range of frequencies, and that its G' is higher than its viscous modulus (G"). This G' dominance and plateau are a hallmark of a cross-linked gel. Pathologic airway mucus, occurring in a range of lung diseases from CF to COPD, is consistently characterized by large increases in G' [5, 32, 37], indicative of a more densely cross-linked gel that is stiffer and harder for the mucociliary escalator to transport. The goal of mucolytic treatment is therefore to normalize the G' of pathologic mucus so as to restore the ability of the mucocilary escalator to transport it. In optimizing methods to test the mucolytic effects of MUC-031 in sputum using rheology methods, we found that the addition of PBS decreased sputum G' and that this effect was largely inhibited by protease inhibitors. Thus, hydration of pathologic mucus gels with saline may liberate proteases that have mucolytic effects, and it is important to control for these protease effects in sputum rheology studies of novel mucolytic agents.

In comparing the effects of MUC-031, rhDNase, and NAC on the G' of CF sputum, we found that MUC-031 potently and quickly decreases the G', and that it has larger and faster acting effects than rhDNase. This finding highlights the importance of disulfide mucin cross-linking vs. free DNA as a mechanism of increased mucus gel elasticity in CF airway mucus. We have previously shown how DNA polymers form a shell around a densely cross-linked mucin core in CF airway mucus [5]. The potent mucolytic effect of MUC-031 shown here supports a

concept in which MUC-031 cleaves disulfide linked mucin polymers in the core of the mucus gel to transform it from its pathologic solid-like state to a more physiologic liquid-like state that is more easily cleared. The superior potency and speed of onset of MUC-031 relative to NAC may relate to the chemical advantages of thiol-saccharides that include neutral electrical charge and interactions with glycosylated mucins through hydrogen bonding. Our finding that MUC-031 lyses mucus twice as quickly as NAC is a therapeutic advantage because residence time in the airway lumen for these two inhaled drugs is likely to be less than 60 minutes [38, 39], and mucolytic drugs with faster speed of effect will be more likely to effectively lyse pathologic mucus. Irrespective of any efficacy advantages, thiol-saccharides also have advantages in terms of tolerability because they can be formulated as iso-osmolar aerosols and, unlike NAC, they are not catabolized by aminotransferases to generate sulfites. Both of these features mean that MUC-031 is unlikely to cause bronchoconstriction as a side effect of treatment. Additionally, thiol-saccharides are highly soluble in water and thus are well suited for inhaled delivery by a nebulizer.

We demonstrated the mucolytic efficacy of MUC-031 *in vivo* in experiments in βENaC-Tg mice that are characterized by airway mucus plugging, chronic airway inflammation, and early mortality [19, 20, 36]. We used single and 14-day treatment protocols in adult mice to model treatment protocols used in management of acute exacerbations of airway disease or as maintenance treatment of chronic stable airway disease. We also included a third protocol in neonatal mice designed to determine if MUC-031 improves survival by preventing mucus pathology and related airway inflammation in the lungs, i.e. this protocol models preventive treatment of pre-symptomatic stages of childhood lung diseases such as cystic fibrosis [40]. A single day of treatment with MUC-031 was sufficient to decrease airway mucus plugs, and 14 days of MUC-031 treatment showed sustained decreases in mucus plugs over time. Preventive treatment of newborn βENaC-Tg mice improved their survival by decreasing airway mucus pathology. Although MUC-031 consistently and effectively decreases intraluminal mucus plugs in neonatal and adult βENaC-Tg mice, we found that 2 weeks of MUC-031

treatment in adult wild-type mice caused an increase in MUC5AC gene expression and an increase in intraepithelial mucin volume. These effects did not occur after single-day treatment of adult wild-type mice with MUC-031 or 14-day treatment with MUC-031 in neonatal wild-type mice. Because we show that MUC-031 can cleave MUC5AC and MUC5B in immunoblot experiments, we speculate that cleavage of secreted or tethered mucins in the normal airway may initiate signals that upregulate MUC5AC expression in the airway epithelium. Because our murine studies only used a single dose of MUC-031, it is possible that lower doses of MUC-031 may not exert these effects on MUC5AC gene expression or intraepithelial mucin stores, but further dose response studies are needed to determine this. Taken together, these *in vivo* data demonstrate that MUC-031, delivered into the airways, effectively lyses airway mucus plugs. Our data are consistent with previous reports in βENaC-Tg mice of the mucolytic efficacy of a different experimental drug (P3001) that also cleaves mucin disulfide bridges [39]. And also relevant here are previous studies we have published showing that hypertonic saline (HS) and amiloride (an ENaC blocker), which improve mucus hydration when delivered to the airways, also have beneficial effects in βENaC-Tg mice [26, 27, 41]. Specifically, treatment of βENaC-Tg mice with HS decreases mucus plugs in adult mice and improves survival in neonatal mice [27], whereas treatment of βENaC-Tg mice with the ENaC blocker amiloride decreases mucus plugging and mortality in neonatal βENaC-Tg mice, but does not decrease established airway mucus plugs in adult mice [26].

An important finding of our study is that treatment with MUC-031 has anti-inflammatory effects including decreases total inflammatory cell numbers and cytokine levels in BAL, especially in the 14-day treatment protocols. The most likely explanation for this finding is that inhaled triggers of airway inflammation (i.e. bacteria, allergens and other irritants) and inflammatory cells embedded in pathologic mucus gels are cleared from the airways as the mucus is lysed and cleared, but this may not be the only mechanism. In our previous work, we found that airway mucus plugs in neonatal βENaC-Tg mice are associated with hypoxic degeneration of airway epithelial cells that causes IL-1α-mediated neutrophilic inflammation in the airway [20,

42]. And more recently, Singanayagam et a [43] showed that MUC5AC has pro-inflammatory actions related to release of adenosine triphosphate (ATP) from airway epithelial cells. Therefore decreasing mucus plugs or airway mucins may lead to reductions in airway inflammation. Indeed, improvements in airway inflammation following improvements in mucus clearance have recently been demonstrated by Morgan et al [44]. In a mouse model of asthma characterized by airway mucus plugging and eosinophilia, these authors showed that mucolytic treatment in the form of nebulized tris-2-carboxyethyl-phosphine (TCEP, 50 and 500 mM solutions) is associated with dose dependent decreases in airway mucus plugs and a 5-fold decrease in lavage eosinophil numbers with the TCEP 500mM dose. In previous studies [20, 34], we reported that neonatal βENaC-Tg mice develop spontaneous inflammation characterized by increases in type 2 cytokines (including IL-13) and increases in eosinophils. Here we show that MUC-031 treatment prevents increases in airway IL-13 and blunts the airway eosinophil effect previously reported. In contrast to these IL-13 effects, we report an increase in TNF-α in MUC-031-treated neonatal βENaC-Tg mice. This finding did not occur in adult βENaC-Tg mice, where MUC-031 treatment reduced TNF-α levels in BAL. It is possible that MUC-031 treatment has different effects on activation of macrophage (i.e. a key source of TNF-α) in neonatal vs. adult βENaC-Tg mice that could explain age-related differences in TNF-α levels in the airways, but further research is needed to determine this. Taken together, our data for the effects of MUC-031 in βENaC-Tg mice, combined with other recently published data, support an emerging concept in which pathologic mucus gels form a scaffold in which mucin rich inflammatory cell niches are sustained in airways. Disrupting these niches may have therapeutic benefits, including decreases in airway inflammation.

In summary, our data support mucolysis via cleavage of disulfide bonds in mucin polymers by MUC-031 as an effective strategy to reduce airway mucus plugging and inflammation in muco-obstructive lung diseases. Collectively our data support clinical development of MUC-031 as a mucolytic drug for patients with muco-obstructive lung diseases, including CF and COPD.

Acknowledgements

We thank the patients with CF for their participation in this study and Mary Ellen Kleinhenz, M.D., and Michelle Yu, M.D. (physicians in the adult CF clinic at UCSF) for their collaboration in this work. We also thank Alexander Rohrbach for excellent technical assistance and Dr. Markus Bardua for supporting animal studies.

Sources of financial support

This study was supported by P01HL128191 and R01HL080414 from the National Heart Lung and Blood Institute, the German Research Foundation (CRC 1449 – 431232613 A01, C04 and Z02) and the German Federal Ministry of Education and Research (82DZL009B1). S.Y.G. is a participant of the BIH-Charité Clinician Scientist Program funded by the Charité – Universitätsmedizin Berlin and the BIH.

Tables

Table 1. Demographic and clinical characteristics of healthy controls and patients with cystic fibrosis who donated sputum for rheology studies.

	Healthy subjects mean ± SD or n (%)	**CF patients** mean ± SD or n (%)
Number of donors	7	25
Age, years	44 ± 11.1	30.8 ± 8.7
Sex, female	3 (43%)	7 (28%)
FEV1 (L)	3.44 ± 0.73	2.2 ± 1.13
FEV1 % predicted	95.8 ± 6.0	54.9 ± 21.8
CFTR genotype		
F508del/F508del		13 (52%)
F508del/other		8 (32%)
Other/other		4(16%)
Pseudomonas Infection		
Negative		2 (8%)
Intermittent		6 (24%)
Chronic		17 (68%)
Pancreatic Insufficiency		23 (92%)

Definition of abbreviations: FEV_1 = forced expiratory volume in one second; SD = standard deviation.

References

1. Boucher RC. Muco-Obstructive Lung Diseases. *N Engl J Med* 2019: 380(20): 1941-1953.
2. Fahy JV, Dickey BF. Airway mucus function and dysfunction. *N Engl J Med* 2010: 363(23): 2233-2247.
3. Kesimer M, Ford AA, Ceppe A, et al. Airway Mucin Concentration as a Marker of Chronic Bronchitis. *N Engl J Med* 2017: 377(10): 911-922.
4. Hill DB, Button B, Rubinstein M, et al. Physiology and Pathophysiology of Human Airway Mucus. *Physiol Rev* 2022.
5. Yuan S, Hollinger M, Lachowicz-Scroggins ME, et al. Oxidation increases mucin polymer cross-links to stiffen airway mucus gels. *Sci Transl Med* 2015: 7(276): 276ra227.
6. Aldini G, Altomare A, Baron G, et al. N-Acetylcysteine as an antioxidant and disulphide breaking agent: the reasons why. *Free Radical Res* 2018: 52(7): 751-762.
7. Winterbourn CC, Metodiewa D. Reactivity of biologically important thiol compounds with superoxide and hydrogen peroxide. *Free Radic Biol Med* 1999: 27(3-4): 322-328.
8. Marrades RM, Roca J, Barbera JA, et al. Nebulized glutathione induces bronchoconstriction in patients with mild asthma. *Am J Respir Crit Care Med* 1997: 156(2 Pt 1): 425-430.
9. Dunican EM, Elicker BM, Gierada DS, et al. Mucus plugs in patients with asthma linked to eosinophilia and airflow obstruction. *J Clin Invest* 2018: 128(3): 997-1009.
10. Dunican EM, Elicker BM, Henry T, et al. Mucus Plugs and Emphysema in the Pathophysiology of Airflow Obstruction and Hypoxemia in Smokers. *Am J Respir Crit Care Med* 2021: 203(8): 957-968.
11. Fuchs HJ, Borowitz DS, Christiansen DH, et al. Effect of aerosolized recombinant human DNase on exacerbations of respiratory symptoms and on pulmonary function in patients with cystic fibrosis. The Pulmozyme Study Group. *N Engl J Med* 1994: 331(10): 637-642.

12. Donaldson SH, Bennett WD, Zeman KL, et al. Mucus clearance and lung function in cystic fibrosis with hypertonic saline. *N Engl J Med* 2006: 354(3): 241-250.
13. Elkins MR, Robinson M, Rose BR, et al. A controlled trial of long-term inhaled hypertonic saline in patients with cystic fibrosis. *N Engl J Med* 2006: 354(3): 229-240.
14. O'Donnell AE, Barker AF, Ilowite JS, et al. Treatment of idiopathic bronchiectasis with aerosolized recombinant human DNase I. rhDNase Study Group. *Chest* 1998: 113(5): 1329-1334.
15. Aaron SD. Mucolytics for COPD: negotiating a slippery slope towards proof of efficacy. *Eur Respir J* 2017: 50(4).
16. Bennett WD, Henderson AG, Ceppe A, et al. Effect of hypertonic saline on mucociliary clearance and clinical outcomes in chronic bronchitis. *ERJ Open Res* 2020: 6(3).
17. Zhang Y, Song A, Liu J, et al. Therapeutic effect of nebulized hypertonic saline for muco-obstructive lung diseases: a systematic review and meta-analysis with trial sequential analysis. *J Investig Med* 2021: 69(3): 742-748.
18. Services DoHaH. National Institutes of Health (NIH), Translational Programs in Lung Diseases (P01). https://grants.nih.gov/grants/guide/pa-files/PAR-14-245.html.
19. Mall M, Grubb BR, Harkema JR, et al. Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med* 2004: 10(5): 487-493.
20. Mall MA, Harkema JR, Trojanek JB, et al. Development of chronic bronchitis and emphysema in β-epithelial Na+ channel-overexpressing mice. *Am J Respir Crit Care Med* 2008: 177(7): 730-742.
21. Stahr CS, Samarage CR, Donnelley M, et al. Quantification of heterogeneity in lung disease with image-based pulmonary function testing. *Sci Rep* 2016: 6: 29438.
22. Oscarson S, Fahy JV, Yuan S, Carrington S. Thiosaccharide mucolytic agents. *PCT/US2014/028656*, 2014.
23. Oscarson S, Fahy JV, Gitlin I, Raymond W. Dithiolsaccharide mucolytic agents and uses thereof. *PCT/US2019/050475*, 2020.

24. Gershman NH, Liu H, Wong HH, et al. Fractional analysis of sequential induced sputum samples during sputum induction: Evidence that different lung compartments are sampled at different time points. *J Allergy Clin Immun* 1999: 104(2): 322-328.

25. Johannesson B, Hirtz S, Schatterny J, et al. CFTR regulates early pathogenesis of chronic obstructive lung disease in βENaC-overexpressing mice. *PLoS One* 2012: 7(8): e44059.

26. Zhou Z, Treis D, Schubert SC, et al. Preventive but not late amiloride therapy reduces morbidity and mortality of lung disease in βENaC-overexpressing mice. *Am J Respir Crit Care Med* 2008: 178(12): 1245-1256.

27. Graeber SY, Zhou-Suckow Z, Schatterny J, et al. Hypertonic saline is effective in the prevention and treatment of mucus obstruction, but not airway inflammation, in mice with chronic obstructive lung disease. *Am J Respir Cell Mol Biol* 2013: 49(3): 410-417.

28. Harkema JR, Plopper CG, Hyde DM, et al. Regional differences in quantities of histochemically detectable mucosubstances in nasal, paranasal, and nasopharyngeal epithelium of the bonnet monkey. *J Histochem Cytochem* 1987: 35(3): 279-286.

29. Livraghi A, Grubb BR, Hudson EJ, et al. Airway and lung pathology due to mucosal surface dehydration in β-epithelial Na+ channel-overexpressing mice: role of TNF-α and IL-4Rα signaling, influence of neonatal development, and limited efficacy of glucocorticoid treatment. *J Immunol* 2009: 182(7): 4357-4367.

30. Ramsey KA, Rushton ZL, Ehre C. Mucin Agarose Gel Electrophoresis: Western Blotting for High-molecular-weight Glycoproteins. *J Vis Exp* 2016(112).

31. Nielsen H, Hvidt S, Sheils CA, et al. Elastic contributions dominate the viscoelastic properties of sputum from cystic fibrosis patients. *Biophys Chem* 2004: 112(2-3): 193-200.

32. Innes AL, Carrington SD, Thornton DJ, et al. Ex vivo sputum analysis reveals impairment of protease-dependent mucus degradation by plasma proteins in acute asthma. *Am J Respir Crit Care Med* 2009: 180(3): 203-210.

33. Genentech. PULMOZYME® (dornase alfa) inhalation solution, for inhalation use. 2021: https://www.gene.com/download/pdf/pulmozyme_prescribing.pdf.

34. Fritzsching B, Hagner M, Dai L, et al. Impaired mucus clearance exacerbates allergen-induced type 2 airway inflammation in juvenile mice. *J Allergy Clin Immunol* 2017: 140(1): 190-203 e195.

35. Brown R, Paulsen M, Schmidt S, et al. Lack of IL-1 Receptor Signaling Reduces Spontaneous Airway Eosinophilia in Juvenile Mice with Muco-Obstructive Lung Disease. *Am J Respir Cell Mol Biol* 2020: 62(3): 300-309.

36. Zhou-Suckow Z, Duerr J, Hagner M, et al. Airway mucus, inflammation and remodeling: emerging links in the pathogenesis of chronic lung diseases. *Cell Tissue Res* 2017: 367(3): 537-550.

37. Patarin J, Ghiringhelli E, Darsy G, et al. Rheological analysis of sputum from patients with chronic bronchial diseases. *Sci Rep* 2020: 10(1): 15685.

38. Rennard SI, Basset G, Lecossier D, et al. Estimation of Volume of Epithelial Lining Fluid Recovered by Lavage Using Urea as Marker of Dilution. *J Appl Physiol* 1986: 60(2): 532-538.

39. Ehre C, Rushton ZL, Wang B, et al. An Improved Inhaled Mucolytic to Treat Airway Muco-obstructive Diseases. *Am J Respir Crit Care Med* 2019: 199(2): 171-180.

40. Stahl M, Wielputz MO, Ricklefs I, et al. Preventive Inhalation of Hypertonic Saline in Infants with Cystic Fibrosis (PRESIS). A Randomized, Double-Blind, Controlled Study. *Am J Respir Crit Care Med* 2019: 199(10): 1238-1248.

41. Mall MA, Graeber SY, Stahl M, et al. Early cystic fibrosis lung disease: Role of airway surface dehydration and lessons from preventive rehydration therapies in mice. *Int J Biochem Cell Biol* 2014: 52: 174-179.

42. Fritzsching B, Zhou-Suckow Z, Trojanek JB, et al. Hypoxic epithelial necrosis triggers neutrophilic inflammation via IL-1 receptor signaling in cystic fibrosis lung disease. *Am J Respir Crit Care Med* 2015: 191(8): 902-913.

43. Singanayagam A, Footitt J, Marczynski M, et al. Airway mucins promote immunopathology in virus-exacerbated chronic obstructive pulmonary disease. *J Clin Invest* 2022: 132(8).

44. Morgan LE, Jaramillo AM, Shenoy SK, et al. Disulfide disruption reverses mucus dysfunction in allergic airway disease. *Nat Commun* 2021: 12(1): 249.

Figure legends

Figure 1. Effect of recombinant human deoxyribonuclease (rhDNase), N-acetylcysteine (NAC) and MUC-031 on the elasticity of sputum from patients with cystic fibrosis (CF). a) Schematic of a cone-and-plate rheometer. Elastic (G') and viscous (G") moduli are calculated from the measured response of the samples to the oscillating angular displacement. b) Schematic of the protocol for testing the mucolytic efficacy of MUC-031, rhDNase and NAC. The G' and G" moduli of CF sputum samples are measured at baseline in a frequency sweep from 0.1 to 50 Hz at 5% strain, followed by addition and manual mixing of the test agents at 10% v/w (PBS control, rhDNase, NAC, and MUC-031). c) Comparison of mucolytic effect of PBS control (n=15), rhDNase (n = 15, 20 µg/mL) and MUC-031 (n = 15, 5 mM), in sputum not treated with protease inhibitors, as measured by change in G' over 30 min and analyzed by generalized additive modeling framework (GAM). d) Percentage (%) of samples in each group (PBS, rhDNase and MUC-031) as function of time for which GAM-normalized G' is decreased by 50%. e) Comparison of mucolytic effect of PBS control (n = 8), NAC (n = 16, 2.5 mM) and MUC-031 (n = 16, 2.5 mM), in sputum treated with protease inhibitors HALT and EDTA, as measured by change in G' over 30 min and analyzed by GAM. f) Percentage (%) of samples in each group (PBS, NAC and MUC-031) as function of time for which GAM-normalized G' is decreased by 50%. In C and E the solid lines represent the model estimates for βj (referred to as normalized G') with the surrounding lighter colors indicate 95% pointwise confidence intervals over the time course. Non-overlapping lines and surround colors are indicative of statistically significant differences between conditions. *P < 0.05, **P < 0.01, and ****P < 0.0001 for comparison of cumulative curves for MUC-031 versus rhDNase, NAC or PBS.

Figure 2. Effect of recombinant human deoxyribonuclease (rhDNase), dithiothreitol (DTT) and MUC-031 on mucin size in sputum from patients with cystic fibrosis (CF). Freshly collected sputum samples from CF patients were treated with rhDNase (20 µg/ml) or increasing concentration (0.1 – 10 mM) of DTT or MUC-031 at 37°C for 30 minutes, mucin were separated

by gel electrophoresis, and Western blots were stained with antibodies against MUC5B and MUC5AC. (a – d) Representative Western blots (a, c) and summary of effect (b, d) of rhDNase, and increasing concentrations of DTT and MUC-031 on high-molecular-weight intensity of MUC5B (a, b) and MUC5AC (c, d) expressed as percentage of untreated sputum aliquots (n = 8 – 10 per group). NT = no treatment. D = rhDNase. *P < 0.0001 compared with NT samples. $^{\dagger}P$ < 0.05 and $^{\ddagger}P$ < 0.001 compared with 0.1 mM concentration of same drug. $^{\#}P$ < 0.05 and $^{\S}P$ < 0.001 compared with 1 mM concentration of same drug.

Figure 3. Acute treatment with MUC-031 reduces airway mucus plugging and inflammation in adult βENaC-Tg mice with chronic muco-obstructive lung disease. Adult βENaC-Tg mice and wild-type (WT) littermate controls were treated with MUC-031 or vehicle alone by intratracheal instillation three times in one day. a) Representative airway histology of βENaC-Tg and WT mice after acute treatment. Sections were stained with Alcian blue-periodic acid Schiff (AB-PAS) to determine the presence of intraluminal and intraepithelial mucus. Scale bars = 100 µm. (b - d) Quantification of total (b), intraluminal (c) and intraepithelial (d) mucus content determined by measuring the volume density of AB-PAS positive material in proximal main axial airways (n = 8 - 17 per group). e) Effects of acute treatment with MUC-031 on inflammatory cell counts in bronchoalveolar lavage (n = 8 - 16 per group). Mac. = macrophages; Eos. = eosinophils; PMN = neutrophils; Lymph. = lymphocytes. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001 compared with vehicle-treated WT mice. $^{\dagger}P$ < 0.05 and $^{\ddagger}P$ < 0.01 compared with vehicle-treated βENaC-Tg mice.

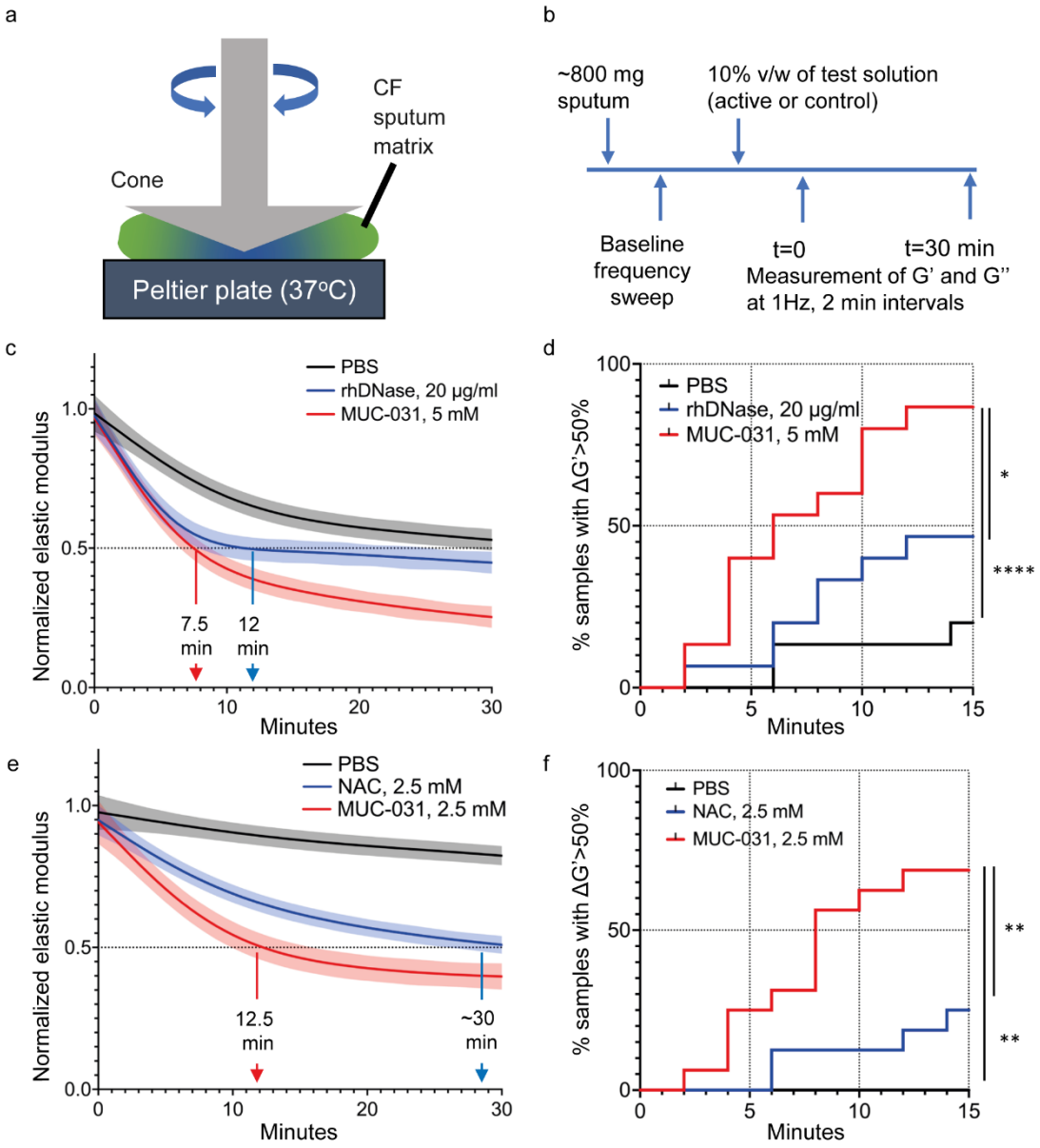
Figure 4. Chronic treatment with MUC-031 reduces airway mucus plugging and inflammation in adult βENaC-Tg mice with chronic muco-obstructive lung disease. Adult βENaC-Tg mice and wild-type (WT) littermates were treated with MUC-031 or vehicle alone by intratracheal instillation twice daily for two weeks. a) Representative airway histology of βENaC-Tg and WT mice after chronic treatment. Sections were stained with Alcian blue-periodic acid-Schiff (AB-PAS) to determine the presence of intraluminal and intraepithelial mucus. Scale bars = 100

µm. (b - d) Quantification of total (b), intraluminal (c) and intraepithelial (d) mucus content determined by measuring the volume density of AB-PAS positive material in proximal main axial airways (n = 15 - 22 per group). (e and f) Effects of chronic treatment with MUC-031 on inflammatory cell counts (e) and concentrations of KC, TNF-α and IL-13 in bronchoalveolar lavage (n = 10 per group). Mac. = macrophages; Eos. = eosinophils; PMN = neutrophils; Lymph. = lymphocytes. *$P < 0.05$, **$P < 0.001$ and ***$P < 0.0001$ compared with vehicle-treated WT mice. $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ compared with vehicle-treated βENaC-Tg mice.

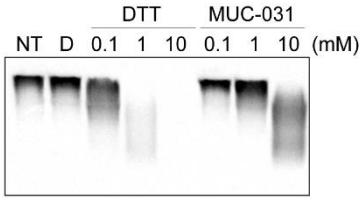
Figure 5. Preventive treatment with MUC-031 reduces mortality and airway mucus plugging in neonatal βENaC-Tg mice. Neonatal βENaC-Tg mice and wild-type (WT) littermates were treated with MUC-031 or vehicle alone by intranasal instillation twice daily from the first day of life for a period of two weeks. a) Effect of preventive treatment with MUC-031 on survival (n = 49 – 95 per group). b) Representative airway histology of βENaC-Tg and WT mice after preventive treatment with MUC-031. Sections were stained with Alcian blue-periodic acid-Schiff (AB-PAS) to determine the presence of intraluminal and intraepithelial mucus. Scale bars = 100 µm. (c - e) Quantification of total (c), intraluminal (d) and intraepithelial (e) mucus content determined by measuring the volume density of AB-PAS positive material in proximal main axial airways (n = 14 - 36 per group). f) Representative agarose gel Western blots and corresponding densitometry of bronchoalveolar lavage samples stained with a murine anti-MUC5B antibody (n = 23 – 32 per group). *$P < 0.05$ and **$P < 0.0001$ compared with vehicle-treated WT mice. $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ compared with vehicle-treated βENaC-Tg mice.

Figure 6. Effects of preventive treatment with MUC-031 on airway inflammation in neonatal βENaC-Tg mice. Neonatal βENaC-Tg mice and wild-type (WT) littermates were treated with MUC-031 or vehicle alone by intranasal instillation twice daily from the first day of life for a period of two weeks. (a - b) Inflammatory cell counts (n = 26 – 53 per group) (a) and concentrations of KC, TNF-α and IL-13 (n = 8 - 12 per group) (b) in bronchoalveolar lavage of βENaC-Tg and WT mice after preventive treatment. Mac. = macrophages; Eos. = eosinophils;

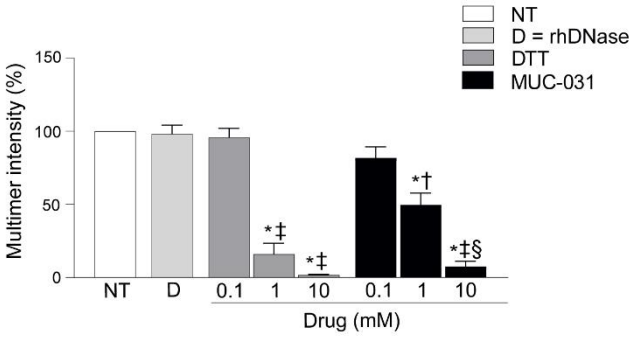
PMN = neutrophils; Lymph. = lymphocytes. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.0001$ compared with vehicle-treated WT mice. $^{\dagger}P < 0.05$ and $^{\ddagger}P < 0.01$ compared with vehicle-treated βENaC-Tg mice.


a
CF
sputum
matrix
Cone
Peltier plate (37°C)
b
~800 mg
sputum
10% v/w of test solution
(active or control)
Baseline
frequency
sweep
t=0
t=30 min
Measurement of G' and G''
at 1Hz, 2 min intervals
c
PBS
rhDNase, 20 µg/ml
MUC-031, 5 mM
Normalized elastic modulus
7.5
min
12
min
Minutes
d
PBS
rhDNase, 20 µg/ml
MUC-031, 5 mM
% samples with ΔG'>50%
*
****
Minutes
e
PBS
NAC, 2.5 mM
MUC-031, 2.5 mM
Normalized elastic modulus
12.5
min
~30
min
Minutes
f
PBS
NAC, 2.5 mM
MUC-031, 2.5 mM
% samples with ΔG'>50%
**
**
Minutes

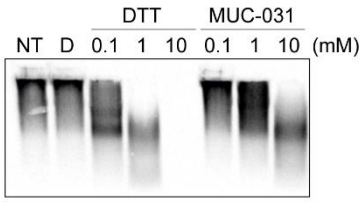
a


DTT
MUC-031
NT D 0.1 1 10 0.1 1 10 (mM)

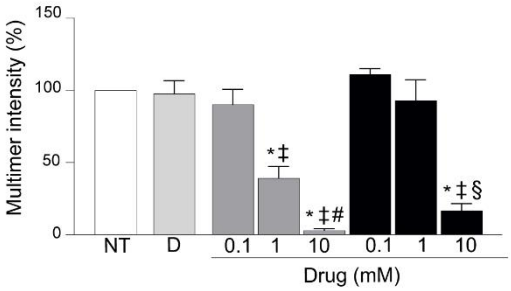
b

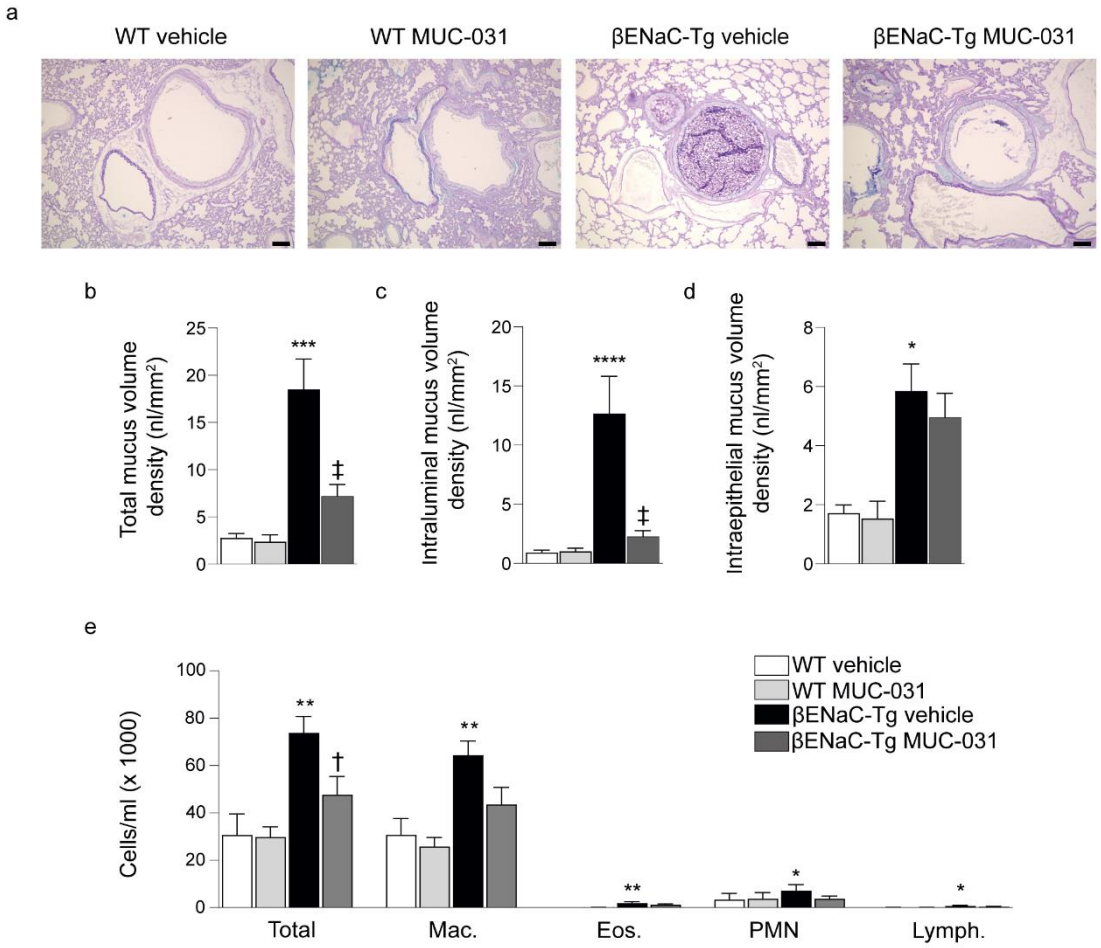

NT
D = rhDNase
DTT
MUC-031
Multimer intensity (%)
150
100
50
0
*‡
*‡
*†
*‡§
NT D 0.1 1 10 0.1 1 10
Drug (mM)

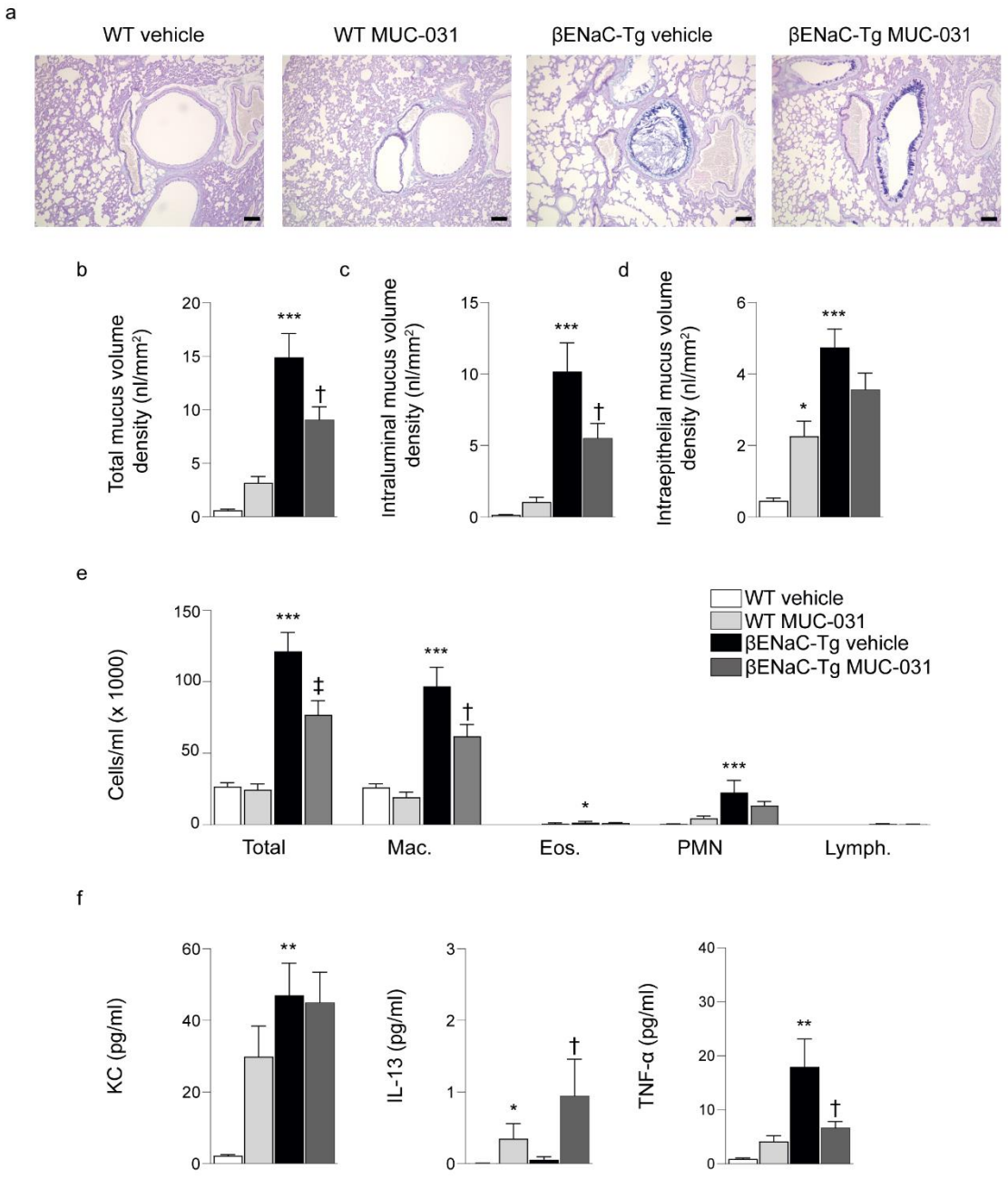
c

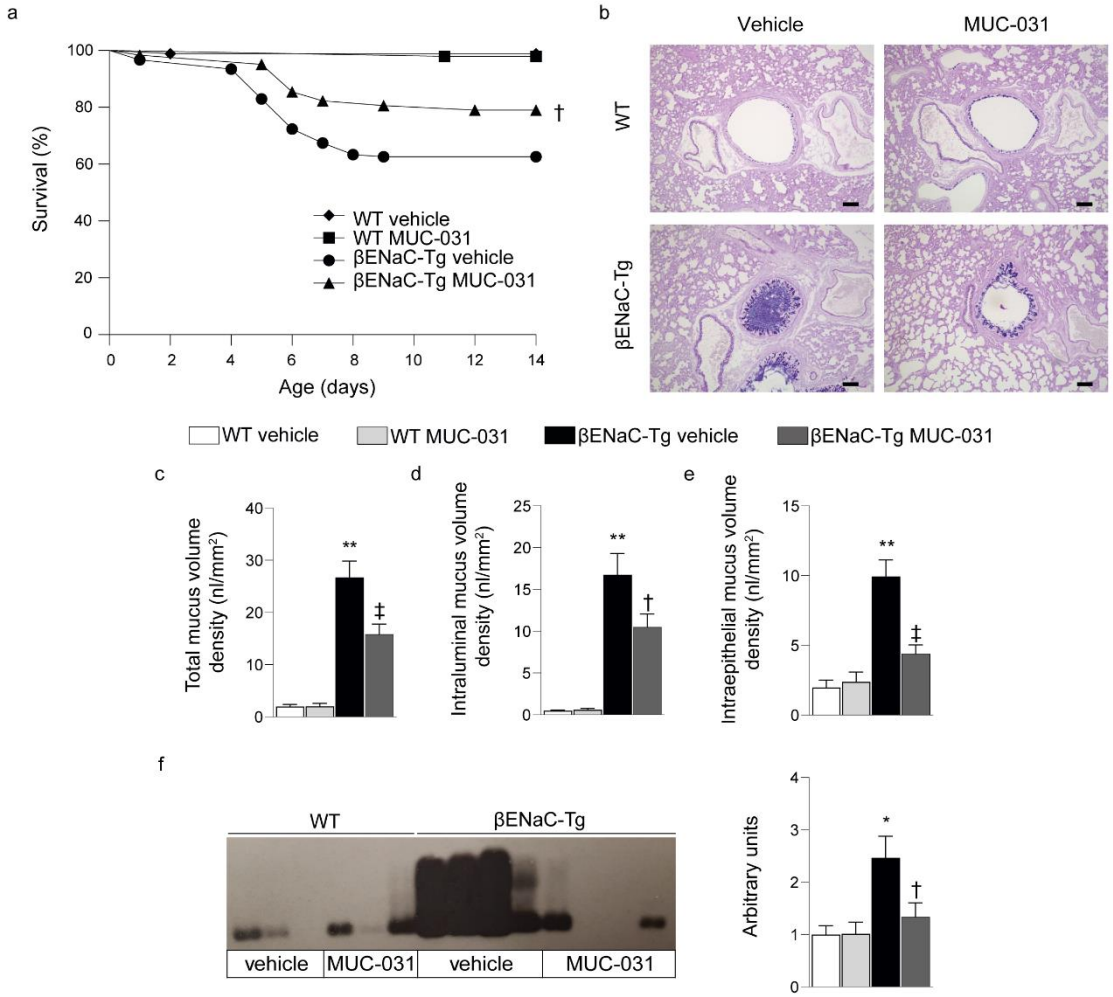

DTT
MUC-031
NT D 0.1 1 10 0.1 1 10 (mM)

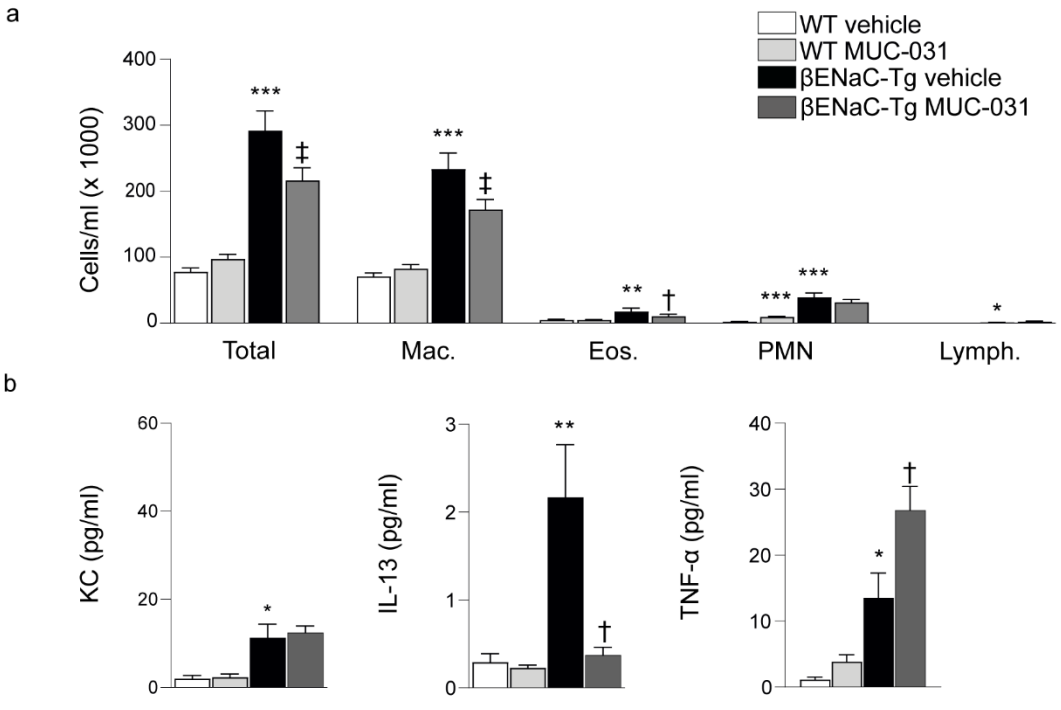
d


Multimer intensity (%)
150
100
50
0
*‡
*‡#
*‡§
NT D 0.1 1 10 0.1 1 10
Drug (mM)


a
WT vehicle
WT MUC-031
βENaC-Tg vehicle
βENaC-Tg MUC-031
b
Total mucus volume density (nl/mm²)
***
‡
c
Intraluminal mucus volume density (nl/mm²)
****
‡
d
Intraepithelial mucus volume density (nl/mm²)
*
e
Cells/ml (x 1000)
**
†
**
**
*
*
WT vehicle
WT MUC-031
βENaC-Tg vehicle
βENaC-Tg MUC-031
Total
Mac.
Eos.
PMN
Lymph.


a
WT vehicle
WT MUC-031
βENaC-Tg vehicle
βENaC-Tg MUC-031
b
Total mucus volume density (nl/mm²)
c
Intraluminal mucus volume density (nl/mm²)
d
Intraepithelial mucus volume density (nl/mm²)
e
Cells/ml (x 1000)
WT vehicle
WT MUC-031
βENaC-Tg vehicle
βENaC-Tg MUC-031
Total
Mac.
Eos.
PMN
Lymph.
f
KC (pg/ml)
IL-13 (pg/ml)
TNF-α (pg/ml)


a
Survival (%)
Age (days)
WT vehicle
WT MUC-031
βENaC-Tg vehicle
βENaC-Tg MUC-031
†
b
Vehicle
MUC-031
WT
βENaC-Tg
WT vehicle
WT MUC-031
βENaC-Tg vehicle
βENaC-Tg MUC-031
c
Total mucus volume density (nl/mm²)
d
Intraluminal mucus volume density (nl/mm²)
e
Intraepithelial mucus volume density (nl/mm²)
f
WT
βENaC-Tg
vehicle
MUC-031
vehicle
MUC-031
Arbitrary units


a
WT vehicle
WT MUC-031
βENaC-Tg vehicle
βENaC-Tg MUC-031
Cells/ml (x 1000)
400
300
200
100
0
***
‡
***
‡
**
†
***
***
*
Total
Mac.
Eos.
PMN
Lymph.
b
KC (pg/ml)
60
40
20
0
*
IL-13 (pg/ml)
3
2
1
0
**
†
TNF-α (pg/ml)
40
30
20
10
0
*
†

A novel thiol-saccharide mucolytic for the treatment of muco-obstructive lung diseases

Annalisa Addante, Wilfred Raymond, Irina Gitlin, Annabelle Charbit, Xavier Orain, Aaron Wolfe Scheffler, Aditi Kuppe, Julia Duerr, Maria Daniltchenko, Marika Drescher, Simon Y. Graeber, Anne-Marie Healy, Stefan Oscarson, John V. Fahy and Marcus A. Mall

Online Supplementary Material

Supplementay methods

Materials and reagents

The compound MUC-031 was synthesized by Centre for Synthesis and Chemical Biology, University College Dublin and by Cascade Chemistry, Inc (Eugene, OR, USA). Commercially available pharmaceutical formulations of recombinant human deoxyribonuclease (rhDNase) (Pulmozyme™, Genentech, South San Francisco, CA, USA) and N-acetylcysteine (NAC) 20% (Fresenius Kabi, Lake Zurich, IL, USA) were used in rheology studies.

Study design and participants

Sputum samples for rheology studies were collected from healthy subjects and adult patients with cystic fibrosis (CF) according to protocols and informed consent procedures approved by the Committee on Human Research at the University of California, San Francisco (UCSF). Collection of sputum samples from CF patients for mucin Western blotting was approved by the ethics committees of the Charité - Universitätsmedizin Berlin (EA2/016/18) and written consent was provided by all participants. Demographics and clinical characteristics of study participants are shown in table 1 and supplementary table E2. Non-smoking adult subjects with no history of lung disease were recruited as healthy controls using community advertising. Adult patients with CF who met the CF Foundation criteria for a diagnosis of CF (E1) were recruited from the adult CF Center at the University of California, San Francisco (UCSF). CF subjects withheld rhDNase treatment on the day of the sputum collection. Table 1 in the main text summarizes demographic and basic clinical characteristics of healthy and CF donors. For repeat CF donors, age and forced expiratory volume in the first second (FEV1) at first visit were used for calculating mean values.

Sputum collection

Healthy subjects provided a sample of induced sputum. Sputum induction involved inhalation of a nebulized solution of 3% saline for 12 minutes, as previously described (E2). CF patients

spontaneously expectorated sputum into a sputum collection cup. Any easily visible saliva layer in the sputum samples were removed by gentle pipetting prior to placement of the sample on the rheometer. The sputum samples were stored at 4ºC and most were analyzed the same day; a subset of samples were analyzed after they had been stored at 4ºC for 24 - 48 hours. Sputa used for comparative studies of MUC-031 and NAC were treated with Halt™ protease inhibitor cocktail and EDTA (ThermoFisher Scientific, Waltham, MA, USA) at 100x dilution per instructions for use immediately after collection. Sputa used for comparative studies of MUC-031 and rhDNase was not treated with Halt+EDTA due to inhibitory effects of Halt+EDTA on rhDNase activity. Sputa pretreated with EDTA only (0.5M stock, 100x dilution) was used for a sub-study of protease effects in rheology and for a subset of dose response studies.

Sputum rheology

The elastic (G') and viscous (G") moduli of sputum samples were measured using a cone and plate rheometer (AR-2000 and DHR-2 devices, TA Instruments, New Castle, DE, USA), using a Peltier plate pre-warmed to 37ºC. The rheometer provides measures of elastic modulus (G'), largely dependent on extent of cross-linking in polymer matrix, and viscous modulus (G"), largely dependent on concentration and length of polymer chains in the gel. Aliquots of sputum (~800 mg) were then interrogated in a strain-controlled mode by oscillating the cone geometry at 5% strain and measuring the torque (figure 1a). Specifically, a six step oscillatory strain sweep from 1 to 10% at 1Hz and a 15 step oscillatory frequency sweep from 0.1 to 50 Hz at 5% strain were performed with a 40 mm 2º cone geometry employing a water solvent trap. The geometry was then raised, and 89 microliters of test compound solution at 10-fold the expected final concentration were added and mixed with the sputum. Timed oscillation measurements at 1 Hz and 5% strain were then taken at 2-minute intervals over 60 minutes. Elastic or stored moduli (G') and viscous or loss moduli (G") were computed from the measured force response of the geometry oscillation on the samples. The 1 Hz measurement from the initial frequency sweep at 5% strain was used as the baseline 0-minute measurement. Mucolytic drugs (MUC-031 or rhDNase) at 10% v/w were added in droplets distributed across the surface of sputum

sample resting on Peltier plate and mixed by gentle swirling using the pipette tip; PBS was used as a control. The measurements at 1Hz / 5% strain made at successive 2 minute intervals after addition of mucolytic drug or PBS were used as outcome measures of the mucolytic efficacy of the test agents and control. Figure 1a provides a schematic illustration of the test system and figure 1b provides a schematic summary of the testing protocol. The number of independent samples tested for each test agent condition ranged from 8 to 16.

The G' and G" values of induced sputum from healthy donors ranged from 0.17 – 0.76 Pa and 0.1 to 0.26 Pa, respectively, whereas the baseline G' and G" of spontaneously expectorated sputum from CF patients ranged from 0.82 to 34.2 Pa and 0.28-9.4 Pa, respectively. Mean G' and G" baseline frequency sweeps for healthy induced sputum samples (n = 7) and CF sputum samples (n = 8) are shown in supplementary figure S2a, with individual G' and G" values at 1Hz for those samples shown in figure S2b. Because test agent effects on G' and G" depend on baseline values which vary considerably among CF sputum samples, we used a varying-coefficient model (VCM) estimated via a generalized additive modeling framework (GAM) to generate normalized G' and G" values (see statistics).

Drug dosing rationale for comparison of mucolytic drugs by rheology

For experiments with rhDNase, we targeted sputum concentrations of 5 μg/mL (equivalent to 170 nM) and 20 μg/ml (equivalent to 680 nM). The rationale for 5 μg/mL concentration came from the drug packet insert which states that mean concentration of drug in sputum after nebulization of 2.5 mg dose is 3 μg/mL (E3). The rationale for the 20 μg/mL concentration was to account for scenarios in which the volume of pathologic mucus in the airways is low or the lung deposition fraction is high (e.g. with higher efficiency nebulizers). For the MUC-031 experiments, we targeted a sputum concentration of 5 mM because this concentration is achievable when small molecules are delivered to the lungs by nebulizer. For example, a unit dose of 150 mg of MUC-031 (e.g. 3 mL of 50 mg/mL solution, MW=374 g/mol) delivered by a nebulizer with 25% efficiency to a lung mucus volume of 10 mL results in 10 mM concentration

of MUC-031 in mucus as an upper bound for achievable concentration ($[MUC\text{-}031]_{mucus}$ (mM) = Dose (mg) * 25% / MW (g/mol) / V_{mucus} (mL).

Statistics applied to the rheological measurements

Comparison of G' and G" values for healthy and CF sputum (figure S2): Comparison was performed using Mann-Whitney test assuming non-parametric distribution of the data.

Varying-coefficient model (VCM) to analyzed G' and G" data in sputum: The VCM model was estimated via a generalized additive modeling framework (GAM) (E4). In VCM-GAM, $y_{ij}(t)$ represents the rheometer measurement (e.g. G') from subject *i*, under condition *j*, at time *t*. The rheometer trajectory $y_{ij}(t)$ defined as: $y_{ij}(t) = \beta_j(t)X(t_{0,ij}) + \varepsilon_{ij}(t)$ where $X(t_{0,ij})$ is the baseline rheology value, βj is a smooth term across *t* that measures the effect of condition *j* on the rheometer trajectory as the percent decrease in the baseline rheometer measure $X(t_{0,ij})$ over time *t*, and $\varepsilon_{ij}(t) \sim N(0,\sigma^2)$ is random measurement error. Inference focuses on the smooth term βj which can be interpreted as fraction of G' (or G") remaining at time *t* across all samples tested at condition *j,* and is referred to as *normalized G'* (or *normalized G"*) in figure 1 and supplementary figure S1 and S3-S6. In this way, VCM allows rheological trajectories to be modeled as a product of condition effects and baseline rheology, allowing for a parsimonious representation which takes into account overall condition effects while accounting for baseline rheology. Each sample tested is treated independently by GAM, including separate aliquots from the same donor. Statistical inference contrasts condition effects based on their 95% pointwise confidence intervals over the time course where a lack of overlap at a fixed time point is evidence of a statistically significant difference. The model is estimated in the statistical software R (E5) using the mgcv package (version 1.8-28, R Foundation for Statistical Computing, Vienna, Austria). Smooth terms are estimated using penalized cubic regression splines with 15 knots via restricted maximum likelihood methods.

Cumulative event curves for time to 50% of starting G': Timepoints for which treatment conditions led to halving of the baseline elastic or viscous moduli of CF sputum samples were used to generate cumulative event curves and analyzed for significant difference with Prism software (Version 9.2, GraphPad Software, LLC). Curve comparisons were made applying the log rank (Mantel-Cox) test for significant difference (figures 1d and 1f).

Animal studies

All animal studies were approved by the animal welfare authority responsible for the Charité – Universitätsmedizin Berlin (Landesamt für Gesundheit und Soziales Berlin, Berlin, Germany; approval number G0045/19). The generation and genotyping of βENaC-Tg mice has been described previously (E6). Mice were housed in a specific pathogen-free animal facility and had free access to chow and water. Treatment studies were performed in hemizygous βENaC-Tg mice and wild-type littermates on the C57BL/6N genetic background (E7). MUC-031 was dissolved in citrate buffer (sodium citrate 20mM, NaCl 38.5 mM, pH 4.5). To test effects of acute treatment, 6- to 8-week-old adult βENaC-Tg mice and wild-type littermates were treated by intratracheal instillation of MUC-031 (131 mg/mL, 1µl/g body weight) or equal volumes of vehicle alone three times in one day at intervals of two hours. Two hours after the last application, mice were sacrificed, bronchoalveolar lavage (BAL) was performed and lungs were removed for histology and morphometry. To test effects of chronic treatment, four 4 week-old βENaC-Tg and wild-type mice were treated by intratracheal instillation of MUC-031 (131 mg/mL, 1µl/g body weight) or equal volumes of vehicle alone two times per day for two weeks. Twelve hours after the last treatment, mice were sacrificed for endpoint analyses. To test effects of preventive treatment, newborn βENaC-Tg and wild-type mice were treated by intranasal instillation of MUC-031 (131 mg/mL, 1µl/g body weight) or equal volumes of vehicle alone two times per day for two weeks. The concentration of MUC-031 of 131 mg/ml (or 350 mM) in the dosing solution in the murine studies was selected to ensure sufficient drug levels of MUC-031 in the airways after intratracheal (adult mice) or intranasal (neonatal mice) delivery and to balance the following factors: i) Lung dose: we aimed to ensure that we reach effective

lung doses of MUC-031, given that intratracheal and intranasal delivery in small animal models is inefficient and inhomogeneous, as shown in our previous deposition studies with other compounds, where intratracheal instillation yielded ~4% intrapulmonary deposition (E8) as well as by work of others (E9). ii) Osmolality: we aimed to avoid the use of strong hyperosmolar solutions as not to confound results with hydration effects. iii) Drug solubility: we ensured to stay below the solubility limit. Based on these considerations, we expected that dosing of mice by intratracheal or intranasal instillation of 131 mg/mL of MUC-031 results in an effective concentration in the airways of βENaC-Tg mice that is in the range of that used for our ex vivo studies on CF patient sputum. Twelve hours after the last treatment (for the two weeks treatment) or two hours after the last application (acute treatment), BAL was performed and lungs were removed for histology and further analysis. During the two week treatment period, survival was monitored daily. All analysis were performed by investigators blinded to the genotype and the treatment of the mice.

BAL cell counts, macrophage size and cytokines measurements

Mice were deeply anesthetized by intraperitoneal injection with ketamine/xylazine (160 mg /20 mg/kg body weight), the trachea cannulated, and the right lung lobes lavaged with PBS to determine total and differential cell counts, and macrophage size as previously described (E10, 11). BAL fluid samples were centrifuged at 600 x g for 5 minutes at 4°C and aliquots of cell-free supernatants were immediately stored at -80°C for further analysis. Supernatants were used to measure concentrations of KC, TNF-α and IL-13 using commercially available cytometric bead array (CBA) kits (BD Biosciences, San Jose, California, USA) according to the manufacturer's instructions.

mRNA expression analysis

Right lungs from mice were stored at 4 °C in RNAlater (Qiagen, Hilden, Germany) over night and afterwards stored at -80 °C for further analysis. Total RNA was extracted using RNeasy Mini Plus Kit (Qiagen, Hilden, Germany). RNA purity and quantity was determined using a

NanoDrop ND100 spectrophotometer (PeqLab, Erlangen, Germany). cDNA was obtained by reverse transcription of 1 µg of total RNA with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, MA USA). To analyze mRNA expression from the gene of interest, quantitative real-time PCR was performed on an Applied Biosystems 7500 Real Time PCR System using TaqMan universal PCR master mix and the inventoried TaqMan gene expression assays for *Muc5ac*, *Muc5b* and *Gapdh* (Applied Biosystems, Waltham, MA USA) listed in supplementary table E1 according to manufacturer's instructions. Relative fold change of target gene expression was determined by normalization to expression of the reference gene glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) (E7).

Histology and airway morphometry

Left lungs were sectioned transversally at the level of the proximal intrapulmonary main axial airway. Sections were cut at 5 µm and stained with alcian blue periodic acid-Schiff (AB-PAS). Airway mucus content was assessed by determining the volume of AB-PAS positive material per surface area (nl/mm^2) of the airway, as previously described (E10, 12). Images of airway sections were taken with an Olympus IX-71 microscope (Olympus, Hamburg, Germany) at a magnification of 10x. All morphometric measurements were performed by an investigator blinded to genotype and treatment of the mice.

Mucin agarose gel electrophoresis

Mucin Western blotting was performed using spontaneously expectorated sputum from patients with CF as previously described (E13). Briefly, fresh sputum from CF patients was diluted at 1:5 in PBS and homogenized using a 1 ml syringe and 20G cannula. Diluted sputum was treated with rhDNase 20 µg/ml (equivalent to 680 nM) or increasing concentration (0.1 – 10 mM) of MUC-031 or DTT at 37 °C for 30 minutes and then quenched with 200 mM iodoacetamide (Merck KGaA, Darmstadt, Germany) and then loaded in the agarose gel. Agarose gel electrophoresis using 0.8% agarose was combined with transfer onto a nitrocellulose membrane via vacuum. After loading the gels, proteins were separated at 80 V

(1.5 hour) with Tris-acetate-EDTA/SDS buffer. For an efficient mucin transfer, the gel was reduced for 20 minutes in a solution containing 10 mM dithiothreitol (DTT) and proteins were then transferred by vacuum blotting (MP Biomedicals, Irvine, CA, USA) to nitrocellulose membranes. Blots were probed with a mouse monoclonal antibody against human MUC5B (sc-393952, Santa Cruz, Dallas, TX, USA) diluted at 1:1000 in 1% milk-PBS and mouse monoclonal antibody against human MUC5AC (MA5-12178, Thermo Fisher Scientific, Waltham, MA, USA) diluted at 1:1000 in 1% milk-PBS. The secondary antibody was a goat anti-mouse immunoglobulin/HRP (P0047, Dako, Glostrup, Denmark), diluted 1:5000 in 1% milk-PBS. Detection was performed using OPTIMAX X-Ray Film Processor (PROTEC Medizintechnik GmbH & Co. KG, Oberstenfeld, Germany) and analysis of specific signals were carried out as previously described (E14). Mucin Western blots of BAL supernatants from mice were performed using the same protocol with some small modifications. Specifically, total protein concentration of BAL fluid was determined using Pierce™ BCA Protein Assay Kit according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA) and was used to control for equivalent loading. Blots were probed with a mouse monoclonal antibody against murine Muc5b (sc21768, Santa Cruz, Dallas, TX, USA) diluted at 1:1000 in 1% milk-PBS.

Statistical analysis

Data were analyzed with GraphPad Prism 8.2.0 (GraphPad Software, San Diego, USA) and are reported as mean ± SEM unless indicated otherwise. Normal distribution of data was assessed prior to statistical analysis. Statistical analyses of groups with normally distributed data sets was performed with one-way ANOVA followed by Tukey's post hoc test or two-way ANOVA. Not normally distributed data were analyzed by Kruskal-Wallis test with Dunn's multiple comparisons test. Survival was compared using Kaplan-Meier survival analysis the log rank test. $P < 0.05$ was accepted to indicate statistical significance.

Supplementary tables

Table E1. List of TaqMan gene expression assays used for transcript analyses

Target gene	Taqman ID
Muc5b	Mm00466391_m1
Muc5ac	Mm01276718_m1
Gapdh	Mm99999915_g1

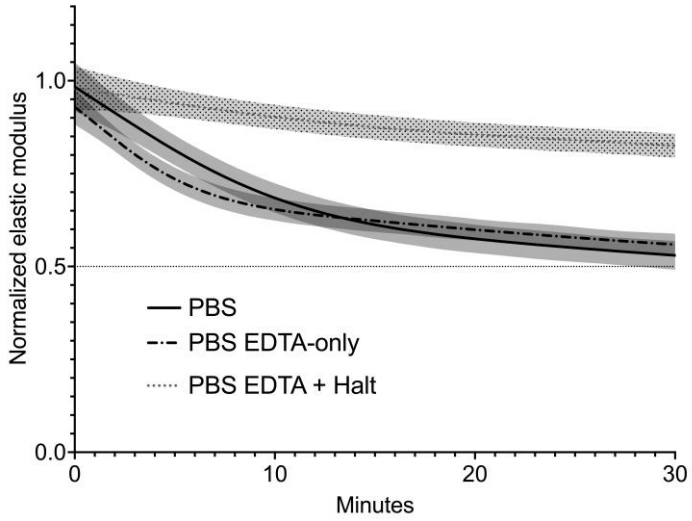
Table E2. Demographic and clinical characteristics of patients with cystic fibrosis who donated sputum for mucin Western blotting

	CF patients Mean ± SD or n (%)
Number of donors	13
Age, years	28.1 ± 6.3
Sex, female	5 (38%)
FEV1 % predicted	44.8 ± 30.3
CFTR genotype	
F508del/F508del	8 (62%)
F508del/other	5 (38%)
Other/other	0 (0%)
Pseudomonas Infection	
Negative	6 (46%)
Intermittent	0 (0%)
Chronic	7 (54%)
Pancreatic Insufficiency	13 (100%)

Definition of abbreviations: FEV1 = forced expiratory volume in one second; SD = standard deviation.

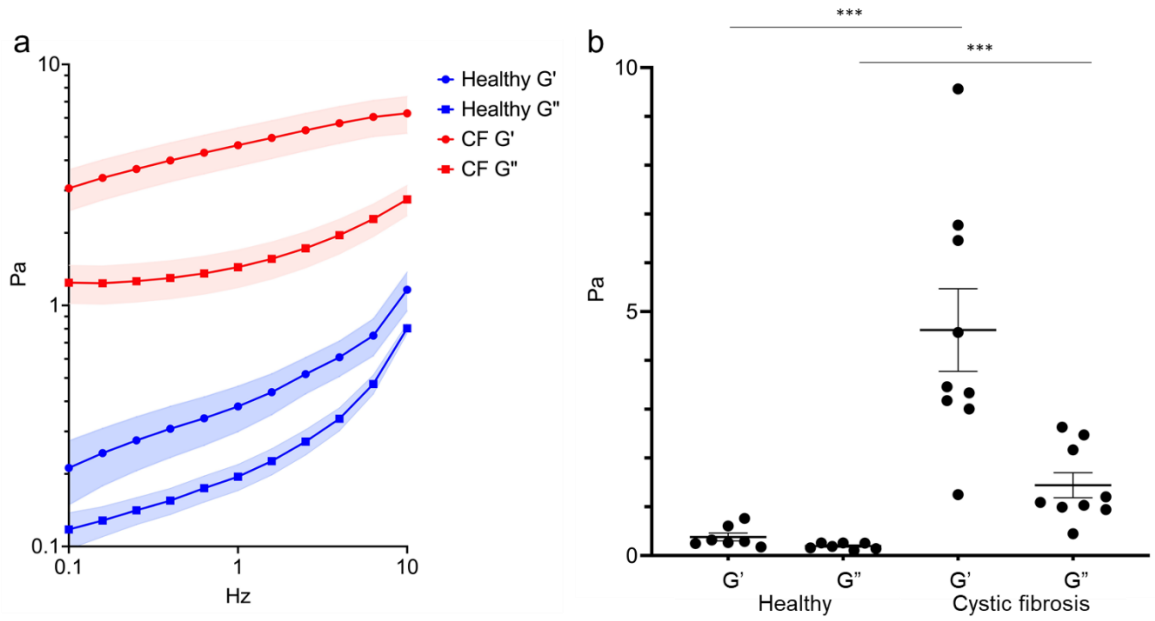
Supplementary figures

Supplementary figure S1



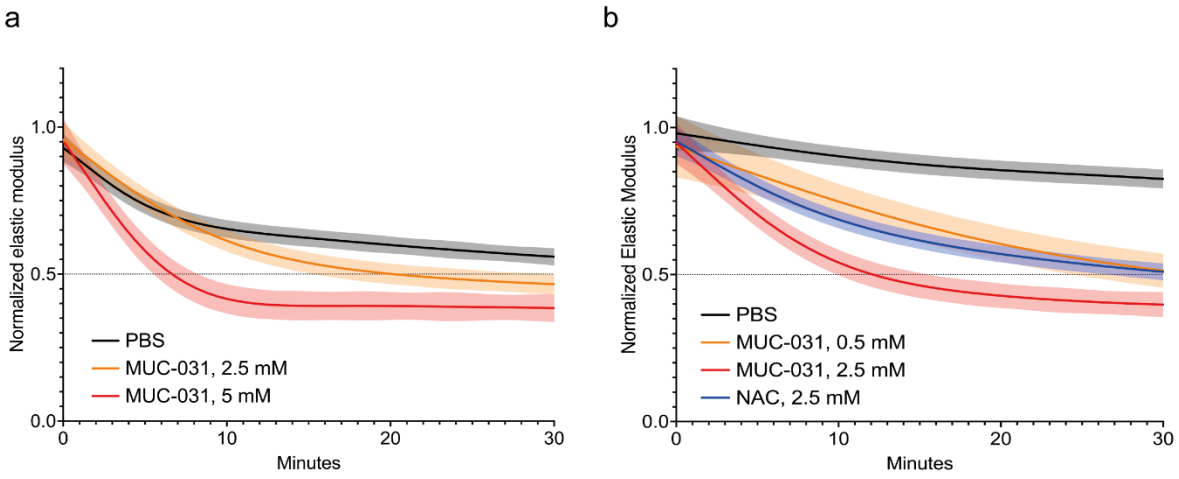
Supplementary figure S1. Effects of protease inhibitors (EDTA and Halt) on the decline in the normalized elastic modulus (G') of cystic fibrosis (CF) sputum after addition of PBS. Untreated (with Halt or EDTA) sputum samples to which PBS was added at 10% v/w show a relatively large time-dependent decline in normalized elastic modulus (G'). Pretreatment with EDTA alone had no effect, but pretreatment with EDTA and Halt effectively inhibited the decline in G' observed in CF sputum samples after adding PBS. n = 16 for untreated (i.e. no protease inhibitor added) sputum samples; n = 21 for EDTA-only treated samples; n = 8 for EDTA+Halt treated samples.

Supplementary figure S2



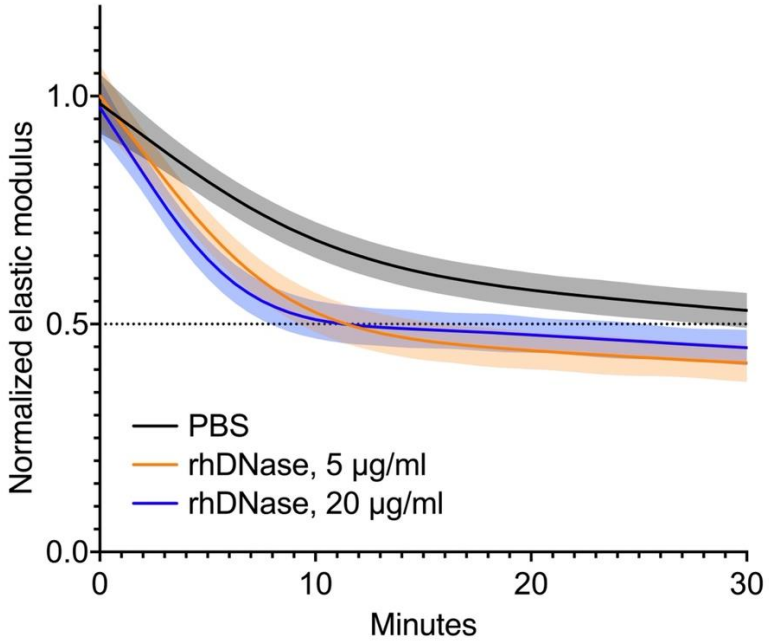
Supplementary figure S2: Comparison of the elastic and viscous behaviour of sputum in health and in cystic fibrosis (CF). a) Mean frequency sweeps of the elastic modulus (G′) and viscous modulus (G″) of induced sputum in health (open symbols, n = 7) and sputum spontaneously expectorated by patients with cystic fibrosis (CF) (closed symbols, n = 8). Error bands are SEM. In healthy sputum, the G' predominates over the G" across a broad range of frequencies. The G' predominance and plateau as well as the identical dependence of G' and G" on frequency (G' and G" are parallel lines) are hallmarks of a cross-linked gel. In CF sputum, the elastic and viscous moduli are higher than normal and the predominant abnormality is the markedly increased elastic response. b) Individual G′ and G″ (at a frequency of 1.0 Hz) in induced sputum from 7 healthy donors and spontaneously expectorated sputum from 8 CF donors. ***$P < 0.001$ for G' and G" values for healthy versus CF sputum.

Supplementary figure S3



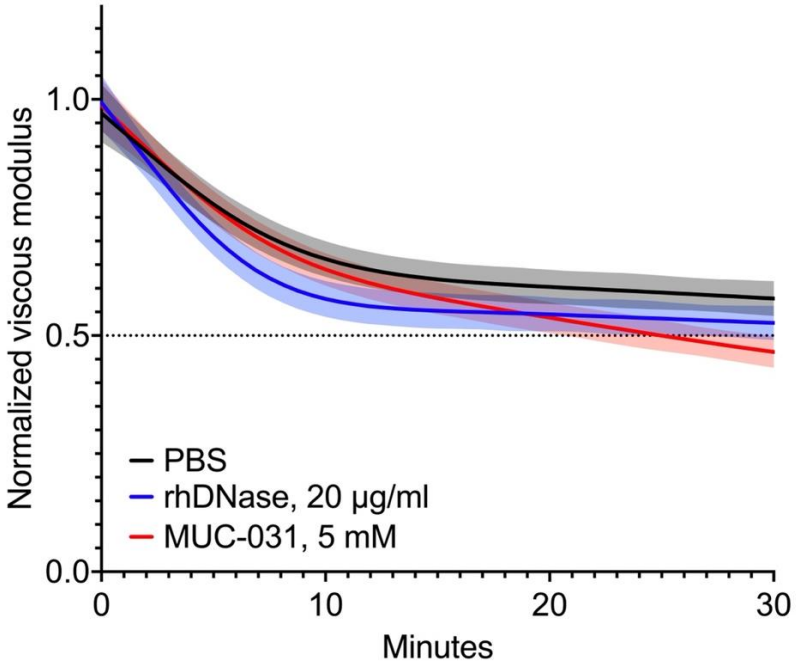
Supplementary figure S3. Effects of increasing concentrations of MUC-031 on the normalized elastic modulus (G') of sputum samples from patients with cystic fibrosis (CF). Measurements were performed after pretreatment of sputum samples with EDTA alone (a) and with a protease inhibitor cocktail of EDTA+Halt (b). Data show a dose response effect between 2.5 and 5 mM concentrations of MUC-031 in EDTA-only treated CF sputa and between 0.5 and 2.5 mM in sputa pretreated with Halt+EDTA. An overlay of NAC effects at 2.5 mM suggest that it is equivalent to the effects of 0.5 mM MUC-031. In (a), n = 21 for PBS control; n = 10 for MUC-031 at 2.5 mM; n = 13 for MUC-031 at 5 mM. In (b), n = 8 for PBS control; n = 5 for MUC-031 at 0.5 mM, n = 16 for MUC-031 and NAC at 2.5 mM condition.

Supplementary figure S4



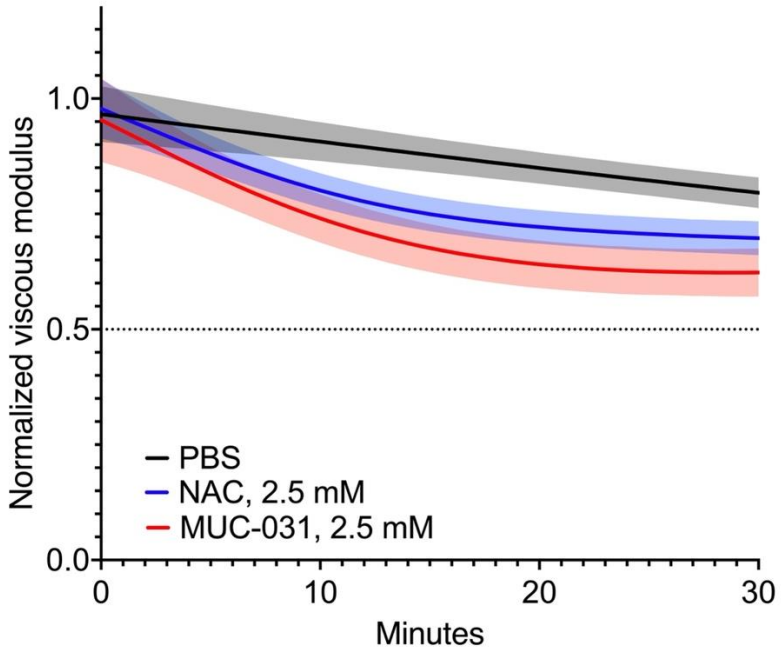
Supplementary figure S4. Effect of low dose and high dose recombinant human deoxyribonuclease I (rhDNase) on the normalized elastic modulus (G') of cystic fibrosis sputum. The data show a lack of a dose response effect, i.e. the overlapping surround colors that show the 95% pointwise confidence intervals over the time course indicate that the differences between conditions are not statistically significant. n = 15 for all groups.

Supplementary figure S5



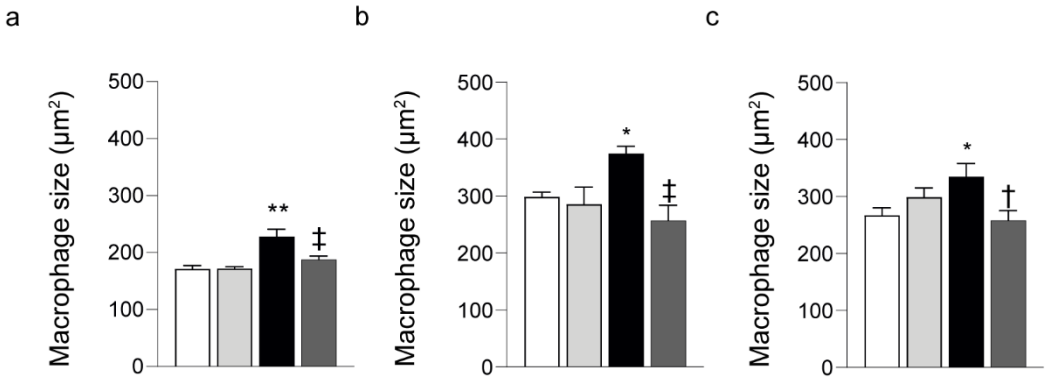
Supplementary figure S5. Effect of MUC-031 and recombinant human deoxyribonuclease I (rhDNase) and the normalized viscous modulus (G") of cystic fibrosis sputum. The data show little difference in the effects of MUC-031 and rhDNase - i.e. the surround colors that show the 95% pointwise confidence intervals over the time course indicate only small differences between drugs. n = 15 for all groups.

Supplementary figure S6



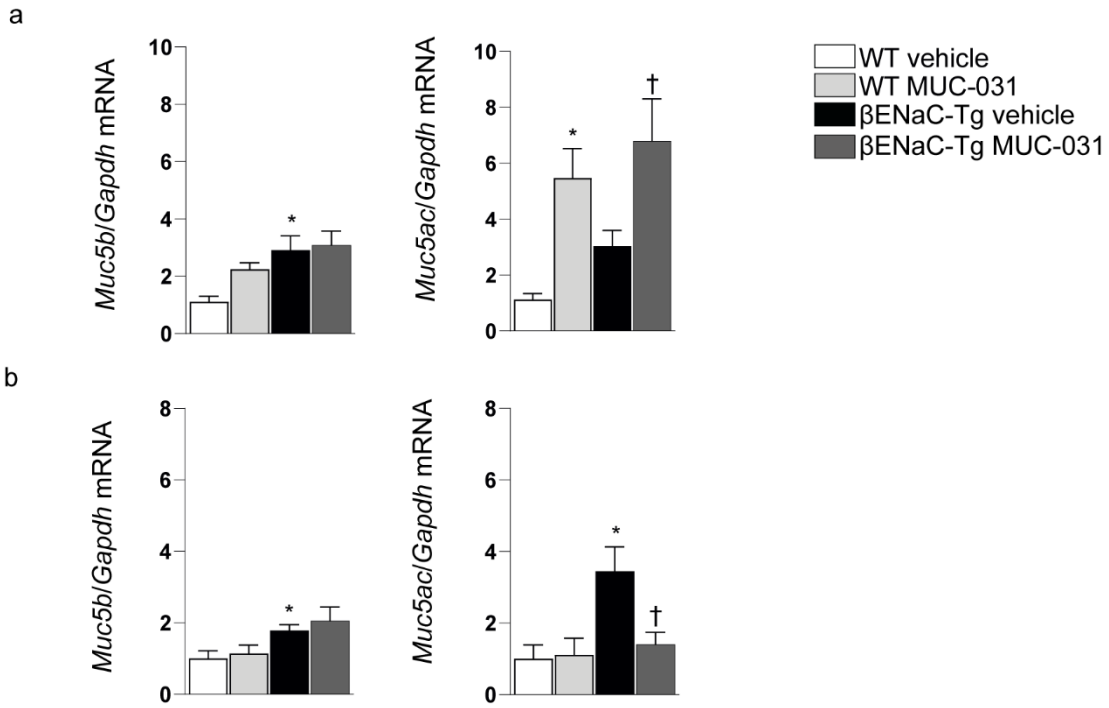
Supplementary figure S6. Effect of MUC-031 and N-acetyl cysteine (NAC) and the normalized viscous modulus (G") of CF sputum. The data show that the effect of MUC-031 on G" is larger than the effect of NAC. n = 16 for NAC and MUC-031 groups; n = 8 for PBS control.

Supplementary figure S7



Supplementary figure S7. Effect of MUC-031 treatment on macrophage size in bronchoalveolar lavage of βENaC-Tg mice. Adult and neonatal βENaC-Tg mice and wild-type (WT) littermates were treated with MUC-031 or vehicle alone as detailed in the online supplement. a, b) Effect of acute (a) and chronic (b) treatment with MUC-031 on macrophage size in adult βENaC-Tg and WT mice (n = 6 - 9 per group). c) Effect of preventive treatment with MUC-031 on macrophage size in neonatal βENaC-Tg and WT mice (n = 13 per group). *P < 0.05 and **P < 0.001 versus vehicle-treated WT mice. $^{\dagger}P$ < 0.05 and $^{\ddagger}P$ < 0.01 versus vehicle-treated βENaC-Tg mice.

Supplementary figure S8



Supplementary figure S8. Expression levels of *Muc5b* and *Muc5ac* in lung tissue of wild-type (WT) and βENaC-Tg mice after treatment with MUC-031. Adult and neonatal βENaC-Tg mice and WT littermates were treated with MUC-031 or vehicle alone for two weeks. a) *Muc5b* and *Muc5ac* transcript levels in lungs of adult (a; n = 8 - 13 per group) and neonatal (b; n = 9 - 29 per group) WT and βENaC-Tg mice after treatment with MUC-031 or vehicle alone for two weeks. *$P < 0.05$ compared with vehicle-treated WT mice. †$P < 0.05$ compared with vehicle-treated βENaC-Tg mice.

Supplemental references

E1. Farrell PM, White TB, Ren CL, Hempstead SE, Accurso F, Derichs N, Howenstine M, McColley SA, Rock M, Rosenfeld M, Sermet-Gaudelus I, Southern KW, Marshall BC, Sosnay PR. Diagnosis of Cystic Fibrosis: Consensus Guidelines from the Cystic Fibrosis Foundation. *J Pediatr* 2017; 181S: S4-S15 e11.

E2. Yuan S, Hollinger M, Lachowicz-Scroggins ME, Kerr SC, Dunican EM, Daniel BM, Ghosh S, Erzurum SC, Willard B, Hazen SL, Huang X, Carrington SD, Oscarson S, Fahy JV. Oxidation increases mucin polymer cross-links to stiffen airway mucus gels. *Sci Transl Med* 2015; 7: 276ra227.

E3. Genentech. PULMOZYME® (dornase alfa) inhalation solution, for inhalation use. 2021; https://www.gene.com/download/pdf/pulmozyme_prescribing.pdf.

E4. Wood SN. Generalized Additive Models: An Introduction with R. 2017.

E5. Team RC. R: A Language and Environment for Statistical Computing. 2019.

E6. Mall M, Grubb BR, Harkema JR, O'Neal WK, Boucher RC. Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. *Nat Med* 2004; 10: 487-493.

E7. Johannesson B, Hirtz S, Schatterny J, Schultz C, Mall MA. CFTR regulates early pathogenesis of chronic obstructive lung disease in betaENaC-overexpressing mice. *PLoS One* 2012; 7: e44059.

E8. Zhou Z, Treis D, Schubert SC, et al. Preventive but not late amiloride therapy reduces morbidity and mortality of lung disease in βENaC-overexpressing mice. *Am J Respir Crit Care Med* 2008: 178(12): 1245-1256.

E9. Miller MA, Stabenow JM, Parvathareddy J, et al. Visualization of murine intranasal dosing efficiency using luminescent Francisella tularensis: effect of instillation volume and form of anesthesia. *PLoS One* 2012: 7(2): e31359.

E10. Mall MA, Harkema JR, Trojanek JB, Treis D, Livraghi A, Schubert S, Zhou Z, Kreda SM, Tilley SL, Hudson EJ, O'Neal WK, Boucher RC. Development of chronic bronchitis and

emphysema in beta-epithelial Na+ channel-overexpressing mice. *Am J Respir Crit Care Med* 2008; 177: 730-742.

E11. Graeber SY, Zhou-Suckow Z, Schatterny J, Hirtz S, Boucher RC, Mall MA. Hypertonic saline is effective in the prevention and treatment of mucus obstruction, but not airway inflammation, in mice with chronic obstructive lung disease. *Am J Respir Cell Mol Biol* 2013; 49: 410-417.

E12. Harkema JR, Plopper CG, Hyde DM, St George JA. Regional differences in quantities of histochemically detectable mucosubstances in nasal, paranasal, and nasopharyngeal epithelium of the bonnet monkey. *J Histochem Cytochem* 1987; 35: 279-286.

E13. Ramsey KA, Rushton ZL, Ehre C. Mucin Agarose Gel Electrophoresis: Western Blotting for High-molecular-weight Glycoproteins. *J Vis Exp* 2016.

E14. Balazs A, Millar-Buchner P, Mulleder M, Farztdinov V, Szyrwiel L, Addante A, Kuppe A, Rubil T, Drescher M, Seidel K, Stricker S, Eils R, Lehmann I, Sawitzki B, Rohmel J, Ralser M, Mall MA. Age-Related Differences in Structure and Function of Nasal Epithelial Cultures From Healthy Children and Elderly People. *Front Immunol* 2022; 13: 822437.