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Original Article

A small molecule neutrophil elastase inhibitor, KRP-109, inhibits cystic fibrosis mucin degradation☆

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Abstract

Background: Neutrophil elastase (NE) rapidly degrades gel-forming airway mucins in cystic fibrosis (CF) sputum. We hypothesized that KRP-109, a small molecule NE inhibitor, would inhibit CF mucin degradation *in vitro*.
Methods: Sputa were collected from CF patients (n = 5) chronically or intermittently infected with *Pseudomonas aeruginosa* (P.a.). Mucin degradation was analyzed using western blot. Protease inhibitor studies were performed using alpha1-proteinase inhibitor (A1-PI Prolastin®) and KRP-109. Elastase activity assays were performed using spectrophotometry.
Results: There were significant differences in the amount of active NE in different CF sputum samples. KRP-109 decreased the NE driven mucin degradation *in vitro*. *Pseudomonas* elastases appeared to blunt elastase inhibition by A1-PI or KRP-109.
Conclusion: Inhibitors of neutrophil and *Pseudomonas*-derived elastases might rescue mucus clearance and reverse airway obstruction in CF.

Keywords: Small molecule inhibitors; Protease and anti-protease balance; Cystic fibrosis; Mucin

1. Introduction

Mutations in the CF transmembrane ion regulator (CFTR) gene impair airway ion homeostasis [1,2] leading to accumulation of sticky secretions on the surface of airways and impairing mucus clearance [3]. Chronic infections are a pathological consequence in CF [4]. Repeated infections caused by bacteria such as *Pseudomonas aeruginosa* (*P.a.*) lead to neutrophil infiltration in the airways [5]. Neutrophils and mucus entrap pathogens *via* neutrophil extracellular traps (NET) [6,7]. These inflammatory cells and pathogens secrete proteases such as neutrophil elastase (NE) [8], cathepsin G (CG), proteinase-3 (PR3) [9], matrix metalloproteases [10], and elastolytic proteases expressed by *P.a.* [11,12]. Increased recruitment and activation of neutrophils and NE production damages the airway architecture

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leading to progressive lung dysfunction [13]. In response to protease secretion, anti-proteases such as alpha1-proteinase inhibitor (A1-PI), secretory leukoprotease inhibitor (SLPI), and elafin are produced in descending abundance in the lung [14].

We have previously reported that these serine proteases cause degradation of CF airway mucins *in vitro* [15]. As well, an impaired anti-oxidative stress response in CF [16] may lead to degradation, oxidation, or oligomerization of anti-proteases decreasing their activity in the airways [17]. During chronic infections in CF, *P.a.*-derived elastases can degrade or neutralize the host anti-proteases in the airways [18]. The restoration of anti-proteases or inhibition of host and pathogen proteases in chronic inflammatory lung diseases like CF might be a valuable therapeutic intervention [19,20]. A1-PI is a potent inhibitor of NE; however, A1-PI is expensive and subject to degradation [17,21]. The administration of novel NE inhibitors may be more effective and efficient than A1-PI [22,23]. KRP-109, a potent, selective neutrophil elastase inhibitor, was shown to reduce lung inflammation in a murine model of severe pneumococcal pneumonia [24]. This drug is water-soluble competitive inhibitor of NE and can concentrate in lung tissue. KRP-109 inhibited NE-induced lung hemorrhage and edema after the intratracheal administration of LPS [25]. Therefore, KRP-109 could be a potent inhibitor of NE for treating airway diseases such as CF.

In this study, we tested the efficacy of KRP-109 on inhibiting CF mucin degradation *in vitro* and compared the effect of KRP-109 to purified A1-PI. We then examined the role of *P.a.*-derived elastases on mucin degradation and tested the efficacy of A1-PI and KRP-109 in inhibiting *P.a.* elastases.

2. Materials and methods

2.1. Subject selection

Sputa were collected from 5 subjects with CF by spontaneous expectoration [15] and quickly frozen at −70 °C for preservation (Supplement Table 1). We selected subjects with chronic or intermittent *P.a.* infection; a patient is considered to be chronically infected if *P.a.* was detected in 3 consecutively collected sputa or in more than 5 out of 8 consecutively collected sputa over a minimum of 2 years; a patient is considered to be intermittently infected if *P.a.* was detected in at least 1 but no more than 4 sputum samples. A "standard mucin" was used as an internal control and as sample with the *Pseudomonas* supernatant. Standard mucin was collected from subjects who had no lung disease and required non-thoracic surgery under general anesthesia. After removing the endotracheal tube (ETT) from the airways, the mucus that coated the ETT was collected by gently scraping the ETT as previously described [15].

2.2. Analyzing functionally active HNE levels in CF sputa

To measure the amount of active HNE, we measured the hydrolysis of the chromogenic substrate *N*-methoxysuccnyl-Ala-Ala-Pro-Val p-nitroanilide (Sigma Aldrich), which is specifically hydrolyzed by NE but not hydrolyzed by cathepsin G. Unless otherwise stated, all measurements were done as described in the elastin products company manufacturer's protocols (Owensville, Missouri, USA). Spectrophotometric measurements were performed using the Nicolet Evolution 100 UV-Vis Spectrophotometer. Results were analyzed using VISIONlife™ software.

2.3. Determining an IC-50 concentration

The half-maximal inhibitory concentration (IC-50) of protease inhibitors (A1-PI and KRP-109) was determined by calculating the half-maximal concentration of the respective inhibitors required to inhibit purified NE (Calbiochem®, product no. 324681) and decrease substrate degradation by 50%. Purified NE was used as positive control. Three independent measurements were considered for all experiments. We incubated purified NE with chromogenic substrate and measured the rate of increase in absorbance at 30-s intervals. The rate of increase was linear for 30 min confirming that the chemical reaction is in a steady state, meaning that there is an excess of substrate and functionally active enzymes. Using these data, we then calculated the IC-50 concentration for both inhibitors. Similarly, we calculated the activity of NE in sputa using the same substrate and accordingly calculated the amount of inhibitor required to achieve the IC-50 in each sputum sample. All samples were thoroughly mixed with the inhibitor in IC-50 concentration and incubated for 2 and 4 h at 37 °C.

2.4. Pseudomonas strains

P.a. mutant strains, lasB (2 strains), lasA and protease IV, and a PA14 wild-type (wt) strain as control were generous gifts from Prof. Dr. Susanne Häußler. Details about the construction of the mutants and their genetic background can be obtained from the website http://ausubellab.mgh.harvard.edu/cgi-bin/pa14/home.cgi last accessed on 05/2014. The strains were revived on LB agar plates containing antibiotics (described in the website) and incubated overnight at 37 °C. Unless otherwise indicated, liquid medium was inoculated from an overnight pre-cultured plate and incubated at 37 °C with constant shaking at 225 rpm. All glassware was washed with double distilled water and thoroughly autoclaved before use. Cells were harvested by centrifugation (Sorval RC 5B plus), and supernatant and pellet fractions were separated. Resulting cell pellets were suspended in cold 0.05 M NaoAc containing 0.1 M NaCl, pH 5, and incubated with standard ETT mucin samples to determine the exogenous protease activity. Alternatively, we also tested the supernatant fractions for protease activity by incubating with standard mucin extracted from ET tubes as described above. Each mutant strain was incubated with and without inhibitors at 37 °C for 4 h, and mucin degradation was analyzed using wet westerns and compared them with standard ETT mucus.

2.5. Gel electrophoresis and blotting

Incubated samples were thoroughly denatured with laemmeli buffer (250 mM Tris, pH 6.8, 4% SDS, 20% glycerol, 0.001% bromophenol blue, 20 mM dithiothreitol [DTT]) and subsequently by denaturing the samples for 5 min at 95 °C. Proteins were separated, blotted, and probed for MUC5AC and MUC5B, NE, and A1-PI as described earlier [15].

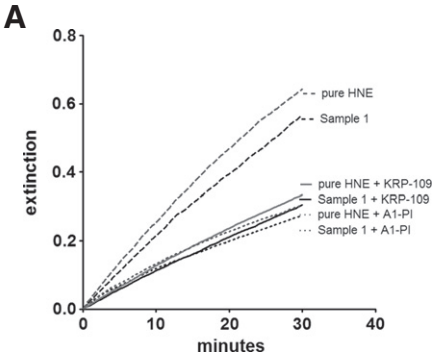
2.6. Statistics

To analyze our data, we used Graph Pad Prism®. Significance was obtained using Student's unpaired *t*-test. A p value <0.05 was considered as significant.

3. Results

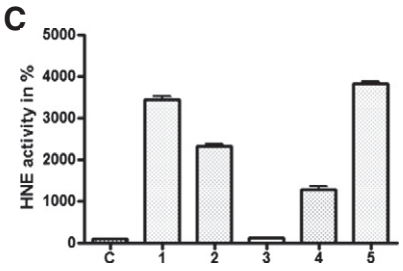
3.1. Quantification of NE activity in CF sputum and measuring the IC-50 for each anti-protease

Before analyzing the inhibition of mucin degradation in CF sputa, we quantified the protease activity in sputum samples. We first measured the elastase activity of pure NE using the synthetic NE substrate, N-MeO-Suc-Ala-Ala-Pro-Val-pNA. To define the half-maximal inhibitor concentration (IC-50), it was necessary to saturate the NE protein. Thus, different dilutions and concentrations of anti-protease were added to the assay mixture, keeping the NE concentration constant (3 μL of a 0.1 mg/mL). The inhibition of NE by A1-PI and KRP-109 was monitored at 410 nm (Fig. 1A). The calculated values of IC-50 were 1.45 μM and 7.57 μM for A1-PI and KRP-109, respectively (Fig. 1B). We then quantified the NE activity in CF mucin samples again by incubating them with synthetic NE substrate and compared it with the pure NE activity. We observed that the levels of functionally active NE varied greatly among our samples. The range was from 121% of our internal standard to 3800% of our internal standard (Fig. 1C). We accordingly diluted the mucin samples and adjusted the NE concentration close to pure NE. To verify the adjustment, we again performed the synthetic NE degradation assay with appropriate dilutions (e.g., 1:100 dilution of sample 1). The activity of NE in diluted sample 1 corresponded to the activity of pure NE (Fig. 1A). Subsequently, we validated the IC-50 calculations of both the inhibitors using CF sample 1. The calculated IC-50 values of A1-PI and KRP-109 using pure NE also correlated with the IC-50 value of the diluted sample 1 (Fig. 1A). Therefore, we diluted the rest of the mucin samples according to the Lambert–Beer law (Supplement 1 formula for calculating in-sample enzyme activity) and calculated the IC-50 for A1-PI and KRP-109 required for inhibiting NE in all the CF samples (Fig. 1C).



B

Sample	IC-50 Concentration of A1-PI [μM]	% of standard	IC-50 concentration of KRP-109 [μM]
Standard (pure HNE)	1.457	100.000	7.575
Sample 1	49.959	3428.906	259.740
Sample 2	33.903	2326.923	176.264
Sample 3	1.763	121.017	9.167
Sample 4	18.846	1293.482	97.981
Sample 5	55.931	3838.797	290.789



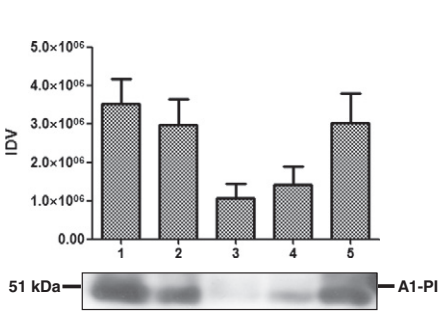


Fig. 1. Quantification of HNE in CF mucin samples *in vitro*. (A) Spectrophotometric determination of NE activity and inhibitory concentration determination (IC-50) of KRP-109 and A1-PI using synthetic NE substrate and pure NE. (B) Calculation of inhibitory concentrations (IC-50) of A1-PI and KRP-109 against active NE within sample sputa based on NE substrate degradation and compared to a defined amount of purified HNE (standard), which was also shown exemplary in Fig. 1A. (C) Representative graphs of NE activity in CF mucin samples calculated in comparison to pure NE, where C represents pure NE activity. The fold change in NE activity is represented as a percentage. Error bars were obtained from 3 independent estimations. (D) Densitometry analysis of A1-PI immunoblots from CF sputa represented as integrated density values. Error bars represent the mean from 3 different immunoblots. Representative image of one of the three immunoblots of A1-PI.

3.2. Altered specificity to anti-proteases in different CF sputum samples over time

CF sputum samples were incubated with the calculated IC-50 concentrations of A1-PI and KRP-109, and we analyzed the reduction in mucin degradation, compared with control mucin samples without inhibitors. After 2 h of incubation, MUC5AC in samples 2, 4, and 5 and MUC5B in all the samples exhibited reduced mucin degradation in comparison to mucin samples without the inhibitor. We observed less MUC5AC degradation in sample 3, which further supports our NE measurements (Fig. 1C) where we observed much less active NE in sample 3 (Supplementary Fig. 1A). Interestingly, sample 1 also did not show any significant degradation of MUC5AC after 2 h with and without inhibitors despite the fact that it has increased elastase activity (Supplementary Fig. 1B). To evaluate this possible discrepancy, we analyzed the anti-proteases in all the mucin samples. We found that sample 1 had significantly high levels of anti-protease when compared to sample 3 (Fig. 1D). It was reported that there exists a positive correlation between sputum levels of active elastase and A1-PI, indicating a compensatory increase in A1-PI in response to increase elastase load [26]. Thus, we reasoned that increased levels of A1-PI in sample 1 might inhibit MUC5AC degradation within 2 h. Surprisingly, the degradation of MUC5AC in samples 2 and 5 and MUC5B in sample 5 was inhibited more effectively with KRP-109 Supplementary Fig. 1A and B).

After 4 h of incubation, the increased degradation of MUC5AC was observed in samples 1, 2, and 4 (Supplementary Fig. 1C), while MUC5B degradation was observed in all samples (Supplementary Fig. 1D). We also observed that KRP-109 decreased MUC5AC degradation in samples 1 and 4 after 4 h, while degradation of MUC5AC in samples 2 and 3 was greater with A1-PI (Supplementary Fig. 1C). We could not quantify the results of sample 5 due to sample size limitation. Consistent with our NE measurements, we did not see much MUC5AC degradation in sample 3 even after 4 h of incubation (Supplementary Fig. 1C). However, after 4 h, we found that KRP-109 reduced MUC5B degradation in 3 out of 5 CF samples (Supplementary Fig. 1D).

KRP-109 was also able to reduce mucin degradation at 2 and 4 h for (Fig. 2). These observations suggest KRP-109 might reduce MUC5B degradation over time. Most importantly, the amounts of active NE present in the sputum samples play an important role in mucin degradation *in vitro*.

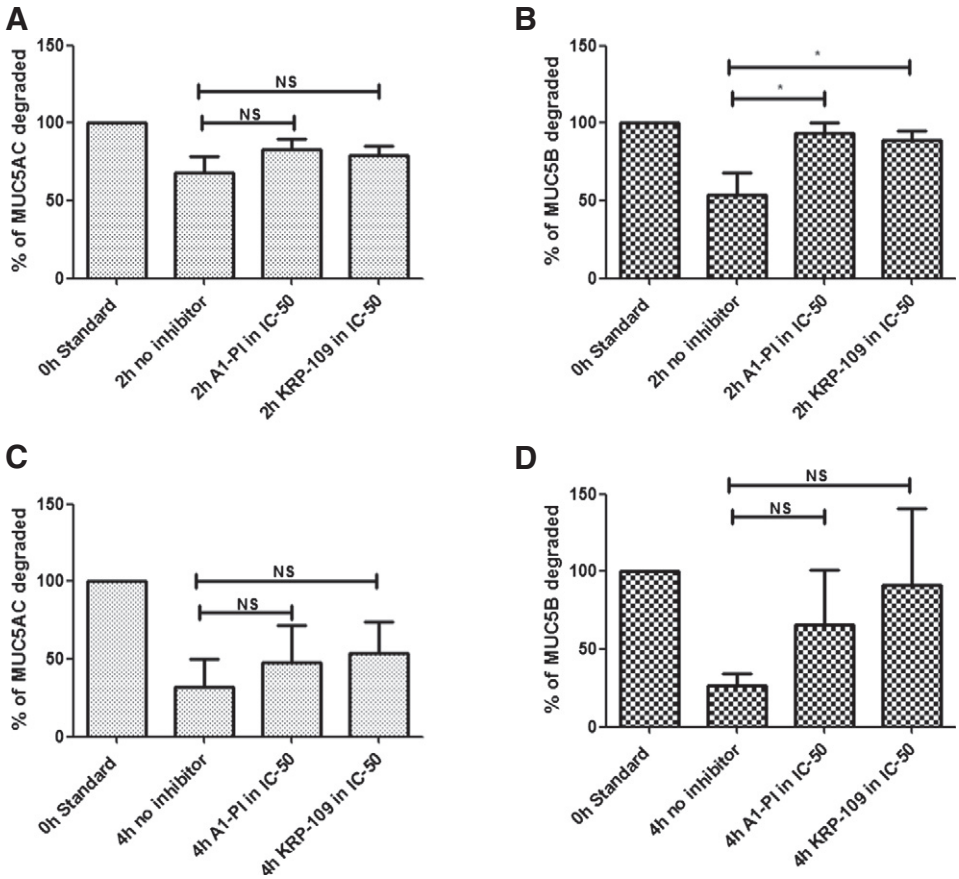


Fig. 2. MUC5AC and MUC5B degradation of all CF sputa *in vitro* after 2 and 4 h. Mean densitometry quantification of mucin degradation from all CF sputa analyzed: $n = 5$ for (A) MUC5AC degradation and (B) MUC5B degradation after 2 h (C) MUC5AC degradation and (D) MUC5B degradation after 4 h incubated with and without protease inhibitors for 4 h. NS; not significant. $^*P < 0.05$.

3.3. *Pseudomonas aeruginosa-derived proteases degrade mucus in vitro*

To gain further insights into the role of *P.a.*-derived proteases on *in vitro* mucin degradation, we performed immunoblots for MUC5AC after incubating with different *P.a.* mutant strains (LasA, LasB1, LasB2, and protease IV) and compared these with wild-type PA14 strain. Different approaches were planned to evaluate the effect of mucin degradation upon incubation with protease deletion mutants. In the first approach, pellet and supernatant fractions of *P.a.* mutants and control strains that were grown to late log phase were separated and incubated with control mucin. In the second approach, pellet and supernatant fractions of cultures grown overnight were incubated with control mucus at 37 °C for 3 h. In comparison to ETT control mucus incubated without *P.a.* knockout strains, we found that LasA and LasB secreting Δ*prpL* mutant degraded mucin efficiently when compared with LasA and Protease IV secretions in Δ*lasB* mutant. Likewise, LasB together with Protease IV in Δ*lasA* mutant degraded mucin much more efficiently when compared with the LasA and LasB in Δ*prpL* mutant. Further, we found that LasA, LasB and Protease IV in wild-type *P.a.* had the maximum degrading effect (Fig. 3). These results suggest that *lasB* encoding protease is efficient in mucus degradation.

3.4. *Protease inhibitors do not inhibit the PA proteases*

To verify the effect of commercially available anti-proteases A1-PI (Prolastin) and the experimental anti-protease KRP-109 in inhibiting *P.a.*-derived proteases, we co-treated control mucus with anti-proteases and protease knockout *P.a.* mutant strains. We observed that these anti-proteases did not inhibit proteases derived from *P.a.* It is possible that pyocyanin, released from *P.a.* strains, inhibited the NE and A1-PI complex formation [27]. However, the *P.a.* elastases were shown to be highly potent in inactivating human A1-PI with a molar ratio of elastase/inhibitor = 1:100 [18]. Most of the results obtained were similar to the incubation of *P.a.* strains as discussed in Section 3.3 (Fig. 4A and B). Results obtained from Sections 3.3 and 3.4 show that LasB knockout *P.a.* strain dramatically

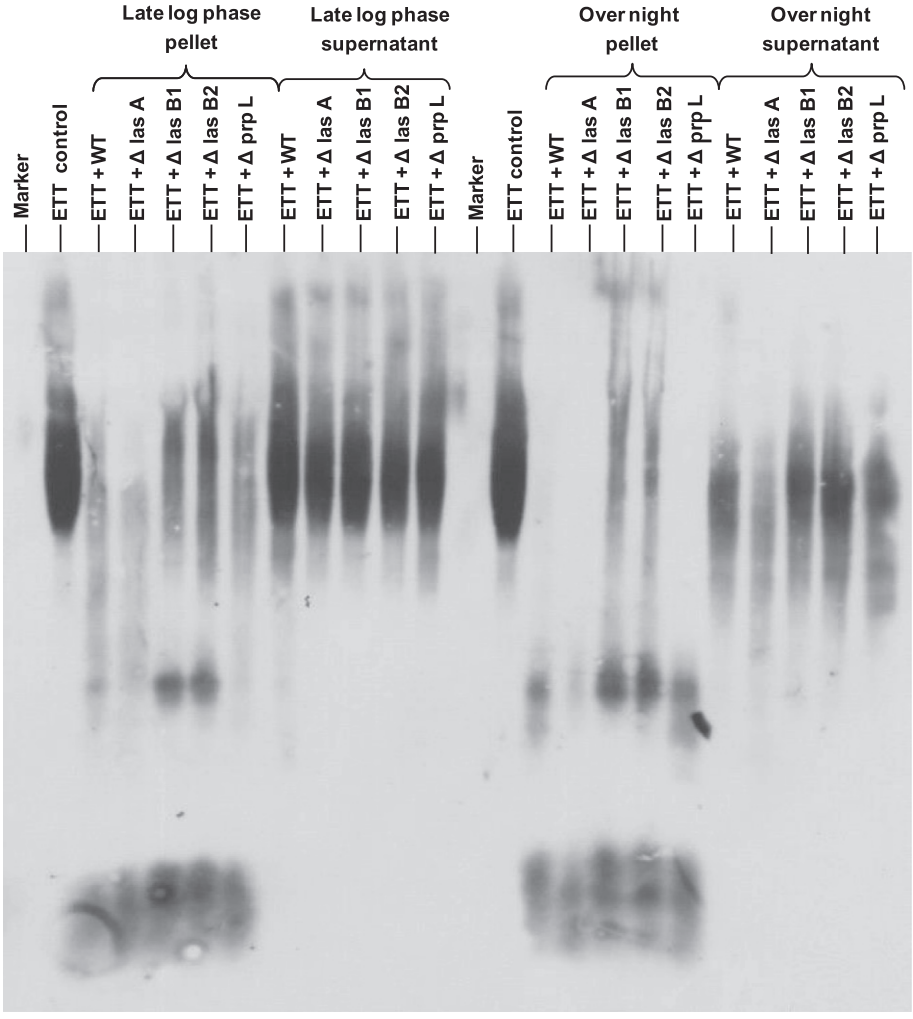


Fig. 3. *Pseudomonas*-derived proteases degrade mucins from CF. Immunoblot analysis of CF mucin degradation (MUC5AC) when incubated with the indicated protease knockout strains from *P.a.* and grown to late log phase or overnight cultures for pellet and supernatant fractions as indicated.

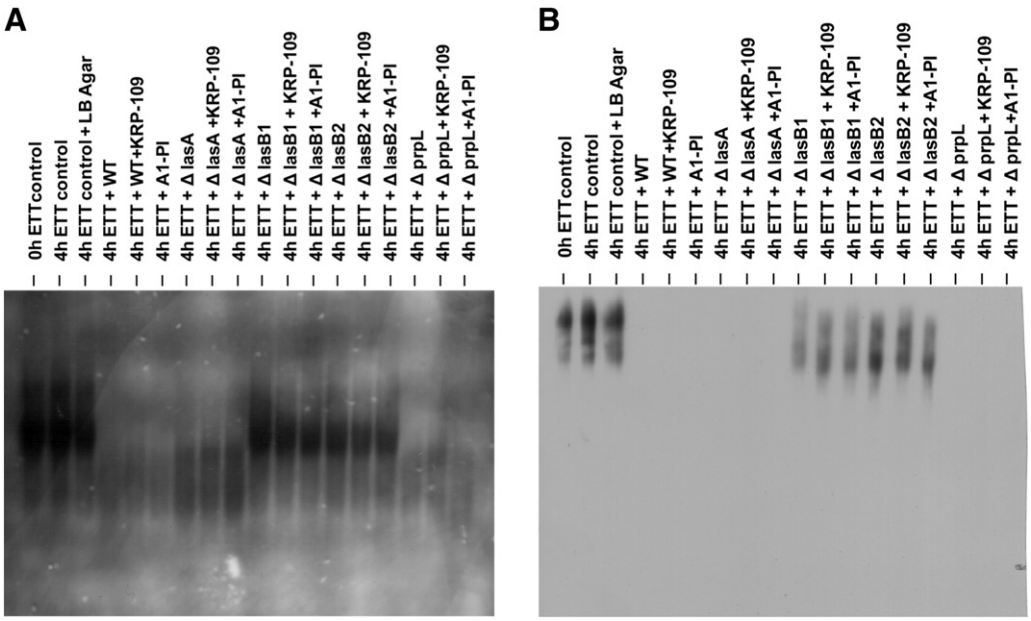


Fig. 4. A1-PI and KRP-109 did not rescue mucin degradation. Immunoblot analysis to determine the ability of A1-PI and KRP-109 to inhibit mucin degradation (A) MUC5AC and (B) MUC5B when incubated with different *P.a.* protease knockout strains for 4 h as indicated.

decreased mucin degradation. In fact, the *LasB* gene encodes an essential metalloproteinase thus making it an important virulence factor for *P.a*.

4. Discussion

Human NE is an important pharmacological target for CF therapy. The goal of this study was to analyze the effectiveness of the NE inhibitor KRP-109, in reducing the excessive protease activity in CF airway, and potentially normalizing intact mucin concentration by inhibiting degradation of gel-forming mucins. In CF patients, there is airway plugging due to accumulation of mucus and DNA [28]. While defective CFTR-mediated altered chloride ion imbalance leads to formation of sticky mucus in the CF airway, increased secretion of NE by neutrophils decreases the concentration of intact MUC5AC and MUC5B mucins [29]. Consistent with this, we reported that serine proteases in CF sputum is a major cause of increased mucus degradation *in vitro* [15]. Airway obstruction due to mucus plugging is sometimes attributed to either mucin overproduction or decreased mucus hydration in CF. MUC5B mucin is essential for mucociliary clearance and innate immune defense in the murine lung [14]. MUC5B-deficient mice died much faster than wild-type mice when their lungs were inoculated with *Staphylococcus aureus,* while MUC5B overexpressing mice had faster mucociliary clearance and improved macrophage function compared to wild-type mice [30]. In light of these studies, we found that the KRP-109 blocked degradation of MUC5B after 2 h (Supplementary Fig. 1B), an effect that was further enhanced after 4 h (Supplementary Fig. 1D).

Earlier studies in CF subjects showed that inhalation of 25 mg A1-PI as Prolastin® daily for four weeks did not improve lung function but was able to decrease free elastolytic activity, neutrophil count, colony forming units of *P.a.*, and proinflammatory cytokines in sputum [31,32]. Studies involving complete knockdown of NE in CF-like murine model "scnn1-TG2" reduced airway neutrophils, mucin expression, goblet cell metaplasia, and emphysema but had no significant effect on airway mucus plugging, bacterial infection, or pulmonary mortality [33]. These studies confirm that changes in the pulmonary protease–anti-protease balance can affect airway inflammation [34,35]. Increased NE and decreased A1-PI was shown to increase the nonheme iron levels in the cells by degrading ferritin [36,37]. Consistent with these studies, decreased hemeoxygenase-1 and ferritin are reported to increase inflammation in CF airways [16]. Anti-inflammatory drugs like ibuprofen and glucocorticoids are unable to overcome the protease burden in CF [38], while inhalation of anti-proteases had some effect reducing proteases and inflammation [31,32], although only a limited effect of recombinant A1-PI on protease inhibition and inflammation was reported in clinical studies [39].

In this study, we analyzed mucus degradation *in vitro* as another effect of proteases that is physiologically importance [30,40]. *P.a.*-derived proteases were able to degrade mucins, but these proteases were not inhibited by these inhibitors in the concentrations used. The *Pseudomonas*-derived proteases, especially the *lasB* gene encoding protease significantly degraded mucin *in vitro*. This suggests that it might be useful to include LasB inhibitors, such as *N*-mercaptoacetyl-Phe-Tyr-amide, to further inhibit protease activity [33] and mucin degradation in the lung.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jcf.2015.10.008.

Conflict of interest statement

The authors report no conflicts of interest.

References

[1] Boucher RC. An overview of the pathogenesis of cystic fibrosis lung disease. Adv Drug Deliv Rev 2002;54:1359–71.

[2] Cheng SH, Gregory RJ, Marshall J, Paul S, Souza DW, White GA, et al. Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. Cell 1990;63:827–34.

[3] Kreda SM, Davis CW, Rose MC. CFTR, mucins, and mucus obstruction in cystic fibrosis. Cold Spring Harb Perspect Med 2012;2:a009589.

[4] Cohen TS, Prince A. Cystic fibrosis: a mucosal immunodeficiency syndrome. Nat Med 2012;18:509–19.

[5] Dakin CJ, Numa AH, Wang H, Morton JR, Vertzyas CC, Henry RL. Inflammation, infection, and pulmonary function in infants and young children with cystic fibrosis. Am J Respir Crit Care Med 2002;165: 904–10.

[6] Manzenreiter R, Kienberger F, Marcos V, Schilcher K, Krautgartner WD, Obermayer A, et al. Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy. J Cyst Fibros 2012;11:84–92.

[7] Papayannopoulos V, Zychlinsky A. NETs: a new strategy for using old weapons. Trends Immunol 2009;30:513–21.

[8] Gifford AM, Chalmers JD. The role of neutrophils in cystic fibrosis. Curr Opin Hematol 2014;21:16–22.

[9] Korkmaz B, Horwitz MS, Jenne DE, Gauthier F. Neutrophil elastase, proteinase 3, and cathepsin G as therapeutic targets in human diseases. Pharmacol Rev 2010;62:726–59.

[10] Gaggar A, Hector A, Bratcher PE, Mall MA, Griese M, Hartl D. The role of matrix metalloproteinases in cystic fibrosis lung disease. Eur Respir J 2011;38:721–7.

[11] Hastie AT, Hingley ST, Kueppers F, Higgins ML, Tannenbaum CS, Weinbaum G. Protease production by *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Infect Immun 1983;40:506–13.

[12] Doring G, Goldstein W, Roll A, Schiotz PO, Hoiby N, Botzenhart K. Role of *Pseudomonas aeruginosa* exoenzymes in lung infections of patients with cystic fibrosis. Infect Immun 1985;49:557–62.

[13] Birrer P. Proteases and antiproteases in cystic fibrosis: pathogenetic considerations and therapeutic strategies. Respiration 1995;62(Suppl. 1): 25–8.

[14] Greene CM, McElvaney NG. Proteases and antiproteases in chronic neutrophilic lung disease—relevance to drug discovery. Br J Pharmacol 2009;158:1048–58.

[15] Henke MO, John G, Rheineck C, Chillappagari S, Naehrlich L, Rubin BK. Serine proteases degrade airway mucins in cystic fibrosis. Infect Immun 2011;79:3438–44.

[16] Chillappagari S, Venkatesan S, Garapati V, Mahavadi P, Munder A, Seubert A, et al. Impaired TLR4 and HIF expression in cystic fibrosis bronchial epithelial cells downregulates hemeoxygenase-1 and alters iron homeostasis in vitro. Am J Physiol Lung Cell Mol Physiol 2014;307: L791–9.

[17] Carp H, Miller F, Hoidal JR, Janoff A. Potential mechanism of emphysema: alpha 1-proteinase inhibitor recovered from lungs of cigarette smokers contains oxidized methionine and has decreased elastase inhibitory capacity. Proc Natl Acad Sci U S A 1982;79:2041–5.

[18] Morihara K, Tsuzuki H, Oda K. Protease and elastase of *Pseudomonas aeruginosa*: inactivation of human plasma alpha 1-proteinase inhibitor. Infect Immun 1979;24:188–93.

[19] Meyer KC, Zimmerman J. Neutrophil mediators, *Pseudomonas*, and pulmonary dysfunction in cystic fibrosis. J Lab Clin Med 1993;121: 654–61.

[20] Stockley RA. Neutrophils and protease/antiprotease imbalance. Am J Respir Crit Care Med 1999;160:S49–52.

[21] Stoller JK, Rouhani F, Brantly M, Shahin S, Dweik RA, Stocks JM, et al. Biochemical efficacy and safety of a new pooled human plasma alpha(1)-antitrypsin, Respitin. Chest 2002;122:66–74.

[22] Dunlevy FK, Martin SL, de Courcey F, Elborn JS, Ennis M. Anti-inflammatory effects of DX-890, a human neutrophil elastase inhibitor. J Cyst Fibros 2012;11:300–4.

[23] Elborn JS, Perrett J, Forsman-Semb K, Marks-Konczalik J, Gunawardena K, Entwistle N. Efficacy, safety and effect on biomarkers of AZD9668 in cystic fibrosis. Eur Respir J 2012;40:969–76.

[24] Yamada K, Yanagihara K, Araki N, Harada Y, Morinaga Y, Izumikawa K, et al. In vivo efficacy of KRP-109, a novel elastase inhibitor, in a murine model of severe pneumococcal pneumonia. Pulm Pharmacol Ther 2011;24:660–5.

[25] Junichi I, Kenji A, Masahiro M, Yoshiaki K, Shigeru I, Eric O, et al. KRP-109, a novel neutrophil elastase inhibitor alleviates LPS-induced lung edema with prevention of decrement in expression levels of ENaC ± and AQP-5 in rats. B69 the endothelial and epithelial barriers in injured lungs. American Thoracic Society International Conference AbstractsAmerican Thoracic Society; 2010 (A3617-A3617).

[26] O'Connor CM, Gaffney K, Keane J, Southey A, Byrne N, O'Mahoney S, et al. alpha 1-Proteinase inhibitor, elastase activity, and lung disease severity in cystic fibrosis. Am Rev Respir Dis 1993;148:1665–70.

[27] Britigan BE, Railsback MA, Cox CD. The *Pseudomonas aeruginosa* secretory product pyocyanin inactivates alpha1 protease inhibitor: implications for the pathogenesis of cystic fibrosis lung disease. Infect Immun 1999;67:1207–12.

[28] Schwab U, Abdullah LH, Perlmutt OS, Albert D, Davis CW, Arnold RR, et al. Localization of *Burkholderia cepacia* complex bacteria in cystic fibrosis lungs and interactions with *Pseudomonas aeruginosa* in hypoxic mucus. Infect Immun 2014;82:4729–45.

[29] Henke MO, Renner A, Huber RM, Seeds MC, Rubin BK. MUC5AC and MUC5B mucins are decreased in cystic fibrosis airway secretions. Am J Respir Cell Mol Biol 2004;31:86–91.

[30] Roy MG, Livraghi-Butrico A, Fletcher AA, McElwee MM, Evans SE, Boerner RM, et al. Muc5b is required for airway defence. Nature 2014; 505:412–6.

[31] Griese M, Latzin P, Kappler M, Weckerle K, Heinzlmaier T, Bernhardt T, et al. alpha1-Antitrypsin inhalation reduces airway inflammation in cystic fibrosis patients. Eur Respir J 2007;29:240–50.

[32] Griese M, Kappler M, Gaggar A, Hartl D. Inhibition of airway proteases in cystic fibrosis lung disease. Eur Respir J 2008;32:783–95.

[33] Gehrig S, Duerr J, Weitnauer M, Wagner CJ, Graeber SY, Schatterny J, et al. Lack of neutrophil elastase reduces inflammation, mucus hypersecretion, and emphysema, but not mucus obstruction, in mice with cystic fibrosis-like lung disease. Am J Respir Crit Care Med 2014;189:1082–92.

[34] Fischer BM, Voynow JA, Ghio AJ. COPD: balancing oxidants and antioxidants. Int J Chron Obstruct Pulmon Dis 2015;10:261–76.

[35] Fischer BM, Pavlisko E, Voynow JA. Pathogenic triad in COPD: oxidative stress, protease-antiprotease imbalance, and inflammation. Int J Chron Obstruct Pulmon Dis 2011;6:413–21.

[36] Fischer BM, Domowicz DA, Zheng S, Carter JL, McElvaney NG, Taggart C, et al. Neutrophil elastase increases airway epithelial nonheme iron levels. Clin Transl Sci 2009;2:333–9.

[37] Ghio AJ, Soukup JM, Richards JH, Fischer BM, Voynow JA, Schmechel DE. Deficiency of alpha-1-antitrypsin influences systemic iron homeostasis. Int J Chron Obstruct Pulmon Dis 2013;8:45–51.

[38] Voynow JA, Fischer BM, Zheng S. Proteases and cystic fibrosis. Int J Biochem Cell Biol 2008;40:1238–45.

[39] Martin SL, Downey D, Bilton D, Keogan MT, Edgar J, Elborn JS. Safety and efficacy of recombinant alpha(1)-antitrypsin therapy in cystic fibrosis. Pediatr Pulmonol 2006;41:177–83.

[40] Ehre C, Worthington EN, Liesman RM, Grubb BR, Barbier D, O'Neal WK, et al. Overexpressing mouse model demonstrates the protective role of Muc5ac in the lungs. Proc Natl Acad Sci 2012;109:16528–33.