Research article

The bacterial serine protease inhibitor ecotin inhibits neutrophil elastase enzymatic activity in cystic fibrosis sputa

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A B S T R A C T

Cystic Fibrosis (CF) airway disease is characterized by impaired mucociliary clearance, chronic, polymicrobial infections and robust, neutrophil-dominated inflammation. Pulmonary disease is the leading cause of morbidity and mortality in people with CF and is due to progressive airflow obstruction and ultimately respiratory failure. One of the earliest abnormalities in CF airway disease is the recruitment of neutrophils to the lungs. Neutrophil activation leads to the release of their intracellular content, including neutrophil elastase (NE), that damages lung tissues in CF. Our goal is to characterize a known bacterial NE inhibitor, ecotin, in the CF airway environment. Our results indicate that ecotins cloned from four Gram-negative bacterial species (*Campylobacter rectus, Campylobacter showae, Escherichia coli* and *Pseudomonas aeruginosa)* inhibit NE activity in CF sputum samples in a dose-dependent manner. Although we observed differences in the NE-inhibitory activity of the tested ecotins with the *Campylobacter* homologs being the most effective in NE inhibition in CF sputa, none of the ecotins impaired the ability of human neutrophils to kill major CF respiratory pathogens, *P. aeruginosa* or *S. aureus, in vitro.* Overall, we demonstrate that bacterial ecotins inhibit NE activity in CF sputa without compromising bacterial killing by neutrophils.

1. Introduction

Cystic Fibrosis (CF) is a recessive genetic disease affecting about 80,000 people worldwide [1]. Severe lung disease causes the majority of the morbidity and mortality in people with CF (PwCF). Progression of CF lung disease has been primarily associated with chronic inflammation, partially mediated by neutrophils, and with the persistence of polymicrobial infections [2]. Persistent inflammatory signals in the CF airways cause activation of recruited neutrophils which contributes to lung tissue damage overtime [3]. Neutrophils contain many antimicrobial proteins and enzymes capable of limiting infections. In general, neutrophils kill and trap microbes in two major ways, via the intracellular mechanism of phagocytosis and the extracellular mechanism of neutrophil extracellular trap (NET) formation [4–6]. In CF, neutrophils accumulate in large numbers in the airways and release their intracellular cargo which has been associated with tissue damage and lung disease progression. Several studies underscore the importance of neutrophils and neutrophil-derived products driving CF airway disease progression. For example, the number of neutrophils and the levels of DNA, myeloperoxidase (MPO), NE, and neutrophil chemoattractants (IL-8, TNF-α, IL-1β) in the sputum all correlate with CF lung disease severity [7–18].

Neutrophil elastase (NE) is a serine protease that is a requisite for the normal antimicrobial function of neutrophils [19].

Extracellular NE degrades interstitial collagen fibers enabling neutrophil cell migration, while intracellular NE kills bacteria within the phagosome [19]. In CF, however, NE is released from neutrophils in the airways in large quantities, causes harm by targeting host proteins within the lung tissue, irreversibly damages the airways over time, and is one of the several host components that are thought to drive chronic inflammation and lung disease progression [20–24]. NE is one of the amplest host enzymes found in the CF lung that degrades elastin, fibronectin, and collagen [25]. NE has also been shown to impair other host defenses by injuring bronchial epithelial cells and destroying the lung extracellular matrix [23,26]. The chronic release of NE by neutrophils causes airway remodeling contributing to worsened lung damage and function in PwCF [23,27]. NE is elevated in the bronchoalveolar fluid of CF infants and toddlers, and is the best predictor of future bronchieactasis [28,29].

There are only a few clinical studies testing NE inhibitors in CF and the results are variable. For example, AZD9668, a reversible and selective NE inhibitor, significantly reduced sputum inflammatory biomarkers in PwCF, but failed to decrease sputum neutrophil counts and NE activity, and did not improve lung function [30]. Another phase II clinical trial using inhaled alpha-1-antitrypsin in CF showed no impact on lung function, but did diminish free NE activity, neutrophil counts, *P. aeruginosa* loads and IL-8 levels [31]. Thus, the potential efficacy of NE inhibitors in CF still needs to be explored, since it remains insufficiently clear whether the recently developed highly effective modulator therapies (HEMT) are effective in controlling the inflammatory response and whether HEMTs

Table 1
Clinical and demographic characteristics of the CF sputum donors participating in this study.

CF sputum donor ID (CFBR-EM-0#)	106	162	185	191	208	306	308	614	683
Sex	Female	Male	Female	Female	Female	Male	Male	Male	Male
Race	Asian	White / Caucasian							
Ethnicity	Not Hispanic or latino						Unknown or NR	Not Hispanic or latino	
CFTR mutation 1	M470V	F508del	W1282X	F508del	F508del	F508del	W1282X	F508del	F508del
CFTR mutation 2	A1319E	F508del	N1303K	F508del	F508del	W846X	3849+10kbC->T	Q493X	F508del
Age at collection (years)	32	40	27	28	25	28	26	22	29
Clinical status	Clinically Stable								
Modulator at time of collection	None	Elexacaftor / Tezacaftor / Ivacaftor	None	Elexacaftor / Tezacaftor / Ivacaftor	Tezacaftor / Ivacaftor	Elexacaftor / Tezacaftor / Ivacaftor	Ivacaftor	None	None
Current modulator	None	Elexacaftor / Tezacaftor / Ivacaftor	Elexacaftor / Tezacaftor / Ivacaftor	Elexacaftor / Tezacaftor / Ivacaftor	Elexacaftor / Tezacaftor / Ivacaftor	Elexacaftor / Tezacaftor / Ivacaftor	Ivacaftor	None	Elexacaftor / Tezacaftor / Ivacaftor
# of Pa+ cultures in last 24 months	8	2	0	6	11	2	0	3	4
Pa+ at time of collection	YES	YES	NO	YES	NO	YES	NO	YES	YES
# of Sa+ cultures in last 24 months	4	0	7	4	0	0	6	6	8
# of MSSA+ cultures in last 24 months	4	0	0	4	0	0	6	6	0
MSSA+ at time of collection	NO	NO	NO	YES	NO	NO	YES	YES	NO
# of MRSA+ cultures in last 24 months	0	0	6	0	0	0	0	0	7
MRSA+ at time of collection	NO	NO	YES	NO	NO	NO	NO	NO	YES
# of SCV+ cultures in last 24 months	0	0	1	0	0	0	0	0	1
SCV+ at time of collection	NO	NO	NO	NO	NO	NO	NO	NO	NO
Other organisms	yeast	yeast	-	-	-	-	*Bb*	*MAC, A. fumigatus*	
Pa: *Pseudomonas aeruginosa*; Sa: *Staphylococcus aureus*; NR: not reported; MAC: *Mycobacterium avium* complex; SCV: small colony variant; MRSA: methicillin-resistant *S. aureus*; MSSA: methicillin-sensitive *S. aureus*; NR, not reported; Bb: *Bordatella bronchiseptica*.									

would require adjunct, anti-inflammatory therapies in the future [32].

Bacteria have also evolved mechanisms to resist the antimicrobial properties of neutrophils. Specifically, Gram-negative bacteria have developed strategies to evade harm by immune cell-derived serine proteases, such as NE, through the production of a protein called ecotin [33,34]. Ecotin was first described in *E. coli* as a protease inhibitor [34,35]. Ecotin is a small, homodimeric protein of approximately 38 kDa that is resistant to boiling and stable at pH 1.0, demonstrating exceptional stability [36]. In bacteria expressing ecotin, the protein translocates to the periplasmic space and protects the bacterium from host proteases. Since its first discovery in *E. coli*, ecotin homologs have been observed in over 300 organisms including *P. aeruginosa*, which is a dominant pathogen in CF airways [34,37–39]. We recently identified and characterized ecotin homologs in *Campylobacter rectus* and *Campylobacter showae* and showed that they inhibit free or NET-bound NE in a dose-dependent manner [37]. Our previous research showed that recombinantly expressed ecotins cloned from two *Campylobacter* species, *C. rectus* and *C. showae,* and cloned from *E. coli* were able to inhibit human NE [37]. In this study we also recombinantly expressed the *P. aeruginosa* ecotin. The objective of this study was to determine whether ecotins from four different, Gram-negative bacteria are capable of inhibiting NE enzymatic activity in CF airway samples.

2. Materials and methods

2.1. Human subjects

All human subject studies were performed according to the guidelines of the World Medical Association's Declaration of Helsinki. Healthy (non-CF) human subjects were recruited at the University of Georgia (UGA) and provided informed consent for blood donation for neutrophil isolation according to the IRB protocol UGA# 2012-10769-06. Based on self-report, the healthy volunteers donating blood in our study did not suffer from CF, chronic obstructive pulmonary disease, tuberculosis, asthma, diabetes or acute airway infections. Volunteers of ages between 18 and 50 years and of both sexes were recruited. Pregnant women were excluded from the study. The vaccination status of the volunteers was not considered as one of the exclusion/inclusion criteria.

2.2. CF subjects

CF subjects were recruited at the Adult CF Clinic at the Children's Healthcare of Atlanta and Emory University CF Care Center (see Table 1). These patients provided informed consent to obtain sputum samples (IRB00042577). All human studies involving sputum collection from CF subjects were approved by the Emory University Institutional Review Board and were in accordance with institutional guidelines. CF diagnosis was confirmed by pilocarpine iontophoresis sweat testing and/or CFTR gene mutation analysis to show the presence of two disease causing mutations. CF subjects were selected for sputum collection only if they were clinically stable (no pulmonary exacerbations at the time of specimen collection) and on no new medications within the previous three weeks of the study visit. The vaccination status of the volunteers was not considered as one of the exclusion/inclusion criteria.

2.3. Sputum processing

Expectorated sputum samples were processed as described earlier [40]. The sputa were kept at 4 °C throughout the processing

Table 2
Bacterial strains used in this study.

Species	Strain	Characteristic	Reference
E. coli	DH5α	F$^-$ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG purB20 φ80dlacZΔM15 Δ(lacZYA-argF)U169, hsdR17(r$_K^-$ m$_K^+$), λ$^-$	Invitrogen
E. coli	BL21(DE3)	E. coli str. B F$^-$ ompT gal dcm lon hsdS$_B$(r$_B^-$ m$_B^-$) λ(DE3 [lacI lacUV5-T7p07 ind1 sam7 nin5]) [malB$^+$]$_{K-12}$(λ^S)	Invitrogen
P. aeruginosa	Wildtype, PAO1	Water and soil	LaBaer et al.
P. aeruginosa	10007	Cystic Fibrosis clinical isolate	Yoo et al.
S. aureus	Wild-type, USA300	Methicillin-resistant	*S. aureus* subsp. *aureus* (ATCC® BAA1717™)
S. aureus	MRSA24	Cystic Fibrosis clinical isolate	Bernardy et al.

procedure. The sputum was weighed and for every 1.0 g of sputum, 3 mL of cold PBS-EDTA (1xPBS with 5 mM EDTA) were added. Sputum was then repeatedly passed through a sterile 18-gauge needle for homogenization and then centrifuged at $800 \times g$ for 10 min at 4 °C to pellet the cells. To ensure removal of cells, mucus, and bacteria, the sputum supernatant was transferred into new tubes and centrifuged at $3000 \times g$ for 10 min at 4 °C. The clear sputum supernatants were stored at −80 °C until further use.

2.4. "Cystic Fibrosis (CF) sputum model"

Our established CF sputum model was used to mimic the CF airway environment [40,41]. Briefly, sputum supernatants obtained from five PwCF were pooled and PMNs were exposed to 30 % (*v/v*) CF sputum cocktail for 3.5 h at 37 °C. Following incubation, PMNs were washed twice with 1 x HBSS and resuspended in "assay medium" (1 x HBSS, 10 mM HEPES, 5 mM glucose, and 1 % (*v/v*) autologous serum). The CF sputum cocktail was also diluted in "assay medium" to reach the desired 30 % concentration. Due to the washes, the CF sputum did not get in direct touch with bacteria.

2.5. Bacterial strains, plasmids and growth conditions for ecotin expression

The bacterial strains used in this study are listed in Table 2. *E. coli* was grown on Luria Bertani (LB) agar or in LB broth at 37 °C with shaking overnight. Expression of ecotin from *E. coli*, *C. rectus* and *C. showae* used in this study has been described in our prior publication (34). The expression plasmid of ecotin from *P. aeruginosa* was constructed as follows. The full length ecotin gene was amplified from genomic DNA of *P. aeruginosa* PAO1 with oligonucleotides PAO1-eco-*Nde*I-F-(5′-AAGGAGATATACA-TATGAAAGCACTACTGATCGCCGCCGGCGTTG-3′) and PAO1-eco-*Sal*I-R-(5′-ATACTCGTGTCGACTTCGCTGACCGCTTTCTC-GACCTTTTCG-3′), introducing *Nde*I and *Sal*I restriction sites, respectively. The obtained PCR product was inserted into plasmid pET24b after restriction with *Nde*I and *Sal*I and candidate plasmids isolated from colonies obtained after transformation of the ligation reaction in *E. coli* DH5alpha were analyzed by restriction digests. One positive clone that carries the PAO1 ecotin-His6 gene under the control of the T7 promoter was used as a template to amplify the PAO1 ecotin-His6 encoding sequence using oligonucleotides ecotin-temp-pET22b-*Bam*HI-F (5′-ATATGGATCCGGCGTAGAGGATCGAGATCTCG-3) and ecotin-temp-pET22b-*Sal*I-R (5′-TAGCAGTCGACT-CAGCTTCCTTTCGGGCTTTGTTAGC-3′), and was subsequently inserted into the *Eco*RV restriction site on plasmid pBBR1-MCS4. One candidate plasmid that expresses the PAO1 ecotin under the control of the constitutive pLac promoter was transferred into the overexpression strain *E. coli* BL21 *eco:kan*. Expression, purification and serine protease activity were confirmed for each ecotin from *P. aeruginosa*, *E. coli*, *C. rectus* and *C. showae* in a trypsin inhibition assay described previously [37].

2.6. Neutrophil elastase activity assay

NE enzymatic activity in CF sputum supernatants was measured with the NE activity kit following the manufacturer's protocol (Cayman Chemical, cat #600610). CF sputum was diluted to a measurable range based upon the NE bulk standard that contained 18 U/ml human NE activity. The standard curve was generated with human NE ranging from 0 mU/ml to 10 mU/ml in NE activity assay buffer. CF sputa and standards were added to a 96-well fluorometric microplate and ecotin was added to the CF sputum at varying doses. Ecotins from *C. rectus*, *C. showae*, and *E. coli* were added at a range from 0 to 1666.67 nM, whereas ecotin from *P. aeruginosa* was added at a range from 0 to 555.56 nM due to its limited availability. Sivelestat (MedChemExpress, cat#127373-66-4), a known NE inhibitor [31,36,42–45], was used as a reference. The NE-specific substrate (Z-Ala-Ala-Ala-Ala) 2Rh110 is selectively cleaved by NE to yield the highly fluorescent compound R110. Fluorescence was read at 485/525 nm (excitation/emission wavelengths) every 2 min for 2 h in a Varioskan Flash™ fluorescent microplate reader (Thermo Fisher Scientific, Waltham, MA). For analysis, the average relative fluorescence unit (RFU) values were recorded for each well (standard and unknown samples), and the blank was subtracted. NE activity (mU/ml) was calculated based upon the equation generated with the help of the standard curve: NE (mU/ml) = [(RFU – y-intercept)/slope] x dilution factor.

2.7. Bacterial killing

Bacterial killing measurement of *P. aeruginosa* and *S. aureus* by neutrophils was performed with a reference strain and a CF clinical isolate for each bacterial species as shown in Table 2. The *P. aeruginosa* strains were streaked on Pseudomonas isolation agar, while the *S. aureus* bacterial strains were streaked on blood agar (TSA II, 5 % sheep blood), and both were grown at 37 °C overnight. Colonies were picked the following day and grown in LB broth for about 3 h with shaking at 37 °C. The bacteria were collected, washed twice with 1xHanks' Balanced Salt Solution (HBSS) and absorbance was measured at 600 nm in a 96-well microplate using the Varioskan Flash™ microplate photometer. As previously published, optical density of 0.6 of the bacterial suspension corresponds to 1.0×10^9 CFU/ml (43). The dose of bacteria was confirmed in each experiment with serial dilution and colony counting. Prior to adding bacteria to neutrophils, bacteria were opsonized with 10 % (v/v) autologous serum of the neutrophil donor for 20 min at 37 °C. After opsonization, serum was removed from bacteria by centrifugation at $10,000 \times g$ for 10 min and the bacterial pellet was resuspended in assay medium [40,46,47].

Neutrophils were isolated from the blood of healthy controls as previously described and resuspended in assay medium (at 1×10^7 cells/ml) [40,46,47]. Next, neutrophils were either left untreated or treated with 555.56 nM of ecotin, and bacteria were added simultaneously to neutrophils at a multiplicity of infection (MOI) of 1 (1:1). Neutrophils were then incubated at 37 °C with shaking every 2–3 min to ensure proper mixing of bacteria and neutrophils. After 30 min of incubation, neutrophils were lysed in saponin (1

mg/ml in 1xHBSS) for 5 min and diluted 100-fold into 1xHBSS. For a bacterial input control, bacteria were added to neutrophils and neutrophils were immediately lysed to enumerate bacteria added at time = 0 (input). Serial dilutions of each bacterial strain were generated in LB broth in each experiment to produce standard curves. Bacterial samples were placed in a 384-well microplate and bacterial growth was measured over time in the Varioskan Flash™ microplate photometer at 600 nm absorbance every 4 min for 16 h with constant temperature (37 °C) and shaking. For quantification of bacterial killing by neutrophils, growth curves for each strain were generated; the initial bacterial concentrations in each sample were determined; and bacterial killing was assessed as the decrease in surviving bacteria over time as described [40,46]. To measure the effect of ecotin on bacterial survival/growth, 555.56 nM of each ecotin was added to 1×10^7 CFU/ml bacteria (that corresponds to the concentration of bacteria when co-cultured with neutrophils) and incubated for 30 min at 37 °C. These samples were diluted 100-fold and added to LB broth on the 384-well microplate – in the exact same manner as the neutrophil-containing samples. Rare experiments in which baseline killing was below 20 % were excluded.

2.8. Statistical analysis

Results for assessing inhibition of NE by ecotin in the CF sputa were analyzed by one-way ANOVA and Dunnett's multiple comparison test (comparison of each ecotin concentration to untreated controls). Results for assessing the differences in inhibition by each ecotin between each CF sputum sample was analyzed by two-way ANOVA and Tukey's multiple comparison test. The assessment of ecotin on neutrophil-mediated bacterial killing was analyzed by one-way ANOVA and Tukey's multiple comparison test. Data are expressed as mean ± S.E.M. Statistically significant differences were considered as *ns*, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. All statistical analyses were performed with GraphPad Prism version 9.01 for Windows software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Neutrophil elastase enzymatic activities vary in CF sputa

To assess whether we can detect NE activities in CF sputa, sputum supernatants from nine PwCF (Table 1.) were assessed for NE activity (mU/ml) using a fluorescent microplate-based kit as done previously [48,49]. Fig. 1A shows representative kinetics of NE enzymatic activities in mU/ml in one CF sputum sample, in purified NE solution, and in assay buffer (control, without NE), over a time period of 2 h. NE activity is evidenced by the rise in fluorescence representing the increased concentration of the fluorescent NE cleavage product of 2Rh110 (Fig. 1A). Fig. 1B shows the comparison of NE activity levels observed in the sputum supernatants of nine PwCF. Freeze/thaw cycles ($\leq$5) had no effect on NE activity in CF sputum (Fig. 1C). Overall, this demonstrates a certain range of variability of NE activities present in the sputa from PwCF.

3.2. C. rectus ecotin inhibits NE in CF sputa

Our primary goal was to determine whether ecotin can inhibit NE present in CF sputa. We have previously cloned and expressed recombinant ecotins from three Gram-negative bacteria: *Campylobacter rectus, C. showae* and *E. coli* [37]. All three ecotins inhibited the enzymatic activities of purified human NE in a dose-dependent and identical fashion under the conditions tested [37]. Based on these observations, we planned to test these recombinant ecotins for their potential NE-inhibitory effect in CF sputa. First, we tested ecotin isolated from *C. rectus*, a bacterium that is typically present in the human oral microbiome and can cause oral abscesses, but is not associated with CF lung disease [50]. *C. rectus* ecotin was added to the sputum supernatants of six CF subjects in increasing doses
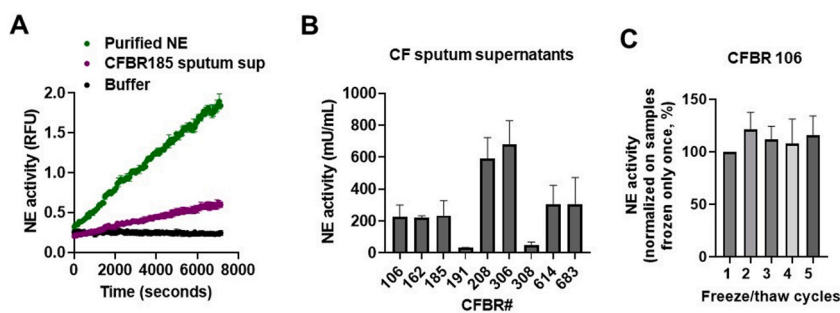

Fig. 1. Neutrophil elastase (NE) activities in sputum supernatants of PwCF. NE enzymatic activity measurements were performed in CF sputum supernatants obtained from nine PwCF using an NE fluorometric assay kit. Fluorescence was measured in a microplate reader (Ex/Em = 485/525 nm). **(A)** A representative kinetic curve of NE activity obtained using the CFBR185 patient sputum. Relative fluorescence units (RFU) of NE activity are shown in diluted (1/500) CF sputum over time compared to purified NE and assay buffer alone. The mean ± S.D of (n = 3) samples is shown **(B)** NE activity in each CF sputum sample. **(C)** The sputum supernatant obtained from CFBR 106 patient was frozen and thawed at the indicated times (freeze/thaw cycles, X-axis) and NE activity was measured. Results are expressed as mean ± S.E.M. of (n = 2–16) samples. RFU, relative fluorescent unit.

(0–1.67 μM) and percent inhibition of NE activity was determined relative to the ecotin-free sputum. *C. rectus* ecotin significantly inhibited NE enzymatic activities in all six CF sputa in a dose-dependent manner (Fig. 2A). Sivelestat, a known NE inhibitor (5 mM), yielded complete inhibition of NE activities in all CF sputum samples tested (data not shown) [51]. NE-inhibitory curves could be generated (Fig. 2B) and allowed us to determine 50 percent inhibitory concentration values (IC$_{50}$ values) for *C. rectus* ecotin in four out of six CF samples. IC$_{50}$ values of *C. rectus* ecotin demonstrated a relatively wide range (5.5–132.1 nM) as indicated in Fig. 2B. IC$_{50}$ values could not be calculated in the case of two CF samples (CFBR 162 and 683) due to wide 95 % confidence intervals. Altogether, *C. rectus* ecotin can achieve complete inhibition of NE activity in CF sputum and the dose needed for 50 % inhibition varies from subject to subject, but was in the nM range.

3.3. C. showae ecotin inhibits NE in CF sputa

C. showae is a species closely related to *C. rectus* also producing ecotin that inhibits the activities of human NE in a similar manner *in vitro* [37,52]. We tested whether *C. showae* ecotin is a potent inhibitor of NE in CF sputa. *C. showae* ecotin also significantly inhibited NE enzymatic activities in the sputa collected from the indicated four PwCF (Fig. 3A). *C. showae* ecotin displays complete inhibition of NE at the high dose of 1666.67 nM in three out of the four tested sputa (Fig. 3A–B). *C. showae* ecotin shows a close-to-complete inhibition of NE activities in the sputum from patient CFBR185 at a dose as low as 55.56 nM (Fig. 3A). In sputum samples CFBR 614 and 185, *C. showae* ecotin significantly inhibits NE at a concentration as low as 16.67 nM (Fig. 3A). The level of inhibition of NE by *C. showae* ecotin does differ between the four CF sputa tested as it is also reflected in their IC$_{50}$ values (16.1, 46.5, 87.5 and 342.1 nM) (Fig. 3B). Overall, *C. showae* ecotin inhibits NE in CF sputa, in a similar nM concentration range observed for the *C. rectus* ecotin.

3.4. E. coli ecotin is a less potent inhibitor of NE in CF sputa than Campylobacter ecotins

Next we compared the efficacy of *Campylobacter* ecotins on NE activity in CF sputa to that of *E. coli* ecotin. *E. coli* ecotin significantly inhibited NE in five of the six CF sputum supernatants tested, showing significant inhibition for CFBR106, 185, 306 and 683 at 1666.7 nM and for CFBR308 at 555.6 nM (Fig. 4A). However, inhibition was only moderate for CFBR185 and just at one dose and minimal to none for sputa from CFBR 614 and 683. This failure of *E. coli* ecotin to inhibit NE for these latter 2 subjects was in sharp contrast to the high degree of inhibition observed for the *Campylobacter* ecotins (Fig. 4 vs. Figs. 2 and 3). Since there are differences in the NE-inhibitory effect of the *E. coli* ecotin among the six CF sputa tested, reliable IC$_{50}$ values could only be calculated for CFBR106 (699.5 nM) and CFBR308 (231.5 nM) (Fig. 4B). Overall, *E. coli* ecotin does inhibit NE in some CF sputa at doses higher relative to the
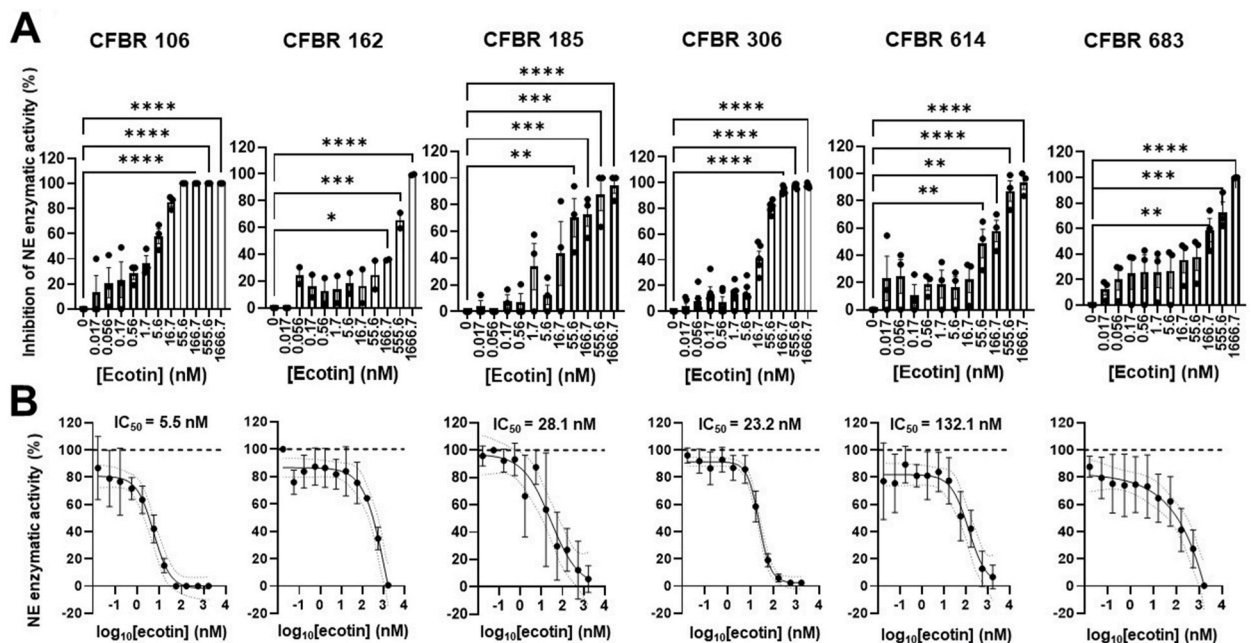

Fig. 2. *C. rectus* ecotin inhibits neutrophil elastase activity in CF sputa. **(A)** *C. rectus* ecotin was added in increasing concentrations (0–1666.7 nM) to the sputum supernatants of six PwCF and NE activity was measured. Percent inhibition of NE activity for each sputum sample was determined by dividing NE activity (mU/ml) at each dose of ecotin with NE activity without ecotin (represented as 0 nM on the graph). Mean ± S.E.M. of (n = 2–5) samples is shown. Data were analyzed by one-way ANOVA, Dunnett's multiple comparison test. **(B)** Summary graphs for the IC$_{50}$ values (nM) for *C. rectus* ecotin on its inhibitory effect of NE activity, represented as percent of untreated CF sputum. IC$_{50}$ values for each CF sputum were determined by interpolation of standard curves, Sigmoidal, 4 PL, X is log(concentration) with 95 % confidence interval bands. Mean ± S.D. of (n = 2–5) is shown. Statistical differences were considered as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.
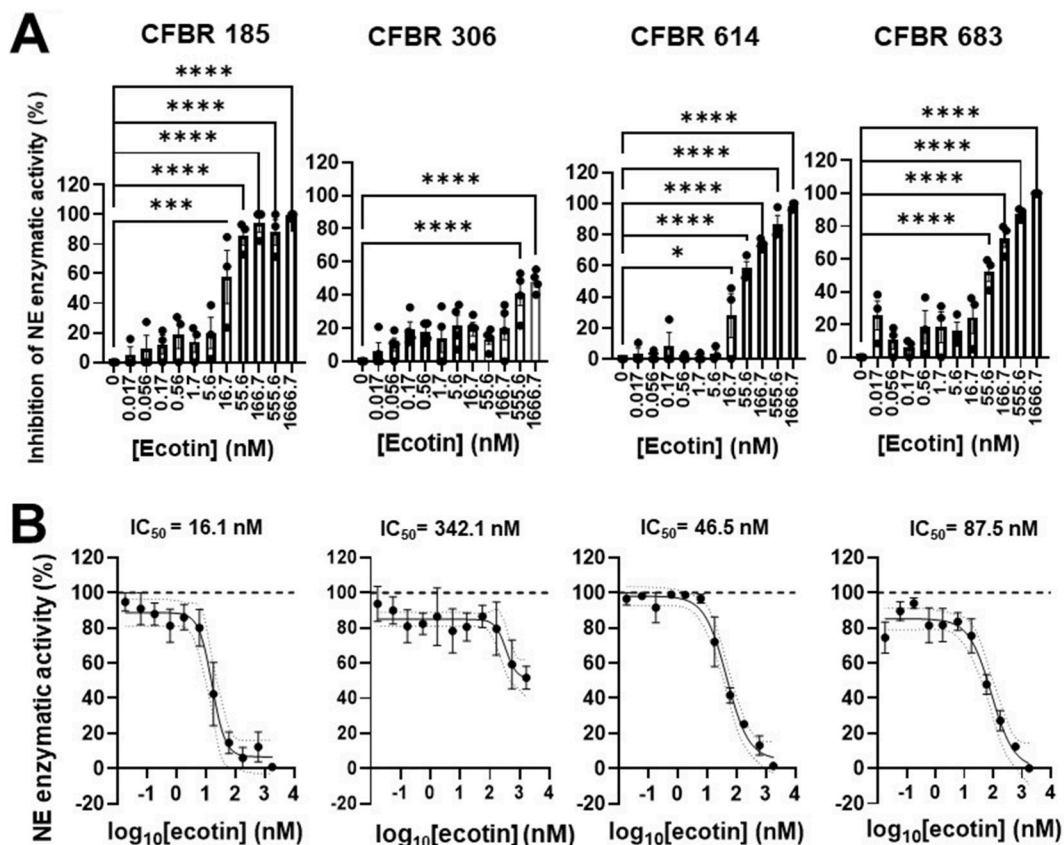
Fig. 3. *C. showae* **ecotin inhibits neutrophil elastase activity in CF sputa. A)** *C. showae* ecotin was added in increasing concentrations (0–1666.7 nM) to the sputum supernatant obtained from four PwCF and NE activity was measured (mU/ml). Percent inhibition of NE activity for each CF sputum sample was determined. Mean ± S.E.M. of (n = 3–4) samples is shown. Data were analyzed by One-way ANOVA, Dunnett's multiple comparison test. **B)** Summary data for percent inhibition of NE activity by the addition of increasing concentrations of *C. showae* ecotin to each CF sputum sample is shown. IC_{50} values for each CF sputum were determined for (n = 3–4) samples. Statistical differences were considered as *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$.

Campylobacter ecotins.

3.5. P. aeruginosa ecotin is the least potent inhibitor of NE activity in the CF sputa

Ecotin from *P. aeruginosa* was found to inhibit NE *in vitro* [52]. Therefore, we cloned and expressed the *P. aeruginosa* ecotin and explored its effect on NE activities in CF sputa. *P. aeruginosa* ecotin significantly inhibited NE activities in three (CFBR 106, 208 and 306) of the five CF sputa tested (Fig. 5A) and displayed inhibitory action at higher concentrations in a dose-dependent manner. In the CFBR 208 sputum, *P. aeruginosa* ecotin was able to significantly inhibit NE at a concentration as low as 1.7 nM (Fig. 5A). While *P. aeruginosa* ecotin significantly inhibited NE in the sputum from CFBR185 at a concentration of 16.7 nM, at higher ecotin concentrations it did not display significant inhibition and overall a clear dose-dependent trend could not be observed (Fig. 5A). *P. aeruginosa* ecotin was also unable to inhibit NE in the sputum of patient CFBR308. *P. aeruginosa* ecotin inhibits NE enzymatic activities by more than 50 % in only two of the five CF sputa tested (CFBR106 and 306), hence IC_{50} values could only be determined for those two (Fig. 5B). Altogether, *P. aeruginosa* ecotin is the weakest NE inhibitor in CF sputa tested in this study. Finally, only two of the six sputa (CFBR106 and 306) appeared to be sensitive to NE inhibition by three ecotins: *Campylobacter*, *P. aeruginosa* and *E. coli*.

3.6. Ecotins do not impair neutrophil-mediated killing of P. aeruginosa or S. Aureus

Data showcased earlier suggest that *Campylobacter* ecotins are potent NE inhibitors in the CF sputum (Figs. 2–5). To investigate whether ecotins added to neutrophils would interfere with their primary immune function, bacterial killing, the effect of ecotins on neutrophil-mediated killing of the two dominant CF pathogens, *P. aeruginosa* and *S. aureus*, was explored. Ecotin was added to neutrophils and killing of bacteria opsonized in the serum of the neutrophil donor was assessed. Two *P. aeruginosa* strains (PAO1 and a CF isolate, PA10007) and two *S. aureus* strains (USA300 and a CF isolate, MRSA24) were tested (Table 2). First, bacterial killing of
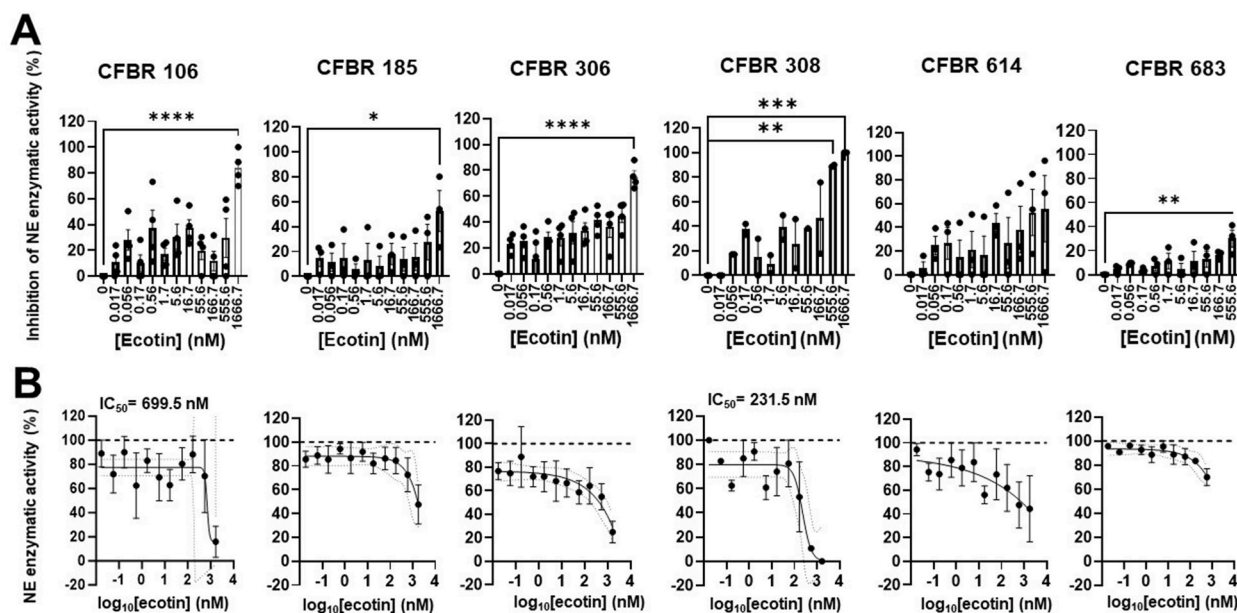
Fig. 4. *E. coli* **ecotin is a weak inhibitor of neutrophil elastase activity in CF sputa. A)** *E. coli* ecotin was added in increasing concentrations (0–1666.7 nM) to the sputum supernatants of six PwCF and NE activity was measured (mU/ml). Percent inhibition of NE activity for each sputum sample was determined. Mean ± S.E.M. of (n = 2–4) samples is shown. Data were analyzed by One-way ANOVA, Dunnett's multiple comparison test. **B)** summary data for inhibition of NE activity by the addition of increasing concentrations of *E. coli* ecotin to each CF sputum sample is shown. IC_{50} values for each CF sputum were determined for (n = 2–4) samples. Statistical differences were considered as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

P. aeruginosa by neutrophils was measured after treatment with ecotins at a dose that inhibits NE activity in most CF sputum samples (555.6 nM). Overall, none of the ecotin species impaired neutrophil-mediated killing of *P. aeruginosa* (Fig. 6A).

Next, bacterial killing of *S. aureus* by neutrophils after ecotin treatment was measured with two strains, the reference stain USA300 and a CF clinical isolate MRSA24. As shown in Fig. 6B, none of the ecotins significantly inhibited *S. aureus* killing by neutrophils.

To mimic the environment of the CF airways, we used our established "CF sputum model" by incubating human neutrophils in a pooled mix of sputum supernatants received from at least 5 PwCF. After 3.5 h, CF sputum was washed away and neutrophils were exposed to ecotin and opsonized bacteria to test the potential effect of ecotin on bacterial killing of neutrophils under "CF airway-like" conditions [40,41]. None of the tested ecotins had any significant effect on killing of *P. aeruginosa* PAO1 or *S. aureus* USA300 by neutrophils under CF airway-like conditions (Fig. 6C).

To ensure that ecotins do not interfere with bacterial growth, ecotin and bacteria alone were incubated (without neutrophils), and the growth of indicated bacterial strains were determined overtime by measuring OD_{600}. As shown in Fig. 7, when compared to untreated bacteria, there were no differences in the growth kinetics of bacteria with or without added ecotins. Altogether, ecotins do not impair neutrophil-mediated killing or growth of *P. aeruginosa* and *S. aureus*.

4. Discussion

This study describes the ability of various ecotins to inhibit NE enzymatic activity in human CF sputum samples without compromising neutrophil-mediated bacterial killing of two dominant CF lung pathogens, *P. aeruginosa* or *S. aureus*. The ecotin homologs from *C. rectus*, *C. showae*, *E. coli*, and *P. aeruginosa* showed variability in their capacity to inhibit NE activity in the CF sputa while none compromised neutrophil-mediated bacterial killing and/or bacterial growth. *C. rectus* and *C. showae* ecotins displayed a strong inhibition of NE activity in CF sputa, with complete inhibition at the higher doses tested. In contrast, *E. coli* and *P. aeruginosa* ecotins were both weak inhibitors of NE activity in CF sputa and in some instances, failed to show any inhibitory action.

The NE-inhibitory patterns of the tested ecotins in the CF sputa are different from our studies using purified human NE where no differences were observed: IC_{50} values (nM) for *E. coli* (4.64 ± 0.23), *C. rectus* (4.49 ± 0.25) and *C. showae* (4.78 ± 0.31) ecotins (34). The reason for this could possibly be explained by differences between purified human NE and the more complex CF sputum samples. Based on protein sequence alignments, the *P. aeruginosa* ecotin is more similar to the *E. coli* ecotin with approximately 60 % identity. There is low amino acid similarity between ecotin homologs from the *Campylobacter* species compared to *E. coli* and *P. aeruginosa* [34, 37]. For example, the *C. rectus* and *C. showae* ecotins have 27 % and 33 % amino acid identity to the *E. coli* and 25 % and 27 % identity to the *P. aeruginosa* ecotin [34]. The two cysteines (Cys50 and Cys87) which form an intra-subunit disulfide bond in the *E. coli* ecotin are conserved [37]. However, the substrate binding pocket residues are different compared to *E. coli* [37] and these might influence the oligomerization capacity of the heterotrimeric *E. coli* ecotin-NE complex [42]. Altogether, these differences may play a role in the
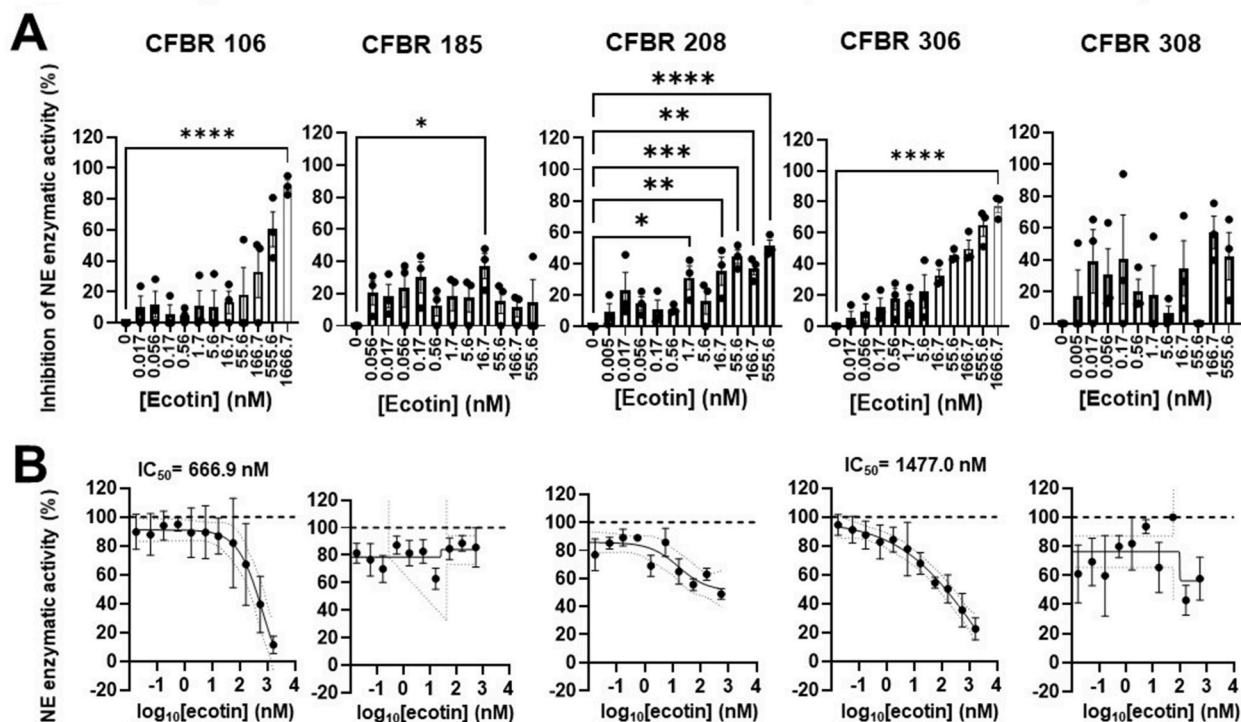
Fig. 5. *P. aeruginosa* ecotin is a weak inhibitor of neutrophil elastase activity in CF sputa. A) *P. aeruginosa* ecotin was added in increasing concentrations (0–555.56 nM) to the sputum supernatant from three PwCF and NE activity was measured (mU/ml). Percent inhibition of NE activity for each sputum sample was determined by the quantification of NE activity (mU/ml) at each dose of ecotin over the quantification of NE activity without ecotin (shown as 0 nM on the graph). Mean ± S.E.M. of (n = 3) samples is shown. Data were analyzed by One-way ANOVA, Dunnett's multiple comparison test. **(B)** Summary data for inhibition of NE activity by the addition of increasing concentrations of *P. aeruginosa* ecotin to each CF sputum. IC$_{50}$ values for each CF sputum were determined for (n = 3) samples. Statistical differences were considered as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

ability to bind and inhibit NE active sites in more complex media such as CF sputa. This idea is supported by our previous data showing that the *C. rectus* ecotin was capable of protecting a *C. jejuni* protein glycosylation mutant from proteolytic attack within chicken cecal samples by approximately 5×10^6 CFU more compared to the *C. showae* ecotin [37].

NE concentrations in CF sputum samples varies across a wide range in our study that is consistent with prior observations made by other groups [53–55]. Unfortunately, the same CF supernatants could not be tested in each figure as only limited amounts of these supernatants were available and some were only used in experiments for one or two readouts. The CF sputum contains NE in multiple compartments such as free NE released from neutrophils as well as NE bound to NETs or exosomes [13,25,56]. The differences between the ecotin species and their ability to inhibit NE in the complete sputa versus NETs could be due to the efficacy of ecotin to inhibit NE in the different compartments and to the differences in NE compartmentalization among different CF sputa. Dissecting the components of the CF sputa and the affinity for each ecotin to inhibit NE found in different compartments could help explain this result. Additionally, PwCF infected with *P. aeruginosa* may contain *P. aeruginosa* ecotin in their lung environment, which has been shown to bind to the biofilm exopolysaccharide matrix and likely protects neighboring *P. aeruginosa* cells from NE-mediated damage [57].

Despite *P. aeruginosa* ecotins being present in the CF lung, NE is active and causes severe damage of lung tissues. Therefore, *P. aeruginosa* ecotins may only be efficient in protecting the microbe from NE within biofilms, but the quantities of ecotin that are released are likely unable to inhibit lung tissue damage caused by NE. Our data suggests that addition of exogenous ecotin inhibits NE activity in the CF sputum while does not interfere with neutrophil-mediated killing of CF clinical isolates *in vitro*. These results raise the question whether ecotin could be of interest for studies on its potential to be a new NE-targeting therapeutic.

There are currently no approved NE inhibitors for human use. Sivelestat was used a positive control in this study as it has been shown to reduce NE activity, however, clinical trials deemed this drug ineffective in PwCF [45]. A meta-analysis analyzing the clinical trials performed with sivelestat in patients with acute respiratory distress syndrome showed that the compound did not improve mortality and did not alter the duration of mechanical ventilation [58]. It has been shown that NE inhibition by sivelestat did not enhance the occurrence of infections, suggesting that NE inhibitors could improve lung function without worsening infections [45,59, 60]. Therefore, new inhibitors against NE with a known mechanism of action are needed. Ecotin from *C. rectus* and *C. showae* showed the same inhibitory effects as sivelestat, with complete NE inhibition in the CF sputa.

Altogether, our study characterizes the effect of four ecotin homologs on their inhibitory activity of NE in CF sputa and neutrophil-
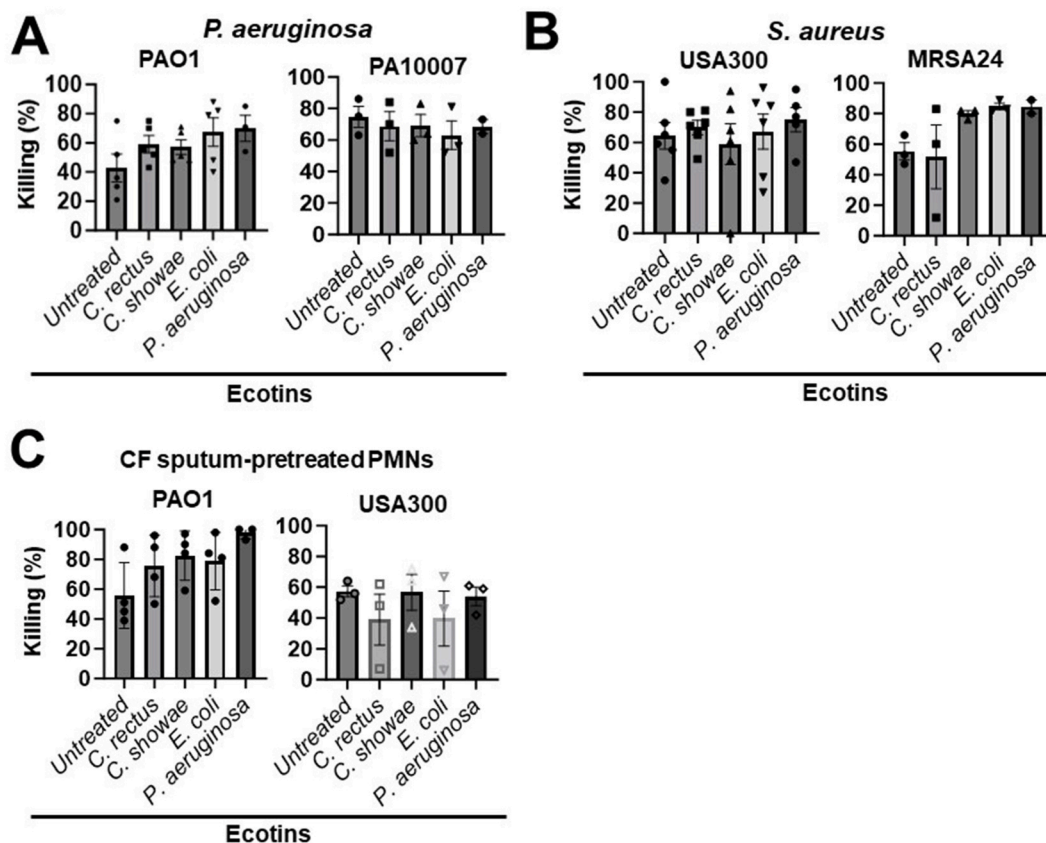
Fig. 6. Ecotin does not impair neutrophil-mediated killing of _P. aeruginosa_ and _S. aureus_. A) The results of _P. aeruginosa_ killing by neutrophils with or without ecotin. Two _P. aeruginosa_ strains (PAO1, n = 5, and the CF clinical isolate, 10007, n = 3) were tested. Neutrophils were either untreated or treated with 555.6 nM of the indicated ecotins and infected with _P. aeruginosa_ at a MOI of 10. Bacterial killing was measured by a microplate-based assay. Each symbol in the bar graph represents one neutrophil donor and the corresponding percent killing of _P. aeruginosa_. Mean ± S.E.M. of samples are shown. Comparison of percent killing by ecotin-treated neutrophils to untreated neutrophils was analyzed by One-way ANOVA, Tukey's multiple comparison test. **B)** The results of bacterial killing of _S. aureus_ by neutrophils with or without treatment of ecotin. Two _S. aureus_ strains were tested, USA300 (n = 6) and the CF clinical isolate, MRSA24 (n = 3). Neutrophils were either untreated or treated with 555.6 nM of each ecotin and infected with _S. aureus_ at a MOI 10. Mean ± S.E.M. of samples is shown. Data were analyzed as described in (A). **C)** Human neutrophils were pre-treated with 30 % (v/v) of pooled CF sputum supernatants for 3.5 h according to the "CF sputum model". After washing out excess sputum, the killing of _P. aeruginosa_ PAO1 (n = 4) and S. aureus USA300 (n = 3) (1 MOI) were measured using the microplate-based killing assay. MOI, multiplicity of infection. Statistical differences were considered as *, $p < 0.05$.

mediated bactericidal responses. This is the first study to characterize the _P. aeruginosa_ ecotin and its limited ability to inhibit NE in the CF sputum. This is also the first study to test ecotin as an NE inhibitor in CF airway disease. Our _in vitro_ data suggest that the ecotins from _C. rectus_ and _C. showae_ have the potential to significantly reduce NE activity in the CF airways, however more studies are needed to determine the effect on the reduction of lung injury. Future studies will also test ecotin in the βENaC-Tg CF mouse model demonstrating a neutrophilic and NE-mediated, CF-like lung disease. Our team has characterized this mouse model and has observed significant lung inflammation mediated by neutrophils (64). The addition of ecotin _in vivo_ to measure its effect on NE activity and lung injury would further confirm that ecotin has potential for therapeutic use in CF lung disease.

Since ecotin is a bacterial protein, administering it to mammalian organisms carries the risk of inducing an inflammatory or antibody response. Therefore, the safety of administering ecotin, or its future derivatives, need to be carefully investigated. Ecotin was reported to exhibit a broad range of inhibitory activity against exogenous serine proteases such as trypsin, chymotrypsin, factor Xa, kallikrein, urokinase, factor XII, and NE [34–37,61–63]. Delivering ecotin directly to airways, therefore, likely represents a better approach to inhibit airway proteases without interfering with proteases in the blood or the gut lumen.

A limitation of the current study is the small number of CF subjects recruited and their sputum samples used. Also, only two strains of each CF pathogen were tested in the neutrophil killing assays. In the future, the effect of ecotins on NE will be expanded to sputum samples of a larger CF study cohort using more bacterial clinical isolates.

Lung function decline is the main cause of morbidity and mortality in PwCF, with NE correlating with decreased lung function. NE has been a target in PwCF for years with no successful, approved therapeutics thus far. Our future work will aim to better characterize the effect of ecotin, and its derivatives, on inhibiting NE activity and its consequences in CF.
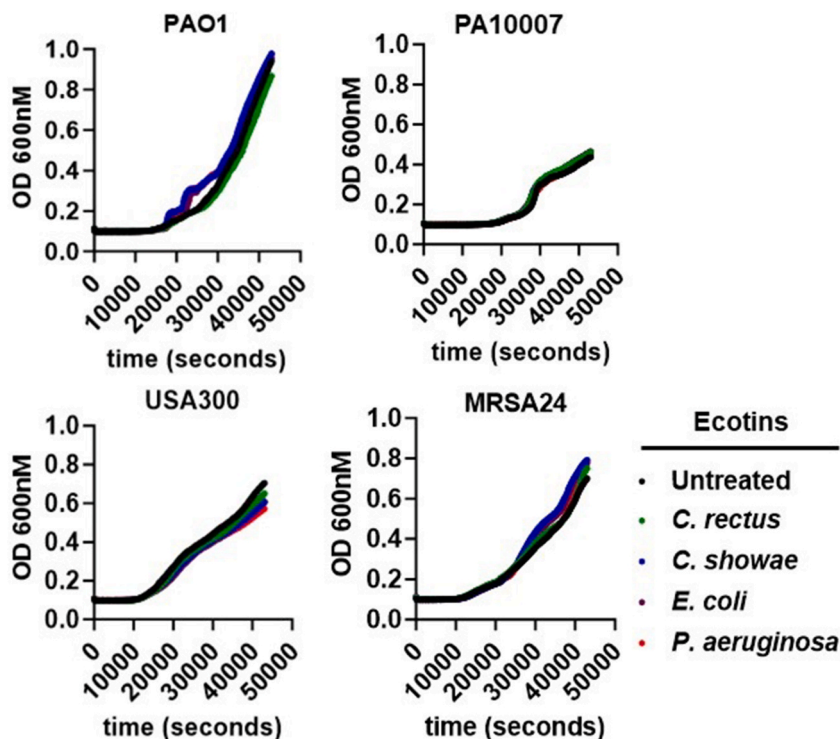
Fig. 7. Ecotin does not impair the growth of *P. aeruginosa* and *S. aureus*. Kinetic growth curves of *P. aeruginosa* bacterial strains, PAO1 and PA1007, and *S. aureus* bacterial strains, USA300 and MRSA24, in the presence of 555.6 nM ecotin (without neutrophils). OD, optical density. Statistical differences were considered as *, $p < 0.05$.

Ethics statement

All human subject studies were performed according to the guidelines of the World Medical Association's Declaration of Helsinki. Healthy human subjects were recruited at the University of Georgia (UGA) and provided informed consent for blood donation for neutrophil isolation according to the protocol UGA# 2012-10769-06 approved by the Institutional Review Board of the University of Georgia.

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Data and code availability statement

Data will be made available on request.

CRediT authorship contribution statement

Kayla M. Fantone: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Harald Nothaft:** Writing – review & editing, Validation, Supervision, Methodology, Formal analysis, Conceptualization. **Yeongseo Son:** Formal analysis, Data curation. **Arlene A. Stecenko:** Writing – review & editing, Methodology. **Christine M. Szymanski:** Writing – review & editing, Resources, Project administration, Investigation, Conceptualization. **Balázs Rada:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

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