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**Exploring Antibiotic and Innate Immune Synergies to
Treat Multi-Drug Resistant Bacterial Infections**

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy

in

Biomedical Sciences

by

Leo Lin

Committee in charge:

Professor Victor Nizet, Chair
Professor Joe Pogliano
Professor Sharon Reed
Professor Stephen Spector
Professor Elizabeth Winzeler

2016

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Chair

University of California, San Diego

2016

DEDICATION

To my family Pannyun & Nathan, parents Athena & Brian, and siblings Clinton & Maya: thank you all for your love, care, and encouragement and to Professor Nizet: I will always remember your selfless mentoring and passion for science.

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ABSTRACT OF THE DISSERTATION

Exploring Antibiotic and Innate Immune Synergies to
Treat Multi-Drug Resistant Bacterial Infections

by

Leo Lin

Doctor of Philosophy in Biomedical Sciences

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Professor Victor Nizet, Chair

Due to the rapid rise of multidrug-resistant bacterial pathogens over the past two decades, the U.S. Centers for Disease Control and the World Health Organization both recently issued major reports warning of the entry of human medicine into a “post antibiotic era”. This growing list of pathogens now includes methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin-resistant *Enterococcus* (VRE), Carbapenem-resistant Enterobacteriaceae (CRE), multi-drug-resistant tuberculosis (MDR-TB), and many others. When treating patients with drug-resistant infections, clinicians have to resort to second and third tier antimicrobials which often have reduced efficacy, increased toxicity, or both, often leading to poorer outcomes.

However, long before a clinician diagnoses an infection and antibiotic treatment is started, our innate immune system responds to pathogens by producing potent endogenous antimicrobial peptides (AMPs) with a broad spectrum of activity. These AMPs are expressed on epithelial cell surfaces and by leukocytes in response to injury or infection. Well-characterized AMPs include cathelicidins, α - and β -defensins, and thrombocidins.

Due to the historic reliance on a single bioassay, the minimal inhibitory concentration (MIC), for testing bacterial pathogen antimicrobial susceptibility, the complex interaction between the innate immune system, antibiotics, and bacterial pathogens is often not well studied: the MIC assay contains only bacteria, bacteriologic broth, and antibiotics without any component of innate immunity.

For this PhD dissertation project, I examined the interactions between components of innate immunity system in combination with conventional antibiotics in the treatment of drug resistant bacterial pathogens. First, I discovered that the most commonly prescribed antibiotic in the United States, azithromycin, has striking efficacy in-vitro and in-vivo against extremely drug resistant Gram-negative pathogens including carbapenem resistant *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. This activity has been overlooked because azithromycin has no activity against these pathogens in standard MIC testing, but becomes extremely potent when tested in eukaryotic media or in synergy with cationic antimicrobial peptides. Secondly, I worked closely with an infectious disease fellow, Monika Kumaraswamy, who spearheaded a logical follow-up to our initial studies and discovered that azithromycin also has potent activity against another emerging multidrug-resistant

pathogen, *Stenotrophomonas maltophilia*. Finally, since preventing infection by drug resistant organisms is even better than finding a good therapy for them, I worked closely with Janie Kim, and discovered a novel formulation of multipurpose contact lens solution that has more antimicrobial efficacy against both the planktonic and biofilm forms of *Pseudomonas aeruginosa* and *Staphylococcus aureus* than anything available to contact lens wearers today. Altogether, this dissertation highlights the importance of studying the interaction between bacterial pathogens and antimicrobial therapy in more physiologic settings, especially in the context of innate immunity, and the importance of trying novel combination therapies in this era of rapidly increasing drug resistance.

CHAPTER 1

INTRODUCTION

Due to historical precedent and the intrinsic appeal of a "gold standard", a single bioassay, the minimum inhibitory concentration (MIC) has come to dominate the evaluation of antibiotic efficacy: the MIC is the key metric used for the screening and development of new antibiotic compounds, the therapeutic management of patients based on clinical isolate testing, and the establishment of hospital formularies. The MIC assay involves exposing a standard concentration of bacteria to different concentrations of antibiotics, and then measuring the optical density of the media at a later time point to determine the effect on bacterial growth.

Even though automated systems can now quickly and inexpensively screen thousands of bacteria-antibiotic combinations a day, the MIC assay has a few critical shortcomings. Since only bacteria and the antibiotics in question are combined, the MIC assay forgoes all of the complex interactions that occur between the human host and pathogens in the setting of an infection. On the host side, there is not a single component of the human immune system critical for fighting infection such as complement, leukocytes, cytokines, antimicrobial peptides, or antibodies. On the pathogen side there is no analysis of changes to basic bacteria physiology or virulence factor production such as motility, adherence to host cells, biofilm formation, toxin production, or changes to the cell membranes or wall.

This introductory review is organized into four main sections: First, we review the effects that antibiotics have on pathogens when used at sub-MIC levels or concentrations that do not alter the rate of bacteria growth (Table 1.1). Second, we review the effects

that antibiotics have on the immune system of the host (Table 1.2). Third, we review the human clinical cases and trials that have been conducted to harness the benefits of these effects (Table 1.3). Finally, due to the ever increasing threat of drug resistant bacterial infections, we review very recent approaches to treating MRSA and VRE with antibiotics that would have been rejected based on standard MIC testing.

Sub-MIC Antibiotic Effects on Bacterial Pathogens

Inhibition or Induction of Bacterial Biofilms

The first strong evidence linking chronic bacterial infection in humans to bacterial aggregation in-vivo came from studying the lungs of cystic fibrosis (CF) patients in the 1970's that were filled with mucoid strains of *Pseudomonas aeruginosa* [1]. Today, we recognize that bacterial biofilms play an extremely important role in bacteria pathogenesis in human infection [2]: biofilms are generated by most species of Gram-positive and Gram-negative pathogens, are produced by bacteria in the majority of human infection cases, confer resistance to killing by the innate and adaptive immune system and pharmacologic antibiotics and antiseptics, and enhance bacterial quorum sensing leading to increased gene transfer and virulence factor expression. Bacterial biofilms are particularly problematic for patients with foreign bodies such as catheters, prosthetic joints, or artificial valves where the only effective method for eradicating the pathogen once a biofilm has formed is the removal of the foreign body. Biofilms also lead to difficult to treat infections in the setting of compromised host defense systems such as in the lungs of CF patients [3] or the wound ulcers of diabetic patients [4]. Recently, it has been discovered that pathogenic bacterial biofilm aggregates can form even without the attachment of the bacteria to a physical surface [5].

Even though the clinical antibiotic susceptibility testing done on bacterial cultures from infected patients does not directly indicate whether or not an antibiotic has any impact on bacterial biofilm formation, multiple studies have demonstrated that sub-MIC antibiotics of different classes can significantly increase or decrease biofilm formation by pathogenic bacteria. Of all the different antibiotics, the macrolides have been found to have the most potent anti-biofilm properties. For example, azithromycin and clarithromycin at concentrations well below the MIC inhibited biofilm formation by *P.aeruginosa* in-vitro [6-8] and erythromycin treatment reduced the amount of biofilm formation in a murine model of *P.aeruginosa* lung infection leading to significantly fewer viable organisms in the mouse lungs [9]. Sub-MIC ciprofloxacin, amikacin, and colistin have also recently been reported to reduce biofilm production by pathogenic *E. coli* isolated from human urine [10]. Finally, the impact of antibiotics on *Staph aureus* biofilms is a little more complex. Sub-MIC azithromycin decreased MRSA biofilm formation in a dose dependent manner [11], while sub-MIC beta-lactams led to increased production of biofilms by clinical MRSA strains, where strains with the highest resistance to methicillin had the strongest induction of biofilm formation [12].

Suppression of Bacterial Virulence Factors

Pathogenic bacteria deploy a large arsenal of virulence factors in their battle against the host. Most of these virulence factors are bacterial toxins and enzymes which are secreted, imbedded in the bacterial membrane, or injected into host cells via multiple specialized secretion systems. These virulence factors enable bacteria to kill and manipulate host cells, resist or escape attack by the host immune system, and alter the local physiology to create a more favorable niche for the pathogen to disseminate or

survive [13]. A large and complex body of literature has emerged in regards to the effect that sub-MIC antibiotics have on bacterial virulence factor expression and production. Results differ based upon both the species of bacteria and the class of antibiotic studied, with the most compelling results coming from the study of cell wall or protein synthesis inhibitors in *S. aureus* and *P. aeruginosa*.

Sub-MIC of macrolides have been shown to suppress virulence factor production by a wide range of clinical *P. aeruginosa* isolates with azithromycin having the most potent effects [14-16]. For *S. aureus*, sub-MICs of the following protein synthesis inhibitors have been demonstrated to reduce virulence factor production: linezolid [17-19], clindamycin [19, 20], macrolides [11, 21, 22] and quinupristin/dalfopristin [22]. In contrast, sub-MICs of the cell wall antibiotics methicillin and vancomycin strongly stimulated virulence factor production in MRSA [19, 20] and VRSA[23] strains respectively. Taken as a whole, these data suggest that protein synthesis inhibitors should be considered as part of the therapeutic regime in settings where *S. aureus* or *P. aeruginosa* toxin production plays a critical role in patients' prognosis.

Inhibition of Bacterial Adherence

A critical first step in a bacteria's ability to establish a host infection is the adherence of the bacteria to host cells. Once attached, bacteria are able to exploit host cell signaling pathways, establish an extracellular presence, and or invade into host cells[24]. The macrolide class of antibiotics has been the most extensively studied for their ability to reduce bacteria adherence at sub-MICs. Low concentrations of erythromycin significantly reduced the adherence of *S. pyogenes* and *S. aureus* to oral and urinary epithelial cells [25] and of *P. aeruginosa* to collagen in-vitro[26]. Sub-MIC

clarithromycin reduced the adherence of *S. aureus* [27] and *B. pertussis* [28] to human epithelial cells. Azithromycin at low concentrations of 0.25ug/ml (1/16 MIC) and below significantly reduced adhesion of *S. aureus* and *E. coli* to human epithelial cells [29]. Finally, in an open label prospective study conducted by U. Baumann et al, 11 children with CF and chronic *P. aeruginosa* infection were given 250mg of azithromycin twice a week for an average of 3 months. Buccal epithelial cells were collected from the patients before and after azithromycin therapy, and *P. aeruginosa*'s ability to adhere to these harvested epithelial cells was tested in-vitro. After low dose azithromycin therapy, the number of *P. aeruginosa* bacteria that were able to attach to each epithelial cell was reduced in all 11 children by an average of 70%, with the effect persisting for 3 months after the therapy was stopped [30]. Overall, these studies indicate that low dose macrolide therapy could improve host barrier function and help to ameliorate the vicious cycle of infection, inflammation, and epithelial cell injury in patients suffering from chronic recurrent bacterial infections.

Reduction of Bacterial Motility and Impairment of Quorum Sensing

The ability of sub-MIC levels of antibiotics to impair of bacterial motility and quorum sensing has been the most extensively studied in *P. aeruginosa* strains exposed to macrolides. Sub-MIC erythromycin, clarithromycin, and azithromycin were all able to impair the mobility of 10 different *P. aeruginosa* clinical isolates [16]. In particular, sub-MIC azithromycin reduced *P. aeruginosa* and *P. mirabilis* motility by causing a loss of flagella in the majority of the bacterial populations [31].

K. Tateda et al first published in 2001 that sub-MIC azithromycin as low as 2ug/ml inhibited the quorum sensing circuitry of *P. aeruginosa*. Addition of synthetic autoinducers restored the activity of the las and rhl quorum sensing systems, and the authors concluded that sub-MIC azithromycin could exerts its effect by reducing *P. aeruginosa* autoinducer synthesis [32]. Sub-MIC azithromycin's impairment of *P. aeruginosa* quorum sensing, biofilm formation, and virulence factor production was subsequently confirmed by Nalca Y et al [33].

Two recent studies using murine models of infection highlighted the therapeutic efficacy of using azithromycin to treat *P. aeruginosa* infections. In a chronic lung infection model of Cfr knockout mice, azithromycin treatment attenuated *P. aeruginosa* pathology by suppressing quorum sensing regulated virulence factor production and improved clearance of pseudomonal alginate biofilms [34]. Even though very little azithromycin is renally excreted, once a day high dose azithromycin given orally or intravenously for 5 days was able to completely eradicate *P. aeruginosa* from the kidneys of mice in an experimental UTI model, while untreated mice still had >1,000 CFU of bacteria per gram of kidney tissue [35].

Enhanced Antibacterial Activity In Non-MIC Assay Conditions

The MIC assay involves mixing bacteria in bacteriologic broth with different dilutions of antibiotics with an optical density reading done at 24 hours or less. Since the macrolides have been shown to have multiple potent sub-MIC effects against *P. aeruginosa* in-vitro and in-vivo, even when the strains tested had extremely high MICs of 64ug/ml or greater, researchers have examined the effects of macrolides against *P.*

aeruginosa in assay conditions that differ from the normal MIC conditions with surprising results.

The first study demonstrating direct anti-pseudomonal activity of macrolides was published in 1996 by Tateda K et al. who showed that after 48 hours, 0.5ug/ml of azithromycin (1/128 MIC), was bactericidal against *P. aeruginosa* strain PA01 and a variety of mucoid and non-mucoid clinical isolates. The authors found that *P. aeruginosa* slowly accumulated azithromycin intracellularly from 12 to 36 hours after being exposed to a low concentration of the antibiotic. Protein synthesis began to decrease in a time dependent manner starting at 12 hours, resulting in 90% or greater kill of the initial inoculum by 48 hours. This time dependent killing was also observed for erythromycin and clarithromycin [36]. In a slightly different approach, it was discovered that 2ug/ml of azithromycin was able to kill >99% of stationary phase PA01 by 8 hours, while there was no effect bacteria in the growth phase [37]. Finally, a recent publication Buyck et al demonstrated that when azithromycin is tested against *P. aeruginosa* in eukaryotic media, bronchial lavage fluid, or human serum instead of bacteriologic broths, the MICs drop dramatically from ≥ 128 ug/ml to pharmacologically attainable concentrations of 1-16ug/ml. This drop in MICs was observed for macrolides and ketolides against multiple species of Gram-negative, but not Gram-positive bacteria [38].

In summary, when one considers all the potent sub-MIC effects that antibiotics have on bacterial pathogens and the dramatically enhanced activity of some antibiotics in non-MIC assay conditions, it is likely that existing antibiotics and novel compounds with therapeutic potential are being overlooked.

Table 1.1 Sub-MIC Antibiotic Effects on Bacterial Pathogens

Antibiotic Class	Biofilms	Virulence Factor Production	Adherence	Motility	Quorum Sensing	Increased Activity in Non-MIC Assay Conditions
Macrolides		↓ <i>S. aureus</i> [11, 21, 22]				<i>P. aeruginosa</i> [36, 37] Gram-Negative Bacteria [38]
Azithromycin	↓ <i>P. aeruginosa</i> [6-8, 34] ↓ MRSA [11]	↓ <i>P. aeruginosa</i> [14-16, 34]	↓ <i>P. aeruginosa</i> [30] ↓ <i>E. coli</i> [29] ↓ <i>S. aureus</i> [29]	↓ <i>P. aeruginosa</i> [16, 31] ↓ <i>P. mirabilis</i> [31]	↓ <i>P. aeruginosa</i> [32-34]	
Clarithromycin	↓ <i>P. aeruginosa</i> [6-8]		↓ <i>S. aureus</i> [27] ↓ <i>B. pertussis</i> [28]	↓ <i>P. aeruginosa</i> [16]		
Erythromycin	↓ <i>P. aeruginosa</i> [9]		↓ <i>S. aureus</i> [25] ↓ <i>S. pyogenes</i> [25] ↓ <i>P. aeruginosa</i> [26]	↓ <i>P. aeruginosa</i> [16]		
Protein Synthesis Inhibitors						
Linezolid		↓ <i>S. aureus</i> [17-19]				
Clindamycin		↓ <i>S. aureus</i> [19, 20]				
Quinupristin/ Dalfopristin		↓ <i>S. aureus</i> [22]				
Beta-lactams						
Methicillin	↑ MRSA [12]	↑ MRSA [19, 20]				
Cell Wall Inhibitors						
Vancomycin		↑ VRSA [23]				
Fluoroquinolones						
Ciprofloxacin	↓ <i>E. coli</i> [10]					
Aminoglycosides						
Amikacin	↓ <i>E. coli</i> [10]					
Antimicrobial Peptides						
Colistin	↓ <i>E. coli</i> [10]					

Antibiotic Effects on the Immune System of the Host

Decreased Inflammation

Excessive inflammation or dysregulation of normal inflammatory pathways play a key role in the pathogenesis of many diseases such as sepsis, acute respiratory distress syndrome, transplant rejection, asthma, cancer, reperfusion injury, and many others. Out of all the different classes of antibiotics, the macrolides and tetracyclines have been the most extensively studied in-vitro and in-vivo for their anti-inflammatory effects.

Erythromycin [39] and clarithromycin [40] reduced the production of IL-8 by cultured human epithelial cells via suppression of NF-kappa B pathway. Clarithromycin [41] or roxithromycin [42] also suppressed cytokine production by human monocytes stimulated by LPS. In a pathogen free model of rat lung injury, Tamaoki J et al demonstrated that inhalation of LPS alone was enough to trigger excessive lung inflammation leading to neutrophil recruitment, microvascular leakage, and acute lung injury. Pre-treatment of the rats with oral erythromycin, but not ampicillin or cefaclor, reduced in a dose-dependent manner the amount of neutrophil infiltration and vascular leakage [43].

These macrolide anti-inflammatory effects observed in cell culture and animal models have also been confirmed in human patient samples. Patients with chronic obstructive pulmonary disease (COPD) have elevated levels of the neutrophil chemoattractant proline-glycine-proline (PGP) in their sputum. Patients treated with chronic low dose azithromycin, but not the untreated controls, demonstrated a reduction sputum PGP that was accompanied by a lower neutrophil burden in the lungs and a reduced frequency of exacerbations [44]. In another study of patients suffering from

neutrophilic chronic lung allograft dysfunction, those who received 3 to 6 months of azithromycin therapy demonstrated a significant decrease in the amount of IL-8 and neutrophils in their bronchoalveolar lavage fluid and had an increase in FEV1 [45].

Similar to the macrolides, the tetracyclines have been shown to have potent anti-inflammatory effects in-vitro and in-vivo. Tetracyclines have been well studied for their ability to inhibit metalloproteinases (MMPs) [46, 47] which is probably due to their ability to act as magnesium and calcium chelators [48]. Excessive connective tissue breakdown by MMPs is a key aspect of many inflammatory disorders such as rheumatoid arthritis (RA), and minocycline therapy in multiple double blind clinical trials proved to be an effective therapy for RA patients [49-51]. Minocycline and doxycycline have also been shown to reduce the synthesis of inflammatory prostaglandins by phospholipase A2 (PLA2) [52] by directly binding to the enzyme [53]. Like the macrolides, tetracyclines inhibited leukocyte chemotaxis [54], LPS induced pro-inflammatory cytokine production [55], and rescued mice from LPS challenge induced shock [56, 57]. Finally, unlike the macrolides, tetracyclines have also been shown to reduce ischemia induced inflammation and damage in the brains of gerbils [58] and rats [59].

Increased Leukocyte Phagocytosis of Bacterial Pathogens

In 1981, Gemmell CG et al were among the first to demonstrate that sub-MIC antibiotic treatment could potentiate opsonization and phagocytosis of bacterial pathogens. The authors found that treatment of an M-protein positive strain of *S. pyogenes* with sub-MIC clindamycin resulted in significantly enhanced phagocytosis and killing by purified human neutrophils and monocytes. Additional assays revealed a more denuded bacterial surface, attributed to decreased M-protein, and increased C3

complement deposition [60]. Follow up studies by the same group demonstrated that sub-MIC clindamycin also increased opsonization and phagocytosis of *S. aureus* and *B. fragilis* [61] and sub-MIC linezolid significantly increased phagocytosis of *S. pyogenes* and *S. aureus* by human neutrophils [17]. More recently, Takeoka K et al demonstrated that the sub-MICs of the macrolide antibiotics, especially azithromycin, increased human neutrophil phagocytosis of *P. aeruginosa* when it was in a biofilm state, but not when it was in its planktonic form [62].

Increased Human Serum and Antimicrobial Peptide Killing of Bacterial Pathogens

Human serum contains complement, antibodies, and antimicrobial peptides, all which play important roles in the host immune defense against bacterial infections. Even though no component of human serum is present in the MIC assay, multiple studies have demonstrated that the macrolide antibiotics can synergize with human serum in the killing of bacterial pathogens. Sub-MIC erythromycin significantly enhanced serum killing of multiple *P. aeruginosa* clinical isolates [63, 64], and Pruul H. et al demonstrated that when MIC assays were conducted in 40% human serum, the MIC of azithromycin against serum-resistant *E. coli* and *S. aureus* decreased by 26 and 15-fold respectively. This synergy between azithromycin and human serum persisted at a wide range of pH values and even when the serum was heat inactivated or antibody depleted [65]. Finally, treating primary cultured human tracheal epithelial cells with erythromycin increased the amount of human beta-defensin 1 and 2 in the airway surface liquid recovered from the cells. This antimicrobial peptide enriched airway surface fluid had >90% increased killing of MRSA and *P. aeruginosa* compared to untreated controls [66].

In summary, the macrolides and tetracycline antibiotics have potent effects on the host immune system that are not assessed by MIC-assays. Of note, even in murine models where bacteria free preparations of LPS are used, the macrolides and tetracyclines can protect the host against excessive inflammatory damage and death [43, 56, 57]. The next section of this review will focus on the clinical trials that have been conducted based upon the anti-inflammatory and sub-MIC antibacterial properties of the antibiotics reviewed above.

Table 1.2 Antibiotic Effects on the Immune System of the Host

Antibiotic Class	Inflammation	Leukocyte Phagocytosis	Human Serum Killing	Antimicrobial Peptide Killing
Macrolides		↑ <i>P. aeruginosa</i> [62]		
Azithromycin	↓ Sputum PGP & myeloperoxidase in COPD [44] ↓ BAL cytokines & PMNs in lung transplant [45]		↑ <i>S. aureus</i> [65] ↑ <i>E. coli</i> [65]	
Clarithromycin	↓ Cytokines [40, 41]			
Erythromycin	↓ Cytokines [39, 43] ↓ PMN chemotaxis [54]		↑ <i>P. aeruginosa</i> [63, 64]	↑ <i>P. aeruginosa</i> [66] ↑ MRSA [66]
Roxithromycin	↓ Cytokines [42]			
Tetracyclines				
Tetracycline	↓ MMP activity [46, 47] ↓ Cytokines [56] ↓ Ischemic Inflammation [58] ↓ PMN chemotaxis [54] ↓ Cytokines [57]			
Minocycline	↓ Inflammation in RA [49-51] ↓ Prostaglandins [52, 53] ↓ Ischemic Inflammation [59]			
Doxycycline	↓ Prostaglandins [52, 53] ↓ Cytokines [55]			
Protein Synthesis Inhibitors				
Clindamycin		↑ <i>S. pyogenes</i> [60] ↑ <i>S. aureus</i> & <i>B. fragilis</i> [61]		
Linezolid		↑ <i>S. pyogenes</i> & <i>S. aureus</i> [17]		

Clinical Trials Harnessing the Sub-MIC Antibacterial and Host Effects of

Antibiotics

Hundreds of completed and ongoing human clinical trials have been conducted to take advantage of the sub-MIC antibacterial and host anti-inflammatory and immune boosting effects of different antibiotics. Due to the tetracyclines well documented anti-inflammatory properties and in particular minocyclines ability to readily cross the blood brain barrier, there are currently over 200 ongoing clinical trials evaluating the efficacy of the tetracyclines in nervous system, immune system, and behavioral and mental diseases [67, 68]. In this review, we will focus on clinical trials of the macrolide class of antibiotics because they have been studied primarily in human disorders in which infection and inflammation play important roles in the disease pathogenesis and patient prognosis.

Azithromycin for Cystic Fibrosis

Cystic fibrosis (CF) is the second most common life-shortening inherited disorder occurring in children in the US. Approximately 30,000 Americans have CF, and the overall birth prevalence in the US is 1 in 3,700 [69]. For each of the ~1,000 new cases of CF diagnosed in the US each year, the average life expectancy of a CF patient is still just 37 years, even with all the recent medical advances that have been made [70]. CF pathogenesis is characterized by the absence of a normal CFTR protein which leads to loss of airway surface liquid and impaired mucociliary clearance in the lungs. Chronic infection, particularly by *P. aeruginosa*, and inflammation of the lungs leads to irreversible bronchiectasis, and 90% of CF patients will die of respiratory failure unless they receive a lung transplant [71].

Even though azithromycin has extremely high and not physiologically attainable MICs against *P. aeruginosa* in standard MIC assay conditions [38], its anti-inflammatory and sub-MIC anti-pseudomonal properties reviewed above led to the first randomized placebo-controlled crossover trial of azithromycin in children with CF published in *The Lancet* in 2002 [72]. This small trial of 41 children demonstrated that oral azithromycin treatment increased by a small but significant amount the FEV1 of treated patients versus controls. Furthermore, the treated patients had fewer exacerbations requiring extra oral antibiotic when they were taking azithromycin. Four larger randomized clinical trials of azithromycin in CF patients then followed [73-76]. When data from all five trials are pooled together and analyzed, azithromycin treatment led to a modest increase in pulmonary function, and patients treated with azithromycin for at least 6 months were twice as likely to be free of respiratory exacerbations compared to placebo [77]. Due to these successful CF clinical trials, azithromycin is now part of the standard of care for children with CF.

Azithromycin for Chronic Allograft Lung Transplant Dysfunction

Lung transplant remains the last life-saving option for many patients suffering from end-stage pulmonary disease such as COPD, idiopathic pulmonary hypertension, and CF. Even though advances in surgical techniques and medical care have steadily improved post-transplant outcomes, long term survival of lung transplant patients still lags behind many other solid organ transplant procedures, with a current median survival rate of only 5.7 years [78]. The major hurdles to long-term graft and patient survival are primary graft dysfunction, infection, and bronchiolitis obliterans syndrome (BOS). BOS is the main culprit, as it will affect ~50% of all lung transplant recipients by 5 years [78].

BOS arises from a host inflammatory response to the transplanted epithelium where excessive repair mechanisms cause narrowing and destruction of the small airways, leading to progressive and irreversible airway obstruction.

Due to azithromycin's anti-inflammatory effects, and the human clinical trials demonstrating the ability of macrolides to improve outcomes and survival in patients with CF [77] and diffuse panbronchiolitis [79], the first 6 patient human pilot study of azithromycin in BOS was conducted in 2003 [80]. 5 of the 6 patients treated with low dose oral azithromycin for an average of 13.7 weeks responded to the macrolide therapy with a significant mean increase in FEV1 of 17%. Multiple follow up studies including more open label trials and larger retrospective cohort studies [45, 81-86] demonstrated that BOS patients treated with azithromycin had improved lung function and increased survival. Compared to non-responders, patients with the best response started macrolide therapy earlier post-transplant and had higher pre-treatment BAL neutrophilia and IL-8 levels [81]. Given these promising results, a large scale randomized controlled trial of azithromycin for BOS is indicated.

Clarithromycin for Sepsis

There are ~1,000,000 cases of sepsis resulting in ~200,000 deaths each year in the United States alone. Sepsis remains the number one cause of death for hospitalized patients, and unlike other major epidemic illnesses, treatment is non-specific and limited to support of organ function through administration of fluids, antibiotics, and oxygen [87]. Sepsis is a complex multi-factorial disorder recently summarized well as "the host's deleterious, non-resolving inflammatory response to infection that leads to organ dysfunction [88]." Due to the anti-inflammatory effects of macrolides reviewed above,

and in particular clarithromycin's ability to reduce inflammation and improve outcomes in Gram-negative animal models of sepsis [89, 90], a double blind randomized clinical trial of clarithromycin use in 200 patients with sepsis and ventilator-associated pneumonia (VAP) was conducted and published in 2008. Giamarellos-Bourboulis EJ et al found that 1 gram of clarithromycin given once a day for 3 consecutive days accelerated the resolution of VAP and weaning from ventilation in surviving patients and delayed death in those who died of sepsis. Although clarithromycin also significantly reduced the risk of death from septic shock and multi-organ dysfunction (MODS), there was no overall difference in mortality between treated patients and controls [91, 92]. This first trial was followed by another larger double blind randomized clinical trials of 600 patients [93] which demonstrated similar results: in patients with Gram-negative infections, clarithromycin treatment shortened the time to resolution of VAP and of mechanical ventilator weaning and improved survival of patients with septic shock and MODS. These trials indicate that clarithromycin holds promise as an adjunctive therapy in patients with severe Gram-negative infections.

Azithromycin for Chronic Bacterial Prostatitis

There are approximately 1.26 cases of chronic bacterial prostatitis (CBP) per 1,000 men per year in the US [94]. CBP has a large negative impact on a patient's quality of life and common symptoms include perineal and testicular pain, irritative and obstructive bladder symptoms, and ejaculatory pain and sexual dysfunction. Even though macrolides are not considered a first line option for patients with CBP, a recent review highlighted the increasing importance of macrolide therapy [95]. Of note, azithromycin achieved high concentrations in prostatic tissue with ~2.5 ug/ml being reached 14 hours

after just two 250mg oral doses spaced 12 hours apart. It also had an extremely long half-life of 60 hours in the prostate [96]. Due to this favorable dosing profile, and the ability of macrolides to address two critical targets in CBP, bacterial biofilms and intracellular bacteria, mono and combination therapy trials of azithromycin for CBP have been conducted. In clinical trials, azithromycin was more effective than ciprofloxacin at eradicating CBP caused by *C. trachomatis* [97], and azithromycin in combination with ciprofloxacin, alfuzosin, and *S. repens* extract resulted in high clinical cure rates (~84%) against both traditional and unusual uropathogens [98, 99]. Finally, a recent case report highlighted the successful treatment of a CBP patient with recurrent infection by a multi-drug resistant strain of *E. coli* [100]. The 69 year old male patient developed *E. coli* prostatitis after a transrectal prostate biopsy with ciprofloxacin prophylaxis. The infection persisted and recurred even after multiple courses of treatment with ceftriaxone, amoxicillin/clavulanate, and gentamicin. During his last treatment course, the patient remained febrile even after receiving 2 grams of ceftriaxone and 7 mg/kg of gentamicin. Gentamicin was discontinued and 500mg of daily oral azithromycin was added. The patient became afebrile within 24 hours, and after 1 week of ceftriaxone and 3 weeks of azithromycin, the patient remained relapse free. 2 months after the finishing his course of azithromycin, the patients prostate was removed due to prostate cancer and histological examination of the prostatic tissue was negative for any evidence of infection.

Azithromycin for COPD, VAP Prevention, and Asthma

Azithromycin has been demonstrated to reduce pathogenic inflammation and have potent sub-MIC antibacterial properties in-vitro and in animal models. These data, along with the positive clinical trials results from azithromycin use in patients suffering from a

variety of disorders such as CF and BOS, has led to its study in very recently published trials where inflammation and infection play critical roles disease pathogenesis. In 2011, data from a clinical trial of 1,142 COPD patients randomly assigned to take low dose oral azithromycin daily for one year versus control was published in the New England Journal of Medicine [101]. Macrolide therapy led to a significant decrease in the frequency of exacerbations and increase in the quality of life, but there was also a small increase in hearing loss in the treated group. In 2012, data from a pilot clinical trial of 85 intubated patients colonized with *P. aeruginosa* randomly assigned to low dose azithromycin for maximum of 20 days was published in Intensive Care Medicine [102]. In this small study population, macrolide therapy resulted in a trend of reduced incidence of VAP in all treated patients, and significantly prevented VAP in patients colonized by more virulent rhamnolipid producing *P. aeruginosa* strains. Finally, a 2013 pilot clinical trial of 109 severe asthma patients randomly assigned to low dose oral azithromycin for 26 weeks was published in Thorax [103]. Though macrolide therapy did not significantly reduce the amount of severe exacerbations and or acute infections requiring antibiotic treatment in the total study population, it did significantly improve outcomes for the predefined sub-group of patients with non-eosinophilic or primarily neutrophilic asthma - 56 out of the total 109 patients. Of note, there was no increased incidence of hearing loss in the treatment group, and though there was a significant increase in colonization of treated patients with erythromycin-resistant streptococci, this did not lead to an increased risk of lower respiratory tract infection or pneumonia.

Table 1.3 Clinical Trials Harnessing the Sub-MIC Antibacterial and Host Effects of Antibiotics.

Disease	Type of Study (Number of Patients) [Ref]	Therapy	Proposed Mechanism	Results of Therapy	Treatment Related Side Effects
Cystic Fibrosis	1. RCT (41) 2002 [72] 2. RCT (61) 2002 [75] 3. RCT (185) 2003 [76] 4. RCT (82) 2006 [74] 5. RCT (260) 2010 [73]	Oral AZM	↓ Inflammation ↓ <i>P. aeruginosa</i> biofilm Anti-pseudomonal activity	1. ↑ FEV1 5.4% ↓ Antibiotic use 2. Maintain FEV1 ↓ Antibiotic use and CRP ↑ QOL 3. ↑ FEV1 0.097-L ↓ Exacerbations ↑ Weight 0.7 kg 4. ↓ Exacerbations ↓ Antibiotic use 5. ↓ Exacerbations ↑ Weight 0.58 kg	1. No serious 2. No serious 3. Minor ↑ in nausea, diarrhea, wheezing 4. No serious 5. No serious
Chronic Allograft Lung Transplant Dysfunction	1. Open label (6) 2003 [80] 2. Open label (8) 2004 [86] 3. RSS (20) 2005 [85] 4. Open label (11) 2005 [84] 5. Open label (14) 2006 [83] 6. RSS (178) 2010 [82] 7. RSS (107) 2010 [81] 8. Open label (20) 2011 [45]	Oral AZM	↓ Inflammation ↑ FEV1 & ↓ Exacerbations in CF patients	1. ↑ FEV1 17% in 5/6 treated patients 2. ↑ FEV1 18% 3. ↑ FEV1 110ml after 3 months of AZM 4. No change in FEV1, may slow disease progression 5. 6/14 patients ↑ FEV1 10% ↓ BAL neutrophilia 6. ↓ Mortality 7. ↑ Pulmonary function ↓ Mortality 8. ↑ FEV1 25% ↓ BAL neutrophilia and MMP-9	No serious
Sepsis	1. RCT (200) 2008 [91] 2. RCT (600) 2014 [93]	IV CLARI	↓ Inflammation ↑ Survival in animal models of Gram-negative sepsis	1. ↓ Time to VAP resolution and ventilator weaning 2. ↓ Time to infection resolution ↓ Mortality in patients with septic shock and MODS	No serious
Chronic Bacterial Prostatitis	1. Randomized Trial (89) 2003 [97] 2. Prospective Trial (137) 2007 [99] 3. Prospective Trial (104) 2007 [98] 4. Case Report (1) 2011 [100]	1. AZM vs Cipro 2. AZM + Cipro 3. AZM + Cipro 4. AZM salvage	High prostate concentrations Good intracellular activity Anti-biofilm properties	1. AZM 69% clinical cure vs Cipro 34% 2. Effective in 20% of initial non-responders 3. Eradicated traditional and unusual uropathogens 4. Addition of AZM cured patient with MDR <i>E. coli</i> that had failed all other therapy	1. No serious 2. Minor ↑ diarrhea 3. No serious 4. No serious
COPD	RCT (1142) 2011 [101]	Oral AZM	↓ Inflammation Activity against lung pathogens	↓ Exacerbations ↑ QOL	Small ↑ in # of pts with hearing loss ↑ AZM resistant nasopharynx colonizers
VAP Prevention	RCT (85) 2012 [102]	IV AZM	Impair <i>P. aeruginosa</i> quorum sensing	Trend of ↓ VAP - significant in pts colonized with more virulent rhamploid producing strains	1 pt with bradycardia and bronchospasm
Asthma	RCT (109) 2013 [103]	Oral AZM	↓ Inflammation ↓ Neutrophilia	↓ Exacerbations and/or LRTI in pts with non-eosinophilic (neutrophilic) severe asthma	No serious
MRSA Bacteremia	Clinical case series (7) 2011 [109]	Beta-lactam + Daptomycin	Beta-lactam/Daptomycin "Seesaw" Effect	Clearance of refractory/persistent bacteremia in all pts within 24-48 hours	No serious
Enterococcal Endocarditis	Case Report (1) 2013 [113]	Cefaroline + Daptomycin	Cefaroline Synergy with Antimicrobial Peptides	Clearance of recurrent aminoglycoside resistant <i>E. faecalis</i> endocarditis	No serious

Abbreviations: RCT = Randomized Controlled Trial, RSS = Retrospective Study, AZM = Azithromycin, CLARI = Clarithromycin, QOL = Quality of Life

Novel Strategies for Treating MRSA and VRE

Recent reports by the U.S. Centers for Disease Control [104] and the World Health Organization [105] describe an ever-worsening antibiotic resistance crisis. Of particular concern to the Infectious Disease Society of America are the drug resistant ESKAPE pathogens: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp [106]. A multifaceted approach of improved antibiotic stewardship, infection control, and new therapies and treatment strategies are needed to stem this rising threat to public health. Here we review very recent strategies for treating two of the drug resistant ESKAPE pathogens *E. faecium* and *S. aureus* which utilize antibiotics that would have been rejected based on MIC results from clinical bacterial culture and susceptibility testing.

S. aureus is a leading cause of human infections worldwide, and in the US, MRSA is a leading cause of death by any single infectious agent [107]. In 2004, it was first published that oxacillin demonstrated synergy with daptomycin against 18 out of 18 randomly selected clinical MRSA isolates, even though all of the isolates were oxacillin resistant with most MICs being >256 ug/ml. Further research by Yang SJ et al demonstrated a “seesaw effect” when oxacillin was used in combination with daptomycin against MRSA. When clinical MRSA strains developed resistance to daptomycin, their susceptibility to oxacillin increased by 3 to 4 fold in-vitro even though *mecA* which confers resistance to beta-lactams was still present. When these daptomycin resistant MRSA strains were used in a rabbit model of aortic endocarditis, daptomycin and oxacillin combination therapy cleared the bacteria from the heart valve, kidneys, and

spleen, when monotherapy with either agent alone was futile [108]. In 2011, these in-vitro and animal results were finally put to the test in a few human patients with stunning results: 7 patients with persistent and or refractory MRSA bacteremia who had failed both 1st line therapy with vancomycin and 2nd line therapy with daptomycin +/- gentamycin were treated with a combination of daptomycin plus a beta-lactam. 7 patients had their bacteremia clear within 24 hours and the last patient's bacteremia cleared within 48 hours [109]. This year, Sakoulas G et al demonstrated that the host innate immune system also acts synergistically with beta-lactams in eradicating MRSA. Even though beta-lactam treatment did not significantly inhibit the growth of MRSA strains, it dramatically increased their susceptibility to killing by human whole blood, neutrophils, and antimicrobial peptides. Furthermore, MRSA treated with sub-MIC beta-lactams became much less virulent in a murine soft tissue infection model [110].

Recent studies have also unveiled a similar story for drug resistant *Enterococcus*. Ampicillin has no effect on the growth rate of VRE. However, it synergized in-vitro with daptomycin against 19 out of 19 VRE clinical isolates [111] and with human cationic human antimicrobial peptides such as LL-37 and alpha defensin by reducing VRE's net positive surface charge [112]. Further in-vitro studies showed that another cell wall active antibiotic ceftaroline was able to achieve synergy with daptomycin at even lower concentrations than ampicillin, even though both antibiotics had MICs that were >32 mg/ml against a clinical vancomycin and gentamycin resistant *E. faecalis* strain [113]. This extremely drug resistant *E. faecalis* isolate was obtained from a patient with *E. faecalis* endocarditis that was successfully treated with daptomycin plus ceftaroline as salvage therapy, after failing all other therapies based on the current standards of care.

SPECIFIC AIMS

The in-vitro, animal, and clinical studies reviewed above demonstrate that if we rely solely on MIC assays to guide our choice of antibiotic therapy and research, then we will fail to harness the benefits of any compounds that synergize with our immune system or other antibiotics, if they do not inhibit bacterial growth rates when tested alone. The main purpose of this dissertation is to further explore the interaction of conventional antibiotics with the human innate immune system in the treatment of multidrug-resistant bacterial pathogens. *I hypothesize that many potential life-saving antibiotic therapies are overlooked because they do not have a significant impact on bacterial growth rates when tested in bacteriologic media alone, without components of innate immunity.* More specifically, I will examine three areas using our established in-vitro assays and multiple mouse models of bacterial infection:

Aim 1: Identify synergistic drug and innate immune interactions between leading pharmaceutical antibiotics and host antimicrobial peptides, serum, and immune cells against leading drug-resistant bacterial pathogens.

Aim 2: Determine whether or not the synergies identified in Aim 1 can be harnessed to treat mice infected with drug-resistant bacterial pathogens.

Aim 3: Explore novel therapeutic combinations of antimicrobial agents in the prevention and treatment of drug-resistant bacterial infections.

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CHAPTER 2

Azithromycin Synergizes with Cationic Antimicrobial Peptides to Exert Bactericidal and Therapeutic Activity Against Highly Multidrug-Resistant Gram-Negative Bacterial Pathogens

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PREFACE TO CHAPTER 2

Chapter 2 in full is an article published in *EBioMedicine*. The work in Chapter 2 addresses both Aim 1: identify synergistic drug and innate immune interactions between leading pharmaceutical antibiotics and host antimicrobial peptides, serum, and immune cells against leading drug-resistant bacterial pathogens; and Aim 2: determine whether or not the synergies identified in Aim 1 can be harnessed to treat mice infected with drug-resistant bacterial pathogens. Using a combination of quantitative in-vitro assays, fluorescent and electron microscopy, and multiple murine models of infection, we demonstrated that azithromycin had potent activity against MDR *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* when tested in eukaryotic media, synergized with cationic antimicrobial peptides such as LL-37 and colistin, and substantially reduced the bacterial burden and mortality of mice infected with MDR Gram-negative rods.

ABSTRACT

Antibiotic resistance poses an increasingly grave threat to the public health. Of pressing concern, rapid spread of carbapenem-resistance among multidrug-resistant (MDR) Gram-negative rods (GNR) is associated with few treatment options and high mortality rates. Current antibiotic susceptibility testing guiding patient management is performed in a standardized manner, identifying minimum inhibitory concentrations (MIC) in bacteriologic media, but ignoring host immune factors. Lacking activity in standard MIC testing, azithromycin (AZM), the most commonly prescribed antibiotic in the U.S., is never recommended for MDR GNR infection. Here we report a potent bactericidal action of AZM against MDR carbapenem-resistant isolates of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *Acinetobacter baumannii*. This pharmaceutical activity is associated with enhanced AZM cell penetration in eukaryotic tissue culture media and striking multi-log-fold synergies with host cathelicidin antimicrobial peptide LL-37 or the last line antibiotic colistin. Finally, AZM monotherapy exerts clear therapeutic effects in murine models of MDR GNR infection. Our results suggest that AZM, currently ignored as a treatment option, could benefit patients with MDR GNR infections, especially in combination with colistin.

INTRODUCTION

Hospital-acquired infections, half caused by drug-resistant bacteria [1], cause ~99,000 deaths annually and increase healthcare costs by \$5-10 billion in the U.S. alone [2]. Recent reports by the U.S. Centers for Disease Control and Prevention [3] and the World Health Organization [4] describe this ever-worsening antibiotic resistance crisis, highlighting the “urgent threat” of emerging carbapenem-resistant Gram-negative rods (GNRs) that forebode the entry of human medicine into a “post-antibiotic era”. Rapid spread of carbapenem resistance in *Pseudomonas aeruginosa* (*PA*), *Klebsiella pneumoniae* (*KP*) and *Acinetobacter baumannii* (*AB*) is of particular concern as effective antibiotic candidates are currently lacking in the development pipeline [5].

Unconventional approaches to infectious disease treatment are gaining more attention, including virulence factor inhibition, bacteriophage therapy, probiotics and immune boosting [6, 7]. Along these lines, we have probed interactions of conventional antibiotics with antimicrobial effectors of the innate immune system, with encouraging results. Drugs with no direct activity in standard minimum inhibitory concentration (MIC) testing nevertheless sensitized multidrug-resistant (MDR) bacterial pathogens to human antimicrobial peptide killing *in vitro*, e.g. nafcillin vs. methicillin-resistant *Staphylococcus aureus* (MRSA) or ampicillin vs. vancomycin-resistant *Enterococcus* (VRE), and contributed to clinical resolution in refractory infections [8, 9].

Our recent experiences with β -lactams and MRSA or VRE indicate that simple MIC testing overlooks potential synergies with cationic antibiotics (e.g. daptomycin) and host AMPs (e.g. human cathelicidin LL-37) that promote bactericidal activity *in vitro* and bacterial clearance in patients [8, 9]. We asked whether similar phenomena could be

identified in MDR-GNRs to challenge conventional antibiotic treatment paradigms. AZM, the most commonly prescribed antibiotic in the U.S. (51.5 million in 2010) [10], is never recommended for inpatient treatment of serious GNR infections because of poor or absent *in vitro* activity by standard MIC testing in bacteriologic media. However, antibacterial activity of AZM is enhanced in mammalian tissue culture media vs. standard bacteriologic media [11], a finding reminiscent of observations we made for LL-37 [12], prompting us to examine its interaction with MDR GNRs more closely.

MATERIALS AND METHODS

2.1. Bacterial Strains

P. aeruginosa (PA) strain PA01, *K. pneumoniae* (KP) strain K700603, and *A. baumannii* (AB) strain AB19606 were obtained from the American Type Culture Collection (ATCC). Human clinical MDR isolates PA P4 (lung) and KP K1100 (lung) [13] were obtained from a tertiary academic hospital in the New York metropolitan area. MDR-AB AB5075 (bone) [14] was obtained from Walter Reed Army Medical Center. All three MDR strains were independently identified and subject to antibiotic susceptibility testing by the clinical microbiology laboratory at the San Diego Veterans Affairs Hospital (**table S1**). Additional MDR GNR clinical isolates tested were also obtained from a tertiary academic hospital in the New York metropolitan area, except for PA USCD P1, which was obtained from the UC San Diego hospital system. Bacteria were grown overnight in Luria Broth (LB), glycerol was added (30% final), and stocks stored at -80°C. Fresh colonies were streaked onto LB plates each week for all experiments.

2.2. Antibiotics and Antimicrobial Peptides

For *in vitro* studies, AZM, colistin sulfate, and ciprofloxacin were purchased from Sigma-Aldrich; erythromycin and clarithromycin were purchased from Fischer Scientific. Stock solutions were prepared in phosphate buffered saline (PBS) at 2,560mg/L for the macrolide antibiotics, 1,000mg/L for colistin, and 10,000mg/L for ciprofloxacin. Trace amounts of glacial acetic acid were used to prepare AZM, erythromycin and clarithromycin stocks for complete solubility [15]. LL-37 and TAMRA-tagged LL-37 were purchased from the American Peptide Company; stock solutions were prepared in molecular quality water (Corning Cellgro) at 640 μ M and 320 μ M respectively and stored at -80° C. For *in vivo* studies, AZM for human injection (Sagent Pharmaceuticals) was reconstituted per manufacturer's guidelines [16]. Pooled human serum was obtained from six healthy consented lab volunteers and immediately aliquoted and stored at -80° C.

2.3. Reagents

Mueller-Hinton Broth (MHB, Spectrum Chemicals) was supplemented with CaCl_2 and MgCl_2 to make cation-adjusted MHB (Ca-MHB) – final cation concentrations (20–25 mg/L Ca^{2+} and 10–12.5 mg/L Mg^{2+}). Luria Broth base (LB) was purchased from Hardy Diagnostics. RPMI-1640 was purchased from Invitrogen. Clear phenol free RPMI-1640 used for microscopy studies was purchased from Corning Cellgro. 1-N-phenylnaphthylamine (NPN) was purchased from Sigma-Aldrich and a fresh stock of 500 μ M in acetone was made each week.

2.4 MIC Determinations

MIC values for AZM, erythromycin, clarithromycin, ciprofloxacin, colistin, and LL-37 were determined using broth microdilution in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines [17][17] using Ca-MHB media, the

recommended bacteriologic broth, or eukaryotic cell culture media RPMI-1640 supplemented with 5% LB [9].

2.5. Time-Kill Curves and Serum Survival Assays

Time-kill studies, $\pm 20\%$ pooled human serum, were performed as previously described [18]. Bacteria were grown overnight in LB at 37°C with shaking. Bacterial stocks in PBS were prepared by washing the overnight cultures twice with PBS via centrifugation at 3220 x g at room temperature with a final re-suspension in PBS to an $OD_{600}=0.40$. Bacterial stocks in PBS were diluted in Ca-MHB or 5%LB-RPMI to an initial inoculum of 1×10^6 CFU/ml (standard time-kill) or 5×10^4 CFU/ml (serum survival). AZM, erythromycin, clarithromycin, colistin, and LL-37 stocks were diluted in Ca-MHB or 5%LB-RPMI to the assay concentrations indicated. For serum studies, AZM 0.5mg/L was chosen in order to approximate human plasma concentrations upon intravenous administration of 500mg of AZM [16]. Assays were conducted in triplicate in a final volume of 200 μ l in 96-well round bottom plates (Costar) $\pm 20\%$ pooled human serum. The 96-well plates were wrapped in paraffin and placed in a shaking incubator at 37°C. Aliquots were collected at the indicated times and serially diluted for CFU enumeration; limit of detection = CFU/ml.

2.6. Electron Microscopy of Bacterial Gross Morphology

Transmission electron microscopy was performed essentially as described [19]. MDR-*AB* was grown overnight in LB at 37°C with shaking. Bacterial stocks in PBS were prepared by washing the overnight cultures twice with PBS and resuspending in PBS to $OD_{600}=0.40$. Then 2.5ml of each bacterial stock was added to 47.5ml of Ca-MHB or RPMI+5%LB media pre-warmed to 37°C. For treatment, the AZM stock was diluted to a

concentration of 0.5mg/L in the final volume of 50 ml. 50ml cultures were placed in a shaking incubator at 37°C for 2h. Cultures were then centrifuged at 3220xg at room temperature for 10 min. The supernatant was aspirated and bacterial pellets re-suspended in 1ml of PBS. These 1ml samples were immersed in modified Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15M sodium cacodylate buffer, pH 7.4) for at least 4h, post-fixed in 1% osmium tetroxide in 0.15M cacodylate buffer for 1h, and stained en bloc in 2% uranyl acetate for 1h. Samples were dehydrated in ethanol, embedded in Durcupan epoxy resin (Sigma-Aldrich), sectioned at 50-60nm on a Leica UCT ultramicrotome, and picked up on Formvar and carbon-coated copper grids. Sections were stained with 2% uranyl acetate for 5min and Sato's lead stain for 1min. Grids were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope and photographs were taken with an Eagle 4k HS digital camera (FEI). Images were taken from multiple random fields at 1,200X, 2,900X, 23,000X; and gross morphology was analyzed in a blinded fashion.

2.7. Fluorescence Microscopy for MDR-AB Cytological Profiling

The fluorescence microscopy studies for MDR-AB were performed as previously described [20]. These microscopy studies required higher concentrations of AZM because the concentration of MDR-AB used was 100X higher (5×10^7 CFU/ml) compared to the concentration used in the MIC assays (5×10^5 CFU/ml). All AZM concentrations used in these studies are pharmacologically obtainable in human tissue. Single MDR-AB colonies were picked from LB plates and grown in LB or RPMI+5%LB overnight. Overnight cultures were then diluted 1:100 into fresh Ca-MHB or RPMI+5%LB media. When an $OD_{600} = 0.20$ was reached, antibiotics were added to

exponentially growing bacteria. Cultures were placed on a shaker at 30°C and collected after 1h or 2h and stained with 1µg/ml FM4-64 2µg/ml DAPI, and 0.5µM SYTOX-Green (Molecular Probes/Invitrogen). In the case of NBD-tagged AZM, SYTOX-Green dye was omitted and cell cultures were washed with fresh media before staining. Stained cultures were centrifuged at 300xg for 30sec in microcentrifuge and resuspended in approximately 5% original volume. 3µl of concentrated cells were transferred onto a pad containing 1.2% agarose and 20% LB medium for microscopy. The exposure time of each excitation was the same for each experimental replicate included in the statistical analysis of all treatments. All images were analyzed using ImageJ software v1.48f and CellProfiler 2.0. Fluorescence intensity analysis was performed on non-deconvolved images. Average DAPI, SYTOX Green, or NBD intensity per cell was measured and respective background intensities subtracted. Finally, the intensity of treated cells was normalized by the intensity of untreated cells from the same experiment set, making intensity data from different experimental sets comparable as relative intensity. Protein translation inhibition phenotype was automatically calculated by the degree of DNA condensation defined by the ratio of DNA area over total cell membrane area. Cells with a ratio less than half of those calculated from untreated control cells were considered to have condensed toroid nucleoids [20]. Fluorescence intensity and protein translation inhibition population data were obtained by counting >500 cells from random microscopy fields per condition per experimental replicate, with 3 independent replicates. The fluorescence microscopy for LL-37 cell binding was performed in a similar manner except that after the MDR-*AB* cultures were incubated in 0.5mg/L of AZM versus control

for 2h, 2 μ M of TAMRA-tagged LL-37 was added and the cultures incubated for an additional 30min before staining and analysis.

2.8. Mouse AZM Dosing

Standard human dosing of AZM is 500 or 1,000 mg administered orally or intravenously q 24 h (7.7 or 15 mg/kg for an average adult). Mice metabolize AZM 29 times faster than humans [21], and 50mg/kg AZM given subcutaneously to mice approximates 500 mg given intravenously to human patients [22]. We therefore chose to administer 50 or 100mg/kg AZM subcutaneously in our murine infection models once every 24h.

2.9. Mouse Pneumonia Models

The murine pneumonia mode was performed with slight modifications as previously described [23]. All intratracheal infections were performed in a blinded fashion with respect to AZM or PBS treatment. For the MDR-*AB* lung infection model, 8-wk-old female C57Bl/6J mice (Jackson Labs) were used. MDR-*AB* cultures were grown overnight in LB at 37°C with shaking and then re-grown in the morning in fresh LB to a concentration of OD₆₀₀=0.40. Bacteria were washed twice with PBS via centrifugation at 3220 x g at room temperature and concentrated in PBS to yield 5x10⁶ CFU in the 30 ul, the inoculation volume. Mice were anesthetized with 100mg/kg ketamine and 10mg/kg xylazine. Once sedated, the vocal chords were visualized using an operating otoscope (Welch Allyn) and 30ul of bacteria or PBS was instilled into the trachea during inspiration using a plastic gel loading pipette tip. Mice were placed on a warmed pad for recovery and given one subcutaneous dose of 50mg/kg or 100mg/kg human AZM for injection reconstituted in PBS. Mice were sacrificed with CO₂ for

bacterial counts or for analysis of their broncho alveolar fluid (BALF) 24h after infection. To enumerate total surviving bacteria in the lungs, both lung lobes were removed and placed in a 2ml sterile micro tube (Sarstedt) containing 1ml of PBS and 1mm silica beads (Biospec). Lungs were homogenized by shaking twice at 6,000 rpm for 1min using a MagNA Lyser (Roche), with the specimens placed on ice as soon as they were harvested. Aliquots from each tube were serially diluted for CFU enumeration on LB plates. For BALF collection and analysis, an incision was made in the trachea and the lungs slowly inflated with 700ul of chilled PBS using a 1ml syringe topped with a 20G blunt needle tip (Harvard Apparatus). The PBS was slowly withdrawn, and the recovered BALF was spun at 1200xg at 4°C for 10 min. The supernatant (350ul) was analyzed for the mouse pro-inflammatory cytokines mIL-1 β , mIL-6, and mMIP-2 (R&D Systems ELISA Kits). Cell pellets were resuspended in chilled PBS to a final volume of 500 μ l. Samples were enumerated with a hemocytometer for total leukocyte number. 100 μ l was spun onto glass microscopy slides using a Shandon Cytospin 3 (Thermo Scientific). Dry glass slides were stained with Wright-Giemsa stain, and >200 leukocytes were counted per animal using a light microscope for neutrophil and alveolar macrophage enumeration. For the MDR-*AB* lung infection survival experiment 5×10^7 CFU were given in 36 μ l of PBS. One dose of 100mg/kg AZM or 100 μ l of PBS control was given subcutaneously immediately after the infection (time 0) and a second dose was given 24h later. The MDR *KP* and *PA* intra-tracheal murine lung infections were performed in a similar manner except that 8-week-old female CD1 mice (Charles River Labs) were used and the animals were sacrificed 36h after infection for enumeration of surviving bacteria. The inoculums for MDR-*KP* and MDR-*PA* infection were 1.5×10^7 CFU and 1×10^7 CFU in 40 ul of PBS, respectively.

The *AB* foreign body infection studies were performed as previously described [24]. All animal studies were performed under protocols approved by the UCSD Institutional Animal Use and Care Committee.

2.10. Statistical Analysis

All statistics were performed using GraphPad Prism version 5.0 2-way ANOVA, 1-way ANOVA, two tailed student's t-test, and log-rank test were performed as described in each figure legend.

RESULTS

3.1. AZM is Highly Bactericidal vs. MDR GNRs in Tissue Culture Media

For each opportunistic GNR pathogen (*PA*, *KP* and *AP*) we tested a common model strain plus a corresponding extremely MDR human isolate (**Table S 2.1**). AZM MICs were determined by Clinical and Laboratory Standard Institute (CLSI) broth microdilution methodology [17] using either the recommended cation-adjusted Mueller-Hinton broth (Ca-MHB) or eukaryotic cell culture media RPMI-1640 supplemented with 5% Luria broth (RPMI+5%LB) (**Table S 2.2**). In every case, a ≥ 30 -fold reduction in MIC was seen in RPMI+5%LB vs. Ca-MHB, rendering all organisms susceptible by the accepted breakpoint of ≤ 8 mg/L for *Campylobacter* and *S. aureus*. In contrast, no marked changes in MIC of fluoroquinolone ciprofloxacin were observed for model strains (all sensitive) or MDR clinical isolates (all resistant) in RPMI+5%LB vs. Ca-MHB (**Table S 2.2**). A similarly profound reduction in MIC in RPMI+5%LB vs. Ca-MHB was also observed for an expanded panel of 11 additional MDR GNR clinical isolates (**Table S 2.3**). Two additional macrolides, erythromycin and clarithromycin, also demonstrated dramatically higher activity against MDR GNRs in RPMI+5%LB versus Ca-MHB,

although AZM was the most potent of the macrolides tested (**Table S 2.2**). Time-kill curves determined the mean bactericidal concentration (MBC, reduction in CFU by $2\log_{10}$) of AZM against the MDR-GNRs in RPMI+5%LB media (**Figure 2.1A-C**). With an initial inoculum of 1×10^6 CFU/ml, the MBC of AZM against MDR-*PA*, -*KP* and -*AB* were 4mg/L (MIC=2mg/L), 1mg/L (MIC=1mg/L) and 0.5mg/L (MIC=0.5mg/L) respectively. While all three MDR-GNRs achieved rapid logarithmic growth in both RPMI+5%LB and Ca-MHB, AZM was markedly more bactericidal in the eukaryotic tissue culture media (**Figure 2.1A-C**): a striking 6-logfold increased killing for *KP* and *AB* and 2-logfold increased killing for *PA*. For MDR-*PA* and -*AB*, AZM 4 mg/L ($<1/16$ MIC) resulted in significant killing even in Ca-MHB once bacteria reached stationary growth phase (**Figure 2.1A and C**), consistent with reported AZM bactericidal activity against stationary phase antibiotic-sensitive *PA* [25]. AZM activity against all three MDR pathogens was further enhanced in the presence of 20% human serum (**Figure 2.1D-F**), mirroring a prior observation of serum potentiation of AZM activity against *E. coli* and *S. aureus* [26]. Serial passage of all three MDR pathogens for 10 consecutive days at sub-minimum inhibitory concentrations of AZM in RPMI+5%LB media demonstrated no increase in resistance to AZM over this time frame (**Figure S 2.1**).

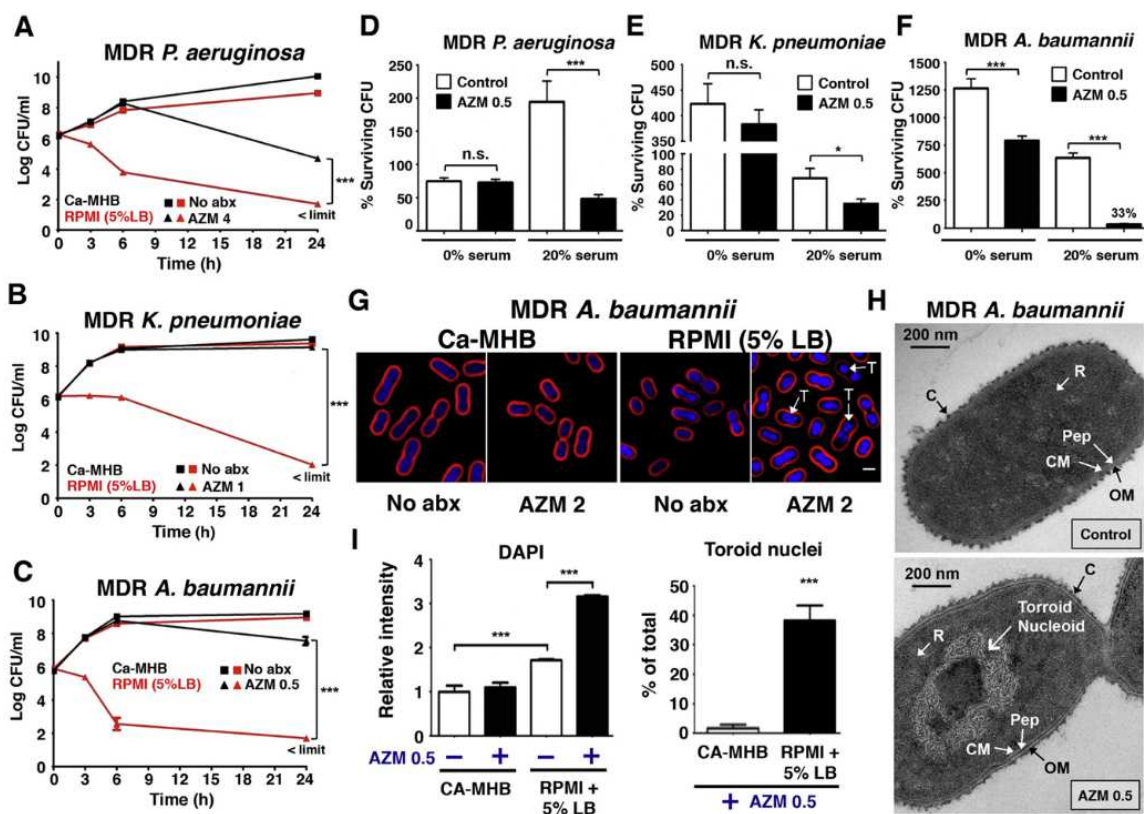


Figure 2.1 AZM bactericidal activity against GNRs in eukaryotic media and human serum. AZM concentrations = mg/L. (A-C) Time-kill curves demonstrating AZM MBC against MDR *PA*, *KP*, and *AB* in eukaryotic media (RPMI+5%LB) vs. bacteriologic broth (Ca-MHB). Mean of triplicates from 3 independent experiments \pm SEM *** P <0.001; two-way ANOVA; limit of detection 50 CFU/ml. (D-F) 5×10^4 CFU/ml bacteria incubated at 37°C for 2h in RPMI alone (0% serum), AZM 0.5, 20% serum, and AZM 0.5+20% serum. Data show % viable CFU vs. initial inoculum; mean of triplicates from 3 independent experiments \pm SEM. * P <0.05, *** P <0.001; two-tailed Student's t-test. (G) MDR *AB* (5×10^7 CFU/ml) grown in Ca-MHB vs. RPMI+5%LB were treated for 2h with AZM 2 and stained for fluorescence microscopy: FM4-64 (red cell membrane stain), DAPI (blue DNA stain), "T" denotes toroid shaped nucleoid. For ready visualization, the concentration of MDR *AB* was 100-fold higher than in MIC assays; thus a higher concentration AZM was used. (H) Transmission electron microscopy images representative of 2 independent experiments with logarithmic growth phase MDR *AB* treated for 2h with AZM 0.5; C = Capsule; CM = Cell Membrane; Pep = Peptidoglycan; OM = Outer Membrane; R = Ribosomes. (I) Bar graphs generated from unbiased software analysis of multiple random microscopy fields with >500 cells counted per condition per experimental replicate. Data representative of 3 independent experiments plotted as mean \pm SEM; additional microscopy details in methods section. *** P <0.001; one-way ANOVA and two tailed student's t-test.

3.2. Enhanced AZM Permeability in Tissue Culture Media Triggers Nucleoid Collapse

AZM binds the 50S large ribosomal subunit at the polypeptide exit tunnel, blocking protein synthesis [27]. We employed fluorescence microscopy-based bacterial cytological profiling (BCP) [20] of MDR-*AB* to identify the cellular pathway perturbed by AZM treatment in RPMI+5%LB media but not Ca-MHB. BCP revealed that nucleoid collapse into a low energy toroid shape, a hallmark of protein synthesis inhibition [20, 28], was markedly increased in *AB* treated with AZM in RPMI+5%LB (**Figure 2.1G**), a finding corroborated by transmission electron microscopy of treated cells (**Figure 2.1H**). Increased staining intensity of the DNA dye DAPI in RPMI+5%LB vs. Ca-MHB even without AZM suggested increased membrane permeability in the cell culture media (**Figure 2.1I**). DAPI staining of MDR-*AB* was further increased upon AZM treatment, possibly reflecting impaired efflux pump function seen in antibiotic-sensitive *PA* treated with AZM in RPMI [11].

3.3. Colistin Permeabilization Markedly Potentiates AZM Bactericidal Activity vs. MDR GNRs

Colistin is an antibiotic of last resort for carbapenem-resistant GNR infections [29], but its use is complicated by marked dose-dependent nephrotoxicity and uncertainties in optimal dosing [30]. We asked whether AZM could act synergistically with colistin, even in standard Ca-MHB media in which AZM alone has little or no activity. At a sub-MIC (**Table S 2.2**) and pharmacologically attainable dose of each drug, marked synergy [31] of AZM+colistin was observed against MDR-*PA* (additional reduction in CFU by $2\log_{10}$), -*KP* (by $5\log_{10}$) and -*AB* (by $3\log_{10}$) (**Figure 2.2A-C**). BCP showed strongly increased DAPI staining and toroid nucleoid morphology in MDR-*AB*

treated with AZM+colistin vs. either agent alone (**Figure 2.2D and E**). A membrane permeability effect was corroborated as bacterial cell entry of the fluorescent dye SYTOX green (MW=600Da vs. 749Da for AZM) was increased 3-fold by colistin and 4-fold by colistin+AZM (**Figure 2.2D and G**). Indeed, colistin markedly enhanced entry of fluorescently tagged AZM (NBD-AZM [32]) into MDR-*AB* cells (**Figure 2.2F and G**). A cationic peptide antibiotic, colistin permeabilizes the bacterial cell envelope [33], and we surmise it facilitates AZM entry whereupon the latter drug can exert its classical ribosomal protein synthesis activity. Erythromycin and clarithromycin also showed significant synergy with colistin (**Figure S 2.2**), with azithromycin the most potent of the three macrolides tested.

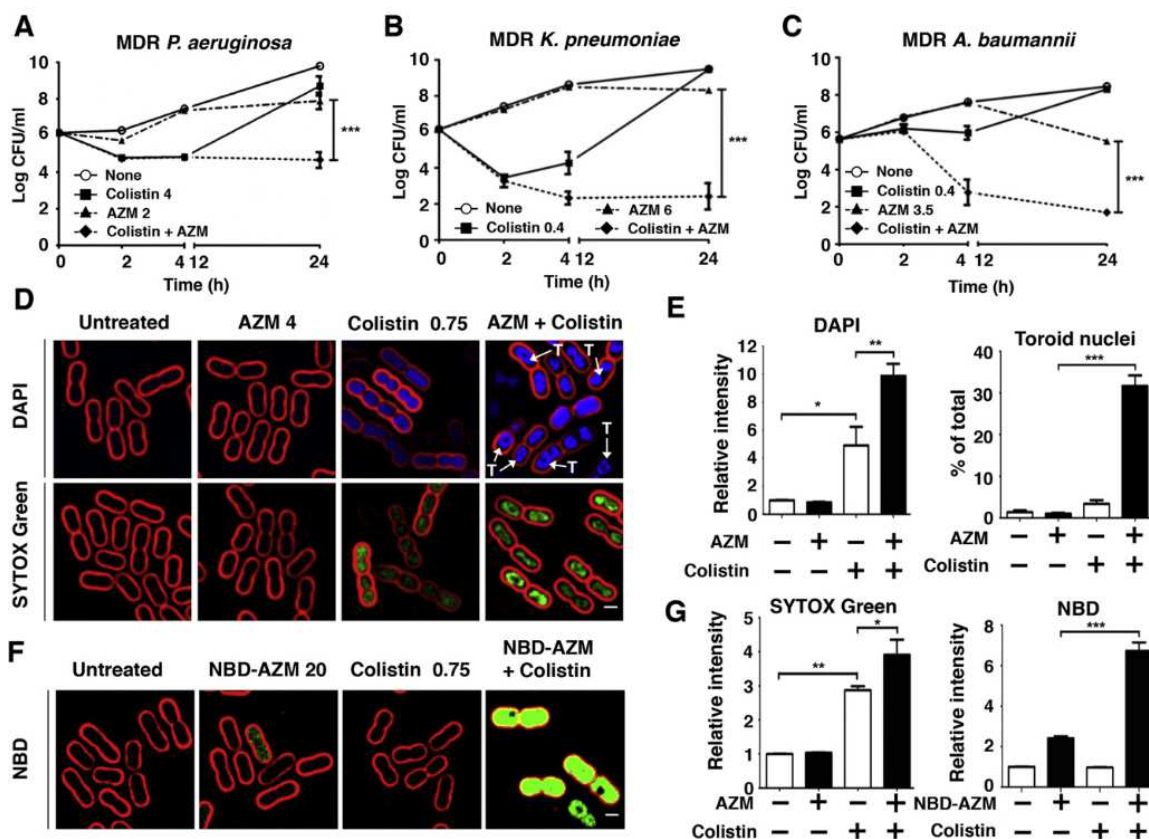


Figure 2.2 Colistin synergizes with AZM by increasing GNR outer membrane permeability. All assays conducted using the bacteriologic media Ca-MHB. (A-C) Time-kill curves demonstrate the effect of AZM, colistin, or both in combination against MDR *PA*, *KP* and *AB*. Data plotted are mean \pm SEM and represent the average of triplicates from 3 independent experiments. *** $P < 0.001$; two-way ANOVA. (D and F) Growth phase MDR *AB* (5×10^7 CFU/ml) treated for 1h with AZM (unlabeled or NBD-tagged), colistin, or a combination of both, then stained for fluorescence microscopy: FM4-64 (red cell membrane stain), DAPI (blue DNA stain), SYTOX Green (green DNA stain), and NBD-AZM (green NBD-tagged AZM), "T" = toroid shaped nucleoid. DAPI blue DNA stain present in "Untreated" and "AZM 4" panels. Since signal from all conditions was normalized to untreated controls and "AZM+Colistin" treated bacteria had 10-fold higher DAPI signal intensity, blue signal was reduced equally across all four displayed DAPI panels to prevent washout and enable visualization of the toroid structures in the cells with high DAPI signal intensity. (E and G) Bar graphs were generated from unbiased software analysis of multiple random microscopy fields with >500 cells counted per condition per experimental replicate. Data representative of 3 independent experiments and plotted as the mean \pm SEM - additional microscopy details in methods section. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; one-way ANOVA.

3.4. Pronounced Bactericidal Synergy of AZM with Human Cathelicidin LL-37 vs. MDR GNRs

Endogenous cationic AMPs are critical to mammalian innate immunity against invasive bacterial infection[34]. Given the observed synergy of AZM with the bacterial-derived AMP colistin, we hypothesized that AZM could be potentiated by LL-37, a cationic AMP produced abundantly by phagocytic and epithelial cells during infection [35]. At sub-MIC doses (**Table S 2.2**), marked synergy of AZM+LL-37 was observed against MDR-*PA* (additional reduction in CFU by 4log₁₀), *KP* (7log₁₀) and *AB* (4log₁₀) (**Figure 2.3A-C**). For MDR-*AB*, AZM 0.0625mg/L (1/8th MIC)+1μM LL-37 (1/4th MIC) resulted in >99% killing within 4h. BCP showed increased DAPI staining and toroid morphology of nucleoids in MDR-*AB* treated with AZM+LL-37 vs. either agent alone (**Figure 2.3D and E**). SYTOX green entry into the bacterial cell was increased 3-fold in the presence of LL-37 and 5-fold in the presence of LL-37+AZM (**Figure 2.3D and G**). LL-37 also markedly enhanced entry of NBD-AZM into MDR-*AB* cells (**Figure 2.3F and G**), consistent with the known activity of LL-37 to interfere with GNR cell wall biogenesis and cytoplasmic membrane integrity [36]. Studies with 1-N-phenyl naphthylamine (NPN), a validated marker for outer membrane permeability of GNRs [37], verified that sub-MIC concentrations of colistin or LL-37 increased outer membrane permeability of the MDR-*AB* strain (**Figure S 2.3**). Interestingly, AZM pretreatment of MDR-*AB* significantly enhanced binding of TAMRA-tagged LL-37 to the bacterial outer membrane, suggesting bidirectional synergy (**Figure S 2.4**).

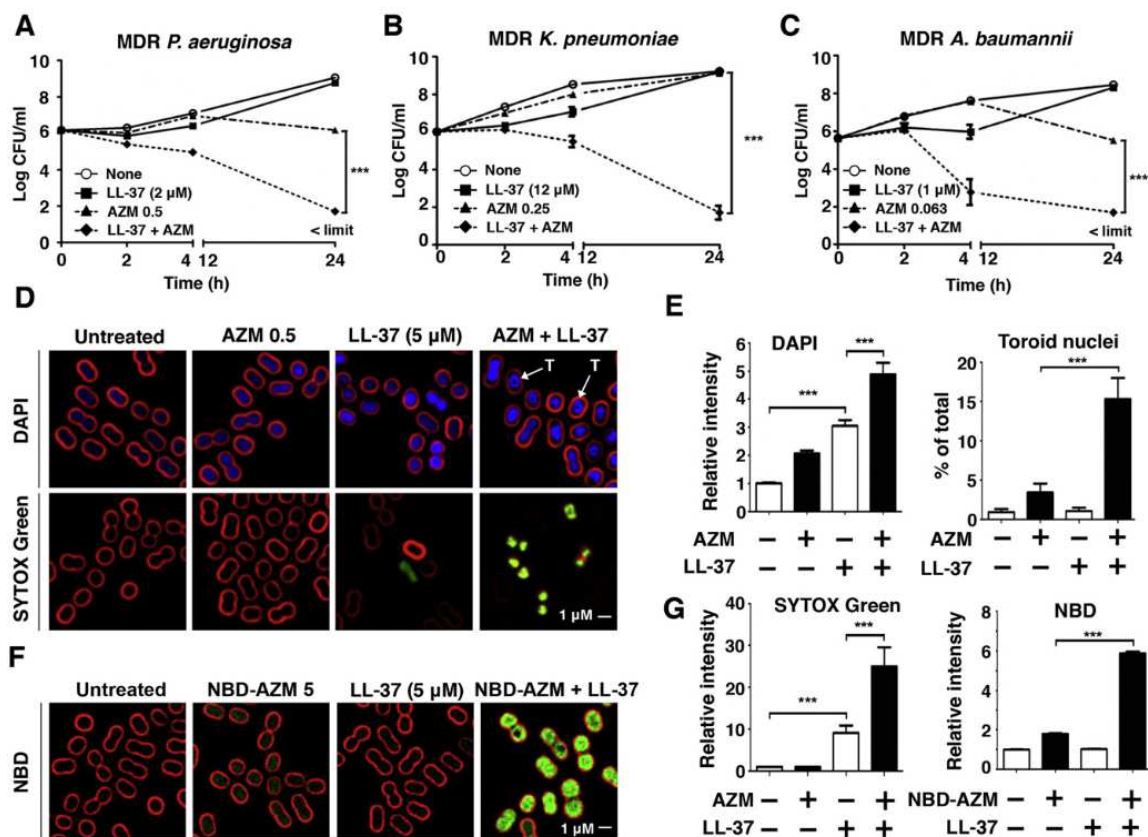


Figure 2.3 Cationic AMP LL-37 synergizes with AZM by increasing the outer membrane permeability of MDR-GNRs. All assays conducted using the eukaryotic media RPMI+5%LB. (A-C) Time-kill curves demonstrating effects of AZM, LL-37, or both in combination against MDR *PA*, *KP*, and *AB*. Data plotted are mean \pm SEM and represent the average of triplicates from 3 independent experiments. *** P <0.001; two-way ANOVA. Limit of detection = 50 CFU/ml (D and F) Logarithmic growth phase MDR *AB* (5×10^7 CFU/ml) were treated for 2h with AZM (unlabeled or NBD-tagged), LL-37, or a combination of both, then stained for fluorescence microscopy: FM4-64 (red cell membrane stain), DAPI (blue DNA stain), SYTOX Green (green DNA stain), and NBD-AZM (green NBD-tagged AZM), “T” denotes toroid shaped nucleoid. (E and G) Bar graphs generated from unbiased software analysis of multiple random microscopy fields with >500 cells counted per condition per experimental replicate. Data representative of 3 independent experiments and plotted as the mean \pm SEM. - additional microscopy details in methods section. *** P <0.001; one-way ANOVA.

3.5. AZM Monotherapy Promotes Clearance of MDR GNR in Murine Infection Models

Activity in mammalian culture media and synergy with host AMPs suggested AZM could be effective against MDR-GNR pathogens *in vivo*. AZM concentrates in soft tissues and phagocytes [38, 39], pharmacodynamic features providing high drug levels at infectious foci where endogenous cationic AMPs are deployed. We studied an intratracheal infection model of MDR-*AB* pneumonia with AZM as the sole therapeutic agent. A single subcutaneous dose of 50 or 100 mg/kg AZM was chosen to achieve serum concentrations mimicking those found with typical 500 or 1,000 mg AZM intravenous dosing given to human patients [22]. A single AZM dose reduced by 2log₁₀ or 99% the amount of MDR *AB* recovered from lungs 24h after challenge (**Figure 2.4A**). Examination of bronchial alveolar lavage fluid (BALF) revealed that AZM-treated animals had 50% fewer neutrophils (alveolar macrophages unchanged) (**Figure 2.4B**), significantly reduced pro-inflammatory cytokines IL-1 β , IL-6, and MIP-2 (**Figure 2.4C**), and histological evidence of reduced neutrophil and bacterial infiltrations (**Figure 2.4D and E**). Increasing the inoculum of MDR-*AB* 10-fold produced significant mortality within 48h. One dose of 100 mg/kg AZM after initial infection, followed by a second dose at 24h, improved 5d survival from 22% to 89% (**Figure 2.4F**). In a subcutaneous foreign body infection model, daily AZM reduced by >90% the quantity of MDR-*AB* recovered after 3d (**Figure 2.4G**). Reductions of up to 10-fold in bacterial counts were also observed for AZM monotherapy in both MDR-*KP* and -*PA* lung infection models (**Figure 2.4H and I**). Low dose colistin therapy alone did not lead to a significant reduction in the amount of MDR *AB* recovered from lungs 24h after challenge. However,

when a low dose of AZM was combined with a low dose of colistin, a significant reduction in lung bacterial CFU was achieved (**Figure S 2.5**).

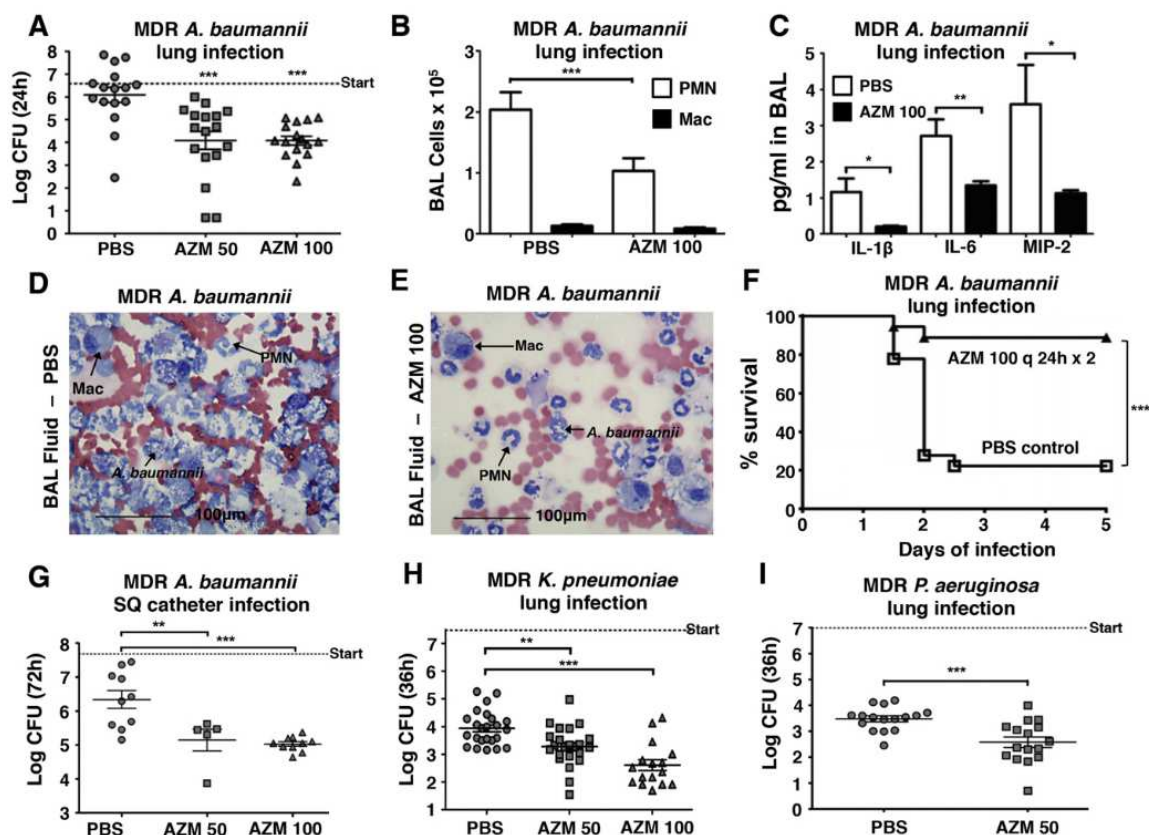


Figure 2.4 AZM activity as monotherapy against MDR-GNRs *in vivo*. (A) C57BL/6J mice were infected intratracheally (i.t.) with MDR *AB*. Lungs homogenized at 24h; n = 16 for PBS control, 17 for AZM 50mg/kg, and 16 for AZM 100mg/kg. (B) Wright-Giemsa stained BALF from C57BL/6J mice infected i.t. with *AB*. Total neutrophils and macrophages enumerated by light microscopy + hemocytometer counts; n = 19 PBS control and 20 AZM 100mg/kg-treated mice. PMN = polymorphonuclear leukocyte/neutrophil, Mac = macrophage. (C) ELISA detection of inflammatory cytokines in the BALF of mice from (b). (D and E) Light microscopy of Wright-Giemsa stained BALF of mice from (b); Mac = alveolar macrophage; PMN = neutrophil. (F) Survival of C57BL/6J mice infected i.t. with 6×10^7 CFU of *AB*. Mice received two total doses of AZM versus PBS spaced 24 h apart. n = 18 PBS control and 18 AZM 100mg/kg-treated mice. (G) 1cm catheter fragment coated with *AB* was implanted subcutaneously into CD-1 mice; results of 2 independent experiments. N = 10 PBS; 5 AZM 50mg/kg-treated, 10 AZM 100mg/kg-treated mice (H) CD-1 mice infected i.t. with MDR *KP*. Lungs homogenized at 36 h. N = 24 PBS control, 23 AZM 50 mg/kg-treated, and 16 AZM 100mg/kg-treated mice (i) CD-1 mice infected i.t. with MDR *PA*. Lungs homogenized at 36h; results of 2 independent experiments. n = 16 PBS control and 16 AZM 50mg/kg-treated mice. Data plotted as mean \pm S.E.M. of 3 independent experiments unless otherwise stated. For *in vivo* mouse studies, AZM was dosed subcutaneously once every 24h. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$; one-way ANOVA *in vivo* studies, log-rank test for survival, two-tailed student's t test *in vitro* studies.

DISCUSSION

The continual emergence and rapid spread of MDR-GNRs in hospitals around the world has alarmed physicians, public health epidemiologists and government agencies, spurring urgent calls to action [3, 4]. Due to historical precedent and the intrinsic appeal of a “gold standard”, a single bioassay, the MIC performed in bacteriological media, has come to dominate evaluation of antibiotic efficacy, from the earliest stages of the drug development process, to management of patients based on clinical isolate testing, to establishment of hospital formularies. However, even before the first diagnostic encounter with a physician, a patient’s infection is already being combatted by numerous endogenous antimicrobial components, including cationic AMPs of the innate immune system. We believe there is value in analyzing the action of pharmaceutical antibiotics in the richer context of these host defense factors.

Using traditional susceptibility testing methods, the familiar antibiotic AZM shows negligible activity against MDR-GNRs. A small number of prior studies examining multiple combinations of antibiotics *in vitro* have provided clues that AZM can exhibit activity against GNRs [40, 41] or synergize with a polymyxin type antibiotic [42], but without mechanistic or *in vivo* analysis or suggestions of clinical application. To this day, AZM remains excluded from the antibiotic testing panel reported to physicians when such bacteria are recovered from the blood, sputum or urine of hospitalized patients. Here we show that AZM has potent bactericidal activity against representative strains of the most fearsome MDR-GNRs when tested in eukaryotic cell media (+/- human serum) and *in vivo* murine models of infection. AZM entry and activity against MDR-GNRs is synergistically enhanced when the bacterial outer membrane is

perturbed by cationic human AMP LL-37 or by the last-line antibiotic colistin. Because mutation of the *oprM* efflux pump system in *P. aeruginosa* has been associated with increased AZM sensitivity, and protein synthesis inhibition by AZM can reduce *oprM* gene expression [11], the enhanced entry of AZM in eukaryotic media and in synergy with colistin or LL-37 that we have demonstrated may initiate a positive feedback loop to increase effective intracellular levels of the antibiotic.

Of note, AZM is touted to have anti-inflammatory effects in lung tissues during infection [43], and several studies of short- or long-term AZM administration in patients with cystic fibrosis, a disease characterized by chronic recurrent pulmonary infection with *PA* and other MDR-GNR bacterial pathogens, have shown reductions in exacerbations and improvement in respiratory capacity (reviewed in [44]). Our findings raise the possibility that these benefits could reflect, at least in part, a direct and otherwise unanticipated bactericidal activity of the drug. The macrolides, and azithromycin in particular, have a number of reported non-bactericidal properties that could further complement the *in vivo* efficacy we observed in our murine models of infection. For example, against various Gram-negative organisms, AZM has been shown to impair bacterial biofilms [45, 46], virulence factor production [45], motility [47], quorum sensing [21], and adherence to host epithelial cells [48, 49].

The major limitation of this study was the use of murine models of MDR GNR infection as opposed to human clinical cases. Laboratory mice are relatively resistant to infection by human clinical isolates of MDR GNRs requiring high inocula to create a disease phenotype. Furthermore, young mice with normal immunity were tested, whereas many human patients that develop MDR GNR infection have multiple co-

morbidities including weakened immunity (e.g. from cancer chemotherapy) or dependence on a medical device (e.g. mechanical ventilator). Future randomized clinical trials will be needed to validate whether or not AZM has therapeutic efficacy in older and sicker human patients suffering from MDR GNR infections.

In summary, our studies provide an experimental rationale to further explore AZM as adjunctive therapy in MDR-GNR infections. In particular, we have demonstrated for the first time that colistin or LL-37 permeabilization of Gram-negative outer membranes facilitates entry of the large AZM molecule. Potentially, additional antibiotics, currently disregarded for various clinical indications due to poor penetration into Gram-negative bacterial membranes in standard MIC testing, may likewise have unrecognized *in vivo* activities when host immune factors perturb bacterial membrane integrity. Most immediately, the AZM synergy with colistin we demonstrate here may allow lower dose, colistin-sparing regimens that reduce adverse drug effects. Continued examination of pharmacodynamic interactions between administered antibiotics and endogenous AMPs of the innate immune system may reveal novel treatment strategies for challenging infections.

SUPPLEMENTAL MATERIALS AND METHODS

Serial Passage of MDR GNR isolates. Cultures of *Pseudomonas aeruginosa*, strain P4, *Klebsiella pneumoniae*, strain K1100, and *Acinetobacter baumannii*, strain AB5075 were grown at 1/2 and 1/4 their AZM MIC in 5ml of RPMI + 5%LB. Cultures were placed in a 37°C shaker, and 24 h later, the highest concentration of AZM in which dense bacterial growth was observed was recorded. 50µl of this dense culture growth was then used to

inoculate three new tubes containing 5ml of fresh media and 2, 1, and 1/2 the original concentration of AZM respectively. Serial passage was performed twice and data represent one of two independent experiments.

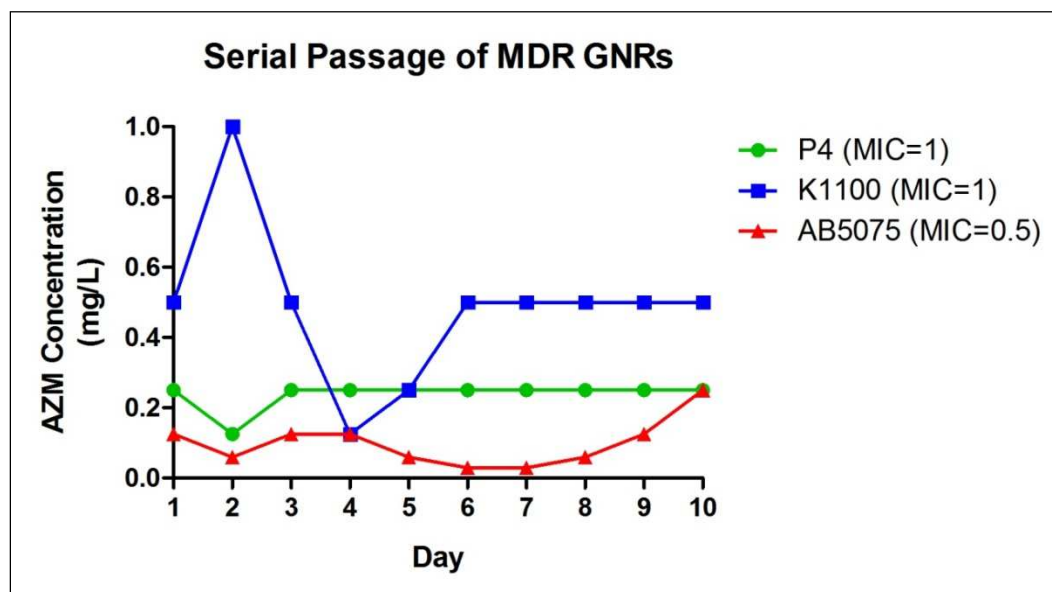
NPN bacterial outer membrane permeability assay. 1-N-phenylnaphthylamine (NPN) is a molecule that becomes fluorescent when it comes into contact with the inner membrane of Gram-negative bacteria, and this property was used to study MDR-*AB* membrane permeability [37]. Overnight cultures of MDR-*AB* grown in LB at 37°C with shaking were washed twice with PBS via centrifugation at 3220xg at room temperature and re-suspended to an OD₆₀₀=0.40 in 8ml of Ca-MHB for colistin assays or 8ml of 5%LB-RPMI, for LL-37 assays. LL-37, colistin, or media control was added to a final concentration of 1μM and 0.5mg/L respectively. The cultures were shaken at 37°C for 1h and then spun at 3000 x g at room temperature for 5 min and re-suspended in 2ml of 10mM Tris buffer pH 8.0. The concentrated 2ml cultures were used to prepare 4ml bacterial stocks at OD₆₀₀=0.40 in 10mM Tris. Assays were conducted in a final volume of 200μl in triplicate in 96-well round bottom plates (Costar). Four conditions were tested. 1) 100μl of bacterial stock + 50μl of NPN (40μM final) + 50μl of 10 mM Tris. 2) 100μl of bacterial stock + 50μl of NPN + 50μl of EDTA (10 μM final). 3) 100μl of bacterial stock + 50μl of EDTA + 50μl of 10mM Tris. 4) 100μl of 10mM Tris + 50ul of NPN + 50ul of EDTA. As soon as all of the components were added and mixed, plates were immediately read in a fluorescent plate reader: excitation 250nm/emission 420nm. The NPN fluorescence signal from conditions 3) and 4), background, were subtracted from the signals measured from conditions 1) and 2). The NPN intensity from condition 1)

bacteria+NPN was divided by the NPN signal measured from condition 2) bacteria+NPN+EDTA to obtain the percentage of permeability recorded in the presence of 10mM EDTA that permeabilizes the outer membrane of Gram-negative bacteria.

***A. baumannii* foreign body infection model.** The *AB* foreign body infection studies were performed as previously described [24]. Briefly, 14-gauge sterile non-pyrogenic intravenous catheters (Excel International) were cut into 1cm segments. These segments were then cut in half, so that the interior lumen was opened. Catheter fragments were sterilized by shaking in 70% ethanol for 2h and then rinsed twice in fresh LB. Catheter fragments were added to a 25ml starting culture of AB5075 in LB and grown overnight at 37°C with shaking. 8-week-old female CD1 mice (Charles River Labs) had the hair on their backs shaved and then removed with Nair (Naircare). The mice were anesthetized in an isoflurane chamber, their nude backs were sprayed with 70% ethanol, and a small 1cm incision was made. Tweezers were gently inserted into the incision site and used to open up a small subcutaneous pouch. A catheter fragment was removed from the overnight culture of MDR-*AB* and inserted into the pouch. The wound site was closed with Tegaderm film (3M) and the mice were placed on a warm heating pad to recover. At 1, 24, and 48h after infection, the mice received a subcutaneous dose of 50mg/kg AZM, 100 mg/kg AZM, or 100µl PBS control. At 72h the mice were sacrificed with CO₂. The catheter fragment and all surrounding infected soft tissue was removed and placed in a 2 ml sterile micro tube (Sarstedt) containing 1ml of PBS and 1mm silica beads (Biospec) and the tissue was homogenized as detailed above for the lungs. Aliquots from each tube were serially diluted for CFU enumeration on LB plates. Three catheter segments per

experimental run were removed from the overnight *AB* culture and homogenized in 1ml of PBS to determine the initial inoculum.

NBD-tagged AZM. 9a-NBD-AZM was synthesized as previously described [32] with slight modifications made in the final step of the synthesis. To a solution of 9a-(3-aminopropyl)-9-deoxo-9a-aza-9a-homoerythromycin A (100mg, 0.126mmol) in dry EtOH (2ml) was added 4-chloro-7-nitrobenofurazan (25mg, 0.126mmol) and the resulting reaction mixture was stirred at room temperature for 5h. Water (5ml) was added to the reaction mixture and extracted with CH₂Cl₂ (3 x 5ml). The organic layers were combined, dried over Na₂SO₄ and concentrated under reduced pressure. Flash chromatography (CH₂Cl₂ to 10:1 CH₂Cl₂/MeOH) afforded the desired compound as a yellow solid (66 mg, 55.1%). NBD-tagged AZM: ¹H NMR (CD₃OD, 500MHz) δ 8.51 (d, *J* = 8.8 Hz, 1H), 6.42 (d, *J* = 9.0 Hz, 1H) –only the diagnostic benzofurazan shifts were noted (**Figure S 2.6A**); ¹³C NMR (CD₃OD, 125 MHz) δ 177.7, 137.3, 102.1, 98.7, 95.6, 83.4, 79.0, 77.7, 77.1, 74.8, 74.5, 74.2, 73.0, 70.5, 67.2, 65.2, 64.5, 48.5, 44.9, 41.7, 40.9, 40.6, 38.8, 34.6, 34.3, 31.7, 31.4, 30.1, 29.4, 28.8, 28.2, 26.2, 24.8, 22.7, 22.3, 21.6, 21.1, 20.4, 20.2, 17.6, 16.4, 14.5, 13.1, 10.1, 8.8, 7.6; HRMS *m/z* calculated for [C₄₆H₇₉N₆O₁₅]⁺: 955.5598, found 955.5597 (**Figure S 2.6B**).



Day	<i>Pseudomonas aeruginosa</i> , Strain P4 (MDR) AZM (mg/L)	<i>Klebsiella pneumoniae</i> , Strain K1100 (MDR, KPC) AZM (mg/L)	<i>Acinetobacter baumannii</i> , Strain AB5075 (MDR) AZM (mg/L)
1	0.25	0.5	0.125
2	0.125	1	0.06
3	0.25	0.5	0.125
4	0.25	0.125	0.125
5	0.25	0.25	0.06
6	0.25	0.5	0.03
7	0.25	0.5	0.03
8	0.25	0.5	0.06
9	0.25	0.5	0.125
10	0.25	0.5	0.25

Abbreviations: AZM = Azithromycin; MDR = multidrug-resistant; KPC = *Klebsiella pneumoniae*, carbapenemase-producing; RPMI = Roswell Park Memorial Institute 1640, a basal mammalian tissue culture medium; LB = Luria broth.

Figure S 2.1 Serial passage for 10 days of MDR GNR isolates at sub-minimum inhibitory concentrations of azithromycin to test for resistance evolution. On Day 0, bacteria were grown at 1/2 and 1/4 of their AZM MIC in 5ml of liquid culture - RPMI + 5%LB. Then 24 h after shaking at 37°C, the highest concentration of AZM in which dense bacterial growth was observed was recorded. 50µl of this dense culture growth was then used to inoculate 3 new tubes containing 5ml of fresh media and 2, 1, and 1/2 the original concentration of AZM respectively.

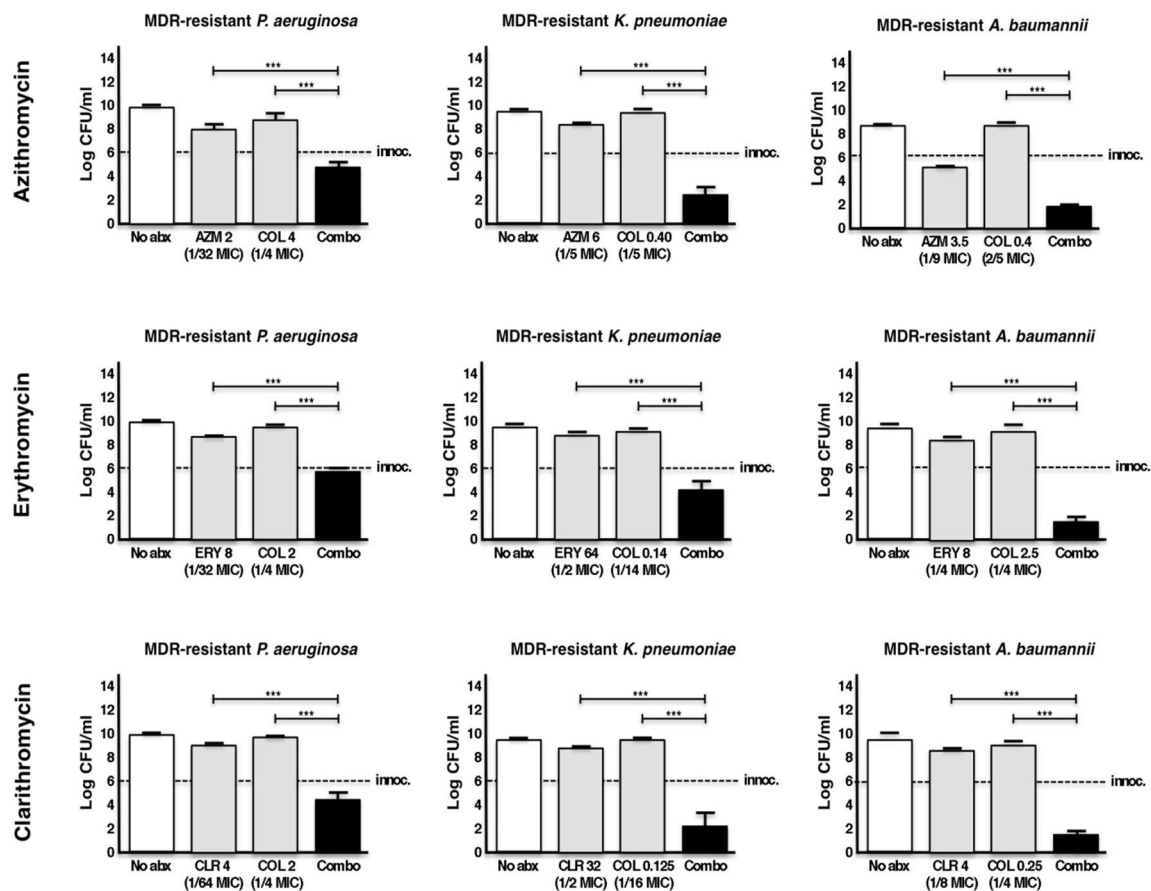


Figure S 2.2 Colistin synergizes with azithromycin, erythromycin, and clarithromycin to kill MDR GNR pathogens *in vitro*. All assays conducted using the standard bacteriologic media Ca-MHB. Time-kill assays demonstrate the effect of AZM, ERY, and CLR alone or in combination with colistin against MDR PA, KP and AB. Data plotted are mean \pm SEM and represent the average of triplicates from two independent experiments. The starting bacterial inoculum ("innoc") is denoted by the dotted line. *** $P < 0.001$; one-way ANOVA.

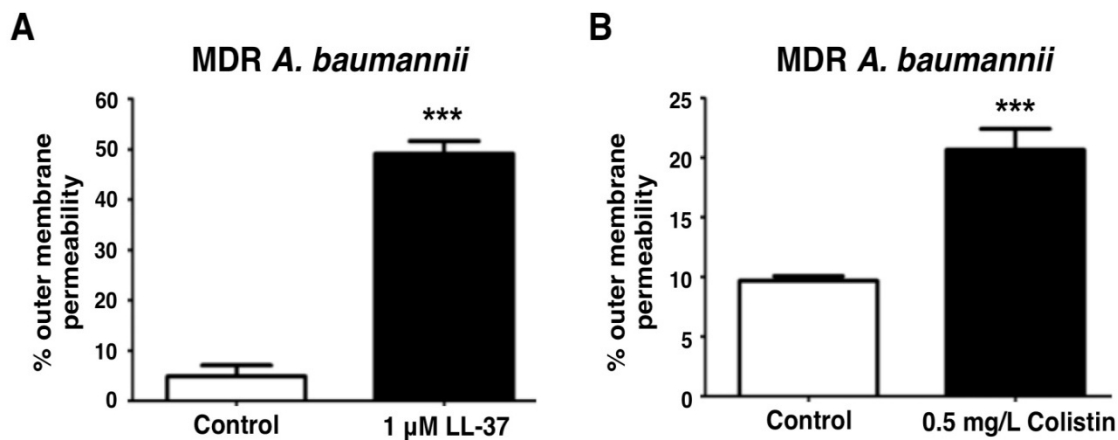


Figure S 2.3 Treating MDR *A. baumannii* with sub-MIC LL-37 or colistin significantly increases outer membrane permeability to 1-N-phenylnaphthylamine (NPN). Logarithmic growth phase MDR *AB* were treated for 1h with 1 μ M LL-37 in RPMI+5%LB (A) or for 1h with 0.5 mg/L colistin in Ca-MHB (B). Bacteria were then washed with 10 mM Tris buffer and treated with 40 μ M NPN. Data are expressed as % of the maximal value recorded in the presence of 10mM EDTA and represent the average of triplicates from 3 independent experiments. *** P <0.001; two-tailed student's t-test.

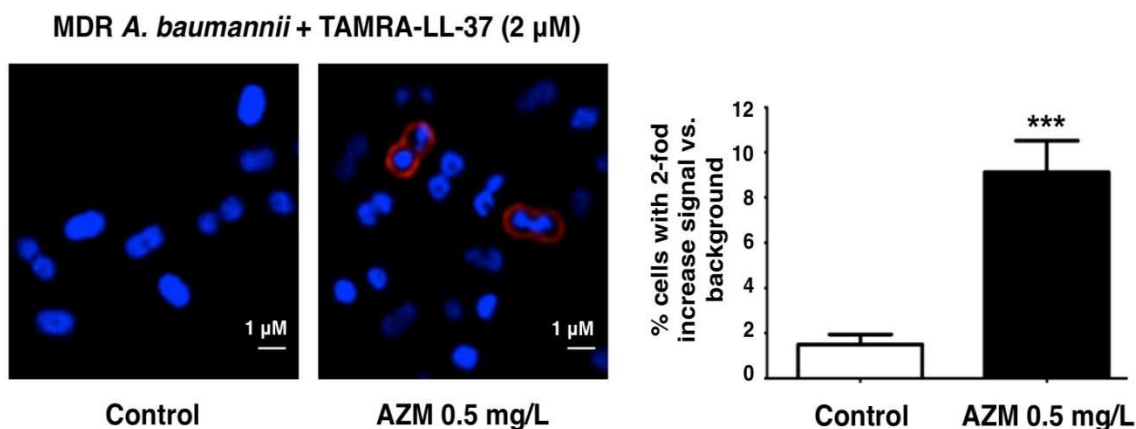


Figure S 2.4 Pre-treating MDR *A. baumannii* with AZM increases outer membrane binding of LL-37. Logarithmic growth phase MDR *AB* in RPMI+5%LB were incubated for 2h + 0.5 mg/L AZM. Bacteria were then treated for 30min with TAMRA-tagged LL-37 (red), washed, and then stained with DAPI (blue DNA stain). The number of MDR *AB* with red membrane signals at least twice the background level were counted using unbiased software analysis of multiple random microscopy fields with >500 cells counted per experimental replicate. Data plotted as the mean \pm SEM and represent the combination of 3 independent experiments. *** P <0.001; two-tailed student's t-test.

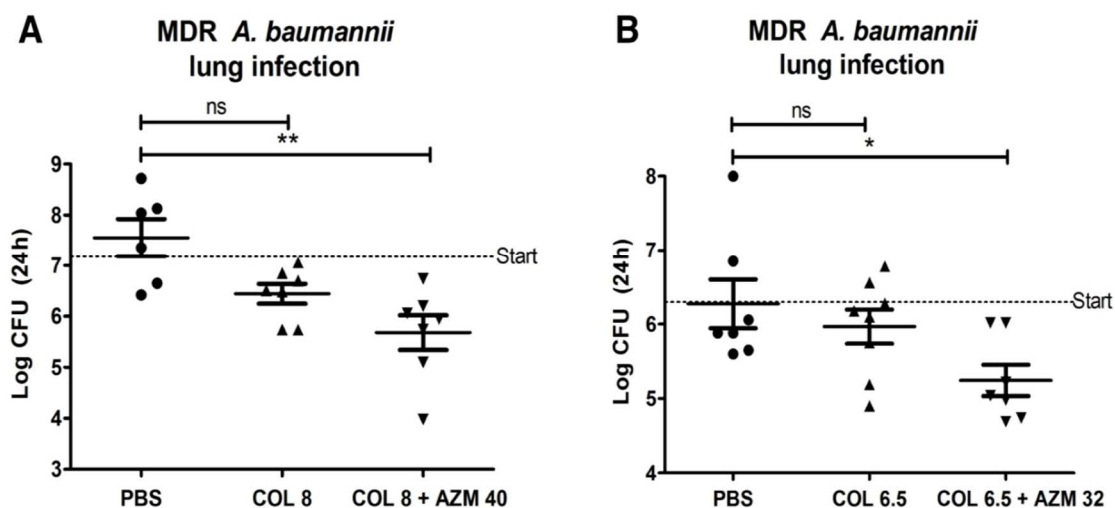


Figure S 2.5 Addition of low dose AZM to reduced dose colistin in treatment of MDR GNRs *in vivo*. (A) 8wk old female C57BL/6J mice were infected intratracheally (i.t.) with MDR *A. baumannii* strain AB5075. Lungs were homogenized at 24h; n = 6 for PBS control, 7 for colistin 8mg/kg, and 7 for AZM 40mg/kg ice. (B) 8wk old female C57BL/6J mice were infected intratracheally (i.t.) with MDR *AB*. Lungs homogenized at 24h; n = 7 for PBS control, 8 for colistin 6.5mg/kg, and 7 for AZM 32mg/kg ice. Data plotted as mean \pm S.E.M. AZM was dosed once subcutaneously immediately after bacterial inoculation. Colistin was dosed once subcutaneously 1 h after the bacterial inoculation. Dashed line “start” denotes the initial inoculum. * $P < 0.05$ and ** $P < 0.01$ based on one-way ANOVA analysis.

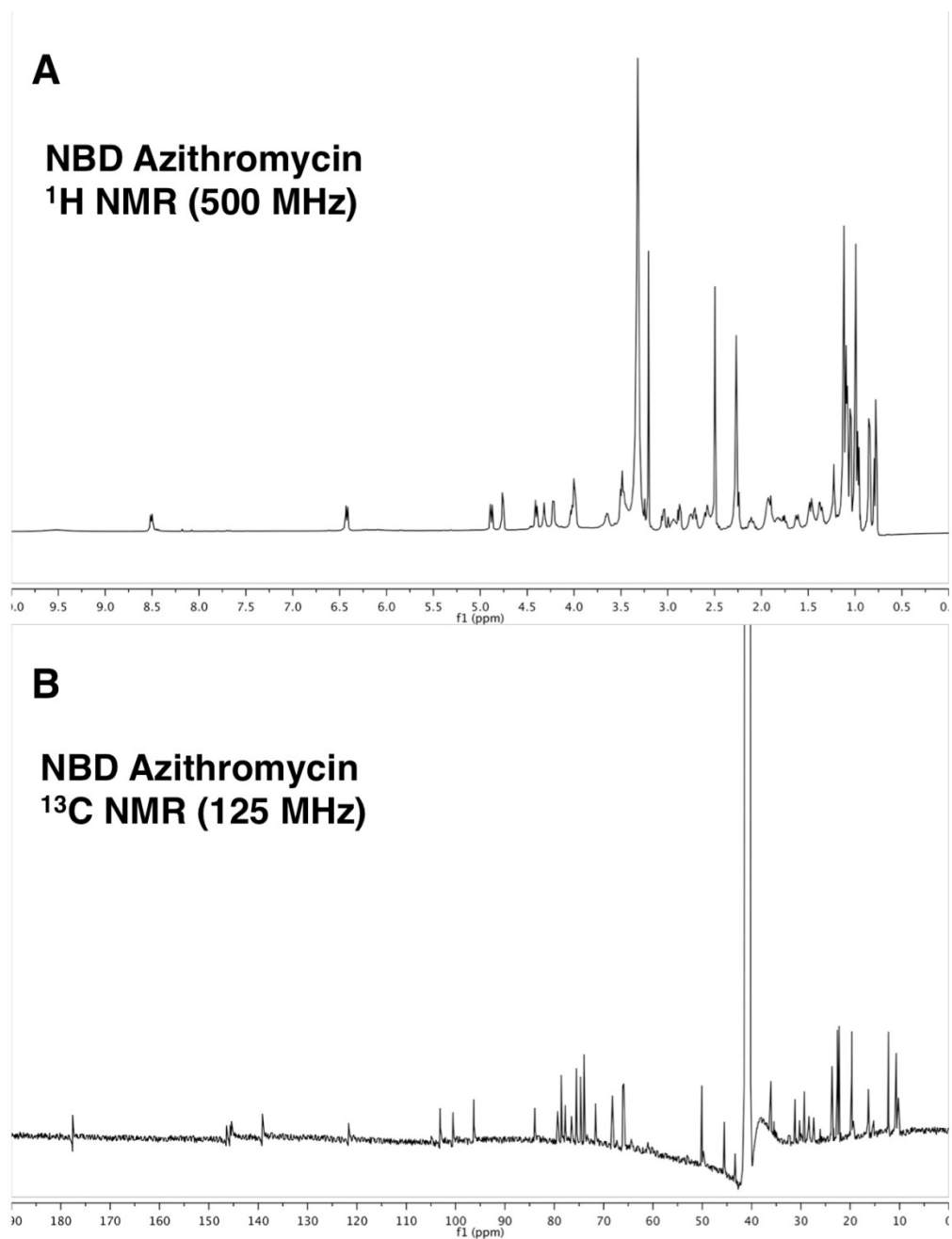


Figure S 2.6 Confirmation of structure and purity of synthesized NBD-tagged AZM. Shown are the spectra of (A) ^1H NMR (500 MHz) and (B) ^{13}C NMR (125 MHz) of NBD- tagged AZM in CD_3OD .

Table S 2.1 Three multidrug (including carbapenem)-resistant Gram-negative bacterial strains examined in this study. Automatic minimum inhibitory concentration (MIC) testing results in cation-adjusted Mueller-Hinton broth by VITEK - 2 testing system.

ANTIBIOTIC	<i>P. aeruginosa</i> , P4 (MDR)		<i>K. pneumoniae</i> , K1100 (MDR, KPC)		<i>A. baumannii</i> , AB5075 (MDR)	
	MIC	Interpret	MIC	Interpret	MIC	Interpret
Ampicillin	≥ 32	R	≥ 32	R	≥ 32	R
Amoxicillin/Clav.	≥ 32	R	≥ 32	R	≥ 32	R
Ampicillin/Sul.	≥ 32	R	≥ 32	R	≥ 32	R
Ticarcillin			≥ 128	R	≥ 128	R
Ticarcillin/Clav.	≥ 128	R				
Piperacillin	≥ 128	R	≥ 128	R	≥ 128	R
Piperacillin/Taz.	≥ 128	R	≥ 128	R	≥ 128	R
Cefalotin	≥ 64	R	≥ 64	R	≥ 64	R
Cefazolin	≥ 64	R	≥ 64	R	≥ 64	R
Cefuroxime	≥ 64	R	≥ 64	R	≥ 64	R
Cefuroxime Axetil	≥ 64	R	≥ 64	R	≥ 64	R
Cefotetan	≥ 64	R	8	R	≥ 64	R
Cefoxitin	≥ 64	R	32	R	≥ 64	R
Cefpodoxime	≥ 8	R	≥ 8	R	≥ 8	R
Cefotaxime	≥ 64	R	8	R	≥ 64	R
Ceftazidime	≥ 64	R	≥ 64	R	≥ 64	R
Ceftizoxime	≥ 64	R	4	R	≥ 64	R
Ceftriaxone	≥ 64	R	≥ 64	R	≥ 64	R
Cefepime	≥ 64	R	4	R	≥ 64	R
Aztreonam	≥ 64	R	≥ 64	R	≥ 64	R
Doripenem	≥ 8	R	≥ 8	R	≥ 8	
Ertapenem			≥ 8	R		
Imipenem	≥ 16	R	8	R	≥ 16	R
Meropenem	≥ 16	R	≥ 16	R	≥ 16	R
Amikacin	32	I	≥ 64	R	≥ 64	R
Gentamicin	8	I	≥ 16	R	≥ 16	R
Tobramycin	≤ 1	S	≥ 16	R	8	I
Nalidixic Acid	≥ 32	R	≥ 32	R	≥ 32	R
Ciprofloxacin	≥ 4	R	≥ 4	R	≥ 4	R
Levofloxacin	≥ 8	R	≥ 8	R	4	I
Moxifloxacin	≥ 8	R	≥ 8	R	≥ 8	R
Norfloxacin	8	I	≥ 16	R	≥ 16	R
Tetracycline	≥ 16	R	4	S	≤ 1	S
Tigecycline	≥ 8	R	4	I	≤ 0.5	S
Nitrofurantoin	≥ 512	R	128	R	≥ 512	R
TMP/SFX	≥ 320	R	40	S	≥ 320	R

Abbreviations: MDR = Multidrug-resistant; KPC = *Klebsiella pneumoniae*, carbapenemase-producing; R = Resistant; I = Intermediate; S = Sensitive; TMP/SFX = Trimethoprim/Sulfamethoxazole

Table S 2.2 Minimum inhibitory concentration (MIC) of Gram-negative bacterial strains used in this study to azithromycin, ciprofloxacin, erythromycin, clarithromycin, colistin and human cathelicidin LL-37. Comparative testing performed in standard bacteriologic testing media or mammalian tissue culture medium.

Bacterial Species/Strain	Azithromycin MIC (mg/L)		Ciprofloxacin MIC (mg/L)		Erythromycin MIC (mg/L)		Clarithromycin MIC (mg/L)		Colistin MIC (mg/L)		LL-37 MIC μ M
	Ca-MHB	RPMI + 5% LB	Ca-MHB	RPMI + 5% LB	Ca-MHB	RPMI + 5% LB	Ca-MHB	RPMI + 5% LB	Ca-MHB	RPMI + 5% LB	RPMI + 5% LB
<i>Pseudomonas aeruginosa</i> , Strain PA01	> 64	2	0.8	0.8							
<i>Pseudomonas aeruginosa</i> , Strain P4 (MDR)	> 64	1	12	12	>256	8	>256	8	8	4	64
<i>Klebsiella pneumoniae</i> , Strain K700603	64	2	< 0.5	< 0.5							
<i>Klebsiella pneumoniae</i> , Strain K1100 (MDR, KPC)	32	1	> 25	> 25	128	4	64	4	2	2	32
<i>Acinetobacter baumannii</i> , Strain AB19606	64	0.25	2	3							
<i>Acinetobacter baumannii</i> , Strain AB5075 (MDR)	32	0.5	> 25	> 25	32	1	32	0.5	1	2	4

Abbreviations: Ca-MHB = Cation-adjusted Mueller-Hinton broth; RPMI = Roswell Park Memorial Institute 1640, a basal mammalian tissue culture medium; LB = Luria broth; MDR = multidrug-resistant; KPC = *Klebsiella pneumoniae*, carbapenemase-producing.

Table S 2.3 Minimum inhibitory concentration (MIC) of azithromycin vs. a panel of contemporary clinical isolates of multidrug-resistant Gram-negative bacteria. Comparative testing performed in standard bacteriologic testing media or mammalian tissue culture medium.

Bacterial Species/Culture Site	Azithromycin MIC (mg/L)	
	Ca-MHB	RPMI + 5% LB
<i>Pseudomonas aeruginosa</i> , WP2 (MDR) - Sputum	32	1
<i>Pseudomonas aeruginosa</i> , WP3 (MDR) - Wound	256	0.5
<i>Pseudomonas aeruginosa</i> , WP5 (MDR) - Sputum	32	<0.06
<i>Pseudomonas aeruginosa</i> , UCSD P1 (MDR) - Urine	128	2
<i>Klebsiella pneumoniae</i> , WK7 (MDR) - Urine	256	32
<i>Klebsiella pneumoniae</i> , WK8 (MDR) - Urine	32	1
<i>Klebsiella pneumoniae</i> , WK9 (MDR) - Urine	32	0.5
<i>Klebsiella pneumoniae</i> , WK10 (MDR) - Sputum	>256	32
<i>Acinetobacter baumannii</i> , WA2 (MDR) - Wound	32	0.125
<i>Acinetobacter baumannii</i> , WA4 (MDR) - Peri-anal	64	0.125
<i>Acinetobacter baumannii</i> , WA5 (MDR) - Sputum	64	0.125

Abbreviations: Ca-MHB = Cation-adjusted Mueller-Hinton broth; RPMI = Roswell Park Memorial Institute 1640, a basal mammalian tissue culture medium; LB = Luria broth; MDR = multidrug-resistant.

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CHAPTER 3

Standard Susceptibility Testing Overlooks Potent Azithromycin Activity and Cationic Peptide Synergy Against Multidrug-Resistant *Stenotrophomonas maltophilia*

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PREFACE TO CHAPTER 3

Chapter 3 in full is an article published in *Journal of Antimicrobial Chemotherapy*. The work in Chapter 3 is a logical extension of the work presented in Chapter 2 and addresses both Aim 1: identify synergistic drug and innate immune interactions between leading pharmaceutical antibiotics and host antimicrobial peptides, serum, and immune cells against leading drug-resistant bacterial pathogens; and Aim 2: determine whether or not the synergies identified in Aim 1 can be harnessed to treat mice infected with drug-resistant bacterial pathogens. Using a combination of quantitative in-vitro assays, fluorescent and electron microscopy, and a murine model of bacterial lung infection, we demonstrated that azithromycin had potent activity against MDR *S. maltophilia* when tested in eukaryotic media, synergized with cationic antimicrobial peptides such as LL-37 and colistin, and enhanced neutrophil killing of MDR *S. maltophilia*. Finally, azithromycin monotherapy led to increased MDR *S. maltophilia* clearance in a murine pneumonia model.

SYNOPSIS

Objectives: The Gram-negative bacillus *Stenotrophomonas maltophilia* (SM) is an emerging multidrug-resistant opportunistic pathogen. Recent studies identify a potentially relevant activity of azithromycin against Gram-negative bacteria overlooked in standard bacteriologic testing. We investigated azithromycin activity against SM in testing conditions incorporating mammalian tissue culture media and host defense factors.

Methods: Minimum inhibitory concentration (MIC) testing, checkerboard assays, time-kill assays and fluorescence microscopy were performed for azithromycin, cationic peptide antibiotic colistin, and human defense peptide cathelicidin LL-37 alone or in combination in cation-adjusted Mueller-Hinton Broth or mammalian tissue culture media. Azithromycin sensitization of SM to host immune clearance was tested in a human neutrophil killing assay and a murine pneumonia model.

Results: We observed potent bactericidal activity of azithromycin against SM in mammalian tissue culture media absent in bacteriologic media. Colistin and LL-37 strongly potentiated azithromycin killing of SM by increasing drug entry. Additionally, azithromycin sensitized SM to neutrophil killing and increased SM clearance in a murine pneumonia model.

Conclusions: Despite lack of activity in standard MIC testing, azithromycin synergizes with cationic peptide antibiotics to kill SM in media mimicking tissue fluid conditions. Azithromycin, alone or in combination with colistin, merits further exploration in therapy of drug-resistant SM infections.

INTRODUCTION

Stenotrophomonas maltophilia (SM) is a ubiquitous multidrug-resistant (MDR) Gram-negative bacillus and opportunistic pathogen. Risk factors for infection include malignancy, neutropenia, HIV, cystic fibrosis, mechanical ventilation, ICU admission, long-term central venous catheter use, recent surgery, trauma and prior broad-spectrum antibiotic administration. Common SM infections are pneumonia and bacteremia, with reported mortality of 18 to 69% [1]. The incidence of SM infections ranges from 7.1 to 14.1 per 10,000 inpatients and is increasing, with intrinsic or acquired antibiotic resistance presenting great therapeutic challenges in severe infection [2,3]. Trimethoprim/sulfamethoxazole and ticarcillin/clavulanic acid (no longer manufactured in the U.S.) are first and second line treatments for serious SM infections, but recent studies have found in vitro resistance to these agents to be as high as 30% and 41%, respectively [4,5].

Clinical data regarding optimal therapy for SM infection are limited. Moreover, in vitro antimicrobial susceptibility testing results may not correctly predict SM clinical treatment response with reported discrepancies among testing methods (e.g. Etest, disc diffusion, agar dilution) and differing guidelines, e.g. European Committee on Antimicrobial Susceptibility Testing (EUCAST) versus Clinical and Laboratory Standards Institute (CLSI). Consequently, SM MIC results vary according to the conditions and methods implemented [3]. There is a great need to identify innovative treatment strategies for SM infection and a consistent antimicrobial susceptibility scheme to guide such therapy.

Macrolides are never recommended for SM therapy based on high MIC values in standard assays. However, recent studies have indicated that conventional antimicrobial susceptibility testing conditions may overlook macrolide activity and antibiotic synergies against other MDR Gram-negative bacteria including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Acinetobacter baumannii* [6,7]. Here we examine the potential of the commonly prescribed macrolide azithromycin to combat SM under more physiologic media conditions, in synergy with colistin or endogenous host defense factors (cathelicidin LL-37, neutrophils) in vitro, and in vivo using a murine pneumonia model.

MATERIALS AND METHODS

Bacterial Strains, Media and Antibiotics

MDR SM isolate K279a (ATCC BAA-2423) was utilized in all experiments. Additional MDR SM clinical isolates were obtained from the UCSD Center for Advanced Laboratory Medicine (3 strains) and the Laboratory of Bacterial Drug Resistance, Gunma University Graduate School of Medicine (5 strains). Isolates were stored in Luria Broth (LB) + 50% glycerol at -80°C until use. Azithromycin and colistin were purchased from Sigma Aldrich and cathelicidin LL-37 from American Peptide. Bacteriologic media Muller Hinton Broth (Spectrum Chemicals) was supplemented with 20-25 mg/L Ca^{2+} and 10-12.5 mg/L Mg^{2+} (CA-MHB). Tissue culture media Roswell Park Memorial Institute 1640 (RPMI) (ThermoFisher Scientific) was supplemented with 10% Luria Broth (LB) (Hardy Diagnostics).

Minimum Inhibitory Concentration, Checkerboard and Time Kill Assays

Broth microdilution antimicrobial susceptibility testing was performed in CA-MHB according to CLSI guidelines and in RPMI+10%LB. Checkerboard panels were likewise performed in both media [8,9]. Synergy, additivity and antagonism were defined by fractional inhibitory concentration index (FICI): FICI ≤ 0.5 defined synergy, >0.5 to <4 indifference, and ≥ 4 antagonism. Time kill assays were performed in CA-MHB and RPMI+10%LB as previously described, with bactericidal activity defined as a reduction in viable bacteria by $\geq 3 \log_{10}$ cfu/mL; synergy, $\geq 2 \log_{10}$ cfu/mL reduction; additivity/indifference, $<2 \log_{10}$ cfu/mL reduction; antagonism, $>2 \log_{10}$ cfu/mL increase [10].

Fluorescence Microscopy

Fluorescence microscopy was performed as previously described with the following modifications [11]. For ready visualization, SM was used at $2 \log_{10}$ cfu/mL higher concentration than in MIC testing, along with higher but pharmacologically achievable concentrations of antibiotics NBD-tagged azithromycin (20X MIC), colistin (8X $\frac{1}{4}$ MIC) and LL-37 (10X $\frac{1}{4}$ MIC) [12]. Bacteria were labeled with $1 \mu\text{g/mL}$ FM4-64 (membrane) and $2 \mu\text{g/mL}$ DAPI (nucleic acid) (Molecular Probes) prior to transfer onto a 1.2% agarose pad containing 20% LB for microscopy and image analysis using ImageJ software v1.48f and CellProfiler 2.0.

Neutrophil Killing Assays

Human neutrophils were isolated from healthy donors using the PolymorphPrep system (Axis-Shield) under protocols approved by the UCSD Human Subjects Institutional Review Board for use in established bacterial killing assays with minor

modifications [13]. Neutrophils were resuspended in RPMI to 2×10^6 cells/mL, stimulated with 25 nM phorbol-12-myristate-13-acetate (PMA) for 2h at 37°C, washed and used to seed a 96-well plate (2×10^5 cells/well). Cells were infected at a multiplicity of infection (MOI) equal to 50 with SM that were untreated or exposed overnight to a sub-bacteriostatic concentration (0.03 mg/L) of azithromycin.

Murine Pneumonia Model

Adapting a published pneumonia model, A/J mice (8-10 weeks old, Jackson Laboratory) were infected intratracheally with 2×10^6 cfu (low inoculum) or 2×10^7 cfu (high inoculum) of SM (K279a) and treated with PBS control or azithromycin 50 µg/g subcutaneously (SQ) every 24h for 1 dose (low inoculum) or 2 doses (high inoculum) [14]. Animals were euthanized and lungs harvested, weighed, homogenized, serially diluted and plated on LB agar to enumerate cfu after 24h incubation at 37°C. In a subset of mice infected at the high inoculum, bronchoalveolar lavage fluid (BALF) was collected at 48h by exposing the trachea, injecting 0.8 mL of cold PBS, and retrieving BALF using an 18 gauge needle connected to a 1mL syringe. BALF was centrifuged at 500xg for 10 min and supernatants frozen at -80°C for later cytokine analysis using commercial ELISA kits for IL-1β (R&D Systems) and MIP-2 (BD Biosciences). Pelleted BALF cells were resuspended in 0.25 mL cold PBS and enumerated utilizing a hemocytometer, light microscopy and Wright-Giemsa staining to determine the differential leukocyte count. Excised lungs from 4 mice in each group were fixed using 4% formalin, embedded in paraffin, sectioned and stained with hematoxylin-eosin for blinded analysis by a pathologist. A graded scale of 0 (absent) to 3 (extensive) was used to assess overall airspace involvement,

perivascular/peribronchial inflammation, evidence of inflammation, and edema/debris or macrophages in the intra-alveolar space. All procedures were performed by a protocol approved by the UCSD Institutional Animal Care and Use Committee.

Statistics

Statistical analyses were performed using GraphPad Prism 6.0f (GraphPad Software). One-way ANOVA, two-way ANOVA or unpaired student's t test were utilized where appropriate. P values <0.05 were regarded to be statistically significant.

RESULTS

Markedly increased activity of azithromycin against SM in tissue culture versus bacteriologic media

Azithromycin MIC assays for several MDR SM strains was determined by CLSI broth microdilution methodology using either the recommended bacteriologic media, CA-MHB, or the common mammalian tissue culture media RPMI supplemented with 10% LB to support equivalent bacterial growth (**Figure S 3.1**). A >500-fold reduction in MIC (≤ 0.25 versus ≥ 128 mg/L) was observed for nearly all isolates tested with RPMI+10%LB compared to CA-MHB (**Table 3.1**). Transmission electron microscopy of SM K279a cells treated with azithromycin, a ribosomal protein synthesis inhibitor, in RPMI+10%LB revealed ribosomal clustering consistent with its known target of action (**Figure S 3.2**). In a kinetic killing assay, azithromycin 0.25 mg/L had no effect on SM K279a growth in CA-MHB, but reduced bacterial cfu below detectable levels within 24h in RPMI+10%LB (**Figure 3.1A**).

Cationic peptides potentiate the bactericidal activity of azithromycin against SM by increasing drug entry

We recently observed synergy of azithromycin with cationic antimicrobial peptides (AMP) against other Gram-negative rods (GNRs) [7]. The cationic peptide antibiotic colistin, a drug of last resort for certain MDR pathogens, is bacteriostatic against SM in RPMI+10%LB but not in CA-MHB (**Figure 3.1A**). At $\frac{1}{4}$ MIC of both drugs, azithromycin showed potent bactericidal synergy with colistin in RPMI+10%LB but not CA-MHB (**Figure 3.1B**). The human cathelicidin LL-37 is a cationic endogenous host defense peptide abundantly produced by neutrophils and epithelial cells. Once again, at $\frac{1}{4}$ MIC of each agent, azithromycin showed bactericidal synergy with LL-37 in RPMI+10%LB but not CA-MHB (**Figure 3.1C**). Using fluorescence microscopy-based bacterial cytological profiling, both colistin and LL-37, which are known to form pores and disrupt bacterial membranes, markedly enhanced entry of fluorescently labeled (NBD-tagged) azithromycin into bacterial cells (**Figure 3.2A and B**), facilitating azithromycin access to the 50S ribosomal subunit.

Table 3.1 MICs of azithromycin for nine different SM strains

SM strain	MIC (mg/L)	
	CA-MHB	RPMI + 10%LB
K279a (ATCC BAA-2423)	256	0.25
SM 5	32	0.06
SM 12	256	0.03
SM 26	128	0.03
SM 57	128	0.03
SM 65	256	0.06
ATCC 51331	256	0.06
SM BWR	256	0.03
SM NP	256	0.25

Comparative MIC testing performed in standard bacteriological (CA-MHB) and supplemented mammalian tissue culture (RPMI + 10%LB) media.

Azithromycin sensitizes SM to neutrophil killing and increases SM clearance in vivo

Azithromycin synergizes with LL-37 against SM suggesting it could sensitize the opportunistic pathogen to killing by neutrophils, which produce the defense peptide as part of their antimicrobial arsenal. SM induced oxidative burst and the formation of neutrophil extracellular traps (in which LL-37 is deployed) from freshly isolated human neutrophils (**Figure S 3.3**). Overnight pretreatment of SM with a sub-bacteriostatic concentration of azithromycin sensitized the bacterium to human neutrophil killing (**Figure 3.3A**). We next assessed the efficacy of azithromycin monotherapy in an adapted murine model of SM pneumonia, using immune susceptible A/J mice that have functional C5 complement deficiency. Under the experimental conditions, azithromycin treatment

did not affect the acute histopathological features of inflammatory pneumonia (**Figure 3.3B and S 3.4A**), nor the amount or composition of BALF leukocyte infiltration (**Figure 3.3C**), nor BALF levels of pro-inflammatory cytokines MIP-2 or IL-1 β (**Figure S 3.4B**) at 48h post-infection. However, the recovered cfu of SM from the lungs was markedly reduced by 58% in azithromycin-treated mice (1629 ± 123 cfu/g) versus PBS controls (3898 ± 1154 cfu/g) at 24h in a lower inoculum challenge, and reduced by 95% in azithromycin treated mice (824 ± 184 cfu/g) versus PBS controls (17915 ± 11186 cfu/g) at 48h in a higher inoculum challenge (**Figure 3.3D**).

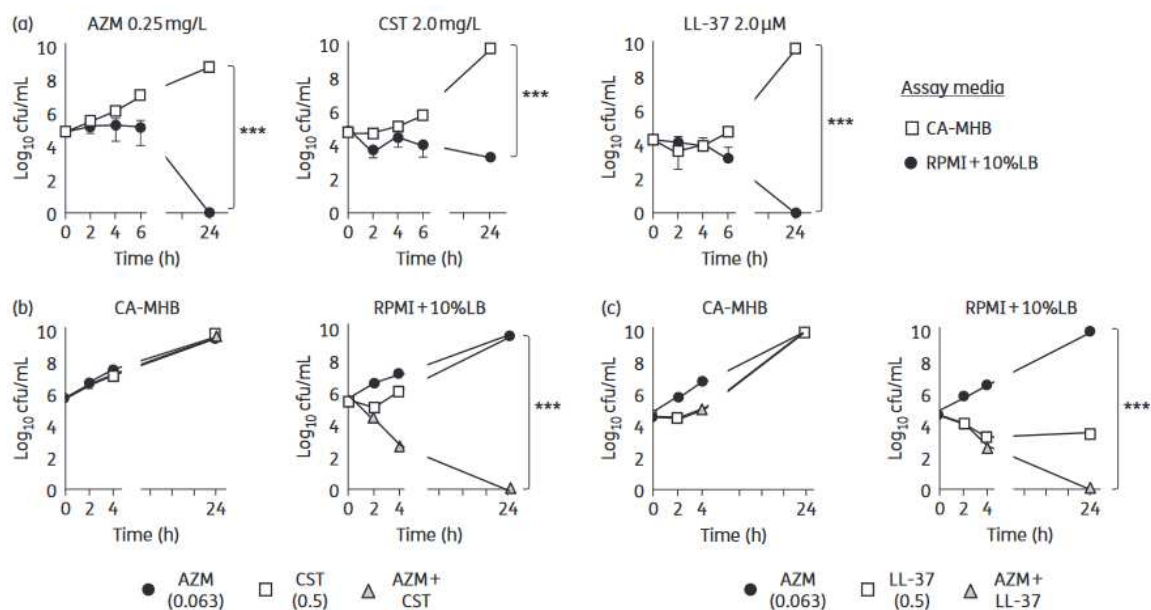


Figure 3.1 Azithromycin bactericidal activity against SM observed in media alone and in synergy with cationic antimicrobial peptides. (a) Time–kill curve demonstrating azithromycin, colistin and LL-37 activity against SM K279a in bacteriological (CA-MHB) versus tissue culture-based (RPMI+10%LB) media. (b and c) Bactericidal synergy, defined as a ≥ 2 log₁₀ decrease in cfu/mL for time–kill assays, was observed for both azithromycin + colistin and azithromycin + LL-37 at 1/4 MIC of all agents (FICI \leq 0.5), but only in RPMI+10%LB. Data are plotted as mean \pm SEM and represent the combination of three experiments performed in triplicate. *** $P < 0.001$ by two-way ANOVA. AZM, azithromycin; CST, colistin.

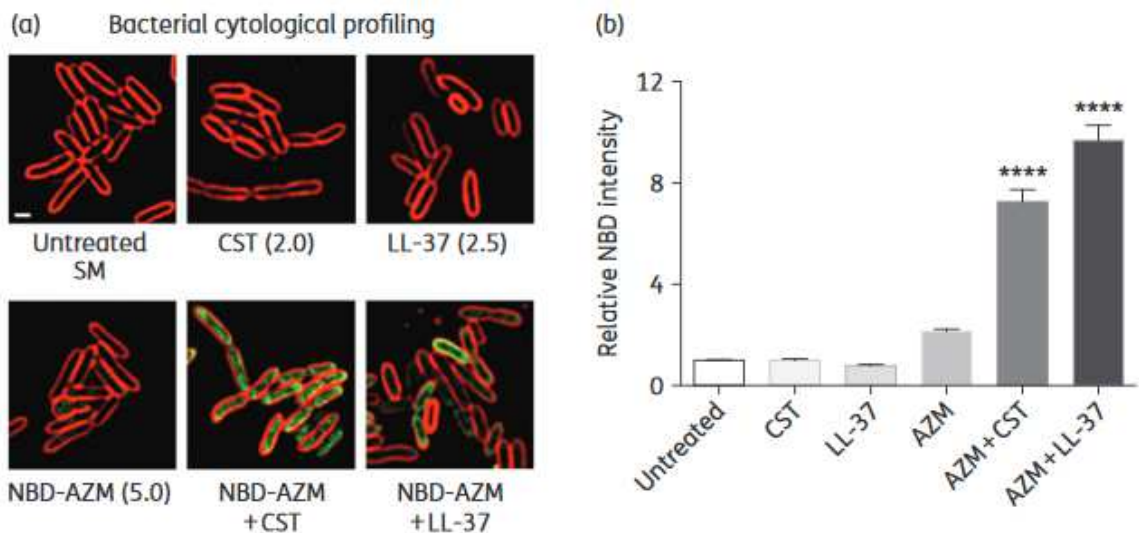


Figure 3.2 Bacterial cytological profiling showing that cationic peptides facilitate azithromycin entry into SM cells. (a) Fluorescence microscopy performed using log-phase SM K279a treated for 1 or 2 h with NBD-tagged azithromycin (5 mg/L) (green), colistin (2 mg/L) or LL-37 (2.5mM) alone or in combination. (b) Bar graphs generated from software analysis of multiple random fluorescent microscopy fields of cells treated with NBD-tagged azithromycin, colistin, LL-37, NBD-tagged azithromycin+colistin and NBD-tagged azithromycin+LL-37 (with >500 cells counted per condition). Azithromycin+colistin and azithromycin+LL-37-treated bacteria had 10-fold higher NBD intensity than azithromycin, colistin or LL-37 alone. Data are plotted as mean \pm SEM and represent the combination of three experiments performed in triplicate. **** P <0.001 by one-way ANOVA. AZM, azithromycin; CST, colistin.

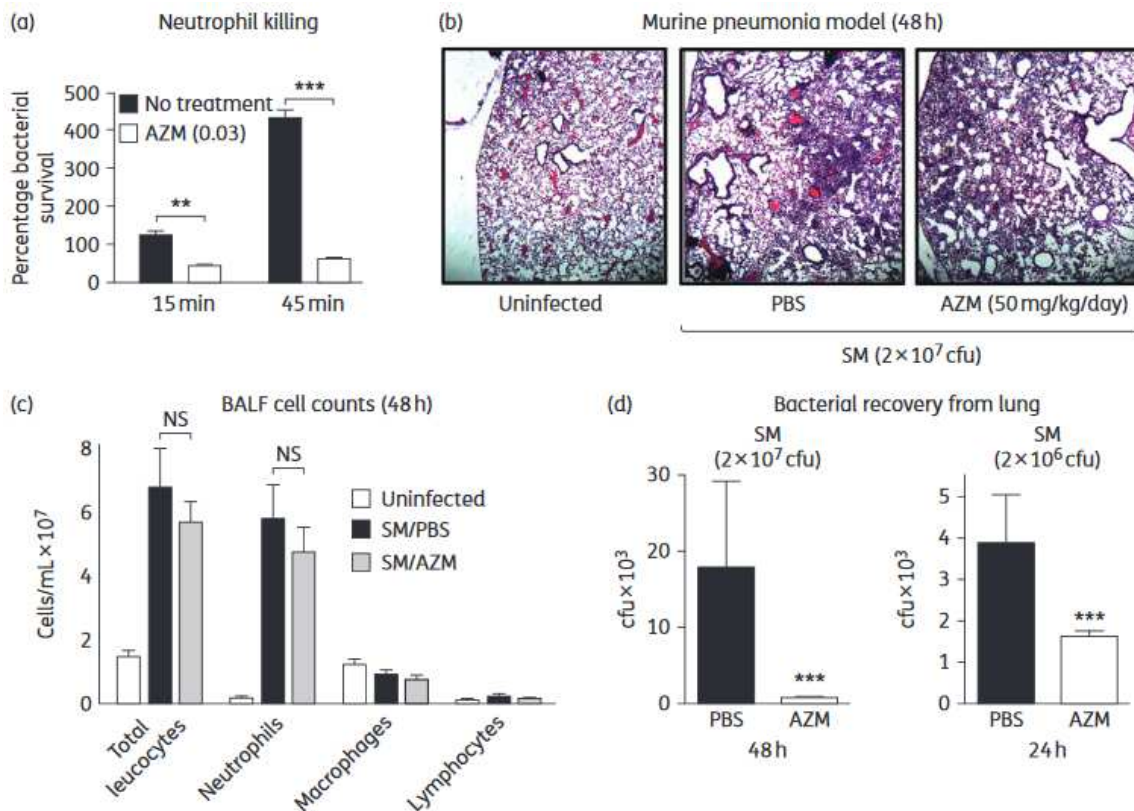


Figure 3.3 Azithromycin sensitizes SM to neutrophil killing and increases SM clearance in vivo. (a) Percentage survival of untreated versus azithromycin-pretreated SM K279a bacteria in a human neutrophil killing assay. (b) Representative lung histopathology of A/J mice infected intratracheally with high-inoculum SM K279a or PBS control and treatment with daily azithromycin or PBS control. (c) Total enumeration of leucocytes in BALF of mice including neutrophils, macrophages and lymphocytes. (d) Bacterial recovery (cfu/g) from lung homogenates of A/J mice intratracheally infected with different inocula of SM K279a and treated with azithromycin (50 mg/kg sc daily) for 24 or 48 h. $n=20$ per group (24 h) and 11 per group (48 h). For all panels (a–d), data represent the mean \pm SEM from the combination of three experiments performed in triplicate. $**P<0.01$ and $***P<0.001$ by two-way ANOVA (a) or unpaired Student's t-test (d). NS, no statistical significance by unpaired Student's t-test (c). AZM, azithromycin.

DISCUSSION

Using SM K279a, a strain with several genes conferring resistance to heavy metals and antimicrobials (including nine resistance-nodulation division type efflux pump genes), and several contemporary clinical SM isolates, this study demonstrated a striking difference of azithromycin potency in standard bacteriologic testing media

(negligible activity, MIC as high as 256 mg/L) compared to mammalian tissue culture media (MIC 0.25 mg/L or lower) supplemented with 10% LB [15]. While there is no current clinical MIC breakpoint for azithromycin and SM, the EUCAST breakpoint used for the GNR *Campylobacter jejuni* is 4 mg/L. Azithromycin also showed potent synergy with colistin, an antibiotic of last resort for MDR GNR infections, and LL-37, an endogenous cationic AMP. As disruptors of bacterial membranes, colistin and LL-37 potentiate azithromycin entry into bacterial cells and enhance azithromycin access to its ribosomal target.

Recent studies suggest different antibiotics may work in concert or at odds with our innate immune system in the setting of infection [13,16]. However, our understanding of the interactions between common antibiotics prescribed in clinical practice and endogenous antimicrobials such as LL-37 and the cells that produce them (e.g. neutrophils) remains limited. Whereas SM was impervious to human neutrophil killing in our ex vivo assay, it was sensitized to neutrophil killing by azithromycin pretreatment. Moreover, azithromycin showed the ability to significantly reduce SM bacterial counts in an in vivo murine pneumonia challenge model in which a predominantly neutrophilic inflammatory infiltrate was elicited in the lungs.

Traditional SM antimicrobial susceptibility testing can be problematic given inconsistencies between the results of different methods, the lack of a gold standard or universal reference method and inability of testing results to reliably and appropriately translate to clinical efficacy. Conventional in vitro studies also inadequately account for the dynamic interactions between conventional antibiotics and the host immune response. Our study and others suggest that current standardized susceptibility testing in

bacteriologic media may fail to adequately detect the bactericidal activity of azithromycin and its interactions with the innate immune system against various MDR GNRs, including SM [6,7].

Limitations of our study include the modest number of SM isolates tested and the reliance on a murine pneumonia model for *in vivo* azithromycin activity against SM without human clinical data. Nevertheless, this preliminary investigation highlights a potential utility of azithromycin, a safe and established antibiotic, used alone or in combination with colistin, in SM infection. The work also emphasizes the importance of accounting for the dynamic interaction between conventional antibiotics and host immunity in the setting of infection. Future randomized clinical trials in humans with azithromycin will be required to determine the true utility of this finding.

TRANSPARENCY DECLARATIONS

The authors declare no conflict of interest.

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J, Sakoulas G, Nizet V. Standard susceptibility testing masks potent azithromycin activity and cationic peptide synergy against multidrug-resistant *Stenotrophomonas maltophilia*. *J Antimicrob Chemother*. 2016; online early. The dissertation author was the second author of this work.

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SUPPLEMENTARY MATERIALS AND METHODS

Growth Curve

SM K279a inoculated into 5 mL of LB was grown overnight to stationary phase (14-16 hrs) at 37°C in a shaking incubator. The following day bacteria were washed twice with PBS and re-suspended in tubes containing 25 mL of CA-MHB or RPMI+10%LB to an initial OD₆₀₀ of 0.05. Tubes were then subsequently placed in a shaking incubator at 37°C with re-growth assessed by measuring OD₆₀₀ at selected time intervals up to 24h.

Electron Microscopy

Electron microscopy was performed to assess the effect of SM K279a (extracellularly and intracellularly) in tissue culture media. SM K279a was grown overnight to stationary phase (14- 16 hrs) in 5mL of LB at 37°C in a shaking incubator.

Bacteria were then washed twice with PBS and re-suspended in RPMI+10% LB prior to being placed in a shaking incubator at 37°C for 2 h. Next cultures were centrifuged at 4000 rpm for 10 min at room temperature. The supernatant was then aspirated and the bacterial pellets re-suspended in modified Karnoversusky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15M sodium cacodylate buffer). These samples were then processed and electron microscopy was performed as previously described [17]. Samples were viewed using a Tecnai G2 Spirit BioTWIN transmission electron microscope and photographed with an Eagle 4k HS digital camera. Images were obtained from random fields at 1900X, 9300X and 13000X.

Neutrophil Extracellular Trap & Oxidative Burst Assays

Neutrophil extracellular trap (NET) induction assays and oxidative burst assays were performed as previously described [18,19].

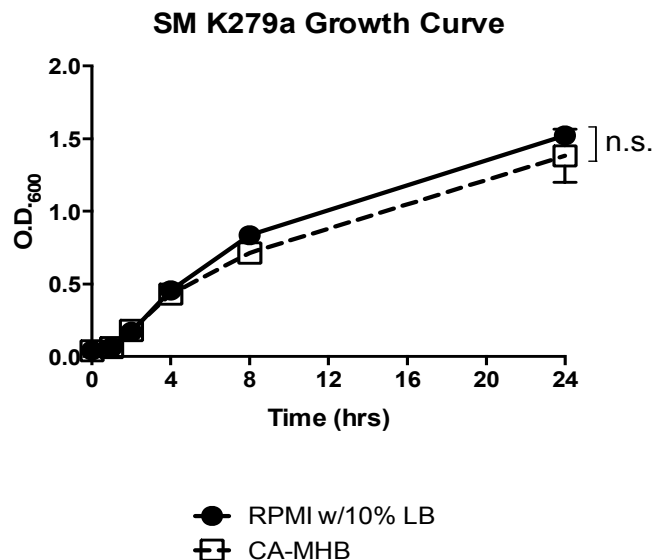


Figure S 3.1 Growth of SM K279a in bacteriologic (CA-MHB) versus supplemented tissue culture (RPMI+10%LB) media. Turbidity as a measure of bacterial growth was determined by OD₆₀₀ at 1, 2, 4, 8 and 24h. Data are plotted as mean \pm SEM and represent the combination of 3 experiments. Statistical analysis by unpaired student's t-test revealed no statistical significance. (ns).

AZM-treated *S. maltophilia* (in RPMI+10% LB)

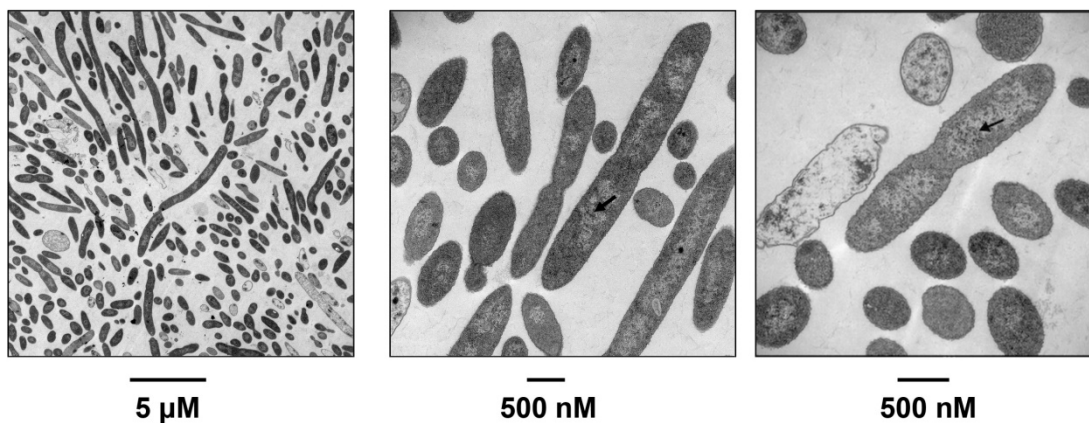


Figure S 3.2 Transmission electron microscopy images (1900X, 9300X and 13000X) of stationary phase SM K279a treated for 2h with AZM 0.25 mg/L. Images reveal ribosomal clustering (indicated by arrow) in bacteria treated with AZM, a protein synthesis inhibitor known to inhibit the translation of mRNA by binding to the 50S subunit of the bacterial ribosome. Images were taken from multiple random fields (>5) and analyzed in a blinded fashion.

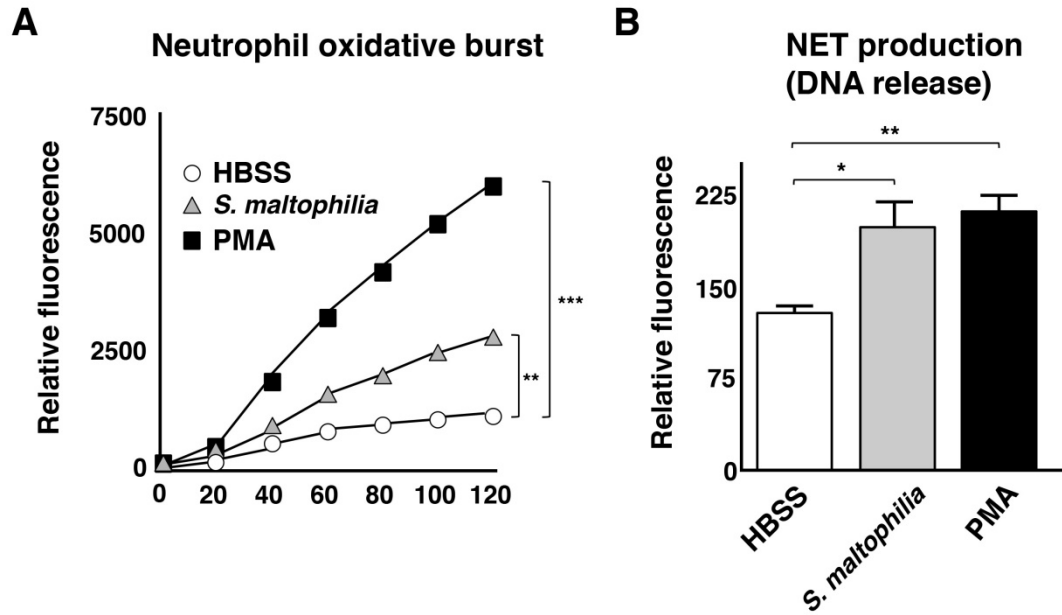


Figure S 3.3 Neutrophil production of reactive oxygen species (ROS) and extracellular traps (NETs) in response to SM. *A*, ROS production induced by SM K279a at an MOI = 10 in comparison to the potent neutrophil agonist PMA. *B*, SM K279a induces NET formation from human neutrophils at an MOI = 10. Data represents the mean \pm SEM of 3 experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ by two-way ANOVA.

A

Murine lung infection model
***S. maltophilia* (2×10^7 CFU), 48 h**

Group	Intra-alveolar edema/debris	Intra-alveolar macrophages	Perivascular/peribronchial inflammation	Intra-alveolar inflammation	Overall % airspace involvement
Uninfected	1.00 ± 0.00	1.25 ± 0.25	0.25 ± 0.25	1.00 ± 0.00	1.00 ± 0.00
PBS	1.75 ± 0.25	2.75 ± 0.25	2.25 ± 0.25	3.00 ± 0.00	22.50 ± 0.00
AZM	2.50 ± 0.30	2.75 ± 0.25	2.50 ± 0.30	3.00 ± 0.00	17.50 ± 2.50

B

Murine lung infection model
***S. maltophilia* (2×10^7 CFU), 48 h**

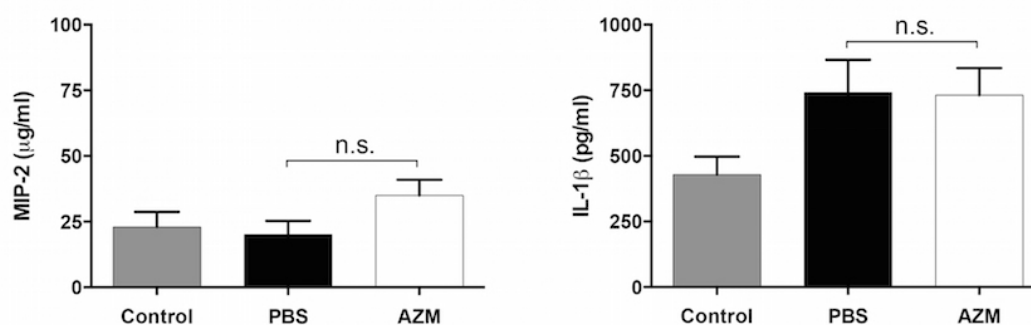


Figure S 3.4 Murine lung infection model. *A*, Histological grading. A/J mice were infected with 2×10^7 cfu of SM K279a or PBS intratracheally and treated with AZM (50 mg/kg) or PBS daily for 48h prior to harvesting lungs; $n = 4$ for control (PBS/PBS), PBS (SM K279a/PBS) and AZM (SM K279a/AZM). *B*, ELISA detection of inflammatory cytokines (IL-1 β and MIP-2) from BALF; $n = 8$ for control (PBS/PBS), PBS (SM K279a/PBS) and AZM (SM K279a/AZM). Results expressed as mean \pm SEM. Statistical analysis by unpaired student's t-test revealed no statistical significance (ns).

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CHAPTER 4

Component Analysis of Multi-Purpose Contact Lens Solutions to Enhance Activity Against *Pseudomonas aeruginosa* and *Staphylococcus aureus*

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Manuscript reviewed, revised, and resubmitted to *Antimicrobial Agents and Chemotherapy*

Running Title: Optimizing activity of contact lens solutions

PREFACE TO CHAPTER 4

Chapter 4 is a manuscript that has been reviewed, revised, and resubmitted to *Antimicrobial Agents and Chemotherapy*. The work in Chapter 4 addresses Aim 3: explore novel therapeutic combinations of antimicrobial agents in the prevention and treatment of drug-resistant bacterial infections. Using an extensive panel of in-vitro assays such as MIC testing, checkerboards, ISO protocol 14729, and biofilm coated contact lenses, we demonstrated that contact lens solutions with the biguanide functional group such as chlorhexidine and polyaminopropylbiguanide had the most antimicrobial activity against *S. aureus* and that EDTA had the most activity against *P. aeruginosa*. Furthermore, based on our in-vitro results we developed a novel contact lens solution formulation that harnessed the synergy we discovered between the biguanide preservatives and EDTA. This new formulation had at least three times more antimicrobial activity against *P. aeruginosa* than anything available on the market today, retained excellent activity *S. aureus*, and was even effective at clearing and killing *P. aeruginosa* and *S. aureus* biofilms.

ABSTRACT

More than 125 million people wear contact lenses worldwide, and contact lens use is the single greatest risk factor for developing microbial keratitis. We tested the antibacterial activity of multi-purpose contact lens solutions and their individual component preservatives against the two most common bacterial keratitis causing pathogens, *Pseudomonas aeruginosa* (PA) and *S. aureus* (SA). In vitro antibacterial activity of five multi-purpose contact lens solutions (Opti-Free GP, Boston Simplus, Boston Advance, Menicare GP, and Lobob) was assayed by the standard broth dilution method. Synergy between the preservative components found in the top performing solutions was assayed using checkerboard and time kill assays. The ISO 14729 criteria and the standard broth dilution method were used to define an optimized contact lens solution formulation against a clinical panel of drug sensitive and drug resistant PA and SA strains. Preservatives with the biguanide function group, chlorhexidine and polyaminopropylbiguanide (PAPB), had the best anti-staphylococcal activity; while EDTA was the best anti-pseudomonal preservative. The combination of chlorhexidine and EDTA had excellent synergy against PA. A solution formulation containing chlorhexidine (30PPM), PAPB (5PPM), and EDTA (5000PPM) had three to seven times more anti-pseudomonal activity than anything available to consumers today. A multi-purpose contact lens solution containing a combination of chlorhexidine, PAPB, and EDTA could help to reduce the incidence of microbial keratitis for contact lens users worldwide.

INTRODUCTION

There are an estimated 38 million contact lens users in the US [1] and 125 million worldwide [2]. Contact lens use is the single greatest risk factor for developing microbial keratitis [3], which can cause vision loss and blindness if not diagnosed and treated promptly. The US Centers for Disease Control estimates that microbial keratitis affects 5 to 10 in every 10,000 contact lens wearers[2] and accounts for ~1 million clinic visits annually in the US[1]. Bacterial infections represent ~90% of all microbial keratitis cases, with *Pseudomonas aeruginosa* (PA) followed by *Staphylococcus aureus* (SA) being the most common pathogens [2]. The remaining 10% are associated with amoeba such as *Acanthamoeba castellanii* or fungi including *Fusarium solani* [2].

In 2008, representatives from the American Academy of Ophthalmology, Cornea Society, American Society of Cataract and Refractive Surgery, and the Contact Lens Association of Ophthalmologists all testified before the Ophthalmic Device Panel of the US Food and Drug Administration about the need to enhance the antimicrobial efficacy of contact lens solutions[4]. Subsequent studies have tested the efficacy of commercially available contact lens solutions against pathogens that cause keratitis[5, 6]. However, these studies test commercial solutions as a whole and have not evaluated the efficacy of each of the component antimicrobial preservative against PA or SA. Furthermore, a recent publication highlights the importance of testing solutions' activity against clinical bacterial isolates rather than the standard laboratory ISO ATCC PA and SA strains [7].

We hypothesized that testing the efficacy of commercially available multi-purpose contact lens solutions, as well as their antimicrobial preservatives alone and in different combinations against clinical PA and SA isolates would allow us to develop a

formulation with more potent antibacterial activity than anything currently available to consumers today.

MATERIALS AND METHODS

Bacterial strains. PA01 and PA103 were obtained from the American Type Culture Collection (ATCC) and MDR PA strain P4 from a tertiary academic hospital in New York. SA strains were methicillin-resistant (MRSA) TCH1516 (USA300) from ATCC, Sanger 252 (USA200) from the Network on Antimicrobial Resistance in SA (NARSA), and methicillin-sensitive UAMS1 from G. Somerville (University of Nebraska). The following clinical keratitis isolates which were fluoroquinolone sensitive (FQs) or fluoroquinolone resistant (FQr) were obtained from R. Kowalski (University of Pittsburg): SA K2751 FQs, K2738 FQr, and K2735 FQr; and PA K2749 FQs, PA13 FQr, and PA16 FQr.

Preservatives and Reagents. Mueller-Hinton Broth (MHB, Spectrum Chemicals) was supplemented with CaCl_2 and MgCl_2 to make cation-adjusted MHB (Ca-MHB) – final cation concentrations (20–25 mg/L Ca^{2+} and 10–12.5 mg/L Mg^{2+}). Other reagents were obtained from the following vendors: Luria Broth base (LB, Hardy Diagnostics); Todd Hewitt Broth base (THB, Neogen); Ethylenediaminetetraacetic acid (EDTA, Sigma); chlorhexidine gluconate (CHD, Sigma); polyaminopropyl biguanide (PAPB, Lotioncrafter); resazurin sodium (Sigma); Difco D/E neutralization broth (BD).

Multi-purpose contact lens solutions. Opti-Free GP (Alcon), Boston Simplus and Boston Advance (Bausch & Lomb), Menicare GP (Menicon), and Lobob (Lobob Labs) were purchased from Amazon.com.

Contact Lenses. Senofilcon A soft silicon hydrogel lenses (Acuvue® Oasys® , Johnson & Johnson Vision) were purchased from Lens.com. These contact lenses were chosen because they represent a leading extended wear silicon hydrogel lens approved by the FDA for up to 14 days of extended wear.

Minimum inhibitory concentrations (MIC) determinations. MIC values for contact lens solutions and their preservative components were determined using broth microdilution in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines[8]. Bacterial viability was determined using an optical plate reader (OD_{600nm}), and indicator dye resazurin as previously described [9].

More specifically, for the individual contact lens solution preservatives such as CHD, PAPB, EDTA, and benzyl alcohol, concentrated stocks were purchased and then diluted down in sterile water to 10X the concentrations used in the contact lens solutions. We then followed the same broth microdilution method outlined in the CLSI guidelines above for the testing of antibiotics. More specifically, in the first row of wells in a 96-well plate, we added 20ul of 5×10^6 cfu/ml of bacteria suspended in Ca-MHB, 20ul of the 10X preservative, and 160ul of Ca-MHB. Thus, the first row of wells on the 96 well plate contained 200ul of liquid total with 5×10^5 cfu/ml bacteria and a 1X concentration of the preservative. Serial dilutions were performed by transferring 100ul from the first well into 100ul of Ca-MHB with 5×10^5 cfu/ml bacteria in the second row of wells, mixing well, and then repeating down the rows.

MIC testing of the contact lens solutions and our optimized formulation was an adaptation of the CLSI broth microdilution guidelines for antibiotics. In the first row of wells in the 96-well plate, 20ul of 5×10^6 cfu/ml of bacteria suspended in Ca-MHB was

combined with 180ul of contact lens solution. Thus, the first well contained 10% Ca-MHB and 90% contact lens solution with 5×10^5 cfu/ml bacteria. Serial dilutions were performed by transferring 100ul from the first well into 100ul of Ca-MHB with 5×10^5 cfu/ml bacteria in the second row of wells, mixing well, and then repeating down the rows. Thus, going down a vertical column of wells in the 96 well plate, every well would have 5×10^5 cfu/ml bacteria, followed by a decreasing percentage of contact lens solution 90%, 45%, 22.5%, 11.3%, 5.6%, 2.8%, 1.4%, 0.7% and a corresponding increase in percentage of Ca-MHB.

Traditional checkerboard and time kill assays. Performed as previously described [10]. Overnight cultures of PA (in LB) and SA (in THB) were grown at 37°C, pelleted, washed twice and resuspended in PBS to $OD_{600}=0.40$. Bacterial stocks were then diluted in Ca-MHB to initial inoculum $\sim 1 \times 10^6$ CFU/ml and contact lens solution preservatives added at the indicated concentrations. Checkerboard assay: 96-well plates were incubated with shaking at 37°C for 20 h, OD_{600} monitored, resazurin added (final concentration 3.38 ng/ml), and color change assessed after 24 h incubation at 37°C. Time kill assays: 96-well plates were incubated with shaking at 37°C. 20 μ l aliquots of test solutions were taken at the indicated time points, serially diluted and plated for CFU enumeration.

ISO 14729 assay. Performed as previously described [11]. Briefly, 500 μ l of washed and concentrated bacteria were added to 4,500 μ l PBS containing CHD 30 PPM, PAPB 5 PPM, and EDTA 5,000 PPM to 1×10^6 CFU/ml, mixed, and incubated x 1 h at room temperature. 100 μ l of test solution was removed, serially diluted in Dey–Engley neutralizing broth, and plated for CFU enumeration.

Biofilm Formation on Silicon Hydrogel Contact Lenses and Evaluation of C30/P5/E5000 Formulation on Contact Lens Biofilms. Performed as previously described [12]. Senofilcon A lenses were washed with PBS and then placed in 12-well tissue culture plates with 4 ml of bacterial cell suspensions: overnight cultures washed twice with PBS and diluted to an absorbance 0.1 at 660nm in PBS. Lenses were incubated at 37⁰C for 120 min to allow adhesion of bacterial to the lens surface (adherence phase). Lenses were then transferred to new 12-well plates containing 4ml of fresh PBS. Each lens was then placed in an eppendorf tube filled with 2ml of 1% THB or 1% LB (weight/volume) for *S. aureus* and *P. aeruginosa* respectively and rotated at 37⁰C for 24 hours (biofilm formation phase). Each lens was then washed in fresh PBS for 5 seconds to simulate the rinsing step and placed in 4ml of C30/P5/E5000 or 4ml of PBS control and incubated at room temperature for 4 hours. Lenses were washed again in fresh PBS for 5 seconds and transferred to a 1.5ml eppendorf tube containing 1ml of PBS and 1mm silicon beads. In order to break up the biofilm on the contact lenses, the tubes were rigorously shaken at 6,000rpm for 1min twice with a 1 min cooldown on ice in between agitations. The bacterial suspensions were serially diluted in Dey-Engley Neutralizing Broth, and serial dilutions were plated on THB and LB agar plates for *S. aureus* and *P. aeruginosa* respectively to evaluate viability.

RESULTS

Activity of five commercial contact lens solutions against methicillin-resistant *S. aureus* (MRSA) and *P. aeruginosa*

We tested five multi-purpose contact lens solutions from major manufacturers in the US: Boston Simplus, Boston Advance, Opti-Free, Menicare GP, and Lobob. Antibacterial preservatives found in each solution are listed in **Figure 4.1a**. The MIC of each solution against MRSA TCH 1516 and *P. aeruginosa* PA01 was determined by CLSI broth microdilution methodology [8]. Boston Simplus had the most potent anti-staphylococcal activity, with MIC = 1.5% (**Figure 4.1b**), while Menicare GP had the most potent anti-pseudomonal activity, with MIC = 23% (**Figure 4.1c**). All multi-purpose solutions tested were less effective against PA than MRSA. The same trends were also observed when we tested the multi-purpose contact lens solutions against three SA and three PA clinical keratitis isolates (**Figure S 4.1** in supplemental material).

Preservatives with a biguanide functional group have the highest anti-MRSA activity, while EDTA has the highest anti-pseudomonal activity

We sought to determine which of preservative(s) found in each top performing solution yielded the antibacterial effects observed. Boston Simplus, with the highest anti-MRSA activity, utilizes the biguanide-containing preservatives CHD and PAPB. CHD and PAPB were equally active against MRSA with MICs = 2.5 parts per million (PPM), less active against PA, with MICs = 15 PPM and 20 PPM, respectively. No synergy of CHD and PAPB in combination was observed for either MRSA or PA (**Figure 4.1d**). Menicare GP, the most active against PA, utilizes EDTA and benzyl alcohol as preservatives. The MIC of EDTA was 2,500 PPM against PA and 300 PPM against MRSA. The MIC of benzyl alcohol was 5,000 PPM against PA and 10,000 PPM against MRSA. Synergy of EDTA and benzyl alcohol was observed against PA but not MRSA (**Figure 4.1e**).

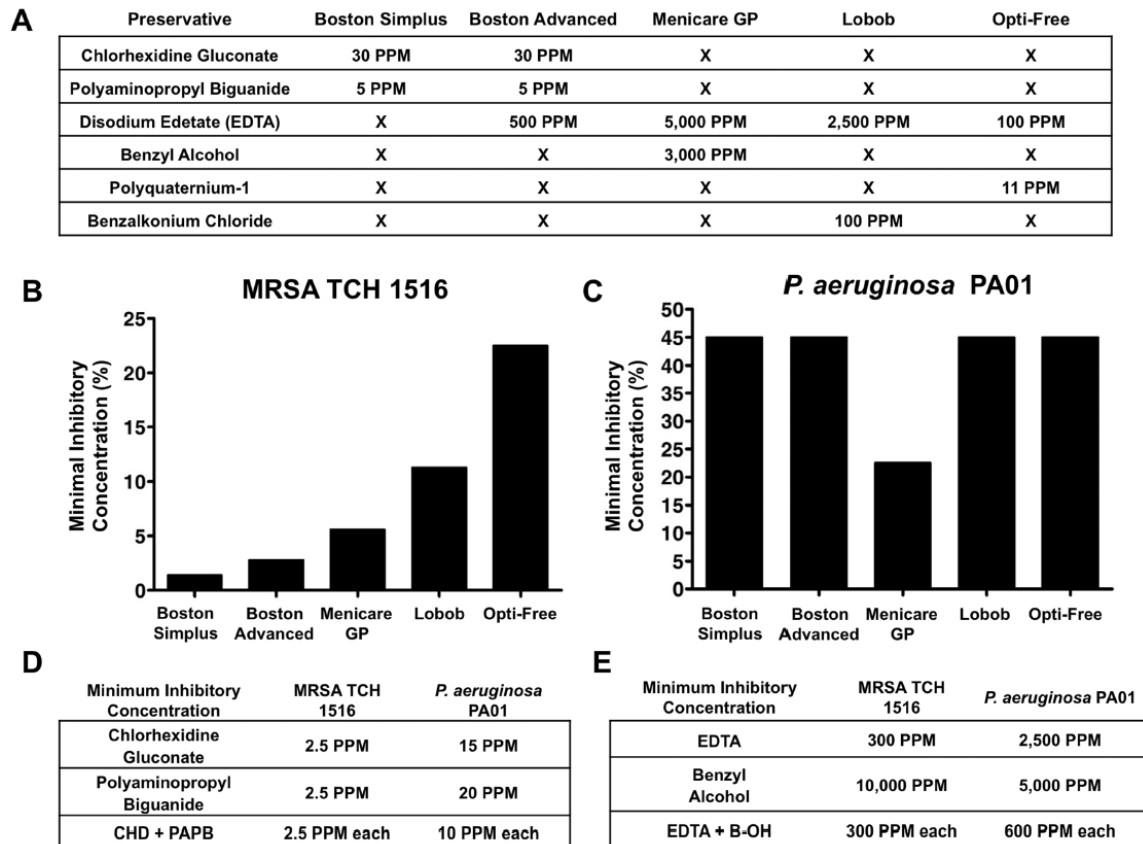


Figure 4.1 Antibacterial efficacy of multi-purpose contact lens solutions and their individual preservative components against *P. aeruginosa* and *S. aureus*. (A) The preservative concentrations of each of the contact lens solutions tested. (B and C) Contact lens solutions were serially diluted in cation-adjusted Muller Hinton broth and minimal inhibitory concentration (MIC) was determined by CLSI broth microdilution methodology. (D and E) The MIC of individual preservatives determined by CLSI broth microdilution methodology. All data are representative of 3 independent experiments.

CHD and EDTA are synergistic against *P. aeruginosa*

Using checkerboard assays to test combinations of component preservatives found in Boston Simplus and Menicare GP, we discovered that the most potent synergistic combination against PA was CHD + EDTA (**Figure 4.2a**). Used together, a solution of 4 PPM CHD (~1/4 MIC) + 300 PPM EDTA (~1/8 MIC) was sufficient to eradicate PA with a corresponding fraction inhibitory concentration index of 0.39. The bactericidal activity of this combination was extremely rapid, with $>4\log_{10}$ reduction in PA in 2 h in quantitative killing assays (**Figure 4.2b**). This CHD + EDTA synergy was also observed against three PA clinical keratitis isolates with fraction inhibitory concentration indexes of less than 0.13 as calculated by checkerboard assays (**Figure S 4.2** in supplemental material).

A formulation of CHD, PAPB and EDTA with excellent antibacterial activity against MRSA and *P. aeruginosa*.

The combination of CHD and PAPB in Boston Simplus had strong activity against MRSA (**Figure 4.1b**), but the EDTA concentration in this product is too low for synergy against PA. Such synergy was achieved by combining the EDTA concentration of Menicare GP with the CHD and PAPB concentrations of Boston Simplus. A formulation of CHD 30 PPM, PAPB 5PPM, and EDTA 5,000 PPM (C30/P5/E5000) satisfies the international criteria for contact lens solution efficacy against bacterial pathogens described in ISO 14729. In just 1 hour, the concentrations of MRSA and PA were reduced by $>4\log_{10}$ (**Figure 4.2c**) far more rapid than the manufacturer's recommended disinfection time for either Boston Simplus (4 h) or Menicare GP (6 h). A $>4\log_{10}$ reduction in CFU/ml was also observed after just 1 hour against all six clinical

keratitis isolates (**Fig S 4.3** in supplemental material). C30/P5/E5000 formulation was also extremely effective against a panel of clinical SA and PA isolates including MRSA and multi-drug resistant PA, as well as our six clinical keratitis strains. The favorable MIC of C30/P5/E5000 was 3 - 6% against all strains tested (**Figure 4.2d**).

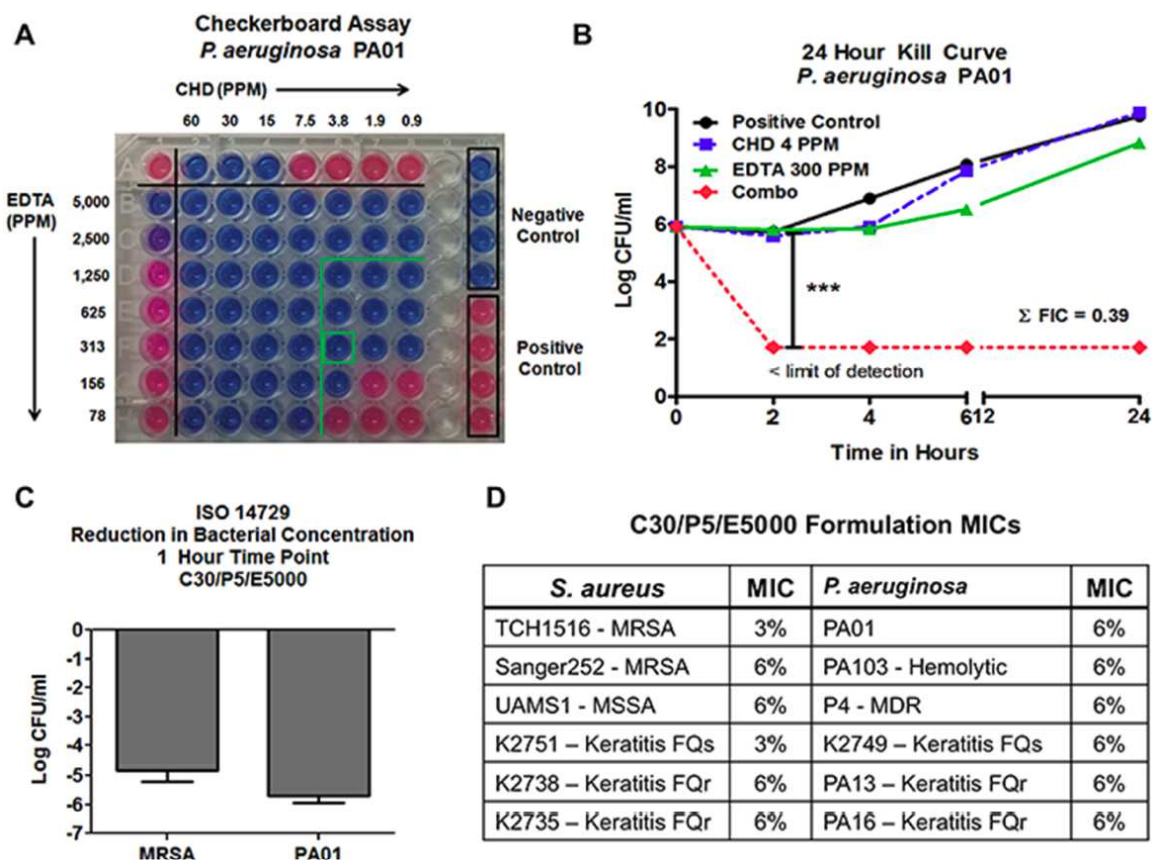


Figure 4.2 Synergy of CHD + EDTA against *P. aeruginosa* and optimized activity of CHD + PAPB + EDTA against both *P. aeruginosa* and *S. aureus*. (A) Checkerboard assay testing the combination of CHD and EDTA against *P. aeruginosa*. Resazurin probe was used to assess bacterial viability: blue color - no viable bacteria; red color - viable bacteria. Blue wells bounded by green bars in bottom right quadrant have FIC values <0.5. Green box demarks approximate concentrations used in the time kill curve in (B): Data plotted are mean \pm SEM and represent the average of triplicates from 3 independent experiments. *** P <0.001; two-way ANOVA. (C) Reduction in bacteria concentrations after 1 h incubation in a phosphate buffered saline solution containing CHD 30 PPM, PAPB 5 PPM, and EDTA 5,000 PPM. ISO 14729 testing guidelines were followed. Data plotted are mean \pm SEM and represent the average of 3 independent experiments. (D) MIC of C30/P5/E5000 formulation against a panel of clinical *P. aeruginosa* and *S. aureus* isolates including multi-drug resistant strains.

The C30/P5/E5000 formulation is able to eradicate SA and PA biofilms that have formed on contact lens surfaces.

Out of all of our clinical keratitis isolates, SA K2738 and PA K2749 were the most mucoid and robust biofilm producers. We used a previously published protocol [12] to grow mature SA K2738 and PA K2749 biofilms on a popular brand of silicon hydrogel lenses. Treatment of these biofilm coated contact lenses with C30/P5/E5000 for 4 hours at room temperature, the minimum recommended disinfection time for most multi-purpose contact lens solutions, resulted in a $>4\log_{10}$ reduction in viable SA and PA (Figure 4.3).

**Silicon hydrogen contact lens, 24 h bacterial biofilms
Treatment with C30/P5/E5000 for 4 h**

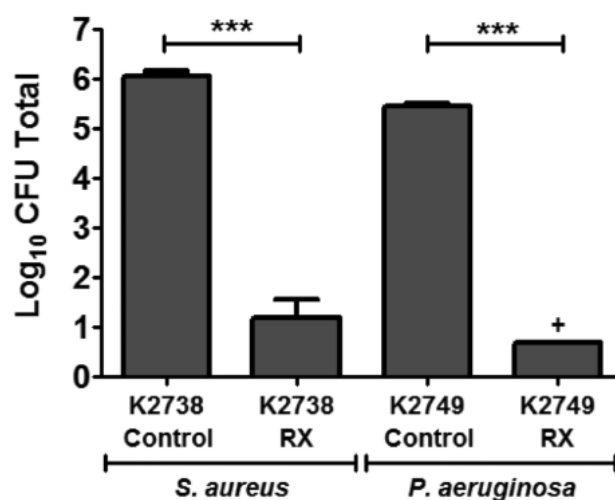


Figure 4.3 Treatment of *S. aureus* and *P. aeruginosa* biofilms formed on silicon hydrogel contact lenses with C30/P5/E5000 formulation. Data plotted are mean \pm SEM with 6 contact lenses per group. *** $P < 0.001$; two-tailed student's T-test. "+" = below the limit of detection.

DISCUSSION

With millions of daily users, contact lens-related microbial keratitis continues to be a significant health problem. Contact lenses interfere with several innate immune defense mechanisms of the eye [13]. Furthermore, most contact lens users are non-compliant with proper lens cleaning and care [14], with significant percentages reporting re-use of old contact lens solution or topping off their existing solution each night. In this setting, a contact lens solution with rapid killing activity against the major keratitis-causing pathogens, even when diluted significantly, could reduce the incidence of keratitis. By harnessing the synergy of CHD and EDTA against PA, the C30/P5/E5000 formulation has 3-7 times more anti-pseudomonal activity than any of the commonly used multi-purpose contact lens solution available today. C30/P5/E5000 is also equivalent to the best solutions tested against SA. Finally, C30/P5/E5000 demonstrates excellent activity against both planktonic and the biofilm associated keratitis isolates of PA and SA. Since CHD and PAPB are effective against acanthamoeba [15] and fungal eye pathogens [16] and EDTA is effective against PA and SA biofilms [17-19] biofilms, a C30/P5/E5000 formulation could provide a one-step solution to reducing contact lens related keratitis of all causes.

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Chapter 4, in full, was reviewed, revised, and has been re-submitted to *Antimicrobial Agents and Chemotherapy*. Lin L, Kim J, Chen H, Kowalski R, Nizet V.

Component Analysis of Multi-Purpose Contact Lens Solutions to Enhance Activity Against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. 2016. The dissertation author was the primary investigator and co-first author of this paper.

SUPPLEMENTARY MATERIALS AND METHODS

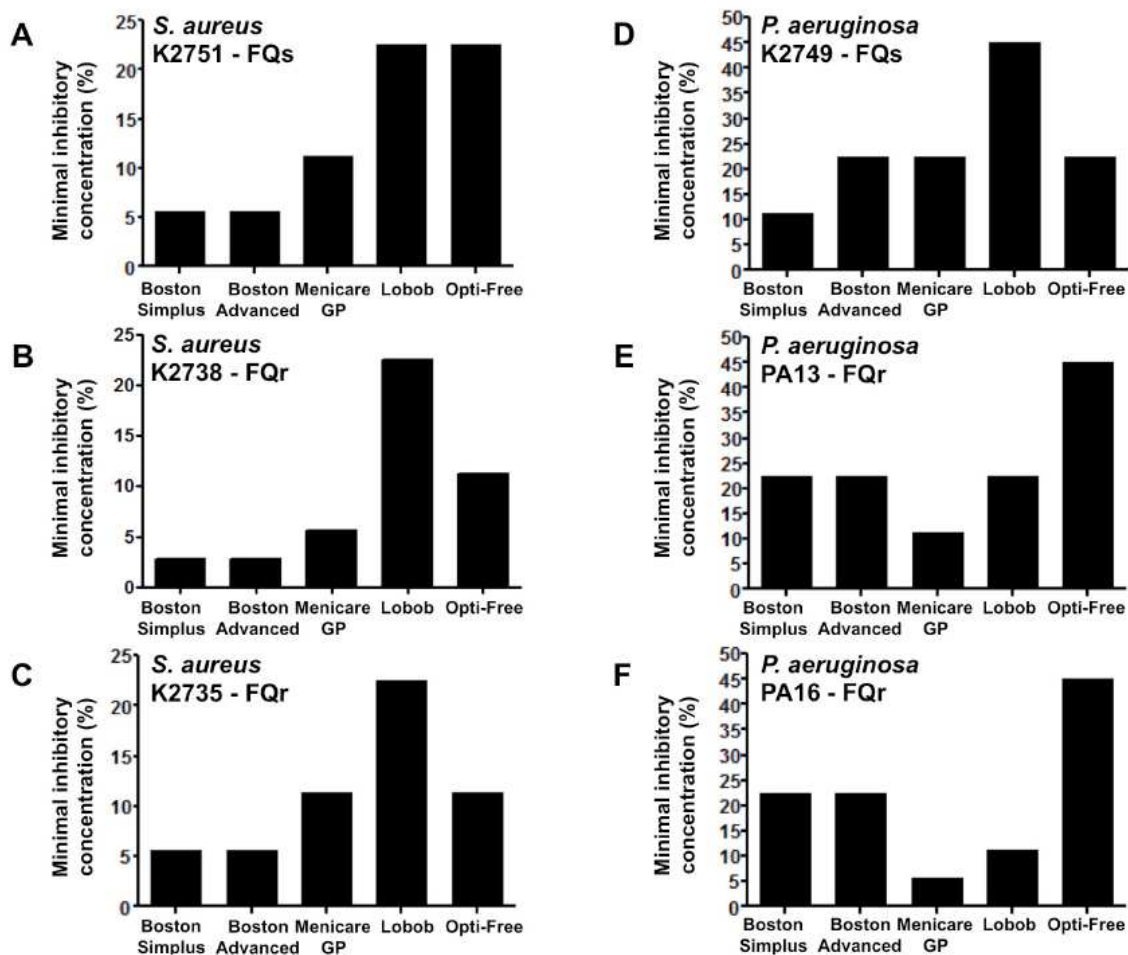


Figure S 4.1 The antibacterial efficacy of multi-purpose contact lens solutions against clinical keratitis isolates of *S. aureus* (A-C) and *P. aeruginosa* (D-F). Contact lens solutions were serially diluted in cation-adjusted Muller Hinton broth and minimal inhibitory concentration (MIC) was determined by CLSI broth microdilution methodology. FQs = Fluoroquinolone sensitive. FQr = Fluoroquinolone resistant. All data points were done in duplicate and are representative of 2 independent experiments.

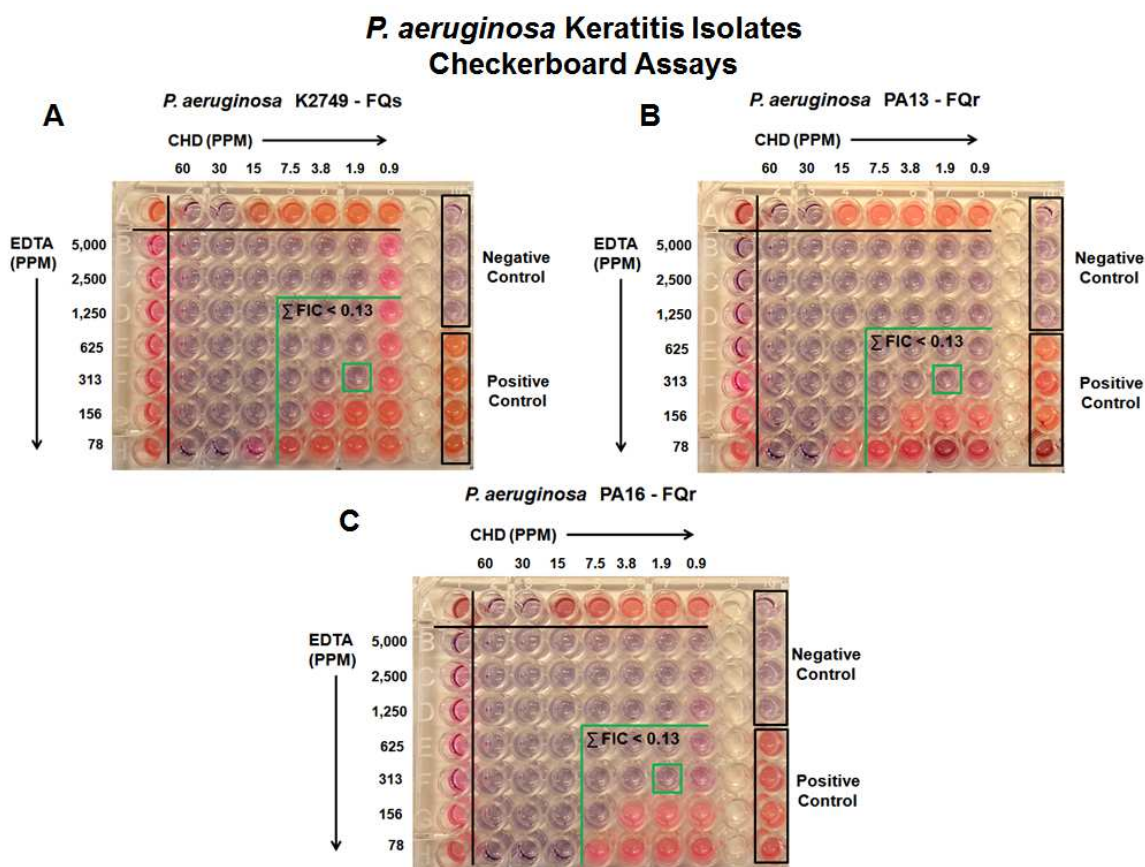


Figure S 4.2 CHD and EDTA have synergistic activity against clinical keratitis isolates of *P. aeruginosa*. (A-C) Checkerboard assays testing the combination of CHD and EDTA against *P. aeruginosa*. Resazurin probe was used to assess bacterial viability: blue color - no viable bacteria; red color - viable bacteria. Blue wells bounded by the green bars in the bottom right quadrant of the plate all have FIC values < 0.5 . Green box demarks well with no viable bacteria with a $\Sigma FIC < 0.13$ indicating synergy of CHD and EDTA against *P. aeruginosa*. FQs = Fluoroquinolone sensitive. FQr = Fluoroquinolone resistant. Data representative of 2 independent experiments.

**ISO 14729: Reduction in bacterial concentration in 1 h
Clinical keratitis isolates, C30/P5/E5000**

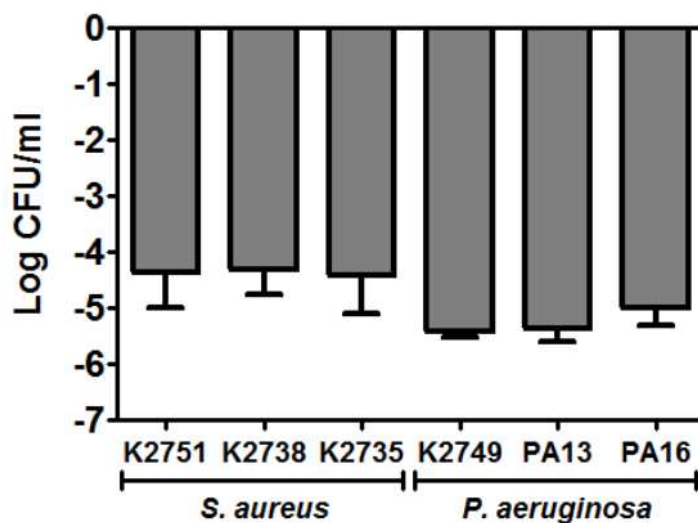


Figure S 4.3 ISO 14729 assay conducted with C30/P5/E5000 against clinical keratitis isolates. Reduction in bacteria concentrations after 1 hour incubation in a phosphate buffered saline solution containing CHD 30 PPM, PAPB 5 PPM, and EDTA 5,000 PPM. ISO 14729 testing guidelines were followed. Data plotted are mean \pm SEM and represent the average of 2 independent experiments.

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CHAPTER 5

CONCLUSIONS

In the last two decades, the percentage of infections caused by antibiotic resistant bacteria has continued to rise steadily. Unfortunately, during this same time frame, the number of new antibiotics discovered and approved has dropped precipitously. Dr. Keiji Fukuda, the WHO's Assistant Director-General for Health Security summed up the situation facing us today well in 2014 with the WHO's release of a comprehensive report highlighting the dire threat of antimicrobial resistance: "Without urgent, coordinated action by many stakeholders, the world is headed for a post-antibiotic era, in which common infections and minor injuries which have been treatable for decades can once again kill. Effective antibiotics have been one of the pillars allowing us to live longer, live healthier, and benefit from modern medicine. Unless we take significant actions to improve efforts to prevent infections and also change how we produce, prescribe and use antibiotics, the world will lose more and more of these global public health goods and the implications will be devastating."

Clearly, meeting this important challenge to human health will require the coordinated effort of governments, doctors and scientists, large pharma and biotech, and the public. In the research domain, much of the low hanging fruit in terms of discovering novel antimicrobial compounds has already been exhausted. However, most of this effort has been focused upon screening giant libraries of small molecules and natural compounds with reliance on a single assay performed in a media that bears almost no resemblance to normal human body fluids and that is

devoid of any human immune components. This final chapter will summarize the key discoveries that have been made in this work which highlight the importance of testing antimicrobial compounds in a more physiologic setting with a focus on how these compounds interact with the innate immune system.

SUMMARY OF RESULTS

Chapter 2 described the astounding discovery that azithromycin, the most commonly prescribed antibiotic in the United States, has tremendous activity against some of the most fearsome drug-resistant bacteria of our time. Azithromycin is never considered for the treatment of multidrug-resistant Gram-negative bacterial infections because it has no activity against these organisms when tested using the standard clinical susceptibility guidelines followed by all clinical and research laboratories. It is only when the bacteriologic broth used in the susceptibility testing is replaced by mammalian tissue culture media, or when components of the innate immune system such as serum or antimicrobial peptides are added that azithromycin's potent bactericidal properties are revealed. Azithromycin was so active against these organisms that a single subcutaneous dose in mice, similar to what a human patient would receive, reduced the bacterial burden in mice infected by MDR GNR's by over 99.9% in just 24 hours versus the saline control.

Chapter 3 extended the discoveries made in Chapter 2 for MDR *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* to a rising MDR nosocomial pathogen *S. maltophilia*. Azithromycin synergized with the cationic antimicrobial peptides LL-37 or colistin and enhanced neutrophil killing of *S. maltophilia*. Furthermore, a single dose of azithromycin significantly reduced the amount of bacteria recovered from the lungs of mice intra-

tracheally infected with *S. maltophilia*. Overall, Chapters 2 and 3 advance the idea that the macrolide class of antibiotics can be used today to help patients suffering from multidrug-resistant bacterial infections and highlight the importance of thinking outside the traditional boundaries of susceptibility testing when searching for new antimicrobial therapies.

Prevention of infection is increasingly important in an era when a patient is often more likely to become infected with a drug-resistant bacterial strain versus a drug-sensitive one. Chapter 4 described the careful analysis of the preservative used in multi-purpose contact lens solution which led to the discovery that chlorhexidine and EDTA have strong synergy against *P. aeruginosa*. We created a novel contact lens solution formulation to harness this synergy and demonstrated that our solution had three times more activity against a wide panel of *P. aeruginosa* isolates than anything available to consumers today. Our formulation also had excellent activity against *S. aureus*, could eradicate *P. aeruginosa* and *S. aureus* biofilms, and the components we chose have been shown to be active against fungal eye pathogens as well. Therefore, adoption of our formulation could help to reduce the incidence of microbial keratitis for the hundreds of millions of contact lens wearers worldwide.

FUTURE STUDIES

It is clear from the data presented in Chapters 2 and 3 that a dramatic change in the historic testing and discovery of antimicrobial compounds needs to take place. We have only just scratched the surface in terms of what can be done to find new therapies for difficult to treat bacterial infections. Future studies will need to test even more antibiotics and combination therapies in physiologic conditions that mimic the site of

infection. Screening existing antibiotic and small molecule collections in eukaryotic media and in the presence of human immune components such as blood, serum, antimicrobial peptides, neutrophils, macrophages, etc has the potential to reveal additional novel antimicrobial therapies. Furthermore, in-depth studies on how bacterial gene regulation and metabolism are changed when they are stressed by the different factors listed above could lead to mechanistic insights which enable novel therapies. Finally, just as in the arena of cancer chemotherapy, a better understanding of host-pathogen interactions could lead to targeted therapies that significantly boost our immune system's ability to clear infections, even though these therapies may not have any direct antimicrobial activity of their own.

With the discoveries that have been described here and the future studies proposed, we also have an obligation to make sure the knowledge is disseminated widely to an audience that can actually utilize and benefit from it. Thus, our findings need to be continually presented at both basic and clinical research conferences, and collaboration with physicians need to be forged, so that clinical case series can be put together and published. By working closing with physicians, research laboratories could in real time obtain difficult to treat bacterial clinical isolates, perform the expanded testing described in this thesis, and then propose novel curative therapies that would have been overlooked by standard susceptibility testing.