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An ‘unlikely’ pair: The antimicrobial synergy of polymyxin B in combination with the cystic fibrosis trans-membrane conductance regulator drugs KALYDECO and ORKAMBI

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Abstract

Novel combination therapies are desperately needed for combating lung infections caused by bacterial ‘superbugs’. This study aimed to investigate synergistic antibacterial activity of polymyxin B in combination with the cystic fibrosis (CF) drugs KALYDECO™ (ivacaftor) and ORKAMBI™ (ivacaftor+lumacaftor) against Gram-negative pathogens that commonly colonize the CF lung, in particular the problematic *Pseudomonas aeruginosa*. The *in vitro* synergistic activity of polymyxin B combined with ivacaftor or lumacaftor was assessed using checkerboard and static time-kill assays against a panel of polymyxin-susceptible and polymyxin-resistant *P. aeruginosa* isolates from the lungs of CF patients. Polymyxin B, ivacaftor or lumacaftor were ineffective when used individually against polymyxin-resistant (MIC, 4 mg/L) isolates. However, when used together the combination of clinically-relevant concentrations of polymyxin B (2 mg/L) combined with ivacaftor (8 mg/L) or ivacaftor (8 mg/L)+lumacaftor (8 mg/L) and

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Supporting Information. The Supporting Information is available free of charge on the ACS Publications website.

displayed synergistic killing activity against polymyxin-resistant *P. aeruginosa* isolates as demonstrated by 100-fold decrease in the bacterial count (CFU/mL) after 24 h. The combinations also displayed excellent antibacterial activity against *P. aeruginosa* under CF relevant conditions in a sputum medium assay. The combination of lumacaftor (alone) with polymyxin B showed additivity against *P. aeruginosa*.

The potential antimicrobial mode of action of the combinations against *P. aeruginosa* was investigated using different methods. Treatment with the combinations induced cytosolic GFP release from *P. aeruginosa* cells and showed permeabilizing activity in the nitrocefin assay, indicating damage to both the outer and inner Gram-negative cell membranes. Moreover, scanning and transmission electron micrographs revealed that the combinations produce outer membrane damage to *P. aeruginosa* cells that is distinct from the effect of each compound *per se*. Ivacaftor was also shown to be a weak inhibitor of the bacterial DNA gyrase and topoisomerase IV with no effect on either human type I or type II α topoisomerases. Lumacaftor displayed the ability to increase the cellular production of damaging reactive oxygen species.

In summary, the combination of polymyxin B with KALYDECO™ or ORKAMBI™ exhibited synergistic activity against highly polymyxin-resistant *P. aeruginosa* CF isolates and can be potentially useful for otherwise untreatable CF lung infections.

Keywords

Ivacaftor; lumacaftor; polymyxin; *Pseudomonas aeruginosa*; cystic fibrosis

Introduction

Antibiotic resistance has evolved into a substantial global health concern.¹ Unfortunately, the ‘magic bullet’ antimicrobials we have gratuitously used over the past decades are rapidly losing their caliber.¹ Modern medicine is clearly entering a critical period, if bacteria continue developing resistance to multiple antibiotics at the present rate and at the same time the antibiotic pipeline continues to dry up; there could be catastrophic costs to healthcare and society globally.² Patients with chronic infections such as CF rely on antibiotics on a daily basis and are particularly at risk. One in 22 people of European descent carry a mutant allele for CF making it the most common recessive genetic disease.³ CF is caused by mutations in a gene that encodes the CF trans-membrane conductance regulator (CFTR) protein.³ The defective CFTR gating function leads to an abnormal transmembrane chloride transport, followed by sodium hyper-absorption with water influx into the cell leading to fluid depletion on the epithelial surface of affected organs and a deficiency in bicarbonate secretion. As a consequence, thick mucus accumulation occurs and promotes bacterial lung infections, most commonly due to *P. aeruginosa* (~80% of patients).⁴ Other bacterial pathogens that less commonly colonize and infect the lungs of CF patients include *Haemophilus influenzae*, *Burkholderia cepacia*, *Burkholderia pseudomallei*, *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae* and *Acinetobacter baumannii*.⁴ *P. aeruginosa* is often the most problematic to treat, as once chronic infection is established the infecting strain becomes increasingly resistant to antibiotics over the lifetime of CF patients, and 80 to 95% of them die of cardiopulmonary failure due to the infection.⁴

Ivacaftor (*syn.* VX-770; KALYDECO™) is the first FDA-approved CFTR modulator, with demonstrated clinical efficacy producing a significant improvement in the lung function of CF patients over placebo in CF patients with certain types of CFTR mutations (which account for 4–5% of cystic fibrosis cases).⁵ Recently, the FDA approved ivacaftor in combination with lumacaftor (ORKAMBI™). Lumacaftor promotes the transport of the defective CFTR protein to the cell surface, where ivacaftor acts to improve its gating function.⁶ Specifically, lumacaftor expands the therapeutic window of ivacaftor to include around 28% of CF patients.

Polymyxin B and colistin (polymyxin E) are lipopeptide antibiotics used to treat infections caused by multidrug-resistant (MDR) Gram-negative pathogens.⁷ Colistin has been commercially available in the clinic as its inactive prodrug colistimethate sodium (CMS) for decades.⁸ CMS nebulizer solution (Coly-Mycin®) and dry powder pod-inhaler (Colobreathe®) has been approved for the treatment of respiratory infections caused by *P. aeruginosa* in CF patients;⁹ however reports of polymyxin-resistant *P. aeruginosa* isolates from CF patient lung samples are becoming more common.¹⁰ Therefore, novel strategies to prolong the efficacy of these important lipopeptide antibiotics are needed. The rise of Gram-negative ‘superbugs’ which are resistant to all available antibiotics implies that even the robust activity of the polymyxins is being lost. The potential synergistic use of antibiotic-nonantibiotic combinations is emerging as a valuable and cost-effective means for improving the clinical efficacy of currently available antibiotics against problematic MDR bacterial pathogens. The focus of the present study was to evaluate the antibacterial synergy of CFTR potentiator drugs KALYDECO and ORKAMBI with polymyxin B against *P. aeruginosa* that commonly infects the lungs of CF patients.

Results and Discussion

Antimicrobial synergy of polymyxin-CFTR potentiator combinations against Gram-negative CF lung pathogens

MICs for polymyxin B, ivacaftor, lumacaftor and VX-661 (a developmental CFTR potentiator Vertex Pharmaceuticals compound) alone, and in combination with polymyxin B, are presented in Table 1. Ivacaftor *per se* were inactive (MIC, >32 mg/L) against all of the Gram-negative isolates tested, except for *P. aeruginosa* FADDI-PA064 non-mucoid (ivacaftor MIC, 4 mg/L). Lumacaftor and VX-661 *per se* were inactive (MIC, >32 mg/L) against all of the isolates including FADDI-PA064 non-mucoid. The broth microdilution checkerboard method results showed the polymyxin B-ivacaftor combination displayed synergism (eight isolates) and additivity (eight isolates) against the sixteen polymyxin-resistant *P. aeruginosa* CF isolates tested (Tables 1 and S2). Across the six polymyxin-susceptible *P. aeruginosa* isolates synergy was only observed for one isolate and additivity for the remaining five. The polymyxin B-ivacaftor combination was also active against most of the *A. baumannii*, *K. pneumoniae* and *S. maltophilia* isolates, displaying either synergy or additivity in most cases. The polymyxin B-lumacaftor and VX-661 combination largely displayed additivity and indifference across the panel of strains, synergy was only observed with one *A. baumannii* (ATCC19606), one *K. pneumoniae* (FADDI-KP081) and one *S. maltophilia* (FADDI-SM006) isolates. Notably, the polymyxin B-ivacaftor combination

displayed a greater degree of synergy against the polymyxin-resistant laboratory strain *A. baumannii* 19606R compared to its polymyxin-susceptible paired wild-type strain ATCC 19606, where additivity was observed. We have previously reported a unique polymyxin resistance mechanism employed by *A. baumannii* 19606R which involves the complete loss of lipopolysaccharide (LPS) from the outer membrane.¹¹ As ivacaftor is a hydrophobic compound (logP=5.76), it is likely that a complete loss of LPS from the outer membrane facilitates the ability of ivacaftor to cross the outer membrane barrier of *A. baumannii* 19606R. This is coincident with our previous report that polymyxin-resistant *A. baumannii* strains are generally more susceptible to a number of antibiotics than their polymyxin-susceptible parent strains.¹² The all the polymyxin B combinations were inactive against all of the *H. influenzae*, *B. cepacia* and *B. pseudomallei* isolates tested. The latter is not surprising as *B. cepacia* and *B. pseudomallei* species are intrinsically highly resistant to polymyxins since their LPS has the 4-amino-4-deoxy-L-arabinose modification on the phosphate groups in the lipid A and in the inner core regions.¹³

The synergistic killing activity of the polymyxin B-ivacaftor combination in static time-kill studies was assessed against four *P. aeruginosa* isolates, the polymyxin-susceptible control strain *P. aeruginosa* ATCC 27853 (polymyxin B MIC, 1 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC, >32 mg/L) and the highly polymyxin-resistant MDR strains FADDI-PA064 (polymyxin B MIC, >128 mg/L; ivacaftor MIC, 4 mg/L; lumacaftor MIC, >32 mg/L), FADDI-PA065 (polymyxin B MIC, >128 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC, >32 mg/L) and FADDI-PA070 (polymyxin B MIC, 64 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC, >32 mg/L) (Figure 1). Clinically relevant concentrations of the drugs were used in the time-kill experiments.^{7, 14} Polymyxin B, ivacaftor and lumacaftor *per se* did not exhibit antibacterial activity against *P. aeruginosa* ATCC 27853, FADDI-PA065 and FADDI-PA070, the bacterial killing curves were essentially indistinguishable from those of the controls. Ivacaftor alone displayed moderate killing kinetics against FADDI-PA064, which is consistent with the superior activity of ivacaftor against this particular polymyxin-resistant strain (FADDI-PA064 ivacaftor MIC, 4 mg/L vs >32 mg/L for all the other *P. aeruginosa* isolates). Against the polymyxin-susceptible strain ATCC 27853, polymyxin B *per se* displayed antibacterial activity up to 1 h (before regrowth occurred) at 2 mg/L. The polymyxin B-ivacaftor combination (polymyxin B 2 mg/L-ivacaftor 8 mg/L) demonstrated synergism in the static time-kill studies against all three polymyxin-resistant CF strains with bacterial killing reaching below the limit of detection at 2–6 h and a 100-fold reduction in CFU/mL at 24 h compared to polymyxin B alone. Regrowth with polymyxin B-ivacaftor over 24 h is seen in ATCC 27853 and FADDI-PA070, whereas, FADDI-PA064 and FADDI-PA065 show complete killing at 24 h (Figure 1). Notably, the general trend we observe with the FIC_I data (Table 1) is that the polymyxin B-ivacaftor combination is less effective against the polymyxin-susceptible *P. aeruginosa* CF isolates. It is therefore tenable to speculate that the regrowth seen with FADDI-PA070 (polymyxin B MIC=64 mg/L) is potentially due to its 2-fold lower MIC (*i.e* greater polymyxin B susceptibility) compared to FADDI-PA064 (polymyxin B MIC=>128 mg/L) and FADDI-PA065 (polymyxin B MIC=128 mg/L), if we recall that the latter two isolates showed complete killing at 24 h. In the case of the polymyxin B+(ivacaftor/lumacaftor [ORKAMBI]) combination, regrowth over 24 h is seen in ATCC 27853 (the polymyxin-susceptible control isolate), FADDI-

PA064 and FADDI-PA070; FADDI-065 is the only isolate that shows complete killing without detectable regrowth at 24 h (Figure 1). Notably, in the case of ATCC 27853 and FADDI-PA064 strains, it appears that lumacaftor is driving the regrowth. We would only go as far to speculate that these complex killing effects may be associated with the different levels of aminoarabinose modified lipid A (a primary mechanism whereby membrane remodeling occurs to modulate the level of polymyxin B resistance in *P. aeruginosa* CF isolates)¹⁵ in the outer membrane of each isolate. To this end, further studies are currently underway to characterize the lipid A dynamics in relation to these complex kinetic killing effects. Overall, the data from the time-kill studies corroborate the findings from the checkerboard experiments, suggesting that polymyxin B in combination with ivacaftor and lumacaftor displays synergistic killing against highly polymyxin-resistant *P. aeruginosa* CF isolates.

We also tested the activity of the combinations against the highly polymyxin-resistant CF isolate *P. aeruginosa* FADDI-065 under planktonic and biofilm growth conditions in a sputum medium assay, the latter of which is relevant to CF lung conditions (Figure 2). The polymyxin B ivacaftor/lumacaftor combinations performed exceptionally well, reducing cell viability to <2% under the planktonic and <20% under the biofilm growth conditions in the sputum medium, respectively. In comparison, polymyxin B alone was only partially active under the planktonic growth condition and completely inactive under the biofilm growth condition. Overall, these findings would suggest that combination therapy of polymyxins with either KALYDECO™ (ivacaftor) or ORKAMBI™ (ivacaftor+lumacaftor) in CF patients may potentially be effective at clearing problematic polymyxin-resistant MDR *P. aeruginosa* lung infections. Notably, a recent clinical study showed that *P. aeruginosa* culture positivity in CF patients was reduced following ivacaftor treatment; whether this was a direct result of an improvement in lung function due to the CFTR regulator activity of ivacaftor or the result of the antibacterial synergy between polymyxin and ivacaftor (many of these patients were likely co-medicated with nebulized or dry powder inhaled colistin) remains unknown.¹⁶

Antibacterial mode of action studies

The Gram-negative outer membrane is a formidable barrier, especially against hydrophobic drugs such as ivacaftor and lumacaftor. Polymyxins exert their antimicrobial action via direct interaction with the lipid A component of the LPS, which leads to disruption of this critical barrier function of the Gram-negative outer membrane⁷. Polymyxins zone into their primary cellular target, LPS through an initial electrostatic interaction between their cationic L- α , γ -diaminobutyric acid (Dab) side chains (*cf.* Figure 6B) with the negatively charged phosphate groups of the lipid A component of LPS, displacing divalent cations (Ca²⁺ and Mg²⁺) that bridge adjacent LPS molecules in the process and resulting in the disruption of the outer membrane.⁷ Therefore, the underlying molecular basis for the synergistic activity of the combination may involve the ability of polymyxin B to permeabilise the outer membrane, thereby allowing ivacaftor or lumacaftor to enter the cell and exert its antibacterial action. This hypothesis was tested and supported in the following sections.

Scanning and transmission electron microscopy (SEM and TEM) imaging of *P. aeruginosa* FADDI-PA065 cells revealed that the bacterial outer membrane structure was drastically disrupted due to the treatment with the combination of polymyxin B with ivacaftor or lumacaftor (Figure 3). This is distinctly different from the cells treated with polymyxin B (8 mg/L) alone where the wide-spread blebbing effect that is commonly associated with polymyxin exposure of Gram-negative cells was observed,¹⁷ and the cells treated with ivacaftor (8 mg/L) alone where large outer membrane protrusions were observed. Treatment with lumacaftor alone resulted in no visible outer membrane damage.

Further evidence that the combinations causes damage to the outer membrane comes from the nitrocefin assay data with the β -lactamase positive strain *P. aeruginosa* FADDI-PA065 (Figure 4A). The assay measures the periplasmic β -lactamase activity using nitrocefin, a chromogenic cephalosporin substrate analogue, which normally penetrates the outer membrane slowly.¹⁸ In the presence of outer membrane permeabilizing agents, nitrocefin can more easily permeate the outer membrane barrier. Figure 4 shows that the combinations of polymyxin B with ivacaftor and with ivacaftor-lumacaftor were more effective at permeabilizing the outer membrane than treatment with either drug *per se*. At this point we would like to emphasize that the nitrocefin assay does not correlate with the killing effect of each drug, and therefore, it is not tenable to draw comparisons with the microbiological antibacterial data. In support of this claim, the time killing kinetics data presented in Figure 1 indicate that the FADDI-065 cells are not killed over 24 h following treatment with each drug alone; at notably 2–4 fold higher concentrations than those used in the nitrocefin assay. Moreover, in light of the SEM/TEM data that clearly shows that ivacaftor causes direct damage to the outer membrane of FADDI-065, it is not surprising that we see a marked response to ivacaftor alone in the nitrocefin assay.

ROS production is purported to be a common bacterial killing mechanism for antibiotics.¹⁹ Figure 4B shows that the combination of polymyxin B with lumacaftor induced a greater increase of ROS production in *P. aeruginosa* FADDI-PA065 cells compared to treatment with each drug *per se*, seen as an increase in the fluorescence emission of the oxidative stress sensitive cell dye CellROX Green. Unfortunately, the impact of ivacaftor on ROS production could not be assayed as this compound produces a quencher effect on the detector dye CellROX Green.

Quantitative analysis of confocal images of the *P. aeruginosa* strain AH298-GFP (expressing green fluorescence protein, GFP) revealed that the combination of polymyxin B with ivacaftor or lumacaftor was more effective at producing outer membrane damage as indicated by the increased cytosolic GFP release compared to treatment with each drug *per se*, (Figure 5).

Ivacaftor has been reported to display poor direct antimicrobial activity against coagulase-negative *Staphylococcus* and methicillin-susceptible *S. aureus* (MSSA) with an MIC of ~8 mg/L.²⁰ In line with our results, these authors reported no direct activity for ivacaftor against Gram-negative bacteria such as *P. aeruginosa*.²⁰ A recent report also noted a decrease in the relative abundance of *Streptococcus* 1 (*S. mitis* group) in the airway micro biota of patients receiving ivacaftor therapy.²¹ Due to the similarity of ivacaftor's chemical structure to the

quinolones (Figure S1B), we tested whether the synergistic activity of ivacaftor was the result of a quinolone-like antimicrobial mode of action (Figure S1A). The antibacterial mode of action of the quinolone antibiotics involves the inhibition of the related DNA gyrase and topoisomerase IV enzymes which are involved in DNA replication.²² The gyrase and supercoiling topoisomerase assay data address two key mechanisms: interfacial poisoning (IFP), i.e., drugs that induce DNA cleavages and catalytic inhibitory compounds (CIC) which simply block catalytic activity of the enzyme. Figure 1A shows that ivacaftor is a relatively weak CIC, but clearly not an IFP. More to it, topoisomerase IV displayed greater sensitivity to ivacaftor, relative to DNA gyrase. The yield of kDNA products (decatenated kDNA) was noticeably reduced by 25 μ M ivacaftor (comparing the yields of decatenated kDNA minicircles). In addition, we screened human topoisomerase I and II α and found ivacaftor had essentially no inhibitory effect on either IFP or CIC (Figure S2). All controls (targeting IFP) worked well, demonstrating the validity of the negative results with ivacaftor. The lack of enzyme inhibition by ivacaftor can be construed in terms of our understanding of quinolone structure-activity relationships (SAR).²² Firstly, ivacaftor has a bulky *N*-(2,4-di-tert-butyl-5-)hydroxyphenyl substituting R₃ of the quinolone nucleus in the place of the carboxylic acid (Figure S1B). Quinolone SAR studies have shown the carboxylic acid moiety is critical for binding to the perturbed DNA, and substitutions of this group produce inactive quinolones.²² Secondly, the ivacaftor structure contains an essentially bare quinolone nucleus, which lacks the fundamental substituents at the R₁, R₆ and R₇ positions that are critical for binding to the enzyme-DNA complex.²² Taken together, our findings would suggest that that antibacterial activity of ivacaftor does not involve a quinolone-like mode of action; indeed, quinolones are strong IFP agents while ivacaftor is not (it is a weak CIC). In support of this claim, it is noteworthy to mention that the polymyxin B-ivacaftor combination was active (showing synergy or additivity) against the CW-XX *P. aeruginosa* strains that are all resistant to the fluoroquinolone antibiotic ciprofloxacin (MICs, 8–16 mg/L) (Table 1). This observation also supports the notion that that the antibacterial activity of ivacaftor involves a novel mode of action, for example, targeting either DNA binding, DNA access or essential ATPase domains. Notably, the action is targeted to the prokaryotic topoisomerases while human enzyme's appear largely unaffected (Figure S2). Selective targeting of prokaryotic activities, vs. eukaryotic enzymes, further validates at the efficacy of a combinatorial therapeutic inroad.

Conclusions

It is acknowledged that MDR Gram-negative lung infections in CF patients represent a major unmet medical need for effective therapies with minimum adverse effects. The off-label use of nonantibiotic drugs for antibacterial purposes can significantly expedite the discovery of new treatment options for MDR lung infections in this population. The findings from the present study suggest that the 'unexpected' combination of polymyxin B with the non-antibiotic CF drugs ivacaftor (KALYDECO™) or ivacaftor-lumacaftor (ORKAMBI™) could potentially prove useful for the treatment of CF lung infections caused by MDR Gram-negative pathogens that are even resistant to the last-line polymyxins. This is especially serendipitous given that both drugs are commonly used in combination in CF patients, i.e., 'kill two birds with one stone'. Moreover, the present findings suggest ivacaftor

and lumacaftor operate via a mode of action (MOA) that is different from that of the polymyxins; which makes the combination even more effective against MDR pathogens, as this scenario presents the pathogen with the difficult task of concomitantly developing resistance against two independent MOAs. Further studies are underway to assess the clinical effectiveness of the combination against MDR polymyxin-resistant *P. aeruginosa* CF lung infections.

Experimental Section

Materials

Polymyxin B (Catalogue number 81334, 6,500 IU/mg) was purchased from Sigma-Aldrich (Australia). Ivacaftor, lumacaftor and VX-661 were purchased from (SelleckChem, USA). All other reagents were purchased from Sigma-Aldrich (Australia) and are of the highest commercial grade available.

Bacterial isolates

A total of twenty two clinical isolates (sixteen polymyxin-resistant and five polymyxin-susceptible) of *P. aeruginosa*, including mucoid and non-mucoid strains, were selected from routine clinical isolates identified from patients with acute exacerbations of CF. Isolates were selected sequentially from different patients as they presented with acute exacerbations; both mucoid and non-mucoid strains came from the same patient. MIC susceptibility screening of fourteen of these CF isolates revealed that five strains were MDR being resistant to at least three of the following agents: aztreonam, ceftazidime, meropenem, piperacillin, ticarcillin, gentamicin, tobramycin, and ciprofloxacin (Table S1).²³ A polymyxin-heteroresistant reference strain, *P. aeruginosa* ATCC 27853 (polymyxin B MIC, 1 mg/L) (American Type Culture Collection, Rockville, USA) was also studied. Resistance to polymyxin B was defined as MICs of ≥ 4 mg/L. Four polymyxin-resistant (polymyxin B MICs, >128 mg/L) *Burkholderia* (*B. cepacia* and *B. pseudomallei*) strains from CF patients were employed. A total of ten *A. baumannii* strains were utilised in this study, including polymyxin-susceptible reference strain ATCC 19606 (polymyxin B MIC, 2 mg/L) and its paired polymyxin-resistant isolate ATCC 19606R (polymyxin B MIC, 64 mg/L). Six polymyxin-resistant *K. pneumoniae* isolates, a polymyxin-susceptible reference strain ATCC 13883 (polymyxin B MIC, 1 mg/L), and eight polymyxin-susceptible strains were employed. Eight clinical *S. maltophilia* isolates, including three polymyxin-susceptible strains and five polymyxin-resistant strains, were included in this study. Two polymyxin-resistant *H. influenzae* isolates were employed.

In vitro antibacterial synergy

MICs were determined in accordance with the recommendations of the Clinical and Laboratory Standards Institute.²⁴ The time-killing kinetics of polymyxin B *per se* and in combination with ivacaftor were examined against *P. aeruginosa* ATCC 27853 (polymyxin B MIC, 1 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC, >32 mg/L), FADDI-PA064 (polymyxin B MIC, >128 mg/L; ivacaftor MIC, 4 mg/L; lumacaftor MIC, >32 mg/L), FADDI-PA065 (polymyxin B MIC, >128 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC, >32 mg/L) and FADDI-PA070 (polymyxin B MIC, 64 mg/L; ivacaftor MIC, >32 mg/L;

lumacaftor MIC, >32 mg/L). Aliquots (~20 μ L) of early log-phase bacterial suspensions were inoculated into sterile 50 mL glass tubes (Greiner Bio-one, Germany) containing 20 mL of cation-adjusted Mueller-Hinton broth (CaMHB, adjusted with 23 mg Ca^{2+} /L and 12.2 mg Mg^{2+} /L), DMSO (2.5%, v/v) and different concentrations of polymyxin B and ivacaftor. Colonies were counted by a ProtoCOL automated colony counter (Synbiosis, United Kingdom) after the incubation of subcultures for 24 h at 35°C. The detection limit was 20 CFU/mL. The definitions for synergism, additivity, indifference and antagonism are foot-noted in Table 1.

Topoisomerases and gyrase enzyme assays

Purified *E. coli* DNA gyrase, *E. coli* topoisomerase IV, human topoisomerase I and human topoisomerase II α were obtained from TopoGEN, Inc. (Buena Vista, CO, USA). These enzymes were purified to >98% purity as judged by over-loading SDS Polyacrylamide gels. Assays for type II enzymes (DNA gyrase, topoisomerase IV and topoisomerase II α) were based on decatenation of kinetoplast DNA (kDNA), which was also purchased from TopoGEN, Inc. The kDNA substrate is composed of a network of interlocked 2.5 kilobase minicircular DNA rings. These minicircular kDNA networks (catenanes) are ideal type II topoisomerase substrates since separation of the large molecular weight kDNA networks from the much smaller 2.5 kilobase minicircles is easily and quickly achieved by agarose gel electrophoresis.^{25, 24, 25} Decatenation assays contained 200 ng of kDNA and 8 units of enzyme (1 unit decatenates 200 ng kDNA in 30 min at 37°C). DNA gyrase assays were carried out in a 1 \times gyrase assay buffer (35 mM Tris-HCl, pH 7.5, 24mM KCl, 4 mM MgCl_2 , 2 mM dithiothreitol, 1.8 mM spermidine, 1 mM ATP, 6.5% (v/v) glycerol and Bovine serum albumin (BSA, 0.1 mg/mL). Topoisomerase IV assays contained 20 mM Hepes-KOH (pH7.6), 50 mM potassium glutamate, 5 mM magnesium acetate, 5mM dithiothreitol, BSA (25 μ g/mL). Topoisomerase II α assays were performed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM MgCl_2 , 0.5 mM dithiothreitol, BSA (30 μ g/mL). Reactions were assembled on ice (20ul final volume) and initiated by adding enzyme. After incubating for 30 min at 37°C, reactions were terminated with SDS (1% w/v final), digested with proteinase K (50 μ g/mL 15 min at 60°C) and following addition of loading (0.2 vol of 0.125% bromophenol blue, 25% glycerol) samples were loaded on 1% agarose gels containing 0.5 μ g/mL ethidium bromide in the gel and running buffer. After electrophoresis (45 min at 3 volts/cm), gels were destained with water (15 min wash) and photographed. Assays for type I enzymes (human topoisomerase I) were based on relaxation of supercoiled DNA (pHOT1, from TopoGEN, Inc.).²⁶ Reactions (20 μ L volume) contained 100 ng of pHOT1 plasmid, and 8 units of topoisomerase I (1 unit will relax 100 ng in 30 min at 37°C) in TGS buffer (10 mM Tris-HCl (pH 7.9), 1 mM EDTA, 150 mM NaCl, 0.1% BSA, 0.1 mM Spermidine, 5% glycerol. Reactions were incubated for 30 min at 37°C, terminated with SDS (1% final) and mixed with a leading dye (0.1 vol of 0.25% bromophenol blue, 50% glycerol) before loading onto agarose gels. Two gel systems were used for this analysis; half of the reaction volume was loaded onto each gel. One gel contained 0.5 μ g/mL ethidium bromide (in gel and running buffer) which is optimal for resolving topo I cleavage products (nicked open circular DNA). The second gel lacked ethidium bromide is allows clear separation of supercoiled DNA and relaxed DNA. The former gel is designed optimally to identify drugs that act as an interfacial poison, inducing DNA cleavages (like camptothecin)

while the latter gel system is optimal for detecting relaxation activity and drugs that inhibit enzymatic activity.

Imaging methods

Scanning and transmission electron microscopy were performed as we have previously described.²⁷ Confocal imaging of the release of cytosolic green fluorescence protein (GFP) release was performed using the *P. aeruginosa* strain Pa AH298-GFP which expresses GFP under the control of the growth-rate dependent *rrnBp₁* promoter, kindly provided by Professor Philip S. Stewart.²⁸ Pa AH298-GFP cells were grown on chamber slides and treated with polymyxin (8 mg/L), ivacaftor (8 mg/L) and lumacaftor (8 mg/L) alone or in combination of polymyxin-ivacaftor and polymyxin-ivacaftor-lumacaftor for 1 h at 37°C. Cell morphology was assessed using an LSM780 confocal microscope (Zeiss) equipped with a 63 × oil objective, excitation/emission filters used were Ex/Em 475/520–560 nm. NIH ImageJ software was used to for quantitative data analysis. Per slide (23.065 × 23.065 micron) 120–130 bacterial cells were counted. The percentage of GFP release was calculated as %GFP release=untreated control cells/treated cells × 100%.

Nitrocefin assays

The outer membrane permeabilizing activity of the test compounds was assessed by the β-lactamase nitrocefin assay. Briefly, the β-lactamase positive *P. aeruginosa* strain FADDI-PA065 was sub-cultured on a nutrient agar and incubated at 35°C overnight. One colony was randomly selected and grown overnight in 10 mL CAMBH at 37°C. The culture was grown to exponential phase and harvested by centrifugation (10000 × *g*, 10 min). The pellet was washed twice in PBS, pH 7.2 and resuspended in PBS to an OD=0.50. Nitrocefin was prepared to a concentration of 2 mM in DMSO. The assay was performed in 96-well plates. The 200 μL reaction mixture consisted of 100 μL bacterial suspension, 96 μL drug (2 mg/L polymyxin B alone or in combination with 4 mg/L ivacaftor) and 4 μL of 2 mM nitrocefin solution. The absorbance (OD 495 nm) was kinetically measured at room temperature for every minute. The values reported are the mean of four independent measurements ± standard deviation (SD).

Artificial sputum media (ASM) biofilm assay

The ASM assays were performed with *P. aeruginosa* strain FADDI-PA065 in a 24-well plate format. ASM culture medium ideal mimics the components of CF patient sputum, and includes f egg yolk, amino acids, mucin and DNA combined in ratios as described in detail by Kirchner *et al.*, prepared fresh and filter sterilized.^{18, 29} *P. aeruginosa* strain FADDI-065 was sub-cultured on a nutrient agar and incubated at 35°C overnight and one colony was randomly selected and grown overnight in 10 mL CAMBH at 37°C. The overnight culture was diluted in CAMBH to an OD of 0.05±0.01 and then further diluted 1:100 in fresh ASM. A total volume of 1.8 mL was added to each well. Plates were secured with parafilm and incubated for 3 days under aerobic conditions at 37°C in which the *P. aeruginosa* biofilms were developed. After 3 days polymyxin B, ivacaftor, and lumacaftor, alone or in combination were added and further incubated for 24 hours. For each antibiotic concentration four replicates were conducted. After the 24 h incubation, biofilms were disrupted with 100 μL of 100 mg/mL, cellulase (diluted in 9.6 g/L citrate; pH adjusted to 4.6

with NaOH) and incubated for 1 h at 37°C. Biofilms were further manually disrupted by pipetting. To determine the cell viability 100 μ L of 0.02% (v/v) resazurin was added to each well and further incubated for 2 h at 37°C. Fluorescence (F) of each well was measured using an ENVISION plate reader (PerkinElmer, Australia) set at an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Fluorescence was corrected by the subtraction of the background noise. The cell viability was calculated as (mean F of treated biofilms/mean F untreated control) \times 100%.

Detection of cellular ROS production

ROS production in exponential phase *P. aeruginosa* FADDI-PA065 cells (OD 600 nm 0.1) following antibiotic treatment were measured using the oxidative stress sensitive dye CellROX Green (Lifetechnologies, Australia). CellROX Green fluorescence emission was measured at an excitation wavelength of 485 nm on a Cary Eclipse Fluorescence spectrophotometer (Varian, Australia) set. The emission spectrum was collected between 490–600 nm and slit widths were set to 5 nm for both the excitation and emission monochromators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ASM	Artificial Sputum Media
CAMBH	Cation adjusted Muller Hinton Broth
MIC	minimum inhibitory concentration
CFU	colony-forming units
GFP	green fluorescence protein
MOA	mode of action
ROS	reactive oxygen species

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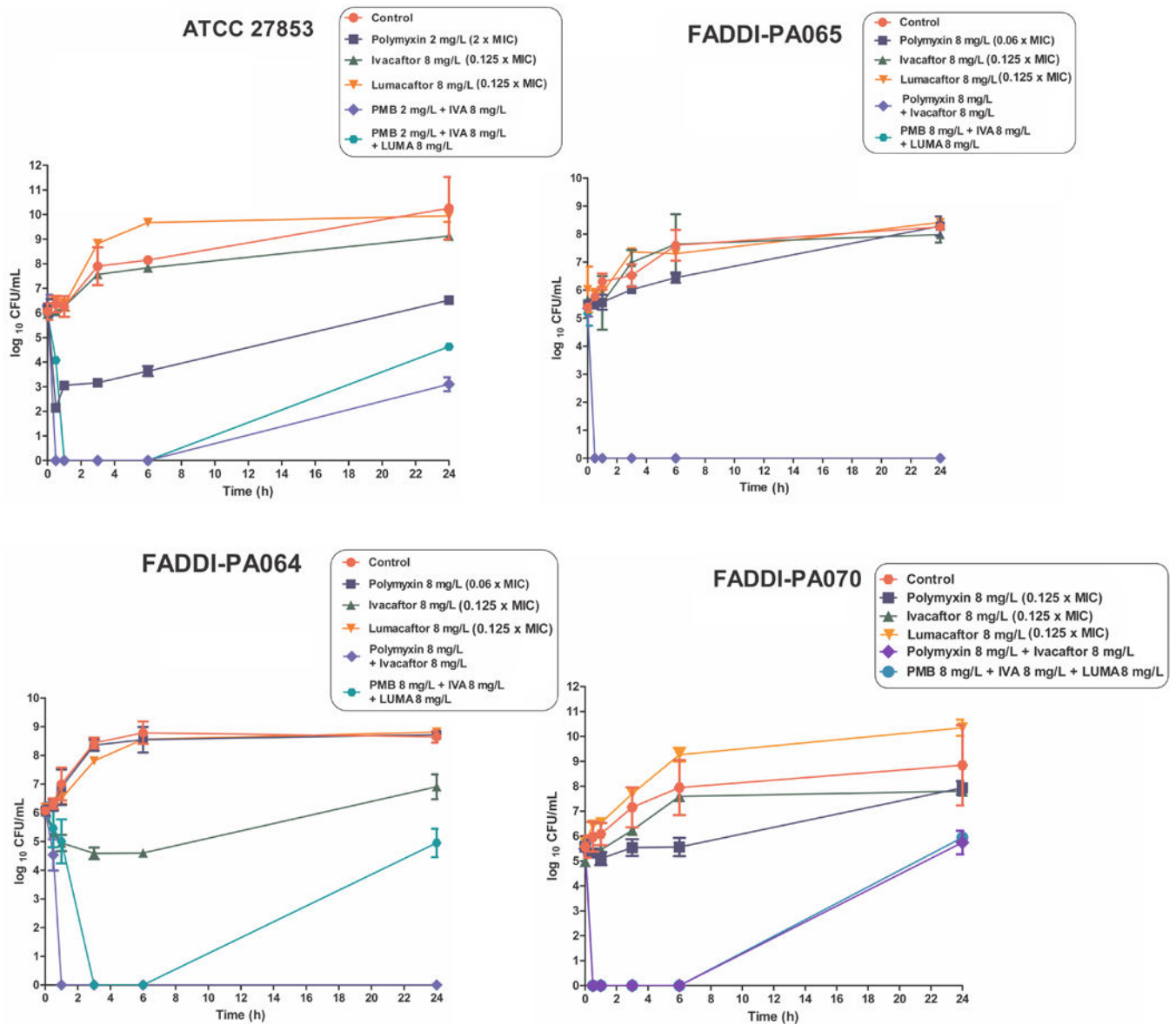


Figure 1.

Time-kill curves for polyxymyxin B, ivacaftor, lumacaftor alone and in combinations against three polyxymyxin-resistant *P. aeruginosa* CF strains FADDI-PA064 (polyxymyxin B MIC, >128 mg/L; ivacaftor MIC, 4 mg/L; lumacaftor MIC, >32 mg/L), FADDI-PA065 (polyxymyxin B MIC, 128 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC, >32 mg/L), FADDI-PA070 (polyxymyxin B MIC, 64 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC, >32 mg/L) and against a polyxymyxin-susceptible *P. aeruginosa* ATCC 27853 strain (polyxymyxin B MIC, 1 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC, >32 mg/L). Data points are plotted as the mean \pm SD of three independent measurements.

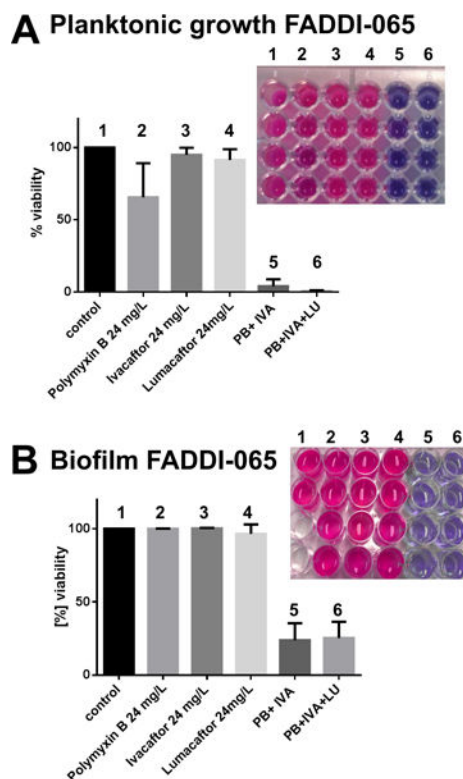


Figure 2.

Antimicrobial activity of polymyxin B (24 mg/L=0.18×MIC), ivacaftor (24 mg/L=0.75×MIC), lumacaftor (24 mg/L=0.75×MIC) alone and in combinations against the polymyxin-resistant *P. aeruginosa* CF strain FADDI-PA065 (polymyxin B MIC=128 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC, >32mg/L) in artificial sputum media under (A) planktonic and (B) biofilm growth conditions. Data points are plotted as the mean ± SD of four independent measurements. The top right-hand corner inset graphics in each bar graph show the raw data from the resazurin fluorescence plate assay; the numbering 1–6 corresponds to the numbering for each condition in the adjacent bar graph. The replicates for each condition are organized vertically in the 96-well plate. The redox sensitive dye resazurin (blue, indicates dead cells) undergoes reduction to resorufin (pink) by NADH from metabolically active (live) cells.

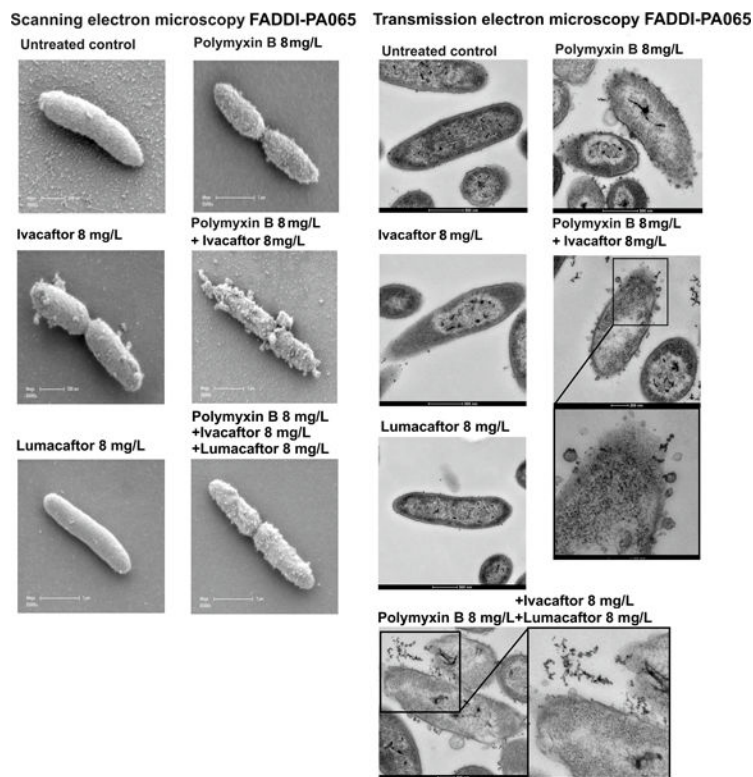


Figure 3. Electron microscopy images of the polymyxin-resistant *P. aeruginosa* CF isolate FADDI-PA065 (polymyxin B MIC, 128 mg/L; ivacaftor MIC, >32 mg/L; lumacaftor MIC > 32 mg/L) treated with polymyxin B, ivacaftor, lumacaftor alone and in combinations.

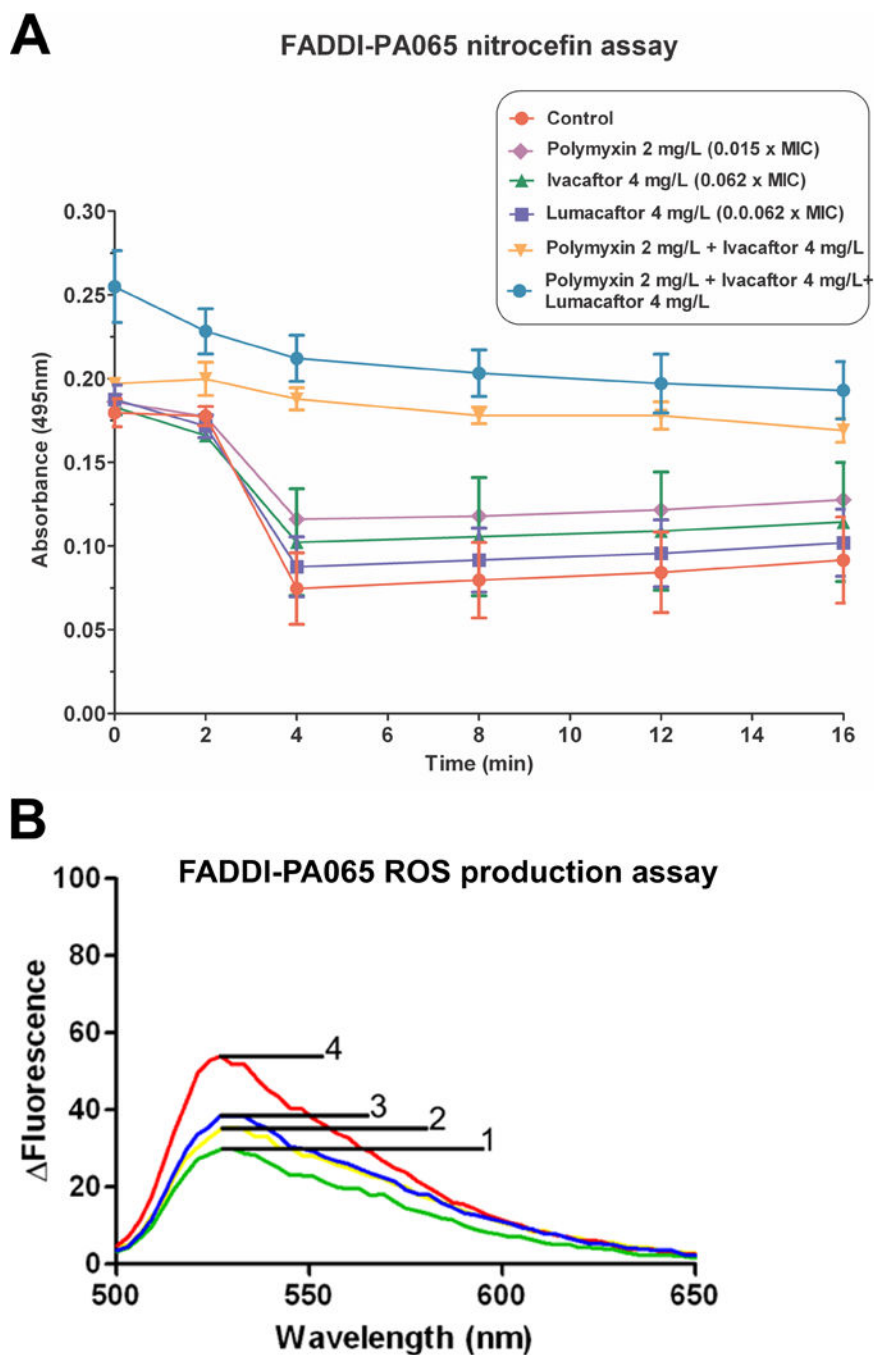


Figure 4. (A) Outer membrane permeabilizing activity of polymyxin B, ivacaftor, lumacaftor alone and in combinations against *P. aeruginosa* FADDI-PA065 measured by the nitrocefin assay. Data points are plotted as the mean \pm SD of four independent measurements. (B) ROS production in *P. aeruginosa* FADDI-PA065 cells following treatment measured by the increased fluorescence emission of the oxidative stress sensitive dye CellROX Green. Treatments: 1, untreated cells; 2, lumacaftor 4 mg/L; 3, polymyxin B 2 mg/L; 4, Combination of polymyxin B 2 mg/L+ lumacaftor 4 mg/L.

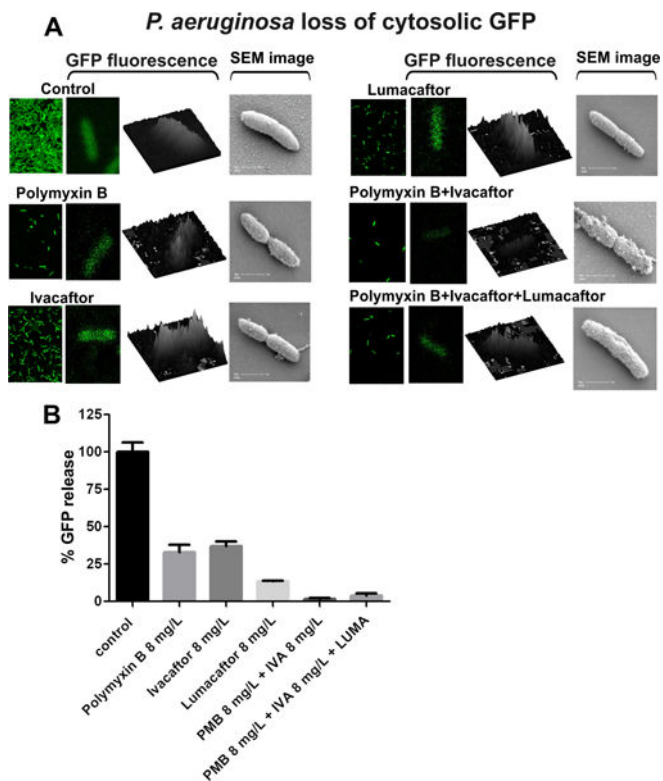


Figure 5. (A) Confocal microscopy images of cytosolic GFP release from *P. aeruginosa* AH298-GFP treated with polymyxin B, ivacaftor, lumacaftor alone and in combinations. The corresponding densitometric analysis and scanning electron microscopy images of the confocal imaged cells are shown. (B) Bar graph showing the % GFP released following drug treatment relative to the untreated control.

Table 1

The antimicrobial activity of polymyxin B (PMB), ivacaftor (IVA), lumacaftor (LUMA), VX-661 alone and in combination.

Bacterial isolate	PMB MIC (mg/L)	IVA MIC (mg/L)	PMB + IVA FIC index ^b	EFFECT	LUMA MIC (mg/L)	PMB + LUMA FIC index ^b	EFFECT	VX-661 MIC (mg/L)	PMB + VX-661 FIC Index ^b	EFFECT
Polymyxin resistant isolates										
<i>P. aeruginosa</i>	>128	4	0.12	synergy	>32	2	Indifference	>32	2	indifference
FADDI-PA064	128	>32	0.18	synergy	^a NA	^a NA		^a NA	^a NA	
FADDI-PA069	128	>32	0.37	synergy	>32	2	indifference	>32	0.75	additivity
FADDI-PA065	>128	>32	0.56	additivity	>32	2	indifference	>32	1	indifference
FADDI-PA068	128	>32	0.75	additivity	^a NA	^a NA		^a NA	^a NA	
FADDI-CW45	128	>32	0.75	additivity	^a NA	^a NA		^a NA	^a NA	
FADDI-CW32	128	>32	0.75	additivity	^a NA	^a NA		^a NA	^a NA	
FADDI-CW22	128	>32	0.75	additivity	^a NA	^a NA		^a NA	^a NA	
FADDI-PA072	64	>32	0.12	synergy	^a NA	^a NA		^a NA	^a NA	
FADDI-PA070	64	>32	0.26	synergy	>32	2	indifference	>32	1	indifference
FADDI-PA066	64	>32	0.50	additivity	>32	2	indifference	>32	2	indifference
FADDI-CW05	64	>32	0.75	additivity	^a NA	^a NA		^a NA	^a NA	
FADDI-CW28	32	>32	0.13	synergy	^a NA	^a NA		^a NA	^a NA	
FADDI-PA067	16	>32	0.26	synergy	^a NA	^a NA		^a NA	^a NA	
FADDI-PA006	8	>32	0.75	additivity	^a NA	^a NA		^a NA	^a NA	
FADDI-PA090	4	>32	0.50	additivity	>32	0.53	additivity	>32	0.75	additivity
FADDI-PA014	4	>32	0.28	synergy	^a NA	^a NA		^a NA	^a NA	
Polymyxin susceptible isolates										
ATCC 27853	1	>32	0.50	additivity	>32	2	indifference	>32	0.75	additivity
FADDI-CW42	0.5	>32	0.28	synergy	>32	1.25	indifference	>32	1	indifference
FADDI-PA021	0.5	>32	0.56	additivity	>32	2	indifference	>32	0.75	additivity
FADDI-CW13	0.5	>32	0.53	additivity	>32	2	indifference	>32	2	indifference
FADDI-CW35	0.25	>32	0.51	additivity	>32	0.51	additivity	>32	2	indifference
FADDI-CW34	0.25	>32	0.53	additivity	>32	2	indifference	>32	2	indifference

Bacterial isolate	PMB (mg/L)	IVA MIC (mg/L)	PMB + IVA FIC index ^b	EFFECT	LUMA MIC (mg/L)	PMB + LUMA FIC index ^b	EFFECT	VX-661 MIC (mg/L)	PMB + VX-661 FIC Index ^b	EFFECT
Polymyxin resistant isolates										
<i>B. pseudomallei</i>										
	>256	>32	2	indifference	>32	2	indifference	>32	2	indifference
	>256	>32	2	indifference	>32	2	indifference	>32	2	indifference
	>256	>32	2	indifference	>32	2	indifference	>32	2	indifference
<i>B. cepacia</i>										
	>128	>32	1	indifference	>32	2	indifference	>32	2	indifference
Polymyxin resistant isolates										
<i>A. baumannii</i>										
	>8	>32	0.28	synergy	>32	0.62	additivity	>32	1	indifference
	>8	>32	0.62	additivity	>32	2	indifference	>32	2	indifference
	8	>32	0.25	synergy	>32	2	indifference	>32	1	indifference
	>4	>32	1	indifference	>32	2	indifference	>32	2	indifference
	4	>32	0.25	synergy	>32	2	indifference	>32	1	indifference
Polymyxin susceptible isolates										
	2	>32	0.50	additivity	>32	0.25	synergy	>32	0.62	additivity
	1	>32	0.52	additivity	>32	0.50	additivity	>32	1	indifference
	0.5	>32	0.50	additivity	>32	0.51	additivity	>32	0.53	additivity
	0.5	>32	0.53	additivity	>32	0.53	additivity	>32	1	indifference
	0.125	>32	0.50	additivity	>32	0.53	additivity	>32	0.62	additivity
Polymyxin resistant isolates										
<i>K. pneumoniae</i>										
	>128	>32	0.62	additivity	>32	2	indifference	>32	2	indifference
	128	>32	0.18	synergy	>32	0.5	additivity	>32	0.75	additivity
	>8	>32	0.25	synergy	>32	2	indifference	>32	2	indifference
	>8	>32	1	indifference	>32	2	indifference	>32	2	indifference
	>8	>32	1	indifference	>32	2	indifference	>32	2	indifference
	>8	>32	1.3	indifference	>32	2	indifference	>32	2	indifference
Polymyxin susceptible isolates										
	2	>32	0.28	synergy	>32	0.51	additivity	>32	0.62	additivity
	2	>32	0.50	synergy	>32	0.51	additivity	>32	0.50	additivity

Bacterial isolate	PMB MIC (mg/L)	IVA MIC (mg/L)	PMB + IVA FIC index ^b	EFFECT	LUMA MIC (mg/L)	PMB + LUMA FIC index ^b	EFFECT	VX-661 MIC (mg/L)	PMB + VX-661 FIC Index ^b	EFFECT	
FADDI-KP033	1	>32	0.25	synergy	>32	2	indifference	>32	0.75	additivity	
FADDI-KP033	1	>32	0.25	synergy	>32	2	indifference	>32	0.75	additivity	
FADDI-KP015	0.5	>32	0.31	synergy	>32	2	indifference	>32	0.50	additivity	
ATCC 13883	0.25	>32	0.52	additivity	>32	0.50	additivity	>32	0.75	additivity	
FADDI-KP081	0.25	>32	0.56	additivity	>32	0.25	synergy	>32	0.62	additivity	
FADDI-KP077	0.25	>32	0.62	additivity	>32	0.50	additivity	>32	0.75	additivity	
Polymyxin resistant isolates											
<i>S. maltophilia</i>	>8	>32	0.50	additivity	>32	1	indifference	>32	0.52	additivity	
FADDI-SM008	>8	>32	0.50	additivity	>32	0.5	additivity	>32	2	indifference	
FADDI-SM006	8	>32	0.18	synergy	>32	0.5	additivity	>32	0.38	synergy	
FADDI-SM007	8	>32	0.50	additivity	>32	0.75	additivity	>32	1	indifference	
FADDI-SM002	4	>32	0.62	additivity	>32	0.62	additivity	>32	1	indifference	
Polymyxin susceptible isolates											
FADDI-SM004	2	>32	0.25	synergy	>32	1	indifference	>32	0.75	additivity	
FADDI-SM003	1	>32	0.37	synergy	>32	0.62	additivity	>32	1	indifference	
FADDI-SM005	0.25	>32	0.37	synergy	>32	0.75	additivity	>32	1	indifference	
Polymyxin resistant isolates											
<i>H. influenzae</i>	>16	>128	2	indifference	>128	1	indifference	>128	0.75	additivity	
FADDI-HI003	>16	>128	2	indifference	>128	1	indifference	>128	0.75	additivity	

^aNA, not accessed^bFICINDEX = (MIC polymyxin B in combination with ivacaftor/MIC polymyxin B alone) + (MIC ivacaftor in combination with polymyxin B/MIC ivacaftor alone); Synergy FIC<0.5; Additivity FIC=0.5–1.0; Indifference FIC=1–4; Antagonism FIC >4.