New Therapeutic Methods to Treat Infections Caused by The ESKAPE Pathogens

by

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Biographical Sketch

Catlyn Elizabeth Blanchard was born in Albany, New York. She attended State University of New York, the College at Brockport and graduated with a Bachelor of Science degree in Biological Sciences in 2008. She then worked as laboratory technician at Children’s Hospital Boston studying the chemotaxis and phagocytic nature of neutrophils. She began her doctoral studies in the Department of Microbiology and Immunology at the University of Rochester in 2010. In 2011, she joined the lab of Dr. Paul Dunman and began her doctoral research on new therapeutics against the ESKAPE pathogens. Catlyn received a Master of Science degree in Microbiology and Immunology from the University of Rochester in 2013. The following publications were a result of work conducted during doctoral study:


*Both authors contributed equally to the work


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Abstract

Over the last three decades, the medical community has seen a drastic increase in the number of antibiotic resistant infections, particularly caused by the ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa and Enterobacter spp.) pathogens. While all six species are known to cause an array of human disease, I have been primarily concerned with bacteremia and skin and soft tissue infections associated with A. baumannii, S. aureus, and P. aeruginosa. Bacteremia and skin based infections have the highest mortality rates and exhibit physiological differences compared to laboratory culture conditions. Indeed, the antibiotic resistant nature of the ESKAPE pathogens, combined with the dwindling antibiotic pipeline, has resulted in a need for new therapeutic options, thus my research has focused on the development of three new antimicrobial therapies. First, a topical ointment containing mupirocin and neomycin has been shown to have a wide spectrum of activity, with efficacy against S. aureus, A. baumannii, and P. aeruginosa, and has been shown to clear colonization of multi-drug resistant S. aureus. A second topical ointment containing compounds found to be effective against Pseudomonas biofilms also demonstrates efficacy against a murine wound model infected with A. baumannii, P. aeruginosa, and S. aureus. The third therapy was the identification and characterization of two compounds with activity as efflux pump inhibitors for A. baumannii and P. aeruginosa that could be used as adjunctive therapy in combination with conventional antibiotics. Seven genes that are likely to be the targets of these compounds have been identified, allowing for an early model of their cellular mechanism of action for future testing. All together the three newly described therapeutic candidates we have developed may provide new strategies for the therapeutic intervention of extremely antibiotic resistant bacterial species.
Contributors and Funding Sources

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List of Abbreviations

ABC ATP-binding cassette

*A. baumannii* Acinetobacter baumannii

CCCP carbonyl cyanide *m*-chlorophenylhydrazone

CDC Centers for Disease Control and Prevention

CFU colony forming units

DMSO dimethyl sulfoxide

*E. coli* Escherichia coli

*E. faecium* Enterococcus faecium

EPI efflux pump inhibitor

ESKAPE *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* species

HAI healthcare associated infection

ICU intensive care unit

*K. pneumoniae* Klebsiella pneumoniae

LB Luria-Bertani

MATE multidrug and toxic compound extrusion

MDR Multidrug resistance

MFS major facilitator superfamily

MH Mueller Hinton

MIC minimum inhibitory concentration

MRSA Methicillin resistant *Staphylococcus aureus*

MSSA methicillin susceptible *Staphylococcus aureus*
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<td>phenylalanine-arginine-beta-naphthylamide</td>
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<td><em>P. aeruginosa</em></td>
<td><em>Pseudomonas aeruginosa</em></td>
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<td>PMF</td>
<td>proton motive force</td>
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<tr>
<td>RND</td>
<td>resistance-nodulation-division</td>
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<td>SMR</td>
<td>small multidrug resistance</td>
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<td>SSD</td>
<td>Silver sulfadiazine</td>
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<td>SSI</td>
<td>Surgical site infection</td>
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<td>SSTI</td>
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<td>WHO</td>
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Chapter 1: Introduction

Antibiotics revolutionized the practice of medicine, mitigating the effects of bacterial infections and allowing for the facilitation of modern organ transplants and oncology treatments [1]. The discovery of penicillin began the Golden age of antibiotics, where 11 classes of antibiotics were identified in a 20-year span (1940-1960). This prompted the Surgeon General to declare the war on infectious disease won. Indeed, most of the drugs used today were discovered before the 1970s, with next generation antibiotics being derivatives of the original chemical scaffolds [2, 3]. However, resistance emerged nearly as quickly as the introduction of each antibiotic into clinical use [4]. The widespread use of antibiotics led to multidrug resistance (MDR) at the same time as a steep decline in the identification and development of novel antibiotics from the pharmaceutical industry [2, 5]. The emergence of MDR bacterial species combined with a dwindling antibiotic pipeline has led to a healthcare crisis with the potential to affect virtually every area of medicine [6, 7].

In response, the Centers for Disease Control and Prevention (CDC), released a report in 2013 entitled “Antibiotic Resistance Threats in the United States”, which categorized bacterial species into levels of importance[8]. The report prioritized 18 species or strains into three levels: urgent, serious, and concerning [8]. Those species in the urgent category are bacteria for which there are limited treatment options and the spread of the species necessitates close monitoring. Bacterial species falling under the serious category are MDR and on the verge of pandrug resistance (resistant to all antibiotic classes), the
exposure to these pathogens is widespread requiring monitoring and necessitating the development of new therapeutics. The ESKAPE pathogens, thus named in 2008 by Louis Rice for the members of the group (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species), were amongst those species classified in the serious level, moving toward the urgent level, and collectively are responsible for the majority of hospital acquired infections (HAIs) [8]. The work within this thesis will be focused on a subset of the ESKAPE pathogens: S. aureus, P. aeruginosa, and A. baumannii.

All three of these species are good examples of MDR bacteria and between the three are known to harbor resistance elements that cover all of the available antibiotics. As exhibited in table 1.1 there are eleven classes of antibiotics, and S. aureus, P. aeruginosa, and A. baumannii have developed resistance mechanisms to each class. Antibacterial drugs can be broken into two overall sets, those that are cidal (bactericidal) or those that inhibit the growth of bacteria (bacteriostatic) and the classification is based upon the action of the antibiotic [9]. The cidal antibiotics outright kill bacterial cells, often by disrupting the integrity of the cell wall or inhibiting DNA replication, the bacteriostatic antibiotics do not kill cells outright; instead they tend to hinder other essential processes such as protein synthesis and cellular metabolism, preventing cells from proliferating [9]. The bactericidal antibiotics include: the 30S ribosomal binding aminoglycosides; cell wall inhibitors: carbapenems, cephalosporins, penicillins, monobactams, glycopeptides; the membrane destabilizing polymyxins; DNA gyrase inhibitors; quinolones; the RNA polymerase inhibitor rifampicin; and mupirocin which inhibits the isoleucine t-RNA
synthetase of Gram-positive bacteria [10, 11]. Bacteriostatic antibiotics include the tetracyclines, known to bind to the 30S ribosomal subunit and prevent the aminoacyl-tRNA from binding and the sulfonamides, which inhibit the synthesis of folate, a necessary component in nucleic acid synthesis [12, 13]. Finally, macrolides, those antibiotics that bind to the 50S subunit of the ribosome are also bacteriostatic antibiotics [14].

In addition to the antibiotic classes mentioned above, non antibiotic compounds have also exhibited antimicrobial activity. These chemical structures include the antiseptics (antimicrobial agents used on the topical surface of animate objects); disinfectants (antimicrobial agents used on the topical surface of inanimate objects); and non-antibiotic antimicrobials. One such compound is silver, which has been used for thousands of years in a variety of products with human contact, including food delivery devices, jewelry, and dental fillings. In addition to the non-medicinal uses for silver, it has been shown that silver particles have broad-spectrum antibacterial activity. Silver ions nonspecifically react with bacterial proteins and DNA, thereby leading to multiple mechanisms of action and[15, 16]. Additional mechanisms of action include cell membrane disruption, electron transport chain inhibition, accumulation of DNA mutations and the production of damaging intracellular silver free-radicals [15, 16]. Thus making silver based compounds effective against both Gram-positive and Gram-negative bacterial species.

My work encompassed within this thesis has focused on three antimicrobial compounds and the identification and development of adjunctive therapies to these three antimicrobials. Described in more detail below, this thesis has focused on the following
antimicrobials: mupirocin, a topical antibiotic that is bactericidal at high concentrations, used to decolonize the nares of *S. aureus*. Silver sulfadiazine, a common therapy in the treatment of *P. aeruginosa* infected wounds or burns. Finally the identification and development of an efflux pump inhibitor as an adjunctive therapy for the tetracycline derivative, minocycline, used in the treatment of *A. baumannii* bacteremia. These three compounds are important because they represent the antimicrobial resistance exhibited by the three ESKAPE pathogens highlighted in this body of work and thus the need for new therapeutic options in the treatment of MDR bacterial species.

**Mupirocin**

The skin is one of the most important immune barriers, preventing bacteria from reaching the internal sterile milieu of the host. However, when this barrier is breached by cuts, abrasions, or surgical procedures, bacteria are able to establish themselves in a number of different clinical disease presentations. Infections of surgical sites (SSI) have recently been recognized as the most common HAI accounting for 31% of all HAIs and treatment accounts for 37% of the annual costs of HAIs [17, 18]. Additionally, the most common bacteria cultured from skin and soft tissue infections (SSTIs) and SSIs, is *S. aureus* [19-23]. In the recently published Antibiotic Threat Report, *S. aureus* infections caused by methicillin resistant (MRSA) strains are responsible for 80,461 invasive infections per year, leading to 11,285 deaths [8]. Historically mupirocin has been used for the prevention and treatment of *S. aureus* SSIs. Specifically, in cardiothoracic surgery, the use of perioperative mupirocin to decolonize the nose has been shown to reduce the incidence of postoperative infections. Indeed, in several multicenter studies the
postoperative rate of SSIs was shown to range between 1.7 and 6% of patients treated with perioperative mupirocin, compared to 4 - 10% of untreated patients [24-26]. As a result, the use of perioperative mupirocin has been advocated for by the Society of Thoracic Surgeons, especially in patients that are known to be carrier of *S. aureus* [27].

Mupirocin, a polyketide antibiotic produced by the bacterium *Pseudomonas fluorescens*, reversibly binds to the bacterial isoleucyl-tRNA synthetase protein, inhibiting Ile-tRNA aminoacylation and protein translation [28-30]. The antibiotic displays antibacterial activity toward most Gram-positive species and the Gram-negative species *Haemophilus influenzae* and *Neisseria gonorrhoeae* and it lacks cross resistance to current antibiotics [31]. However, it is unstable systemically, due the rapid metabolism of the drug and thus is used exclusively as a topical antimicrobial agent [31]. Mupirocin-based ointment has emerged as the standard of care for pre-surgical nasal decolonization [Reviewed in [32]] in the U.S. and UK prior to surgical procedures as a means to mitigate *S. aureus* transmission and, subsequently colonization of wound sites [33]. As noted above, the application of mupirocin preoperatively, has shown a significant reduction in postoperative SSIs [24-26]. Furthermore, nasal decolonization has been shown to be effective in reducing infections in compromised patient populations, particularly those with burn wounds, on dialysis, and surgical patients, as well as bacterial transmission amongst healthcare workers and intensive care unit (ICU) patients [25, 34-39]. Decolonization has the ability to prevent *S. aureus* infection in susceptible patients, however topical applications of mupirocin can also treat an established infection. Success has been shown in the treatment of hemodialysis central venous catheter exit sites,
impetigo, eczema, surgical wound sites, skin and soft tissue wounds, mastitis, and tympanic membrane lesions [40-45]. While effective, the wide-spread use of mupirocin to limit *S. aureus* colonization and infection has had deleterious effects in regards to the emergence of mupirocin resistance and has led to a concern about the future use of this antibiotic.

Point mutations in the native isoleucyl t-RNA synthetase gene (*ileRS*) confer low-level mupirocin resistance, defined as strains with a minimum inhibitory concentration (MIC) of 8 to 256 µg ml\(^{-1}\) [46]. These strains have been observed to develop rapidly in both the laboratory and clinical settings [46]. Although low-level resistant strains may initially respond to treatment, a subpopulation that is not eradicated by mupirocin exposure is thought to be the reason for the re-emergence of such strains. However, resistant strains with an MIC greater than 256 µg ml\(^{-1}\) are noted as exhibiting high-level mupirocin resistance. Strains exhibiting high-level resistance occur less frequently than low-level resistant mutants and are attributable to the acquisition of a mobile genetic element harboring either *mupA*, which codes for an alternate isoleucyl tRNA synthetase, or the less-characterized *mupB* gene [47, 48]. Due to the presence of an alternate synthetase, high-level resistant strains do not respond to treatment with mupirocin ointment [49, 50].

The rate of mupirocin resistance varies by institution and region in the U.S., and can be influenced by the infection control practices of the individual hospitals and the strains found in the region. One study showed that in 23 US hospitals the rate of MRSA strains exhibiting high-level mupirocin resistance was 3% of nasal isolates and 5% of MRSA isolated from blood cultures between 2009 and 2010 [51]. Yet, single center studies have
reported higher than average mupirocin resistance rates. For instance a New York City hospital pediatric unit recently reported a prevalence rate of 31% of MRSA isolates tested and a Korean neonatal ICU reported a rate of 79% of hospital-associated MRSA isolates tested positive for high-level mupirocin resistance [52, 53]. Further, in compromised populations such as nursing home residents, high-level mupirocin resistance was identified in 12% of MRSA isolates from 2008 to 2011 in the U.S. [54]. Globally, high-level mupirocin resistance is even more varied than it is in the U.S.; the rate of resistance ranges from as low as 1% in French hospitals to as high as 47% in Turkish hospitals [55-59]. Similarly, the resistance rate of low-level mupirocin resistance was found to vary from 0 to 80% within in US hospitals [60-64]. The high rates of mupirocin resistance and the commonality of mupirocin-based ointments highlight the need for new therapeutic options for the treatment and decolonization of S. aureus, which is the focus of chapter 3 of this thesis.

The above example of mupirocin resistance in MRSA strains is an all too often occurrence among bacterial species. As elaborated below, the Gram-negative pathogen Pseudomonas aeruginosa has developed high levels of antibiotic resistance and is known to fail to respond to antibiotic based ointment treatment. Additionally, P. aeruginosa readily forms biofilms, a growth state known to confer antibiotic resistance to bacterial species. This growth state and the impact of biofilms on the healthcare industry has prompted the need for new antimicrobial therapies. Further, the development of resistance to the commonly used anti-Pseudomonal therapy, silver sulfadiazine, has driven the identification and development of an adjunct compound.
Silver Sulfadiazine

*Pseudomonas aeruginosa* is a ubiquitous bacterial species that has emerged as a major human pathogen. Indeed, it was found that 8% of all HAIs are the result of *P. aeruginosa* infection, of which 13% were caused by MDR strains [8]. Acute skin infections, such as SSIs are more likely to be colonized by pathogens such as *S. aureus*, however, *P. aeruginosa* is more commonly associated with wounds that have a more chronic nature such as burns and nonhealing wounds (diabetic and pressure ulcers) [65-69].

Burn site infections increase patient mortality by 5%, whereas diabetic wound infections are associated with increased risk of limb amputation and a 12.7% increase in mortality rate [70-72]. To further complicate these infections, there is increasing evidence that nonhealing wounds are a result of biofilm formation within the wound site, an antibiotic and host defense recalcitrant bacterial growth state [73-76]. Thus the control and maintenance of biofilm within wounds is particularly important due to the prolonged healing process and potential systemic spread of the infection, leading to further antibiotic use [69, 77].

Silver based compounds are amongst the most commonly used topical therapeutics, usually a silver salt formulated into either a cream or impregnated into wound dressings. This element has been used for centuries as an antimicrobial compound, with documented usage as early as the 17th century and in the wound setting since the 18th century [78]. As noted above silver has multiple mechanisms of antimicrobial action
accounting for the broad spectrum of activity and is thought to contribute to the antibiofilm activity. Medicinal silver is most commonly formulated into silver sulfadiazine ointments, creams, bandages, and coatings on indwelling devices and represents a 237 million dollar industry in the US [79-81]. However, the adverse side effects of argyria (toxic accumulation of silver particles), leucopenia, hepatic, and renal toxicity along with the lack of statistical improvement upon similar non-silver treatments such as collagenase ointment, hydrocolloid dressing and even bee honey suggest that new therapeutic options are necessary [82-88]. Moreover, reports of emerging silver resistance have prompted research into improved topical treatments [89]. Thus the focus of chapter 4 is the identification and development of an adjunctive antimicrobial to treat bacterial infections in wounds.

In addition to the wound and skin associated infections caused by *S. aureus* and *P. aeruginosa*, my work has also focused on bloodstream infections caused by *A. baumannii*. Furthermore, this thesis is concerned with the adaptive antibiotic resistance developed in response to growth in human serum. Bloodstream infections, where bacteria establish infection in an otherwise sterile environment, results in high rates of mortality [90]. In addition to increased mortality, bloodstream infections are particularly difficult to treat due to the antibiotic binding capacity of proteins such as albumin found in the bloodstream [91, 92]. Furthermore, bacterial species have been shown to upregulate the expression of antibiotic resistance mechanism including efflux pumps during growth in the bloodstream component serum [93]. Due to the combination of serum binding
antibiotics and the adaptive antibiotic resistance exhibited by *A. baumannii*, adjunctive therapy of antibiotics with an efflux pump inhibitor is the focus of the next section.

**Efflux Pump Inhibitors**

*A. baumannii* is responsible for 12,000 infections per year in the U.S., accounting for 7\% of all HAIs within critically ill populations and within this subpopulation, mortality rates have reached as high as 80\% and average around 50\% [8, 94-96]. Numerous mechanisms are thought to contribute to the organism’s propensity to circumvent antibacterial agents. While *A. baumannii* exhibits an extraordinary ability to acquire antibiotic resistance determinants, which include enzymatic functions such as β-lactamases and aminoglycoside-modifying factors, it is the expression of the organism’s expansive catalog of efflux pumps that largely contribute to the observed clinical antibiotic failure [97-99].

The repertoire includes representatives of each of the five bacterial drug efflux pump families: CraA and AmvA are major facilitator superfamily (MFS) pumps that are proposed to efflux chloramphenicol and erythromycin, respectively [100, 101]. AbeM is a multidrug and toxic compound extrusion (MATE) family protein that effluxes aminoglycosides, quinolones, and chloramphenicol [102]. AbeS is a small multidrug resistance (SMR) family pump that confers resistance to erythromycin and novobiocin as well as low level tolerance to aminoglycosides, quinolones, tetracycline and trimethoprim [103]. AdeABC, AdeFGH, and AdeIJK are resistance nodulation division (RND) family pumps that have been associated with resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, tigecycline, macrolides, chloramphenicol, and
trimethoprim [104-108]. Furthermore, *A. baumannii* is also known to harbor several horizontally acquired Tet efflux pumps belonging to the MFS family that confer tetracycline resistance [109, 110] and express ATP-binding cassette (ABC) transporters in response to subinhibitory concentrations of tigecycline [111]. Recent publications, have also shown that in addition to the characterized efflux systems, strains 983709, ATCC 17978, and AC12 have at least 32 additional efflux genes that may aid in the antibiotic resistance exhibited by this species [93, 112, 113].

Efflux pump inhibitors (EPIs) have been proposed to offer a solution to the antimicrobial resistance presented by efflux pumps. This is based on the premise that EPIs are small molecules capable of preventing the activity of efflux pumps, thereby allowing intracellular antibiotic accumulation. Adjunctive therapy with EPIs can combat the activity of efflux pumps and potentiate the activity of antibiotics in an otherwise efflux-pump mediated resistant strain. Corresponding EPI searches have been successful to varying degrees and have led to the identification of EPIs that have six ways to inactivate efflux pumps. The most common mechanisms of inhibition being the competitive binding of the substrate pocket or by blocking the access to the pump itself [114]. However, there are several other ways that EPIs have been theorized and shown to inactivate efflux pumps. These inhibition mechanisms include: 1) altering the regulation and expression of the efflux pump components, 2) inhibiting the assembly or placement of the pump into the cell membrane, 3) inhibiting or blocking the outer membrane channel by jamming the channel, 4) collapsing/dissolving the energy source of the pump, 5) competitive binding of substrates, and 6) altering the structure of the antibiotic to decrease the affinity for
recognition by the pump as exhibited in Fig. 1.1 [115-118]. A number of compounds have been identified and developed as efflux pump inhibitors including Phenylalanine-arginine-beta-naphthylamide (PAβN), globomycin, carbonyl cyanide m-chlorophenylhydrazone (CCCP), reserpine, and verapamil [115, 119-122].

Initially identified in 1999, PAβN potentiates the activity of fluoroquinolones and every other class of antibiotic effluxed by the RND pumps in Gram-negative bacterial species [114]. Addititionally, it is able to permeabilize the outer membrane, and is now widely used to study efflux in bacterial species [114, 123]. PAβN is a substrate of the RND efflux pumps and competitively binds in the same pocket as antibiotics, thus it will be preferentially pumped out over antibiotics such as fluoroquinolones [115]. By competing the antibiotic for the binding pocket, PAβN ensures that the antibiotic accumulates within the cell allowing it to interact with its cognate target. However, clinical usage of this molecule is prohibited by the toxicity associated with exposure to human serum [124, 125].

The EPI globomycin has a much different mechanism of action, it can prevent the assembly of the efflux protein complex [119]. More specifically, globomycin is an inhibitor of signal peptidase II which removes the lipoprotein signal sequence, directing the efflux pumps to the membrane, consequently preventing the membrane insertion of the machinery [119, 126, 127]. The removal of the antibiotic from the intracellular space is counteracted through the insertion failure of the inner membrane protein of the RND family efflux pump [126]. However, this action is thwarted by the expression of non-
target RND pumps that recognize many of the membrane modifying inhibitors, globomycin, fatty acid inhibitors, and antimicrobial polypeptides as a few examples, as substrates and will extrude them. The efflux of the above-mentioned inhibitors prohibits their use clinically.

Disruption of the power source enabling the pumping activity to efflux antibiotics is also a common mechanism of action of EPIs. More specifically, most efflux pump families are powered by the cell’s proton motive force (PMF), ion antiporters exchanging either sodium or hydrogen ions for the substrates of the pumps [114, 124, 125, 128]. However, a single family of pumps (the ABC family) utilizes ATP to power the exchange of drugs from within the cell to the external environment [129]. There are several EPIs that have been developed that are able to disrupt the power sources of these pumps, including carbonyl cyanide m-chlorophenyl hydrazone (CCCP), reserpine, and verapamil, however they each have their own unique mechanisms [130, 131]. CCCP is a known PMF inhibitor that has also been shown to induce the accumulation of antibiotics such as fluoroquinolones, tetracyclines and ethidium bromide within the cell [130, 131]. Much like CCCP, reserpine and verapamil are also power source inhibitors. Reserpine inhibits the hydrolysis of ATP by P-glycoprotein transporters and the efflux activity of many MFS pumps such as Bmr and NorA potentiating the activity of ciprofloxacin and linezolid [122, 132-134]. Verapamil is also known to inhibit the activity of bacterial efflux pumps with the inhibition mediated through disruption of either the PMF or calcium channels [121, 135]. Largely studied in the context of Mycobacterial efflux
inhibition, verapamil has been shown to potentiate the activity of ethidium bromide and erythromycin [121].

While EPIs have proven valuable laboratory tools for characterizing bacterial efflux systems, they have unfortunately not translated to clinical use. As indicated above, PAβN was shown to be unstable in biological fluids and in fact becomes toxic to eukaryotes when exposed to human serum [125]. Furthermore, like PAβN, the energy uncoupling EPIs are clinically prohibited due to the interference with the PMF or ATP hydrolysis. It cannot be strictly determined if it is the uncoupling of the energy source of the pump itself that confers the increased susceptibility or if the inhibitors are disrupting the overall energy of the cell and thereby decreasing the viability of the cells themselves. In addition EPIs such as verapamil and reserpine are known to have targets in eukaryotic cells, reserpine has been used to treat hypertension and verapamil is a common beta-blocker, as such, the toxic concentrations necessary to inhibit the bacterial efflux pumps would do more harm than good if used as adjunctive therapy [135, 136].

Inhibitors of efflux are well documented to potentiate the activity of antibiotics in MDR bacteria, however current structures are not clinically applicable due to their adverse effects. New chemical backbones lacking the inherent problems of toxicity and adverse effects of established EPIs would provide new classes of bacterial efflux pump inhibitors. These compounds could be used as adjunctive therapy for current and future antibiotics in combination therapy. Two such compounds will be explored further in chapter 5, as the identification and characterization of EPIs was one focus of my thesis.
Exploiting Combination and Adjunctive Therapy to Contend with Antibiotic Resistance

As indicated above, there is a need for new treatment options for the increasing number of resistant infections; unfortunately there is a lack of new antimicrobials in development [1, 137]. Popular target based screening approaches in which small molecule inhibitors of a enzyme target are improved by medicinal chemistry based optimization have failed to yield a single antibiotic. Further, classic whole cell based screening approaches designed to identify novel chemical scaffolds that display antibacterial activity toward pathogens of interest have continually identified the same antibiotics that are already in use and for which resistance is already circulating. One alternative strategy is to develop novel antimicrobial combinations of existing FDA approved drugs or to find adjuncts to currently available antibiotic that are able to overcome resistance. Indeed, this is a concept that is already in place for the clinical treatment of diseases including cancer, hypertension, malaria, and HIV infections [138, 139]. In fact, several current antibiotics represent combination or adjunctive therapeutics, two such examples are Augmentin and Bactrim [140, 141]. Augmentin, the combination of a β-lactam and β-lactamase inhibitor is a commonly used antibiotic to treat a wide range of bacterial infections and the combination of sulfamethoxazole and trimethoprim (Bactrim) has been widely used since its release in the 1970s [142-146]. Additionally, Augmentin and Bactrim are on the World Health Organization (WHO) model list of essential medicines, or medications that are needed for a basic health-care system, thereby underlining the importance of combinational and adjunctive antimicrobial therapy [147]. New drug combinations and
adjuncts can provide an advantage over MDR bacterial strains that is desperately needed in the current environment of antibiotic failure. Thus, the focus of my thesis is the identification and development of new combination therapies.

**Summary of Thesis**

Herein I describe three new potential therapeutic options to be used against antibiotic resistant ESKAPE pathogens. Each of these options is designed based on the combination/adjunct premise and include two combination antimicrobial ointments and new chemical backbones with efflux pump inhibitor activity. Each of these therapies is described in more detail below and in chapters 3 through 5 of this thesis.

One ointment combination contains the topical antibiotic mupirocin with neomycin, and overcomes both low- and high- level mupirocin resistance. It was found that the combination of mupirocin and neomycin was able to clear methicillin susceptible *S. aureus* (MSSA) and MRSA strains as well as decrease the burden of a *mupA* harboring strain of *S. aureus*. The efficacy of this combination was observed *in vitro* via an increased zone of inhibition when the two drugs were used in combination. Additionally, the additive effect of mupirocin and neomycin was observed *in vivo* in a murine nasal colonization model and a murine wound model. While mupirocin treatment alone generated resistant colonies recovered from the murine models, there was no resistance developed during treatment when mupirocin and neomycin were used in combination. Altogether we were able to conclude that the combination of mupirocin and neomycin ointment was not only able to clear bacteria from wounds, but it was also able to prevent
the development of mupirocin resistance. A combination ointment made up of mupirocin and neomycin provides a potential new therapeutic for the preoperative decolonization of the nares as well as the treatment of *S. aureus* infected wounds.

A second combination, which combines silver with the anti-fungal drug zinc pyrithione, displays improved efficacy in comparison to either agent alone. More specifically, zinc pyrithione (ZnPT) was initially identified as an anti-*Pseudomonal* biofilm agent. In addition to activity against *P. aeruginosa* biofilms, ZnPT was found to have *in vitro* activity against *A. baumannii* and *S. aureus* biofilms as well as planktonic cells. However, I found that ZnPT was even more effective when it was used in combination with the known antibiofilm agent silver sulfadiazine (SSD). When used in combination ZnPT and SSD were found to elicit a response in an *in vitro* assessment of antimicrobial activity and in a murine wound model, the combination was found to clear *S. aureus* and *A. baumannii* from the wound. To see a reduction in bacterial burden in *P. aeruginosa* infected wounds, the concentration of SSD was increased. Despite this, the combination was shown to be effective against both *in vitro* biofilms and as a treatment for infected wounds. This provides a new therapeutic option in the arsenal to treat infected burns and other chronic wounds.

The third therapeutic development involved the potential for systemic adjunctive therapy. As was mentioned before, the use of an EPI in conjunction with antibiotics is a widely held belief; however up to now the EPIs identified have had intrinsic problems that have prevented their use. Herein, I describe the identification of two novel backbones that
exhibit efflux pump inhibitory activity. One of the most interesting and important aspects of the two identified compounds is that they possess activity in human serum. The two compounds were found during a screen of a small molecule library with the purpose to select for compounds that aid minocycline in preventing the growth of *A. baumannii*. Characterization of the two compounds illuminated their potential as EPIs and their broad spectrum of activity. In addition I used a genetic approach to identify the target of the putative EPIs. From a screen of an *A. baumannii* transposon mutant library, seven unique loci were determined to be potential targets for the identified EPIs. Altogether, the combination of EPI and minocycline is a potential therapeutic in the treatment of *A. baumannii* bacteremia and the transposon mutants offer potential factors mediating serum-specific antibiotic resistance.

Although each section of this project has been unique, the overall theme of new antimicrobial therapies has remained constant. All three of the organisms studied are known to possess a variety of resistance mechanisms and have the potential to cause serious disease. With the work described in this thesis, I have identified new compounds and combinations of drugs that have shown efficacy in the treatment of the most concerning pathogens of our age.
<table>
<thead>
<tr>
<th>Antibiotic class</th>
<th>S. aureus</th>
<th>A. baumannii</th>
<th>P. aeruginosa</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Main classes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolones</td>
<td>Point mutations in grlA and grlB or gyrA and gyrB, overexpression of efflux pump (NorA) [152-154]</td>
<td>Point mutations in gyrA/parC, efflux pumps [155, 156]</td>
<td>Point mutations in gyrA, efflux pumps [157]</td>
</tr>
<tr>
<td>β-lactams (including carbapenems and cephalosporins)</td>
<td>PBP2a encoded by SCCmec, β-lactamases, mecA [158, 159]</td>
<td>β-lactamases (all four Ambler classes), AmpC, efflux pumps, down regulation of CarO [160]</td>
<td>Carbapenemases (Ambler class B), efflux pump (MexAB-OprM, MexCD-OprJ), loss of OprD [161]</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>TetM or TetO (ribosomal protection proteins), TetK or TetL (efflux pumps) [158, 164, 165]</td>
<td>Tetracycline efflux pumps, ribosomal protection [166, 167]</td>
<td>Tetracycline efflux pumps [168, 169]</td>
</tr>
<tr>
<td><strong>Other/ Derivative classes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glycopeptides</td>
<td>Expression of Van genes (substitution of alternate peptidoglycan precursor) [170, 171]</td>
<td>Gram-positive antibiotics</td>
<td>Gram-positive antibiotics</td>
</tr>
<tr>
<td>Lincosamides</td>
<td>Efflux pump MsrA, methylation of 23s rRNA [162, 163]</td>
<td>Gram-positive antibiotics</td>
<td>Gram-positive antibiotics</td>
</tr>
<tr>
<td>Polypeptides</td>
<td>Gram-negative antibiotics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other (Trimethoprim/ Tigecycline/ Mupirocin)</td>
<td>Mutations in dhfr (trimethoprim), efflux pump (tigecycline), and mutations or alternative ile tRNA synthetase (mupA/mupB mupirocin) [46-48, 177, 179]</td>
<td>Expression of dhfr, dfr, and folA (trimethoprim), efflux pump (tigecycline) [98, 107, 108, 160]</td>
<td>Efflux pumps (trimethoprim, tigecycline) [169, 180]</td>
</tr>
</tbody>
</table>
**Figure 1.1 Model of efflux pump inhibition.** Efflux pump inhibitors are capable of acting in six ways. They can change the antibiotic structure so that the pump no longer recognizes it, they can interfere with the regulation of the efflux pump expression, they can collapse the energy source of the pump, prevent the functional assembly of the pump components, they can compete with antibiotics for the binding pocket and finally, they can block the channel on the outer membrane protein.
Chapter 2: MATERIALS AND METHODS

Bacterial Strains, Human Cell Lines and Growth Conditions

The bacterial strains and human cell lines used in this thesis are listed in Table 2.1. Briefly, Staphylococcus aureus strain UAMS-1, a well-characterized antibiotic susceptible clinical isolate commonly used to study the organism’s biofilm formation and colonization properties [181], USA300, a neomycin and methicillin resistant community-acquired clinical isolate [182] or BAA-1708 a high level mupirocin resistant strain containing mupA obtained from the American Type Culture Collection (ATCC, Manassas, VA) were all maintained in tryptic soy broth (TSB). Acinetobacter baumannii strains 983709, 07061, and 011205, clinical isolates acquired from the Centers for Disease Control and Prevention (CDC) [183] and Pseudomonas aeruginosa strain PA01, a prototype laboratory strain generously provided by Dr. Barbara Iglewski (University of Rochester, Rochester, NY) were maintained in either Mueller Hinton (MH) or Luria-Bertani (LB) broth as necessary. Hepatic carcinoma cell line HepG2 was obtained from ATCC and human embryonic kidney HEK293T cells were a generous gift from Yoshihiko Murata (University of Rochester, Rochester, NY), both strains were maintained in Dulbeccos Modified Eagle Medium (DMEM, ThermoScientific, Waltham, MA) supplemented with 10% Fetal bovine serum (FBS), 500 units Pen/Strep (ThermoScientific) and 50 mg/ml Gentimicin (ThermoScientific). All strain stocks were maintained in TSB or LB medium containing 50% glycerol at -80°C. Antibiotic used in this body of work include: mupirocin and neomycin (0 – 32 µg ml⁻¹); minocycline, amikacin, gentamicin, kanamycin, meropenem, ceftriaxone, erythromycin, colistin,
polymyxin B, ciprofloxacin, levofloxacin, nalidixic acid, sulfamethoxazole, trimethoprim, and tigecycline (0 – 2 µg ml\(^{-1}\), ciprofloxacin 20 µg ml\(^{-1}\)); tetracycline (0 – 16 µg ml\(^{-1}\)); silver sulfadiazine and zinc (0 - 128 µg ml\(^{-1}\)).

**Animals**

All *in vivo* work was performed with female Balb/C mice 4 to 6 weeks of age, obtained from Charles River (Wilmington MA) and housed according to approved University of Rochester Medical Center Council on Animal Research (UCAR) protocol UCAR-2013-024. Samples from mice were processed as described below.

**Chemicals and Compound Libraries**

A library of 853 Food and Drug Administration (FDA)-approved drugs was obtained from Selleck Chemical (Houston, TX). Ciprofloxacin and zinc pyrithione were purchased from Sigma-Aldrich (St. Louis, MO). SSD was obtained from TCI chemicals (Philadelphia, PA). ToxiLight BioAssay kits were acquired from Lonza (Basel, Switzerland). The TimTec ActiProbe-25K diversity-set and Natural Product compound libraries were acquired from TimTec (Newark, DE).

**Mupirocin Synergism Screen**

Members of the Selleck Library of FDA approved drugs were screened for agents that potentiate the antimicrobial activity of mupirocin toward *S. aureus* strain UAMS-1. To do so, 1x10\(^5\) colony forming units of UAMS-1 were added to individual wells of a 96-well microtiter plate, mixed with 0.03 µg ml\(^{-1}\) mupirocin (0.5x minimum inhibitory
concentration) and 50 µM of test agent in Mueller Hinton broth (MHB; 100 µL total well volume). Microtiter plates were incubated at 37°C for 16 hr, and individual wells were inspected for growth. Wells lacking growth were considered to represent agents that either potentiated the antimicrobial properties of mupirocin or mupirocin-independent antimicrobial microbial properties. All drugs that resulted in no growth were confirmed in duplicate and were plated without mupirocin to measure their inherent antimicrobial activity.

**Antimicrobial Susceptibility Testing**

Minimum inhibitory concentration (MIC) was tested in accordance with the Clinical and Laboratory Standards Institute guidelines [184]. Briefly, 1x10⁵ CFU of the indicated bacterial strain was added to individual wells of a microtiter plate containing 88 µL of MHB media and two-fold increasing concentrations of known antibiotic or test agent: mupirocin and neomycin (0 – 32 µg ml⁻¹); minocycline, amikacin, gentamicin, kanamycin, meropenem, ceftriaxone, erythromycin, colistin, polymyxin B, ciprofloxacin, levofloxacin, nalidixic acid, sulfamethoxazole, trimethoprim, and tigecycline (0 – 2 µg ml⁻¹, ciprofloxacin 20 µg ml⁻¹); tetracycline (0 – 16 µg ml⁻¹); silver sulfadiazine and zinc (0 - 128 µg ml⁻¹). Plates were incubated for 16 hr at 37°C and wells were visually inspected for growth. The lowest concentration of antibiotic or test agent that inhibited bacterial growth was considered to be the minimum inhibitory concentration. Fractional inhibitory concentration index (FIC) testing was performed to measure interactions between mupirocin and neomycin or silver sulfadiazine (SSD) and zinc pyrithione (ZnPt), as previously described [185]. Briefly, in checkerboard format each row of the
plate contained increasing concentrations of mupirocin (2-fold increments; 0 to 32 µg ml\(^{-1}\)) or SSD (2-fold increments; 0 to 64 µg ml\(^{-1}\)), whereas each column contained increasing concentrations of neomycin (2-fold increments; 0 to 32 µg ml\(^{-1}\)) or ZnPt (2-fold increments; 0 to 64 µg ml\(^{-1}\)). To every well (100 µl total volume) MHB containing 3 \times 10^5 CFU of a bacterial strain was added and the plate was incubated at 37°C for 16 hr. The FIC was determined using the following formula: \((\text{MIC of Drug A in Combination}/\text{MIC of Drug A Alone}) + (\text{MIC of Drug B in Combination}/\text{MIC of Drug B Alone}) = \text{FIC}\). A synergistic interaction was defined as an FIC value ≤ 0.5, additive as FIC value 0.5 – 1.0, no interaction as an FIC of 1-4, or an antagonistic interaction FIC > 4 [185].

**Biofilm Bactericidal Screen**

An adenylate kinase (AK) reporter assay of bacterial cell death was used to screen Selleck compound library members for agents that displayed bacteriocidal activity toward established *P. aeruginosa* biofilms, as previously described [186, 187]. As a prerequisite to doing so, the assay’s performance in the high throughput setting was evaluated by measuring the Z-factor score of mock (DMSO; negative) and ciprofloxacin (20 µg ml\(^{-1}\); positive control) treated *P. aeruginosa* biofilm cells in 96-well plate format. To do so, overnight cultures of *P. aeruginosa* strain PAO1 were used to seed individual wells of a flat bottom plate (Falcon, Corning Life Sciences; Durham, NC) containing 200 µl of fresh LB medium (~1 \times 10^7 CFU ml\(^{-1}\) final concentration). Plates were incubated at 37°C for 72 hours to allow for biofilm formation. Non-adherent cells were aspirated, biofilms were washed twice with phosphate-buffered saline (PBS) and alternating columns were
treated with fresh media containing either 2 µl of Dimethyl sulfoxide (DMSO) or ciprofloxacin. Plates were incubated for 24 hours at 37°C then processed to compare the effects of antibiotic treatment on biofilm-associated bacteria by measuring adenylate kinase release into the biofilm supernatant and enumerating the number of bacteria remaining. More specifically, biofilm supernatants were transferred to a 96 well-white-walled flat-bottomed plates (Corning, Inc.) for adenylate kinase detection, as described below, whereas, the number of biofilm-associated bacteria remaining in DMSO and ciprofloxacin treatment conditions were enumerated by resuspending the biofilm in 100 µl LB and plating for colony forming units. The amount of adenylate kinase released into the supernatant was measured in the dark by adding AK detection reagent (100 ul) for 30 min at room temperature as luminescence all wavelengths (from 250 nm– 850 nm) with an integration time of 1000 ms per well on a SpectraMax M5 plate reader (Molecular Devices, Sunnyvale, CA). A comparison of the AK-based luminescence of DMSO and ciprofloxacin treated biofilms revealed a Z’-factor score of 0.90 ± 0.11, which corresponded to a ≥ 9-log reduction in P. aeruginosa biofilm-associated cells, indicating that the assay is amenable to identifying agents that display bactericidal activity toward P. aeruginosa biofilms [188]. For Selleck library screening, 72 hr P. aeruginosa PA01 biofilms were grown and processed as described above, except that biofilms were treated with 50 µM of individual members of the library; the first and last position of each plate were treated with 20 µg ml⁻¹ ciprofloxacin (positive control) or DMSO (1% final concentration; negative control).
**Biofilm Antimicrobial Susceptibility**

A modified MIC protocol was used to measure the antimicrobial effects of test agents toward established *S. aureus*, *A. baumannii*, and *P. aeruginosa* biofilms. To do so, 24-72 hr biofilms were created as previously described for *S. aureus* [189] and *A. baumannii* [190], or as described above for *P. aeruginosa*. Biofilms were washed to remove non-adherent cells and 100 µl of fresh media was added to each well such that the wells contained increasing concentrations of SSD (2-fold increments; 0 to 128 µg ml⁻¹), ZnPT (2-fold increments; 0 to 128 µg ml⁻¹) or the combination (2-fold increments; 0 to 128 µg ml⁻¹). Plates were incubated for 24 hours at which point the supernatant was removed, the biofilm was resuspended in 0.8% NaCl and the number of biofilm-associated cells were enumerated by plating on MH agar (Fischer Scientific, Pittsburgh, PA).

**Efflux Pump Inhibitor Screen**

The TimTec ActiProbe-25K diversity-set and Natural Product compound libraries (29,900 compounds total; TimTec) were screened for putative efflux pump inhibitors (EPIs), by identifying compounds that potentiated the antimicrobial property of a sub-inhibitory concentration of minocycline toward *A. baumannii* grown in human serum. To do so, *A. baumannii* strain 983709 was grown for 16 hours in LB medium at 37°C on a rotary shaker at 225 rpm. Approximately 1 x10⁵ CFU were then transferred to individual wells of a 96-well round-bottom plate (Corning Costar, Tewksbury, MA) containing 100 µl of human serum supplemented with minocycline (0.5 µg ml⁻¹; 0.5X minimum inhibitory concentration in serum) and individual members of the TimTec ActiProbe or Natural product library (50 µM). Plates were then incubated at 37°C for 48
hours. Putative efflux pump inhibitors (EPI) were identified as compounds that inhibited A. baumannii growth in human serum containing minocycline and were subsequently retested in triplicate, as indicated above. Positive and negative controls constituted untreated A. baumannii grown in serum supplemented with minocycline plus known EPI phenylalanine-arginine-beta-napthylamide (PaβN) or minocycline alone [104]. To distinguish TimTec compounds with inherent antimicrobial properties from putative efflux pump inhibitors, compounds were directly assessed for antibacterial activity in human serum in the absence of minocycline. To do so, 1 x10^5 CFU of A. baumannii strain 983709 were inoculated into individual wells of a microtiter plate containing 100 µl 100% human serum supplemented with increasing concentrations of the test compound (0 to 128 µg ml⁻¹) and incubated at 37°C for 48 hr.

**Serum Adaptive Antibiotic Resistance and Minimum Effective Concentration (MEC)**

To assess the adaptive antibiotic resistance of A. baumannii and P. aeruginosa, the antibiotic susceptibility of these species LB medium and 100% human serum was measured, as previously described [191]. Briefly, the indicated bacterial species/strain was grown overnight in LB medium, diluted into fresh medium (1:100 dilution) and grown to mid-exponential phase (OD₆₀₀nm = 0.4 to 0.5) at 37°C with aeration. A total of 1 x 10^5 colony forming units (CFU) were transferred to individual wells of a 96-well round bottom plate containing 100 µl of LB or 100% human serum supplemented with 2-fold increasing concentrations (0 to 2 µg ml⁻¹) minocycline, amikacin, gentamicin, kanamycin, meropenem, ceftriaxone, erythromycin, colistin, polymyxin B, ciprofloxacin,
levofloxacin, nalidixic acid, sulfamethoxazole, trimethoprim, tigecycline or 0 to 16 μg ml\(^{-1}\) tetracycline and incubated at 37°C for 48 h. To quantify the antimicrobial effects of each antibiotic toward bacteria grown in LB or serum, well constitutes were serially diluted in PBS and plated on LB agar to enumerate the CFU ml\(^{-1}\). Where indicated, antimicrobial susceptibility assays were also performed in the presence of 50 μg ml\(^{-1}\) the efflux pump inhibitors, verapamil, reserpine, or phenylalanine arginine beta naphthylamide (PA\(\beta\)N) or the minimum effective concentration (MEC) of putative efflux inhibitors ABEPI1 and ABEPI2.

The MEC is the lowest concentration of a test compound that potentiates the activity of an antibiotic. Thus, compounds that inhibited bacterial growth in combination with minocycline were considered putative efflux inhibitors and the MEC toward serum-grown \textit{A. baumannii} was determined. For MEC determination, individual wells of 96-well round-bottom plates containing 100% human serum supplemented with 0.5X MIC minocycline (0.5 μg ml\(^{-1}\)) and increasing concentrations of test compound (0 to 128 μg ml\(^{-1}\)) were inoculated with approximately \(1 \times 10^5\) CFU of \textit{A. baumannii} strain 983709 and incubated for 48 hr at 37°C. The MEC was defined as the lowest concentration of test compound required to inhibit the growth of \textit{A. baumannii} in serum in the presence of 0.5 μg ml\(^{-1}\) minocycline.

**Cellular Accumulation of Minocycline**

High pressure liquid chromatography and triple-quadrupole mass spectrometry were used to measure the \textit{A. baumannii} intracellular levels of minocycline during growth in human
serum in the absence and presence of each putative efflux pump inhibitor. To do so, \textit{A. baumannii} strain 983709 was grown in 5 ml of 100% human serum supplemented with 0.5 \( \mu \)g ml\(^{-1}\) minocycline, in the absence and presence of 0.5X MEC each putative efflux pump inhibitor (test compound) or the known efflux pump inhibitor, verapamil [192]. Cultures were incubated for 48 hours with shaking, at which point an aliquot was removed, serial diluted, and plated to determine the number of viable CFU per mixture. The remaining cells were pelleted by centrifugation at 900 x g at 4\(^\circ\)C, washed twice in PBS, mechanically lysed with a FastPrep cell disrupter (MP Biomedicals, Santa Ana, CA) for 20 seconds at 5 m s\(^{-1}\) and the cellular debris was pelleted via centrifugation at 900 x g at 4\(^\circ\)C. The amount of minocycline present within the supernatant of ruptured cells was measured, as previously described [193]. Briefly, doxycycline (0.5 \( \mu \)g ml\(^{-1}\)) was first added to each supernatant to serve as an internal control to account for sample-to-sample preparation variability. The supernatant was then combined with acetonitrile (ACN) at a 1:10 ratio and centrifuged at 16,000 x g, at 4\(^\circ\)C, to collect minocycline and doxycycline. Supernatants were discarded and residual liquid was evaporated in a speed vacuum for 2 hours at 8,000 x g. To quantify the amount of antibiotics retained, sample materials were suspended in 50% acetonitrile, filtered through a 0.2 \( \mu \)m low protein binding hydrophilic membrane (Millipore, Billerica, MA) then separated on Shimadzu high performance liquid chromatography instrument (Fisher Scientific) using a BetaBasic C18 reverse phase column (ThermoScientific). Separation was carried out with two mobile phase solutions consisting of solution (A) water with 0.1% formic acid and solution (B) 100% ACN. The gradient profile of the chromatography runs was as follows: from 0 to 0.1 min 8% solution B, ramp step wise (37% to 60% solution B) from 0.1 to 6.5 min then holding
for one minute before ramping down from 60% to 10% from 7.5 to 8 min. This was followed by a hold at 10% solution B for one minute. From 10 to 13 min the gradient was ramped to 100% solution B and held until 11.5 minutes and then reduced to 8% solution B and in these conditions for an additional four minutes. The column was equilibrated in 8% solution B at 40°C and the flow rate was set to 0.2 ml min⁻¹. Mass spectrometry analysis of fractions was carried out using a Thermo TSQ Quantum Ultra triple quadrupole mass spectrometer (Fisher Scientific). Data was analyzed using Xcalibur software (ThermoScientific); the following parameters were used to detect minocycline and the internal control doxycycline: 458.208 m/z → 282.971m/z (collision energy = 43, tube lens = 119) for minocycline and 445.144 m/z→ 266.900 m/z (collision energy = 39, tube lens = 127) for doxycycline. Analysis of the raw data was conducted by using the area under the curve calculations with the Genesis algorithm to determine the concentration in each sample. The difference between the peak intensity and the y intercept of pre-established minocycline and doxycycline standard curves was divided by the slope of the standard curve to quantify the amount of minocycline within each sample. The total concentration of minocycline within each cell was calculated by normalizing to the number of cells within each corresponding culture.

**Cytotoxicity Assay**

The human cytotoxicity of putative efflux pump inhibitors was measured alone and in combination with minocycline using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell viability assays, according to the manufacturer’s recommendations (ATCC, Manassas, VA). Briefly, human HepG2 cells were grown to
approximately 1 x 10^6 cells per well in Dulbecco’s Modified Eagle Media supplemented with 10% fetal bovine serum (Invitrogen) then treated with 1X or 4X the MEC of the indicated compound alone or in combination with 0.5 µg ml^-1 minocycline for 24 hours. Cell viability was measured following the addition of the tetrazolium salt (MTT) as per the manufacturer’s recommendations; cells challenged with 50 µg ml^-1 Mitomycin C (Sigma-Aldrich) and mock-treated cells served as positive and negative controls, respectively.

**Ethidium Bromide Efflux Assay**

Standard bacterial ethidium bromide efflux activity assays were used to measure the efflux inhibitory properties of compounds of interest, as previously described [97, 105, 115, 194, 195]. For assays, an overnight culture of *A. baumannii* strain 983709 was diluted (1:100) into 100% human serum or fresh LB and grown to mid-exponential phase. Cell pellets were collected via centrifugation 900 x g for 20 minutes, washed 3x with 20 mM sodium phosphate buffer and resuspended to an OD_{600nm} = 0.2 in sodium phosphate buffer. Approximately 1 X 10^6 CFU were loaded into individual wells of 96-well white-bottom plates, mixed with 10 µg ml^-1 ethidium bromide and ethidium fluorescence (excitation 530 nm; emission 600 nm) was measured every 5 min for 90 min on a Spectramax5 fluorimeter (Molecular Devices). To determine if the putative efflux pump inhibitors affected ethidium bromide efflux, cells were treated with the indicated amount of compound of interest or the efflux pump inhibitor, PAβN, two minutes after fluorescence monitoring began. Mock treated cells served as a negative control; plating confirmed that the test conditions used did not affect cell viability.
**Mammalian Calcium Channel Assays**

Fluo-4 Direct Calcium channel assay kits were used to determine whether putative EPIs affect human Ca^{2+} channel activity, according to the manufacturer’s recommendations (Life Technologies, Carlsbad, CA). Briefly, 5 x 10^4 human HEK 293T embryonic kidney cells were grown in individual wells of 96-well black-walled plates (Corning Costar). Next 2X Fluo-4 dye supplemented with Probenecid (5 mM) was added to each well and allowed to equilibrate for 1 hr at 37°C. To determine whether putative efflux pump inhibitors ABEPI1 or ABEPI2 affect Ca^{2+} channel activity, Fluo-4 fluorescence measures (excitation 495 nm; emission 516 nm) were taken every second for 15 sec. At that time point cells were treated with either DMSO (mock), 50 µg ml^{-1} of the Ca^{2+} channel inhibitor verapamil (positive control) or 1X MEC ABEPI1 or ABEPI2 followed by the calcium channel stimulator carbamylocholine chloride (50 µg ml^{-1}; Fisher Scientific) at 60 seconds and fluorescence was measured for an additional 120 seconds on a FlexStation 3 benchtop multimode microplate reader (Molecular Devices).

**Transposon Mutant Screen**

The genetic factors mediating adaptive antibiotic resistance in A. baumannii were of particular interest, not only do they allow bacterial species to negate the efficacy of antibiotics; they are also likely to be the targets of EPIs. Previously, a collection of 6,000 transposon mutants were generated in A. baumannii strain 983709, using the EZ-Tn5 <R6Kγ ori/Kan-2> Tnp transposome system [183]. All members of the library were
screened for the loss of antibiotic tolerance during growth in serum. For mutant assays, members were grown for 16 h in individual wells of 96-well round-bottom plates (Costar 3788; Corning) containing 150 µl TSB and 5 µg ml\(^{-1}\) kanamycin. Approximately \(1 \times 10^7\) cells of each library member were subsequently transferred to new 96-well flat-bottom plates containing 100 µl human serum (Falcon 3072; Becton Dickinson, Franklin Lakes, NJ) supplemented with 0.25X MIC (0.25 µg ml\(^{-1}\)) minocycline. Plates were statically grown at 37°C for 48 h, at which point all plates were inspected visually for the loss of growth. Those wells that lacked visual growth were plated on LB agar to enumerate the cells in each well. Mutants that exhibited at least a two log decrease in growth compared to wild type cells were isolated to identify the transposon insertion point.

**Inverse PCR**

Inverse PCR was used to identify the transposon insertion site of mutants exhibiting reduced growth in human serum supplemented with minocycline, as shown in Fig. 2.1. To do so, total bacterial DNA was purified from each mutant of interest using a DNeasy Blood and Tissue Kit, following the manufacturer's recommendations (Qiagen, Valencia, CA). Two micrograms of purified DNA was then digested with the restriction enzyme MfeI (10 U; New England Biolabs, Ipswich, MA) at 37°C for 1 h. The restriction fragments were circularized by ligation using 1.5 U of T4 DNA ligase (Invitrogen, Carlsbad, CA) at 16°C for 16 h. Following enzyme heat inactivation at 65°C for 20 minutes, 10 µl of each ligation mixture was subjected to inverse PCR using Platinum PCR Supermix High Fidelity (Invitrogen) and transposon-specific primers (forward, 5′-
ACCTACAACAAAGCTCTCATCAACC-3’; reverse, 5’-CTACCCTGTGGAACACCTACATCT-3’) supplied in the EZ-Tn5 <R6Kγori/KAN-2> Tnp transposome kit (Epicentre Biotechnologies, Madison, WI). Inverse PCR was performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems, Foster City, CA) with the following parameters: 94°C for 10 min and 50 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 6 min, followed by an extension at 72°C for 10 min. The PCR products were electrophoresed in a 1% UltraPure Agarose gel (Invitrogen) at 75 V for 40 min and gel purified using a QIAquick Gel Extraction Kit (Qiagen). To determine the sequence of each PCR product, approximately 40 ng of gel-purified PCR products was ligated into 25 ng of pCRII-Topo vector and transformed into 50 µl Escherichia coli One Shot® Top 10 cells, following the manufacturer’s recommendations for dual-promoter TA cloning (Invitrogen). One Shot® Top10 E. coli cells were used as they have improved efficiency in transformations, reduced nonspecific recombination, no nonspecific digestion by Endonuclease I, and the presence of the lacZ for easy of insertion screening. Transformants were selected on LB agar containing 50 µg ml⁻¹ kanamycin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (80 µg ml⁻¹; Sigma-Aldrich); the latter allowed an efficient screen of plasmids containing an insert within the vector lacZ gene. Following propagation, plasmid DNA was purified using QIAprep Spin Miniprep kits (Qiagen) and sequenced through ACGT, Inc. using vector-specific primers (forward, 5’-GTAAAACGACGGCCAG-3’; reverse, 5’-CAGGAAACAGCTATGAC-3’).
RNase P ptRNA Processing Assay

Neomycin was previously found to inhibit the activity of RNase P in Gram-negative bacterial species *E. coli*, thus we wanted to determine if it was able to inhibit the *S. aureus* RNase P holoenzyme. *S. aureus* RNase P activity assays were performed as previously described [196]. Briefly, RNase P was first reconstituted by mixing an equimolar ratio of denatured rnpB and RnpA for 15 min at 37°C then added (2.5 pmol) to 5 pmol of ptRNA_{Tyr}, and increasing concentrations of the indicated concentration of neomycin or the known RNase P inhibitor, RNPA2000 [196] in a total volume of 20 µl. Mixtures were incubated for 5 min at 37°C, stopped by adding 20 µL of 2x RNA loading dye (95% formamide, 0.025% SDS, 0.025% bromophenol blue, 0.025% xylene cyanol FF, 0.5 mM EDTA), and 30 µL of each sample was electrophoresed in a 7M urea–8% polyacrylamide gel and stained with ethidium bromide (0.5 µg ml⁻¹). A FluorChem 5500 imaging system was used to visualize RNA products and quantified using ImageJ software (National Institutes of Health, Bethesda MD). The percent RNase P activity was then calculated using the following equation: test compound tRNA_{Tyr} signal/mock tRNA_{Tyr} signal.

Preparation of Test Articles

Polyethylene glycol (PEG) ointment-base was prepared by mixing PEG 400 (70% w/v) with PEG 3350 (30% w/v) as described by the United States Pharmacopeia and The National Formulary [197]. Mupirocin (AppliChem, Chicago IL) and neomycin (Sigma-Aldrich) were suspended in 250 µl of dimethyl sulfoxide (DMSO) to create working
concentrations of 100 mg and 50 mg, respectively. Mixtures were then added directly to 5 g of PEG ointment pre-liquefied by heating at 60°C for 30 min to create 2% mupirocin, 1% neomycin suspensions then cooled to room temperature to solidify the suspension. The same procedure was used to create DMSO vehicle control and 2% mupirocin/1% neomycin PEG mixtures by adding a combination of 100 mg mupirocin and 50 mg neomycin in a total 250 µl DMSO. Additionally, SSD and ZnPT were suspended in 250 µl of DMSO to create working concentrations of 100 mg and 12.5 mg, respectively. Mixtures were then added directly to 5 g of PEG ointment pre-liquefied by heating at 60°C for 30 min to create 2% SSD, 0.25% ZnPT suspensions then cooled to room temperature to solidify the suspension. The same procedure was used to create DMSO vehicle control and 1% SSD/0.25% ZPT PEG mixtures by adding a combination of 50 mg SSD and 12.5 mg ZPT in a total 250 µl DMSO.

In vitro Ointment Antimicrobial Testing

Antimicrobial zones of inhibition were measured for PEG ointment compilations using the indicated bacterial strains. To do so, 100 µL of 1x10^8 CFU ml⁻¹ of the indicated strains was spread on TSA or MH plates. Plates were dried for 10 min and 40 µL of ointment was pipetted onto the center of the plate. Plates were incubated at 37°C for 16 hr and zones of bacterial clearance were measured using ImageJ software (NIH). Briefly, each plate was imaged using the FluorChem 5500 imager from Alpha Innotech (San Leandro, CA) then imported into ImageJ. The contrast was adjusted to produce a distinct border between the zone of inhibition and lawn of bacterial growth. The area of the zone of inhibition was outlined using the tracing tool and the area was determined using the
measuring function. The scale was set by determining the number of pixels in a standard 1 cm².

**Nasal Colonization and Treatment of Mice**

Mupirocin and neomycin containing ointments were evaluated for *in vivo* antimicrobial activity using a *S. aureus* nasal colonization model as previously described [198], but with modifications. The nostrils of awake mice were inoculated with 1 x 10⁷ of the indicated *S. aureus* strain by pipetting 10 µL of culture directly into the nostrils and confirmed by the visualization of air bubbles appearing as the mouse breathed in and out. Mice nostrils were then treated with 10 µL PEG ointment (brought to 55°C in a heat block to liquefy) containing either vehicle alone or 2% mupirocin, 1% neomycin, or the combination, 45 min post inoculation and treatments were repeated every 8 hr for three days. Mice were then euthanized via CO₂ asphyxiation and cervical dislocation. The full nares from the back of the soft palate to the tip of the nostrils was collected by gross dissection and placed in microcentrifuge tubes containing 1 mL of freshly made PBS. Samples were homogenized for five minutes, serially diluted, and plated on Mannitol Salt agar (MSA, ThermoScientific). Plates were incubated for 16 hr and the number of *S. aureus* were enumerated.

**Dermal Wound Model of Infection and Treatment of Mice**

The effects of the mupirocin/neomycin ointments and SSD/ZnPT ointments were evaluated for *in vivo* antimicrobial activity using a *S. aureus* dermal wound model [199], but with modifications. Mice were anesthetized by intraperitoneal injection with a
mixture of 100 mg ml\textsuperscript{-1} Ketamine (Hospira Inc., Lake Forest IL) and 20 mg ml\textsuperscript{-1} Xylazine (Lloyd Laboratories, Shenandoah IA) in 0.9% NaCl at 5 µl per 1 g body weight. Pain relief in the form of 20 µl 0.5% Sensorcaine (APP Pharmaceuticals, Schaumburg, IL) was administered prior to dermal wounding. The dorsal mid-section of the mouse was shaved and cleaned with a series of betadine scrub (Fisher Scientific), povidone-iodine pads (Professional Disposables International Inc; Orangeburg, NY) and isopropyl alcohol pads (Fisher Scientific) for a total contact time of 2 minutes. A single wound was created in this sterile field on the mouse with a 6 mm biopsy punch (Fisher Scientific) to remove only the dermal layer and not disrupt the underlying musculature. The wounds of the mice were inoculated with 1X10\textsuperscript{6} of \textit{A. baumannii} or \textit{P. aeruginosa} or 1x10\textsuperscript{7} of the indicated \textit{S. aureus} strain by pipetting 10 µL of culture directly onto the wound. Mice were then treated with ointment formulations (50 µL) containing either vehicle alone, 2\% mupirocin, 1\% neomycin, the combination, or 1\% SSD, 2\% SSD, 0.25\% ZnPT, 1\% SSD plus 0.25\% ZnPT, 2\% SSD plus 0.25\% ZnPT, 45 min post inoculation; treatments were repeated every 12 hr for three days. Mice were then euthanized via CO\textsubscript{2} asphyxiation and cervical dislocation, as per UCAR approved methodology, the wound and underlying muscle was excised with an 8 mm biopsy punch and placed in microcentrifuge tubes containing 1 mL of freshly made PBS. Samples were homogenized for five minutes, serially diluted, and plated on MSA (\textit{S. aureus}) or MH (\textit{A. baumannii} and \textit{P. aeruginosa}). Plates were incubated for 16 hr and the number of \textit{S. aureus} was enumerated.
In vivo Toxicity Testing

Ointment toxicity was tested in a modified dermal wound model [199]. Mice in groups of three per indicated treatment group were wounded as described above but were not inoculated with bacteria. The wound was treated with vehicle, 2% mupirocin, 1% neomycin, or 2% mupirocin plus 1% neomycin combination ointments twice daily for 14 days. Separately, mice were also treated with vehicle, 2% SSD, 0.25% ZnPT, 1% SSD plus 0.25% ZnPT, or 2% SSD plus 0.25% ZnPT combination ointments twice daily for 14 days. Mice were weighed, assessed for grooming and alertness, and images of the wound were obtained daily to measure wound contraction using Image J (NIH). Wound contraction was calculated as percentage of wound area reduction using the formula: \[ WC_d = (1 - WA_d/WA_0) \times 100 \], where WC is wound contraction, WA is wound area, \( d \) is day, and 0 indicates initial day, as previously described [200].
<table>
<thead>
<tr>
<th><strong>Bacterial Strain</strong></th>
<th><strong>Description</strong></th>
<th><strong>Source</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em> UAMS-1</td>
<td>Antibiotic susceptible laboratory strain</td>
<td>[181]</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> USA300</td>
<td>Neomycin and methicillin resistant strain</td>
<td>[182]</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em> BAA1708</td>
<td>High level mupirocin resistant strain</td>
<td>Obtained from ATCC</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em> 983709</td>
<td>CDC clinical isolate</td>
<td>[183]</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em> 07061</td>
<td>CDC clinical isolate</td>
<td>[183]</td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em> 011205</td>
<td>CDC clinical isolate</td>
<td>[183]</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> PA01</td>
<td>Characterized laboratory strain</td>
<td>Gift of Dr. Barbara Iglewski</td>
</tr>
<tr>
<td><em>Enterobacter cloacae</em></td>
<td>Clinical strain</td>
<td>Obtained from the clinical microbiology laboratory</td>
</tr>
<tr>
<td><em>Escherichia coli</em> 8307</td>
<td>Laboratory strain</td>
<td>Obtained from the Yale <em>E. coli</em> collection</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> CKP4</td>
<td>Laboratory strain</td>
<td>Gift of Dr. Thomas Russo</td>
</tr>
<tr>
<td><em>Enterococcus faecium</em> 824-05</td>
<td>Laboratory strain</td>
<td>Gift of Dr. Jose Lemos</td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em> V583</td>
<td>Laboratory strain</td>
<td>Gift of Dr. Jose Lemos</td>
</tr>
<tr>
<td><strong>Mammalian Cell Lines</strong></td>
<td><strong>Description</strong></td>
<td><strong>Source</strong></td>
</tr>
<tr>
<td>HepG2</td>
<td>Liver carcinoma cell line</td>
<td>Obtained from ATCC</td>
</tr>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney cell line</td>
<td>Gift of Dr. Yoshi Murata</td>
</tr>
</tbody>
</table>
Figure 2.1. Inverse PCR schematic. Genomic DNA was isolated from each of A.
baumannii strain 983709 transposon mutants that displayed a growth defect in serum
supplemented with minocycline and exhibited ethidium bromide accumulation. The
genomic DNA was then digested with the restriction enzyme \textit{MfeI}. Digested DNA was
then ligated onto itself and the DNA on either side of the transposon was amplified. The
amplified DNA was then sequenced at ACGT, Inc. to determine the gene with the
transposon insertion.
Chapter 3: Neomycin Improves the Antimicrobial Activity of Mupirocin Against *Staphylococcus aureus*

**INTRODUCTION**

*Staphylococcus aureus* has been designated as one of the six ESKAPE bacterial pathogens of greatest U.S. healthcare concern [1]. The organism is a predominant cause of nosocomial- and community- associated bacterial infections and has developed resistance to all currently available antibiotics [201]. *S. aureus* annual U.S. mortality rates have already surpassed that of HIV/AIDS and are predicted to worsen given the downsizing of most pharmaceutical antimicrobial programs [202, 203]. Consequently, new strategies are needed for the prevention and treatment of staphylococcal infections.

The anterior nares of humans is a principle ecological niche for *S. aureus* and nasal carriage is a recognized risk factor for staphylococcal disease, particularly among patient populations undergoing surgical procedures, hemodialysis, or requiring long term intensive care unit stays [reviewed in [204]]. *S. aureus* nasal decolonization reduces colonization of other body sites, the risk of transmission, and subsequent infection [204]. Consequently, infection control practices routinely include nasal decolonization procedures as a means to prevent staphylococcal disease.

Mupirocin is an antimicrobial agent that inhibits bacterial isoleucyl-tRNA synthetase mediated Ile-tRNA aminoacylation and protein translation [28-30]. The agent displays excellent antibacterial activity toward most Gram-positive species, lacks cross resistance to current antibiotics, but is also unstable *in vivo* and thus not well-suited for systemic use.
in humans [31]. However, mupirocin based ointments have proven effective for the treatment of *S. aureus* skin and wound infections [31, 205-208] and have also recently emerged as the standard of care for pre-surgical nasal decolonization [Reviewed in [32]]. Indeed, mupirocin mediated nasal decolonization has been shown to be effective in reducing infections in burn wound, dialysis, and surgical patient populations, as well as *S. aureus* transmission among healthcare workers and intensive care unit patients [25, 34-39]. In addition to nasal decolonization, topical mupirocin has been used to successfully treat hemodialysis central venous catheter exit sites, impetigo, eczema, surgical wound sites, skin and soft tissue wounds, the breasts of breast-feeding mothers, and tympanic membrane lesions [40-45]. However, the emergence of *S. aureus* mupirocin resistance has reduced the agent’s efficacy both as a nasal decolonization agent and as a treatment option for skin and wound infections.

Low level mupirocin resistant (LL-MR) *S. aureus* strains are defined as exhibiting an MIC of 8 to 256 µg ml\(^{-1}\) due to point mutations in the organism’s native isoleucyl tRNA synthetase gene (*ileRS*) and develop rapidly in both the laboratory and clinical settings [46]. High level mupirocin resistance (HL-MR; MIC of > 256 µg ml\(^{-1}\)) occurs less frequently and is attributable to the acquisition of a mobile genetic elements harboring either *mupA*, which codes for an alternate isoleucyl tRNA synthetase, or the less-characterized *mupB* gene [47, 48]. Both LL-MR and HL-MR lead to mupirocin treatment failure [50]. Indeed, while low level resistant strains initially respond to therapy they frequently re-emerge quickly; relapse is hypothesized to be due to latent LL-MR subpopulations that are not eradicated by mupirocin dosing [49, 50]. Conversely,
HL-MR are recalcitrant to mupirocin ointments [50]. Thus, the emergence of mupirocin resistance has prompted renewed interest in developing alternative decolonization and wound infection treatment strategies.

*S. aureus* RNase P is an essential riboprotein complex consisting of RnpA and ribozyme *rnPB* that acts upstream of tRNA synthetases in the transfer RNA maturation pathway [196, 209]. More specifically RNase P is hypothesized to catalyze removal of the 5’ leader sequences from precursor tRNA species thereby creating mature tRNA substrates for tRNA synthetases, including isoleucyl tRNA synthetase (the cellular target for mupirocin) [196, 209-214]. Recognizing that two antimicrobials targeting independent steps in the same metabolic pathway can have combined antibacterial effects it has been hypothesized that combination therapies involving mixtures of RNase P inhibitors together with mupirocin would display increased antimicrobial efficacy and the potential to overcome mupirocin resistance [196, 215].

Herein, we report the results of a screen of a Food and Drug Administration (FDA) approved drug library for agents that potentiate the antimicrobial properties of mupirocin toward *S. aureus*. The antibiotic neomycin sulfate, which is approved for topical use and previously shown to inhibit *Escherichia coli* RNase P, was among the hits identified [216]. Assays revealed that neomycin also inhibits *S. aureus in vitro* RNase P function, confers an additive antimicrobial advantage to mupirocin and the combination could be effectively formulated in ointment format. Topical application of the combination displayed significantly improved murine nasal decolonization toward a panel of *S. aureus*
strains, in comparison to either agent when tested alone. Likewise, the combination led
to the near eradication of contemporary methicillin susceptible, methicillin resistant, and
high-level mupirocin resistant strains in a murine wound model of colonization.

RESULTS

Agents that potentiate the antimicrobial activity of mupirocin. Members of the
Selleck library of 853 FDA approved drugs were screened for agents that potentiate the
activity of mupirocin. To do so, the antibiotic susceptible *S. aureus* strain UAMS-1 was
inoculated into individual wells of a microtiter plate containing 0.5X the strain’s
mupirocin minimum inhibitory concentration (MIC; 0.0625 µg ml⁻¹) and 50 µM of
library material. A total of 101 library members (11.8%), including 61 antibiotics,
inhibited bacterial growth suggesting that they may represent agents that: 1. potentiate the
antimicrobial activity of mupirocin, 2. exhibit mupirocin-independent antimicrobial
activity, or 3. both (*Table 3.1*).

To distinguish between these possibilities, the MIC of each compound was determined in
medium lacking or containing 0.5X the strain’s mupirocin MIC. Ninety-eight of the 101
compounds (97%) evaluated displayed similar antimicrobial activities regardless of
whether mupriocin was present, indicating that they do not potentiate the antibacterial
effects of mupirocin. Conversely, the antimicrobial activity of nitazoxanide,
nitrofurazone, and neomycin sulfate, increased in the presence of mupirocin. Fractional
inhibitory concentration index (FIC) measures confirmed that each agent displayed an
additive effect (FIC’s = 0.75) when combined with mupirocin indicating that they have
the capacity to potentiate the activity of mupirocin (Table 3.2). More specifically, nitazoxanide and nitrofurazone reproducibly displayed modest antimicrobial activities of 16 µg ml\(^{-1}\) and 8 µg ml\(^{-1}\) in the absence and presence of 0.5X MIC mupirocin, respectively. The aminoglycoside antibiotic neomycin sulfate exhibited the most potent activity against the test strain in the absence (0.5 µg ml\(^{-1}\)) and presence (0.125 to 0.25 µg ml\(^{-1}\)) of 0.5X MIC mupirocin (0.0625 µg ml\(^{-1}\)). Given that no other antibiotics within the Selleck library, including other aminoglycosides, displayed improved antimicrobial properties in the presence of mupirocin and expanded FIC testing revealed that neomycin did not improve the antimicrobial activity of rifampicin, vancomycin, sulfamethoxazole, meropenem, minocycline, ciprofloxacin, ceftriaxone, or erythromycin (data not shown), the additive effects between neomycin and mupirocin appeared to be combination specific.

**Neomycin inhibits *S. aureus* RNase P in vitro activity.** As noted above, it has been hypothesized that inhibitors of RNase P function would display improved antimicrobial effects when combined with mupirocin. In that regard, aminoglycoside antibiotics bind the major groove of the 16S rRNA to disrupt the fidelity of tRNA selection and block protein translation, but recent studies have revealed that they can also bind and affect the function of mRNAs, tRNAs, and catalytic RNAs [216-219]. Indeed, neomycin B and/or derivatives have been shown to bind to the *rnpB* component of RNase P and/or precursor tRNA molecules in a manner that inhibits *Escherichia coli*, *Neisseria gonorrhoeae*, *Porphyromas gingivalis*, *Streptococcus pneumoniae* and *Bacillus subtilis* RNase P function [216, 220, 221]. Accordingly, we evaluated whether neomycin also inhibits *S.*
aureus RNase P activity using an *in vitro* precursor tRNA processing assay [196]. As shown in Fig. 3.1, results revealed that high concentrations (250 µM) of neomycin inhibit *S. aureus* RNase P’s ability to catalyze the maturation of precursor tRNA\textsubscript{Tyr}, suggesting that the agent’s ability to potentiate mupirocin may, in part, be mediated by its ability to inhibit the organism’s RNase P activity.

**Antimicrobial effects of mupirocin and neomycin combination in ointment formation.** Because neomycin improves the antimicrobial potency of mupirocin and the two antibiotics have differing mechanisms of action, we reasoned that combination ointments containing both agents would overcome mupirocin resistance. As a first test of this hypothesis, antimicrobial plate assays were used to monitor the antimicrobial effects of PEG-based ointments containing either DMSO (vehicle), 2% mupirocin, 1% neomycin, or combination (2% mupirocin + 1% neomycin) toward a neomycin and mupirocin susceptible clinical isolate (UAMS-1), a neomycin resistant clinical isolate (USA300; MIC > 128 µg ml\textsuperscript{-1}; data not shown), and a strain containing the *mupA* gene that confers high level mupirocin resistance (BAA-1708; MIC > 256 µg ml\textsuperscript{-1}; data not shown).

As shown in Fig. 3.2A, measures of each treatment’s zone of inhibition revealed that while vehicle alone did not affect UAMS-1 growth, both antibiotics, alone and in combination, produced zones of growth inhibition, suggesting that the ointment formulation did not antagonize the antimicrobial properties of either agent. More specifically, 2% mupirocin generated a zone of inhibition of 20 (± 2) cm\textsuperscript{2}, whereas 1%
neomycin exhibited an average zone of clearance of 9.4 (± 1.1) cm². The combination of 2% mupirocin and 1% neomycin displayed the greatest zone of inhibition (24.3 ± 1 cm²), which was statistically improved over that of mupirocin or neomycin alone. We considered that the improved activity of the combination could be attributed to either the additive effects of the specific antibiotic combination or merely reflect an overall increase in active antimicrobial ingredients. However, similar improvements in antimicrobial clearance were not observed in tests of 2% mupirocin in combination with 1% of kanamycin, vancomycin, erythromycin, or oxacillin. Representative results for vancomycin and erythromycin, which exhibited antagonistic and no improvement in combination, respectively, toward the strain are shown in Figs. 3.2D and 3.2E. These results indicate that the additive effects of the mupirocin + neomycin combination observed in liquid culture conditions also occur in ointment format.

As shown in Fig. 3.2B, tests of the neomycin resistant strain USA300 revealed that mupirocin elicited a 14.0 (± 4) cm² zone of growth inhibition. Interestingly, 1% neomycin ointment produced a small (4.3 (± 0.01) cm²) halo-like zone of inhibition despite the strain’s resistance to the agent, indicating that the concentration tested is able to overcome the organism’s resistance phenotype to a certain extent. Moreover, the combination treatment showed a significantly increased inhibition zone (24.0 (± 3.4) cm²) in comparison to either agent alone. Testing of the high level mupirocin resistant strain BAA-1708 (Fig. 3.2C.) demonstrated that the strain was resistant to 2% mupirocin ointment in comparison to both UAMS-1 and USA300 but did generate a small zone of growth inhibition (3.6 (± 0.86) cm²). Conversely, 1% neomycin ointment elicited a clear
zone of inhibition (4.9 (± 1.1) cm²), which was significantly increased by combination
treatment (7.3 (± 0.4) cm²).

Taken together, these results indicate that mupirocin and neomycin are compatible in the
ointment format tested here. Further, the combination of 2% mupirocin + 1% neomycin
exhibited increased antimicrobial activity in comparison to either agent alone and
displayed activity against all strains irrespective of their resistance profile. From these
perspectives, we hypothesized that the combination would be similarly therapeutically
beneficial in host-environments that mupirocin (alone) is typically used for the
prevention and/or therapeutic intervention of staphylococcal infections.

The effects of mupirocin and neomycin on *S. aureus* nasal decolonization. A murine
model of *S. aureus* nasal colonization was used to compare the antimicrobial efficacy of
mupirocin, neomycin, and the two agents when applied in combination. To do so, the
nasal passages of Balb/C mice were inoculated with ~1 x 10⁷ colony forming units of *S.
aureus* then treated three times a day for a total of three days, at which point the bacterial
burden was measured and the antibiotic susceptibility of ten isolates from each animal
was measured by MIC testing.

Consistent with previous reports, 2% mupirocin treatment resulted in a 1.1-log reduction
in *S. aureus* strain UAMS-1 nasal colonization Fig. 3.3A [222]. However, two mice
displayed uncharacteristically high-burdens; upon testing, these isolates were found to
exhibit 4-fold increase in mupirocin resistance (MIC of 0.5 µg ml⁻¹) in comparison to the
inoculating strain as well as isolates from the other animals within the treatment group (MIC of 0.125 \( \mu g \) ml\(^{-1}\)), suggesting that mupirocin (alone) dosing selected for low-level resistant derivatives. One percent neomycin treatment displayed a slight, although not statistically significant, 0.5-log reduction in bacterial burden in comparison to vehicle alone, whereas combination treatment with 2% mupirocin + 1% neomycin resulted in the greatest reduction in \textit{S. aureus} colonization (1.7-log) and did not appear to select for low level mupirocin resistance. Similar results were observed for USA300 nasal decolonization (Fig. 3.3B). More specifically, 2% mupirocin treatment resulted in a 1-log decrease in bacterial burden, whereas treatment with 1% neomycin (alone) resulted in nearly a 1.8-log reduction in USA300 burden. The combination of mupirocin and neomycin appeared to consistently reduce bacterial burden to the greatest extent (1.7-log reduction). Likewise, combination treatment exhibited increased efficacy toward \textit{S. aureus} strain BAA-1708, in comparison to each agent alone (Fig. 3.3C). Despite displaying a high-level mupirocin resistant phenotype, the strain exhibited a moderate reduction in burden (0.54-log) following mupirocin (alone) treatment, a 0.9 log reduction in 1% neomycin treated animals and a 1.2-log reduction following combination treatment. The observed improved activity of the combination toward each strain, combined with the notoriously low resolution of the nasal models available [198, 222, 223], prompted us to evaluate the combination’s ability to reduce \textit{S. aureus} wound site colonization.

\textbf{The effects of mupirocin and neomycin on \textit{S. aureus} wound clearance.} A murine dermal wound model was used to evaluate the decolonization properties of 2%
mupirocin, 1% neomycin and 2% mupirocin + 1% neomycin. To do so, dermal wounds were created on the backs of Balb/C mice, inoculated with either *S. aureus* strain UAMS-1, USA300, or BAA-1708, and then treated with test agent suspended in PEG-based ointment twice a day for a total of 3 days, at which point bacterial burden was measured.

As shown in Fig. 3.4A, three day treatment with 2% mupirocin resulted in an approximately 6-log reduction in UAMS-1 colonization (8.7 x 10^1 CFU per lesion) of the wound site in comparison to animals that were treated with vehicle alone (4.8 x 10^7 CFU per lesion). One percent neomycin treatment exhibited improved clearance in comparison to mupirocin (alone), resulting in a 1.4 x 10^1 CFU per lesion with no bacteria recovered from 5 of the 10 (50%) of the animals within the treatment group. Combination treatment displayed the greatest efficacy. No bacteria were recovered from 9 of the 10 animals (90%) treated with 2% mupirocin + 1% neomycin, whereas a single UAMS-1 colony was recovered from the remaining animal.

Testing of the neomycin resistant strain, USA300, showed that 2% mupirocin was effective, resulting in a 5-log reduction in bacterial wound site burden, with no bacteria recovered from 4 of the 10 (40%) animals in the treatment group (Fig. 3.4B). As expected, neomycin treatment (alone) had minimal effects on decolonization, presumably due to the strain’s neomycin resistance phenotype, while the greatest efficacy was observed for the combination treated group, in which no USA300 cells were recovered from 7 of 10 (70%) of the animals tested. Similarly, the combination of mupirocin and neomycin displayed the greatest efficacy in tests of the mupirocin resistant strain BAA-
1708 (Fig. 3.4C). More specifically, as expected, 2% mupirocin treatment (alone) did not reduce wound site colonization in comparison to vehicle treated cells, whereas neomycin treatment (alone) resulted in a 4.9-log decrease in recoverable bacteria. The combination of mupirocin + neomycin produced the greatest reduction in colonization, resulting in a 6.1-log decrease in wound site bacteria and no recoverable bacteria in 3 of the 10 (30%) animals tested. These results indicate that mupirocin + neomycin ointments are more effective in reducing wound site S. aureus burden than either agent alone and that the combination is capable of overcoming resistance to either agent.

The antimicrobial potential of mupirocin and neomycin combination ointment toward other bacterial species. Mupirocin and neomycin are predominantly active toward Gram-positive and Gram-negative species, respectively. Consequently, we predicted that the combination would display increased spectrum of activity, in comparison to either agent alone, and could improve treatment options for polyclonal wound site infections composed of mixtures of both Gram-positive and negative organisms.

As a preliminary test of that hypothesis, zone of inhibition assays were performed for 2% mupirocin, 1% neomycin and 2% mupirocin + 1% neomycin using A. baumannii and P. aeruginosa, two Gram-negative organisms that are frequent causes of wound site infections. As shown in Fig. 3.5, 2% mupirocin ointment did not appear to restrict growth of A. baumannii strain 983709 or P. aeruginosa strain PA01. Conversely, neomycin, both alone and in combination with mupirocin, restricted growth of both
organisms, indicating that the combination of 2% mupirocin + 1% neomycin may be useful in the prevention and/or treatment of complicated wound infections. Both agents, independently and in combination, also limited growth of *S. epidermidis, Escherichia coli*, and *Streptococcus pyogenes* strains tested (data not shown).

**Effects of mupirocin and neomycin on wound healing.** The above results indicate that combination ointments comprised of mupirocin and neomycin display improved antimicrobial efficacy, overcome mupirocin resistance, and are likely to exhibit increased spectrum of activity toward other bacterial species, in comparison to mupirocin (alone). Such a combination therapeutic would most likely be of value in the context of the wound setting. In that regard, although both mupirocin and neomycin are FDA approved antibiotics for topical use, we evaluated whether the mixture of both agents exhibited overt detrimental side effects at the wound site. To do so, dermal wounds were created and animals were treated with either vehicle, 2% mupirocin, 1% neomycin, or the combination twice daily for a total of 14 days. Each day, animals were assessed for alertness and grooming, weight and wound size.

No significant differences in wound contraction were observed for any of the treatment groups (N=3 for each treatment), in comparison to vehicle containing ointment (Figs. 3.6A and 3.6B). Regardless of ointment used, wound size increased 3 days post-lesion formation and was followed by a linear increase in wound contraction, such that the wound healing was completed and hair growth had been restored at 14 days of treatment.
Likewise, no significant differences in weight were recorded for any animals in any of the treatment groups (Fig. 3.6C).

**DISCUSSION**

More than 30 million patients undergo surgery in the U.S. annually and up to 20 percent of those patients acquire a postoperative nosocomial infection, resulting in increased rates of morbidity and mortality, systemic antibiotic use, and healthcare costs of $5 to $10 billion [224, 225]. Mupirocin-based ointments (2% mupirocin) have proven successful in the prevention and/or treatment of staphylococcal disease. Indeed, in the United Kingdom it is recommended that MRSA carriers should undergo nasal decolonization with mupirocin as a prophylactic measure prior to surgical intervention [33]. However, mupirocin use has predictably selected for resistance that has, in-turn, mitigated the agent’s efficacy.

The incidence of *S. aureus* low- and high- level mupirocin resistance within individual healthcare institutions is highly variable and is presumably influenced by differences in corresponding infection control practices and between the strains circulating at local and regional levels. One retrospective survey of methicillin resistant *S. aureus* (MRSA) nasal and blood isolates collected from 23 U.S. hospitals revealed that 3% and 5% of the strains tested displayed high level mupirocin resistance, respectively [226]. However, single-center studies have recorded higher prevalences both in the U.S. and abroad. For instance, one New York hospital recently reported that 31% of pediatric isolates tested exhibited high-level resistance [52] and, in one extreme case, 47% and 79% of
community and hospital-associated MRSA isolates collected from a Korean neonatal intensive care unit exhibited high-level mupirocin resistance [53]. Single center low-level mupirocin resistance rates of 0-80% have been recorded in the U.S. [226]. From these perspectives it is not surprising that recent studies have called into question the advantageous effects of mupirocin ointments, highlighting the need for new approaches for *S. aureus* decolonization and wound care management.

Drug combinations are a mainstay therapeutic strategy in the treatment of cancer, HIV, asthma, hypercholesterolemia malaria, and tuberculosis [138]. Several current antibiotics represent combination therapeutics, such as sulfonamides and trimethoprim and β-lactam antibiotics in conjunction with β-lactamase inhibitors [140, 141]. A central tenet of the combination approach is that the sum of the ingredients is greater than the individual components themselves and a highly successful strategy for development of multicomponent drugs has been to combine single-compound drugs that already exist; early examples include Advair (fluticasone + salmeterol), Advicor (niacin + lovastatin), Combivir (azidothymidine + lamivudine) and Trizivir (azidothymidine + lamivudine + abacavir) [227-229]. In that regard, we set out to improve the performance of mupirocin ointment via the addition of an FDA approved agent with the goal of creating an improved antimicrobial ointment with increased antimicrobial efficacy and capable of overcoming high-level mupirocin resistance.

Numerous studies have made it apparent that the simple addition of two agents does not reliably correlate with improved combined activity. Indeed, that has also been our
experience. Screening of an 853 member F.D.A approved drug library identified 101 agents that displayed antimicrobial activity against the antibiotic susceptible test strain, UAMS-1. Yet only three of those agents, nitazoxanide, nitrofurazone, and the antibiotic neomycin sulfate, were found to exhibit increased anti-staphylococcal activity when combined with mupirocin (FIC 0.75). Of these, neomycin displayed the greatest potency, both alone (MIC 0.5 µg ml\(^{-1}\)) and in the presence of mupirocin (MIC 0.25 µg ml\(^{-1}\)), and is currently available as 0.25%-4% weight per volume ointment for topical antimicrobial use. Thus we chose to focus effort on characterizing the effects of combinations of mupirocin and neomycin, with the anticipation that they may have the greatest likelihood of having a clinical impact and ease of advancement.

While neomycin is known to bind 16S rRNA and inhibit bacterial protein translation, more recent studies indicate that it also has off-target effects that may contribute to its antimicrobial activity. In that regard while other translational inhibitors, including several aminoglycosides, exhibited antimicrobial activity toward the test strain they did not potentiate the activity of mupirocin. Thus, it seemed reasonable to predict that neomycin’s off-target effects contribute to its potentiation of mupirocin. Neomycin binding to the \(rnpB\) component of the RNase P holoenzyme interferes with the enzyme’s ability to catabolize precursor tRNA processing and consequently generation of mature tRNA substrates for tRNA synthetases, including the primary cellular target of mupirocin, isoleucyl-tRNA synthetase. Consequently, neomycin may limit \(S. aureus\) cellular RNase P activity resulting in a limited supply of mature tRNA\(^{lle}\) species, thereby requiring less mupirocin to generate an antimicrobial phenotype. As a first test of that
prediction, it was found that *S. aureus* RNase P activity is inhibited by neomycin (250 µM) during *in vitro* conditions that admittedly may be vastly different than are expected of the enzyme within bacterial cells (buffer conditions and co-factors, Fig. 3.1). Even so, neomycin’s RNase P inhibitory activity approximates the concentration required to potentiate mupirocin in liquid format (50 µM) and is well below its potentiating activity in topical format (16 mM), suggesting that the agent’s ability to improve mupirocin’s antimicrobial effects may be, in part, mediated by the cellular inhibition of RNase P. Further, neomycin did not increase the antimicrobial properties of other antibiotics such as vancomycin or erythromycin in combination, supporting the notion that the agent’s off-target effects may account for its ability to potentiate the antimicrobial activity of mupirocin and that these results are specific to mupirocin.

Combinations of 2% mupirocin and ≥ 1% neomycin proved to display improved antimicrobial activity in zone of inhibition assays designed to measure the combination’s performance in topical format, in comparison to either agent when tested alone. For that reason, all studies were conducted with 2% mupirocin and/or 1% neomycin. As noted earlier, combinations of 2% mupirocin and 1% of other antibiotics evaluated did not exhibit improved antimicrobial effects or caused an antagonistic effect, suggesting that the improved performance of the combination was specific to neomycin and mimicked their performance in liquid format.

Using a murine nasal colonization model that has admittedly proven highly variable in terms of establishing *S. aureus* colonization and measuring the performance of
antimicrobial agents, such as mupirocin, in the past we found that the combination of mupirocin + neomycin displayed greater efficacy than either agent alone. In initial studies designed to measure the model’s performance using *S. aureus* strain UAMS-1, it was found that optimal colonization was achieved using $1 \times 10^7$ CFU and when animals were allowed to breathe in-and-out the inoculum, whereas colonization occurred in ~70% of the animals challenged with less cells and/or were anesthetized at the time of inoculation. Moreover, testing of various dosing regiments showed that optimal mupirocin decolonization was observed following 3 nasal treatments per day (data not shown), and consequently served as the standard dosing for nasal dosing studies. Complete antimicrobial-associated decolonization was rarely observed and may reflect poor distribution of the test agents throughout the nasal passage. In the model, mupirocin treatment displayed efficacy to varying degrees for the three strains evaluated, with greatest decolonization observed for strains UAMS-1 and USA300 and less activity measured for the high level mupirocin resistant strain BAA-1708. Presumably, the dosing regimen used may partially over-ride the resistance phenotype of the strain and/or the mupirocin resistant determinant may only be partially expressed during nasal colonization. Similar effects were observed for neomycin (alone treatment) for all strains, including neomycin resistant USA300. In all cases, the combination of mupirocin and neomycin resulted in the greatest extent of nasal decolonization and this occurred regardless of the strain used, suggesting that the combination may have greater promise in decolonizing at-risk patient populations than mupirocin (alone) ointments.
Similarly, the combination exhibited pronounced improvement in a murine wound model of *S. aureus* decolonization, in comparison to either mupirocin or neomycin alone. In this model, twice a day mupirocin dosing consistently exhibited efficacy toward the mupirocin susceptible strains evaluated, thus, each topical formulation was tested twice daily (as opposed to 3x daily for nasal decolonization studies). Indeed, while twice a day 2% mupirocin treatment dramatically reduced *S. aureus* strain UAMS-1 and USA300 wound site colonization, the agent lacked efficacy toward the high-level mupirocin resistant strain tested, mimicking what occurs in the clinical setting. One percent neomycin (alone) exhibited excellent decolonization activity toward UAMS-1 and BAA1708 but no significant activity toward the neomycin resistant strain USA300. The combination nearly eradicated each *S. aureus* strain tested, with either no measurable viable colony forming units or a single colony detected in 100% of UAMS-1, 90% of USA300 and 60% of BAA-1708 inoculated wounds.

Interestingly, as noted above, we observed differing antimicrobial effects of neomycin (alone) and mupirocin (alone) toward strains USA300 and BAA-1708, respectively, in the two animal model systems. The application of neomycin three times a day exhibited mild antimicrobial activity toward the neomycin resistant strain, USA300, in the nasal decolonization model but no activity toward the strain in the wound model when applied twice daily. Similarly, three times a day mupirocin dosing was associated with reduction in BAA-1708 nasal colonization, but had no effect on wound decolonization. While there are likely to be vast differences between the bacterial physiology and host-pathogen dynamics in these two settings that may account for the observed differences in
antibiotic susceptibility these results could also suggest that more frequent antibiotic application may allow drug accumulation to an extent that overrides each strain’s resistance phenotype and may have corresponding clinical implications. Likewise, it is possible that extended time-course or more frequent dosing may further improve the combination’s effects. Wound contraction and overt cytotoxic measures indicate that each agent, when used alone or in combination, is well tolerated over the course of 14 days when applied either twice or three times (not shown) a day to wound sites.

Taken together the results presented indicate that the topical combinations of mupirocin and neomycin are likely to be superior to currently available mupirocin ointments in terms of promoting *S. aureus* nasal and wound site decolonization, and may be particularly valuable in areas where high-level mupirocin resistance has emerged. Such combination therapies may offer a much needed option for improving *S. aureus* infection prevention, limiting disease progression and, consequently, systemic antibiotic usage. Further, by virtue of the increased spectrum of activity toward problematic Gram-negative organisms, such as *A. baumannii* and *P. aeruginosa*, neomycin and mupirocin combinations may provide the option to develop similar strategies for reducing the incidence of these organisms as well as additional options for treatment of polymicrobial infections.

In considering the development of any clinical candidate, including a combination ointment, one must also take into account that resistant isolates can and will emerge (if they don’t already exist). In that regard, neither mupirocin or neomycin sulfate are
routinely used for systemic treatment purposes, thus corresponding resistance surveillance data is sparse. However, a comprehensive assessment of gentamycin resistant isolates collected between 1997 and 2002 in the U.S. revealed that all high-level mupirocin resistant isolates collected were susceptible to neomycin, indicating that they would be responsive to mupirocin and neomycin combination therapeutics [230]. The study also indicated that while neomycin resistance was observed frequently (31%) within *S. aureus* isolates collected less than 1% of those strains were capable of tolerating 1:100th the level of neomycin present in topical formulations and would thus ostensibly be treatable by mupirocin + neomycin ointments. Moreover, as noted above, neither agent is routinely used for systemic purposes or is associated with cross resistance to currently used systemic antibiotics. From these perspectives, it is anticipated that combination neomycin and mupirocin ointments may hold great promise in the prevention and treatment of currently circulating *S. aureus* strains, and that resistance to the multicomponent mixture will be slow to develop and unlikely to compromise the current anti-staphylococcal armament. We also recognize that there will be limitations in the use of such a combination ointment. Indeed, one widely referenced study reported that neomycin-related contact allergy developed in 34% of patients with chronic dermatoses, who were patch tested with 20% neomycin [231]. Thus, others have noted that neomycin containing topical preparations use should be avoided or closely monitored for multiallergic individuals but advocated for the use of neomycin in the majority of the general population, in whom the incidence of neomycin sensitivity is estimated to be 0.9% [232, 233].
Table 3.1 Primary Hits from Selleck Library

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**Antifungals**

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<td>Fluoropyrimidine</td>
<td>Antineoplastic</td>
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<td>Multichannel blocker</td>
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<td>Monoketo Acid</td>
<td>Integrase inhibitor</td>
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<td>Pyrimidine 2’-deoxyribonucleoside</td>
<td>Antineoplastic</td>
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<td>Aminobenzoic acid</td>
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<td>N-phenylbenzamide</td>
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<td>Salicylamide</td>
<td>Coccidiostat</td>
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<td>Quaternary ammonium</td>
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<td>Phenylmethylamine</td>
<td>Stem cell mobilizer</td>
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Table 3.1 Con’t

<table>
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<th>Drug</th>
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<td>Antineoplastic</td>
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<td>Strontium ranelate</td>
<td>Ranelic acid</td>
<td>Osteoporosis</td>
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<td>Stilbene</td>
<td>Estrogen receptor modulator</td>
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<td>Teniposide</td>
<td>Podophyllotoxin</td>
<td>Antineoplastic</td>
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<td>Triazolopyrimidine</td>
<td>Platelet aggregation inhibitor</td>
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<td>Benzophenone</td>
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<td>Stilbene</td>
<td>Estrogen receptor modulator</td>
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<td>Zafirlukast</td>
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Table 3.2. Selleck Library Members with Mupirocin-Associated Improved Activity

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<th>Drug</th>
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<th>(-) Mup</th>
<th>(+) Mup(^1)</th>
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<td>8</td>
<td>8</td>
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<tr>
<td>Nitrofurazone</td>
<td>16</td>
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<td>8</td>
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<td>Neomycin sulfate</td>
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<td>0.25</td>
<td>0.25</td>
<td>0.75</td>
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</table>

\(^1\) Performed in the presence of 0.5x Mupirocin MIC (0.0625 µg ml\(^{-1}\))
**Figure 3.1. Effects of neomycin on *S. aureus* RNase P mediated ptRNA\(^\text{Tyr}\) processing.** Shown are the mobility of precursor tRNA\(^\text{Tyr}\) in the absence and presence of *S. aureus* RNase P enzyme and the indicated concentration of neomycin. Densitometry measured percent activity shown (tRNA product formed) normalized to DMSO treated enzyme alone.
Figure 3.2. Antimicrobial zone of inhibition measures. Plotted are the average zones of inhibition (y-axis; cm$^2$) of PEG-based ointments containing the indicated antibiotic or antibiotic mixture (x-axis) toward S. aureus strain UAMS-1 (Panel A, D, and E), USA300 (Panel B) or BAA-1708 (Panel C). Significant increases in growth inhibition zones, in comparison to 2% mupirocin, are indicated (Student’s t-test (N=4); * $P \leq 0.1$; ** $P \leq 0.05$).
**Figure 3.3. Murine nasal decolonization measures.** Plotted are the numbers of colony forming units (CFU) per mouse nasal passage (y-axis) following 3 days dosing with PEG-based ointment containing the indicated antibiotic or antibiotic mixture (x-axis). Results for *S. aureus* strain UAMS-1 (Panel A), USA300 (Panel B), and BAA-1708 (Panel C) are shown; red data points indicate low-level mupirocin resistant isolates. Significant reductions in bacterial burden, in comparison vehicle are indicated (one-way ANOVA; *P* ≤ 0.05; **P** ≤ 0.01; ***P** ≤ 0.001; ****P** ≤ 0.0001).
Figure 3.4. Murine wound decolonization measures. Shown are the numbers of colony forming units (CFU) per lesion (y-axis) following 3 days dosing with PEG-based ointment containing the indicated antibiotic or antibiotic mixture (x-axis). Results for S. aureus strain UAMS-1 (Panel A), USA300 (Panel B), and BAA-1708 (Panel C) are shown. Significant reductions in bacterial burden between treatment groups are indicated (one-way ANOVA; *$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$).
Figure 3.5. Antimicrobial effects of PEG-based ointments toward *A. baumannii* and *P. aeruginosa*. Shown are the antimicrobial effects of PEG ointments containing vehicle, 2% mupirocin, 1% neomycin and the combination of 2% mupirocin + 1% neomycin toward *A. baumannii* strain 983709 (Top Panels) or *P. aeruginosa* strain PA01 (Bottom Panels).
Figure 3.6. Effects of PEG-based ointments on wound healing and animal health.

Representative wound healing images following 0, 3, 7, and 14 days of treatment with PEG-base ointments containing vehicle, 2% mupirocin, 1% neomycin or combination (Panel A). Panel B shows average measures (N=3) of corresponding wound contraction corresponding to Panel A conditions. Panel C average body weight of animals (y-axis) at the indicated day (x-axis) post lesion formation and treatment with PEG-based ointment supplemented with the indicated agent.
Chapter 4: Zinc pyrithione Improves the Antimicrobial Activity of Silver sulfadiazine

INTRODUCTION

Wound sites frequently serve as foci for bacterial colonization and invasive disease, particularly among post-surgical, burn and diabetic patient populations. Indeed, in the U.S. surgical site wound infections are reported to occur in approximately 11% of patients undergoing major elective surgery, resulting in a 5-fold increase in hospital remittance and a 2 to 3-fold increase in mortality [234]. Similarly, approximately 15% of patients seeking medical treatment for burns suffer an infectious complication with 4% of those succumbing to wound infection-related sepsis in the U.S. [70]. Diabetic wound infections worldwide are especially complex, leading to increased risk of limb amputation and 12.7% mortality rate [71, 72, 76]. The development of novel topical bacterial decolonization strategies has the potential to decrease the incidence of wound infection and corresponding rates of morbidity and mortality.

The ESKAPE pathogens *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Acinetobacter baumannii* are among the most common causative agents of acute and chronic wound infections worldwide [67, 235]. Retrospective studies consistently list these organisms as among the five most frequent causes of bacterial burn wound infections, collectively accounting for approximately 57% of worldwide burn wound isolates collected [236, 237]. Moreover, *P. aeruginosa* and/or *S. aureus* are also the predominant causes of surgical wound site infections among patients undergoing spine [238], coronary artery bypass grafting [239], abdominal [240], and orthopedic procedures
[241], whereas *A. baumannii* is a common cause of combat associated traumatic extremity wound infections and limb amputation due to infectious complications [242, 243].

The importance of *P. aeruginosa*, *S. aureus* and *A. baumannii* biofilms has been well documented in the setting of chronic diabetic ulcers and may also play a role in mediating infection in burn and post-surgical patients. In the biofilm growth state these organisms display recalcitrance to conventional antibiotics posing particular clinical challenges [76]. The relatively limited systemic antibiotic efficacy toward biofilm-associated bacteria accentuates the importance of effective topical antimicrobials during wound maintenance. In that regard, silver-based topical antimicrobials are widely used as an adjunctive to systemic antibiotics for the prevention and treatment of wound infections (reviewed in [78]).

Silver ions nonspecifically react with bacterial proteins and DNA, thereby leading to multiple mechanisms of antimicrobial action [15, 16]. Such promiscuity accounts for its broad spectrum antimicrobial toward both Gram-positive and -negative bacterial species and is thought to contribute to its antibiofilm activity. One of the most common preparations of medicinal silver, silver sulfadiazine, is the active component of numerous wound care associated products including ointments, creams and bandages as well as coatings on indwelling catheters representing a $237 million dollar industry in the United States [79-81]. Despite such wide-spread use, numerous clinical studies have called into question the impact of silver sulfadiazine treatment. Although silver sulfadiazine shows
improved antimicrobial activity compared to placebo, there does not appear to be a statistical advantage in the prevention of infection compared to alternative wound dressing agents [86-88]. Such reports combined with rising concerns over the emergence of silver resistance have prompted interest in developing improved topical therapeutic options [89].

Toward that goal we screened an FDA approved drug library for agents with bactericidal activity toward established P. aeruginosa biofilm-associated cells. The anti-fungal, zinc pyrithione (ZnPT), was active against P. aeruginosa but its antimicrobial potency was less than that of silver sulfadiazine (SSD). Conversely, ZnPT’s antimicrobial activity was superior to SSD toward S. aureus, A. baumannii and other six other bacterial pathogens tested during planktonic and biofilm growth, suggesting that ZnPT + SSD combinations would exhibit broader spectrum antimicrobial activity than either agent alone. Interestingly, the combination of ZnPT and SSD exhibited additive antimicrobial effects toward planktonic and biofilm-associated S. aureus, P. aeruginosa and A. baumannii. Likewise, in topical formulation the combination of ZnPT + SSD displayed significantly improved antimicrobial activity toward each bacterial species in a murine model of acute wound infection. Considering both ZnPT and SSD are already active antimicrobial components of numerous topical formulations, their combination represents a novel and potentially more effective treatment alternative for wound infections.
RESULTS

**Identification of zinc pyrithione as an antibiofilm agent.** A library of 853 FDA approved drugs was screened for agents that displayed antimicrobial activity toward 72-hr established *P. aeruginosa* biofilms using an adenylate kinase-based bactericidal assay [187]. Screening results revealed that a total of 34 library members exhibited \( \geq 2 \) fold increase in adenylate kinase (AK) signal in comparison to mock treated biofilms, indicating that they elicited a bactericidal response toward biofilm-associated *P. aeruginosa* cells (**Table 4.1**). Among these were 28 antibiotics belonging to eight distinct classes, four anti-tubercular agents and two non-antibiotics that have been previously developed for other therapeutic indications. Ten of the identified antibiotics, tobramycin, doripenem, aztreonam, ciprofloxacin, besifloxacin, ofloxacin, norfloxacin, lomefloxacin, moxifloxacin and levofloxacin, are known anti-*Pseudomonal* agents with current clinical use in the United States. Eight additional fluoroquinolones, two quinolones, three macrolides, tigecycline, spectinomycin, streptomycin, three tetracyclines, four anti-tubercular agents and two non-antibiotics, bleomycin sulfate and zinc pyrithione, were also identified, suggesting that they may be valuable *P. aeruginosa* antibiofilm agents.

As an initial means to validate our screening results, seven compounds that were readily available were re-tested for antimicrobial activity toward established *P. aeruginosa* biofilms (ciprofloxacin, spectinomycin, tobramycin, rifampicin, levofloxacin, tetracycline, and zinc pyrithione). To do so, biofilms were formed, challenged with increasing concentrations of each agent (0 to 256 \( \mu \text{g ml}^{-1} \)) and then plated to enumerate
the remaining number of viable biofilm-associated bacteria. Results revealed that all of the compounds tested did indeed result in $\geq 0.37$ $\log_{10}$ reduction in biofilm-associated cells at the initial screening concentration (50 $\mu$M; 16 to 32 $\mu$g ml$^{-1}$ depending on the agent tested) and exhibited a dose-dependent decrease in biofilm cell viability at the higher concentrations tested, confirming that our initial screen performed as expected (Table 4.2). Differences in antibiofilm potency were also observed, suggesting that subsets of antibiotics are likely to outperform others in the treatment of $P. aeruginosa$ biofilm-associated infections. Ciprofloxacin and levofloxacin exhibited the most potent antimicrobial activity toward biofilm-associated cells at virtually all concentrations tested, resulting in a 1.8 to 4.6-log and 1.1 to 6.8-log decrease in biofilm cells, respectively. Tobramycin and zinc pyrithione produced more modest 0.7 to 2.4-log and 0.3 to 2.2-log reduction in biofilm associated cells. The antibiotics spectinomycin, rifampicin, and tetracycline, produced only marginal decreases in biofilm cells, yielding maximum reductions of 0.75 to 1.5-log in biofilm associated cells at the highest concentrations tested (164 to 256 $\mu$g ml$^{-1}$).

Considering that one of our overarching goals was to identify novel therapeutics for the treatment of wound-associated bacterial infections we considered that zinc pyrithione (ZnPT) may be a viable topical candidate to explore further because: i. it is currently a successful topical treatment of seborrheic dermatitis with a favorable safety profile [244], ii. bacterial resistance to ZnPT is very slow to develop and unlikely to confer cross-resistance to systemic antibiotics [245], and iii. It has been previously shown to have
excellent antibiofilm activity toward A. baumannii, another common wound infection associated organism [186].

**Spectrum of activity of Zinc Pyrithione (ZnPT).** As an initial means to evaluate the potential of ZnPT based wound-infection intervention approaches, we measured the agent’s spectrum of antimicrobial activity against other bacterial species commonly associated with wound infections including Escherichia coli and each of the ESKAPE pathogens. As shown in Table 4.3, standard minimum inhibitory concentration (MIC) testing revealed that ZnPT demonstrated antimicrobial activity toward all species evaluated. The agent exhibited the most potent activity (MICs between 1 and 2 µg ml$^{-1}$) toward, *E. coli*, *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Enterococcus faecium* and *E. faecalis*, whereas it exhibited less antimicrobial activity toward both *Enterobacter cloacae* (MIC 4 µg ml$^{-1}$) and *P. aeruginosa* (16 µg ml$^{-1}$). Moreover, with the exception of *P. aeruginosa*, ZnPT exhibited between 2 and 16-fold increased antimicrobial activity in comparison to silver sulfadazine (SSD). Given that SSD exhibited improved activity against *P. aeruginosa*, whereas ZnPT displayed increased activity toward the other bacteria species tested, we considered that the combination of zinc pyrithione and silver sulfadiazine may demonstrate a broader spectrum antimicrobial than either compound in isolation.

**Zinc pyrithione demonstrates additive activity with silver sulfadiazine toward P. aeruginosa, S. aureus and A. baumannii.** As a first test of the antimicrobial effects of ZnPT and SSD in combination, fractional inhibitory concentration (FIC) testing was
performed in checkerboard format using planktonic *P. aeruginosa*, *S. aureus* and *A. baumannii* to evaluate whether the combination displayed equivalent, antagonistic, or improved effects toward three organisms frequently associated with wound infections. Results revealed additive antimicrobial effects toward *P. aeruginosa* (FIC = 0.55 ± 0.02), *S. aureus* (FIC = 0.52 ± 0.02), and *A. baumannii* (FIC = 0.66 ± 0.13) suggesting that the combination of ZnPT and SSD may be more effective than either agent alone. Next we evaluated whether the additive effects of ZnPT and SSD in combination extended toward biofilm associated *P. aeruginosa*, *A. baumannii* and/or *S. aureus*. To do so, biofilms were established for each organism then treated with increasing concentrations of each agent alone or in combination (0 to 128 µg ml⁻¹), and corresponding biofilm-associated viability was enumerated for each treatment condition.

As shown in **Fig 4.1A.**, *S. aureus* biofilms treated with the combination of ZnPT and SSD displayed a greater reduction in biofilm-associated cell numbers than biofilms treated with an equivalent amount of either agent alone at concentrations of 64 and 128 µg·ml⁻¹. More specifically, SSD (alone) exhibited consistent, yet modest 0.5 to 1 log reduction of biofilm-associated cells at virtually all concentrations tested (2 to 128 µg ml⁻¹, p< 0.05). ZnPT (alone) demonstrated dose response dependent increase in antibiofilm activity, which reached a maximum 3.7-log (±0.93, p< 0.0005) reduction in biofilm associated cells at 32 µg ml⁻¹ but no further decrease at higher concentrations tested. However, the combination of ZnPT and SSD demonstrated a consistent dose response-dependent increase in antimicrobial activity throughout all concentrations tested, resulting in a maximum of 5-log (±0.14, p< 0.0001) reduction of biofilm-associated *S.*
*aureus* at 128 µg ml\(^{-1}\) (64 µg ml\(^{-1}\) ZnPT and 64 µg ml\(^{-1}\) SSD). Although not statistically significant, the combination at 32 µg ml\(^{-1}\) (a mixture of 16 µg ml\(^{-1}\) SSD and 16 µg ml\(^{-1}\) ZnPT) exhibited the same or a greater reduction in biofilm cells and continued to exhibit this phenotype at all higher concentrations tested.

Similar results were observed for *A. baumannii* treated biofilms ([Fig. 4.1B](#)). SSD (alone) demonstrated a modest dose-dependent decrease in *A. baumannii* biofilm associated cells with a maximum of 3-log (±0.94, p < 0.005) reduction at 128 µg ml\(^{-1}\). ZnPT (alone) also demonstrated a dose-dependent increase in antimicrobial activity, which reached a maximum of approximately 5-log (±2.60, p < 0.005) reduction at 128 µg ml\(^{-1}\). The combination of ZnPT and SSD exhibited the most potent activity toward *A. baumannii* biofilms at 8 µg ml\(^{-1}\) and this improvement in antimicrobial activity continued in a dose-response manner, resulting in an 6-log (±1.95, p < 0.0001) reduction at the highest concentration tested (128 µg ml\(^{-1}\); 64 µg ml\(^{-1}\) ZnPT and 64 µg ml\(^{-1}\) SSD). Furthermore, the combination at 8 µg ml\(^{-1}\) exhibited a greater, though not significant, reduction in biofilm cells than either compound at 4 µg ml\(^{-1}\).

Measures of ZnPT and/or SSD treatment effects on *P. aeruginosa* biofilms revealed that the combination also improved upon the activity of either compound alone ([Fig. 4.1C](#)). Indeed, both SSD (alone) and ZnPT (alone) treatment demonstrated a modest dose-dependent reduction in biofilm-associated cells, reaching a maximum of a 4-log (±0.37 and 0.31, respectively with a p < 0.0001) decrease in biofilm-associated cells at 128 µg ml\(^{-1}\). The combination proved to have a dose-dependent additive effect beginning at 32
µg ml⁻¹ and ultimately resulting in a 5.7-log (±0.32, p< 0.0001) reduction at 128 µg ml⁻¹ (64 µg ml⁻¹ ZnPT and 64 µg ml⁻¹ SSD). Much like *S. aureus* and *A. baumannii*, there was a greater reduction in biofilm cells at a combination concentration of 16 µg ml⁻¹ compared to 8 µg ml⁻¹ of SSD or ZnPT, although this was not significant until the aforementioned reduction at 128 µg ml⁻¹.

Taken together the above results suggest that the combination of ZnPT and SSD demonstrated more potent activity toward *P. aeruginosa*, *S. aureus* and *A. baumannii* in both the planktonic and biofilm growth states, suggesting that topical application of ZnPT + SSD combination ointments may have promising therapeutic value.

**Ointment Zone of Inhibition measures.** To evaluate whether ZnPT and SSD were compatible in ointment format, both agents were formulated alone and in combination in standard polyethylene glycol-based (PEG) vehicle and assessed for activity in antimicrobial plate assays. To do so, *P. aeruginosa*, *S. aureus* or *A. baumannii* cells were spread on agar plates, PEG-based ointment containing either 1% DMSO (vehicle), 1% SSD, 0.25% ZPT, or the combination (1% SSD + 0.25% ZnPT) was applied to the center of the plate and the resulting zone of growth inhibition was measured following overnight incubation. It should be noted that 1% SSD was selected for these studies to reflect the concentration commonly provided in commercially available ointment preparations, whereas 0.25% ZnPT was selected for these studies because higher concentrations of the agent appeared insoluble in PEG-ointment (not shown).
Measures of each treatment’s zone of inhibition revealed that while vehicle alone did not affect the growth of any species tested, SSD and ZnPT, when applied both alone and in combination, produced similar antimicrobial properties, suggesting that the formulation did not antagonize, or only slightly diminished, the activity of either agent. More specifically, against *S. aureus*, 1% SSD generated a 3.49 (± 0.33) cm$^2$ zone of inhibition whereas 0.25% ZnPT generated an average zone of inhibition of 7.68 (± 0.19) cm$^2$. The combination of 1% SSD and 0.25% ZnPT displayed a 6.12 (± 0.35) cm$^2$ zone of inhibition indicating a slight reduction in the combination’s activity in comparison to ZnPT alone (Fig. 4.2A). Similarly, tests of *A. baumannii* revealed that 1% SSD and 0.25% ZnPT elicited a 3.12 (± 0.02) cm$^2$ and 4.51 cm$^2$ (± 0.30) zone of inhibition, respectively. The combination of 1% SSD and 0.25% ZnPT produced a similar zone of inhibition of 3.98 cm$^2$ (± 0.38) (Fig. 4.2B). Surprisingly, 1% silver sulfadiazine did not produce a measurable zone of inhibition toward *P. aeruginosa* strain PA01 cells (data not shown). As a consequence, the concentration was increased to 2% SSD to achieve a measurable zone of clearance of 3.16 (± 0.82) cm$^2$, which was similar to 0.25% ZnPT (3.30 cm$^2$ (± 0.55) and ZnPT and SSD in combination (3.48 cm$^2$ (± 0.48); Fig. 4.2C). Taken together, these data indicate that in combination ZnPT and SSD are compatible in PEG-based ointment formulations tested here. Yet, the effects of the combination did not appear additive (i.e. SSD + ZnPT did not produce an increased zone of growth inhibition in comparison to either agent alone). We considered that the lack of improved zone of inhibition measures could reflect limitations in the release characteristics of the two agents in ointment formulation or that the physicochemical properties of the co-existence of the two agents in ointment formulation somehow negate the additive properties
observed earlier in liquid format. To, in part, distinguish between these two possibilities the antimicrobial activity of ointment compilations was tested in a murine dermal wound model and compared.

**Silver sulfadiazine and zinc pyrithione combination-based ointment demonstrates effective antimicrobial activity in vivo.** A murine acute dermal wound model was used to evaluate the topical antimicrobial properties of PEG-based ointments containing either vehicle, SSD, ZPT, or the combination of SSD + ZPT. To do so, dorsal wounds were created and inoculated with either *P. aeruginosa*, *A. baumannii*, or *S. aureus*, and subsequently treated twice daily for 3 days, at which time bacterial burden was enumerated.

As shown in Fig. 4.3B, treatment of *A. baumannii* inoculated wounds with 1% SSD (alone) resulted in 3-log (p <0.0001) decrease in bacterial burden (2.2 x 10^4 CFU per lesion) in comparison to animals that were treated with the vehicle alone (2.1 x 10^5 CFU per lesion). ZnPT treatment resulted in 2.8 x 10^2 CFU per lesion with no bacteria recovered from 2 of the 10 (20%, p ≤0.002) of the animals within the treatment group. The combination of 1% SSD and 0.25% ZnPT displayed the greatest antimicrobial efficacy. Although there was no statistical significance between the combination and the SSD treated mice, no bacteria were recovered from 7 of the 10 animals (70%, p <0.0001) treated compared to the 5 of 10 animals in the SSD (alone) treated group. Tests of *S. aureus* inoculated wounds revealed that treatment with 1% SSD (alone) resulted in an approximately 2-log (p ≤0.05) decrease in bacterial burden 3.34 x 10^5 CFU per lesion, in
comparison to vehicle alone. Similarly, 0.25% ZnPT resulted in a 3-log (p ≤0.002) decrease in *S. aureus* colonization (3.9 x 10^4 CFU per lesion), with no recoverable colony forming units recovered from one animal in the treatment group (10%). The greatest antimicrobial efficacy was observed for the combination treated group, which displayed an approximately 5.3-log (p <0.0001) decrease in *S. aureus* burden (1.7 x 10^2 CFU per lesion) in comparison to vehicle treated wounds (3.3 x 10^7 CFU per lesion) and resulted in no recoverable colony forming units from 4 (40%) of the animals within the group (*Fig 4.3A*). Treatment of *P. aeruginosa* inoculated wounds revealed that while 0.25% ZnPT (alone) treatment did not result in a significant reduction in bacterial burden in comparison to vehicle (2.5 x 10^7 CFU vs 2.4 x 10^7 CFU), 2% SSD (alone) elicited an approximately 1.2-log decrease in *P. aeruginosa* colonization (1.4 x 10^6 CFU per lesion). The combination of 2% SSD + 0.25% ZnPT displayed the greatest efficacy, resulting in an approximately 2-log (p <0.0005) decrease in bacterial burden (2.3 x 10^5 CFU per lesion; *Fig 4.3C*). Considering that the combination of SSD and ZnPT demonstrated the most antimicrobial efficacy toward *S. aureus* and *P. aeruginosa*, mixtures of the two agents may provide an improved topical agent for the prevention and/or treatment of wound site infections. In addition to the efficacy of 1% SSD against *A. baumannii*, the combination of ZnPT and SSD, exhibited a greater number of mice with bacterial clearance (five vs. seven mice). Accordingly, next we sought to explore this possibility further by evaluating whether the combination-ointment negatively affects the normal wound healing process.
**Effects of silver sulfadiazine and zinc pyrithione on wound healing.** Both silver sulfadiazine and zinc pyrithione are FDA approved topical medications with established, favorable safety profiles. However, their combination has not been evaluated with respect to sterile wound healing. Thus, sterile dermal wounds were created and mice were treated with either vehicle, 2% SSD, 0.25% ZPT, or the combination twice daily for 14 days. Each day, animals were assessed for overt signs of wound toxicity including alertness and grooming weight and wound contraction. It should be noted that although 1% silver sulfadiazine is typically used in commercial preparations as much as 10% SSD can be used clinically [246]; 2% SSD was chosen for evaluation here as it was the concentration required for sufficient activity against *P. aeruginosa*.

Results revealed no significant difference in body weight for any of the treatment groups (N=3), in comparison to vehicle ointments (**Fig 4.4A**). All mice, regardless of treatment, demonstrated full wound contraction and healing by day 14. However, mice treated with ZnPT alone demonstrated a prolonged initial healing time with a maximum wound size found at day 7 in contrast to day 3 in all other treatment groups (**Fig 4.4B-C**). Additionally, ZnPT ointment resulted in fur matting adjacent to the wound (personal observation), which may have been due to licking. Interestingly, the combination ointment did not exhibit a prolonged healing time nor did it cause fur matting. These early toxicity results support the potential safety of the ZnPT and SSD combination in an ointment preparation.
DISCUSSION

The treatment of both acute and chronic wound infections are of central importance to hospitals and health care systems that are actively striving to meet current patient safety benchmarks. The nature and complexity of wound infections are highly dependent on the type of wound as well as underlying patient co-morbidities although common etiologic organisms identified in most wounds include *S. aureus, P. aeruginosa, A. baumannii, E. coli* and *Enterococcus sp.* [67, 235, 247]. While acute wound infections are often treated successfully with systemic antibiotics, frequently a topical drug is warranted, particularly in cases of burn-associated wounds, ischemic wound beds with limited vascular support, chronic colonization or infection by a multidrug resistant organism. Non antibiotic topical medications have the advantages of minimizing systemic side effects, ease of use, direct application to infected tissue, and activity toward otherwise antibiotic resistant bacteria.

Silver sulfadiazine-based products including ointments, creams and impregnated dressings have become a common prophylactic and adjunctive treatment strategy for many wounds, particularly burn-associated. Silver provides the advantage of broad-spectrum bactericidal activity with the ability to disrupt biofilms and has been shown to promote wound decolonization [78, 89, 248]. However, despite widespread use of silver based products and supporting *in vitro* and animal model data, there is continued debate regarding clinical infection outcomes in humans and concerns related to delayed wound healing [249, 250]. This has prompted efforts to improve the antimicrobial performance of silver sulfadiazine by the addition of cerium [251], 0.2% chlorhexidine [252] and
hyaluronic acid [253] with varied success. As such there remains a need for improved topical antimicrobial therapeutics.

In efforts to improve silver sulfadiazine as a topical antimicrobial, our work initially focused on utilizing an adenylate-kinase based reporter of bacterial cell death [186, 187] to identify FDA approved compounds that exhibit bactericidal activity toward \textit{P. aeruginosa} biofilms. As noted above, the presence of biofilms in chronic wounds has been well established and creates a bacterial physiological state that is tolerant to most antibiotics. Thus identifying compounds with bactericidal activity toward bacteria within established biofilms has particular relevance. Zinc pyrithione, a compound well known for its anti-fungal activity in the clinical setting of seborrheic dermatitis and dandruff was identified in our initial screen and subsequently confirmed to have \textit{in vitro} antimicrobial activity toward planktonic cultures of the ESKAPE pathogen group. Of note ZnPT demonstrated improved activity compared to SSD for \textit{E. coli}, \textit{S. aureus}, \textit{K. pneumonia}, \textit{A. baumannii}, \textit{E. faecium}, \textit{E. Faecalis}, and \textit{E. cloacae}. Interestingly, SSD was more effective against planktonic \textit{P. aeruginosa} cultures than ZnPT. This finding, though, provided the rationale for evaluating the combination of ZnPT with SSD \textit{in vitro} and \textit{in vivo}.

Our characterization of the antimicrobial effects of the combination of ZnPT and SSD focused on \textit{S. aureus}, \textit{P. aeruginosa}, and \textit{A. baumannii} based on their propensity to cause severe wound site infections. In that regard, the combination proved to have an additive effect toward all three organisms during planktonic growth and toward established \textit{S.}}
*Staphylococcus aureus* and *A. baumannii* biofilms suggesting that mixtures of ZnPT + SSD may represent a more effective treatment compared to either agent in isolation. Given that the combination suspended in an ointment preparation did not inhibit the activity of the ZnPT and SSD combination *in vitro*, it was then applied to a murine dermal acute wound infection model. In 70% of the mice infected with *A. baumannii*, the bacteria was completely eradicated from the wound site and in mice infected with *S. aureus* 40% of mice showed eradication of bacteria. Results were more modest in *P. aeruginosa* wounds with a 2-log reduction in CFU burden following treatment with a ZnPT and SSD combination ointment.

As both ZnPT and SSD are current FDA approved drugs with favorable safety profiles, significant adverse effects were not anticipated. The rate of wound contraction was not affected by SSD alone or ZnPT + SSD in combination although was mildly delayed in mice exposed to ZnPT alone. However by day 14 of exposure all wounds had closed and fully reepithelization suggesting there was no overt cytotoxicity for the combination treatment. Furthermore, there was no overt cytotoxicity observed at 2% SSD and 0.25% ZPT, alone or in combination.

Taken together our results support the combination of ZnPT and SSD as an improved alternative compared to either compound in isolation towards common wound-associated organisms. Furthermore, given that both compounds are already FDA approved topical therapeutics, their combination is particularly attractive for patient safety and expedited drug development. Silver-based products are currently a major component of topical
wound therapy with expansion into impregnated dressings and coatings on medical devices. Given the existing limitations of silver-based therapies, the additive antibacterial effects of SSD and ZnPT may allow for more effective treatment and improved patient outcomes.
<table>
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<tr>
<th>Class</th>
<th>Drug</th>
<th>Average Fold Change in AK Signal$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aminocyclitol</td>
<td><em>Spectinomycin hydrochloride</em></td>
<td>6.6 (±3.5)</td>
</tr>
<tr>
<td>Aminoglycoside</td>
<td><em>Streptomycin sulfate</em></td>
<td>44 (±0.7)</td>
</tr>
<tr>
<td></td>
<td><em>Tobramycin</em></td>
<td>50 (±10.4)</td>
</tr>
<tr>
<td>Beta-Lactam</td>
<td><em>Doripenem Hydrate</em></td>
<td>56 (±1.4)</td>
</tr>
<tr>
<td></td>
<td><em>(Carbapenem)</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Aztreonam (Azactam, Cayston)</em></td>
<td>12 (±9.9)</td>
</tr>
<tr>
<td></td>
<td><em>(Monobactam)</em></td>
<td></td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td><em>Ciprofloxacin (Cipro)</em></td>
<td>29 (±0.7)</td>
</tr>
<tr>
<td></td>
<td><em>Clinafloxacin (PD127391)</em></td>
<td>36 (±24)</td>
</tr>
<tr>
<td></td>
<td><em>Balofloxacin</em></td>
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</tr>
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<td></td>
<td><em>Besifloxacin HCl</em></td>
<td>34 (±12)</td>
</tr>
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<td></td>
<td><em>(Besivance)</em></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>Danofloxacin Mesylate</em></td>
<td>46 (±15)</td>
</tr>
<tr>
<td></td>
<td><em>Enrofloxacin</em></td>
<td>41 (±17)</td>
</tr>
<tr>
<td></td>
<td><em>Levofoxacin (Levaquin)</em></td>
<td>38 (±2.8)</td>
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<tr>
<td></td>
<td><em>Lomefloxacin</em></td>
<td>59 (±23)</td>
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<td></td>
<td><em>Moxifloxacin hydrochloride</em></td>
<td>43 (±5.7)</td>
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<tr>
<td></td>
<td><em>Nadifloxacin</em></td>
<td>35 (±20)</td>
</tr>
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<td></td>
<td><em>Norfloxacin (Norxacin)</em></td>
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<tr>
<td></td>
<td><em>Ofloxacin (Flaxin)</em></td>
<td>39 (±9.8)</td>
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<td><em>Sitafloxacin hydrate</em></td>
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<td>Quinolone</td>
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<td><em>Clarithromycin (Biaxin, Klacid)</em></td>
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<td><em>Erythromycin (E-Mycin)</em></td>
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<td></td>
<td>Tetracycline HCl</td>
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</tr>
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<td>Anti-tubercular</td>
<td>Rifabutin (Mycobutin)</td>
<td>33 (±5.4)</td>
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<td></td>
<td>Rifampin (Rifadin, Rimactane)</td>
<td>16 (±9.8)</td>
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<td></td>
<td>Rifapentine (Priftin)</td>
<td>4.5 (±1.6)</td>
</tr>
<tr>
<td></td>
<td>Rifaximin (Xifaxan)</td>
<td>20 (±20)</td>
</tr>
<tr>
<td>Non-antibiotics</td>
<td>Bleomycin sulfate (Anti-cancer)</td>
<td>3.1 (±0.5)</td>
</tr>
<tr>
<td></td>
<td>Pyrithione zinc (Anti-fungal)</td>
<td>24 (±11)</td>
</tr>
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<sup>a</sup>- Average fold change in adenylate kinase signal compared to background with the standard deviation in parenthesis.
Table 4.2 *P. aeruginosa* strain PA01 biofilm antimicrobial treatment

<table>
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<tr>
<th>Biofilm-Associated Cells (log_{10})</th>
<th>0 µg ml^{-1}</th>
<th>4 µg ml^{-1}</th>
<th>8 µg ml^{-1}</th>
<th>16 µg ml^{-1}</th>
<th>32 µg ml^{-1}</th>
<th>64 µg ml^{-1}</th>
<th>128 µg ml^{-1}</th>
<th>256 µg ml^{-1}</th>
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<td>Ciprofloxacin</td>
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<td>6.81 (± 0.2)</td>
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<td>6.19 (± 0.9)</td>
<td>5.05 (± 1.2)</td>
<td>4.13 (± 0.2)</td>
<td>4.27 (± 1.9)</td>
<td>4.03 (± 0.2)</td>
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<td>Levofoxacin</td>
<td>9.26 (± 0.1)</td>
<td>8.14 (± 0.2)</td>
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<td>5.91 (± 0.9)</td>
<td>5.74 (± 0.5)</td>
<td>3.57 (± 0.2)</td>
<td>5.12 (± 1.9)</td>
<td>2.41 (± 0.6)</td>
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<td>Tobramycin</td>
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<td>7.77 (± 0.1)</td>
<td>7.46 (± 0.02)</td>
<td>6.85 (± 0.8)</td>
<td>5.86 (± 0.3)</td>
<td>6.18 (± 0.3)</td>
<td>6.28 (± 0.8)</td>
<td>6.09 (± 1.5)</td>
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<td>Zinc Pyrithione</td>
<td>8.78 (± 0.1)</td>
<td>8.46 (± 0.02)</td>
<td>7.70 (± 0.1)</td>
<td>7.32 (± 0.6)</td>
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<td>7.78 (± 0.2)</td>
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<td>Bacterial Species</td>
<td>Zinc Pyrithione (µg/ml)</td>
<td>Silver Sulfadiazine (µg/ml)</td>
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<td></td>
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<tr>
<td>983709</td>
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<td><em>Pseudomonas aeruginosa</em></td>
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<tr>
<td><em>Enterococcus faecium</em></td>
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Figure 4.1: Antibiofilm effects of Zinc pyrithione, silver sulfadiazine, and the combination against *S. aureus*, *A. baumannii*, and *P. aeruginosa*. The log difference in bacterial viability from established biofilms treated with two-fold increasing concentrations of silver sulfadiazine (gray bars), zinc pyrithione (black bars), or the combination (white bars) for *S. aureus* (A), *A. baumannii* (B), and *P. aeruginosa* (C). Significant difference in biofilm growth, in comparison to mock treated biofilms are indicated (Student’s t-test (N=2); *P < 0.05, **P <0.005, ***P <0.0005, ****P <0.00001
Figure 4.2: Antimicrobial zone of inhibition measures. Plotted are the average zones of inhibition (y-axis; cm^2) of PEG-based ointments containing the indicated antibiotic or antibiotic mixtures (x-axis) toward *S. aureus* (A), *A. baumannii* (B), and *P. aeruginosa* (C).
Figure 4.3: Murine wound decolonization measures. Shown are the recovered number of colony forming units (CFU) per lesion (y-axis) following 3 days treatment with PEG-based ointment containing the indicated antibiotic or mixture (x-axis). Results shown are for *S. aureus* (A), *A. baumannii* (B), and *P. aeruginosa* (C). Significant reductions in bacterial burden between treatment groups are indicated (one-way ANOVA (N=10); * P ≤ 0.05, **P ≤ 0.002, ***P ≤ 0.0005, ****P < 0.0001
Figure 4.4: Effects of PEG-based ointments on wound healing and animal health.

Panel A is the average (N=3) body weight of animals (y-axis) at the indicated day (x-axis) of treatment with the PEG-based ointments containing the indicated antibiotic. Panel B shows representatives wound healing images following days 0, 3, 7, 14 of treatment with PEG-based ointments containing 2% silver sulfadiazine, 0.25% zinc pyrithione, and the combination. Panel C shows average measures (N=3) of wound contraction corresponding to the images in panel B.
Chapter 5: Efflux pump inhibitors of Serum-specific *Acinetobacter baumannii* efflux pumps

INTRODUCTION

*Acinetobacter baumannii* has emerged as a major nosocomial pathogen that can cause ventilator-associated pneumonia (VAP) and bacteremia, with associated mortality rates as high as 80% among susceptible patient populations [94, 254-258]. The high rates of *A. baumannii* associated morbidity and mortality have been largely attributed to the emergence of antibiotic resistance that has compromised the effectiveness of currently available antibiotics. Indeed, the Centers for Disease Control and Prevention recently reported that 63% of all U.S. *A. baumannii* infections are caused by multi-drug resistant strains that are resistant to three or more classes of antibiotics and strains that are resistant to all classes of antibiotics have recently been identified in the U.S. and elsewhere [259-262].

Therapeutic intervention of *A. baumannii* infections has been compromised by an alarming increase in the organism’s resistance to front-line therapies. Indeed, multidrug resistance in *Acinetobacter* spp. increased from 6.7% in 1993 to 29.9% in 2004, more than twice that observed in any other Gram-negative bacillus causing nosocomial intensive care infections [263]. Moreover, strains that are resistant to all currently available antibiotics have been isolated from patients both in the U.S. and abroad [259, 264]. Numerous mechanisms are thought to contribute to the organism’s propensity to circumvent antibacterial agents. *A. baumannii* exhibits an extraordinary ability to acquire antibiotic resistance determinants, which include enzymatic functions such as β-
lactamases and aminoglycoside-modifying factors [99]. Additionally, the organism harbors a repertoire of efflux pumps that have also been hypothesized to contribute to clinical antibiotic failure [97-99]. The repertoire includes representatives of each of the five so-called bacterial drug efflux pump families: CraA and AmvA are major facilitator superfamily (MFS) pumps that are proposed to efflux chloramphenicol and erythromycin, respectively [100, 101]; AbeM is a multidrug and toxic compound extrusion (MATE) family protein that effluxes aminoglycosides, quinolones, and chloramphenicol [102]; AbeS is a small multidrug resistance (SMR) family pump that confers resistance to erythromycin and novobiocin as well as low level tolerance to aminoglycosides, quinolones, tetracycline and trimethoprim [103]; AdeABC, AdeFGH, and AdeIJK are resistance nodulation division (RND) family pumps that have been associated with resistance to aminoglycosides, β-lactams, fluoroquinolones, tetracyclines, tigecycline, macrolides, chloramphenicol, and trimethoprim [104-108]. Furthermore, *A. baumannii* is also known to harbor several horizontally acquired Tet efflux pumps belonging to the MFS that confer tetracycline resistance [109, 110]. While progress has been made in characterizing the organism’s antibiotic resistance determinants, little is known about their expression patterns or the mechanism(s) by which they are acquired or controlled.

We initially set out to comprehensively assess the expression properties of exponential- and stationary-phase *A. baumannii*, with the expectation that doing so may provide an important step toward identifying *A. baumannii* virulence factors that are regulated in a cell density-dependent manner and simultaneously provide researchers with a reference database of the organism’s expression properties during laboratory culture conditions.
Accordingly, custom-made Affymetrix GeneChips® were used to compare the expression properties of two genetically diverse *A. baumannii* strains, ATCC 17978 and 983709 during exponential and stationary phase of growth in laboratory culture medium. Results revealed that, in addition to expected growth phase-associated metabolic changes, biological systems ostensibly associated with biofilm formation and tolerance to desiccation were upregulated during stationary phase and may constitute *A. baumannii* virulence factors. Further, using these data as a baseline, microarray studies were expanded to define the expression profile of *A. baumannii* grown in human serum. A comparison of the transcriptomes of cells cultured in laboratory media versus serum revealed that many biological processes are commonly employed during growth in both substrates. However, growth in serum also dramatically upregulated *A. baumannii* iron acquisition systems, genes associated with epithelial cell adherence and DNA acquisition, as well as numerous putative drug-efflux pumps. As a preliminary validation of those observations, reverse-transcriptase polymerase chain reaction (RT-PCR) verified the expression levels of genes associated with the aforementioned cellular processes, and antibiotic susceptibility testing confirmed that the organism exhibits increased antibiotic tolerance when cultured in human serum, as compared to laboratory medium. Taken together, results of these studies provide a reference for *A. baumannii*’s expression properties in laboratory medium and serum, as well as identify biological processes that may contribute to the organism’s ability to tolerate desiccation, form biofilms on abiotic surfaces, and resist antimicrobial agents. The study was continued to further our understanding of the adaptive antibiotic efflux potential of *A. baumannii* during growth in human serum and to identify small molecule inhibitors of these efflux properties. Results
revealed that in addition to minocycline, serum-induced efflux pumps are associated with *A. baumannii*’s ability to tolerate ciprofloxacin, meropenem, tetracycline, and tigecycline. Further, using a high-throughput screening strategy and secondary assays, we identified two structurally distinct classes of novel efflux pump inhibitors that restore the antibiotic susceptibility of serum-grown *A. baumannii* and lack the inherent problems commonly associated with other classes of antibiotic efflux pump inhibitors, namely, mammalian cytotoxicity and calcium channel inhibition. These compounds may represent promising structural scaffolds for the development of new classes of bacterial antibiotic efflux pump inhibitors that can be used as adjunctive therapy to potentiate the activity of current and future antibiotics for the therapeutic intervention of *A. baumannii* infections.

**RESULTS**

**Serum-induced efflux pumps extrude multiple antibiotics**

We characterized the transcriptional response of the serum-resistant *A. baumannii* strain 983709 during growth in human serum. To do so, 983709 was cultured to exponential or stationary phase in human serum, RNA was extracted, and microarrays were used to compare the expression profiles of cells grown in serum to those of cells grown in LB medium, allowing for the identification of genes that most likely contribute specifically to growth in serum, as opposed to growth in general. A total of 547 genes exhibited higher transcript levels (≥ two fold; *t*-test; *p* ≤ 0.05) during exponential phase of growth in serum, in comparison to exponential growth in LB medium. Further, 85 transcripts were predominantly expressed within stationary phase 983709 cells grown in serum, in comparison to stationary phase growth in LB. A more thorough assessment of these
genes revealed several potentially important aspects about *A. baumannii* physiology. Results indicated that during growth in human serum the organism upregulates biological systems that allow it to tolerate stress, acquire iron and resist antibiotic challenge. Multi-drug efflux pumps are broad Specificity exporters involved in bacterial antibiotic resistance. A total of 22 ORFs associated with efflux pumps or drug transport were upregulated greater than two-fold during exponential phase in human serum (Table 5.1). Due to the relatedness of the ORFs, it is likely these 22 genes represent up to eight efflux systems. The observed dramatic upregulation of efflux pumps and drug transporters prompted us to ask if the *A. baumannii* cells would then be naturally primed to become more resistant to antibiotics when grown in serum.

To test this, minocycline susceptible strain 983709 was cultured in Mueller-Hinton, LB or 100% human serum in the presence of increasing concentrations of minocycline (0.25 – 2 µg mL\(^{-1}\)). As shown in Fig. 5.1 in comparison to growth in Mueller-Hinton (or LB) 983709 cells cultured in serum were significantly less susceptible (*p* < 0.002) to minocycline at concentrations ≥ 0.5 µg ml\(^{-1}\). Moreover, this observed serum-specific antibiotic tolerance phenotype was also seen in other *A. baumannii* strains tested (Fig. 5.2). Further, growth in the presence of the efflux pump inhibitor, PAβN, reduced the serum-dependent increase in minocycline tolerance and restored the organism’s susceptibility to minocycline (Fig. 5.2). Collectively, these data suggest that during growth in serum *A. baumannii* upregulates an array of drug efflux pumps that allow otherwise antibiotic susceptible strains to tolerate antibiotic challenge and could, consequently, contribute to the clinical failure of antibiotics.
As a means to evaluate this phenomenon further, we assessed whether *A. baumannii* growth in serum elicits drug efflux-mediated tolerance to other tetracyclines, quinolones (ciprofloxacin, levofloxacin, and nalidixic acid), aminoglycosides (amikacin, gentamicin, and kanamycin), a carbapenem (meropenem), a cephalosporin (ceftriaxone), a macrolide (erythromycin), polypeptides (colistin and polymxin B), a sulfonamide (sulfamethoxazole), glycylcycline (tigecycline), and trimethoprim (data not shown). To do so, *A. baumannii* strain 983709 was cultured in LB or 100% human serum in the presence of increasing concentrations of each antibiotic and the antimicrobial effects of each antibiotic were measured by plating for colony forming units.

As expected based on our previous results, *A. baumannii* 983709 cells grown in human serum were significantly (*P < 0.01*) less susceptible to minocycline at concentrations ≥ 0.25 µg ml⁻¹ in comparison to cells cultured in LB (Fig. 5.1A). Further, minocycline susceptibility could be restored to cells grown serum supplemented with the known efflux, PAβN, suggesting that the observed serum-associated minocycline tolerance was efflux-mediated as opposed to antibiotic inactivation by serum components. Similar susceptibility assays using other antibiotics revealed that this serum-associated adaptive resistance phenomenon is not minocycline specific. Indeed, serum grown 983709 cells were less susceptible to the antibiotic ciprofloxacin at concentrations ≤ 1 µg ml⁻¹, in comparison to LB grown cells; ciprofloxacin susceptibility could be restored to cells grown in serum supplemented with the known efflux pump inhibitor, reserpine (Fig. 5.1B). Likewise, *A. baumannii* grown in human serum displayed efflux-mediated
tolerance to tetracycline at antibiotic concentrations $\geq 2 \ \mu g \ \text{ml}^{-1}$ (Fig. 5.1C). More specifically, treatment of LB-grown *A. baumannii* with $4 - 16 \ \mu g \ \text{ml}^{-1}$ tetracycline reduced cell viability to undetectable levels ($< 1 \times 10^1$ colony forming units; CFU), whereas cells grown in serum displayed considerable antibiotic tolerance, equaling between $1 \times 10^6$ and $1 \times 10^4$ CFU. Serum-grown *A. baumannii* tetracycline susceptibility was partially restored in the presence of the drug efflux pump inhibitor PAβN, suggesting that efflux pumps, in part, modulate the organism's tetracycline tolerance during serum growth. Similar phenotypes were also observed for representatives of three of eleven previously characterized *A. baumannii* lineages evaluated [183], indicating that serum-associated efflux pump mediated minocycline and ciprofloxacin tolerance is a semi-conserved *A. baumannii* response that is presumably dependent on the genetic composition of the organism evaluated (data not shown).

While we did not observe significant differences between the susceptibility of serum- and LB-grown 983709 cells to other classes of antibiotics tested, during the course of our investigations it was observed strains representing seven of the eleven other lineages tested displayed albeit varying, but significantly increased efflux-mediated tolerance to the antibiotic tigecycline during serum growth (representative results shown in Fig. 5.1D). As an example, tigecycline displayed clear antimicrobial activity toward *A. baumannii* strain 01-12-05 during growth in LB medium at concentrations $\geq 1 \ \mu g \ \text{ml}^{-1}$, but the strain appeared to be highly-resistant to the antibiotic during growth in serum. Susceptibility could be partially restored by addition of the efflux pump inhibitor,
reserpine, suggesting that serum-associated efflux pump activity(ies) contributes to the strain’s ability to tolerate tigecycline during growth in serum.

The observed serum-dependent efflux pump mediated antibiotic tolerance may, in part, account for the clinical failure of antibiotics toward clinically defined susceptible *A. baumannii* strains; during adaptation to host-associated environmental conditions, such as serum, the organism may induce efflux pumps that allow clinically defined antibiotic susceptible organisms to tolerate antibiotic challenge *in vivo*. Accordingly, adjunctive therapy with corresponding efflux-pump inhibitors may provide a valuable strategy to limit antibiotic tolerance within the host and, consequently, pose as an attractive therapeutic approach for both current and future antibiotics.

**High-throughput screening for agents that potentiate the antimicrobial activity of minocycline toward serum-grown *A. baumannii*.** As a means of identifying new chemical classes of *A. baumannii* antibiotic efflux inhibitors we exploited the finding that during growth in human serum *A. baumannii* expresses efflux pumps that mediate cellular tolerance to minocycline ([191]; **Fig. 5.1A**). Accordingly, the 29,900 member TimTec ActivProbe small molecule and Natural product libraries were screened for agents that eliminated *A. baumannii*’s tolerance to minocycline during serum growth. To do so, approximately $1 \times 10^5$ *A. baumannii* 983709 cells were inoculated into individual wells of a microtiter plate containing 100% human serum supplemented with 0.5 µg ml$^{-1}$ minocycline (0.5X serum minimum inhibitory concentration; MIC). A total of 50 µM of each library member were added, plates were incubated for 48 hr at 37°C, and growth was measured as a function of turbidity. Most compounds (99.6%; 29,806 compounds)
did not affect the organism’s growth, whereas 94 compounds (0.4%) inhibited the strain’s ability to grow in serum supplemented with minocycline. Repeat testing in which well constituents were serial diluted and plated on LB agar plates verified that 85 compounds did indeed limit *A. baumannii* growth in serum supplemented with minocycline, resulting in a 2-6 log reduction in the number of viable colony forming units in comparison to minocycline alone treated cells.

To distinguish whether compounds of interest potentiate the activity of minocycline, as opposed to displaying antibacterial properties on their own, each compound was subsequently evaluated for antimicrobial activity toward *A. baumannii* in serum or LB in the absence of minocycline. Twelve of the 85 compounds tested (12.7%) exhibited antimicrobial activity toward *A. baumannii* grown in serum and/or LB in the absence of minocycline and may represent novel antimicrobial agents (data not shown). The remaining 73 compounds did not display antimicrobial activity in the absence of minocycline, suggesting that a subset of these compounds may represent efflux pump inhibitors that potentiate the antimicrobial activity of minocycline toward serum-grown *A. baumannii*. Accordingly, their minimum effective concentration (MEC) was defined as a means to both rank-order compounds based on their potency and also as a prerequisite for more extensive characterization, as described below. To define each compound’s MEC, *A. baumannii* strain 983709 was inoculated into individual wells of a microtiter plate containing 100% human serum supplemented with 0.5 µg ml⁻¹ minocycline and increasing concentrations (0 to 125 µg ml⁻¹) of test compound then incubated for 48 hours. The lowest amount of compound required to potentiate the
antimicrobial activity of minocycline, as defined by growth inhibition, was considered the MEC. Plating confirmed that addition of 1X MEC of each compound elicited \( \geq 1.9 \)-log reduction in \( A. baumannii \) cells grown in serum supplemented with minocycline alone (Table 5.2).

**A. baumannii Minocycline Accumulation.** As an initial means of determining whether compounds of interest displayed characteristics expected of an \( A. baumannii \) efflux pump inhibitor, and simultaneously prioritize compounds for further characterization, we considered that efflux pump inhibition would lead to intracellular antibiotic accumulation in comparison to cells in which efflux was active. Accordingly, triple quadrupole mass spectrometry was used to measure the cellular antibiotic concentration of \( A. baumannii \) cells grown in human serum supplemented with 0.5 \( \mu g \) ml\(^{-1} \) minocycline in the absence and presence of 0.5X MEC test compound. These concentrations were used, and plating of each culture validated, that the conditions did not affect \( A. baumannii \) viability (data not shown).

During growth in serum (efflux active conditions), the cellular minocycline concentration was determined to be \( 1.58 \times 10^{-10} \) femtomoles per bacterial cell, whereas addition of the known efflux pump inhibitor, verapamil, increased the cellular concentration nearly 1,000-fold (\( 3.56 \times 10^{-7} \) femtomoles cell\(^{-1} \)), indicating that the approach is appropriate to measure efflux-pump dependent cellular antibiotic accumulation. As shown in Fig. 5.3, while virtually all of the compounds evaluated appeared to induce minocycline accumulation in comparison to mock treated cells, 41 compounds stimulated minocycline
accumulation within serum-grown *A. baumannii* cells to levels equaling or exceeding that of the known antibiotic efflux pump inhibitor, verapamil, and were considered to be highest priority agents that presumably include efflux inhibitors as well as compounds that lead to antibiotic accumulation via unappreciated means. Regardless of mechanism, these 41 compounds were considered putatively clinically valuable agents that potentiate the antimicrobial activity and cellular accumulation of minocycline toward *A. baumannii* in serum and were carried forward for further characterization.

**Human cytotoxicity measures.** Given that our overarching goal was to identify therapeutically relevant novel compounds for future medicinal chemistry based improvement and refinement, we considered that the most desirable compounds would display little or no human cytotoxicity. Indeed, while PAβN has proven a valuable bacterial drug efflux inhibitor tool-compound in the laboratory setting, the compound displays toxicity at concentrations required for antimicrobial efficacy in the host and, consequently, has limited therapeutic promise [265]. Thus, to distinguish putatively non-toxic from cytotoxic compounds conventional 3-(4,5-dimethythiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assays were performed for each compound of interest at 1X and 4X their MEC. As shown in Table 5.2., 19 (46.3%) of the compounds tested elicited significant toxicity toward HepG2 cells, which was defined has < 75% cellular survival during 48 hr treatment at 4X MEC, and were deprioritized. Conversely 22 (53.6%) compounds displayed ≥ 75% survival (75.1 to 100%) and were considered to either exhibit no- or low-level human HepG2 cytotoxicity, the latter of which may be reduced by future medicinal chemistry campaigns. It should be noted that
75% human cell survival was used as a culling criterion because it approximates the toxicity measures of the antibiotic, minocycline, when tested alone in these assays conditions (77.9% HepG2 survival at 2 µg ml⁻¹). Thus, we expect that molecules displaying human cytotoxicity measures correlating with minocycline in these assays conditions represent the most promising scaffolds for future medicinal chemistry based optimization.

**Spectrum of activity.** As a means to further prioritize non-toxic compounds of interest based on their therapeutic promise we considered that broad-spectrum antimicrobial efflux pump inhibitors may be more clinically valuable than narrow-spectrum agents that only potentiate the activity of a limited number of antibiotics or that display activity toward a single bacterial species. As described above, in addition to minocycline, *A. baumannii* growth in human serum also facilitates the efflux- and the organism’s tolerance- of ciprofloxacin ([Fig. 5.1B.](#)). Consequently, we evaluated whether each compound potentiated the activity of ciprofloxacin toward serum-grown cells. To do so, 1 X 10⁵ CFU *A. baumannii* strain 983709 were inoculated into individual wells of microtiter plates containing 100% human serum supplemented with 0.125 µg ml⁻¹ ciprofloxacin and 0, 1X, or 2X the compound’s MEC, as defined by the lowest concentration needed to potentiate minocycline’s antimicrobial effects (above). Plates were incubated for 48 hours at which point each compound’s ability to potentiate ciprofloxacin was measured, as growth inhibition. Nineteen of the compounds evaluated did not affect the organism’s tolerance to ciprofloxacin during these conditions, suggesting that they are narrow spectrum agents that limit minocycline efflux, but not
ciprofloxacin efflux, and were deprioritized. Conversely, 3 compounds potentiated ciprofloxacin’s antimicrobial activity, suggesting that they may represent broad-spectrum antibiotic drug efflux pump inhibitors. Plating confirmed that, when administered in combination with ciprofloxacin, each compound reduced \textit{A. baumannii} viability at least 1.5 log at 1X the compound’s MEC, in comparison to cells treated with ciprofloxacin alone (Fig. 5.4A; Table 5.2).

During the course of our studies we also investigated whether the Gram-negative pathogens \textit{Klebsiella pneumoniae} and \textit{Pseudomonas aeruginosa} exhibit antibiotic tolerance to minocycline and/or ciprofloxacin during growth in human serum. While \textit{K. pneumoniae} strain CKP4 did not, it was found that serum-grown \textit{P. aeruginosa} PA01 cells exhibit efflux-mediated tolerance to ciprofloxacin, as described below. Thus, as an additional means to evaluate the spectrum of activity, and simultaneously identify highest priority compounds of interest that potentiate the activity of antibiotics across bacterial species, we evaluated whether the aforementioned three putative broad-spectrum \textit{A. baumannii} efflux pump inhibitors also inhibited \textit{P. aeruginosa} serum-dependent ciprofloxacin tolerance. To do so, PA01 was inoculated into individual wells of a microtiter plate containing 100% human serum supplemented with 1X MEC of test compound and increasing concentrations of ciprofloxacin (0 to 2 µg ml\(^{-1}\)) and cell viability was measured. Results revealed that two of the three putative broad-spectrum efflux pump inhibitors also potentiated the activity of ciprofloxacin toward serum-grown \textit{P. aeruginosa} (Fig. 5.4B), suggesting that these compounds, ABEPI1 and ABEPI1 (Fig. 5.4C), represent broad-spectrum agents that may potentiate the antimicrobial properties
of antibiotics toward at least two bacterial species of immediate healthcare concern, *A. baumannii* and *P. aeruginosa*. PAβN alone (absence of ciprofloxacin) did not display antimicrobial properties toward serum-grown PA01 cells in these assay conditions (data not shown).

**ABEPI1 and ABEPI2 inhibit *A. baumannii* efflux properties.** To distinguish whether the antimicrobial potentiation of ABEPI1 and ABEPI2 correlates to the inhibition of *A. baumannii*’s efflux properties, conventional ethidium bromide efflux assays were performed in the presence and absence of each compound, as previously described [97, 105, 115, 194, 195]. The assay is predicated upon the fluorescent properties of ethidium bromide during intercalation into cellular nucleic acids, whereby efflux active cells display limited intracellular ethidium bromide accumulation and, consequently, low fluorescence. Conversely, efflux inhibition leads to increased cellular ethidium bromide levels and correspondingly high fluorescence relative to efflux proficient cells.

As shown in Fig. 5.5, mock treated cells displayed low-level ethidium bromide fluorescent signal that slowly increased during the course of the experiment, presumably reflecting the slow dye accumulation over time despite efflux pump activity. Conversely, efflux deficient PAβN treated cells exhibited significantly increased cellular ethidium bromide accumulation in comparison to mock treated cells, confirming that the assay conditions were appropriate to measure the efflux properties of *A. baumannii* cells. Likewise, both ABEPI1 and ABEPI2, displayed significantly increased signal in comparison to mock treated cells at all measured time points, indicating that they act as
A. baumannii efflux pump inhibitors. More specifically, APEPI1 dramatically increased cellular fluorescence to levels exceeding that of PAβN within the first 20 min of treatment, at which point the compound’s potency appeared to level off. APEPI2 treatment measures were essentially identical to those of PAβN until approximately 35 min post-treatment at which point efflux inhibition appeared to drop below PAβN levels although the observed differences were not considered significantly different. Thus, ABEPI1 and ABEPI2 appear to represent novel A. baumannii drug efflux inhibitors.

Mammalian Ca$^{2+}$ channel blocking assays. In considering whether ABEPI1 and/or ABEPI2 represent attractive antibiotic efflux pumps worthy of future medicinal chemistry-based improvement we were cognizant of the fact that many laboratory bacterial efflux inhibitor tool compounds cannot be used in the clinical setting because they limit mammalian ion channel activity. Verapamil is one such agent, which effectively limits bacterial antibiotic efflux pumps, but also elicits human neurotoxicity due to the inhibition of host Ca$^{2+}$ channels [266]. Thus, we measured the effects of ABEPI1 and ABEPI2 on mammalian calcium channel functions using Fluo-4 Direct Calcium Channel Assay kits, in which the dye Fluo-4 was used to measure changes in mammalian cytoplasmic Ca$^{2+}$ levels in response to the calcium channel stimulator, carbachol, in the absence and presence of test compound. Fig. 5.6A, shows the profile of human embryonic kidney (HEK 293T) intracellular Ca$^{2+}$ levels prior to- and following- the addition of carbachol, which stimulates endoplasmic calcium-channel activity and, consequently, release of Ca$^{2+}$ into the cytoplasm. As expected, carbachol treatment induced an approximately 2.3-fold increase in cytoplasmic Ca$^{2+}$ levels.
Conversely, treatment of HEK 293T cells with the known calcium channel blocker, verapamil, virtually eliminated Ca\(^{2+}\) channel activity and cytoplasmic accumulation, indicating that the system was appropriate for measuring mammalian cytoplasmic channel activity and inhibition (Fig. 5.6B). As shown in Fig. 5.6C and 5.6D, HEK 293T treatment with 1X MEC or 4X MEC (not shown) of either ABEPI1 or ABEPI2 did not appear to significantly affect mammalian cell Ca\(^{2+}\) channel stimulation in response to carbachol.

Taken together, it appears as though ABEPI1 and ABEPI2 represent novel, structurally distinct molecules that potentiate the activity of antibiotics toward serum-grown bacterial cells by inhibiting the organism’s drug efflux properties, leading to cellular antibiotic accumulation and, consequently, antimicrobial effects. Moreover, the compounds did not display significant human cytotoxicity measures or Ca\(^{2+}\) channel blocking activity that has plagued the development of antibiotic drug efflux inhibitors. Such compounds may represent attractive starting scaffolds for medicinal chemistry based improvement and refinement with the ultimate goal of creating adjunctive efflux pump inhibitors to be used in combination with current antibiotics for improving the treatment of bacterial infections.

**Potential targets of ABEPI1 and ABEPI2.** The target of EPIs is of great interest as the identification can lead to further information about the mechanism of action. We took a genetic approach to identify the target site(s) of ABEPI1 and ABEPI2, our new EPIs and used a transposon mutant library to identify factors necessary for the adaptive antibiotic
resistance during growth in serum. Six thousand arrayed mutants were grown in 100% human serum supplemented with a subinhibitory concentration (0.25 µg ml\(^{-1}\)) and those that were unable to grow in this condition were deemed of interest. I reasoned that the inability to thrive in this condition was indicative of an interruption in efflux mechanism. In total fifty mutants were identified by their lack of growth in the presence of a subinhibitory concentration of minocycline supplemented human serum. The individual mutants were indicative of either an efflux mutation or a mutation conferring minocycline resistance. Secondary testing as efflux mutants was assessed through the increased accumulation of ethidium bromide, as one of the most common assays used to establish efflux in bacterial species, the accumulation of ethidium bromide is indicative of an interruption of an efflux system. Thus the transposon mutants with increased ethidium bromide accumulation were determined to be the most likely factors mediating minocycline efflux in human serum. There were a total of fourteen mutants that exhibited loss of growth in minocycline-supplemented serum and accumulated ethidium bromide to a greater degree than cells treated with a known inhibitor (Fig. 5.7), suggesting that the insertion of a transposon interrupted an efflux gene. These fourteen mutants were then subjected to inverse PCR to identify the insertion point and thus the factors mediating the adaptive resistance. All together seven unique loci were identified (Table 5.3) and included proteins known to transport amino acids and ammonium. The first transposon mutant identified had an insertion crossing two proteins, a heat shock protein and an amino acid efflux protein. The amino acid efflux protein belongs to the LysE family of proteins which have been characterized in other species and are known to efflux amino acids and antiseptics [267]. Additionally, two other genes with transposon insertions were
transport proteins, an amino acid transport protein and an ammonium transport protein. Transport proteins are the most likely genetic element to contribute to efflux in *A. baumannii*, as these proteins are transmembrane proteins that can move “toxic” compounds such as antibiotics from the internal environment to the external environment. Furthermore, the putative lytic murein transglycosylase gene identified could be indicative of the failure of the efflux pumps to insert into the membrane thus preventing the activity thereof. Finally, the remaining genes identified encompass a reductase that has is known to reduce xenobiotic compounds; an isomerase in the amino acid biosynthesis pathway; and a ribosomal RNA. Despite the identification of factors that mediate the adaptive resistance response of *A. baumannii* grown in human serum, further study on the individual targets is necessary. The role each factor plays in serum-specific efflux of antibiotics has not been fully elucidated and most of the preliminary work has primarily focused on the heat shock protein and the amino acid efflux protein, thus necessitating further study on the remaining factors.

**Discussion**

Antibiotic resistance is a general term used to describe the phenomenon whereby antibiotics used to treat a bacterial pathogen are rendered useless or less effective by the target organism. The so-called bacterial drug efflux pumps have been shown to contribute to antibiotic resistance by modulating the cellular concentration of a given antibiotic within bacterial cells and can generally be divided into five classes of transporters: small multidrug resistance (SMR) pumps of the drug/metabolite transporters super family, ATP-binding cassette (ABC) transporters, Major facilitator superfamily (MFS), and
Resistance nodulation division (RND) superfamily, and Multidrug and toxic compound extrusion (MATE) transporters (see [268] for a recent review). While some members of these families selectively extrude specific antimicrobial agents, the majority efflux a variety of antimicrobial agents. For instance, the *A. baumannii* MATE family transporter, AbeM, is believed to efflux fluoroquinolones, aminoglycosides, quinolones, chloramphenicol, and erythromycin [102]. Thus, AbeM inhibitors may be valuable therapeutic agents that restore the utility of several antibiotic classes. However, such a strategy is complicated by the fact that many antibiotics are extruded by multiple efflux pumps. Thus, in considering the development of an antimicrobial efflux-inhibitor program, clinically relevant inhibitors must limit the activity of multiple efflux pumps as a prerequisite for efficacy.

*A. baumannii* harbors representatives of all the major efflux families, and this is thought to contribute to the organism’s antimicrobial resistance, a phenotype that has, in part, led to its designation as one of the six bacterial ESKAPE pathogens of immediate healthcare concern [1]. While much has been learned about the substrates affected by previously characterized efflux pumps, studies regarding their expression and biological consequences are in their infancy. Likewise, bioinformatics analysis has revealed that the organism is likely to express a plethora of previously uncharacterized factors that may also contribute to clinical antibiotic failure. Within the genome of *A. baumannii* strain 17978, there are thirty-seven genes that are annotated as drug efflux pumps or transporters. Additionally, twenty-nine additional genes are described as heavy metal, cation, and amino acid efflux pumps. Microarray analysis of strains 983709, ATCC
17978, and AC12 have at least 32 additional efflux genes specific to growth conditions that may aid in the antibiotic resistance exhibited by this species [93, 112, 113]. The growth conditions represented in these studies are indicative of the major diseases caused by *A. baumannii*.

Ventilator-associated pneumonia and bacteremia are the two most severe types of *A. baumannii* infection, both of which are thought to include dissemination of the organism to visceral organs via the circulatory system [254, 255, 258, 269]. For that reason, much effort has been devoted toward defining the organism’s cellular components that modulate growth and persistence in blood by using human serum as a convenient growth medium for such studies. In that regard, several *A. baumannii* virulence factors including, phospholipase D (PLD), penicillin binding protein 7/8, K1 capsule polysaccharide and outer membrane protein A (OmpA), have been shown to augment the organism’s ability to survive in human serum and to contribute to the organism’s ability to cause disease in animal models of infection, validating serum as a biologically relevant medium for *A. baumannii* study [183, 270-273]. More recently, a transcriptional-profiling based study revealed that during growth in human serum *A. baumannii* differentially expresses several putative virulence factors. Perhaps most striking, that study also showed that serum growth induces high-level expression of at least 22 previously uncharacterized putative drug efflux-associated proteins and corresponds to the organism’s efflux-mediated resistance to the antibiotic minocycline in serum [191]. Such a phenomenon has recently been recently termed adaptive resistance, a connotation used to describe processes by which clinically defined susceptible bacterial species temporally alter gene
expression in response to an environmental cue(s) in a manner that confers tolerance to a given antibacterial agent [268].

One of the goals of the immediate work was to expand our understanding of the adaptive efflux-mediated antibiotic resistance potential of *A. baumannii* during growth in human serum. The results presented indicate that serum growth-associated antibiotic efflux is conserved across all genetic lineages that we have evaluated thus far, although strain-to-strain differences clearly do occur in terms of the specific antibiotics extruded. For instance, the *A. baumannii* clinical isolate, 983709, that was used for most of these studies displays tolerance to minocycline, tetracycline, and ciprofloxacin during growth in serum. While serum growth-associated minocycline resistance has also been observed for a subset of strains evaluated, it is not completely conserved across isolates. Serum growth of strain 07-09-54 does not exhibit resistance to minocycline, but does display efflux-mediated high-level tolerance to meropenem (data not shown). Likewise, strain 983709 does not exhibit serum dependent tigecycline tolerance, but seven representatives of the other eleven lineages evaluated do. Taken together these results suggest that during serum growth, *A. baumannii* has the capacity to express a multitude of antibiotic efflux pumps that confer antibiotic resistance, but tolerance is dependent on the genetic composition, and consequently efflux pump repertoire, of the strain evaluated. Notably, the same phenomenon was observed for the prototypical *P. aeruginosa* laboratory strain, PA01, which displayed efflux-mediated tolerance to ciprofloxacin during serum growth in comparison to laboratory medium. It remains to be seen whether this correlates to other *P. aeruginosa* strains or other antibiotics. Likewise, it is not yet clear what serum-
dependent factors activate antibiotic efflux. However, the efflux of antibiotics is presumably a by-product of the transporters themselves, which are likely functioning to aid in cellular adaptation to nutrient limiting conditions. By extension, one would predict that other host-associated environments are likely to also elicit such a phenotype, either upregulating the efflux pumps orchestrating serum antibiotic tolerance and/or other so-called antibiotic efflux pumps. In support of that hypothesis, it has been shown that growth of *A. baumannii* in physiologically relevant salt concentrations induces antibiotic efflux-mediated tolerance to the antibiotics amikacin and levofloxacin [274].

Given the biological importance of these findings, we reasoned that small molecule inhibitors of serum-associated antibiotic efflux functions would represent valuable therapeutics to be used in combination with current and possibly future antibiotics. Such agents would potentiate the antimicrobial agents despite *A. baumannii*'s efflux potential in serum, thereby improving the potency of such antibiotics. Thus, a whole-cell high-throughput assay was performed to identify agents that potentiate the antimicrobial activity of a subinhibitory concentration of minocycline toward serum-grown cells, with the expectation that a subset of these compounds would represent serum-associated minocycline efflux pump inhibitors.

Our screening campaign and secondary assays identified two structurally distinct compounds, ABEPI1 and ABEPI2, which display the early characteristics of promising *A. baumannii* serum-associated efflux pump inhibitors. Both compounds restore the antimicrobial activities of minocycline and ciprofloxacin toward serum-grown *A.
*baumannii* but did not affect the antibiotic susceptibility of LB grown cells, suggesting that they affect serum-associated efflux factors. Further, both compounds limit the organism’s efflux properties, as measured in standard ethidium bromide assays, and lead to minocycline accumulation within treated cells. Moreover, neither compound displayed cytotoxicity toward human cells or human Ca\(^{2+}\) channel inhibitory activities, two issues that have limited the development of other bacterial efflux pump inhibitors.

Sulfonamide is a convenient and widely used functional group in medicinal chemistry that improves the physicochemical properties, such as solubility, of small molecule organic scaffolds and has been exploited in anti-bacterial, anti-viral, anti-inflammatory, and anti-cancer agent development (reviewed in [275]). In that regard, ABEPI1 (*Acinetobacter baumannii* efflux pump inhibitor 1; (E)-4-((4-chlorobenzylidene)amino)benezenesulfonamide) is a sulfonamide derivative that does not display antimicrobial activity on its own, but strongly potentiates the activity of minocycline and ciprofloxacin, leads to minocycline accumulation within bacterial cells, and has low cytotoxicity toward human cells. Twenty structurally similar analogs were found to be putative efflux pump inhibitors in our screening campaign (Table 5.2), affording some insight into a preliminary structure-activity relationship for ABEPI1. Certain analogs showed that substitutions at the 4-position of the benzylidene influence the chemical series’ activity. The 4-methylbenzylidene analog increased the MEC 2-fold and displayed a 100,000-fold reduction in accumulation of minocycline within *A. baumannii* cells. Further, the 4-ethylbenzylidene analog had reduced activity as well, measured by an 8-fold increase in MEC, and roughly a 30-fold reduction in minocycline
accumulation in comparison to EPI1. The 4-bromobenzylidene analog has only about a 5-fold decrease in minocycline accumulation, and has the same MEC as EPI1, indicating that certain halogens may have increased activity over hydrocarbons in this position. The 4-fluorobenzylidene, in contrast with the other two halogens, shows a 40-fold reduction in minocycline accumulation and a 32-fold increase in MEC. Other functional groups in this position also displayed measurable effects on activity; the 4-ethoxybenzylidene and the 4-(dimethylamino)benzylidene analogs both had increased MEC values, decreased minocycline accumulation, and increased toxicity. Clearly, additional analoging is required to develop a refined SAR, but those tested thus far indicate the 4-substituted benzylidenes to be at least one path to improving upon the scaffold for a more effective ABEPI1-derived efflux pump inhibitor. In comparison to ABEPI1, ABEPI2 (N-tert-butyl-2-(1-tert-butyltetrazol-5-yl)sulfanylacetamide) appears to be less potent with a relatively high MEC of 32 µg ml\(^{-1}\) but also displays promising activities, leading to an accumulation of \(6.84 \times 10^{-6}\) femtomoles of minocycline cell\(^{-1}\), potentiating the activity of ciprofloxacin toward both \textit{A. baumannii} and \textit{P. aeruginosa}, low cytotoxicity toward human cells, and was confirmed to inhibit efflux pump activity. Additionally, the compound offers a scaffold amenable to possible structural alterations which, in future studies, could lead to analogs that further improve the compound’s efficacy.

While ABEPI1 and ABEPI2 exhibit similar antibiotic potentiation profiles with regard to minocycline and ciprofloxacin, early extended spectrum of activity studies indicate that they also display differing activities and consequently are likely to both target a common efflux pump (or set) but also affect differing subsets of antibiotic efflux pumps, and
consequently, display broad-spectrum activity. More specifically, ABEPI1 is capable of improving the activity of tigecycline toward the carbapenem-resistant *A. baumannii* strain 07-09-61 to levels equaling that of PAβN, whereas ABEPI2 does not improve tigecycline’s activity (Fig. 5.8A). Conversely, ABEPI2 was found to improve the activity of tobramycin toward *P. aeruginosa* strain PA01 during growth in human serum to levels equaling PAβN (Fig. 5.8B), but ABEPI1 did not demonstrate tobramycin potentiating activity (Fig. 5.8B).

Taken together, results of these studies indicate that *A. baumannii* and presumably *P. aeruginosa*, impart adaptive efflux resistance mechanisms during growth in human serum that may, in part, contribute to antibiotic treatment failure toward laboratory defined susceptible strains. Further, ABEPI1 and ABEPI2 may represent attractive compounds for future medicinal chemistry based campaigns designed to limit Gram-negative bacterial efflux. Additionally, the seven loci identified in the genetic screen of *A. baumannii* transposon mutants provide likely targets for the EPIs are active.

Further study into the role each of the seven loci has in adaptive antibiotic resistance is needed, while it has been shown that there is a growth defect in serum supplemented with minocycline and that the transposon mutants accumulate ethidium bromide, the direct link between efflux of antibiotics and each of the loci has not been fully elucidated. The interrupted genes annotated as transporters have homology to transmembrane proteins that are known to exchange toxic compounds from the internal milieu to the environment. Genetic loci A1S_0365 is annotated as a LysE family protein, which have been characterized in other species and are known to efflux amino acids and antiseptics [267].
Additionally, A1S_2793 and A1S_0219 are known to be an amino acid transport protein and an ammonium transport protein, respectively. However, each of these transposrt protein need to be isolated and overexpressed to fully comprehend the role they play in adaptive antibiotic resistance. Furthermore, the putative lytic murein transglycosylase gene identified could be indicative of the failure of the efflux pumps to insert into the membrane thus preventing the activity thereof. Transglycosylase activity assays are needed to assess the importance of this gene in the *A. baumannii* grown in human serum. Additionally, studies connecting the activity of the murein transglycosylase and the insertion of efflux pumps need to be done. Moreover, the activity of the reductase mentioned above needs to be investigated. It needs to be shown that the product of this gene is capable of reducing compounds such as antibiotics and either preventing their efficacy or establishing that efflux pumps recognize the reduced form. Finally, the role of the isomerase in the amino acid biosynthesis pathway and the ribosomal RNA identified above, needs to be explored.
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1 Minimal Effective Concentration (MEC) required to potentiate the antimicrobial effects of 0.5 µg ml\(^{-1}\) minocycline toward serum grown *A. baumannii* strain 983709
2 Log reduction in colony forming units of 983709 cells during growth in serum supplemented with 0.5 mg ml\(^{-1}\) minocycline at 1x MEC in comparison to minocycline alone.
3 Femtomoles of minocycline cell\(^{-1}\) during growth in human serum supplemented with 0.5 µg ml\(^{-1}\) minocycline and 0.5x MEC.
4 Percent human HepG2 cell survival following 24 hr exposure at 4x MEC in the presence of 0.5 mg ml\(^{-1}\) minocycline.
Figure 5.1. Antimicrobial effects of minocycline, ciprofloxacin, tetracycline, and tigecycline toward LB and serum-grown *A. baumannii*. **Panel A.** Plotted are the colony forming units of strain 983709 following incubation in LB or serum (-/+ the efflux pump inhibitor, PAβN) supplemented with 0 – 2 µg ml\(^{-1}\) of minocycline. **Panel B.** Colony forming units of strain 983709 following incubation in LB or serum (-/+ the efflux pump inhibitor, reserpine) supplemented with 0 - 1 µg ml\(^{-1}\) of ciprofloxacin. **Panel C.** Colony forming units of strain 983709 following incubation in LB or serum (-/+ the efflux pump inhibitor, PAβN) supplemented with 0 - 16 µg ml\(^{-1}\) of tetracycline. **Panel D.** Colony forming units of strain 01-12-05 after incubation in LB or serum (-/+ the efflux pump inhibitor, reserpine) supplemented with 0 – 4 ml\(^{-1}\) tigecycline. Colony forming units are plotted on the y-axis; antibiotic concentration is indicated on the x-axis; asterisks indicate statistically significant differences in colony forming units between growth in LB and serum (Student’s t-test; * P<0.05, ** P<0.01, *** P<0.001).
Figure 5.2. Minocycline tolerance of other *A. baumannii* strains after exposure to human serum. *Acinetobacter baumannii* strains were grown in LB media or human serum in the presence of minocycline as described in Materials and Methods. Bacteria were serially diluted and plated for colony forming units (CFUs). Depicted are log CFUs per milliliter after 48 hr of growth at each concentration of minocycline in LB medium (white diamonds) or human serum (black diamonds). At least two biological replicates were included for each sample point. The asterisks indicated statistically significant differences between LB growth and serum growth as determined by Student’s *t*-test (*, *p* < 0.01; **, *p* < 0.01).
**Figure 5.3.** *A. baumannii* intracellular minocycline concentration measures. Shown are the femtomoles of minocycline per cell following growth of strain 983709 in serum supplemented with 0.5 µg ml⁻¹ minocycline (y-axis) in the absence (white bar) or presence of the efflux pump inhibitor, verapamil (grey bar), or presence of 0.5X minimum effective concentration of the indicated putative efflux pump inhibitor (x-axis). The dashed grey line represents the concentration of minocycline per cell grown in human serum supplemented with minocycline and verapamil for comparison.
Figure 5.4. Spectrum of activity.  
Panel A.  Graphed are the colony forming units of strain 983709 grown in human serum supplemented with 0.125 µg ml⁻¹ ciprofloxacin in the absence or presence of 1X MEC the putative efflux pump inhibitors ST009675 (2 µg ml⁻¹), ST058165 (32 µg ml⁻¹), or ST060273 (32 µg ml⁻¹); standard deviations shown.  
Panel B.  Colony forming unit measures of P. aeruginosa strain PA01 following incubation in LB or serum -/+ the efflux pump inhibitor, PAβN, ST009675 (2 µg ml⁻¹) or ST060273 (32 µg ml⁻¹) supplemented with 0 - 2 µg ml⁻¹ of ciprofloxacin.  
Panel C.  Structures of ABEPI1 (ST009675) and ABEPI2 (ST060273).
Figure 5.5. Ethidium bromide (EtBr) efflux assay. EtBr fluorescence properties of strain 983709 following growth in human serum and treatment with PAβN (10 µg ml⁻¹; white squares), ABEPI1 (ST009675; 2 µg ml⁻¹; grey triangles), or ABEPI2 (32 µg ml⁻¹; grey X). Mock treated cells are also shown (black diamonds).
Figure 5.6. Mammalian calcium channel inhibition assays. Cytoplasmic Ca\(^{2+}\) measures of mock treated cells (Panel A) pre- and post- carbachol treatment (grey arrow; 60 sec). Panels B-D. Same as Panel A except that cells were treated with verapamil (50 µg ml\(^{-1}\); Panel B) or 1X MEC ABEPI1 (ST009675; 2 µg ml\(^{-1}\); Panel C) or ABEPI2 (ST060273; 32 µg ml\(^{-1}\); Panel D) at 45 sec (black arrow) pre-carbachol treatment.
Figure 5.7. Transposon mutants have decreased efflux activity. EtBr fluorescence properties of WT 983709 A.baumannii or representatives of the transposon mutant strains following growth in human serum and treatment with PAβN (10 µg ml⁻¹; white squares). Mock treated cells are also shown (black diamonds).
Table 5.3. Identity of the transposon insertions.

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Figure 5.8. Differential antibiotic potentiation effects of ABEPI1 and ABEPI2.

Panel A. Colony forming units of *A. baumannii* strain 07-09-61 after incubation in LB or serum supplemented with 0 – 4 ml⁻¹ tigecycline. Also shown are tigecycline antimicrobial effects in serum-grown cells in the presence of PAβN (10 µg ml⁻¹), ABEPI1 (ST009675; 2 µg ml⁻¹) or ABEPI2 (ST060273; 32 µg ml⁻¹). Panel B. Colony forming units of *P. aeruginosa* strain PA01 after incubation in LB or serum supplemented with 0 – 4 ml⁻¹ tobramycin; the antimicrobial effects of tobramycin toward serum-grown cells in the presence of PAβN (10 µg ml⁻¹), ABEPI1 (ST009675; 2 µg ml⁻¹) or ABEPI2 (ST060273; 32 µg ml⁻¹) also shown.
Chapter 6: Final Conclusions and Discussion

As we return to the pre-antibiotic age of treatment, the need for new therapeutic measures are vital to defend against the rising toll of antibiotic resistant bacterial species, especially the ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species) pathogens. Combination therapy has been commonly used to treat non-bacterial diseases ranging from HIV, malaria, and hypertension [138, 139]. However, the focus of this thesis has been on combination and adjunctive therapy in relation to bacterial infections; indeed there are several combination/adjunctive therapies already on the market that have been shown to be vital in the treatment of such infections. Augmentin and Bactrim have both been shown to be effective combinations of amoxicilin with clavulinic acid and sulfamethoxazole with trimethoprim, respectively, in the treatment of bacterial infections such as otitis media and urinary tract infections [143-146]. In addition, the treatment of Mycobacterium tuberculosis requires there to be multiple antibiotics such as Isonaizid and Rifampin, administered at the same time [276].

Despite the common usage of drugs such as Augmentin, Bactrim and the necessary use of multiple drugs in the treatment of tuberculosis, combination/adjunctive therapy is still a controversial therapeutic option. Nearly all of the combination therapies used in the clinic are based upon empirical data, observations made by clinicians on the antibiotics that work best together [277, 278]. Furthermore, the addition of two or more antibiotics or drugs together can increase the toxicity towards patients and the adverse side effects exhibited by said patients. For example, empirical data has suggested that the
combination of an aminoglycoside antibiotic and a β-lactam antibiotic can lead to increased efficacy against serious Gram-negative infections, however this combination can also lead to nephrotoxicity or ototoxicity [279, 280]. Finally, the greatest concern about using combination/adjunctive therapy to treat bacterial infections is the inadvertent creation of a multidrug resistant “superbug” [281]. With the use of two or more antibiotic classes in combination, a bacterial species may be able to develop resistance to both classes, thus prohibiting further use of these antibiotics.

Conversely, combination and adjunctive therapy has several advantages over the traditional method of single drug therapy (monotherapy). Monotherapy fails for a variety of reasons, but in regards to Gram-negative the impermeability of the membrane combined with the expression of promiscuous efflux pumps has led to greater antibiotic resistance [282]. Adjunctive therapy can directly address these problems, through the use of a potentiating molecule that improves the permeability of the membrane or, as we have shown in this thesis (Chapter 5), by directly inhibiting the efflux of antibiotics [123, 283]. Furthermore, as previously shown and further described in Chapter 3 of this thesis, the combination of two synergistic drugs have more efficacy than either in single use and are able to prevent the emergence of resistance [283]. In addition to the synergism, resistance prevention, and improved penetration of antibacterial agents, combination therapy has also been shown to prevent the formation of biofilms, toxin reduction, and has been shown to cause a reduction in antibiotic modifying enzymes [283]. Combination therapy can also broaden the spectrum of activity and can provide coverage to polymicrobial infections [284]. Overall, there have been several studies where it has been shown that
Empiric combination therapy has reduced the mortality rate associated with serious infections compared to monotherapy [285, 286].

Treatment of bacterial infections with more than one antibiotic is generally an empirical choice made by physicians in the treatment of serious infections. However, there is limited in vitro data that the combinations used in a clinical setting are synergistic or can prevent resistance development. Towards that end, I have identified two new combination therapeutics, which have in vitro combination activity and have been shown to have in vivo activity against bacterial infections. Additionally, I have identified a new adjunctive chemical backbone that has in vitro efficacy against Gram-negative bacterial species.

Mupirocin and silver sulfadiazine (SSD) are standard treatment options against skin wounds and burn infections [40-42, 79, 80]. Indeed, mupirocin ointment is used before surgery in patients that are known carriers of methicillin-resistant Staphylococcus aureus (MRSA) in the UK and is further used in combination with antiseptics in the localized treatment of skin and soft tissue infections [33, 226]. Additionally, Silvadene, the commercially available ointment containing 1% silver sulfadiazine, is the standard topical antimicrobial for partial thickness burns [31, 79]. Both ointments (2% mupirocin and 1% silver sulfadiazine) represent the standard therapy to prevent S. aureus and Gram-negative bacterial soft tissue infections. Internally, A. baumannii bacteremia can be treated with systemic antibiotics such as tetracyclines. While the empirical treatment option against A. baumannii infections remains carbapenems, minocycline has been
shown to be an effective treatment [166]. Furthermore, with the increase in MDR *A. baumannii* infections minocycline has gained popularity as an alternative antibiotic [287].

As base therapies these three drugs have shown efficacy, however resistance has quickly developed and called into question the future use of each of these drugs [46, 89]. Indeed, resistance post-antibiotic treatment to mupirocin was found in 3-5% of nasal or blood clinical isolates from 23 different US hospitals [226]. In some conditions, it has been shown that MRSA strains exhibiting resistance to mupirocin ointment has reached as high as 81% [288]. Silver resistance has largely been characterized in enteric bacteria where the rate of resistance is 10%, however recently, resistance to silver sulfadiazine has been shown in *P. aeruginosa* burn isolates [89, 289]. Finally, 63% of *A. baumannii* infections are caused by MDR strains and the expression of efflux pumps accounts for both the intrinsic and acquired resistance observed during treatment [8, 106, 109, 290].

Thus I reasoned that adding synergistic or additive compounds together would be able to produce a new therapeutic for the treatment of resistant infections. As stated in Chapter 3, we were able to identify the antibiotic neomycin as an additive compound in a screen of FDA approved drugs. It has recently been shown that in *Escherichia coli*, neomycin exhibits off target inhibition of the processing ribonuclease RNaseP, a protein in the same pathway as isoleucyl tRNA synthetase, the antibiotic target of mupirocin [216]. The combination of the mupirocin and neomycin inhibit the two proteins that are directly responsible for cleaving the leader sequence of isoleucyl tRNA and the charging of the amino acid to the tRNA molecule. The combination of these two antibiotics have
exhibited additive activity against *S. aureus* in FIC in vitro assays as well as in *in vivo* murine models of infection (Chapter 3). Furthermore the efficacy has been shown against not only susceptible strains of *S. aureus* (UAMS-1), but also those strains that have been shown to be methicillin or mupirocin resistant (USA300 and BAA1708).

Biofilm growth has long been associated with antibiotic resistance and has a three dimensional structure made up of an exopolysaccharide matrix, extracellular DNA, proteins and enzymes. Additionally, the structure created by the exopolysaccharide matrix (EPS) creates microenvironments with nutrient and oxygen gradients. DNA in the extracellular matrix can upregulate the expression of a two-component system that modifies the LPS production in Gram-negative bacteria, conferring resistance to antibiotics such as colistin [291]. It has also been found that cells in this state have an increased mutation rate, which can facilitate a resistance phenotype [292-294]. Perhaps the most important mechanism of resistance in biofilms is the altered metabolic state of cells due to the development of nutritional microenvironments [295, 296]. The nutrient content and concentration varies greatly between the microenvironments found within biofilms. This causes a gradient of both nutrients and oxygen throughout the 3D structure of a biofilm, accounting for the different growth states of bacteria and the subsequent antibiotic resistance.

Furthermore, biofilms also prove to be important for the chronicity and severity of diseases, which can lead to an increase in antibiotic usage. However, the protective nature of biofilms necessitates new antimicrobials that are able to kill cells already living in the
biofilm and prevent the reemergence of infections. To this end we identified an anti-
biofilm agent zinc pyrithione (ZnPT) from a screen of F.D.A approved drugs that were
able to destroy established *P. aeruginosa* biofilms. Zinc pyrithione, is a known antifungal
compound that is most commonly used in the clearance of the fungal etiology of
dandruff. The mechanism of action of this antifungal is largely under studied, however,
recent evidence suggests that ZnPT is able to inhibit the growth of fungi by inducing the
accumulation of copper with the cell [297, 298]. By damaging proteins in metabolism
pathways, which contain iron-sulphur clusters, ZnPT causes an increase in cellular levels
in copper, which proves to be toxic to fungal cells [297]. While this compound is largely
known as an antifungal, one study has shown that it has activity against *S. aureus* strains
[299]. With that said, the antibiofilm /antibacterial activity of this compound is largely
unknown. As the known mechanism of silver sulfadiazine is the binding of silver ions to
DNA and membrane proteins, the addition of a compound that inhibits metabolic
pathways is appealing due to the potential for bacterial cell death.

In our studies, ZnPT was active against *P. aeruginosa*, but its antimicrobial potency was
less than that of the known therapeutic option SSD. Conversely, ZnPT’s antimicrobial
potency was superior to SSD toward *S. aureus, A. baumannii* and the other ESKAPE
pathogens, thus we chose to focus on this compound from the screen. Furthermore, the
combination of ZnPT with the standard of treatment SSD, showed that the combination
was active against *P. aeruginosa, S. aureus*, and *A. baumannii* in planktonic and biofilm
growth states. Similarly, in a topical formulation of ZnPT + SSD, an improved
antimicrobial activity was observed when compared to either drug individually. As both
ZnPT and SSD are available in commercial antimicrobial topical formulations, the combination represents a new and potentially more efficacious therapeutic option, particularly for wounds such as burns or chronic nonhealing wound sites.

As stated before resistance in response to growth conditions has a great number of implications for ESKAPE pathogens. Bacteremia attributed to *A. baumannii* has been shown to have the highest mortality rates amongst all the infections caused by this species with the highest recorded rate of 81% mortality in a neonatal unit [94]. Unfortunately, the proteins found in human serum often pose insurmountable problems for antibiotics [91, 92]. The proteins in human serum are known to bind to antibiotics and prevent their activity through inactivation or a reduction in available concentration. Two such examples are fluoroquinolones and penicillins, which have both been shown to bind to the major human serum protein albumin [91, 300, 301]. Other factors found in human serum have been found to influence the activity of antibiotics such as complement factors, transferrin and other minor proteins such as lipoproteins, α₁-acid glycoproteins, and α-, β-, γ- globulins [302]. I have been able to show that in addition to these known problems, *A. baumannii* specifically upregulates the expression of antibiotic efflux pumps in response to growth in human serum, thus exhibiting adaptive antibiotic resistance (Chapter 5 and exhibited in **Fig. 6.1**) [93]. The adaptive antibiotic resistance observed in *A. baumannii* includes resistance to the tetracyclines, tigecycline, fluoroquinolones, and aminoglycosides, which was ablated with the use of an efflux pump inhibitor (EPI). Given the preclusion of EPI use in the clinical setting, I set out to identify new chemical backbones that have EPI activity against Gram-negative bacterial species. Thus I
identified two individual chemical structures, which exhibited EPI activity expressly in human serum. Future directions of this include developing these chemical backbones to identify the best possible compound for use in combination therapy. Towards that end, one of the structures identified has led us to begin structure activity relationship assays to improve upon the activity and to further develop compounds for the use in adjunctive therapy. Additionally, I are interested in defining the target of the EPIs. In a genetic screen of transposon mutants, the target of our two compounds was narrowed down to seven unique loci (Chapter 5). Interruption of the identified loci exhibited decreased efflux with recapitulation of the gene leading to the restoration of wild type phenotypes, suggesting that the seven loci are factors leading to adaptive antibiotic resistance. Ethidium bromide accumulated within each of the transposon mutants, as shown in Fig. 5. 7, suggesting that the efflux of this compound was inhibited by the insertion of a transposon into a specific gene. Additionally, each of the mutants tested were hypersusceptible to minocycline, exhibiting a growth defect at concentrations of antibiotics lower than wild type cells treated with an EPI. Complementation of A1S_0365 (amino acid efflux protein) resulted in ethidium bromide efflux and antibiotic tolerance similar to that of wild type cells. Studies with the rest of the genes are ongoing, but it is likely that complementation will result in wild type levels of efflux. Finally, these seven genes are likely to be the target of the new EPI chemical backbones. Moving forward I wish to confirm the target of each EPI and define the factors conferring adaptive antibiotic resistance.
Combinational and Adjunctive Therapy is the Future

Traditional therapy for bacterial infections has constituted of one drug with a single target within the cell. This has been the basis of our antibacterial drug development for more than 50 years, however we have reached a time where this may not be the best approach anymore. Furthermore, the antibacterials that have been developed and approved by the FDA in the last 20 years, are largely modifications on the same basic structures that were identified during the Golden age of antibiotics. Two major options exist for the future of antibiotics, the identification of inhibitors to new targets or combinational therapy with drugs that exhibit synergism. While there has been some success with the first option, new targets are difficult to identify and new inhibitors to said targets involve lengthy development, for example our own use of RNases as a new antibiotic target. Additionally, this process still has the pitfalls of currently available antibiotics; one drug for one target has potential for rapid resistance developed. Combinational therapy has potential to be a relatively quick process, when we use F.D.A approved drugs and has been theorized to decrease the likelihood of resistance, although combination therapy is still controversial in the treatment of bacterial infections due to the lack of empirical data.

The use of multiple drugs at the same time is not a new therapeutic method, it has been utilized in the treatment of multiple diseases from hypertension and HIV to mycobacterium infections [303-306]. Furthermore, this same methodology has been applied to nonmycobacterial infections with great success. Two combination drugs are some of the most commonly used antibacterials in the world, in fact as stated before Augmentin and Bactrim are on the World Health Organizations list of drugs that
constitute a basic healthcare system. Augmentin is the combination of a beta-lactam antibiotic with a beta-lactamase inhibitor and has been in use in the clinic since the late 1980s with indications towards skin and soft tissue infections, sinusitis, otitis media, and urinary tract infections [140, 281, 307-309]. The other commonly used combinational therapy against bacterial species is Bactrim, a combination of sulfamethoxazole and trimethoprim and has similar indications as Augmentin with the addition of disease such as Shigellosis and other gastro-intestinal diseases [142-144]. With the amount of success using combinational therapy in treating non-bacterial diseases as well as bacterial disease, the future development of new antimicrobials should utilize this method as a mainstay.

**Ongoing Studies**

One of the most important steps of drug discovery is studying analogs of the lead compounds. With ABEP11 I was able to perform initial structure activity relationship (SAR) studies with the compounds within the collection as discussed in Chapter 5. Twenty similarly structured compounds allowed us to make some conclusions towards where to chemically modify the lead compound. Certain analogs showed that substitutions at the 4-position of the benzylidene influence the chemical series’ activity. The 4-methylbenzylidene analog increased the MEC 2-fold and displayed a 100,000-fold reduction in accumulation of minocycline within *A. baumannii* cells. The 4-ethylbenzylidene analog exhibit reduced activity as well, as measured by an 8-fold increase in MEC, and roughly a 30-fold reduction in minocycline accumulation in comparison to EPI1. The 4-bromobenzylidene analog has only about a 5-fold decrease in minocycline accumulation, and has the same MEC as EPI1, indicating that certain
halogens may have increased activity over hydrocarbons in this position. The 4-fluorobenzylidene, in contrast with the other two halogens, shows a 40-fold reduction in minocycline accumulation and a 32-fold increase in MEC. Other functional groups in this position also displayed measurable effects on activity; the 4-ethoxybenzylidene and the 4-(dimethylamino)benzylidene analogs both had increased MEC values, decreased minocycline accumulation, and increased toxicity. Going forward, more related chemical structures need to be developed for additional SAR studies. As of the writing of this dissertation, two additional compounds that have modifications to the amine bond have been identified. The modifications are meant to stabilize the compound since it is known that ABEPI1 breaks down into two metabolites in the cytoplasm of bacterial cells, Cl-Benzaldehyde and sulfanilamide. Further, it has been observed that the metabolites have differing activities, the sulfanilamide component is necessary for uptake into the cell for growth inhibition (Fig. 6.2). However, the Cl-Benzaldehyde component is necessary for the inhibition of efflux pumps (Fig. 6.3). With the additional compounds the lab will assess their activity using the same experiments from Chapter 2. These studies will allow us to determine whether the stabilization of the molecule will alter the activity of the compound. Additionally, with the seven unique loci identified as potential targets of our EPIs, we have genetic elements to investigate in relation to the serum induced efflux activity of A. baumannii as well as the mechanism of action of EPIs.

The in vivo efficacy exhibited by the combination ointments have shown the potential for use in the clinical setting and studies to this effect are ongoing in our laboratory. The development of resistance to the combination of mupirocin and neomycin described in
Chapter 3 is currently under investigation. Concurrently, the efficacy in treating a resistant infection has caused an interest in the rate of resistance among clinical isolates treated with mupirocin. Using the same and similar techniques described in Chapter 2 and applied in Chapter 3, we are able to evaluate the base mupirocin MIC of clinical isolates collected around the world and to explore how quickly these isolates can develop resistance to mupirocin or the combination of mupirocin and neomycin. This will allow us to evaluate the potential use of this treatment in a clinical setting. In the future we work on the continuation of preclinical studies with the hope to take the combination ointment to clinical trials.

Although this body of work has focused on the ointment combination of mupirocin/neomycin and SSD/ZnPT, there are other potential combinations that can be explored for the treatment of bacterial infections. Currently, in the lab we are exploring other drugs that may show improved activity in combination with a synergistic compound. We have recently begun to focus on eye infections and using combinational therapy in the form of eye drops to treat infections caused by *S. aureus* and *P. aeruginosa*.

**Final Perspectives**

Antibiotics are vital to the practice of medicine; they allow physicians to progress the treatment of a number of other conditions. Without them an everyday skin infection very
well might be deadly. Additionally, with the difficulty of identifying new targets and the development of novel compounds added to the growing problem of resistance, combinational therapy is appearing more likely to be the future of antibacterials. A method that has proven successful in the past in treating not only viral and homeostatic disease, but also bacterial infections, herein we have described three new combination therapeutic options. The ointments have application in the treatment of skin and soft tissue infections as well as chronic wounds and the EPIs have application in the treatment of Gram-negative bacteremia. These studies show great promise for the future of combinational therapy to treat bacterial infections in many different host sites.
Figure 6.1 Adaptive Antibiotic Resistance Model. Exponential growth of A. baumannii strain 983709 does not exhibit efflux activity, but stationary phase cells are known to express efflux pumps such as AdeABC. Growth in human serum induces the expression of uncharacterized efflux pumps that cause antibiotic resistance in these cells.
Figure 6.2. Growth inhibition of Sulfanilamide and Cl-Benzaldehyde. A subinhibitory concentration (0.5 μg ml\(^{-1}\)) of minocycline in serum is able to kill *Acinetobacter baumannii* in LB. Supplementation with ABEP11 and 0.5 μg ml\(^{-1}\) minocycline cause a growth reduction in human serum. Sulfanilamide and Cl-Benzaldehyde do not potentiate the activity of minocycline.
Figure 6.3. Cl-Benzaldehyde inhibits efflux in *A. baumannii*. EtBr fluorescence properties of WT 983709 *A.baumannii* following growth in human serum and treatment with PAβN (10 μg ml⁻¹; white squares), ABEPI1 (2 μg ml⁻¹; grey triangles), sulfanilamide (grey circles), and Cl-benzaldehyde (black xs). Mock treated cells are also shown (black diamonds).
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