RANKING OF MAJOR CLASSES OF ANTIBIOTICS FOR ACTIVITY AGAINST STATIONARY PHASE GRAM-NEGATIVE BACTERIA PSEUDOMONAS AERUGINOSA AND CARBAPENEMASE-PRODUCING KLEBSIELLA PNEUMONIAE AND IDENTIFICATION OF DRUG COMBINATIONS THAT ERADICATE THEIR PERSISTENT INFECTIONS

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ABSTRACT

From the earliest identification of different bacterial phenotypic states, researchers found under antibiotic exposure, there are some bacteria that can keep dormant in a non-growing state as persister cells. These dormant persister bacteria can revert back to the growing population when the antibiotics are removed. The formation of bacterial persister cells establishes phenotypic heterogeneity within a bacterial population and is important for increasing the chances of successfully adapting to environmental change. Persister cells were first discovered in *Staphylococcus sp.* in 1944 when penicillin failed to kill a small subpopulation of bacterial cells. Persisters exhibit temporary antibiotic-tolerant phenotype and the underlying mechanisms involved in the induction and regulation of persister cells formation have been investigated by the previous lab members regarding mechanisms of persistence in *Borrelia burgdorferi* and with Yin-Yang Model to illustrate persistent infection. This investigation focuses on the optimal treatment for persistent infection. Because current treatments for such chronic persistent infections are not effective and antibiotic phenotypic resistance is a significant issue.

The discovery of antibiotics and their widespread use represent a significant milestone in human history since the 20th century. However, their efficacy has declined at an alarming rate due to the spread of antibiotic resistance, and persistence and the evidence is accumulating that persister cells can contribute to the emergence of antibiotic resistance. Effective treatments for bacterial persistent infections can greatly improve patient outcome. A comprehensive overview

of anti-persister treatments suggests that development of drug combination treatments may represent a useful therapeutic approach. A typical drug for treating tuberculosis persistent infection includes pyrazinamide (PZA) which is combined with rifampin and isoniazid which kill growing bacteria. PZA is an anti-persister drug that inhibits unconventional drug targets such as proteins involved in energy metabolism and trans-translation. PZA in this triple drug combination therapy demonstrates a strong activity against persister cells, and based on this principle, we screened for pesister drugs like PZA and ranked drugs from six typical classes of antibiotics for their activity against non-growing *Pseudomonas aeruginosa*. Based on the ranking results, we formulated drug combinations that can effectively kill the heterogeneous population of Gram-negative bacteria in biofilm model. To further evaluate the activity of drug combinations in a relevant mouse model, we established a chronic pulmonary murine infection model. We found that consistent with our findings *in vitro*, the drug combinations are more effective against the persistent lung infection than the current standard of care treatment. Finally, we conclude that drug combinations consisting of drugs targeting both actively growing bacteria and non-growing persister cells can eradicate the Gram-negative bacteria biofilm related chronic infections. These findings lay the groundwork for possible improved treatment of persistent infections in the clinic.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

DRUG COMBINATIONS TARGETING GROWING AND PERSISTER

CELLS ERADICATE CHRONIC PSEUDOMONAS AERUGINOSA

INFECTION

Introduction

Clinical Significance of Pseudomonas aeruginosa

Pseudomonas aeruginosa is a hardly eradicated, opportunistic Gram-negative bacterium [1] that causes serious infections in hospitalized patients or people with compromised immune systems. Patients with burn wounds, cystic fibrosis, acute leukemia, organ transplants, and intravenous-drug users are at high risk for infections [2]. Because *P. aeruginosa* causes many nosocomial infections, epidemics within the hospitals have been reported [3, 4]. *P. aeruginosa* is a major pathogen in the cystic fibrosis (CF) lung, and it causes a persistent infection that cannot be eradicated by even the most aggressive antibiotic therapy [5]. This has been attributed to bacterial biofilms which are resistant or tolerant to antibiotic treatments and can evade host immune defense [6].

Treatment of *P. aeruginosa Infections* and persisters

Based on the Johns Hopkins Antibiotics Guide [7], high doses of synergistic antibiotic combinations (β-lactam + aminoglycoside) may improve outcomes of serious *P. aeruginosa* infections in immunocompromised hosts clinically and these combinations were determined against *Pseudomonas aeruginosa* from the sputum of patients with cystic fibrosis [8]. For multidrug resistant strains, colistin can be added to the above treatment [9]. Due to the increased

antibiotic resistance, the repertoire of effective agents against *P. aeruginosa* is limited [10], and thus better therapies are needed.

Bacterial cells may escape the effects of antibiotics due to epigenetic changes; these cells are known as persisters [11]. Many chronic infections are associated with the ability of the bacteria to predominantly colonize body surfaces and tissues as multicellular aggregates such as biofilms [5, 12]. Formation of these sessile communities and their inherent resistance to antimicrobial agents are at the root of many persistent and chronic bacterial infections. The number of persisters in a growing population of bacteria rises in mid-log and reaches a maximum of approximately 1% at stationary state [13]. Similarly, slow-growing biofilms produce substantial numbers of persisters. The ability of a biofilm to limit the access of the immune system components, and the ability of persisters to sustain an antibiotic attack could then account for the recalcitrance of such infections *in vivo* and can cause frequent relapse after treatment.

The Yin-Yang Model of Persistence

To better describe a dynamic and complex heterogeneous bacterial population consisting of growing (Yang) and non-growing persister cells (Yin) that are in varying growth and metabolic states in continuum [14]. There are small numbers of Yin bacterial population called persisters in the growing Yang bacterial population, and persisters can cause latent or persistent infections and there are Yang growing bacterial population in Yin non-growing bacterial cells which can cause active disease at the host level. This Yin-Yang model can also be attributed to the

heterogeneous population in a biofilm model because persisters can adopt varying sizes and shapes from regular morphology to altered morphologies as found in biofilms and L-form bacteria [15-17]. A typical persistent form of *P. aeruginosa* is mucoid biofilms in CF lung colonization. The biofilm form is a rather complex continuum with growing and dormant states that can be described and simplified using the Yin-Yang model [14].

Mechanisms of Persisters and Biofilm Forms of *P. aeruginosa*

The differentiation or maturation of *P. aeruginosa* biofilms *in vitro* depends on intercellular signaling systems or quorum sensing (QS) [18, 19]. QS systems in many Gram-negative bacteria rely on acylated homoserine lactones (AHLs), which are produced at high levels when cell density is high and act as ligands for transcriptional regulators. *P. aeruginosa* has been shown to form biofilm-like microcolonies in the lungs of CF patients and researchers found that quorum sensing signals found in patients' lungs were only produced by isolated strains when they were grown in biofilms [20, 21]. These findings suggest that *P. aeruginosa* forms biofilms in the CF lung, possibly explaining the difficulty of treating this hardly-eradicated chronic infection.

An optimized SYBR Green I/PI Assay for Rapid Viability Assessment for *P. aeruginosa*

The most common method to assess the killing activity of antibiotics against stationary phase bacteria is through counting of viable cells grown on agar plates in colony forming unit (CFU) assay. A major disadvantage of CFU counting is the lengthy time (1-3 days) for bacteria

to grow on agar plates and CFU counting may miss the subpopulation of viable but non-culturable bacteria that do not form CFUs. To more rapidly quantify the amount of live cells after drug treatment, we used a SYBR Green I/Propidium Iodide (PI) viability assay [22] which was developed to evaluate antibiotic susceptibility and to perform high-throughput drug screens in *B. burgdorferi* [23]. SYBR Green I is a high affinity dye that binds double-stranded DNA (dsDNA) and is commonly used to stain nucleic acids in PCR and flow cytometric analysis [24-26]. SYBR Green I is a permeable dye that stains all live cells green, whereas PI is an impermeable dye that stains dead or damaged cells with compromised cell membrane red [27]. Therefore, fluorescence microscopy or fluorescence microplate readers can be used to measure the live/dead ratio of a bacterial sample as a rapid method to assess bacterial viability after drug treatment without CFU counts. Here, we applied the SYBR Green I/PI viability assay to *P. aeruginosa* and ranked six major classes of antibiotics in killing growing and stationary phase non-growing forms of *P. aeruginosa* compared with conventional CFU based viability assay. Using these methods, we also identified drug combinations that could more effectively eradicate persisters in stationary phase culture of *P. aeruginosa* as well as in biofilms *in vitro* and more importantly, in a persistent lung infection mouse model *in vivo*.

Materials and Methods

Bacterial strain, culture media and growth condition

P. aeruginosa strain PAO-1 was obtained from Colin Manoil Lab, University of Washington, Seattle, WA. *P. aeruginosa* strain was cultured in tryptic soy broth (TSB) and tryptic soy agar (TSA) from Becton Dickinson (Franklin Lakes, NJ, USA) at 37 ℃. The culture was incubated at 37 ℃ with shaking at 200 rpm. After overnight incubation, stationary phase cultures were exposed to different antibiotics and incubated at indicated times before washing and plating on TSA plates for colony forming unit (CFU) enumeration. The plates were incubated at 37 ℃ incubator overnight.

Preparation of antibiotics

Stock solutions of antibiotics Cefuroxime, Colistin, Gentamicin, Clinafloxacin, Rifampicin, Sulfamethoxazole and Nitrofurantoin (Sigma Aldrich Co.) were prepared in the laboratory, filter-sterilized and with appropriate solvents at 10 mg/ml. Each antibiotic (25 μM) was added into 500 μL of stationary phase culture of *P. aeruginosa* and was incubated at 37 ℃ without shaking for various times. In addition, appropriate concentrations of the antibiotics were used for MIC determination using the microdilution method in 96-well plates (see below).

Drug exposure assay by CFU counting on plates

Viable bacterial cells were determined by CFU count after drug exposure at days 2, 4, 6 and 10 as described [28]. First, bacterial suspensions (50 µL) after drug exposure were washed with fresh TSB medium twice and serial dilutions were prepared. 10 µL of each dilution was dropped onto TSA plates in triplicate followed by incubation at 37 ℃ overnight. The CFU/mL was calculated accordingly.

SYBR Green I/PI assay for *P. aeruginosa*

The staining dyes were prepared by mixing SYBR Green I/PI (10,000X stock, Invitrogen) with propidium iodide (20 mM, Sigma-Aldrich) in distilled water and the ratio of SYBR Green I to propidium iodide was 1:3 in 100 µl distilled H_2O [23]. The drug treated samples were aliquoted and diluted 1:50 with fresh TSB medium. The SYBR Green I/PI dye mix (10 µL) was added to each 100 µL of sample. Each sample was vortexed and incubated at room temperature in the dark for 20 minutes. After incubation, each sample was transferred into 96-well plate. With excitation wavelength of 485 nm and 538 nm and 612 nm for green and red emission, respectively, the green and red fluorescence intensity was determined for each sample using a Synergy H1 microplate reader by BioTek Instruments (VT, USA). To generate the regression equation and regression curve of the relationship between the percentage of live bacteria and green/red fluorescent ratios, different proportions of live and 70% isopropyl alcohol killed dead cells were prepared (0:10, 1:4, 5:5, 4:1, 10:0) and both live and dead samples were diluted 50 times with fresh TSB medium first. Each proportion of live/dead *P. aeruginosa* was mixed with

SYBR Green I/PI dye into each well of 96-well plate and the green/red fluorescence ratios were measured as described above, generating standard curve and equation with least-square fitting analysis. We used this equation to calculate the percentage of live cells of *P. aeruginosa*. Fluorescence microscopy imaging visualizing live and dead cells was performed using a Keyence BZ-X710 Fluorescence Microscope and was processed by BZ-X Analyzer provided by Keyence (Osaka, Japan).

The MIC (minimum inhibitory concentration) determination

The standard microdilution method was used to determine the MIC of each antibiotic as described [29-31]. *P. aeruginosa* (1×10^9 CFU/mL) was inoculated (10 µL) into each well of 96-well plate containing 90 µL fresh TSB medium per well. Then each antibiotic was added into the well and the serial dilutions of drug treatment were made from 16, 8, 4, 2, 1 and 0 µg/mL. All experiments were run in duplicate or triplicate. The 96-well plate was incubated in 37 ℃ incubator overnight. The MIC is the lowest concentration of the antibiotic that prevented visible growth of *P. aeruginosa*.

***P. aeruginosa* biofilm preparation**

The *P. aeruginosa* biofilm model was prepared based on the protocol as described [32]. We first inoculated *P. aeruginosa* PAO-1 strain in a 5 mL fresh TSB culture and let it grow to stationary phase overnight. Then this stationary phase culture was diluted 1:100 in fresh TSB

medium, and 100 µL diluted culture was pipetted into each well of 96-well plate and then the covered plate was put into 37 ℃ incubator overnight and *P. aeruginosa* biofilm attached on the bottom of the plate after removing the supernatant medium by pipetting on the side of the well while leaving the biofilm formed at the bottom of the plate intact.

Drug combination assay on *P. aeruginosa* stationary phase persister model and biofilm model *in vitro*

Based on the Johns Hopkins Hospital Antibiotics Guideline [7], using high doses of synergistic antibiotic combination (β-lactam + aminoglycoside) is considered to be able to improve outcomes of serious *P. aeruginosa* infections in immunocompromised hosts [33]. As for the multi-drug resistant Gram-negative bacterial strains, colistin can be added to the above double combination [9]. The fluoroquinolones are broad-spectrum antibiotics with particular activity against gram-negative organisms, especially *Pseudomonas aeruginosa* [34]. But the commonly used ciprofloxacin and levofloxacin always cause increasing antibiotic resistance [7], so they are not recommended as empiric monotherapy in serious *P. aeruginosa* infection. Thus, we tried to investigate the function of some new fluoroquinolone derivatives like clinafloxacin. We evaluated Clinafloxacin (1.5 µg/mL) in combination with Cefuroxime (Cmax: 5 µg/mL), Gentamicin (Cmax: 10 µg/mL) and Colistin (Cmax: 5 µg/mL) separately. Colistin was replaced by Clinafloxacin in the clinically used triple drug combination Cefuroxime + Gentamicin + Colistin. The designed drug combinations or their single or two drug controls were added

directly to stationary phase culture and CFU count was performed after 2 day and 4 day drug treatment. For the biofilm model, the designed drug combinations were prepared with MOPS buffer (1X) (diluted from 10X MOPS from Sigma-Aldrich) to the final drug concentration and then transferred in to 96-well plate with *P. aeruginosa* biofilm attached to the bottom. Biofilm was washed with phosphate buffered saline (PBS) before plating for CFU count.

Preparation of inoculum for infection of mice

P. aeruginosa PAO-1 was cultured in 5 mL TSB at 37 ℃ with shaking overnight. The culture was centrifuged 2,700 x g for 15 minutes at 4 ℃ and the bacterial cell pellet was resuspended in 1 ml PBS. To create a persistent infection of the lung, the concentrated bacterial culture was mixed with 9 ml of liquid TSA pre-equilibrated at 50 ℃, and was mixed with heavy mineral oil to embed bacteria in agar beads to be used for the infection, using the procedure as described [35]. The challenge inoculum of *P. aeruginosa* was established by a pilot experiment to be 10^7 CFU/mL for C57BL/6 mice.

Persistent *P. aeruginosa* lung infection mouse model

C57BL/6 male mice (22-22g, 6-8 week old) from Charles River were used for infection as described [35]. Before challenge, mice were anesthetized with ketamine (50 mg/mL) and xylazine (5 mg/mL) in 0.9 % NaCl administered at a volume of 0.002 mL/g body weight by intraperitoneal injection. After mice were fully anaesthetized, they were placed in supine

position, followed by intra-tracheal instillation with 50 µl of agar beads containing bacterial

suspension (10^7 CFU/mL) to infect mice. A persistent lung infection that is difficult to heal is

created due to the use of agar beads, mineral oil and stationary phase culture used for infection.

Drug treatment in mouse persistent lung infection

Based on a pilot experiment, at 3 day post-infection, mice would have the highest 10^8

CFU/mL count and establish a stable persistent lung infection model as shown in the previous

study [35]. To evaluate different drugs and drug combinations, the following groups of mice

were used with each group having 5 mice per group: (1) drug free control (PBS); (2) cefuroxime

(40 mg/kg) + gentamicin (30 mg/kg) treatment; (3) cefuroxime + clinafloxacin (40 mg/kg)

treatment; (4) cefuroxime + gentamicin + levofloxacin (40 mg/kg) treatment; (5) clinafloxacin

treatment; (6) cefuroxime + gentamicin + clinafloxacin treatment. Mice were treated daily

intraperitoneally. After 7 day treatment, the mice were sacrificed and the whole lung of each

mouse was excised aseptically and homogenized in 1 mL PBS, and 100 µL of appropriately

serial diluted lung homogenates were plated on TSA plate, followed by incubation at 37 ℃

overnight for CFU count.

Statistical Analysis

Statistical analysis was performed using two-tailed Student's t-test and two-way ANOVAs

where appropriate. Mean differences were considered statistically significant if p was <0.05. All

experiments were performed in triplicates. Analyses were performed using GraphPad Prism and

Microsoft Office Excel.

Results

Use of SYBR Green I/PI assay to assess the viability of *P. aeruginosa*

The SYBR Green I/PI assay is a rapid and convenient method to assess bacterial viability under drug exposure at different time points [23]. Thus, we first optimized the SYBR Green I/PI assay for use in determining *P. aeruginosa* viability. To generate a standard curve, we combined live and isopropyl-killed *P. aeruginosa* samples in ratios of 0:10, 1:4, 5:5, 4:1, 10:0 and after staining with SYBR Green I/PI, the green (live) and red (dead) fluorescence intensities of the samples were measured using a microplate reader (BioTek Instrument). A linear relationship (R^2 values of 0.90561) between the ratios of green /red fluorescence and the percentages of live *P. aeruginosa* bacterial cells was established (Figure 1A). Fluorescence microscopy imaging confirmed the varying proportions of live and/or dead bacteria (Figure 1B).

Ranking of major classes of antibiotics for activity against stationary phase *P. aeruginosa*

To determine the relative activity of different classes of antibiotics against stationary phase *P. aeruginosa*, we performed drug exposure assays (all at 25 μM) with cell wall inhibitor (e.g. Cefuroxime), cell membrane inhibitor (Colistin), DNA synthesis inhibitor (e.g. Clinafloxacin), protein synthesis inhibitor (e.g. Gentamicin), RNA synthesis inhibitor (e.g. Rifampicin), Sulfa drug (e.g. Sulfamethoxazole), and Nitrofurantoin against *P. aeruginosa* stationary phase bacteria (Figure 2A). After 2-day exposure, Clinafloxacin showed the highest activity in killing stationary

phase bacteria, resulting in 0 CFU (Figure 2B). Cefuroxime and Colistin, cell wall and cell membrane inhibitors, respectively, and Sulfamethoxazole had high activity against stationary phase bacteria compared with the drug free control. In contrast, Gentamicin, Rifampicin and Nitrofurantoin showed poor activity against the stationary phase *P. aeruginosa*.

Although the standard CFU assay can be used to evaluate the activity of antibiotics against stationary phase *P. aeruginosa*, we wanted to rank the same antibiotics by using the SYBR Green I/PI assay which is a more rapid method that can be used for high-throughput drug screens [23]. Our results generated by SYBR Green I/PI viability assay correlated with the results of the CFU counting assay (Figure 3A). Cefuroxime and Colistin, Sulfamethoxazole and Clinafloxacin had among the highest activity against the stationary phase *P. aeruginosa.* The other three classes of antibiotics as represented by Gentamicin, Rifampicin and Nitrofurantoin had poor activity. We then calculated the residual viable bacteria cells after 10-day drug exposure through the regression equation (Table 1). After 10 days of treatment, Colistin killed the highest number of stationary phase bacteria, and Cefuroxime, Sulfamethoxazole and Clinafloxacin also showed remarkable effects with low percentages of residual viable bacterial cells remaining (Figure 3B). Like our results from CFU counting, the other three classes of antibiotics as represented by Gentamicin, Rifampicin and Nitrofurantoin killed fewer stationary phase bacteria with considerable numbers of residual viable cells remaining after treatment.

Fluorescence microscopy analysis also confirmed that Cefuroxime, Colistin, Clinafloxacin and Sulfamethoxazole had the highest activities against stationary phase bacteria (Figure 4). At

10 day post-treatment, the majority of the bacterial cells were dead (as depicted in red PI stain) and the numbers of viable live cells (depicted in green SYBR Green stain) were minimal. In contrast, stationary phase bacteria treated with Gentamicin, Rifampicin or Nitrofurantoin had more viable bacterial cells than dead cells, indicating their poor activity in killing stationary phase bacteria. Although these six classes of antibiotics all had activity against stationary phase bacteria compared to the drug free control, their relative activity against *P. aeruginosa* was quite different. From our CFU counts and our SYBR Green I/PI results, the six classes of antibiotics ranked from highest to lowest activity are as follows: clinafloxacin > colistin > gentamicin > cefuroxime > sulfamethoxazole > rifampicin > nitrofurantoin (Table 1).

Cefuroxime exhibits greater activity against stationary phase *P. aeruginosa* than other cell wall inhibitors

Cell wall inhibitors usually do not have good activity against persisters. Here, we found that cefuroxime at 25 μM reduced stationary phase bacteria from 10^9 to 10^6 CFU/mL (Figure 2B). To determine if it is the specific activity of cephalosporin cefuroxime against persisters, we tested other cell wall inhibitors such as amoxicillin and meropenem and found that compared to the dramatic persister killing activity of cefuroxime, amoxicillin and meropenem had limited activity against *P. aeruginosa* persisters as more than 10^7 CFU/mL bacteria remaining after 7-day drug exposure (Figure 5A).

Clinafloxacin exhibits the highest activity against stationary phase *P. aeruginosa* among different quinolone antibiotics

Since we found clinafloxacin had excellent activity against stationary phase bacteria (Figure 2B), we wanted to compare its activity with that of currently recommended quinolone antibiotics ciprofloxacin and levofloxacin in the treatment of *P. aeruginosa* infection [7], in terms of their activity against stationary phase *P. aeruginosa*. With the same 25 μM concentration, we found that only clinafloxacin could eradicate *P. aeruginosa* persisters only after 2 days by CFU count (Figure 5B). In contrast, ciprofloxacin and levofloxacin treatment still had 10^3 and 10^4 CFU/mL remaining, respectively.

Relationship between the MIC values of antibiotics and their activity against stationary phase *P. aeruginosa*

Antibiotics with a low MIC value may have great activity against growing bacteria but may not have strong activity against stationary bacteria and vice versa in previous studies with *S. aureus* and *E coli* [36-38]. Thus, we also tried to make a ranking among the six major classes of antibiotics (cell wall inhibitor, cell membrane disruptor, protein synthesis inhibitor, DNA synthesis inhibitor, RNA synthesis inhibitor, and anti-metabolite folate inhibitor) for their ability to kill growing log-phase bacteria. Based on the MIC values determined, our data showed that Colistin and Clinafloxacin had the lowest MIC values for *P. aeruginosa* PAO-1 strain (Table 2), which demonstrated that these two antibiotics can effectively kill both log phase and stationary

phase bacteria. We also observed that Gentamicin was highly active against log phase bacteria but had low activity against stationary phase bacteria. Conversely, Cefuroxime and Sulfamethoxazole were less effective against growing PAO-1 bacteria, both with MICs above 16 µg/mL, but were effective in killing stationary phase bacteria (Tables 1 & 2). Rifampicin and Nitrofurantoin had low activity against both growing and stationary phase *P. aeruginosa*.

Development of drug combinations to eradicate *P. aeruginosa* biofilms *in vitro*

The bacterial cells within a biofilm structure are heterogeneous and contain persister cells that are tolerant to antibiotics [39]. Our previous work with *B. burgdorferi* biofilm-like structures indicated that a drug combination approach utilizing drugs active against growing bacteria and non-growing persister bacteria respectively is required to more effectively eradicate biofilm bacteria [40]. Using the drugs that we have ranked with high activity against *P. aeruginosa* stationary phase persister cells, we tested the activity of various two-drug combinations (at clinically relevant Cmax concentrations) in killing stationary phase cells and biofilms. Our data demonstrate that only Clinafloxacin as a single drug could kill stationary phase cells completely (10^9 CFU/mL) after 4-day treatment, while single drugs such as colistin, cefuroxime, and gentamicin could kill only about 2-logs of stationary phase cells (Figure 6A). By adding Cefuroxime (a drug that has great activity killing growing bacteria) to the combination with anti-persister drug clinafloxacin, the time to kill all stationary phase cells and biofilms was shortened from 4 days to 2 days. Compared to the clinical treatment cefuroxime + gentamicin + colistin,

our designed drug combination cefuroxime + gentamicin + clinafloxacin could kill all biofilm bacteria (10^9 CFU/mL) after 4-day drug treatment, while single drugs or two drug combinations without clinafloxacin could kill only about 3-logs of biofilm cells and the two drug combinations with clinafloxacin could kill biofilm bacteria significantly with 10^2 CFU/mL remaining (Figure 6B). In contrast, two-drug combinations such as colistin + gentamicin or cefuroxime + gentamicin, a combination currently used clinically, and even a three-drug combination cefuroxime + gentamicin + colistin only killed about 10^3-10^4 stationary phase and biofilm cells in 4 days, suggesting that a combination without an anti-persister drug (e.g. clinafloxacin) is not as effective. While the two-drug combination of colistin + gentamicin could also kill stationary phase cells by 10^8 CFU/mL after 4-day treatment, this combination is ineffective against biofilms which still remains 10^6 CFU/mL bacterial cells.

Cefuroxime + Gentamicin + Clinafloxacin drug combination successfully eradicated persistent *P. aeruginosa* infection in cystic fibrosis model in mice

Since we found that cefuroxime + gentamicin + clinafloxacin completely eradicated stationary phase culture and biofilms *in vitro*, we wanted to test if this triple persister drug regimen could also eradicate the persistent *P. aeruginosa* infection *in vivo*. To do so, we utilized a cystic fibrosis persistent *P. aeruginosa* infection model in mice as described [35]. Mice were infected with stationary phase *P. aeruginosa* PAO-1 mixed in beads through intra-tracheal instillation as described in Methods. After 3 day infection, bacteria reached peak at about 10^8

CFU/g of the infected lungs when different treatments were started and continued for 7 days.

There was a modest decrease of bacterial load (10^3 fold) in the un-treated group after 7 days due

to the host immune clearance of the bacterial infection [35]. After 7-day drug treatment, the

current clinical recommended drug combination Cef + Gen only reduced the bacterial load from

10^8 CFU/g to 10^4 CFU/g (Figure 7). Clinafloxacin (Clin) alone, or Cef + Clin, reduced the

bacterial load to 10^4 or 10^3 CFU/g, respectively, but were unable to completely clear the bacterial

load in the lungs. In contrast, only Cef + Gen + Clin combination cleared all bacteria in the

infected lungs. When we replaced Clin with levofloxacin (Lev) in the drug combination, we

found that Cef + Gen + Lev combination was unable to clear the bacterial load and still had close

to 10^4 CFU/g remaining, indicating that the complete sterilization of the bacterial load is a

property of Clinafloxacin in the context of combination with Cef and Gen. Thus, drug

combination with clinafloxacin in this persistent lung infection model validated our *in vitro* drug

combination results on biofilm bacteria.

Drug Name	Residual viable cells (%)
Drug free control	58.096
Colistin	11.696
Cefuroxime	15.456
Clinafloxacin	18.016
Sulfamethoxazole	18.096
Gentamicin	20.976
Rifampicin	22.096
Nitrofurantoin	25.296

Table 1. Ranking of antibiotic activity from highest to lowest based on the amount of residual viable cells of *P. aeruginosa* (Drug concentration: 25 μM)

*The residual viable *P. aeruginosa* cells were calculated from a regression standard curve and equation from the SYBR Green I/PI assay as described by Feng et al. (2014) [23].

Class	Drug name	MIC (μg/mL)	Cmax (μg/mL)[a]	Activity against stationary phase bacteria
Cell wall inhibitor	Cefuroxime	16	4.1-7.0	+
Cell membrane disruptors	Colistin	4	2.4	+++
Protein synthesis inhibitor	Gentamicin	1	5-12	+
DNA synthesis inhibitor	Clinafloxacin	1	1.5-2.1	+++
RNA synthesis inhibitor	Rifampicin	16	8.2-11.7	-
Sulfa drug	Sulfamethoxazole	16	46.3	++
Nitrofurantoin	Nitrofurantoin	>16	0.72	-

Table 2. Comparison of major classes of antibiotics and their respective activity against growing

and stationary phase of *P. aeruginosa* ([a] Cmax values are derived from the literature)

A

B

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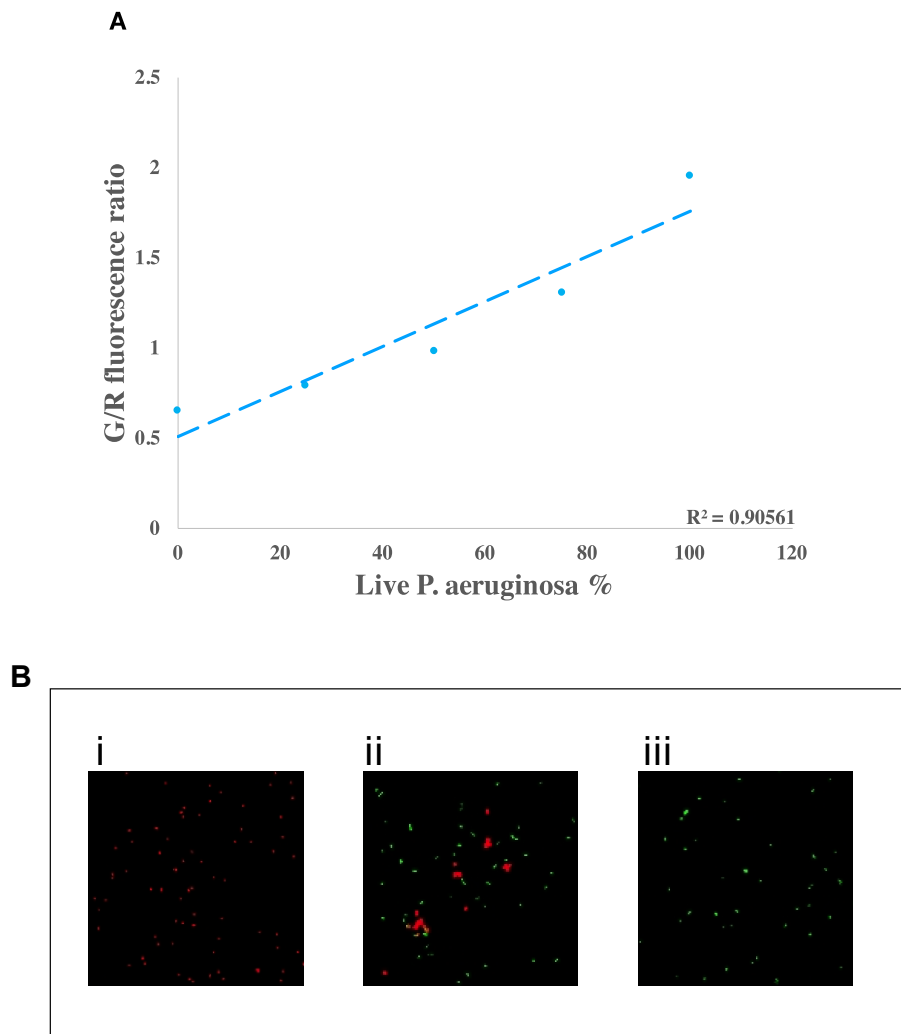
Figure 1. SYBR Green I/PI staining can assess the viability of *P. aeruginosa* (A) A standard curve revealed linear relationship between the Green/Red fluorescence ratios from SYBR Green I/PI viability assay and the percentage of live *P. aeruginosa* PAO-1 cells. The dead organisms were prepared with 70 % isopropyl alcohol and different proportions of live and dead cells were mixed and stained with SYBR Green I/PI and the green/red fluorescence ratios were measured by a microplate reader. **(B)** Representative microscope images of 0%, 50% and 100% of live *P. aeruginosa* cells using SYBR Green I/PI stain (200 X magnification). Green cells represent live cells and Red cells represent dead cells.
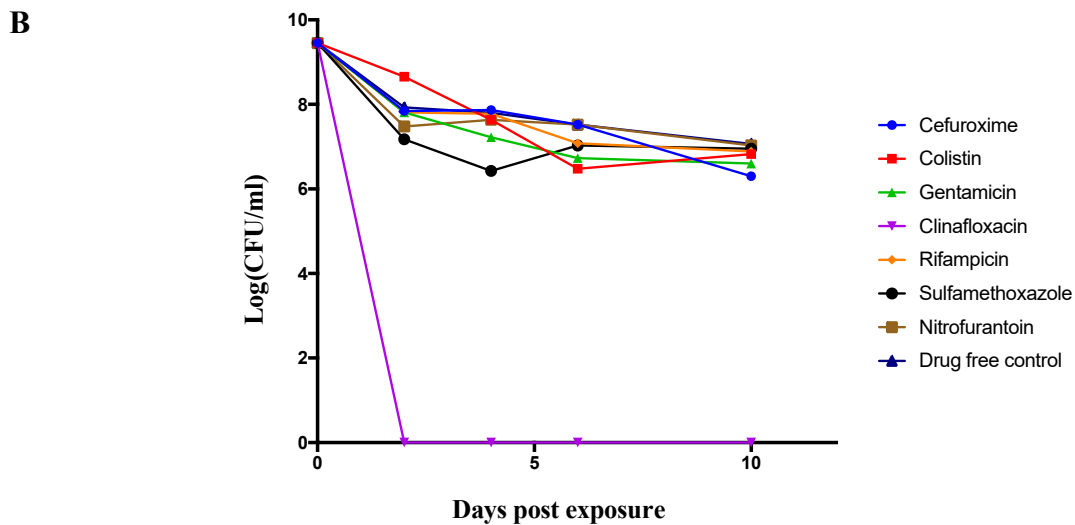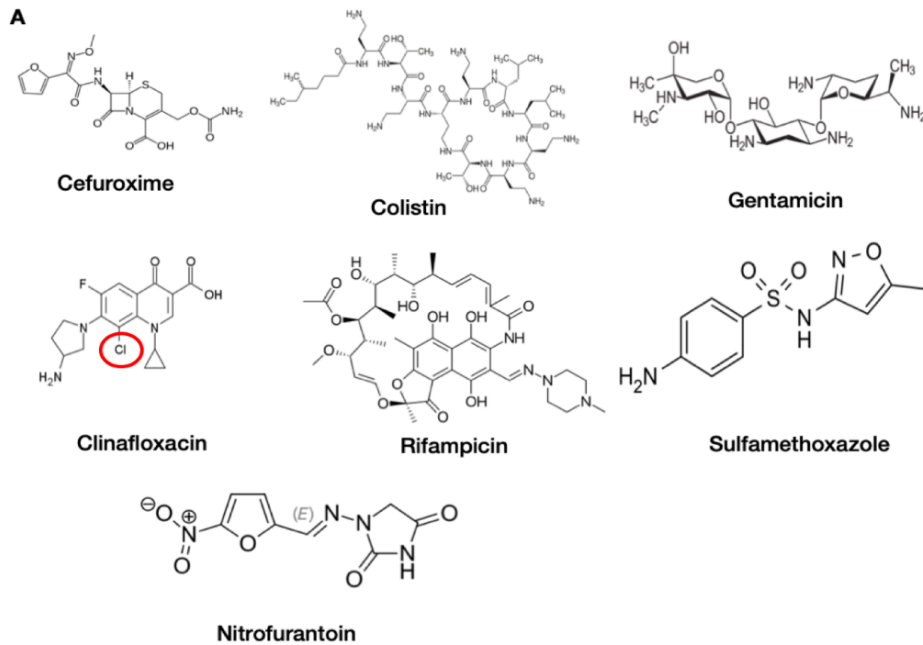
Figure 2. Ranking of the six classes of antibiotics by their activities against stationary phase *P. aeruginosa* determined by CFU assay (A) Structures of the selected six different classes of antibiotics tested against stationary phase *P. aeruginosa* PAO-1 strain. **(B)** After 10-day drug exposure (25 μM), the activity of each antibiotic against stationary phase *P. aeruginosa* was determined by CFU count.
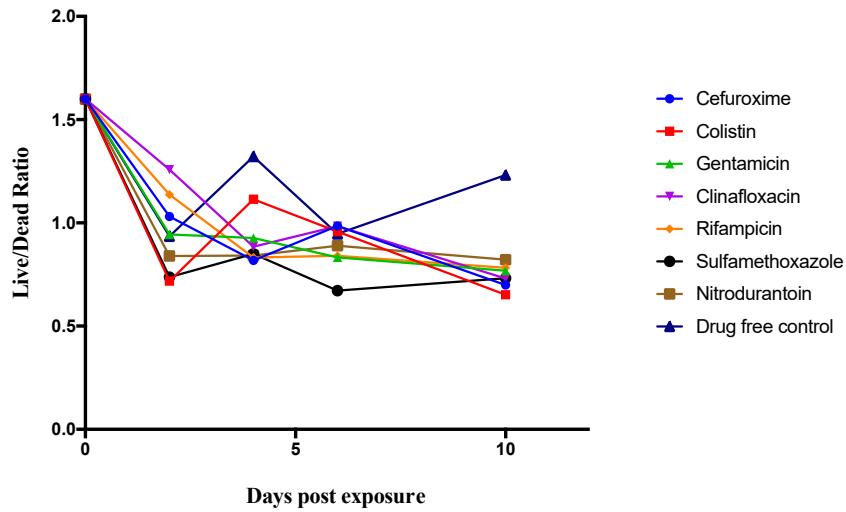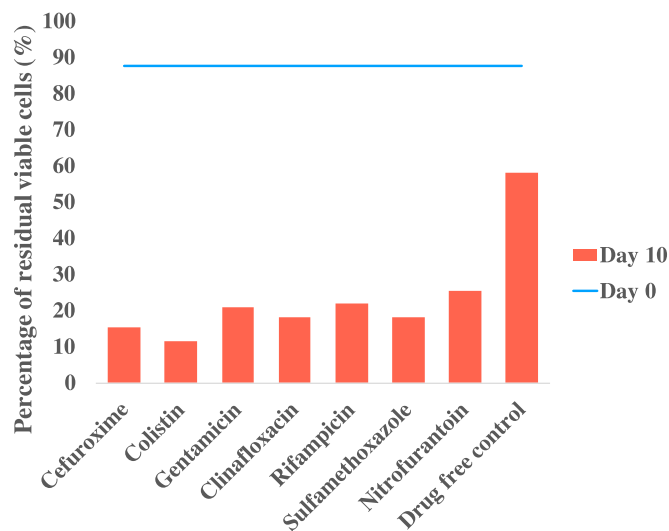
A

B

Figure 3. Ranking of the six classes of antibiotics by their activity against stationary phase *P. aeruginosa* determined by SYBR Green I/PI viability assay. (A) After staining the bacterial culture with SYBR Green I/PI in the 96-well plate, the green/red fluorescence ratios were measured. SYBR Green I/PI viability assay revealed drug activity results in concordance with the CFU assay. Cefuroxime and Colistin, Sulfamethoxazole and Clinafloxacin had good activity against stationary phase *P. aeruginosa*. **(B)** The percentage of residual viable cells after 10-day antibiotic treatment was calculated with the green/red ratios and the *P. aeruginosa* standard curve.
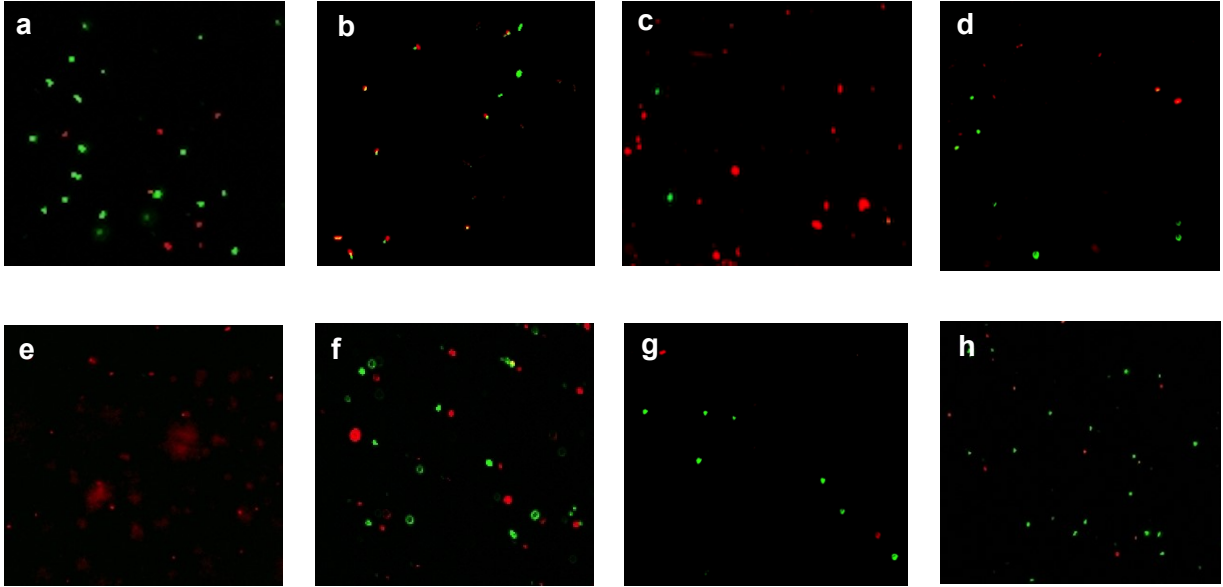
Figure 4. Fluorescence microscopy confirmation of stationary phase *P. aeruginosa* PAO-1 treated with six different classes of antibiotics (25 μM) followed by staining by SYBR Green I/PI assay (200X magnification). **(a)** Drug-free control, **(b)** Cefuroxime, **(c)** Colistin, **(d)** Gentamicin, **(e)** Clinafloxacin, **(f)** Rifampicin, **(g)** Sulfamethoxazole, and **(h)** Nitrofurantoin.

A
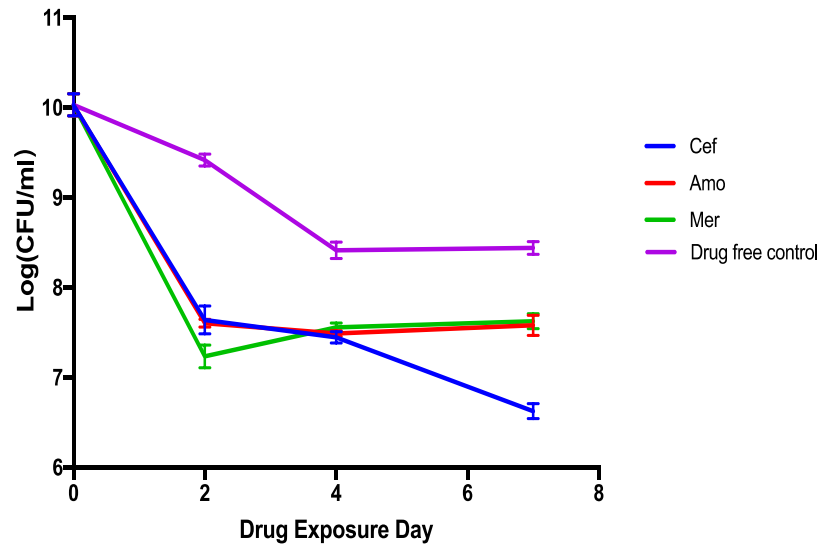
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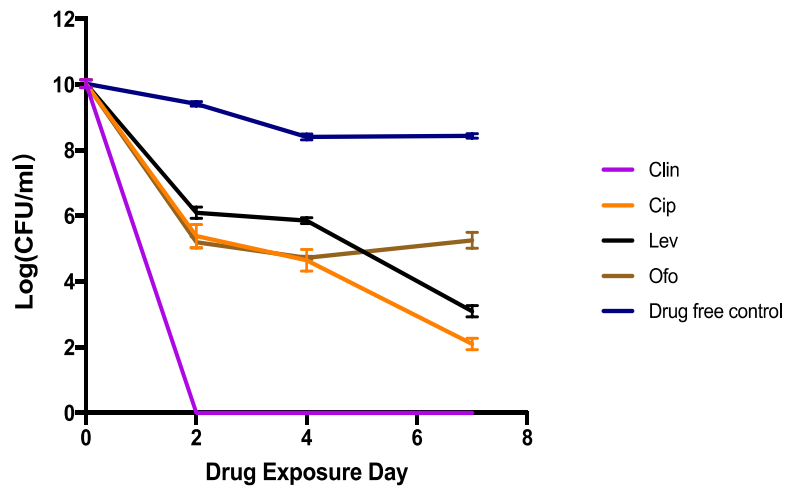
Figure 5. Comparison of relative activity of different cell wall inhibitors and different quinolone antibiotics against stationary phase *P. aeruginosa* by CFU assay. Stationary phase cultures of *P. aeruginosa* were exposed to different cell wall inhibitors (25 μM) (A) or different quinolone antibiotics (25 μM) for 0, 2, 4, 7 days when the CFU count was performed after washing and serial dilutions as described in Methods. Cefuroxime (Cef); Amoxicillin (Amo); Meropenem (Mer); Clinafloxacin (Clin); Ciprofloxacin (Cip); Levofloxacin (Lev); Ofloxacin (Ofo).
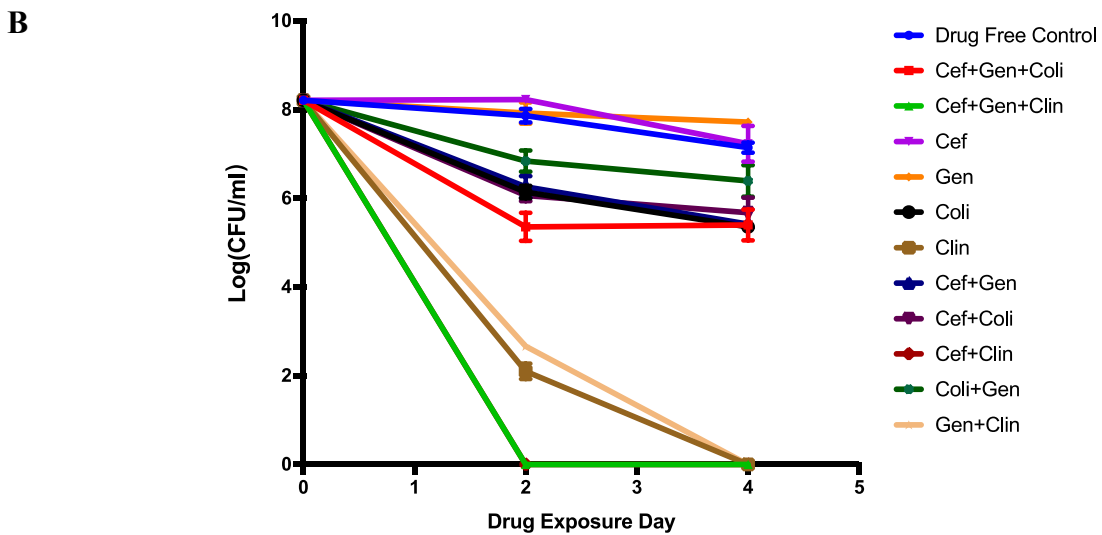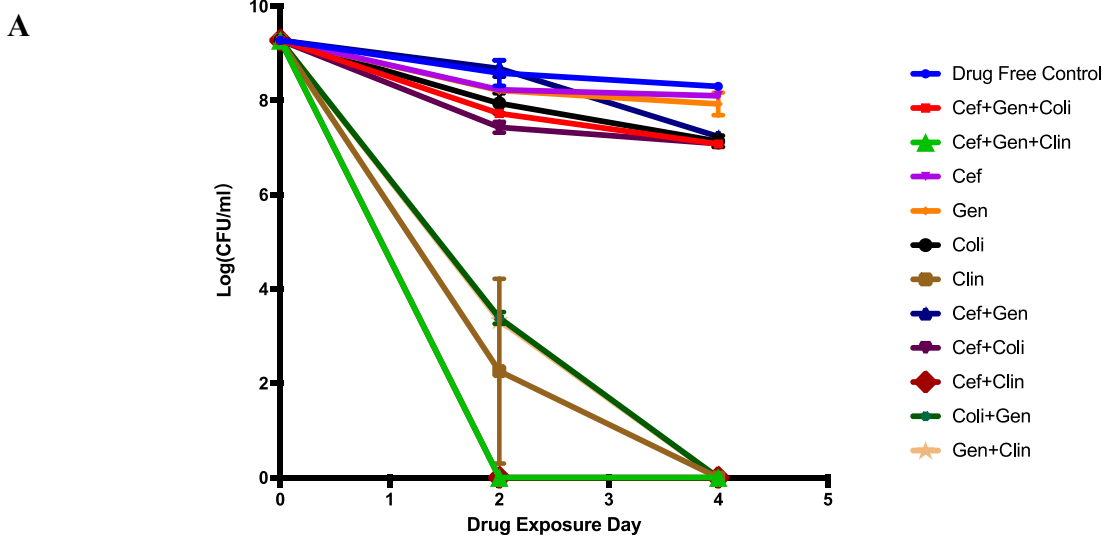
Figure 6. Comparison of the relative activity of different antibiotic combinations against stationary phase *P. aeruginosa* and biofilm bacteria by CFU assay. The effects of Clinafloxacin in drug combinations against stationary phase *P. aeruginosa* (A) and against *P. aeruginosa* biofilm model (B) were evaluated in drug exposure assays as described in Methods. CFU assay was performed on Day 0, 2, 4 after washing and serial dilutions. Cefuroxime (Cef): 5 μg/mL; Gentamicin (Gen): 10 μg/mL; Colistin (Coli): 5 μg/mL; Clinafloxacin (Clin): 1.5 μg/mL.
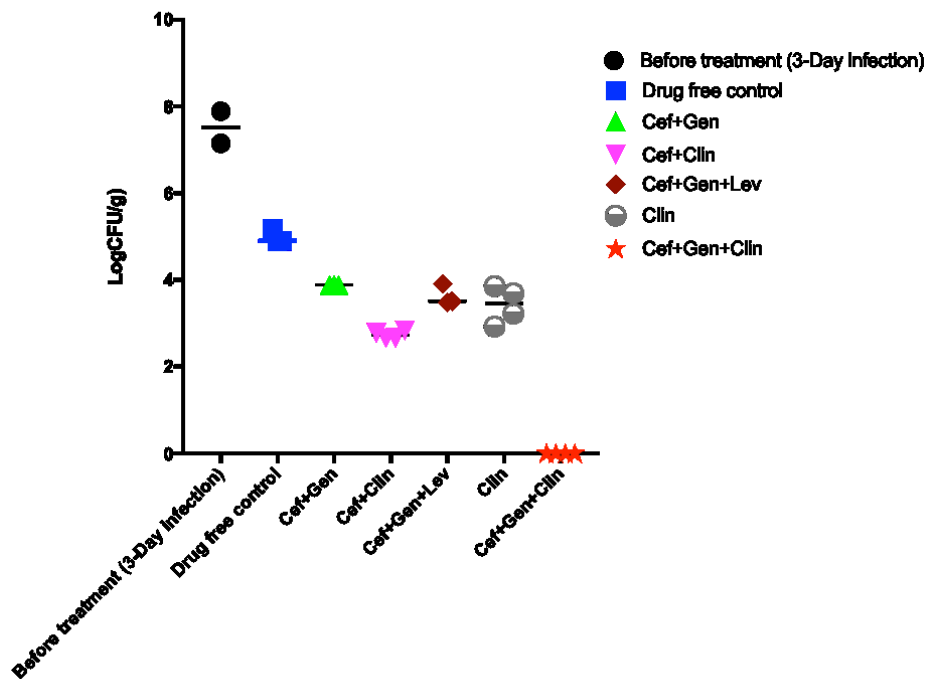
Figure 7. Cefuroxime + Gentamicin + Clinafloxacin drug combination successfully eradicated *P. aeruginosa* persistent infection in mouse cystic fibrosis model. Persistent *P. aeruginosa* lung infection in mice was established as described in Methods. The treatment was started 3 days after infection and continued for 7 days when the CFUs from the lungs were determined. The treatment groups are as follows: before treatment group; drug-free control; Cef+Gen; Cef+Clin; Cef+Gen+Lev; Clin; Cef+Gen+Clin. Drug dosages used were Cef 40 mg/kg; Gen 30 mg/kg; Clin 40 mg/kg; Lev 40 mg/kg.

CHAPTER 2

DRUG COMBINATIONS TARGETING GROWING AND PERSISTER CELLS ERADICATE CHRONIC CARBAPENEMASE-PRODUCINT KLEBSIELLA PNEUMONIAE INFECTION

Introduction

Carbapenemase-Producing *Klebsiella pneumoniae*

Klebsiella pneumoniae is a Gram-negative bacterium that can cause both nosocomial and community-acquired pneumonia [41]. *K. pneumoniae* is now the second most common cause of Gram-negative bacteremia and a major pathogen in hospital-acquired infections, particularly in immunocompromised patients [42]. *K. pneumoniae* is an opportunistic pathogen that can cause severe hospital-acquired infections such as septicemia, pneumonia, urinary tract infection and soft tissue infection in debilitated individuals [43]. Since 1980s, the management of infections due to *K. pneumoniae* has been complicated by the emergence of antimicrobial resistance [44]. From early this decade, Enterobacteriaceae that produce *Klebsiella pneumoniae* carbapenemases (KPC) were reported in the USA and subsequently worldwide [45]. These KPC-producing bacteria are predominantly involved in nosocomial and systemic infections.

Antibiotic Resistant KPC and KPC Biofilms

Based on the Johns Hopkins Antibiotic Guide [7], KPC confers broad antibiotic resistance and is associated with high mortality rate [46], so their susceptibility is limited to gentamicin, colistin and tigecycline. However, monotherapy such as single tigecycline treatment is associated with higher treatment failure rates compared to combination therapy when managing infections due to KPC-producing bacteria, particularly when treating respiratory infections [47]. Thus,

combination therapy is recommended. But, which antimicrobial combination is superior has yet to be established.

There are many different mechanisms of resistance to carbapenems in *K. pneumoniae* [48]. The co-occurrence of permeability defects, together with the production of β-lactamases that possess very weak carbapenemase activity, may lead to reduced susceptibility to carbapenems, particular ertapenem [49]. In addition, the mechanisms involving β-lactamases (e.g., ESBLs, AmpC), which are not considered significant carbapenem-hydrolyzing enzymes, true carbapenemases are also responsible for nonsusceptibility to carbapenems without additional permeability defects in *K. pneumoniae* [50]. Infections with *K. pneumoniae* strains with the ability to form biofilms are more difficult to treat [51]. Previous researchers found that nearly half of 40 examined KPC strains were able to form obvious biofilms, including 13 that exhibited high levels of biofilm formation [52]. The antibiotic resistance of mature bacterial biofilm is 10–1,000 times that of planktonic bacteria, and bacteria in biofilms can resist phagocytosis as well as host immune responses, making them very challenging to be eliminated [53].

An optimized SYBR Green I/PI Assay for Rapid Viability Assessment for KPC

Then conventional antibiotic susceptibility test is the Kirby–Bauer disk diffusion assay used to determine the susceptibility of the various strains to antibiotics. Instructions and interpretation of zone diameters were made using the established standards as listed in the Clinical and Laboratory Standards Institute [54]. In order to expedite the process of antimicrobial

susceptibility testing, we developed a novel approach using SYBR Green I/Propidium iodide (PI) staining viability assay for KPC viability assessment [55], which the lab initially developed for quantifying the viability and drug screens of *Borrelia burgdorferi* [23]. The advantages of using SYBR Green I/PI Assay to assess the viability of bacterial pathogens and susceptibility to antibiotics have been described in Chapter 1. Here, we applied the SYBR Green I/PI viability assay to *k. pneumoniae* and ranked six major classes of antibiotics in killing growing and stationary non-growing forms of KPC compared with conventional CFU based viability assay. Using these methods, we also identified drug combinations that could more effectively eradicate persisters in stationary phase culture of KPC as well as in biofilms *in vitro*.

Materials and Methods

Bacterial strain, culture media and growth condition

K. pneumoniae carbapenemase strain (Isolate 7) was obtained from ATCC (Manassas, VA, USA). KPC strain was cultured in tryptic soy broth (TSB) and tryptic soy agar (TSA) from Becton Dickinson (Franklin Lakes, NJ, USA) at 37 ℃. The culture was incubated at 37℃ with shaking at 200 rpm. After overnight incubation, stationary phase cultures were exposed to different antibiotics and incubated at indicated times before washing and plating on TSA plates for colony forming unit (CFU) enumeration. The plates were incubated at 37 ℃ incubator overnight.

Preparation of antibiotics

Stock solutions of antibiotics Cefuroxime, Colistin, Gentamicin, Clinafloxacin, Rifampicin, Sulfamethoxazole and Nitrofurantoin (Sigma Aldrich Co.) were prepared in the laboratory, filter-sterilized and with appropriate solvents at 10 mg/ml. Each antibiotic (25 µM) was added into 500 µL of stationary phase culture of KPC and was incubated at 37 ℃ without shaking for various times. In addition, appropriate concentrations of the antibiotics were used for MIC determination using the microdilution method in 96-well plates (see below).

Drug exposure assay by CFU counting on plates

Viable bacterial cells were determined by CFU count after drug exposure at days 2, 4, 6 and 10 as described [28]. First, bacterial suspensions (50 μL) after drug exposure were washed with fresh TSB medium twice and serial dilutions were prepared. 10 μL of each dilution was dropped onto TSA plates in triplicate followed by incubation at 37 ℃ overnight. The CFU/ml was calculated accordingly.

SYBR Green I/PI assay for KPC

SYBR Green I (10,000X stock, Invitrogen) was mixed with PI (20 mM, Sigma) in distilled H_2O. The staining dye for *K. pneumoniae* was made by mixing SYBR Green I to PI (3:1) in 100 μl distilled H_2O which was developed by the previous lab members [55]. The drug treated samples were aliquoted and diluted 1:50 with fresh TSB medium. The SYBR Green I/PI dye mix (10 μL) was added to each 100 μL of sample. Each sample was vortexed and incubated at room temperature in the dark for 20 minutes. After incubation, each sample was transferred into 96-well plate. With excitation wavelength of 485 nm and 538 nm and 612 nm for green and red emission, respectively, the green and red fluorescence intensity was determined for each sample using a Synergy H1 microplate reader by BioTek Instruments (VT, USA). To generate the regression equation and regression curve of the relationship between the percentage of live bacteria and green/red fluorescent ratios, different proportions of live and 70% isopropyl alcohol killed dead cells were prepared (0:10, 1:4, 5:5, 4:1, 10:0) and both live and dead samples were diluted 50 times with fresh TSB medium first. Each proportion of live/dead KPC was mixed with

SYBR Green I/PI dye into each well of 96-well plate and the green/red fluorescence ratios were measured as described above, generating standard curve and equation with least-square fitting analysis. We used this equation to calculate the percentage of live cells of KPC. Fluorescence microscopy imaging visualizing live and dead cells was performed using a Keyence BZ-X710 Fluorescence Microscope and was processed by BZ-X Analyzer provided by Keyence (Osaka, Japan).

The MIC (minimum inhibitory concentration) determination

The standard microdilution method was used to determine the MIC of each antibiotic as described [29-31] and was used the same as we did on *P. aeruginosa* in Chapter 1. KPC (1×10^9) was inoculated (10 µL) into each well of 96-well plate containing 90 µL fresh TSB medium per well. Then each antibiotic was added into the well and the serial dilutions of drug treatment were made from 16, 8, 4, 2, 1 and 0 µg/mL. All experiments were run in duplicate or triplicate. The 96-well plate was incubated in 37 ℃ incubator overnight. The MIC is the lowest concentration of the antibiotic that prevented visible growth of KPC.

KPC biofilm preparation

The KPC biofilm model was prepared based on the protocol as described [32]. We first inoculated KPC bacterial cells in a 5 mL fresh TSB culture and let it grow to stationary phase overnight. Then this stationary phase culture was diluted 1:100 in fresh TSB medium, and 100 µl

diluted culture was pipetted into each well of 96-well plate and then the covered plate was put into 37 ℃ incubator overnight and KPC biofilm attached on the bottom of the plate after removing the supernatant medium by pipetting on the side of the well while leaving the biofilm formed at the bottom of the plate intact.

Drug combination assay on KPC stationary phase persister model and biofilm model *in vitro*

Based on the Johns Hopkins Hospital Antibiotics Guideline [7], using high doses of colistin (Coli) in the combination of tigecycline (Tige) is considered to be able to improve outcomes of serious infections in immunocompromised hosts [46]. However, there is a large concern for nephrotoxicity and neurotoxicity caused by colistin treatment [7]. We evaluated Clinafloxacin (1.5 μg/ml) (Clin) in combination with Cefuroxime (Cmax: 5 μg/ml) (Cef), Rifampin (Cmax: 10 μg/ml) (Rif), Clarithromycin (Cmax: 2 μg/ml) (Cla) and Colistin (Cmax: 5 μg/ml) separately. Based on our findings on triple drug combinations against *P. aeruginosa* chronic infection, we also tested the triple drug combinations of Cef + Gen + Clin, Cef + Rif + Clin, Cef + Cla + Clin and Cef + Coli + Clin for their activities against KPC in biofilm model in vitro. The designed drug combinations or their single or two drug controls were added directly to stationary phase culture and CFU count was performed after 2-day and 4-day drug treatment. For the biofilm model, the designed drug combinations were prepared with MOPS buffer (1X) (diluted from 10X MOPS from Sigma-Aldrich) to the final drug concentration and then transferred in to 96-

well plate with KPC biofilm attached to the bottom. Biofilm was washed with phosphate buffered saline (PBS) before plating for CFU count.

Statistical Analysis

Statistical analysis was performed using two-tailed Student's t-test and two-way ANOVAs where appropriate. Mean differences were considered statistically significant if p was <0.05. All experiments were performed in triplicates. Analyses were performed using GraphPad Prism and Microsoft Office Excel.

Results

Use of SYBR Green I/PI assay to assess the viability of KPC

The SYBR Green I/PI assay is a rapid and convenient method to assess bacterial viability under drug exposure at different time points [23]. Thus, we first optimized the SYBR Green I/PI assay for use in determining KPC viability. To generate a standard curve, we combined live and isopropyl-killed KPC bacterial cells samples in ratios of 0:10, 1:4, 5:5, 4:1, 10:0 and after staining with SYBR Green I/PI, the green (live) and red (dead) fluorescence intensities of the samples were measured using a microplate reader (BioTek Instrument). A linear relationship (R^2 values of 0.84553) between the ratios of green /red fluorescence and the percentages of live KPC bacterial cells was established (Figure 1A). Fluorescence microscopy imaging confirmed the varying proportions of live and/or dead bacteria (Figure 1B).

Ranking of major classes of antibiotics for activity against stationary phase KPC

To determine the relative activity of different classes of antibiotics against stationary phase KPC, we performed drug exposure assays (all at 25 μM) with cell wall inhibitor (e.g. Cefuroxime), cell membrane inhibitor (Colistin), DNA synthesis inhibitor (e.g. Clinafloxacin), protein synthesis inhibitor (e.g. Gentamicin), RNA synthesis inhibitor (e.g. Rifampicin), Sulfa drug (e.g. Sulfamethoxazole), and Nitrofurantoin against KPC stationary phase bacteria. After 2-day exposure, Clinafloxacin showed the highest activity in killing stationary phase bacteria,

resulting in 0 CFU (Figure 2). In contrast, the clinical used Colistin showed poor activity against the stationary phase KPC.

Although the standard CFU assay can be used to evaluate the activity of antibiotics against stationary phase KPC, we wanted to rank the same antibiotics by using the SYBR Green I/PI assay which is a more rapid method that can be used for high-throughput drug screens [23]. Our results generated by SYBR Green I/PI viability assay correlated with findings from the CFU counting assay (Figure 3A). Clinafloxacin had the highest activity against the stationary phase KPC. We then calculated the residual viable bacteria cells after 4-day drug exposure through the regression equation (Table 1). After 4 days of treatment, Clinafloxacin killed the highest number of stationary phase bacteria, and Colistin and Gentamicin also showed remarkable effects with low percentages of residual viable bacterial cells remaining (Figure 3B).

Fluorescence microscopy analysis also confirmed that Clinafloxacin, Gentamicin and Colistin had the highest activities against stationary phase bacteria (Figure 4). At 10 day post-treatment, the majority of the bacterial cells were dead (as depicted in red PI stain) and the numbers of viable live cells (depicted in green SYBR Green stain) were minimal. In contrast, stationary phase bacteria treated with Rifampicin or Nitrofurantoin had more viable bacterial cells than dead cells, indicating their poor activity in killing stationary phase bacteria. Although these six classes of antibiotics all had activity against stationary phase bacteria compared to the drug free control, their relative activity against KPC was quite different. From our CFU counts and our SYBR Green I/PI results, the six classes of antibiotics ranked from highest to lowest

activity are as follows: clinafloxacin > colistin > gentamicin > cefuroxime > sulfamethoxazole > rifampicin > nitrofurantoin (Table 1).

Clinafloxacin exhibits the highest activity against stationary phase KPC among different quinolone antibiotics

Since we found clinafloxacin had excellent activity against stationary phase bacteria (Figure 2B), we wanted to compare its activity with that of currently recommended quinolone antibiotics ofloxacin and levofloxacin in the treatment of gram-negative bacterial persistent infection[56]. With the same 25 μM concentration, we found that only clinafloxacin could eradicate KPC persisters only after 2 days by CFU count (Figure 5). In contrast, ofloxacin and levofloxacin treatment still had 10^6 CFU/mL remaining, respectively. And the clinically recommended treatment with colistin or tigecycline could only kill 10^1 CFU/mL stationary phase KPC.

Relationship between the MIC values of antibiotics and their activity against stationary phase KPC

Antibiotics with a low MIC value may have great activity against growing bacteria but may not have strong activity against stationary bacteria and vice versa in previous studies with *S. aureus* and *E coli* [36-38]. Thus, we also tried to make a ranking among the six major classes of antibiotics (cell wall inhibitor, cell membrane disruptor, protein synthesis inhibitor, DNA synthesis inhibitor, RNA synthesis inhibitor, and anti-metabolite folate inhibitor) for their ability

to kill growing log-phase bacteria. Based on the MIC values determined, our data showed that Colistin and Clinafloxacin had the lowest MIC values for KPC (Table 2), which demonstrated that these two antibiotics can effectively kill both log phase and stationary phase bacteria. We also observed that Rifampin was highly active against log phase bacteria but had low activity against stationary phase bacteria. Conversely, Cefuroxime and Gentamicin were less effective against growing KPC bacteria, both with MICs above 16 μg/mL, but were effective in killing stationary phase bacteria (Tables 1 & 2). Sulfamethoxazole and Nitrofurantoin had low activity against both growing and stationary phase KPC.

Development of drug combinations to eradicate KPC biofilms *in vitro*

The bacterial cells within a biofilm structure are heterogeneous and contain persister cells that are tolerant to antibiotics [39]. Our previous work with *B. burgdorferi* biofilm-like structures indicated that a drug combination approach utilizing drugs active against growing bacteria and non-growing persister bacteria respectively is required to more effectively eradicate biofilm bacteria [40]. Using the drugs that we have ranked with high activity against KPC stationary phase persister cells, we tested the activity of various two-drug combinations (at clinically relevant Cmax concentrations) in killing biofilms. Compared to the clinical treatment colistin + tigecycline , our designed drug combination cefuroxime + rifampin + clinafloxacin could kill all biofilm (10^9 CFU/mL) after 5-day drug treatment, while single drugs or two drug combinations without clinafloxacin could kill only about 3-logs of biofilm cells and the two drug combinations

with clinafloxacin could kill biofilm bacteria significantly to 10^4 CFU/mL remaining (Figure 6), suggesting that a combination without an anti-persister drug (e.g. clinafloxacin) is not as effective.

Drug Name (Concentration 25uM)	Residual viable cells (%)
Drug free control	95.30
Clinafloxacin	32.14
Colistin	38.78
Gentamicin	39.97
Cefuroxime	40.08
Sulfamethoxazole	43.81
Nitrofurantoin	47.72
Rifampicin	49.23

Table 1. Ranking of antibiotic activity from highest to lowest based on the amount of residual viable cells of KPC (Drug concentration: 25 μM)

*The residual viable KPC cells were calculated from a regression standard curve and equation from the SYBR Green I/PI assay as described by Feng et al. (2014).

Class	Drug name	MIC (µg/mL)	Cmax (µg/mL)	Activity against stationary phase bacteria
Cell wall inhibitor	Cefuroxime	>16	50-100	+
Cell membrane disruptors	Colistin	4	2.4	++
Protein synthesis inhibitor	Gentamicin	>16	5-12	++
DNA synthesis inhibitor	Clinafloxacin	1	2.1	+++
DNA synthesis inhibitor	Rifampicin	6	8.2-11.7	-
Sulfa drug	Sulfamethoxazole	16	46.3	+
Nitrofurantoin	Nitrofurantoin	8	0.72	-

Table 2. Comparison of major classes of antibiotics and their respective activity against growing and stationary phase of KPC ([a] Cmax values are derived from the literature)

A

B

0%   50%   100%
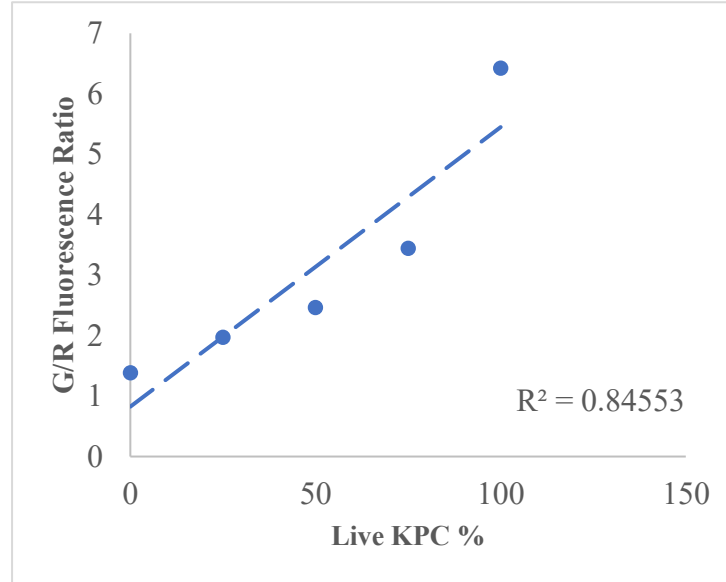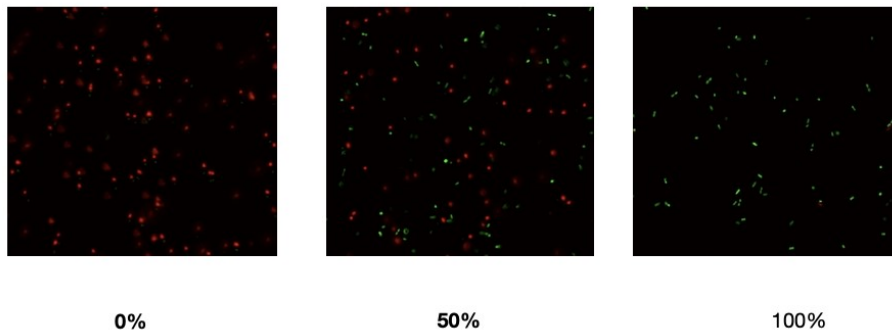
Figure 1. SYBR Green I/PI staining can assess the viability of KPC. (A) A standard curve revealed linear relationship between the Green/Red fluorescence ratios from SYBR Green/PI viability assay and the percentage of live KPC cells. The dead organisms were prepared with 70 % isopropyl alcohol and different proportions of live and dead cells were mixed and stained with SYBR Green I/PI and the green/red fluorescence ratios were measured by a microplate reader. **(B)** Representative microscope images of 0%, 50% and 100% of live KPC cells using SYBR Green I/PI stain (200 X magnification). Green cells represent live cells and Red cells represent dead cells.
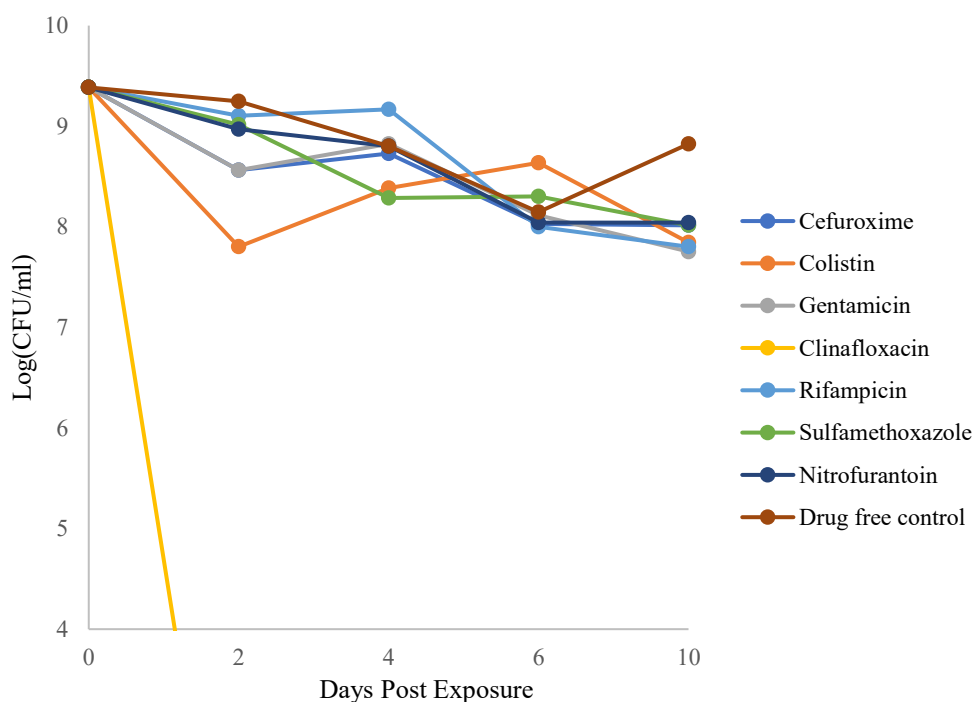
Figure 2. Ranking of the six classes of antibiotics by their activities against stationary phase KPC determined by CFU assay (A) Structures of the selected six different classes of antibiotics tested against stationary phase KPC. **(B)** After 10-day drug exposure (25 μM), the activity of each antibiotic against stationary phase KPC was determined by CFU count.
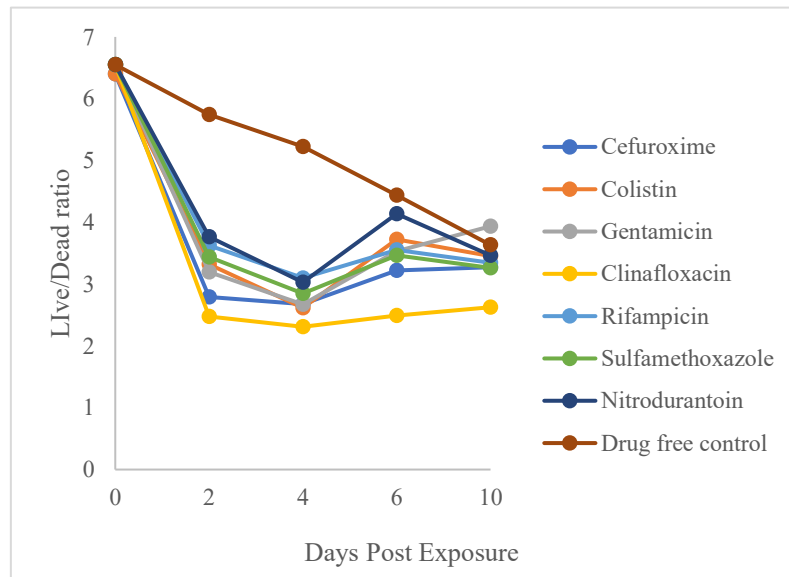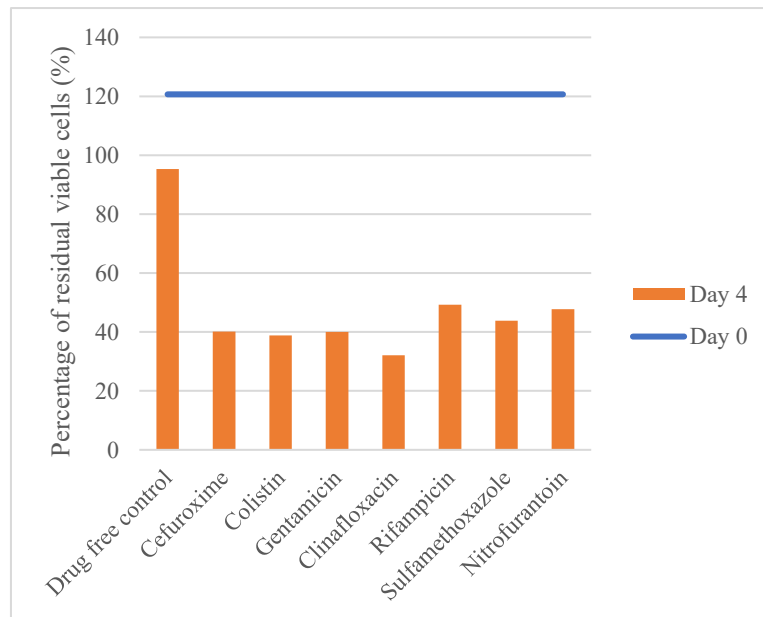
A

B

Figure 3. Ranking of the six classes of antibiotics by their activity against stationary phase KPC determined by SYBR Green I/PI viability assay. (A) After staining the bacterial culture with SYBR Green I/PI in the 96-well plate, the green/red fluorescence ratios were measured.

SYBR Green/PI viability assay revealed drug activity results in concordance with the CFU assay. Cefuroxime and Colistin, Sulfamethoxazole and Clinafloxacin had good activity against stationary phase KPC. **(B)** The percentage of residual viable cells after 10-day antibiotic treatment was calculated with the green/red ratios and the KPC standard curve.
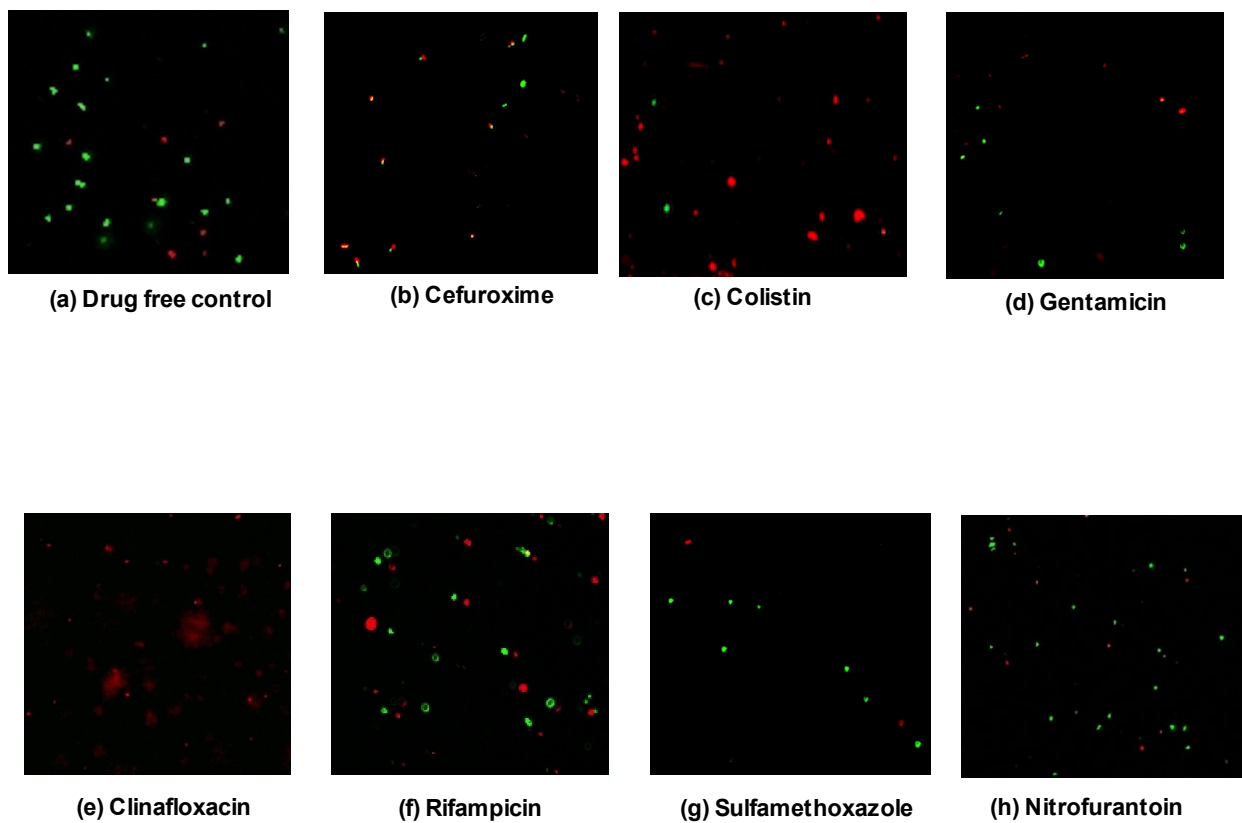
Figure 4. Fluorescence microscopy confirmation of stationary phase KPC treated with six different classes of antibiotics (25 μM) followed by staining by SYBR Green I/PI assay (200X magnification). **(a)** Drug-free control, **(b)** Cefuroxime, **(c)** Colistin, **(d)** Gentamicin, **(e)** Clinafloxacin, **(f)** Rifampicin, **(g)** Sulfamethoxazole, and **(h)** Nitrofurantoin.
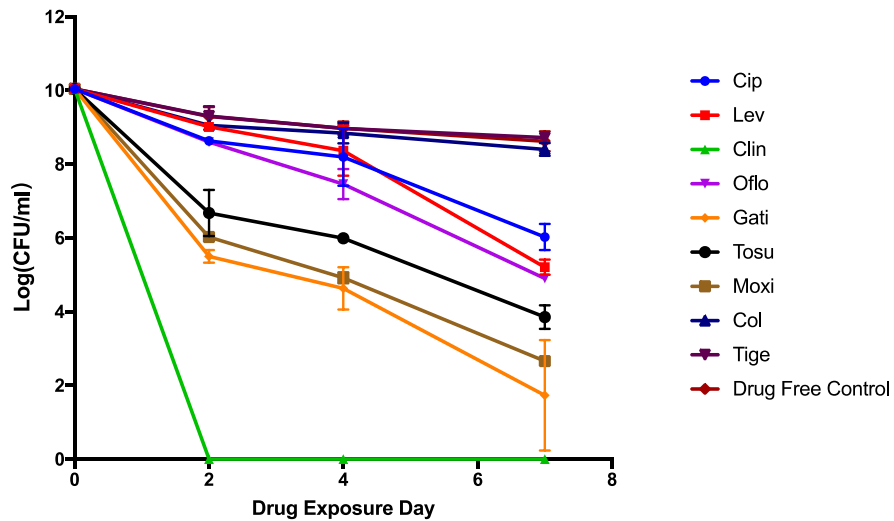
Figure 5. Comparison of relative activity of different quinolone antibiotics against stationary phase KPC by CFU assay. Stationary phase cultures of KPC were exposed to different cell wall inhibitors (25 μM) (A) or different quinolone antibiotics (25 μM) for 0, 2, 4, 7 days when the CFU count was performed after washing and serial dilutions as described in Methods. Cip (Ciprofloxacin); Lev (Levofloxacin); Clin (Clinafloxacin); Oflo (Ofloxacin); Gati (Gatifloxacin); Tosu (Tosufloxacin); Moxi (Moxifloxacin); Col (Colistin); Tige (Tigecycline).
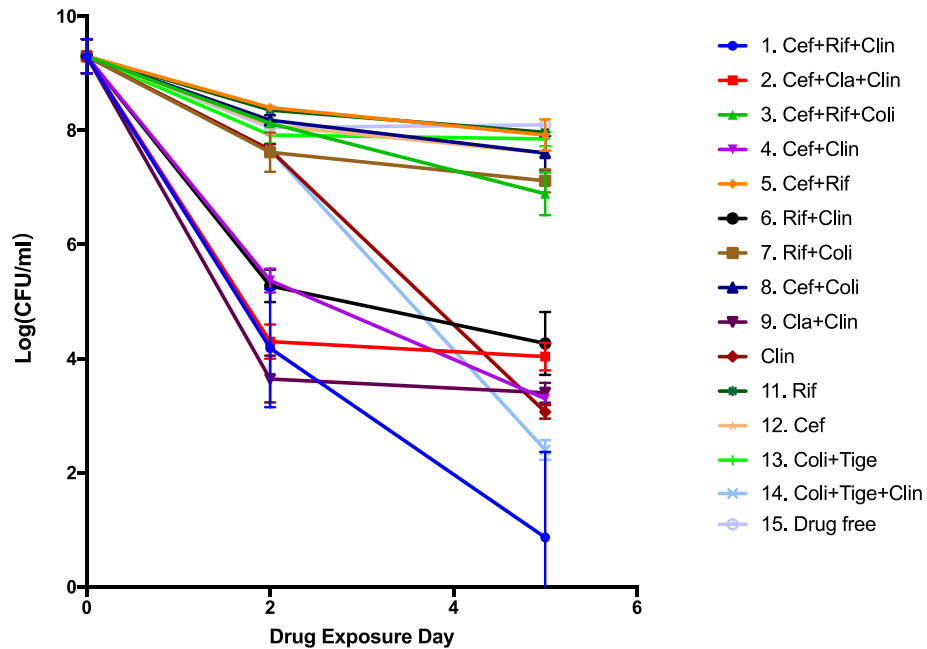
Figure 6. Comparison of the relative activity of different antibiotic combinations against KPC biofilm bacteria by CFU assay. The effects of Clinafloxacin in drug combinations against stationary phase KPC biofilm model were evaluated in drug exposure assays as described in Methods. CFU assay was performed on Day 0, 2, 5 after washing and serial dilutions. Cefuroxime (Cef): 5 μg/mL; Rifampin (Rif): 6 μg/mL; Colistin (Coli): 5 μg/mL; Clinafloxacin (Clin): 1.5 μg/mL; Clarithromycin (Cla): 2 μg/mL; Tigecycline (Tige): 2 μg/mL).

DISCUSSION

Clinically, it has been challenging to cure persistent infections like *P. aeruginosa* lung infections in cystic fibrosis patients and gastrointestinal colonization by KPC. The difficulty to cure the persistent infections is thought to be mainly due to persister bacteria that are not effectively killed by the current antibiotics used to treat such infections [14]. To address this hypothesis, here, we first ranked the six major classes of antibiotics for their activity against *P. aeruginosa* persisters in vitro and found a ranking order based on both CFU count and our SYBR Green I/PI results: clinafloxacin > colistin > gentamicin > cefuroxime > sulfamethoxazole > rifampicin > nitrofurantoin. Among different cell wall inhibitors, cefuroxime showed greater activity to kill *P. aeruginosa* persisters than amoxicillin and meropenem. Among different quinolone antibiotics, clinafloxacin killed all persister bacteria after 2-day drug exposure but other quinolones were not able to eradicate all persisters even after 7-day drug exposure, suggesting that clinafloxacin could be a potential persister drug candidate for inclusion in drug combinations to kill persisters and biofilm bacteria. Based on the Yin-Yang model [14] utilizing drugs with activity against growing bacteria (MICs) and anti-persister activity results, we designed a series of drug combinations to evaluate their ability to eradicate stationary phase or biofilm bacteria *in vitro*. Although the double drug combination of cefuroxime + clinafloxacin and gentamicin + clinafloxacin could eradicate persisters on day 4 treatment which has the same

activity as the triple therapy cefuroxime + gentamicin + clinafloxacin (Cmax as concentration of each drug) and effectively killed stationary phase bacteria, only the triple drug combination cefuroxime + gentamicin + clinafloxacin could kill all persisters after 2-day drug exposure in the *P. aeruginosa* biofilm model. To further confirm the efficacy of the triple drug combination cefuroxime + gentamicin + clinafloxacin *in vivo*, we successfully established the *P. aeruginosa* lung persistent infection mouse model using stationary phase bacteria mixed with agar beads for intra-tracheal instillation infection. Using this persistent lung infection model, we were able to validate that clinafloxacin in combination with cefuroxime and gentamicin cleared all bacterial infection in the mouse lungs, whereas other single drugs or two drug combinations failed to eradicate the *P. aeruginosa* persistent lung infection. We then confirmed our antibiotics ranking methods on KPC persistent infection *in vitro*. we ranked the six major classes of antibiotics for their activity against KPC persisters *in vitro* and found a ranking order based on both CFU count and our SYBR Green/PI results: clinafloxacin > colistin > gentamicin > cefuroxime > rifampicin > sulfamethoxazole > nitrofurantoin. Among different quinolone antibiotics, clinafloxacin killed all persister bacteria after 2-day drug exposure but other quinolones were not able to eradicate all persisters even after 7-day drug exposure, suggesting that clinafloxacin could be a potential persister drug candidate for inclusion in drug combinations to kill persisters and biofilm bacteria which correspond to our findings in antibiotics screenings for *P. aeruginosa*. After we tested different drug combinations for their activities against KPC biofilm model, we found compared to the clinically used colistin + tigecycline, only triple drug cefuroxime +

rifampin + clinafloxacin can eradicate the KPC biofilm model in 5-day treatment *in vitro*. And we found that when adding clinafloxacin to the colistin + tigecycline treatment, there would be a great decrease of bacterial cells due to the dramatic anti-persister effect from clinafloxacin.

Based on the Johns Hopkins Hospital Antibiotics Guideline [7], drugs that are included in routine treatment of *P. aeruginosa* infections range from β-lactam, aminoglycosides, fluoroquinolones to polymyxins. The majority of cystic fibrosis patients suffer from chronic infections of the airway with *P. aeruginosa* and the empiric treatment for *P. aeruginosa* currently uses two active agents from two different classes of antibiotics [33]. The two drug combination using high doses of synergistic antibiotic combinations (β-lactam + aminoglycoside) has been considered to improve outcomes of serious infections in immunocompromised hosts [8]. For multidrug resistant strains, colistin can be added to the above treatment [9]. However, in our study, we found that the current recommended treatment β-lactam + aminoglycoside (Cef + Gen) did not completely kill *P. aeruginosa* stationary phase and biofilm bacteria *in vitro* and failed to sterilize the lungs in the persistent lung infection in mice. In contrast, our persister drug Clinafloxacin in combination with Cef + Gen completely eradicated biofilm bacteria *in vitro*, and more importantly, the persistent lung infection in mice.

Our effective drug combination findings successfully apply the 'Yin-Yang' model to kill both replicating log phase bacteria (Yang) and also non-replicating persister bacteria(Yin) [14]. In the Tuberculosis (TB) therapy, isoniazid (INH) is only active for growing mycobacteria, rifampin (RIF) is used to kill both growing and non-growing bacteria, and pyrazinamide (PZA)

is added to kill exclusively non-growing persister bacteria in the treatment of TB [15]. The importance of PZA in killing persister TB bacteria and shortening the TB therapy without relapse is mainly recognized in the TB field and not appreciated in other persistent infections. We proposed to apply the PZA persister drug principle for the treatment of other infections [57]. This PZA principle has been shown to be valid in subsequent studies with different bacteria such as *B. burgdorferi* [40, 58], *S. aureus* (Yee R et al. to be published)*, E. coli* [59] both *in vitro* and *in vivo*. In this study, our findings with Clin + Cef +Gen combination both *in vitro* and *in vivo* further validate this PZA principle and the Yin-Yang model for developing more effective treatment of persistent infections, where Clin is an excellent drug targeting dormant persister bacteria (Yin) when used together with Cef and Gen which target growing bacterial populations for effective eradication of *P. aeruginosa* persistent lung infection. In our separate study with *S. aureus* pesistent skin infection model, we were also able to show that persister drug combinations utilizing the Yin-Yang model could achieve eradication of biofilm persistent infections (Yee, et al., to be published). Thus, we believe the PZA principle and Yin-Yang model can be applied for more effective treatment of other persistent infections in general.

There remain several areas for further investigation. First, the structural basis for the anti-persister activity in clinafloxacin fluoroquinolone drug is not clear but may be related to the unique chloride group in the quinolone ring. Further synthetic chemistry studies are needed to determine if the unique anti-persister activity is due to the chloride group and to further optimize the activity of clinafloxacin for anti-persister activity and to reduce its potential toxicity. Second,

the mechanism of the unique anti-persister activity in clinfloxacin is not known but could be due to its activity on the bacterial cell membrane, as it is known that several persister drugs such as pyrazinamide [60], daptomycin [58], and colistin [37, 59] have activity on disrupting the bacterial membranes [61]. Further studies are needed to understand how Clinafloxacin kills persisters more effectively than other fluoroquinolones. Third, despite the impressive anti-persister activity of clinafloxacin and its compassionate use for treatment of *Burkholderia cenocepacia* infection in a cystic fibrosis patient [62], it is not an FDA approved drug because of its adverse drug reactions such as photosensitivity and hypoglycemia [63]. Thus, although our findings are encouraging, further studies are needed to address the above issues with its unique mechanism of anti-persister activity and its safety concerns in the future.

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