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Activity of innate antimicrobial peptides and ivacaftor against clinical cystic fibrosis respiratory pathogens

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ABSTRACT

There is a clear need for new antimicrobials to improve current treatment of chronic lung infection in people with cystic fibrosis (CF). This study determined the activity of antimicrobial peptides (AMPs) and ivacaftor, a novel CF transmembrane regulator potentiator for treatment of CF. Antimicrobial activity of AMPs (LL37, Human β-Defensins [HβD] 1-4 and SLPI) and ivacaftor against clinical respiratory isolates (*Pseudomonas aeruginosa, Staphylococcus aureus*, *Streptococcus spp.*, *Achromobacter spp.* and *Stenotrophomonas maltophilia*) were determined using radial diffusion and time-kills assays, respectively. Synergy of LL37 and ivacaftor with tobramycin was determined by time-kill with *in vivo* activity of ivacaftor and tobramycin compared using a murine infection model. LL37 and HβD3 were the most active AMPs tested with MICs for genera ranging from 1.1-51.9 mg/L and 1-35.4 mg/L, respectively, with the exception of *Achromobacter* which was resistant. HβD1 and SLPI demonstrated no antimicrobial activity. LL37 demonstrated synergy with tobramycin against 4/5 *S. aureus* and 2/5 *Streptococcus* spp. isolates. Ivacaftor demonstrated bactericidal activity against *Streptococcus spp.* (mean $\log_{10}$ decrease 3.31 CFU/ml), bacteriostatic activity against *S. aureus* (mean $\log_{10}$ change 0.13 CFU/ml) but no activity against other genera. Moreover, ivacaftor demonstrated synergy with tobramycin with a mean $\log_{10}$ decrease of 5.72 CFU/ml and 5.53 CFU/ml at 24 hours for *S. aureus* and *Streptococcus* spp., respectively. Ivacaftor demonstrated immunomodulatory but no antimicrobial activity in a *P. aeruginosa in vivo* murine infection model. Following further modulation to enhance activity, AMPs and ivacaftor offer real potential as therapeutics to augment antibiotic therapy of respiratory infection in CF.

Key words: Ivacaftor, Cystic Fibrosis, antimicrobial, *Pseudomonas aeruginosa*, innate antimicrobial peptides

1. Introduction

Cystic fibrosis (CF) is a hereditary disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene. Respiratory failure caused by repeated cycles of infection and inflammation is the leading cause of morbidity and mortality in people with CF and is responsible for 80% of deaths [1]. Although *Pseudomonas aeruginosa* is the most frequently isolated CF respiratory pathogen [2], infection caused by other bacteria including *Staphylococcus aureus*, *Burkholderia cepacia* complex, *Stenotrophomonas maltophilia, Achromobacter spp.* and *Streptococcus spp.* [3,4] also occur. As a result, primarily due to increased use of antibiotics, bacteria causing respiratory infection are becoming progressively more resistant to conventional antibiotics with up to 45% of CF patients colonised with multidrug resistant pathogens [5–7]. Furthermore, when chronic infection is established, pathogens such as *P. aeruginosa* grow within polymicrobial biofilms in the CF lung and exhibit increased resistance to antibiotics [8,9]. Tobramycin is the most frequently prescribed inhaled antibiotic for CF patients with chronic *P. aeruginosa* infection [2], with ciprofloxacin frequently prescribed as an oral antibiotic [10]. However, in a recent study of CF *P. aeruginosa* isolates in Northern Europe, 60% were multidrug resistant with 28% and 56% resistant to tobramycin and ciprofloxacin, respectively [11].Therefore, there is a clear need for novel antimicrobial agents or combinations of antimicrobials to treat respiratory infection in CF patients.

Antimicrobial peptides (AMPs) form part of the non-specific innate immune response and have been shown to have antibacterial activity [12]. Some of the most well characterised AMPs include the human cathelicidin LL37, Human β-defensins 1-4 (HβD1-4) and secretory leukocyte protease inhibitor (SLPI) which are all produced by the lung epithelium [13–15]. The antibacterial properties of these peptides have been

83 previously demonstrated [16–19], but only LL37 has been specifically tested against
84 clinical CF respiratory isolates.
85 Ivacaftor is a first-in-class CFTR potentiator that potentiates defective CFTR at the
86 apical membrane of lung epithelial cells, thus increasing the probability of successful
87 chloride transport across the membrane [20]. Treatment results in sweat chloride
88 correction, decreased exacerbation frequency and an improvement in lung function
89 and quality of life [21]. Furthermore, Reznikov et *al.* reported that ivacaftor
90 demonstrated some antimicrobial activity against laboratory and non-CF clinical
91 methicillin susceptible and resistant *S. aureus* (MSSA and MRSA) and *Streptococcus*
92 *pneumoniae* isolates [22] and suggested that this may be due to the presence of a
93 quinolone ring.

94 The aim of this study was to determine the antimicrobial activity of a number of AMPs
95 and ivacaftor against clinical CF respiratory isolates from a range of genera.
96 Furthermore, we investigated if there was synergy between LL37 or ivacaftor and
97 tobramycin. Finally, a murine infection model was used to compare the *in vivo*
98 antimicrobial and immunomodulatory activity of ivacaftor and tobramycin.

99

2. Materials and Methods

2.1. Bacterial isolates

Eighteen clinical bacterial isolates [*P. aeruginosa*, n=4; *S. aureus* n=4 (MRSA, n=3; MSSA n=1); *Streptococcus* spp., n=4; *Achromobacter* spp., n=3 and *S. maltophilia*, n=3] were used for susceptibility testing. The isolates were cultured from sputum samples or bronchoalveolar lavage fluid collected from CF patients attending CF clinics in Belfast and the University of North Carolina at Chapel Hill. Samples were cultured on non-selective agar plates and individual colonies sub-cultured to obtain pure bacterial culture. Bacterial isolates were identified using 16S rRNA sequencing as described previously [23]. Bacterial isolates were stored at -80°C. *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 29213 and *Streptococcus anginosus* NCTC 10713 were included as quality control and reference strains. In biofilm assays, *S. epidermidis* ATCC 35984 was used as a strong biofilm former (positive control) and a laboratory *S. capitis* isolate was used as a weak biofilm former (negative control). *P. aeruginosa* strain PAO1 was used for in *vivo* experiments.

2.2. Antimicrobials and reagents

Etest® strips were purchased from bioMerieux (North Carolina, USA). Ivacaftor was purchased from Selleckchem (Houston, USA), synthetic LL37 and synthetic HβD1-4 from Innovagen (Lund, Sweden) and recombinant human SLPI from R&D Systems (Minneapolis, USA); All AMPs were active. Tobramycin was obtained from Hospira (Warwickshire, UK). Ciprofloxacin (≥98% HPLC grade), monobasic and dibasic sodium phosphate, agarose (Type 1, low EEO), methanol (≥99.9% HPLC grade), crystal violet (for biofilm staining), Dulbeco's phosphate buffered saline (PBS) (endotoxin tested) and trypan blue were all purchased from Sigma-Aldrich (Gillingham,

UK). Mueller Hinton agar (MHA), Mueller Hinton broth (MHB), Cetrimide agar, Anaerobe Basal agar (ABA) and Brain Heart infusion (BHI) broth were all purchased from Oxoid Ltd (Basingstoke, UK). Sterile, defibrinated horse blood was purchased from TCS Biosciences (Buckingham, UK). Xylazine (Xylacare 2% w/v) and Ketamine (Narketan 10, 100g/L) were obtained from Animalcare (York, UK) and Ventoquinol (Buckinghamshire, UK), respectively. IL-6 and KC enzyme-linked immunosorbent assay (ELISA) kits were purchased from eBioscience (Hatfield, UK) and R&D Systems (Minneapolis, USA), respectively.

2.3. MIC testing

The MICs of antibiotics (Supplementary Table 1) routinely used in the treatment of CF lung infection were determined by Etest® according to the manufacturer's instructions. The MICs of AMPs (LL37, HβD1-4 and SLPI) were determined using a radial diffusion assay (RDA) as previously described [24]. BHI supplemented agar was used with *Streptococcus* spp*., S. maltophilia* and *Achromobacter* spp. with MHA used for *P. aeruginosa* and *S. aureus*. Peptides were tested at concentrations of 200, 150, 100 and 50 mg/L.

2.4. LL37 and tobramycin synergy

A modified time-kill assay was used to determine if there was synergy between LL37 and tobramycin against selected isolates (*P. aeruginosa* [n=5]; *S. aureus* [n=5] and *Streptococcus spp.* [n=5]). Overnight cultures were washed with 10mM sodium phosphate and adjusted to approximately $1x10^5$ CFU/ml. In a 96-well plate, the bacterial suspension was incubated with LL37 (64–1 mg/L) alone or in combination with tobramycin (0.5 MIC for each isolate) for 3 hours at 37°C under aerobic or microaerophilic conditions. Killing activity was assessed by enumerating on MHA or

ABA following serial dilution (10^{-1} to 10^{-3}) in sterile saline. The MBC of LL37 was determined as the lowest concentration at which there was no growth on the plate. Synergy was defined as a ≥2 $\log_{10}$ decrease in total viable count (TVC) compared to the starting inoculum and as a ≥2 $\log_{10}$ decrease in TVC by the combination compared to the most active single agent [25]. Results are expressed as mean CFU/ml ±SD.

2.5. Time-kill studies

All time-kill experiments were performed according to CLSI standards [25]. Initial time-kill studies were performed using a range of ivacaftor concentrations (32, 16, 8, 4 and 1mg/L) against selected isolates (*P. aeruginosa* [n=5]; *S. aureus* [n=5] and *Streptococcus spp.* [n=5]); the highest concentration of ivacaftor tested (32 mg/L) was that previously used by Reznikov *et al.* [22]. Ivacaftor was dissolved in DMSO and time-kills were also performed using DMSO (0.32%) as a reagent control. Subsequently, time-kill assays were performed with these isolates to compare the activity of ivacaftor (32 mg/L) with ciprofloxacin (5 mg/L; concentration above MIC for the majority of isolates), a comparator fluoroquinolone. Time-kill assays were performed in MHB for *P. aeruginosa* and *S. aureus* isolates and BHI broth for *Streptococcus spp.*

For synergy studies, ivacaftor was used at a concentration of 32 mg/L in combination with tobramycin at 0.5 MIC. Tobramycin was chosen for synergy studies as it is the most frequently prescribed inhaled antibiotic in CF and is also frequently prescribed for treatment of acute infective exacerbations [2]. A no drug control was used in each assay.

Bacterial cultures and antibiotics were incubated at 37°C under aerobic or microaerophilic (5% CO_2 for *Streptococci*) conditions. Killing activity was assessed at

0, 2, 4, 6 and 24 h by carrying out serial dilutions in sterile saline (10^{-1} to 10^{-6}) followed by enumerating on MHA or ABA agar. Bactericidal activity was defined as a ≥3 $\log_{10}$ reduction in colony forming units (CFU/ml) in the original inoculum [25]. Synergy was defined as described above [25] with results for time-kill assays expressed as mean CFU/ml ±SD.

2.6. Biofilm Studies

The effect of ivacaftor on bacterial adherence and biofilm formation by all isolates (clinical isolates n=18; reference strains, n=3) was determined using a previously described method that quantifies the adherence of bacteria to microtitre plates (ThermoFisher Scientific, Waltham, USA) [26]. An overnight culture of bacteria was adjusted to $1x10^6$ CFU/ml with 200 µl added to each well. Ivacaftor (32 mg/L), ciprofloxacin (5 mg/L) or DMSO (0.32%) were added to the wells and the plates were incubated for 24 hours at 37°C. The contents of the wells were aspirated, washed three times with 200 µl sterile PBS and adherent bacteria stained using crystal violet. Bacterial adherence was quantified by measurement of OD_{570} (FLUOstar Omega microplate reader) and compared with an untreated control. Limits for non, weak, moderate and strong biofilm formation were defined as previously described [26].

2.7. In vivo activity using a mouse model of intraperitoneal infection

The *in vivo* activity of ivacaftor was determined using a systemic sepsis model of infection in mice. Age and sex matched B6 mice (B6N-$^{Tyrc\text{-}Brd}$/BrdCrCrl [Charles River]) mice (n=5 per group; 4 male, 1 female) were inoculated intraperitoneally (IP) with 100 µl of *P. aeruginosa* (PAO1) ($6x10^8$ CFU/ml). PAO1 was used as our group and others have shown that it reproducibly produces intraperitoneal infection in mice [27]. Ivacaftor (60 µg/mouse), negative control (endotoxin free PBS) or tobramycin (140

μg/mouse), were administered IP immediately following infection. The dose of ivacaftor used in this model was calculated based on a single adult dose (150mg) adjusted for mouse weight (20 g) and the assumption that an adult with CF weighs 50 kg. The dose of tobramycin used was calculated based on the intravenous once daily dose paediatric regimen of 7 mg/kg adjusted for mouse weight (20 g). The bacterial load inoculated was chosen to ensure systemic infection was achieved with mice sacrificed after 4 hours to prevent significant deterioration in health. A peritoneal lavage was performed with 5 ml of ice-cold sterile PBS, with collected samples stored on ice. Total viable count of PAO1 from the lavage was determined by enumerating on cetrimide agar, a *P. aeruginosa* selective agar. Total cell count and cell viability were determined following staining with trypan blue using the Countess™ Automated Cell Counter (Invitrogen). The lavage fluid was centrifuged at 600 xg and the supernatant used to determine IL-6 and KC (chemokine CXCL1, a functional homologue of human IL-8) levels by ELISA. Housing and experimentation was carried out in accordance with the Animal (Scientific Procedures) Act 1986 and current guidelines approved by the Queen's University Ethical Review Committee.

2.8. Statistical analysis

One-way ANOVA and Bonferroni's Multiple Comparison Tests were used to determine statistical significance in the biofilm assays and in the *in vivo* experiments. All analysis was performed using GraphPad software with a P-value of <0.05 considered statistically significant.

3. Results

3.1. MIC testing

The MICs of antibiotics routinely used in the treatment of CF lung infection were determined for all isolates (Supplementary Table 1) with differences in susceptibility apparent both within and between genera. The majority of *P. aeruginosa* (4/5; 80%) and *S. aureus* (4/5; 80%) isolates were susceptible to tobramycin; however, high tobramycin MICs were demonstrated for *Streptococcus* (3/5; 60%), *Achromobacter* (3/3; 100%) and *S. maltophilia* (4/5; 80%) isolates. With the exception of *Streptococci,* where all isolates displayed intermediate resistance, based on the breakpoint for *S. pneumoniae*, resistance and susceptibility to ciprofloxacin was apparent within each genera.

The MIC for each antimicrobial peptide against clinical isolates from each genus is summarised in Table 1 with the MICs of type strains presented for comparison. The MICs for individual isolates are also presented in Supplementary Table 2. Overall, LL37 and HβD3 were the most active peptides tested with MICs for genera ranging from 1.1-51.9 mg/L and 1-35.4 mg/L, respectively, with the exception of *Achromobacter* which was resistant. In contrast, HβD1 and SLPI demonstrated no activity against any isolates at the concentrations tested. Peptide MICs for type strains were within the range of those for clinical isolates, with the exception of *S. anginosus* (NCTC 10713) which was resistant to both HβD2 and HβD4.

3.2. Synergy between LL37 and tobramycin

When combined with tobramycin, LL37 demonstrated no synergistic activity against *P. aeruginosa* (Table 2). In contrast, LL37 demonstrated synergistic activity in combination with tobramycin against 4/5 *S. aureus* and 2/5 *Streptococcus* spp.

isolates (Table 2). Change in CFU/ml for individual isolates is presented in supplementary Table 3.

3.3. Bactericidal activity of ivacaftor

When tested alone, ivacaftor demonstrated no antimicrobial activity against *P. aeruginosa* at any concentration tested (Fig. 1A). In contrast, at the highest concentration tested, 32 mg/L, bacteriostatic activity was apparent against all *S. aureus* isolates tested (Fig. 1B). Similarly, bacteriostatic (n=3 isolates) and bactericidal (n=2 isolates) activity was apparent against *Streptococcus* spp. isolates (Fig. 1C) at this concentration with no effect apparent against any genera at lower concentrations. DMSO (0.32%) had no effect on bacterial growth in any assay. Individual time-kill curves are presented for *P. aeruginosa* (Supplementary Fig. 1), *S. aureus* (Supplementary Fig. 2) and *Streptococcus* spp. isolates (Supplementary Fig. 3.).

The change in CFU/mL in the presence of ivacaftor, ciprofloxacin or untreated control at 24 hours for isolates within each genus is summarised in Table 3. Ciprofloxacin demonstrated bactericidal activity against 16/21 isolates tested with no growth detected at 24 hours. In contrast, ivacaftor only demonstrated bactericidal activity against 2/21 isolates tested, both of which were clinical *Streptococcus spp.*

There was no synergy between ivacaftor and tobramycin against *P. aeruginosa* (Fig. 2A, Table 4). In contrast, synergy was apparent for 4/5 *S. aureus* and 4/5 *Streptococcus* spp. isolates (Fig. 2B & 2C, Table 4). Tobramycin (0.5 MIC) had no effect on growth of the isolates.

3.4. Effect of ivacaftor on biofilm formation

Biofilm formation was classified as non-adherent, weak, moderate or strong; 2/5 *P. aeruginosa*, 1/5 *Streptococcus* and 1/3 *Achromobacter* species were non-adherent and were excluded from further analysis. Of the remaining 17 isolates, 10, demonstrated weak adherence, 4 demonstrated moderate adherence and 3 were strong biofilm formers. Treatment with ivacaftor resulted in a significant decrease (P <0.001) in biofilm formation for 1/3 *P. aeruginosa* and 2/4 *Streptococcus* spp. biofilm forming isolates. However, ivacaftor had no effect on biofilm formation by the remaining isolates tested (n=14; Fig. 3). Similarly, ciprofloxacin did not decrease biofilm formation by *S. aureus* isolates (n=5). However, ciprofloxacin caused a significant decrease (P <0.001) in biofilm formation for 7/12 isolates (*P. aeruginosa*, n=2/3, *Streptococcus* spp., n=1/4, *Achromobacter* spp., n=1/2; *Stenotrophomonas* spp., n=3/3) across the other genera tested (Fig. 3). DMSO (0.32%) had no effect on bacterial adherence in any assay.

3.5. In vivo activity of ivacaftor

There was no significant difference between the control group (PBS) and any of the treatment groups in the total number of cells recovered from the IP lavage or cell viability (Fig. 4A & 4B). However, compared with control, treatment with tobramycin caused a significant decrease in the TVC of *P. aeruginosa* recovered from the peritoneal lavage (Fig. 4C). In contrast, there was no significant difference when the mice were treated with ivacaftor. Both treatments caused a significant reduction in IL-6 levels (Fig. 4D); however, KC was only significantly reduced in the group treated with tobramycin (Fig. 4E).

4. Discussion

As bacteria causing pulmonary infection in CF become progressively more resistant to conventional antibiotics, interest in the use of AMPs as antimicrobials for treatment has increased considerably. In the present study, we have shown that LL37 and HβD3 possess antibacterial properties against CF respiratory pathogens such as *P. aeruginosa*, MRSA and *S. maltophilia*. Moreover, both of these AMPs demonstrated antibacterial activity against clinical isolates which were resistant to antibiotics routinely used in the treatment of CF pulmonary infection such as ciprofloxacin, tobramycin and meropenem. SLPI had no activity against any genera tested in the present study. These results contrast to those previously published which reported activity of SLPI against both *P. aeruginosa* and *S. aureus* [16]. However, in the study by Wiedow et al. [16], antibacterial activity of SLPI was assessed using a time-kill assay with a single dermatological *P. aeruginosa* and *S. aureus* isolate. No data were provided with respect to the antibiotic susceptibility of these two isolates. It is likely that the isolates used in the present study demonstrated greater inherent antimicrobial resistance due to prolonged and repeated exposure to antibiotics, which may account for the lack of concordance between studies.

If used clinically, it is likely that AMPs would need to be administered by inhalation in combination with an antibiotic to directly target the site of infection. Therefore, we determined the activity of the most potent AMP, LL37, in combination with tobramycin, the most frequently prescribed inhaled antibiotic for treatment of *P. aeruginosa* pulmonary infection in CF [2]. This combination demonstrated greater antimicrobial activity than either agent alone against both clinical *S. aureus* and *Streptococcus* spp. isolates. However, no synergistic activity was apparent against *P. aeruginosa*. In

contrast to our findings, it has been previously reported that LL37 and tobramycin in combination demonstrate enhanced killing of *P. aeruginosa* biofilms [28]. However, the concentration of both LL37 (640 mg/L) and tobramycin (160-2560 mg/L) used in this biofilm killing study were considerably higher than those used in the present study which ranged from 1-64 mg/L and 0.25-3 mg/L for LL37 and tobramycin, respectively. Synergy may also have been apparent in our study if we had used both LL37 and tobramycin at higher concentrations. Despite the excellent antimicrobial activity demonstrated by LL37 and HβD-3, we were unable to perform further work such as biofilm assays due to the high cost of these AMPs.

As previous studies have reported that ivacaftor, a first-in-class CFTR potentiator, has some antimicrobial activity against non-CF clinical isolates such as *S. aureus*, we determined its antimicrobial activity against clinical CF respiratory isolates from a range of genera. Using quantitative culture time-kill assays, we demonstrated activity, at a concentration of 32 mg/L, against MSSA, MRSA, and *Streptococcus* spp. isolates but no activity against *P. aeruginosa*, *S. maltophilia* and *Achromobacter* species. This result is consistent with the findings of Reznikov et al. who reported that ivacaftor had some antimicrobial activity against *Streptococcus spp.* and *S. aureus*, but was not active against *P. aeruginosa* [22]. Similar to Reznikov et al., who reported enhanced antimicrobial activity when ivacaftor was used in combination with vancomycin or ciprofloxacin, we also found a synergistic effect against *S. aureus* and *Streptococcus* spp. when ivacaftor was combined with tobramycin.

Reznikov et al. suggested that the antimicrobial activity of ivacaftor may be due to the presence of a quinolone ring in its structure, similar to that of fluoroquinolone antibiotics such as ciprofloxacin [22]. Quinolones are broad spectrum antibiotics and

are typically more active against Gram negative bacteria. In the present study, ciprofloxacin demonstrated bactericidal activity against both Gram-positive bacteria (*S. aureus* and *Streptococcus* spp.) and Gram-negative bacteria (*P. aeruginosa*, *S. maltophilia* and *Achromobacter* spp.), consistent with broad-spectrum activity expected from a conventional quinolone antibiotic. In contrast, ivacaftor had no activity against any of the Gram negative isolates tested, suggesting that its antimicrobial effect may not be directly related to the quinolone ring in the structure. Alternatively, lack of activity against Gram negative bacteria could be due to a number of other potential mechanisms such as inability to cross the outer membrane and enzymatic inactivation.

In addition to clinical outcomes, the effect of ivacaftor treatment on the CF lung microbiota was also determined in the GOAL study, a longitudinal cohort study of 151 CF patients before and up to 6 months after ivacaftor initiation. A significant reduction in the number of patients from whom *P. aeruginosa* was cultured from sputum samples either through a decrease in *P. aeruginosa* culture positivity over 6 months [29] or change from *P. aeruginosa* culture positive to negative over the course of a year [30] was reported. In contrast, there was no significant change in sputum culture positivity for MRSA, MSSA, *Stenotrophomonas* spp. or *Achromobacter* spp. [29,30]. Similarly, in a small study of three paediatric CF patients, a reduction in the relative abundance of *Streptococcus spp.* was reported following treatment with ivacaftor. The results of our study suggest that these changes in *Streptococcus* relative abundance could be due to the antimicrobial activity of ivacaftor on this genus; however, the increased abundance of other genera detected could also account for this change [31]. In contrast, our results, which clearly show that ivacaftor has no direct antimicrobial activity against *P. aeruginosa*, growing planktonically or in biofilm, suggest that the

363 change in *P. aeruginosa* culture positivity reported in the GOAL study is more likely
364 attributed to increased mucociliary clearance resulting in increased clearance of
365 biofilm from the airways. The concentration of ivacaftor achieved in sputum following
366 oral administration has not been reported to date; however, it is likely to be significantly
367 lower than the reported serum levels of ~1.4 mg/L [32]. Therefore, the concentration
368 of ivacaftor (32 mg/L) which demonstrated antimicrobial activity in both our study and
369 that of Reznikov et al. [22] is likely to be considerably higher than that achievable in
370 sputum following oral administration. This further supports the hypothesis that
371 changes in pathogen culture positivity reported post-ivacaftor treatment are not as a
372 result of a direct antimicrobial effect.

373 Given that pulmonary infection in the CF airways involves bacterial growth in biofilms
374 [8,9], we also determined the effect of ivacaftor on biofilm formation. The effect of
375 ciprofloxacin on biofilm formation was also determined to enable comparison between
376 ivacaftor and a fluoroquinolone antibiotic with a related chemical structure. In general,
377 ivacaftor had limited effect on biofilm formation. However, as the majority of isolates
378 demonstrated weak adherence, any change attributable to the use of ivacaftor was
379 difficult to detect. The decrease in adherence for 2/5 *Streptococcus* spp. isolates is
380 likely due to the bactericidal activity of ivacaftor against these isolates. In contrast,
381 ivacaftor had no antimicrobial activity against *P. aeruginosa* and thus the decrease in
382 biofilm formation with one *P. aeruginosa* isolate is indicative of inhibition of adherence.
383 Ciprofloxacin demonstrated bactericidal activity against the majority of isolates tested;
384 therefore, inhibition of biofilm formation could be as a result of either a direct effect on
385 bacterial adherence or bacterial cell death before adherence could occur.

386 It has been previously reported that ivacaftor has some immunomodulatory activity
387 with Bratcher et al. (2015) demonstrating a decrease towards normalisation of blood
388 leukocyte activation following ivacaftor treatment [33]. To establish whether ivacaftor
389 exhibited any antimicrobial or immunomodulatory activity *in vivo,* an acute systemic
390 mouse infection model was used; mice were administered ivacaftor or tobramycin at
391 doses approximately reflecting those used in humans. Ivacaftor demonstrated no
392 antimicrobial activity in this model; however, there was some indication of modulation
393 of the innate immune response, based on the reduced production of cytokines and
394 chemokines. The immunomodulatory effect of fluoroquinolones has been widely
395 documented [34]; furthermore, in a mouse injury model, treatment with ciprofloxacin
396 decreased production of IL-6 and KC [35]. A subset of fluoroquinolones with the
397 cyclopropyl moiety have been linked to this immunomodulatory activity, although the
398 mechanism has yet to be elucidated [34]. The similarity in structure between ivacaftor
399 and fluoroquinolones could potentially explain the immunomodulatory activity
400 demonstrated here.

401 There are a number of limitations to this study. Firstly, the AMPs used were expensive
402 restricting the volumes that could be used for testing; therefore, we could not
403 determine MIC by the preferred microbroth dilution method or bactericidal activity
404 using time-kill assays. This also limited the number and range of isolates which could
405 be tested. Physiological conditions may also be important when evaluating AMPs as
406 potential novel antimicrobial therapies. It has been suggested that higher salt
407 concentration in CF airway surface liquid could reduce the antimicrobial activity of
408 AMPs [36] by affecting the ionic interaction between the AMPs and bacterial
409 membranes. Moreover, It has also been demonstrated that in CF sputum, LL37 is
410 inactivated by binding to DNA, F-actin and cell debris bundles [37]. In contrast, it has

411 also been demonstrated that the presence of carbonate, which is found in many
412 microenvironments of the body including the respiratory tract, can greatly enhance
413 bacterial susceptibility to AMPs under physiological ionic conditions [38]. Therefore,
414 further work testing AMP activity under conditions more reflective of the environment
415 present in the CF airways would be required if these compounds were being
416 considered as potential therapeutics to treat CF pulmonary infection. Furthermore,
417 tobramycin was the only antibiotic used in synergy studies with LL37 and ivacaftor.
418 Given the wide range of antibiotics used in the prophylaxis and treatment of CF
419 pulmonary infection, future work to determine synergy between an extended range of
420 antibiotics and LL37/ivacaftor could be of potential clinical benefit.

421

422

5. Conclusion

In summary, we have shown that the AMPs, LL37 and HβD3, demonstrate antimicrobial activity against CF pathogens from a range of genera with LL37 also demonstrating synergistic activity, in combination with tobramycin, against *S. aureus* and *Streptococcus* spp. isolates. Similarly, ivacaftor demonstrated bactericidal activity against *S. aureus* and *Streptococcus* spp. isolates but no activity against Gram-negative bacteria. There is a low propensity for the development of resistance to AMPs due to the interaction of the peptides with the cytoplasmic membrane of bacteria and their bactericidal nature [12]. Therefore, AMPs could potentially be developed as novel therapeutic options but further work is required to enhance their activity.

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Table 1
Antimicrobial activity (MIC range) of antimicrobial peptides against CF respiratory isolates and reference strains *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 29213) and *S. anginosus* (NCTC 10713).

	MIC Range (mg/L)					
Genus	LL37	HβD1	HβD2	HβD3	HβD4	SLPI
***Pseudomonas* (n=4)**	3.9-22.8	>200	43.4->200	6.4-17.6	39.1-49.3	>200
ATCC 27853	3.2	>200	28.6	13.9	17.8	>200
***Staphylococcus* (n=4)**	15.7-29.9	>200	>200	8.9-9.6	>200	>200
ATCC 29213	21.5	>200	>200	12.7	>200	>200
***Streptococcus* (n=4)**	2.4-33.2	>200	3.8-49.9	4.8-18.9	32.4-45.7	>200
NCTC 10713	28.5	>200	>200	18.6	>200	>200
***Achromobacter* (n=3)**	36.6->200*	>200	>200	50.6->200*	>200	>200
***S. maltophilia* (n=3)**	3.3-34.6	>200	47.7->200*	9.6-29.4	51.5->200*	>200

*only active against 1 isolate

Table 2.
Mean log change in CFU/ml (±SD) at 3 hours to determine synergistic activity between LL37 and tobramycin.

	Log change in CFU/ml (mean ± SD)			
Genus	LL37 (0.5 MIC)	Tobramycin (0.5 MIC)	LL37 (0.5 MIC) + tobramycin (0.5 MIC)	Control
***Pseudomonas* (n=5)**	-0.31 (±0.22)	-0.58 (±0.31)	-0.92 (±0.37)	0.02 (±0.02)
***Staphylococcus* (n=5)**	-1.37 (±0.26)	-0.04 (±0.07)	-3.82 (±1.05)	0.01 (±0.01)
***Streptococcus* (n=5)**	-0.13 (±0.15)	-1.12 (±1.51)	-2.53 (±1.96)	0.03 (±0.03)

Table 3
Mean log change in CFU/mL (± SD) at 24 hours following challenge with ivacaftor or ciprofloxacin.

	Log change in CFU/mL (mean ± SD)		
Genus	Ivacaftor (32 mg/L)	Ciprofloxacin (5 mg/L)	Control
***Pseudomonas* (n=5)**	4.44 (±0.30)	-5.83 (±0.28)	3.84 (±0.79)
***Staphylococcus* (n=5)**	-0.51 (±0.61)	-2.06 (±4.33)	3.37 (±0.44)
***Streptococcus* (n=5)**	-3.16 (±2.25)	-4.50 (±1.35)	3.63 (±0.51)
***Achromobacter* (n=3)**	2.83 (±0.27)	-1.63 (±3.84)	3.01 (±0.17)
***Stenotrophomonas* (n=3)**	2.69 (±0.16)	-5.83 (±0.29)	3.11 (±0.01)

Table 4
Mean log change in CFU/ml (± SD) at 24 hours to determine synergistic activity between ivacaftor and tobramycin.

	Log change in CFU/mL (mean ± SD)			
Genus	Ivacaftor (32 mg/L)	Tobramycin (0.5 MIC)	Ivacaftor (32 mg/L) & Tobramycin (0.5 MIC)	Control
***Pseudomonas* (n=5)**	3.91 (±1.57)	3.69 (±0.66)	2.46 (±1.61)	3.84 (±0.49)
***Staphylococcus* (n=5)**	0.13 (±0.45)	3.53 (±0.15)	-5.72 (±0.17)	3.51 (±0.27)
***Streptococcus* (n=5)**	-3.31 (±2.13)	2.38 (±1.42)	-5.53 (±0.61)	3.49 (±0.59)

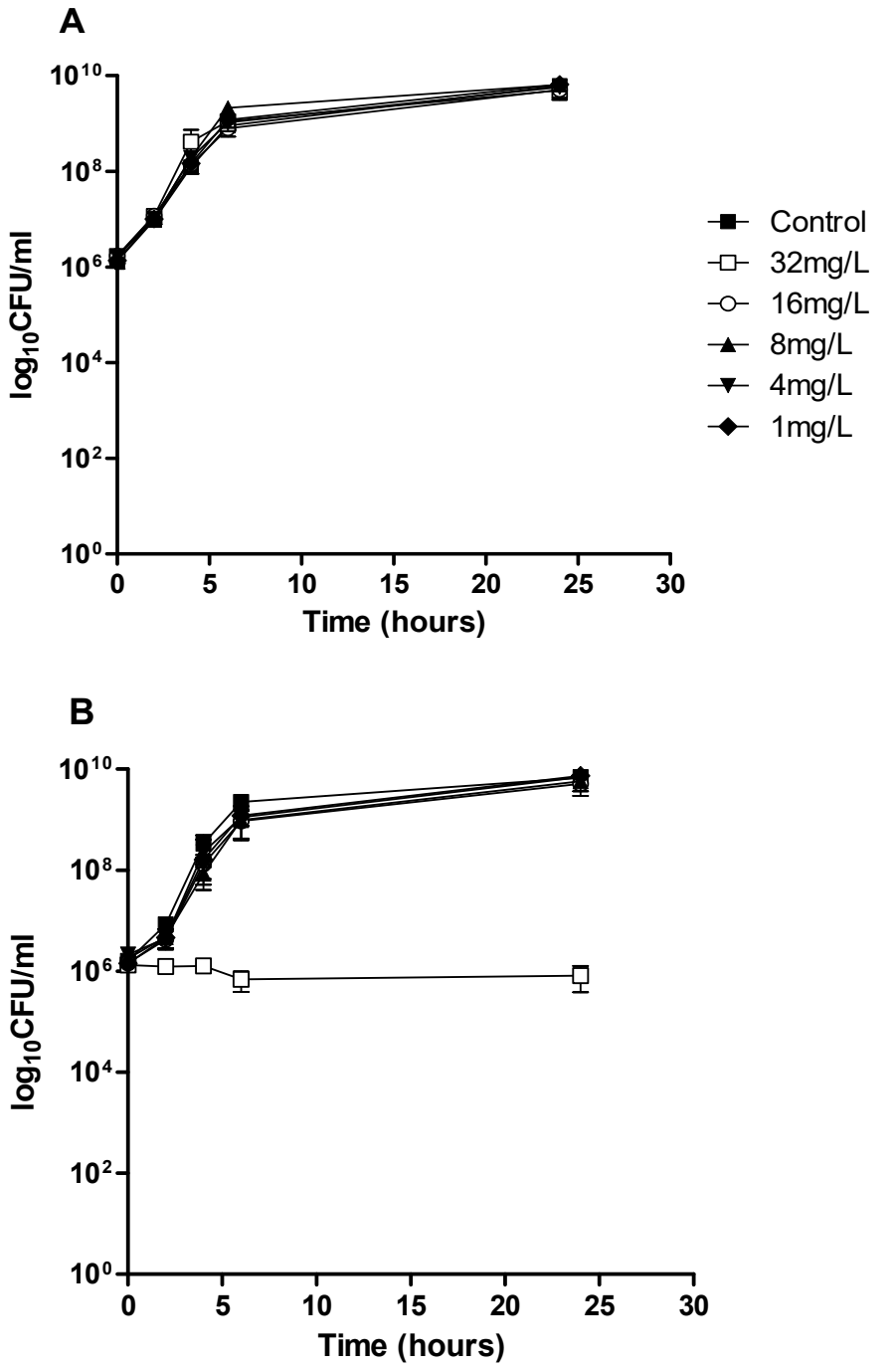
Fig. 1. Time-kill curves for A) *P. aeruginosa* (n=5), B) *S. aureus* (n=5) and C) *Streptococcus spp.* (n=5) challenged with ivacaftor at a range of concentrations (1-32 mg/L) plotted as the mean change in CFU/ml (±SD).

Fig. 2. Activity of ivacaftor and tobramycin (0.5 MIC) in combination against A) *P. aeruginosa* (n=5), B) *S. aureus* (n=5) and C) *Streptococcus spp.* (n=5); the mean change in CFU/ml (±SD) for each genera is shown.

Fig. 3. Effect of ivacaftor (32mg/L) and ciprofloxacin (5mg/L) on biofilm formation by *P. aeruginosa* (n=3), *S. aureus* (n=5), *Streptococcus spp.* (n=4), *Achromobacter spp.* (n=2) and *Stenotrophomonas spp.* (n=3). Adherence is plotted as the mean of 8 replicates (±SD). Non-adherent bacteria were not included in the analysis. P≤0.01**, P≤0.001***.

Fig. 4. Effect of ivacaftor in comparison to tobramycin on intraperitoneal infection using *P. aeruginosa* (PAO1) in C57bl6 albino mice. A) total number of cells recovered from the peritoneal lavage, B) cell viability, C) Total viable count (TVC) of *P. aeruginosa* recovered from the peritoneal lavage, D) IL-6; E) KC. Results are plotted as the mean value from 5 mice (±SD). P≤0.05*, P≤0.01**, P≤0.001***.

Fig. 1.


A
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
$\log_{10}$CFU/ml
0
5
10
15
20
25
30
Time (hours)
Control
32mg/L
16mg/L
8mg/L
4mg/L
1mg/L
B
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
$\log_{10}$CFU/ml
0
5
10
15
20
25
30
Time (hours)

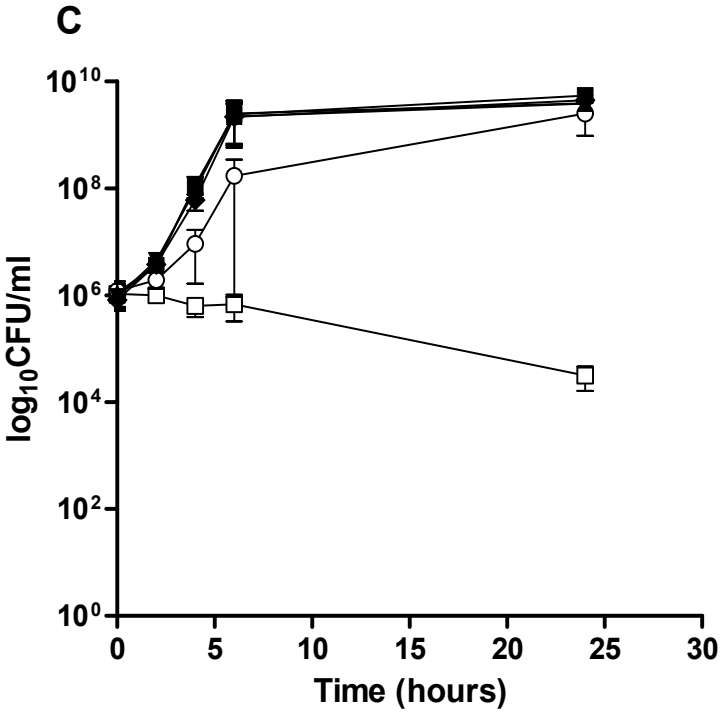
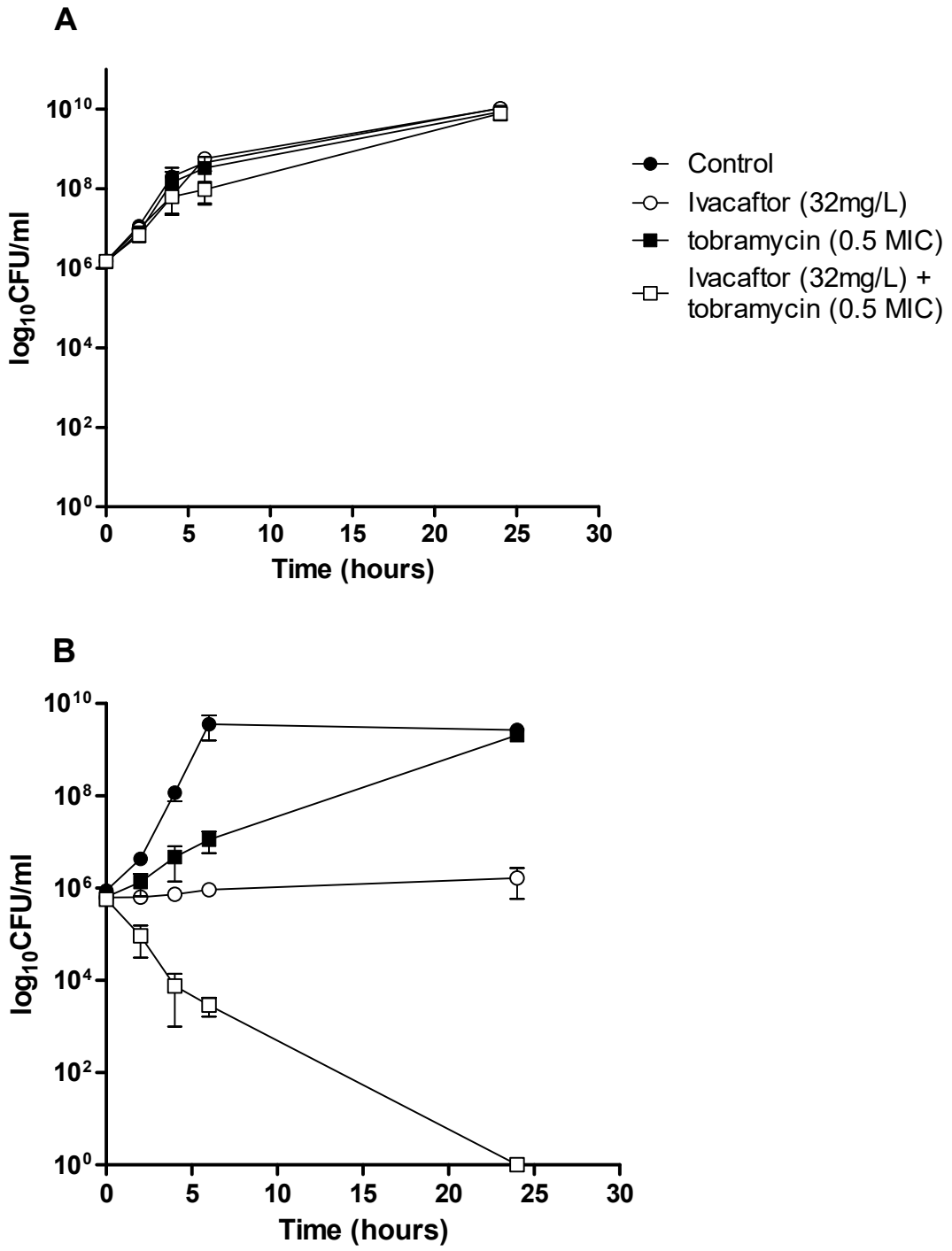

C
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
$\log_{10}$CFU/ml
0
5
10
15
20
25
30
Time (hours)

Fig. 2.


A
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
$\log_{10}$CFU/ml
0
5
10
15
20
25
30
Time (hours)
Control
Ivacaftor (32mg/L)
tobramycin (0.5 MIC)
Ivacaftor (32mg/L) +
tobramycin (0.5 MIC)
B
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
$\log_{10}$CFU/ml
0
5
10
15
20
25
30
Time (hours)

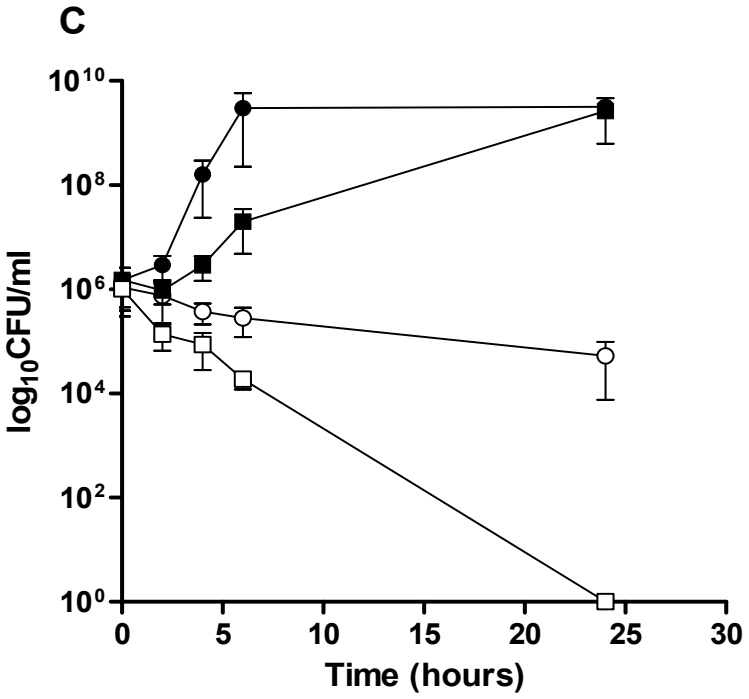

C
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
$\log_{10}$CFU/ml
0
5
10
15
20
25
30
Time (hours)

Fig. 3.

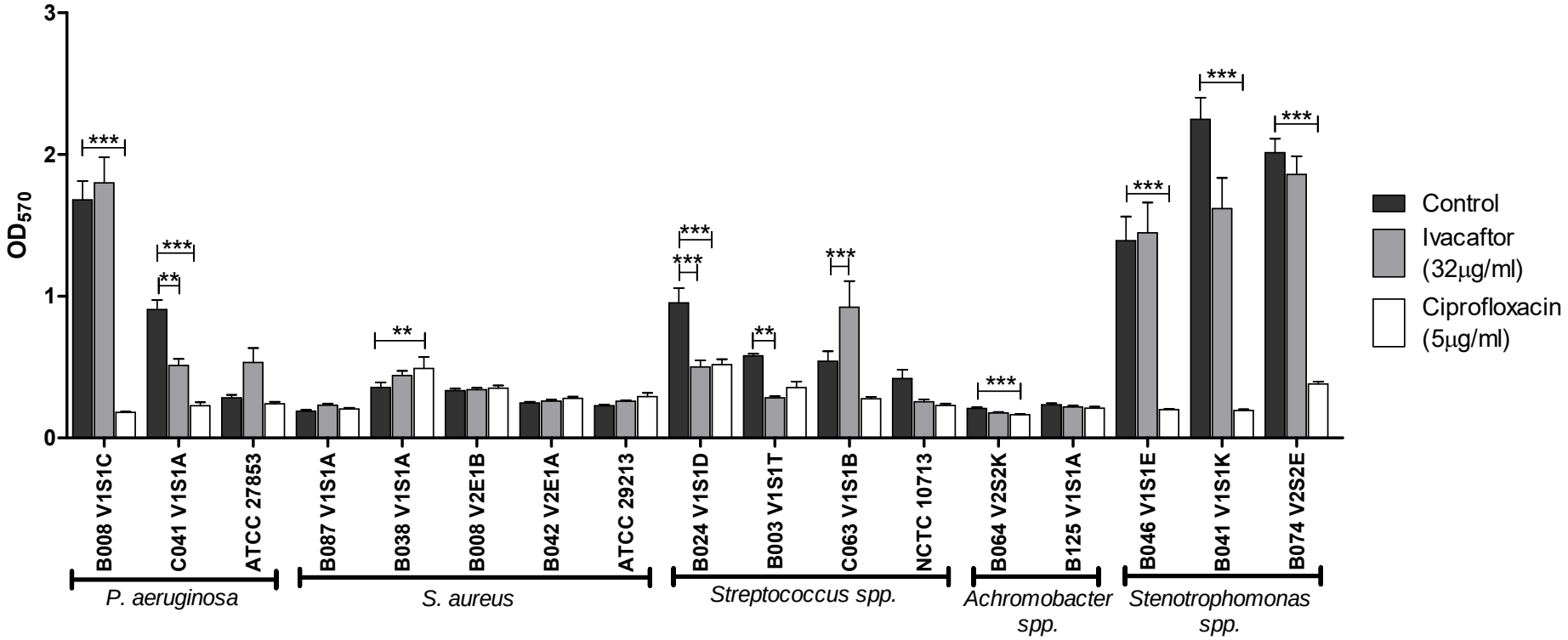
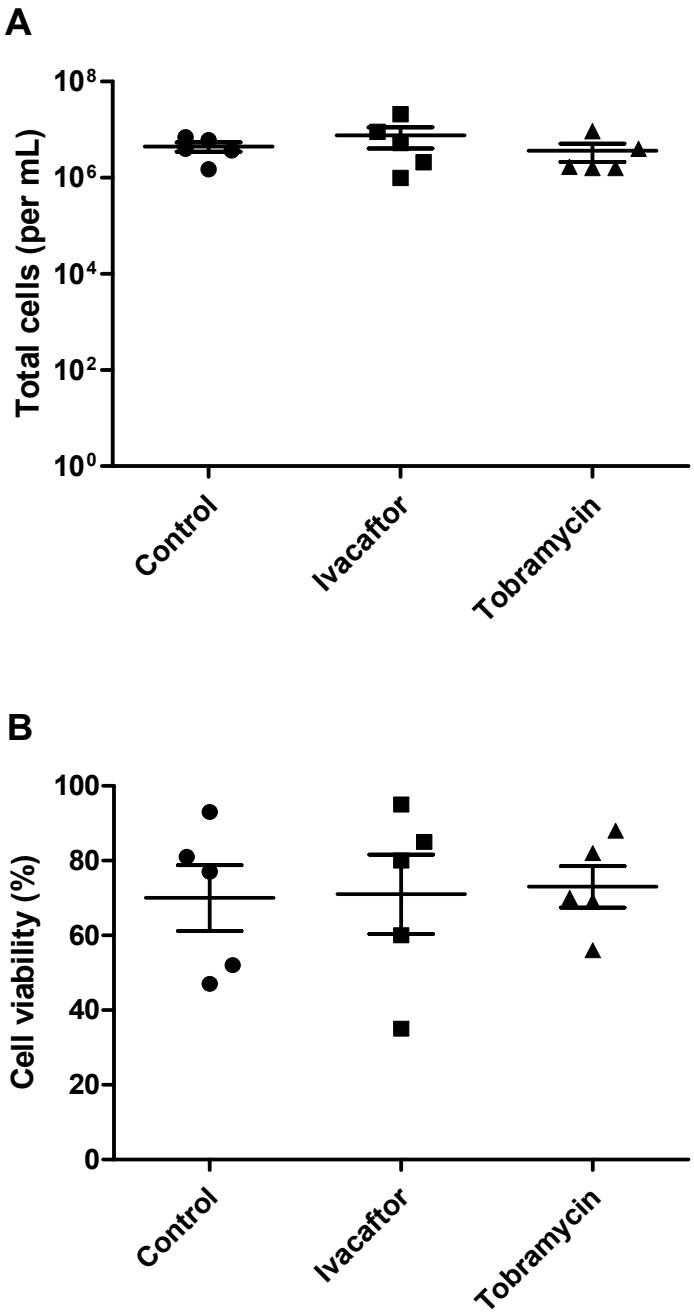
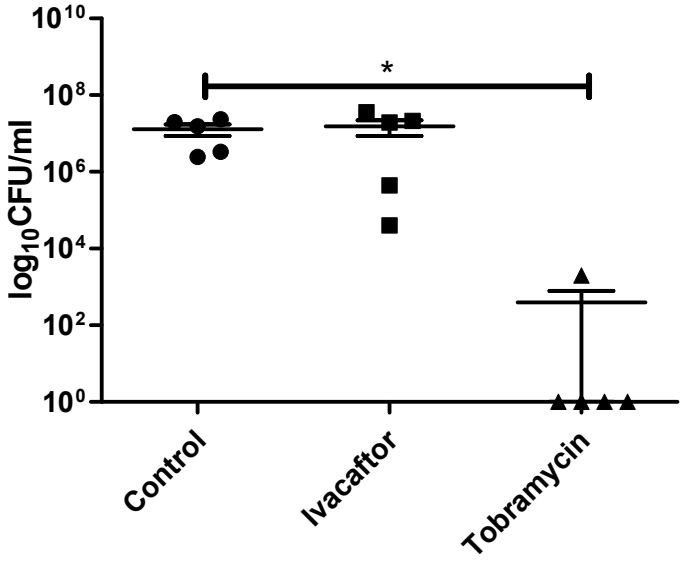

OD570
3
2
1
0
***
**
Control
Ivacaftor (32μg/ml)
Ciprofloxacin (5μg/ml)
B008 V1S1C
C041 V1S1A
ATCC 27853
B087 V1S1A
B038 V1S1A
B008 V2E1B
B042 V2E1A
ATCC 29213
B024 V1S1D
B003 V1S1T
C063 V1S1B
NCTC 10713
B064 V2S2K
B125 V1S1A
B046 V1S1E
B041 V1S1K
B074 V2S2E
P. aeruginosa
S. aureus
Streptococcus spp.
Achromobacter spp.
Stenotrophomonas spp.

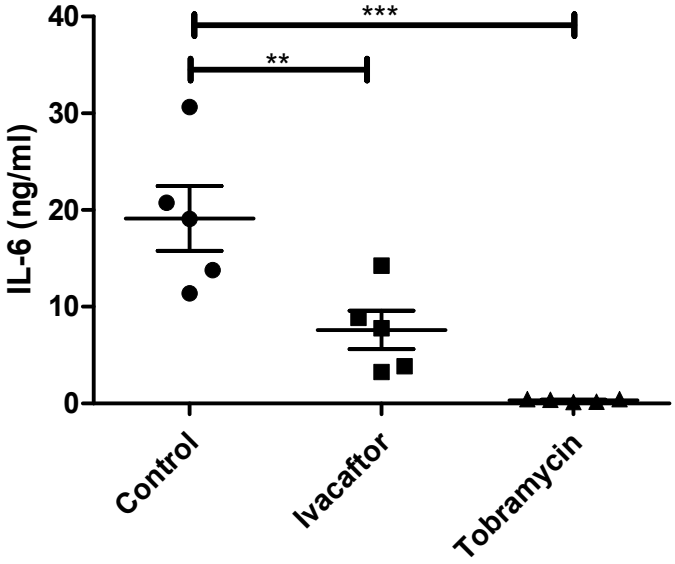
Fig. 4.

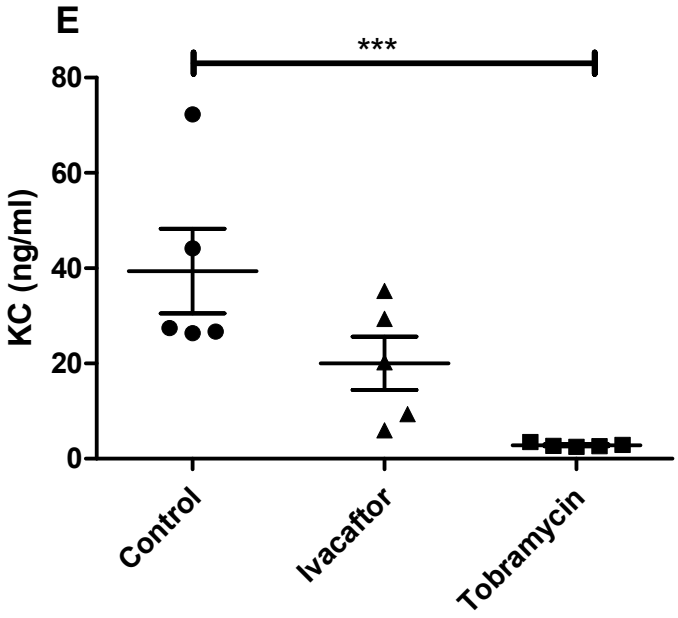

A
Total cells (per mL)
10^8
10^6
10^4
10^2
10^0
Control
Ivacaftor
Tobramycin
B
Cell viability (%)
100
80
60
40
20
0
Control
Ivacaftor
Tobramycin

C


10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
10^{0}
log_{10}CFU/ml
*
Control
Ivacaftor
Tobramycin

D


40
30
20
10
0
IL-6 (ng/ml)
***
**
Control
Ivacaftor
Tobramycin


E
***
80
60
40
20
0
KC (ng/ml)
Control
Ivacaftor
Tobramycin

Supplementary Table 1. MIC (mg/L) of a panel of antibiotics against the isolates tested. Breakpoints, where available, are taken from the EUCAST guidelines [39].

	Amoxicillin	**Azithromycin**	**Ceftazidime**	**Chloramphenicol**	**Ciprofloxacin**	**Clindamycin**	**Co-amoxiclav**	**Colistin**	**Doxycycline**	**Meropenem**	**Tobramycin**
P. aeruginosa											
B004 V2S2B	>256	>256	>256	12	2	>256	48	3	24	12	6
B008 V1S1C	3	48	1.5	16	3	>256	3	1	>256	0.094	1.5
C041 V1S1A	>256	48	1	>256	0.125	>256	>256	1.5	>256	0.38	0.75
C070 V1S1A	>256	128	1	>256	0.5	>256	256	1	>256	0.094	1
ATCC 27853	>128	32	1	>256	0.25	>256	128	4	48	0.25	0.5
Breakpoints			**R>8**		**S≤0.5, R>1**			**R>4**		**S≤2, R>8**	**R>4**
S. aureus											
BO38 V1S1A[#]	24	>256	64	3	0.38	0.047	12	>256	4	0.5	32
BO87 V1S1A	1.5	>256	16	3	0.19	0.25	8	>256	0.094	0.38	0.38
BO42 V2E1A[#]	24	>256	>256	2	>32	8	48	96	0.094	>32	0.75
BOO8 V2E1B[#]	8	>256	256	2	>32	0.047	12	32	0.064	1	0.094
ATCC 29213	0.5	0.12	8	2	0.5	0.06	0.25	>256	0.19	0.06	0.5
Breakpoints		**S≤1, R>2**		**R>8**	**R>1**	**S≤0.25, R>0.5**			**S≤1, R>2**		**R>1**
Streptococcus sp.											
B012 V1S1Q (*S. anginosus)*	0.064	0.38	3	2	1.5	0.094	0.094	>256	3	0.064	96
B024 V1S1D (*S. mitis)*	0.032	6	1	1.5	4	0.094	0.047	>256	1.5	0.064	96
B003 V1S1T (*S. constellatus*)	0.5	>256	4	2	1	0.125	0.25	64	12	0.094	0.125
C063 V1S1B (*S. constellatus*)	0.094	0.19	2	1.5	1.5	0.094	0.094	>256	1.5	0.094	3
NCTC 10713 (*S. anginosus)*	0.19	0.25	3	2	1	0.064	0.38	>256	1	0.064	192
Breakpoints	**S≤0.5, R>2**	**S≤0.25, R>0.5***		**R>8***	**S≤0.25, R>0.5***	**R>0.5**			**S≤1, R>2***	**R>2**	
Achromobacter sp.											
BO64 V2S2K *(A. insolitus)*	4	32	2	2	1.5	>256	8	0.38	1.5	0.38	12
B125 V1S1A *(A. insolitus)*	>256	>256	>256	3	>32	>256	>256	0.25	198	>32	>1024
BO32 V2E1D *(A. spanius)*		>256	16	6	>32	>256	3	>256	16		>1024
Breakpoints	**No breakpoints available**										
S. maltophilia											
BO46 V1S1E	4	>256	0.75	2	4	>256	1.5	32	1.5	2	1.5
BO74 V2S2E	>256	32	32	3	2	>256	>256	4	3	>32	48
BO41 V1S1K		>256	96	8	>32	>256	64	0.38	48	>32	96
Breakpoints	**No breakpoints available**										

[#]MRSA; *Indicates breakpoints for *S. pneumoniae* as no breakpoints available for *S. viridans* group

Supplementary Table 2. MIC (mg/L) of antimicrobial peptides against CF respiratory isolates

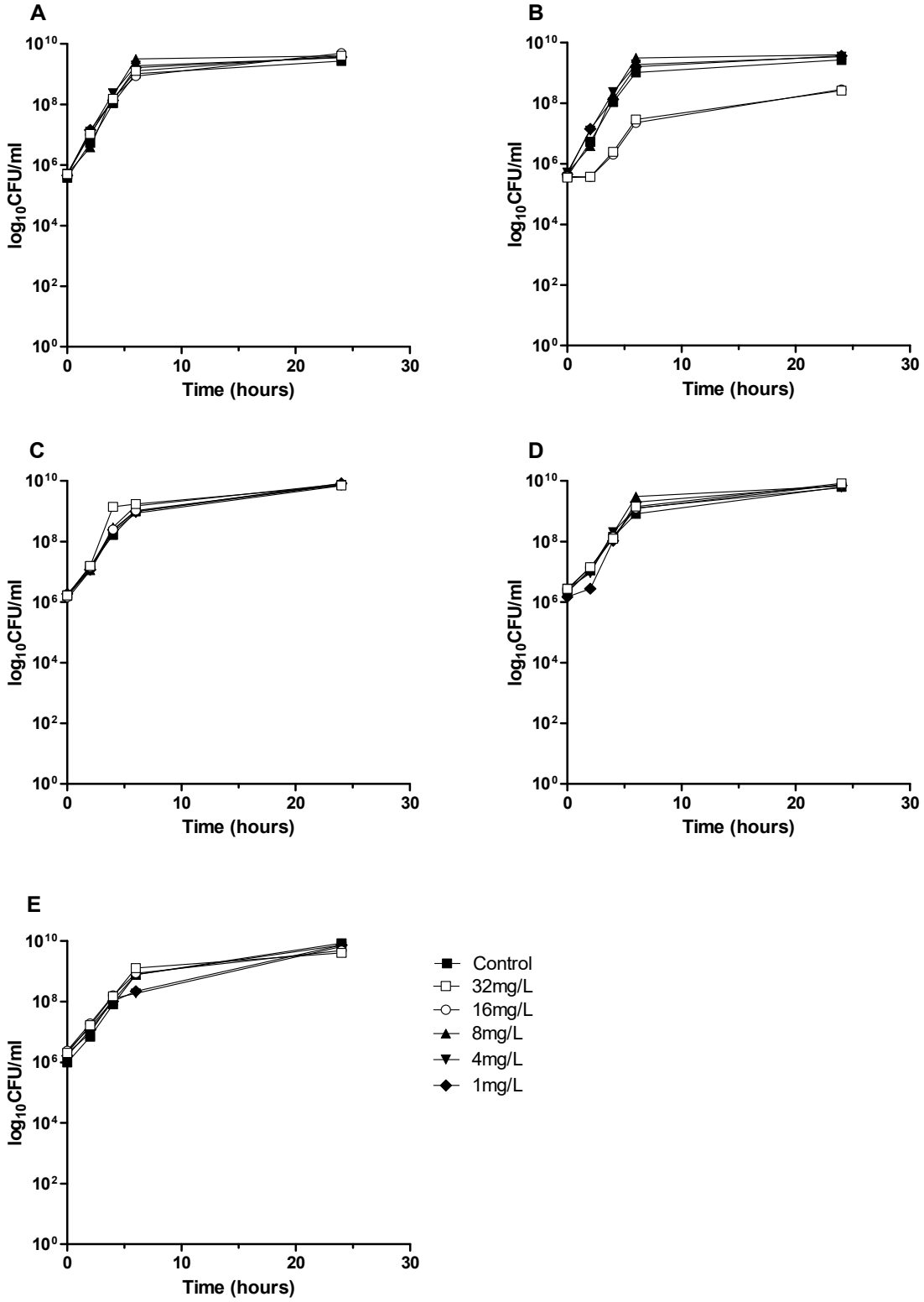
Isolate	LL37	HβD1	HβD2	HβD3	HβD4	SLPI
P. aeruginosa						
B004 V2S2B	22.8	≥200	≥200	8.1	49.3	≥200
B008 V1S1C	3.9	≥200	43.4	6.4	46.6	≥200
C041 V1S1A	9.5	≥200	51.3	9.6	44.1	≥200
C070 V1S1A	9.7	≥200	47.7	17.6	39.1	≥200
ATCC 27853	3.2	≥200	28.6	13.9	17.8	≥200
S. aureus						
BO38 V1S1A	29.9	≥200	≥200	9.2	≥200	≥200
BO87 V1S1A	17.8	≥200	≥200	8.9	≥200	≥200
BO42 V2E1A	22.4	≥200	≥200	9.4	≥200	≥200
BOO8 V2E1B	15.7	≥200	≥200	9.6	≥200	≥200
ATCC 29213	21.5	≥200	≥200	12.7	≥200	≥200
Streptococcus spp.						
B012 V1S1Q (*S. anginosus)*	2.4	≥200	49.9	8.9	45.7	≥200
B024 V1S1D (*S. mitis)*	33.2	≥200	42.9	13.9	32.4	≥200
B003 V1S1T (*S. constellatus*)	6.5	≥200	3.8	4.8	38.5	≥200
C063 V1S1B (*S. constellatus*)	29.0	≥200	43.0	18.9	36.0	≥200
NCTC 10713 (*S. anginosus)*	28.5	≥200	≥200	18.6	≥200	≥200
Achromobacter sp.						
BO64 V2S2K *(A. insolitus)*	36.6	≥200	≥200	50.6	≥200	≥200
B125 V1S1A *(A. insolitus)*	≥200	≥200	≥200	≥200	≥200	≥200
BO32 V2E1D *(A. spanius)*	≥200	≥200	≥200	≥200	≥200	≥200
S. maltophilia						
BO46 V1S1E	3.3	≥200	≥200	23.6	≥200	≥200
BO74 V2S2E	34.6	≥200	≥200	29.4	≥200	≥200
BO41 V1S1K	18.6	≥200	47.7	9.6	51.5	≥200

Supplementary Table 3. Synergy between LL37 and tobramycin against CF respiratory isolates (change in log CFU/ml) at 3 hours.

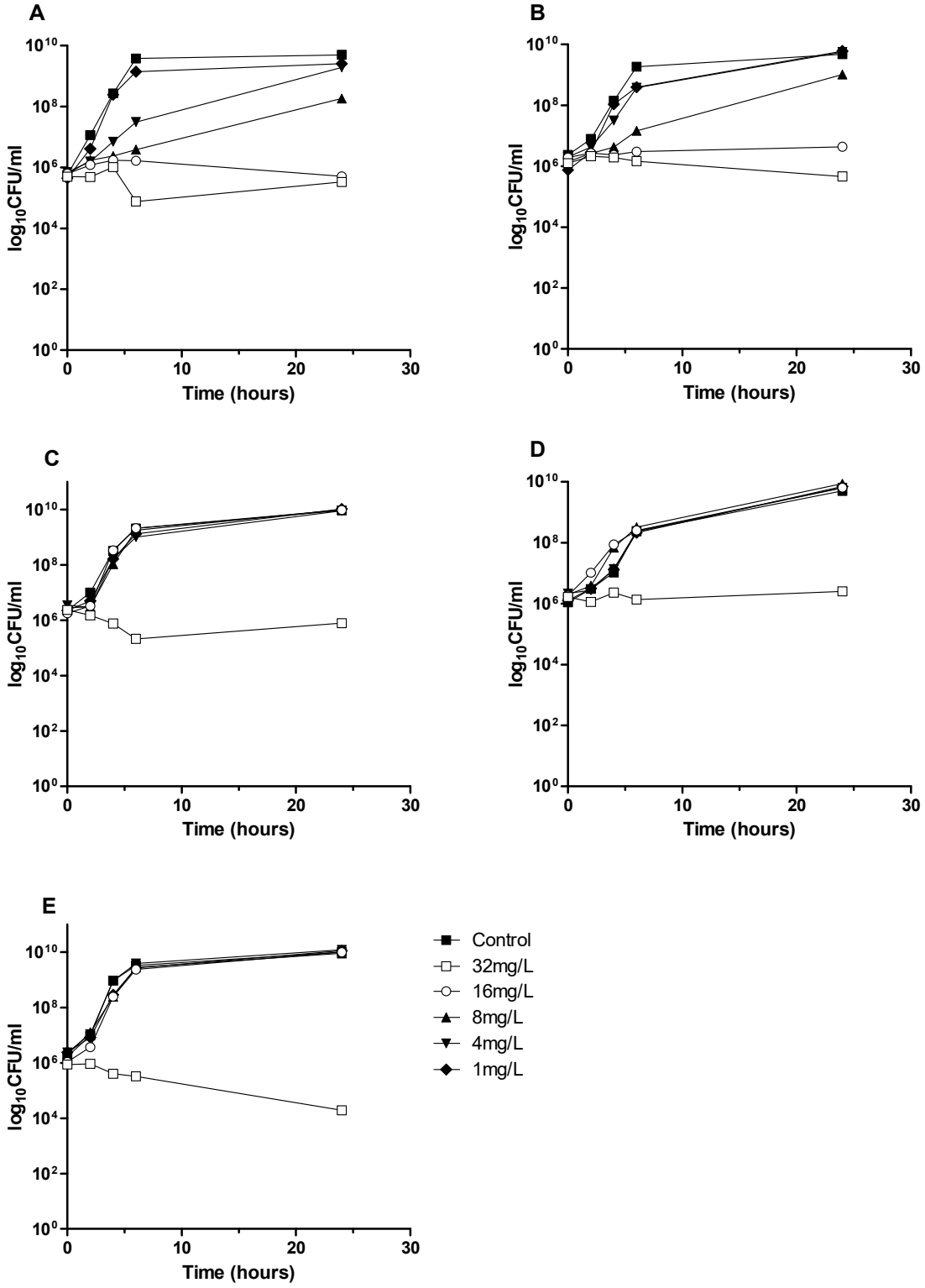
	Log change in CFU/ml (±SD)			
	Control	LL37 (0.5 MIC) + tobramycin (0.5 MIC)	LL37 (0.5 MIC)	Tobramycin (0.5 MIC)
P. aeruginosa				
B004 V2S2B	0.02	-1.17	-0.17	-0.90
B008 V1S1C	0.00	-0.70	-0.55	-0.46
C041 V1S1A	0.01	-1.52	0.05	-0.96
C070 V1S1A	0.07	-0.58	-0.41	-0.40
ATCC 27853	0.01	-0.62	-0.48	-0.16
Mean	0.02 (±0.02)	-0.92 (±0.37)	-0.31 (±0.22)	-0.58 (±0.31)
S. aureus				
BO38 V1S1A	0.01	-1.72	-0.91	-0.15
BO87 V1S1A	0.02	-4.30*	-1.61	0.02
BO42 V2E1A	0.02	-4.48*	-1.64	0.03
BOO8 V2E1B	0.01	-4.30*	-1.40	0.00
ATCC 29213	0.00	-4.30*	-1.30	-0.08
Mean	0.01 (±0.01)	-3.82 (±1.05)	-1.37 (±0.26)	-0.04 (±0.07)
Streptococcus spp.				
B012 V1S1Q (*S. anginosus)*	0.01	-4.18*	-0.10	-0.17
B024 V1S1D (*S. mitis)*	0.02	-4.05*	-0.26	-4.05
B003 V1S1T (*S. constellatus*)	0.08	0.21	0.14	0.03
C063 V1S1B (*S. constellatus*)	0.01	-0.49	-0.18	-0.38
NCTC 10713 (*S. anginosus)*	0.01	-4.14*	-0.24	-1.01
Mean	0.03 (±0.03)	-2.53 (±1.96)	-0.13 (±0.15)	-1.12 (±1.51)

*Synergy (defined as a ≥2 $\log_{10}$ decrease in total viable count (TVC) compared to the starting inoculum and as a ≥2 $\log_{10}$ decrease in TVC by the combination compared to the most active single agent)

Supplementary Figure 1. Antimicrobial activity of ivacaftor at a range of concentrations against *P. aeruginosa* isolates; A) B004 V2S2B, B) B008 V1S1C, C) C041 V1S1A, D) C070 V1S1A and E) ATCC 27853.



Supplementary Figure 2. Antimicrobial activity of ivacaftor at a range of concentrations against *S. aureus* isolates; A) B038 V1S1A, B) B042 V2E1A, C) B087 V1S1A, D) B008 V2E1B and E) ATCC 29213.



Supplementary Figure 3. Antimicrobial activity of ivacaftor at a range of concentrations against *Streptococcus* spp. isolates; A) B012 V1S1Q (*S. anginosus)*, B) C063 V1S1B (*S. constellatus*), C) B003 V1S1T (*S. constellatus*), D) B024 V1S1D (*S. mitis)*, E) NCTC 10713 (*S. anginosus)*.