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Discovering Antibacterial and Anti-Resistance Agents Targeting Multi-Drug Resistant ESKAPE Pathogens

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Discovering Antibacterial and Anti-Resistance Agents Targeting Multi-Drug Resistant ESKAPE
Pathogens

by

Renee Fleeman

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Cell Biology, Microbiology & Molecular Biology
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ABSTRACT

Antibiotic resistance has been a developing problem for mankind in recent decades and multi-drug resistant bacteria are now encountered that are resistant to all treatment options available. In 2014, the World Health Organization announced that this problem is driving us towards a “post-antibiotic era” that will change the face of modern medicine as we know it. If lack of novel antibiotic development and FDA approval continues, by the year 2050, 10 million people will die each year to an antimicrobial resistant bacterial infection. With lack of pharmaceutical industry involvement in developing novel antibiotics, the responsibility now lies within the academic institutions to identify potential novel therapeutics to fuel the antibiotic drug discovery pipeline. Combinatorial chemistry is one technique used to expedite the discovery process by assessing a large chemical space in a relatively short time when compared to traditional screening approaches. Combinatorial libraries can be screened using multiple approaches and has shown successful application towards many disease states. We initially discovered broad spectrum antibacterial bis-cyclic guanidines using combinatorial libraries and expanded on the knowledge of the physiochemical attributes necessary to inhibit Gram negative bacterial pathogens. Following this success, we continued to assess the combinatorial libraries for adjunctive therapeutics that potentiate the activity of obsolete clinical antibiotics. The polyamine efflux pump inhibitors discovered in this subsequent study prove the benefits of using the large chemical space provided in the combinatorial libraries to identify a variety of therapeutics. Our studies always begin with identifying an active compound and active compounds undergo hit-to-lead optimization. This optimization studies are of utmost importance in developing a novel antibacterial agent for therapeutic applications. Our medicinal

chemistry work described here is proof of the success of careful structure activity analyses to optimize a hit scaffold to create a more effective antibacterial agent. Overall, our work described here reveals the potential role of academic institutions in fending off the impending “post-antibiotic era”.

CHAPTER 1: INTRODUCTION

Infectious Diseases. Infectious diseases have plagued mankind throughout our history.(1) Evidence of these epic battles can be deciphered when reading ancient texts and unearthing historical artifacts.(1) Ancient hieroglyphics have been translated to reveal antimicrobial treatment methods to cure infectious diseases.(2) The plagues of *Yersinia pestis* had devastating effects to Asia, Africa, and Europe beginning with the Justinian plague of 541 AD until the bubonic plague or “Great Plague” that began in 1334 and is said to have killed 60% of the European population.(3, 4) As mankind developed throughout the years, the study of infectious diseases and transmission alleviated the mortality rate of many diseases.(5) For example, the paramount work of biologists such as John Snow and William Budd demonstrated the transmission of the respective diseases of cholera and typhoid fever in order to stop their relentless spread.(1, 6) Today, we are much more capable of treating and surviving infection diseases. However, bacterial pathogenesis and virulence factors have allowed for bacterial infections to persist and become problematic.(7) Bacterial infections are of particular interest because without all the complexity that multi-cellular species have, bacteria have found a unique way to survive.(8) As such, they are the most ancient organisms on our planet and continue to thrive today. The secret to their success is the extreme plasticity of their genome, which allows them to very quickly adapt.(9) Bacteria have the innate ability to spontaneously mutate their DNA while replicating in response to deleterious circumstances and therefore pass this survival instinct to their progeny to ensure survival.(8, 10) With this simplicity

and plasticity they are able to survive and continue to be problematic for a highly intelligent species such as ourselves.

Bacterial pathogenesis. Bacterial infections have become a major health issue for mankind once again in the 21st century.(11) The bacteria that cause human infections are able to invade and persist because of the development of pathogenesis.(12) As humans, we have as many bacteria living commensally inside of us as we do our own cells.(13, 14) These commensal bacteria are referred to as our microbiome and do not cause infections as they do not have the developed pathogenesis that invading bacteria use to cause disease.(15) Furthermore, these pathogenic invading bacteria can be distinguished from non-pathogenic bacteria by their degree of virulence.(12) Virulence is the ability to evade the host immune system and cause infections in healthy human hosts.(16) These virulence determinants can be acquired horizontally or vertically to aid in survival of the bacteria.(17-19) One of the most important factors for invasion and persistence in the human host is immune evasion.(20, 21) Pathogenic bacteria are recognized as not part of the host microbiome and must hide or protect themselves from the human immune factors used to fight off invading bacteria.(15) The human host has innate and adaptive immune factors that normally identify and eradicate invading bacteria to avoid illness. These factors are why we have survived at all as bacterial species evolved before humans. However, when the balance between invading bacterium and the immune defense becomes unbalanced, infectious disease is the result.(22)

Virulence factors. Our innate immune system is designed to destroy invading bacteria to protect us from infectious disease, however bacterial pathogens have developed complex and efficient ways to evade our immune system.(16) The factors that make this invasion possible are referred to as virulence factors. These include, but are not limited to: i) adherence factors, ii) capsule formation, and iii) secretion of endotoxins, exotoxins, and siderophores.(23-28) These virulence factors allow the bacteria to invade the host, cause disease, and evade the host immune system.(12, 29-31) The invading bacteria use a combination of these virulence factors to successfully survive and thrive within the host.(32, 33) For example, *Escherichia coli* has evolved the ability to resist the extreme acidic environment of the human stomach through the use of three inducible Acid Resistance Systems (AR): AR1, AR2, and AR3.(34) Once past the harsh acidic stomach environment into the alkaline intestinal environment, *E. coli* can then turn acid resistance off and use that conserved energy to activate the type III secretion system encoded on the locus of enterocyte effacement (LEE) pathogenicity island, which allows for intestinal cell invasion and colonization of an immune competent individual.(35) Of course one cannot discuss virulence without discussing the diverse repertoire of *Staphylococcus aureus* virulence factors.(36-38) *S. aureus* is one of the most common infectious agents afflicting the United States and this is largely because of its ability to cause disease in a healthy human host.(39, 40) *S. aureus* virulence factors include but are not limited to: Agr-mediated survival and escape from the macrophage environment,(41-43) proteases designed to deactivate host immune proteins,(44, 45) and robust biofilm formation to hide from the traditional immune recognition factors.(46-48) The virulence of *S. aureus* has become extremely problematic for isolates known as “Community acquired” Methicillin Resistant *Staphylococcus aureus* (CA-MRSA), which is extremely efficient at causing infection in young immune competent individuals.(49) CA-MRSA has become notorious as the

leading cause of bacterial infections and death in healthy adults in the United States.(50) With the efficiency of the virulence and pathogenesis of the bacterial species that cause human infections, it is paramount that we have a tool chest of therapeutics to aid in recovery when an infection occurs.(51, 52)

Bacterial Infection Treatment. Antibiotic chemotherapy is the most common form of treatment for bacterial infections and has been very successful in the past at curing bacterial infections.(53) Antibiotics can be used to treat an already existent infection,(54) or be used prophylactically to prevent infections when having surgery or travelling abroad.(55) These therapeutics have given healthcare workers an opportunity to stop otherwise fatal infections by hypervirulent bacterial species found in our hospitals today.(56) Vaccine therapy for bacterial pathogens has focused on the antigenic protein expressed on the surface of the pathogenic species and has only shown success for a few bacterial species.(57) New approaches are being pursued that involve biological agents such as antibodies(58) and bacteriophages(59), however these new approaches have not shown enough promise to be implemented into clinical use in the United States.(60, 61)

Antibiotic history. There is evidence of tetracycline found in skeletal remains dating back to 350-550 CE revealing that natural products played an important role in the pre-antibiotic era.(62) Evidence found in the remains of the Sudanese Nubia population, which were relatively free of bacterial infections, suggests they were ingesting tetracycline from something in their diet, not necessarily taking a therapeutic agent.(54) Although the history of bacterial infections dates to the beginning of our time, it was not until 1940 with the discovery of penicillin that we began to use

chemotherapeutic agents to treat bacterial infections.(63) In the early 1900's, Dr. Paul Ehrlich proclaimed that chemicals could be developed that would selectively kill invading pathogenic bacteria.(54) After this benchmark, Alexander Fleming serendipitously discovered on September 3, 1928 that a *Penicillium* species he found growing on a petri dish could inhibit bacterial growth.(54, 63) Following through on this novel discovery, it took Dr. Fleming 12 years to find a chemist that would purify and create mass distribution in 1945.(54) The subsequent mass production of penicillin helped the allies win World War II, as previously many of the soldiers fighting lost their lives to bacterial infections after surviving their combat wounds.(64) However, the success of penicillin was short lived because resistance was first discovered in 1942, even before mass production for general population distribution was complete.(65)

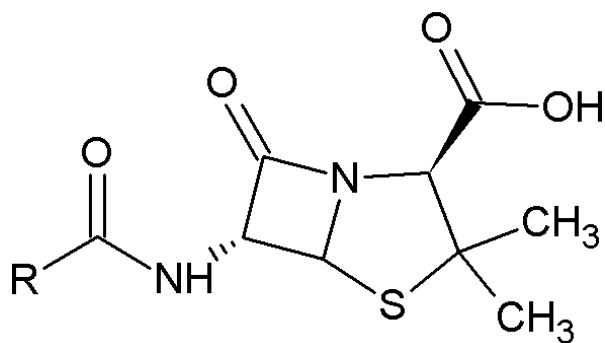


Figure 1. Structure of penicillin. Shown here is the chemical structure of penicillin that was discovered in 1928 by Alexander Fleming. This is the first of a class called beta-lactams, which is characterized by the fused ring structure.

Dr. Selman Waksman coined the term “antibiotic” and developed important screening techniques leading to the discovery of streptomycin found to cure *Mycobacterium tuberculosis* infections.(66) The screening approaches developed by Dr. Waksman led to a Nobel prize and the advent of the golden age of antibiotics, where many of our current antibiotics were discovered from screening of natural products.(67) Following this era of discovery, finding novel antibiotics became more difficult to identify from natural products as many discoveries had already been made.(67) Today, many natural product chemists are investigating unexploited areas and organisms or identifying novel screening or cultivating methods to probe the remaining hypothetical chemical space.(68, 69)

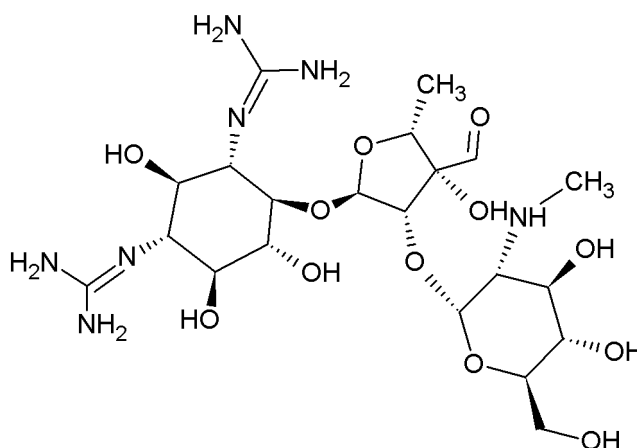


Figure 2. Structure of streptomycin. Shown here is the chemical structure of streptomycin that was discovered by Dr. Selman Waksman. It was isolated from *Streptomyces griseus* in 1943 and found to effectively treat *Mycobacterium tuberculosis* infections.

Mechanisms of action. Antibiotics work by disrupting an essential cellular function in bacteria.(70) Antibiotics are classified in several different ways: by the chemical structure; by the target or system they inhibit; or by whether the antibiotic action is bacteriostatic or bactericidal.(70) Antibiotics that inhibit bacterial replication are generally termed bacteriostatic antibiotics, as the bacteria are able to recover after the antibiotic has been washed away.(70) On the other hand, antibiotics that lyse the cell or induce cellular death are generally termed bactericidal antibiotics because the damage to the bacterial cell cannot be overcome following removal.(70) While both bactericidal and bacteriostatic antibiotics are effective, the bacteriostatic antibiotics are reliant on an active immune system, therefore bactericidal antibiotics are preserved for infections in immunocompromised patients or extremely difficult to treat systemic infections.(71) Furthermore, the distinctions between bacteriostatic and bactericidal are not exactly clear cut. For example, chloramphenicol activity is bactericidal towards *Streptococcus pneumoniae* but bacteriostatic towards *S. aureus*.(71) Similarly, tetracycline is classified as a bacteriostatic antibiotic, however when the concentration is increased it becomes bactericidal.(72) Generally, bacteriostatic antibiotics target the metabolic pathways of replication and require the bacteria to be replicating in order to be effective.(73) On the other hand, bactericidal antibiotics that target the bacterial cell membrane integrity do not necessarily need the bacterium to be effective and are therefore can also be effective towards non-replicating bacteria.(73) Ultimately, both bactericidal and bacteriostatic antibiotics are slowly becoming obsolete as bacterial resistance is ever so steadily increasing in our world.(74-76)

Mechanisms of drug resistance. The occurrence of antibiotic resistant nosocomial infections has been increasing steadily in recent decades.(76) The selective pressure that antibiotics place on

bacteria causes an increased occurrence of resistant species that cause nosocomial infections.(77) The consequence of this antibiotic resistance is increased length and complexity of treatment methods, which in turn favors more resistant isolates.(74) Mortality rates are on the rise due to multi-drug resistant (MDR) pathogens as there are few treatments left to eradicate these isolates.(11) With the increase in MDR pathogens the steady rise in mortality is becoming a global health crisis.(11) The World Health Organization (WHO) has declared that if measures are not taken to divert this increase in MDR infections we will enter a “post-antibiotic era” where bacterial infections will be the leading cause of death worldwide.(11) The most recent statistics reveal that if the spread of antibiotic resistance is not thwarted, antimicrobial resistant infections will be the leading cause of death by the year 2050.(78) Currently, clinicians are turning to last resort antibiotics that have been abandoned decades ago due to toxicity issues in order to treat MDR bacterial isolates.(79) The increased reliance on last resort antibiotics is creating bacterial resistance towards these last resort toxic antibiotics, therefore creating pan-drug resistant (PDR) isolates. There are no reliable treatment options for PDR bacterial isolates and an infection is an almost certain death sentence. Recently, resistance to the last resort antibiotic colistin has been discovered in the United States.(80) The resistance gene *mcr-1* is encoded on a plasmid carried by *Escherichia coli* and has allowed resistance to colistin, which is the only antibiotic left that will eradicate some extreme Gram negative MDR infections. This marks a turning point towards the post-antibiotic era and will have devastating effects on modern medicine.(11)

Resistance has developed for every antibiotic mechanism of action, consequently creating resistance towards all classes of antibiotics developed to date.(79) Twenty classes of antibiotics were discovered between 1940 and 1962 (**Table 1**),(81, 82) while only two new classes have been

discovered since 1962, creating a void in novel therapeutic development that has allowed MDR organisms to increase in numbers as the years continue.(83) Unfortunately, resistance to an antibiotic is usually identified before or just after its introduction to the public. Moreover, of the antibiotics discovered to date, there is a general lack of Gram negative therapeutic options. This is a compounding factor adding to the dangerous rise in resistance with these species.(84) For decades now, pharmaceutical companies have focused on discovery of analogues of antibiotic classes rather than novel classes because there is less toxicity issues with these compounds.(83) Multi-drug resistant bacteria are able to develop resistance very quickly to these analogs created from the existing antibiotic classes and therefore novel targets must be pursued in order to break the cycle of resistance development.(74, 77, 83) Most antibiotic classes inhibit actively replicating bacteria by disrupting DNA, RNA, protein, and cell wall synthesis, or by inhibiting an essential metabolic pathway.(70)

Table 1. Antibiotic classes, their discovery, introduction, and resistance development

Antibiotic Class	Example	Discovery	Introduction	Resistance	Activity
β-Lactams	Penicillins	1928	1936	1942	Broad spectrum
Sulfadrgs	Sulfamethoxazole	1932	1936	1945	Gram positive
Aminoglycosides	Gentamicin	1943	1946	1946	Broad spectrum
Tetracycline	Doxycycline	1944	1952	1950	Broad spectrum
Rifamycins	Rifampicin	1957	1958	1962	Gram positive
Macrolides	Erythromycin	1948	1951	1955	Broad spectrum
Lincosamides	Lincomycin	1962	1964	1956	Gram positive
Glycopeptides	Vancomycin	1953	1958	1960	Gram positive
Lipopeptides	Daptomycin	1986	2003	1987	Gram positive
Streptogramins	Streptogramin B	1963	1998	1964	Gram positive
Oxazolidinones	Linezolid	1955	2000	2001	Gram positive
Quinolones	Levofloxacin	1961	1968	1968	Broad spectrum
Chloramphenicol	Chloramphenicol	1946	1948	1950	Broad spectrum
Trimethoprim	Trimethoprim	1961	1962	1972	Broad spectrum
Polymyxins	Colistin (polymyxin E)	1947	1958	2011	Gram negative

There are two general classifications of bacterial resistance development, endogenous and exogenous. Endogenous resistance occurs by mutation and selection, while exogenous resistance occurs through horizontal gene transfer.(82) Bacteria can spontaneously mutate to gain resistance towards antibiotics or acquire the genetic material through horizontal transfer methods (i.e. transduction, transformation, and transposon insertion).(85) The acquisition of genetic material has allowed for the greatest increase in resistance worldwide. Plasmid resistance can allow the bacterium to modify the antibiotic target, produce enzymes to inactivate the antibiotic, alter the cell wall composition, or efflux the antibiotic before it can interact with the target (**Figure 1**).(86) Once bacteria have developed resistance towards an antibiotic class, analogs developed thereafter become ineffective much more rapidly.(70)

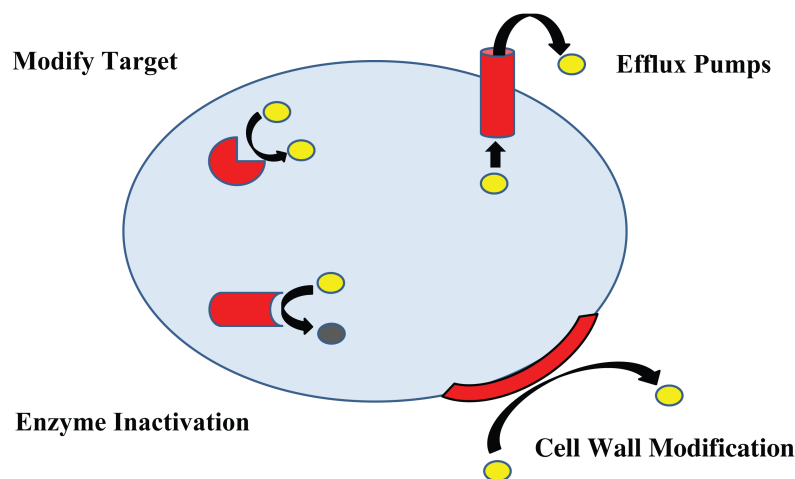


Figure 3. Mechanisms of antibacterial resistance. The four main mechanisms of antibacterial resistance acquired by plasmid acquisition and create MDR isolates. Bacterial isolates may have one or a combination of these mechanisms to persist following treatment.

Efflux resistance. Bacterial efflux pumps create the greatest resistance for MDR bacterial isolates. These pumps create resistance towards multiple classes of antibiotics, in addition to general toxins such as ethidium bromide and dyes.(87, 88) Bacteria have intrinsic constitutively expressed efflux pumps and acquire genetic material that allows for increased efflux pump expression.(89) The ability to extrude a large variety of chemically diverse agents makes efflux pumps an attractive drug target to increase the availability of clinically effective antibacterial agents. Efflux pumps are found in both Gram positive and negative pathogens creating broad spectrum resistance towards multiple classes of antibiotics across many species.(90)

There are five main classes of efflux pump types; Small multidrug resistance (SMR), major facilitator (MF), resistance nodulation division (RND), multidrug and toxic compound extrusion (MATE), and ATP-binding cassette (ABC).(91) MDR bacterial species use multiple efflux pumps from more than one class. In addition, the substrates extruded from each efflux pump ranges widely, therefore creating multidrug resistance in each isolate that produces these pumps. Many efflux pumps use the proton gradient of the bacterial membrane to gain energy for extrusion of their substrates. The efflux pumps that use the proton gradient to extrude their substrate include: SMR, RND, and MF families.(91) On the other hand, the MATE efflux pumps are driven by coupling sodium import to export toxins and the ABC pumps use ATP to drive efflux.(91) Substrate extrusion using efflux pumps always begins with the substrate binding to the recognition region of the efflux protein. This binding causes a conformation change in the efflux pump structure consequently allowing the toxin to be released into the extracellular environment.(91) The diversity of efflux pumps that respond to similar substrates is advantageous for the development of novel inhibitors that mimic the efflux substrates, but have more efficient binding

affinity. This opens the possibility for broad spectrum inhibition of multiple efflux pumps across many bacterial species with one efflux pump inhibitor. With the diversity of substrates extruded by efflux pumps, inhibition by a novel efflux pump inhibiting compound would allow the return of many obsolete antibiotics and help battle the difficult to treat MDR pathogens.

The importance of efflux pumps in MDR bacterial species extends beyond antibiotic extrusion and this must not be overlooked. In addition to extruding many toxins, efflux pumps of many species are also involved in extrusion of quorum sensing molecules.(92) In particular, it was found that *P. aeruginosa* has additional purposes for the MexAB-OprM efflux system, including quorum sensing molecule extrusion. Specifically, MexAB-OprM exports 3-OC12-HSL molecules to communicate and coordinate cells in a community to begin biofilm formation.(93) Efflux inhibition of the MexAB-OprM efflux pump therefore decreases the robust formation of biofilms of *P. aeruginosa*.(94) It has also been revealed that *E. coli* uses efflux pumps while establishing catheter biofilms, and it is these infections that are most difficult to treat.(95) This reveals efflux pumps play an important role in the formation of biofilms, which allow for the bacterium to protect itself from the immune system as well as antibiotic treatment.(93)

Biofilm formation. Biofilm development during a bacterial infection is the leading cause of chronic reoccurring infections, creating high medical costs and elevated mortality rates in hospitals today.(96) It has been determined that most chronic infections are from bacteria embedded in a biofilm formation during a bacterial infection.(96) Biofilms are bacterial aggregates that are physically fused together by excreting a collection of biomolecules called extracellular polymeric

substances (EPS) to protect themselves from identification from the host immune and eradication with antibiotics. In fact, it has been reported that around 90% of biofilm mass is the EPS, not the bacteria themselves.(97) Biofilm formation occurs in a series of steps: attachment, cell to cell adherence, exopolymer production, maturation, and dispersal (**Figure 2**).(98) During the attachment phase, a single bacterium begin to attach to a solid surface, while the second phase is cell to cell attachment to bind the bacteria together as a community.(98) Once cells are in close proximity and attached to each other, they begin to excrete EPS, such as polysaccharides and extracellular DNA.(99) The final steps are maturation and dispersal during which the biofilm has become a complex community of cooperative bacteria that are resistant to both antibiotics and the human immune responses.(100)

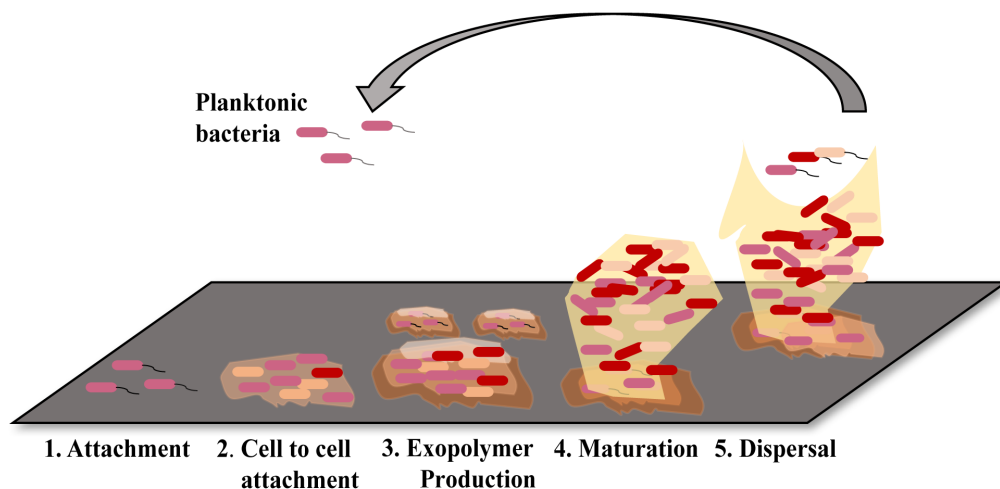


Figure 4. Steps of biofilm formation. The figure above shows the 5 main steps leading to complex biofilm formation. The grey arrow emerging from step 5 indicated biofilm dispersal of planktonic cells.

The role of biofilm formation on drug resistance and chronic infections is paramount. These biofilm communities are found on implanted devices such as catheters, implants, and heart valves, as well as being found on non-implanted surfaces of the lungs of cystic fibrosis patients.(96) As of today, there are no clinical antibiotics that are able to effectively penetrate and eradicate bacteria living within a mature biofilm.(96, 101) Furthermore, with advances in medical technology more implanted devices are being used resulting in more biofilm infections. Biofilms slow the rate of diffusion of antibiotics to the cells within the matrix and allow for only a fraction of the treatment to reach the cells, which is key to their survival.(100) In addition, the biofilms have a diverse heterogeneity that includes persister cells within the biofilm that grow at a decreased rate creating an intrinsic resistance to antibiotics targeting the mechanisms of replication.(102) The heterogeneous biofilm community has diverse roles for each bacterium. Although efflux pumps play a major role in drug resistance of planktonic cells, they are also very important for biofilm development and drug resistance.(93) As the biofilm is forming, the cells communicate through quorum sensing to sense that they are in a high population of cells, begin to attach and excrete EPS.(103) Once the biofilm is formed, the persisters remain deep within the biofilm while the metabolically active cells found on the surface layers overexpress efflux pumps to aid in inhibition of antibiotic penetration into the biofilm matrix.(93)

ESKAPE pathogens. Nosocomial infections are caused by bacteria that infect immune compromised patients in hospitals and experience increased exposure to the available antibiotics used in clinical settings.(104) Therefore, antibiotic resistance is most prominent in these nosocomial infections. These bacteria have developed the most resistance and they are therefore the most difficult to eradicate, in turn causing the highest mortality within the population.(104,

105) A group of six pathogens have been identified to cause the majority of nosocomial infections and resist the actions of clinical antibiotics.(76) These pathogens are collectively termed the ESKAPE pathogens: *E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. cloacae*.(106) The ESKAPE acronym is based on the first initial of their genus names. All of these bacterial species cause fatal infections because their growth cannot be inhibited by the common clinical antibiotics.(76)

The first two bacterial species of the ESKAPE pathogens are Gram positive organisms that have a thick peptidoglycan cell wall but no outer membrane.(76, 106) Specifically, *E. faecium* has been reported to cause 40% of catheter infections and the majority of MDR strains are resistant to vancomycin, ampicillin, and aminoglycosides.(107) This species is significantly more resistant than its sister species *Enterococcus faecalis*.(107, 108) Furthermore, in U.S. hospitals today, *S. aureus* bacteremia has a 20 – 40% mortality rate despite treatment using the available antibiotics.(109) This pathogen is of particular concern because it can cause a variety of life-threatening infections, is highly virulent, and can adapt to environmental changes easily.(109)

The Gram negative species included in the ESKAPE pathogens have a thin cell wall and an outer membrane that causes decreased penetration by antibiotics. These species have become so resistant that the last resort antibiotic colistin is often prescribed because nothing else will eradicate the infection.(76) *K. pneumoniae* is the origin of *Klebsiella pneumoniae* carbapenemases (KPC), which is carried on a mobile transposon, and has been successfully transferred to many other Gram negative bacteria. The *K. pneumoniae* isolates that produce KPCs cause infections are resistant to

carbapenems.(75) These are one of the last classes of antibiotics effective against this species and therefore KPC producing *K. pneumoniae* isolates have a much higher mortality rate. Furthermore, multidrug resistant (MDR) and Pan-drug resistant (PDR) isolates of *A. baumannii* have increased in occurrence and accompanied by 30-75% mortality rates.(110) This pathogen is of importance because of the devastating infections that occur in U.S. soldiers with combat related injuries.(111) Soldiers fighting in Iraq become infected while recovering from traumatic injuries and acquire deep wound infections, respiratory infections, osteomyelitis and bacteremia.(111, 112) *A. baumannii* is a genetically diverse species due to its natural competence and ability to integrate exogenous DNA.(113, 114) In addition, *P. aeruginosa* is referred to as the “holy grail” target for antimicrobial testing because of its extreme resistance.(106) This pathogen has gained multi-drug resistance quickly because it has inherent biofilm mediated resistance and a developed ability to acquire resistant determinants.(115) For *A. baumannii* and *P. aeruginosa*, there are isolates identified that are already resistant to every antibiotic with the exception of colistin.(106) Lastly, *E. cloacae* is the most common *Enterobacter* species, and is the cause of the majority of nosocomial bloodstream infections. This species has multiple resistance determinants, including extended β -lactamases and carbapenemases, which render the majority of the antibiotic classes ineffective.(116, 117)

The search for effective treatment options for the multi-drug resistant ESKAPE pathogens continues as the occurrence of PDR isolates rises.(106) These pathogens all have high levels of intrinsic resistance and the ability to accumulate individual resistance determinants. However, the absence of novel drugs to combat the ESKAPE pathogens in combination with the increasing resistance rates has created a nightmare scenario.(106) The problem is so severe that the measures

of targeting these pathogens must be shifted from discovering novel antibiotics to preserving the antibiotic arsenal we are rapidly losing.(106)

Post antibiotic era. With the high level of resistance development in bacteria today, many of the antibiotics we have discovered in the golden age of antibiotics are no longer effective to treat MDR species.(118) The steady rise in antibiotic resistance can be attributed to many factors such as: the overprescribing of antibiotics in the clinical setting, lack of public knowledge about the overprescribing, increased use in the agricultural industry, and innate resistance development that would occur even before human interaction.(118) In addition to the increased resistance, the simultaneous decrease in pharmaceutical development of antibiotics has created a catastrophic problem that will threaten mankind in the 21st century.(79) For example, in 2004 a mere 1.2% of drugs in clinical development in the top 15 pharmaceutical companies were antibiotics.(118) The combination of increased resistance and lack of therapeutic development has created an apocalyptic scenario for our future, coined the post-antibiotic era. This is the point where we will no longer have treatments for bacterial infections and minor infections will become lethal again.(11) This will effectively change the face of modern medicine as we know it because much of our medical surgical advances are dependent on prophylactic antibiotic treatment.(55, 76) Recent statistical analyses have estimated that 10 million people will die each year due to MDR infections by 2050 if we do not find more effective therapeutic options.(119) Unfortunately, for certain isolates of the Gram negative species *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* this “post-antibiotic era” has already become a reality.(75, 120) For these species, polymyxins have been the last line of therapeutics to treat the extremely resistant infections.(121) However, the *mcr-I* gene encoded on a plasmid has allowed for mobilized efficient transfer of polymyxin resistance

across between species and genus.(122) The *mcr-1* gene was first identified in China in 2015, and has since been rapidly spreading across the world leading to the spread to at least 18 countries as of 2016.(121, 123)

Antimicrobial Agents. The golden age of antibiotic drug discovery began when Selman Waksman developed methods for screening *Streptomyces* species for antibacterial activity after Alexander Fleming accidentally discovered penicillin.(81) Resistance toward these natural products began to appear rapidly. Consequently, chemists began creating modified versions of the original antibiotic and these new analogs were more effective than the parent compounds.(81) During the 1960's the discovery rate was keeping up with the resistance development, but discovery of novel classes of antibiotics have been on the decline ever since. The discovery of the lipopeptide daptomycin in 1986 was the last new class of antibiotic to be discovered even though it was not approved for clinical use until 2003.(81) The effects of the lack of discovery began to take hold in the 1990's when challenges with *in vitro* cell free target screening methods translating to activity in cell based assays became problematic. Additionally, the pharmaceutical industry's adoption of Lipinski's rule of five created a major challenge to identify novel antibacterials because in order to inhibit bacteria effectively the compound physiochemical properties must fall outside the rule of five.(81) (124) Furthermore, the toxicity of antibiotics is problematic with the high doses needed to eradicate infections and the income earned from these discoveries was modest with the high rate of resistance development and short term treatment.(81)

Synthetic small molecules. Synthetic small molecules are organic compounds with a molecular weight of no more than 500 daltons that are designed to aid in a biological process.(125) This allows for rapid diffusion across cellular membranes and more effective oral bioavailability.(126) Most pharmaceutical drugs are small molecules due to these excellent physiological properties. Many natural products are small molecules, for example polyketides, terpenes, and phenazines.(127) The first therapeutics used to chemically treat illnesses were small molecule natural products.(125) One would assume that synthetic small molecules would be better at fending off bacterial resistance, however bacterial efflux pumps are ubiquitous and promiscuous and small molecules fare no better than the natural products.(128) This is a major challenge with small molecule development, and it is difficult to achieve biological relevance, a trait inherent to natural products.(129) To address this issue, chemists are using the diverse chemical space found with bioactive natural products to develop Diversity-Oriented Synthesis (DOS) techniques to increase the molecular diversity of small molecules and increase the antimicrobial properties.(129)

Natural products. The exploration of natural products for the discovery of novel antibacterials agents has historically been a remarkably productive approach.(130) Nature is replete with a large number of pharmacophores and high degree of stereochemistry, which leads to a greater number of hits in screening libraries. In addition to being biologically active, natural products typically have drug-like properties, which allows for successful entrance into clinical trials.(130) Indeed, natural products extracted from fungal or bacterial species have been the primary source of antibiotics since the discovery of penicillin in the 1940s. During the golden age of antibiotic development (1940-1960) a wealth of new antibiotics, with outstanding properties and efficacies were discovered, many of which are still in use today. Following this time, synthetic chemistry

began to find a foothold in antimicrobial drug development, but largely based on chemical scaffolds isolated from nature.(131) Despite this, even up to the 1990s, it was still the case that >80% of antibiotics being discovered were from natural products or analogs designed from them.(68) More recently, in the last 20 years, there have been 12 new natural product antibiotics, from five different structure classes, launched into clinical trials; while 10 new synthetic compounds were developed, but from only two structure classes (and with quinolones accounting for 9 of these 10).(132) Collectively, however, regardless of the source, a void now exists in the antibacterial drug discovery space, with only three new antibacterial classes having been identified since 1970: mupirocin, linezolid, and daptomycin. Ultimately, without new strategies, and new approaches for the discovery of novel therapeutics targeting drug resistant pathogens, the prospect of a post-antibiotic era is close at hand.

A major explanation for the current lack of available antimicrobial therapeutics stems from the observation that natural product drug discovery campaigns require increasing numbers of samples to be screened in order to find novel compounds using traditional methods.(133, 134) This has resulted from the relative exhaustion of obvious natural product reservoirs, leading to the continued re-identification of known chemistry. Encouragingly, it is predicted that only 10% of the world's biodiversity has been assessed to date, leaving many potential therapeutics waiting to be discovered.(134)

Antimicrobial peptides. Like natural products, antimicrobial peptides evolved within all living organisms to combat infections.(135) The primary mechanism of these small cationic molecules

is to rapidly disrupt the bacterial cell membrane.(135) Human antimicrobial peptides including defensins and cathelicidin (LL-37) have roles in not only antimicrobial killing, but also inflammation, immune activation, and wound healing.(135) Therefore, antimicrobial peptide development can be targeted towards: anti-infectives, synergistic therapeutics with conventional antibiotics, immunostimulatory agents, and endo-toxin neutralizers to decrease septic shock.(135) However, our understanding of antimicrobial peptides needs to be further developed in order to fully exploit this chemical class.(135) Peptide antibiotics have two subsets: non-ribosomally synthesized peptides and ribosomally synthesized peptides.(136) The ribosomally synthesized peptides are host defense molecules that organisms produce to protect themselves from invading bacteria.(136) On the other hand, non-ribosomally synthesized peptides such as gramicidin, polymyxins, bacitracin, and glycopeptides are mainly produced by bacteria to eradicate surrounding bacteria in order to compete for survival.(136) These peptides contain two or more amino acid moieties within their structures and are synthesized on multi-enzyme complexes rather than ribosomes.(136) Although not all are broad spectrum, these antibiotics inhibit Gram positive and negative species by disrupting the cell membrane.(136) Problems inherent with antimicrobial peptides have made it difficult to progress through clinical trials. These problems include but are not exclusive to: toxicity problems, pharmacokinetic issues, and decreased activity in vivo because of proteolysis and pH changes.(135)

Combinatorial chemistry. Solid phase synthesis was developed in 1963 by Merrifield and colleges to allow for the synthesis of libraries of small organic molecules.(137) Twenty years later this approach was utilized to synthesize combinatorial libraries.(138) In 1990, Dr. Richard Houghten developed the tea bag solid phase synthesis method to create combinatorial peptide

libraries and since then this approach has been broadly utilized to assess large chemical space relatively quickly.(139, 140) The purpose of this approach is to allow for the synthesis of structurally diverse chemical libraries to be screened at once in a high throughput therapeutic screening.(138) This effectively allows for the synthesis of millions of compounds in the same amount of time it takes to synthesize one compound. Therefore, this increases the speed of the discovery process of generating a lead or optimizing a previous lead compared to traditional approaches that screen one compound at a time.(138) In general, there are two approaches to synthesize combinatorial libraries: the biological approach and the spatially addressable parallel solid phase approach.(138) Solid phase combinatorial libraries are synthesized on a monolithic support to allow the chemist to identify the composition of the molecule from its position.(141) There are four types of spatially addressable parallel solid phase synthesis methodologies: multi-pin, tea bag, SPOTS membrane, and light directed peptide synthesis on resin support.(138) Even though these approaches were developed and initially used to screen peptide antigens for recognition by monoclonal antibodies, it was quickly realized that these approaches could be utilized to synthesize different compounds, including heterocycles.(142) Additionally, Houghten and colleagues were able to develop positional scanning libraries to allow for extensive structure activity relationship (SAR) analysis.(142) This allows for information about the activity created from each functionality for each position of the library.

Screening Approaches and Methods. High throughput discovery of hit molecules that could lead to antibiotic candidates mainly occurs in academic setting and not by the pharmaceutical industry.(82) Conversely, drug development to ensure efficacy and safety is performed mainly in a clinical setting by the pharmaceutical industry in order to bring an Investigational New Drug

(IND) to the market.(82) From the decrease in both discovery and development, we are now slowly reaching a post antibiotic era where clinical antibiotics used today will no longer be effective and the mortality rates due to resistant infections will reach astronomical heights.(11, 76, 143) In recent years there has been a shift in drug discovery to increase innovation using genomics, and this is where academic based drug discovery groups are beginning to make their mark.(144, 145) It is now accepted that industry has not realized the potential of academic discovery in the past and there is great promise in linking industry with academia to fully exploit the strengths of both sides.(144) There are two general approaches to screening chemicals for antimicrobial activity: bioactive guided screening and target-oriented screening.(146)

Bioactive-guided screening. Most antibiotics to date have been discovered using bioactive guided screening of natural products.(130) This approach is also called classical pharmacology, forward pharmacology, or phenotypic drug discovery.(146) Bioactive-guided screening involves testing crude natural extracts or purified chemicals for bacterial inhibition using whole cell-assays. Using this approach, the target of the chemical is not known as the minimal inhibitory concentration is used to identify the most active chemicals.(146) The benefits of bioactive-guided screening is that the activity can be effectively translated into therapeutic treatments for bacterial infections.(147) A fallback of this approach is that it is difficult to determine the molecular mechanism of action of these therapeutics once a lead agent is discovered.(147) This approach is optimal for natural product screening, however recent advances in drug discovery platforms have been introduced to apply a more hypothesis driven approach to natural product screening to decrease the occurrence of re-discovery that comes with bioactive-guided screening.(148)

Target-based screening. Target-based antibiotic screening has been widely used since the advent of genomic analyses in the 1990's.(147) The goal of this approach is to identify purified chemicals that inhibit the activity of a known target in an in vitro cell free assay. This approach is not optimal for screening crude natural product extracts but has been used with combinatorial chemistry screening.(146) The difficulty in using target-based screening is the identified hit compounds often do not translate into therapeutically relevant compounds that inhibit the bacteria in whole cell assays.(147) Furthermore, the pharmaceutical industry has been successful in finding effective therapeutics using bioassay-guided screening in the past and therefore it has been suggested that the target-based screening methods are contributing to the decrease in success of research and discovery (R&D).(147)

Project Aim. In recent decades, the decrease in effective treatments for drug resistant bacterial infections has created a catastrophic problem for the future of mankind. In the 21st century we will reach a point where the antibiotics that were used in the 20th century will no longer be effective towards treating pan-drug resistant bacterial species. With this information at hand, the pharmaceutical companies are still not investing the time and finances needed to revert this situation. Therefore, the innovative screening necessary to discover novel therapeutics for drug resistant bacteria is in the hands of academic institutions. Combinatorial chemistry is one approach used by academic institutions to increase the rate of discovery of novel therapeutics by assessing a large diversity of chemical space in a short amount of time when compared to traditional approaches. Furthermore, collaborative efforts of medicinal chemists and microbiologists are needed to facilitate the progression of novel therapeutics into clinical trials. Accordingly, the aim of this project is to reveal the power of combinatorial chemistry in expediting the discovery of

novel therapeutics in the form of antibacterial agents and anti-resistance agents. In addition, the project will highlight the importance of collaborative efforts with medicinal chemists to increase the spectrum of activity of novel therapeutics. Together, these efforts show the necessary approaches for academia to successfully bring new antibiotics to replace the obsolete 20th century alternatives.

CHAPTER 2: GUANIDINE ANTIBACTERIALS

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CHAPTER 3: POLYAMINE ANTI-RESISTANCE AGENTS

Note to Reader. This chapter has been submitted to PLoS ONE journal and is currently in review (Fleeman et al., 2017). The submitted manuscript can be found in Appendix 2.

CHAPTER 4: QUINAZOLINE ANTIBACTERIALS

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CHAPTER 5: FINAL DISCUSSION

Final Discussion. In this work, we have shown the wide applicability of combinatorial chemistry when discovering both antibacterial agents and anti-resistance agents. The strength of this approach lies within the positional scanning library utilized by Torrey Pines to create structure activity relationship profiling assessing the active components of combinatorial libraries.(139) This important step in combinatorial chemistry creates advantages above previous iterations of deconvolution methods designed to progress from complex libraries to individual compounds.(151, 152) Our initial analysis of the Torrey Pines combinatorial libraries revealed the bis-cyclic guanidine scaffold as the most broad spectrum antibacterial library that inhibited all six ESKAPE pathogens at low concentrations. Continuing assessment of the combinatorial libraries led us to discover that the same set of libraries can be used to develop a completely different type of therapeutic agent, therefore revealing the benefits of using combinatorial libraries to assess a large chemical space.(153) Finally, our medicinal chemistry approaches proved successful to increase the spectrum of novel quinazolines from Gram positive *S. aureus* inhibitors to Gram negative *A. baumannii* inhibitors. This is a great success in medicinal chemistry considering the difficulties with penetration and retention into the Gram negative intracellular space.(154-156)

In our initial study of the Torrey Pines combinatorial libraries we utilized bioassay-guided or phenotypic assays to determine bacterial growth inhibition. This revealed that of the 37 combinatorial libraries assessed, the bis-cyclic guanidine scaffold had the best broad spectrum

inhibition of the ESKAPE pathogens.(149) This was very intriguing because of the vast utilization of guanidine molecules in biological systems stemming from a rich chemodiversity, allowing for a wide range of therapeutic applications.(157) The biological functions of natural guanidino compounds discovered to date include: convulsant activity; hypoglycaemic activity; antihypertensive activity; and most relevant to our study, antibacterial and antitumor activities.(158) Heterocyclic guanidine compounds, including derivatives of imidazoles, pyrroles, pyrimidines, and purines, have displayed the best antibacterial and antitumor activities.(158) Furthermore, it is the lysine and arginine substituents on natural occurring antimicrobial peptides that allows them to target bacterial cells, specifically the highly negative charged Gram negative bacterial outer membrane.(159) The most common therapeutic guanidines are Polyhexamethyleneguanidine (PHMG) derivatives that are widely used as antiseptics. PHMG can be detoxified to create polyhexamethylene biguanide hydrochloride (PHMB-H) and incorporation of this scaffold with anions increases water solubility.(160) PHMB-H clinical studies have revealed this agent consistently inhibits oral bacterial counts and subsequent dental plaque regrowth.(161)

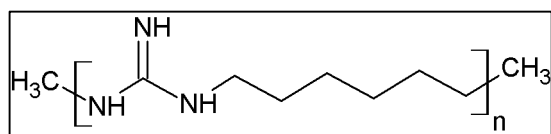


Figure 5. Structure of PHMG. The figure above shows the structure of polyhexamethyleneguanidine (PHMG). There are many derivatives of this compound with varying chain length, indicated by []_n. These derivatives are used as antiseptics.

The most interesting clinical guanidine with great similarity to our bis-cyclic guanidines from this work is pentamidine, a derivative of synthalin, used to treat African sleeping sickness.(162) Synthalin was used in 1926 as an antidiabetic drug and its structure consists of two non-cyclic diguanines groups separated by a polymethylene chain.(163) Further synthalin research in 1937, lead to the discovery of trypanocidal properties towards *Trypanosoma brucei*.(164) Modifications by the English chemist Arthur James Ewins, lead to less toxic cyclic synthalin derivatives and the most promising of these diamidines was found to be pentamidine.(165) Pentamidine Isethionate (NebuPent) is used today in Africa to treat *Trypanosoma brucei gambiense* and *Pneumocystis jirovecii* in Aids patients.(166, 167) In addition, NebuPent is approved by the FDA for an oral inhalant treatment of fungal lung infections caused by *Pneumocystis jirovecii*.(167) With the similarities to the approved NebuPent, we decided the bis-cyclic guanidine would be the best combinatorial scaffold to develop as broad spectrum antibacterial agents.

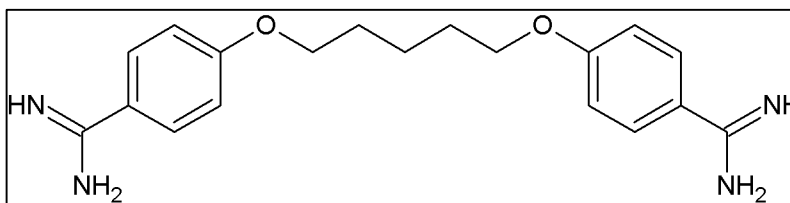


Figure 6. Structure of pentamidine. The figure above shows the structure of pentamidine. This compound is the active agent of the FDA approved NebuPent used to treat fungal lung infections. The compound is a derivative of synthalin, an antidiabetic drug used in 1926. The structure of pentamidine is similar to the bis-cyclic guanidines discussed in Chapter 2. The ends of the compounds have amines and aromatic rings, which are separated by an alkyne linker.

To ensure our identification of broad spectrum activity was not limited to a few bacterial isolates, we tested and found our lead agents to be equally effective towards ten clinical isolates of each ESKAPE pathogen.(149) This is particularly important for the Gram negative species *A. baumannii* and *P. aeruginosa*, where clinical isolates are found to vary greatly in their clinical antibiotic susceptibility.(168) The inconsistent therapeutic treatment outcomes of these organisms with penicillins, cephalosporins, and carbapenems are said to be because of the site of action location within the impenetrable periplasmic space.(169) The bis-cyclic guanidine antibacterials were not only effective at low concentrations, but were extremely bactericidal towards all 6 ESKAPE pathogens at concentrations just above their respective MICs. This is a beneficial attribute because bacteriostatic antibiotics are known to have little efficacy treating endocarditis, therefore creating a need for bactericidal antibiotics.(170) It is for this reason that the most recent bactericidal antibiotics approved by the FDA, ceftolozane/tazobactam (Zerbaxa) and ceftazidime/avibactam (Avycaz), are referred to as the *superheroes of Gram negative bacteria*.(171)

The positional scanning libraries were the key to understanding how to increase the antibacterial activity of the guanidine scaffold towards Gram negative species.(142) Using Canvas cheminformatics techniques to generate physiochemical properties necessary for broad spectrum activity, we were successful in determining the broad spectrum activity of the bis-cyclic guanidines was linked to increased molecular weight, AlogP (lipophilicity) values, and rotatable bonds.(172) This was a crucial finding to link the superiority of our guanidines over the approved pentamidine. Pentamidine has been shown to permeabilize Gram negative cells with its cationic nature but does not have the lipophilicity that is necessary for growth inhibition.(173) Specifically, pentamidine

has a molecular weight of 340 Da, an AlogP of 2.66, and 10 rotatable bonds;(174) while our work revealed the physiochemical properties for Gram negative activity are molecular weights above 600 Da, AlogP values higher than 8, and more than 16 rotatable bonds. Our findings are in line with recent work revealing that amines and hydrophobicity are necessary physiochemical attributes for antibiotic penetration and retention into the Gram negative intracellular environment.(175) The most famous example of a clinical antibiotic that adheres to these physiochemical rules is colistin, the last resort polypeptide that attaches to the Gram negative outer membrane utilizing a positive charge and penetrates the inner membrane with its lipophilic tail.(176) Investigation of colistin led to the finding that both chemical properties are necessary for antimicrobial activity towards Gram negative species.(176) This hydrophobicity dependency is also observed with cationic antimicrobial peptides, where increased activity towards *E. coli* was found to be a direct effect of the hydrophobic properties of the peptides.(135, 177) Recently, the interest in antimicrobial peptides has grown because of their strong Gram negative antibacterial activity.(136) However, the toxicity and instability of cationic peptides is currently inhibiting progression of this class of antibiotics through clinical trials.(178) Perhaps our bis-cyclic guanidine can offer a less toxic small molecule counterpart to such cationic antimicrobial peptides.

The ESKAPE pathogens had a low propensity for resistance development towards the bis-cyclic guanidines compared to clinical antibiotics.(82) This is perhaps suggestive that multiple spontaneous mutations are necessary to overcome their antimicrobial actions.(82, 179) This is of interest for new therapeutic development and compounds with more than one mechanism of action are now actively pursued.(82, 179, 180) This multi-targeting was first shown with cationic antimicrobial peptides that have similar physiochemical properties to our guanidine lead

agents.(181) Cationic antimicrobial peptides have been found to disrupt the membrane and inhibit intracellular synthesis of biomolecules.(182-184) In fact, it is the amphipathic nature of cationic antimicrobial peptides that allows for these two mechanisms of action.(185) This would explain the lack of bis-cyclic guanidine spontaneous mutations and the low propensity for resistance accumulation. With our knowledge of bacterial resistance development towards antibacterials having more than one mechanism of action, we hypothesize the bis-cyclic guanidine antibacterials have more than one molecular mechanism of action. In addition to low resistance development, we revealed the bis-cyclic guanidines had great specificity towards bacterial cells resulting in a lack of toxicity towards eukaryotic cells. This is another similarity to antimicrobial peptides, which are more attracted to the negatively charged bacterial cell than the neutral charged eukaryotic cell.(183) Furthermore, it has been shown that this specificity can be increased by systematically modifying the cationic and hydrophobic properties.(178)

Our *in vivo* efficacy studies with the bis-cyclic guanidine antibacterial agents were successful in rescuing mice from an otherwise lethal dose of *S. aureus*.(149) This is the ultimate success of the bis-cyclic guanidine over cationic antimicrobial peptides because there are many problems associated with translating *in vitro* activity of amphipathic peptides into *in vivo* efficacy.(186) Overall, this work reveals the potential therapeutic applications of the bis-cyclic guanidines. They have similar attributes to the already approved NebuPent, however the bis-cyclic guanidines physiochemical attributes create potential for Gram negative lung infection applications, opposed to the fungal lung infections for which NebuPent is prescribed.

Next, to fully exploit the utilization of combinatorial chemistry, we screened the same set of combinatorial libraries in a modified bioassay to identify anti-resistance agents towards *P. aeruginosa*, a species that attains much resistance because of efflux pumps.(187, 188) The modified bioassay used was a checkerboard assay that allows for the identification of adjuvant compounds with no antibacterial properties alone but potentiate the activity of ineffective antibiotics.(189) The checkerboard bioassay is widely used to define synergy between two inhibitory agents and has been successful for identifying synergistic antibiotics.(190-192) However, we modified the readout of the checkerboard assay by utilizing a potentiation calculation, not the fractional inhibitory calculation (FIC) that traditional checkerboards use to quantify synergy because we were interested in adjuvant compounds.(193) This was important because efflux inhibition does not lead to antibacterial activity alone, therefore any antibacterial properties would be the result of off-target effects.(194) Of the three classes of antibiotic adjuvants, efflux pump inhibitors belong to class 1.A, which are compounds that inhibit mechanisms of resistance.(195) However, the only adjuvants that have successfully progressed into clinic therapeutics are β -lactamase inhibitors and aminoglycoside kinase inhibitors.(195) Currently there are few clinical studies on efflux pump inhibitors and these are limited to the adjuvant activity of omeprazole to activate amoxicillin towards *Helicobacter pylori*.(196)

Our checkerboard bioassay of the combinatorial libraries lead to the discovery of potential adjuvant polyamine library. Polyamines are cationic and aliphatic small molecules that have been found to have many therapeutic benefits.(197) Naturally occurring polyamines are essential for many biosynthetic pathways and play a role in fighting off infections in host organisms, however the catabolism of these molecules are toxic and therefore tight regulation is necessary.(198, 199)

In fact, targeting polyamine biosynthesis of spermine and spermidine in cancer cells has shown promise in cancer therapeutics.(200) More relevant to our study, the search for potentiators of antibacterial activity has revealed the natural polyamines, spermidine and putrescine were able to potentiate antimicrobial activity of chloramphenicol, nalidixic acid, and trimethoprim towards *P. aeruginosa* without having membrane deleterious effects.(201) This, in combination with the finding that natural polyamines are extruded from cells through efflux pumps, suggest polyamines inhibit efflux through competitive inhibition, not membrane depolarization.(202) It is important to note that some polyamines have been shown to permeabilize membranes, however this function is largely dependent on the acyl chain length of the polyamine.(203) The potential membrane activities of exogenous polyamine treatment lead us to be meticulous in our secondary assay screening of the polyamine efflux pump inhibitors.(203) Taking note of potential toxicity issues and keeping in mind the many therapeutic benefits of polyamines, we were confident the pursuit of this library could be beneficial, and that the application of medicinal chemistry during the hit-to-lead process could reduce toxicity issues by protecting the molecules from degradation that creates reactive aldehydes.(199, 204)

Lead polyamines were chosen with the information learned from the positional scanning library and when tested they revealed a dose dependent potentiation of tetracycline activity towards *P. aeruginosa*. This was interesting because our polyamines resemble the known efflux pump inhibitor phenylalanine arginine β -naphthylamide (PA β N) more than the other well-known control efflux pump inhibitor 1-(1-naphthylmethyl)-piperazine (NMP), which has been shown to be ineffective towards *P. aeruginosa*.(205) In fact, ligand binding of MexAB and AcrB in co-crystal structures suggest that there are potential mechanistic differences between these two RND family

efflux pumps.(206) This would explain the findings by Sjutts and colleges, who identified a pyranopyridine inhibitor, MBX2319 with potent activity towards Enterobacteriaceae, but no activity towards *P. aeruginosa*.(207) However, in a different study recently published in Nature, pyridopyrimidine derivatives were shown to bind the distal pocket of both MexAB and AcrB.(208) This suggests that pyridopyrimidine efflux inhibitors have broader applications than pyranopyridine inhibitors.(207) Further investigation revealed our lead polyamines have broad spectrum activity similar to pyridopyrimidines that have been found to not only potentiate tetracycline but also chloramphenicol and norofloxacin, whereas the pyranopyridine MBX2319 was shown to only inhibit ciprofloxacin, levofloxacin, and piperacillin.(209)

Our initial secondary validation began with a widely-used direct measure of efflux inhibition following ethidium bromide florescence with and without the efflux pump inhibitors.(197, 210-214) Our polyamine efflux pump inhibitors were successful to increase accumulation of ethidium bromide, similar to the positive control PA β N. Another important factor in using this assay is that is has wide applicability in both Gram positive and negative bacterial species.(215) We found a similar increase in fluorecence across both Gram negative and positive species. This is exciting because only a few adjuvants, namely antipsychotic phenothiazines (promazine and chloropromazine), have shown broad spectrum anti-resistance properties.(216-218)

Membrane depolarization assays validated our polyamines are not disturbing the *P. aeruginosa* cell membrane like PA β N that behaved similar to the positive depolarizing agent nisin.(219) This is an extremely important to revealing the specificity of our polyamines towards efflux pumps

because many efflux pump inhibitors identified depolarize the membrane gradient that efflux pumps use to function.(220-224) In line with our findings, PABN has been shown to disrupt the bacterial cell membrane, which in addition to toxicity issues, stopped further development of this agent into clinical development.(222, 225, 226) Furthermore, a 2017 study by Machado and colleges identified a phenylquinoline efflux pump inhibitor PQQ4R that inhibits efflux through depolarization of the bacterial cell membrane similar to PABN.(224) In addition to secondary effects on bacterial membranes, many identified efflux pump inhibitors, such as verapamil, thioridazine, and reserpine have activity towards both prokaryotic efflux and eukaryotic channels.(227) Calcium channel inhibitors can be extremely toxic, as increased dosages can cause cardiac arrest, making them problematic as therapeutic agents.(228) Therefore, we were pleased to see that our polyamine efflux pump inhibitors still allowed for calcium channel activity in a eukaryotic cell line when probed with a calcium channel stimulator. In addition to lack of inhibition of calcium channels, the polyamines had little general toxicity towards both HepG2 and Hek293T cell lines. This was very important to show the polyamines are not creating reactive aldehydes, which leads to general eukaryotic cellular death.(199) The work shown here will help guide the future hit-to-lead optimization of polyamine adjuvants in order to reactivate obsolete therapeutics.(90, 102)

Following the initial discovery of hit compounds that inhibit multi-drug resistant bacteria, it is necessary to maintain an iterative collaboration with medicinal chemists for hit-to-lead optimization. For this approach, the medicinal chemist modifies the scaffold of the original hit in a systematic manner to obtain a well-developed SAR. This can be done through target-based assays using known binding pocket physiochemical properties or by following bio-assay results of the

modified compounds.(229) Chapter 4 is an example of a bio-assay guided hit-to-lead endeavor. Quinazolines have been known to have a wide range of therapeutic applications towards both eukaryotic and prokaryotic organisms.(230-238) This can lead to problems because this wide range activity can cause significant off-target effects and toxicity in humans.(239, 240) It is therefore necessary to have a strong medicinal chemistry approach to optimize the scaffold for antibacterial activity. This study was based on the utilization of bio-assay guided SAR to expand the activity of our original MRSA active quinazolines to include Gram negative species inhibition.(241). Recent efforts in synthesis of benzoxazolyl, benzothiazolyl, and benzimidazolyl quinazoline derivatives found benzothiazolyl derivatives were active towards *S. aureus* and benzimidazolyl derivatives were active towards *Aspergillus niger*, however none of these analogs inhibited the Gram negative species tested.(242)

Our N^2, N^4 -disubstituted quinazoline-2,4-diamines are unique from other quinazoline DHFR inhibitors because the 2- and 4-amino groups are alkylated causing steric hindrance and prevents deep insert into the mammalian DHFR enzyme pocket.(243) Another study probed benzenoid ring of the sterically hindered quinazoline scaffold, which is hypothesized to be the bacterial dihydrofolate reductase (DHFR) binding region, and successfully identified 5-substituted 2,4-diaminoquinazolines with activity towards *E. coli*. (244) However, the acquired activity towards *E. coli* did not exceed that of the clinical DHFR inhibitor trimethoprim. Perhaps this is because of lack of penetration into the Gram negative intracellular space as it has been found that quinazolines acting as DNA gyrase inhibitors are more active when adding 6-position substituted allowing for better cell penetration.(245, 246) Therefore, our analysis began by systematic probing of the 6- and 7- positions instead of the 5-position of the benzenoid ring of the N^2, N^4 -disubstituted

quinazoline-2,4-diamine scaffold to optimize coverage the hypothesized DHFR binding region while simultaneously increasing Gram negative inhibition. Our collaborative efforts were successful to create activity towards *A. baumannii*, revealing the promise for continuing efforts to identify effective therapeutics towards additional Gram negative pathogens

Our initial SAR revealed placement of any substituent at the 6-position of the benzenoid ring when compared to placement at the 7-position resulted in increased antimicrobial activity towards clinical isolates *A. baumannii*. Similar studies with antibacterial 2,4-diaminoquinazolines with steric hindrance by Lam and colleges in 2014, focused on 7-substituted 2,4-diaminoquinazolines based on the success of iclaprim (ICL).(247) However, their SAR was guided by molecular docking specifically avoided the 6-position substituents to increase specificity to the bacterial enzyme and therefore these efforts only succeeded in increased activity towards the Gram positive species *S. aureus*.(247) However, this 7-position may not lead to the specificity mentioned above because in an anti-parasitology study, Van Horn and colleges found antileishmanial activity with 7-position substituents of the N^2, N^4 -disubstituted quinazoline-2,4-diamine scaffold.(150, 235, 248-250)

With confidence that the 6-position would increase the Gram negative activity, we continued to optimize the 6-position by first adding lipophilic modifications to further probe the chemical space necessary to create a significant increase in antibacterial activity. These lipophilic groups would probe the potential hydrophobic pocket of the bacterial DHFR target that has led to trimethoprim specificity in the past.(251-253) We were successful with these modifications to increase activity

towards the most resistant *A. baumannii* clinical isolate #1403. Specifically, we found that large lipophilic groups, n-pentyl, n-cyclohexyl, and cyclohexenyl at the 6-position were more effective than the vinyl and ethyl groups, revealing a bulkier group at this position is more effective towards the extremely resistant *A. baumannii* isolates. This was in line with the 7-substituted 2,4-diaminoquinazolines discovered using molecular docking that focused on the hydrophobic region of this pocket to increase bacterial DHFR enzyme binding.(247) Furthermore, a discovery by Bourne and colleges, found that a large hydrophobic moiety substituted on phthalazine scaffold allowed for specificity to *Bacillus anthracis* DHFR.(252) With this information, we felt confident to choose lead agents and continue forward assessing the additional antibacterial characteristics of our quinazolines towards *A. baumannii*.

When testing the lead quinazolines for bactericidal effects on *A. baumannii*, we found it interesting that compounds **4** and **5**, substituted with 6-bromo and 6-methyl group respectively, had the greatest bactericidal effects. These two electron donating chemical substituents when added as *ortho* or *para* directing groups, effectively create a more reactive compound.(254) These electron donating groups are nucleophilic and are known to form hydrogen or covalent bonds with biological targets.(255) In addition, it has been determined that electron donating groups allows for better DHFR inhibitors and this would explain the increased effectiveness of these lead agents.(256, 257) Similar to our findings, Shaikh and colleges discovered when targeting *P. falciparum* DHFR, increased bioactivity when substituting electron donating groups to the 6-position on the phenyl ring of a pyrimethamine scaffold. They found that although the bulky hydrophobic groups are favored, the most beneficial substituents are electron donating groups.(258) Furthermore, because electron withdrawing groups allowed for increased inhibition

of the mammalian DHFR enzymes, those substituents were avoided in our study to avoid off target inhibition of the mammalian DHFR counterpart.(256, 257)

We next tested the quinazolines for the ability to eradicate a pre-formed biofilm of our clinical *A. baumannii* isolate collection and found lead quinazoline **5** was the most effective This was particularly exciting for us because it has previously been shown that 2, 4 quinazoline analogs inhibited biofilm formation of another Gram negative species, *Vibrio cholerae*.(259, 260) Therefore, although not common, our quinazolines appear to be able to disrupt the biofilm matrix and perhaps this is in part because of the electron donating group on lead quinazoline **5**. In fact, it has recently been explained that electron donating groups can create singlet state oxygen, a very reactive state, leading to oxidation of biomolecules (DNA, protein, polysaccharide), which are the main components of a biofilm.(261)

Further testing revealed the lead quinazoline **4** and **5** were the most effective to fend off resistance development than trimethoprim. Trimethoprim resistant comes from a F99Y mutation leading to a tyrosine hydrogen bond in the binding pocket of the DHFR enzymes and this cannot be disrupted with a hydrophobic substituent.(247) Therefore hydrophobic substituted quinazolines are more efficient towards inhibition of wild type *S. aureus* DHFR than the enzymes carrying this mutation, whereas if the substituent is able to hydrogen bond it could disrupt this tyrosine bond.(247) This is a promising finding warranting further development of these two analogs as DHFR inhibitors because trimethoprim resistance is widespread.(262, 263) We also revealed the lead quinazolines displayed low toxicity towards a human HepG2 human liver cell line. Based on the cytotoxicity

LD₅₀ values, it was determined that the largest therapeutic windows belonged to quinazoline lead agents 4 and 5. This was a great success for our SAR analyses because the 6-position substitution was previously said to create greater eukaryotic cytotoxicity.(247)

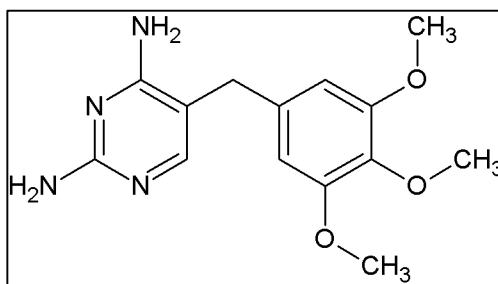


Figure 7. Structure of trimethoprim. The figure above shows the structure of trimethoprim. This compound has been revealed to specifically bind and inhibit bacterial dihydrofolate reductase (DHFR). This is an essential enzyme to produce folic acid and facilitate bacterial replication.

This in addition to the low hemolytic ability of the quinazolines lead us to move forward and test the efficacy of one of our lead agents for treating in vivo murine peritonitis infection. We were pleased to observe that a limited dose of compound **5** (2 mg kg⁻¹) successfully rescued the mice from an otherwise lethal dose of *A. baumannii*. This reveals the benefit of probing the 6-position of the *N*²,*N*⁴-disubstituted quinazoline-2,4-diamine benzenoid ring for Gram negative inhibition because the recent in vivo success of 4(3H) Quinazolines was limited to *S. aureus*.(264) This is possibly because of limited probing of the benzenoid ring as they only tested two analogs with 6-position substituents because they were basing SAR on molecular modeling for PBP inhibitors, not bioassay-guided DHFR inhibitors.(264) This was a very strong conclusion to our initial study to increase the spectrum of quinazoline antibacterials by probing the 6-position of the benzenoid ring of the *N*²,*N*⁴-disubstituted quinazoline-2,4-diamine scaffold to inhibit Gram negative species.

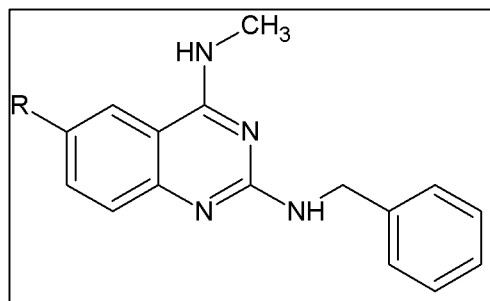


Figure 8. Structure of N^2,N^4 -disubstituted quinazoline-2,4-diamine scaffold. The figure above shows the structure of N^2,N^4 -disubstituted quinazoline-2,4-diamine scaffold with the variant 6-position displayed as R. This variant position is hypothesized to probe the binding pocket of DHFR bacterial enzyme and inhibit folic acid synthesis.

Future Directions. The success of the studies described in this work are a direct result of collaborating efforts of biologist and chemists. The studies outlined in this work are part of a continuing effort to fuel the drug discovery pipeline and each will continue to develop through the upcoming years. Once the initial discovery and hit-to-lead optimization is complete, pharmacokinetic analyses must be performed to create a target product profile (TPP) for the IND application, stating how the compound can safely be administered once accepted into phase I clinical trials.(265) The TPP covers not only the target population intended to be treated by the novel therapeutic, but also the route of administration, dose range, frequency, and duration of treatment.(265) This is a huge endeavor for any therapeutic agent as it is necessary to know all mechanisms of antibacterial activity before the FDA will approve an IND application.(266) To avoid unnecessary expenses, this must be performed in the initial stages of discovery to identify problems before the investment increases.(267)

The guanidine antibacterials are currently being studied using molecular analyses to determine their primary mechanism of action as well as any off-target mechanisms. Our hypothesis is that the bis-cyclic guanidine is inhibiting protein synthesis after penetration of the bacterial outer membrane. Previous work with guanidine anti-infective agents has shown the guanidine moiety is important for ribosomal binding.(268-270) Biaryl guanidines have been shown to inhibit viral translation by blocking the internal ribosome entry site (IRES) and this activity is specifically attributed to the protonated guanidinium groups because activity was lost when a methyl, urea, or thiourea substituent replaced the guanidine core.(268) In an effort to target bacterial translation proteins with little eukaryotic homology, a high throughput screening of chemical scaffolds for inhibition of the essential elongation factors of *S. aureus* was successful to discover N-substituted guanidines, benzimidazole amidines, and Indole dipeptides inhibit activity of *S. aureus* EF-Tu and EF-Ts.(270) Furthermore, in a 2017 study by Komarova and colleges identified guanidylated quinazolines inhibited activity of bacterial ribosome translation.(269) Their SAR analyses revealed modifications to the quinazoline core had small effects on activity while modifications to the guanidine moiety lead to decreased antibacterial activity.(269) Our preliminary data suggests that protein synthesis may be inhibited with our guanidines, both bis-guanidine and mono-guanidine analogs. (Fleeman et al. Unpublished) To validate our initial investigations we plan to use a global proteome cellular thermal shift assay (CETSA).(271-273) This experiment was developed to determine direct and indirect effects of cancer therapeutics in eukaryotic cells. We have optimized this assay for bacterial cells and hope to identify the intracellular binding proteins of the guanidine antibacterials. In addition to this specific in depth analysis, we hope to complement this experiment with cytological profiling to determine the general bacterial response following treatment with both the bis-guanidine and mono-guanidine analogs. Florescence

microscopy has been used for years to investigate cellular responses to inhibitory agents.(274-277) However, in 2013 Pogliano and colleagues found they could identify cellular pathways inhibited by the different classes of antibiotics using principal component analyses that and further use the knowledge gained to identify potential pathways inhibited by a novel therapeutic.(278) Following analysis of the guanidine analogs using these approaches, continuing SAR analyses will be performed in order to develop novel guanidine bacterial protein synthesis inhibitors.

For our polyamine efflux pump inhibitors, we plan on screening individual compounds synthesized based on the polyamine positional scanning library. Once we have confidently identified lead agents, we plan on assessing the lead efflux inhibitor polyamines for potentiation of multiple classes of antibiotics towards multiple bacterial isolates to increase the knowledge gained in this work.(279) Continued secondary validation efforts will include all of the experiments utilized in this work, in addition to mass spectrometry accumulation assessment for the best direct method of assessing compound accumulation, and a hERG potassium channel assessment said to be more sensitive than the calcium channel assay.(280) With this increased knowledge, we hope to progress the individual compounds into in vivo efficacy studies for utilization for combination therapy with an obsolete antibiotic towards a *P. aeruginosa* isolate.

The quinazoline antibacterials discussed in this work are currently being optimized for specificity towards bacterial DHFR enzymes and elimination of the toxicity for developing a TPP. For continuing SAR analogs are being synthesized to eliminate the reactive furfuryl group and replace it with more benign groups in hopes to reduce the toxicity associated with singlet oxygen

production.(281) Molecular docking analyses will be performed to increase the specificity of the quinazolines to the bacterial DHFR enzyme and decrease the affinity to the mammalian DHFR counterpart. There have been differences identified in the pocket dimensions of the human versus bacterial DHFR and we hope to exploit these differences in future quinazolines analogs to increase the specificity of our agents. As we have already identified inhibition of both *S. aureus* and *A. baumannii* clinical isolates, we are approaching formulation for TPP to begin to move past the hit-to-lead stage into pre-clinical investigations. The development of this profile will include multi-parameter optimization (MPO) algorithm application and ADMET analyses to ensure optimal physiochemical properties. This MPO will focus specifically on partition coefficient clogP (≤ 3.0) and distribution coefficient $\text{clogD}_{7.4}$ (≤ 0.2), total polar surface area (100-200 Å²), number of hydrogen bond donors (6-11), hydrogen bond acceptors (2-6), and pK_a (6.1-8.7). ADMET properties for the TPP will include stability using mouse microsomes, absorption testing a caco-2 cell line, and toxicity using human cytochrome P450 inhibition.

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APPENDIX 1:

Combinatorial Libraries as a Tool for the Discovery of Novel, Broad-Spectrum Antibacterial Agents Targeting the ESKAPE Pathogens.

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Page 1 of 1

Authors Contributions:

RF: Performed experiments, collected data, analyzed data, drafting and revision of manuscript

TL: Chemical synthesis

RS: Mathematical data analysis

AM: Chemical synthesis

AN: Chemical synthesis

GW: Acquisition of funding, data interpretation

JM: Chemical synthesis

MG: Concept and design, data interpretation, drafting of manuscript

RH: Acquisition of funding, concept and design, final approval of manuscript

LS: Acquisition of funding, concept and design, data interpretation and analysis, drafting, revision, and final approval of manuscript

Combinatorial Libraries As a Tool for the Discovery of Novel, Broad-Spectrum Antibacterial Agents Targeting the ESKAPE Pathogens

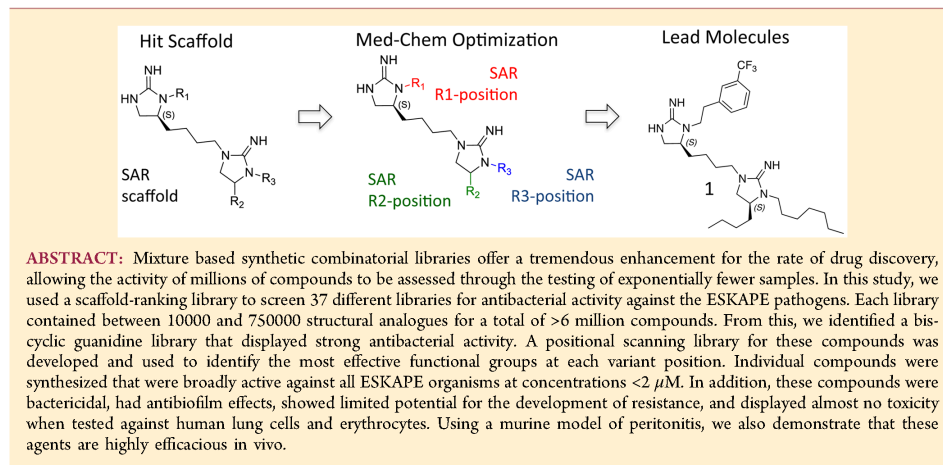
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Supporting Information



INTRODUCTION

Nosocomial infections are a significant cause of human morbidity and mortality. In the United States alone, there are 2 million such infections every year caused by bacterial pathogens, leading to approximately 100000 deaths.¹ These infections are a significant public health concern as they are typically caused by broadly multidrug resistant organisms, which have become virtually unmanageable with existing antibacterial chemotherapeutics.² It is thus no surprise that the World Health Organization has identified antimicrobial resistance as one of the three greatest threats to mankind in the 21st century.³ In light of this, the Infectious Disease Society of America (IDSA) coined the term ESKAPE pathogens almost a decade ago, referring to the six bacterial species that collectively cause around two-thirds of all US nosocomial infections and have effectively escaped the ability to be treated by existing drugs.⁴ These bacteria are *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter*

baumannii, *Pseudomonas aeruginosa*, and *Enterobacter* species.

In spite of the rapid and continued emergence of drug resistant ESKAPE pathogen isolates, there has been an alarming decline in drug discovery efforts in the pharmaceutical industry, resulting in a 75% reduction in FDA approval of antibacterial agents from 1983 to 2007.⁴ For three of the Gram-negative ESKAPE organisms (*K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*), a post antibiotic era has effectively been realized, with pan-resistant isolates identified on numerous occasions over the past decade.⁵ As a result, the IDSA recently issued a call to action, indicating the urgent necessity of developing sustainable antibacterial research and development that responds to current resistance trends and anticipates the development of resistance in the future.⁴

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The use of positional scanning libraries (PSL) provides a fundamental shift in the drug discovery processes for diseases. These libraries allow the evaluation of thousands to millions of synthetic compounds through the use of exponentially fewer test samples compared to traditional approaches of screening individual agents against a given target. PSLs contain diverse chemical structures, and large numbers of compounds in each library, which increases the rate of identifying compounds with useful chemical characteristics.⁶ Such approaches expedite the screening process, allowing for rapid selection of specific PSLs that can be deconvolved, generating strong and detailed SAR data due to high structural density of the libraries.⁷ Furthermore, it has been demonstrated through chemoinformatic approaches that PSLs have the ability to expand currently known medicinal chemistry space.⁸ Taking all these considerations together, the use of such libraries can rapidly enhance the drug discovery process, which is of significant benefit in trying to keep pace with increasing rates of antibacterial resistance.

In this study, we began with a scaffold ranking library containing 37 different combinatorial libraries composed of 10000 to 750000 compounds each, leading to a total of >6 million compounds tested. From this initial screening, a PSL based around a core bis-cyclic guanidine scaffold was selected for further evaluation. The library contained 45864 different bis-cyclic guanidines systematically formatted into 110 mixture samples. By screening this library, we identified a series of individual bis-cyclic guanidine compounds that have strong antibacterial activity against both Gram-positive and Gram-negative organisms.

Guanidine-based compounds have been found to possess extensive functional bioactivities.⁹ In the late 1960s, guanidine hydrochloride was used to treat *Clostridium botulinum* infections as it was found to block presynaptic potassium channels, which stimulated neurotransmitter release and alleviated toxin mediated paralysis.¹⁰ Guanidine related compounds like bisbiguanides (such as chlorhexidine) and bisamidines (such as hexamidine) have shown broad spectrum activity against both Gram-negative and Gram-positive pathogens and are commonly used as antiseptics. Recently, the attachment of guanidinium groups to both neomycin B and kanamycin A has been shown to restore and extend their activity toward gentamicin-resistant *Pseudomonas aeruginosa* and methicillin-resistant *Staphylococcus aureus*.¹¹ Finally, a highly novel antimicrobial agent (teixobactin) containing a cyclic guanidine substituent was recently discovered, demonstrating excellent activity against Gram-positive organisms and displaying essentially no propensity for the development of resistance.¹²

More relevant to this study, in work by Rideout et al.,¹³ pyrrolidine bis-cyclic guanidines were identified, with antibacterial activity toward Gram-positive and Gram-negative species. These agents were found to interfere with DNA replication and induce envelope stress in target organisms. Interestingly, the bis-cyclic guanidine library tested herein was also screened in these holiday junction accumulation assays and found to bind these structures as well, although at a much lower frequency, than the pyrrolidine-based counterparts. Further to this, the pyrrolidine bis-cyclic guanidines were actually part of the combinatorial library screened in the present study (library 1955); however the simpler bis-cyclic guanidine scaffold identified herein possessed a broader spectrum of activity at more promising concentrations.

As such, in this study, we have identified a novel series of bis-cyclic guanidine compounds that have broad activity against all of the ESKAPE pathogens, limited toxicity to human cells, a strong

ability to eradicate bacterial biofilms, and show promising efficacy in mammalian models of infection. We contend that employing positional scanning approaches, and the accompanying strategies described herein, create a fundamental shift away from traditional antibacterial testing methodologies by introducing a rapid approach to discover novel compounds that possess broad spectrum activity.

RESULTS AND DISCUSSION

Scaffold Ranking Library. To rapidly assess the available chemical scaffolds in our combinatorial collection for their potential broad-spectrum antibacterial activity, a scaffold ranking library approach was utilized. We have previously described in detail the construction,^{6,14} advantages, and limitations of the scaffold ranking library,⁶ as well as its successful implementation for the discovery of several classes of novel ligands for a range of targets and indications,^{15–17} including antimicrobials that inhibit tyrosine recombinases and Holliday junction-resolving enzymes.^{13a,18} In the current project, we utilized a scaffold ranking library containing 37 mixture samples, each of which was comprised of approximately equal molar concentrations of individual compounds containing the same common core scaffold (Supporting Information Table S1). The 37 mixtures were screened for antimicrobial activity against all six ESKAPE pathogens using a microbroth dilution assay. From the initial scaffold ranking data (Figure 1), we determined that the most potent broad spectrum

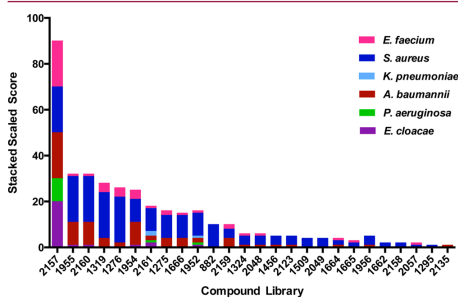
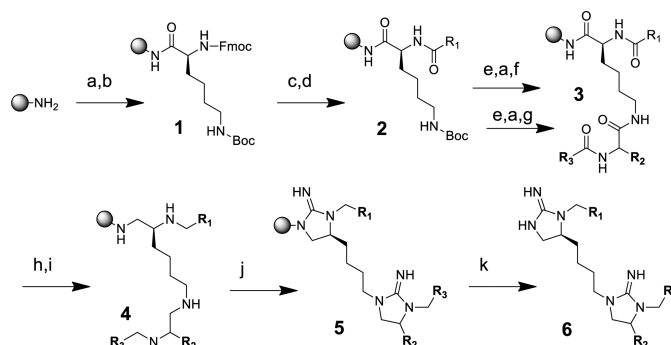


Figure 1. Screening the scaffold ranking library for antibacterial activity against the ESKAPE pathogens. Compound mixtures were assayed against the ESKAPE pathogens using a micro broth dilution assay. Data are presented as a stacked scaled score, which is determined by dividing 100 μM (the maximum concentration tested) by the individual doses tested. Each library is given a scaled score for each pathogen, and these are then stacked to determine the library with the broadest activity at the lowest concentration.

library was 2157. This sample (Figure 1) effectively inhibited *E. faecium*, *S. aureus*, *A. baumannii*, *P. aeruginosa*, and *E. cloacae* at 100 μM . More importantly, the sample retained broad spectrum activity at 5 μM , where it inhibited *E. faecium*, *S. aureus*, *A. baumannii*, and *E. cloacae*. Two other samples, 2161 and 1952 (both polyamines), were active against all six ESKAPE pathogens at 100 μM but did not retain broad spectrum activity at lower concentrations, which led to a less significant stacked scale score. In general, scaffolds containing cyclic guanidines, piperazines, and polyamines were among the most active scaffolds (see Supporting Information Table S1 for list of core scaffolds). However, the broad antimicrobial activity, even at low concentrations, led us to further investigate the 2157 positional scanning library.

Scheme 1. Synthetic Scheme of Bis-cyclic Guanidines^a

^a(a) 5% DIEA/DCM; (b) Fmoc-Lys(Boc)-OH, DIC, HOBT, DMF; (c) 20% piperidine/DMF; (d) R₁COOH, DIC, HOBT, DMF; (e) 55% TFA/DCM; (f) Boc-AA(R₂), DIC, HOBT, DMF; (g) R₃COOH, DIC, HOBT, DMF; (h) BH₃-THF, 65 °C, 96 h; (i) piperidine, 65 °C, 24 h; (j) CNBr, DCM; (k) HF, anisole, 0 °C.

R1 + R3	R2
1 phenylethyl	a hydrogen
2 2-phenylbutyl	b 5-methyl
3 3-phenylbutyl	c 6-methyl
4 m-tolylethyl	d 8-propyl
5 2-(3-fluoro-phenyl)-ethyl	e R-propyl
6 2-(3-bromo-phenyl)-ethyl	f S-butyl
7 2-(3-trifluoromethyl-phenyl)-ethyl	g R-butyl
8 2-(3-methoxy-phenyl)-ethyl	h S-isopropyl
9 4-methyl-benzyl	i R-isopropyl
10 p-tolylethyl	j S-2-butyl
11 2-(4-fluoro-phenyl)-ethyl	k R-2-butyl
12 2-(4-bromo-phenyl)-ethyl	l S-isobutyl
13 2-(4-methoxy-phenyl)-ethyl	m R-isobutyl
14 2-(4-ethoxy-phenyl)-ethyl	n 5-cyclohexylmethyl
15 2-(4-isobutyl-phenyl)-propyl	o R-cyclohexylmethyl
16 2-biphenyl-4-yl-ethyl	p R-hydroxymethyl
17 3,4-dichlorophenethyl	q S-hydroxymethyl
18 3-(3,4-dimethoxy-phenyl)-propyl	r (R,R)-1-hydroxyethyl
19 2-(3,5-bis-trifluoromethyl-phenyl)-ethyl	s (S,S)-1-hydroxyethyl
20 3,4,5-trimethoxy-benzyl	t S-phenyl
21 hydrogen	u S-benzyl
22 butyl	v R-benzyl
23 heptyl	w S-2-naphthylmethyl
24 isobutyl	x R-2-naphthylmethyl
25 2-methylbutyl	y S-4-hydroxybenzyl
26 3-methylbutyl	z R-4-hydroxybenzyl
27 3-methylpentyl	
28 4-methylpentyl	
29 2-methylcyclopropyl-methyl	
30 cyclobutyl-methyl	
31 cyclopentyl-methyl	
32 3-cyclopropyl-propyl	
33 cyclohexyl-methyl	
34 cyclohexyl-ethyl	
35 cyclohexyl-propyl	
36 cyclohexyl-butyl	
37 4-methyl-1-cyclohexyl-methyl	
38 4-tert-butyl-cyclohexyl-methyl	
39 cycloheptyl-methyl	
40 2-Bicyclo[2.2.1]hept-2-yl-ethyl	
41 adamantan-1-yl-methyl	
42 adamantan-1-yl-ethyl	

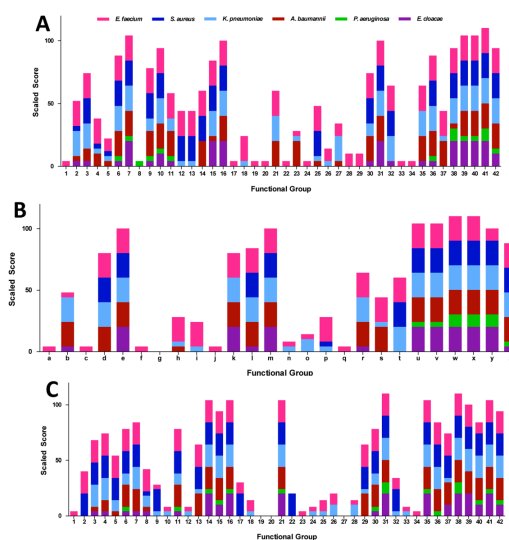
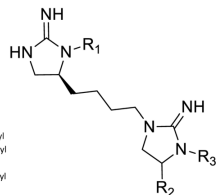


Figure 2. Deconvolving the antibacterial activity of the bis-cyclic guanidine library. The bis-cyclic guanidines were systematically synthesized into a positional scanning library containing 110 samples (shown in Supporting Information Table S2). These were fixed at: (A) = the R₁ (42 samples), (B) = R₂ (26 samples), or (C) = R₃ (42 samples) positions. For example, the first sample in (A) is an approximate equal molar mixture of 1092 compounds. The 1092 compounds contain phenylethyl fixed in the R₁ position and all 1092 combinations of the 26 R₂ and 42 R₃ functionalities. Similarly, the first sample in (B) is 1764 compounds generated from fixing R₂ with hydrogen and utilizing all 1764 combinations of the 42 R₁ and 42 R₃ functionalities. The height for each color of individual bars is determined by dividing 100 μM (the maximum concentration tested) by the individual MIC for each agent. Libraries are then given a scaled score for each pathogen, and these are stacked to determine the library with the broadest activity at the lowest concentration.

Deconvolution of the 2157 Library. Library 2157 is a positional scanning library containing 45864 individual bis-cyclic guanidines (Scheme 1 and Figure 2) systematically synthesized into 110 mixture samples (Supporting Information Table S2).

These separate 110 mixtures were next screened against the ESKAPE pathogens to deconvolve specific antibacterial activity and begin to generate a structure–activity relationship. The first 42 of these 110 samples contain the 45864 bis-cyclic guanidines

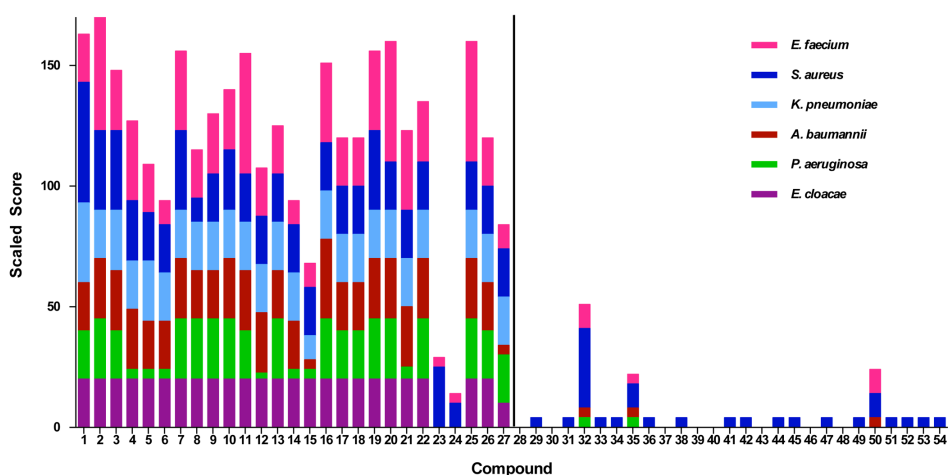


Figure 3. Assessing the antibacterial activity of individual bis-cyclic guanidines synthesized based on library SAR data. Fifty-four individual compounds were synthesized for testing against the ESKAPE pathogens. 1–27 were generated based on SAR data from ESKAPE testing with the combinatorial libraries; 28–54 were included as they were predicted to be significantly less active based on PSL data. Data is presented as stacked, scaled scores, with the height for each color of individual bars determined by dividing 100 μM (the maximum concentration tested) by the individual MIC for each agent. Compounds are then given a scaled score for each pathogen, and these are then stacked to determine which have the broadest activity, at the lowest concentration. Note data is generated using “crude” compounds (see Materials and Methods section for details).

arranged by fixing the R_1 position (Figure 2A, Supporting Information Table S2), the next 26 samples are arranged by R_2 position (Figure 2B), and the last 42 samples are arranged by R_3 (Figure 2C). By way of example, the first sample in Figure 2A contains an equal molar amount of the 1092 individual compounds in the library that have phenethyl fixed at the R_1 position; likewise, the last sample in Figure 2C contains an equal molar amount of the 1092 individual compounds in the library that have adamantan-1-yl-ethyl fixed at the R_3 position.

The 110 samples from Library 2157 were screened for antimicrobial activity against all six ESKAPE pathogens in a similar manner to the Scaffold Ranking Library, generating MIC data for each sample (Figure 2, Supporting Information Table S3). From this, we determined a clear differentiation in the potency of mixtures. For example, those fixed with large aromatic or aliphatic substitutions, such as 2-(3-trifluoromethyl-phenyl)-ethyl and adamantan-1-yl-ethyl, respectively, at R_1 , were more potent than any of the mixtures fixed with small aliphatic groups such as butyl and isobutyl. However, we noted that samples fixed at the R_2 position with different butyl functionalities are actually among the most potent, although as the butyl group is shortened to a propyl and then a methyl, there appears to be stepwise reduction in potency. Additionally, there is no apparent preference for absolute configuration at this position. For the R_3 position, a number of samples with aliphatic (cyclic and acyclic) and aromatic functionalities fixed at the R_3 position show activity, however, there were a few trends that seem to affect activity at this position such as the size of the aliphatic group (larger favored) as well as the preference for aromatic electron withdrawing groups over electron donating groups. For example, changing from a heptyl, six-carbon chain functionality to a butyl, four-carbon chain in R_3 effectively eliminates activity of the sample, and switching from a weak meta-electron donating group

such as 2-(3-fluoro-phenyl)-ethyl or 2-(3-bromo-phenyl)-ethyl to a strong meta-donating group such as 2-(3-methoxy-phenyl)-ethyl at the R_3 position reduced the overall activity of the sample.

From this data, we could have chosen a number of different functionalities (active samples) to fix at each of the positions; however, to reduce the number of compounds produced, we selected 27 individual compounds for synthesis. These compounds were selected by combining the functionalities of the most potent mixtures from each of the R positions, while biasing to include as much structural diversity as possible (Supporting Information Table S4, samples 1–27).

Screening of Individual Compounds. The 27 individual compounds were synthesized (Scheme 1, Figure 3) and screened for antimicrobial activity against all six ESKAPE pathogens, again using MIC assays, and are reported using stacked scaled scores (Figure 3, 1–27). There are several SAR trends of note. Using a *S*-cyclohexylmethyl instead of a *S*-butyl (ie 7 vs 4, 9 vs 6, and 17 vs 14) corresponds with a decrease in total activity score and the decrease in total activity score for each pair is most significantly driven by a decrease in activity against *Pseudomonas aeruginosa*. Additionally, when R_2 was fixed with either *S*-butyl or *S*-cyclohexylmethyl, there was a stepwise decrease in total activity when R_1 was fixed with 2-(3-trifluoromethyl-phenyl)-ethyl versus cyclohexyl-butyl versus adamantan-1-yl-ethyl (ie 4 vs 5 vs 6). When R_2 was fixed with *R*-2-naphthylmethyl, the trend was slightly different as there was a stepwise decrease in total activity when R_1 was fixed with cyclohexyl-butyl versus 2-(3-trifluoromethyl-phenyl)-ethyl versus adamantan-1-yl-ethyl (ie 2 vs 1 vs 3). Finally, in general, substituting the R_3 2-biphenyl-4-yl-ethyl group with either a cyclohexyl-butyl (1–9 vs 10–18) or a heptyl (1–9 vs 19–27) resulted in a reduction in total activity and this reduction in activity was not necessarily bacteria specific but rather attributed to a slight loss in activity against several bacteria.

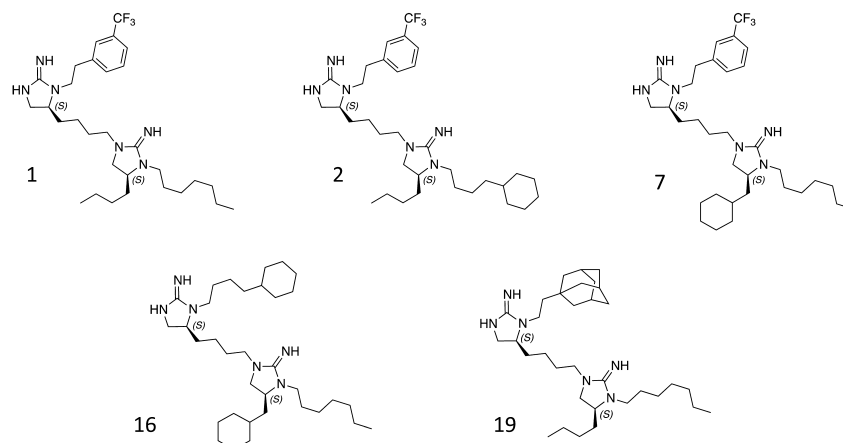


Figure 4. Lead bis-cyclic guanidine compounds.

Table 1. Antimicrobial Activity of Front-Runner Bis-Cyclic Guanidines^a

compd	MIC (μM)						MBC ₅₀ (μM)						MBEC ₅₀ (μM)						IC ₅₀ (μM)	
	E	S	K	A	P	E	E	S	K	A	P	E	E	S	K	A	P	E	A549	AI
1	1.6	1.6	1.6	1.6	1.6	1.6	1.9	3.1	2.4	3.1	4.0	3.1	39	14	7.0	15	20	13	164	100
2	1.5	1.5	1.5	1.5	1.5	1.5	1.7	2.0	4.0	2.8	2.3	4.1	29	2.2	4.7	16	31	13	125	82
7	1.5	1.5	1.5	1.5	1.5	1.5	3.3	3.3	2.2	3.2	14	6.4	26	4.4	4.8	13	46	15	66	43
16	1.6	1.6	1.6	1.6	1.6	1.6	2.9	3.9	4.5	2.8	4.0	9.6	28	7.6	6.3	14	34	13	>225	>139
19	1.7	1.7	1.7	1.7	1.7	1.7	3.6	2.9	2.6	4.7	2.8	2.7	13	6.4	8.6	24	5.0	15	146	88

^aThe in vitro antibacterial and cytotoxic properties of the lead bis-cyclic guanidines were assessed. Shown are the antibacterial activity (MIC), the bactericidal capacity (MBC₅₀), anti-biofilm properties (MBEC₅₀), and toxicity towards human A549 cells (IC₅₀). Selectivity windows were also determined in the form of an activity index (AI, IC₅₀/MIC); note that only 1 value is given because all compounds have the same MIC against each of the ESKAPE pathogens. All data in table generated with purified compounds.

Additionally, a separate set of 27 structural analogues predicted not to be potent based on the SAR of the positional scanning library data were tested to verify as much and that the SAR generated holds value for predicting potent inhibitors (Supporting Information Table S4a and Figure 3, 28–54). Although these additional 27 compounds (28–54) are close structural analogues to the 27 compounds selected for synthesis in the ESKAPE project (1–27), based on the screening of library 2157, the additional compounds were predicted to be significantly less active toward the ESKAPE pathogens. We included these compounds to validate that the activity observed is being driven by the correct combination of functionalities around the core bis-cyclic guanidine scaffold and not just generally by any compound from this library. The 27 novel compounds synthesized for the ESKAPE project displayed an increase in broad spectrum antibacterial activity at low concentrations. At a concentration of 45 μM , 25 of the 27 compounds inhibited growth of all six ESKAPE organisms, with 14 of these retaining activity against all organisms when the concentration decreased to 10 μM . Even more promising, five of the individual compounds tested (1, 2, 7, 16, and 19; Figure 4) had antibacterial activity against all six species at concentrations <2 μM (Table 1). Conversely, and as expected, the 27 additional compounds (28–54) displayed almost no activity toward the ESKAPE pathogens (Figure 3, 28–

54), further validating our structure-guided design of individual compounds.

Preliminary SAR Based on Rapid Deconvolution and Potential Activity Cliffs for the Individual Compounds. As previously described, the 27 compounds (1–27) were selected based on SAR information inherently contained in positional scanning library 2157 as the individual compounds most likely to be active. While compounds 28–54, having the same scaffold as all the compounds in 2157, are close structural analogues to compounds 1–27, they clearly possess little-to-no activity when compared to compounds 1–27. An exploration of compound structures, in order to identify parameters that distinguished these two sets of analogues, is therefore warranted. As a first step, for each of the 54 compounds, Canvas¹⁹ was used to generate six physicochemical properties commonly used to characterize and compare compound data sets in drug discovery:^{19,20} molecular weight (MW), polar surface area (PSA), AlogP (logP as calculated by Canvas), number of rotatable bonds (RB), number of hydrogen bond acceptors, and donors (HBA and HBD, respectively). A list of all computed values for each compound can be found in Supporting Information Table S4; the average and standard deviation for each of the six properties for the two sets, as well as the subset of five leads (1, 2, 7, 16, and 19), are shown in Table 2. From these data, it is evident that the average MW, AlogP,

Table 2. Physicochemical Properties of Individual Bis-cyclic Guanidines^a

set	MW	AlogP	RB	HBA	HBD	PSA
1–27	618.24 ± 50.99	8.72 ± 0.66	16.33 ± 1.27	6 ± 0	3 ± 0	69.45 ± 0
28–54	421.32 ± 55.46	4.78 ± 1.35	11.67 ± 2.59	6 ± 0	3.3 ± 0.5	72.38 ± 4.22
lead 5	566.03 ± 23.33	8.00 ± 0.62	17.40 ± 0.55	6 ± 0	3 ± 0	69.45 ± 0

^aShown are data for the active set (1–27), inactive set (28–54), and front-runner compounds (lead 5; 1, 2, 7, 16, and 19). Molecular weight (MW), number of rotatable bonds (RB), number of hydrogen bond acceptors (HBA) and donors (HBD), and polar surface area (PSA).

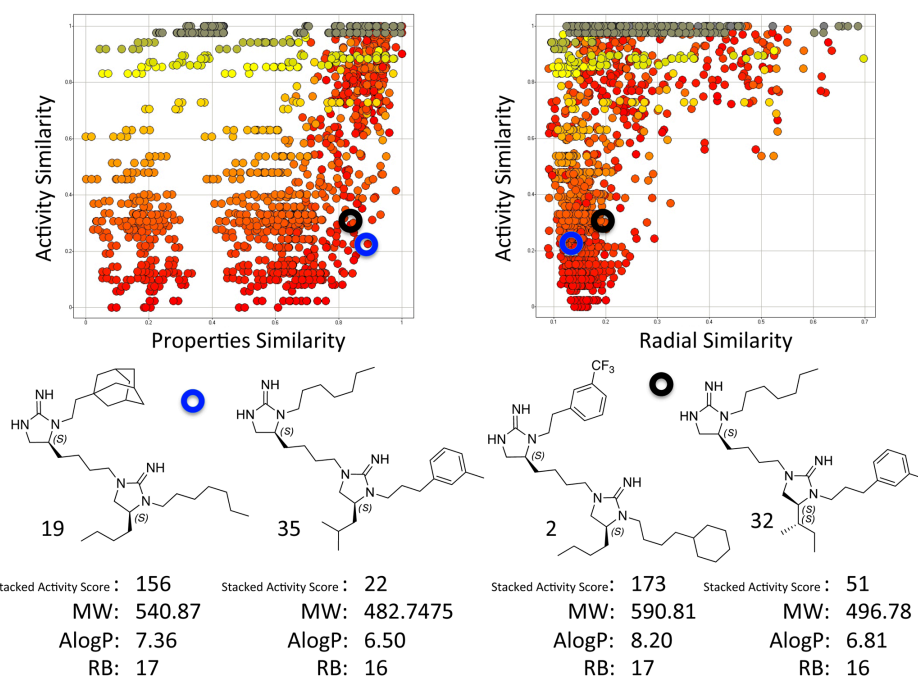


Figure 5. Computational exploration of physicochemical properties. Each of the 54 compounds (1–54) are compared against each of the remaining 53 compounds for differences in potency (y-axis both left and right panel) and molecular representation (physicochemical properties, x-axis left panel; radial, x-axis right panel). Each pair is represented by a dot. In this way, a pair of compounds with similar activity potencies and physicochemical properties will be shown by a dot in the upper-right-hand quadrant of the left panel. The dots are colored by activity of the most potent compound in a pair, using a continuous color of: gray (no activity), yellow (low activity), orange (moderate activity), and red (high activity). Shown below the panels are structures for two such pairs. The pair in the left location on both panels (19–35) is identified by open blue circles, while the pair in the right location (2–32) is indicated by open black circles. Under each structure is the total activity value used for each compound, as well as the three physicochemical values (MW, AlogP, and RB) associated with a given agent.

and RB for the two sets is markedly different, with the active group (1–27) having a higher average MW, AlogP, and number of RB than the inactive set. However, it is important to note the five lead compounds (1, 2, 7, 16, and 19) had, on average, slightly lower MW and AlogP values, making the most potent compounds more similar (based on properties) to the inactive group than the other members of the active group. Thus, it is clear that size, lipophilicity, and flexibility do not fully capture the SAR of these data sets; even though the groupings are grossly categorized by these properties (Table 2), there are numerous examples of compounds with similar physicochemical properties having large activity differences, e.g., activity cliffs with respect to property similarity.²¹ To investigate the potential presence of activity cliffs

in a systematic manner, we used structure–activity similarity (SAS) maps,^{7,20,22} which were one of the first methods developed to characterize SARs by using the concept of activity landscape modeling. The impact of activity landscape modeling and activity cliffs in medicinal chemistry has been extensively discussed as well as the principles of the computational approaches. The interested reader is referred to recent reviews of this topic.^{23–25} SAS maps systematically compare a given representation of molecular similarity with activity similarities for all possible combinations of compounds in a data set.²⁶ Because the SAR observed with any data set is highly dependent on the molecular representation used,²⁷ different structural similarity methods can have drastically different behavior with regard to activity cliffs. Using SAS maps,

each of the 1431 nonredundant pairs of the 54 compounds in the series was evaluated for potency, similarity, and relative molecular similarity (Figure 5). The left panel of Figure 5 shows a SAS map with molecular similarity computed using the six physicochemical properties (following a method we have previously described²⁷ and summarized in the Materials and Methods) on the *x*-axis and potency similarity on the *y*-axis. The data points in the lower right-hand quadrant of this plot indicate pairs of compounds with high similarity in the six physicochemical properties used but low activity similarity (i.e., large differences in potency). Such points thus represent activity cliffs. Unsurprisingly, it is clear from the large number of similar points in the plot that property differences alone are not sufficient to explain the activity differences in these 54 compounds. Two example pairs are highlighted in Figure 5: pair **19** (an active compound) and **35** (a relatively inactive compound) are shown by open blue circles, while pair **2** (an active compound) and **32** (a relatively inactive compound) are shown by open black circles. The right panel shows a SAS map where the molecular similarity was computed using a different molecular representation: radial fingerprints. Radial fingerprints entail growing a set of fragments radially from each heavy atom over a series of iterations²⁸ and are equivalent to extended connectivity fingerprints (ECFPs).²⁹ In sharp contrast to the SAS map obtained with physicochemical properties, the SAS map generated with radial fingerprints does not show activity cliffs. For example, the two pairs of compounds **19–35** and **2–32** are now appropriately located in the lower left quadrant of the SAS map (right panel Figure 5). Notably, we recently reported the superior performance of radial fingerprints over other fingerprint-based methods for activity landscape studies.⁶ We would like to emphasize that, despite the fact that a comprehensive description of the activity landscape study can be performed with the 54 compounds (using SAS maps and/or other approach), the discussion of the two SAS maps presented in Figure 5 is focused on showing the relevance of changing the representation to explore the SAR from continuous physicochemical properties (SAS map on the left), the atom connectivity (SAS map on the right). By no means, the distribution of the data points in the SAS maps are meant to suggest specific substitutions to improve potency. However, the fact that it successfully explains the structure–activity relationship extant in this data set means this fingerprint method will be a useful tool in the ongoing more intensive exploration of the SAR associated with the lead compounds.

The disparity between the SAS maps is a strong argument for the exploration of dense portions of the chemical space; here, representative compounds based on physicochemical properties would have been ill-equipped to properly characterize the active compounds found. Indeed, because of the high structural density of positional scanning libraries,²⁰ they are inherently very rich in SAR information and well suited to assess the presence of activity cliffs.^{21,30} The SAR information from the positional scanning libraries was thus not only able to communicate that in general the active compounds (**1–27**) are more hydrophobic and have more rotatable bonds than the inactive compounds (**28–54**) but was also able to capture the specific atom connectivity, as described by radial fingerprints, that plays a key role in the activity of the molecules.

Exploring the Antibacterial Activity of Frontrunner Agents Using a Library of ESKAPE Pathogen Isolates. Thus far, all data was derived using individual, albeit highly drug resistant, isolates. To assess the full antibacterial potency of front runner agents, additional data was collected using a panel of

clinical ESKAPE isolates (Supporting Information, Tables S5–S6). We determined that all Gram positive strains (*E. faecium* and *S. aureus*), as well as those isolates of the Gram negative organism *A. baumannii*, were sensitive to frontrunner agents at 2 μM , with absolutely no variation. Furthermore, the growth of 95% of all isolates (regardless of species) was inhibited by the five lead bis-cyclic guanidines at concentrations of $\leq 10 \mu\text{M}$. *K. pneumoniae* and *E. cloacae* strains displayed slight variation in MIC values, with agents **2** and **19** inhibiting the growth of 90% of isolates for both species at 5 μM , and 70% of strains at 2 μM . Lead agent **16** had an MIC of 2 μM against 90% of *E. cloacae* strains and 5 μM against 90% of *K. pneumoniae* strains. Lead agent **7** was found to be similar to **16** in activity toward *K. pneumoniae*, inhibiting 90% of strains at 5 μM and 90% of *E. cloacae* strains at 2 μM . Compound **1** had the most variation in MIC against *K. pneumoniae* and *E. cloacae*, with growth of 90% of clinical isolates for each pathogen inhibited at 10 μM . Against the *P. aeruginosa* panel of strains, the lead bis-cyclic guanidines had slightly higher MIC values. Lead agents **2** and **16** inhibited 90% of isolates at 5 μM , while agents **1**, **7**, and **19** inhibited 90% at 10 μM .

Given the minor variations observed in MIC for three of the Gram-negative organisms and that these particular bacteria are renowned for efflux detoxification of antibacterial agents, we hypothesized that the differences observed likely relate to variation in efflux pump activity between strains. To test this contention, we reanalyzed MICs for all five front runner agents against our full panel of *K. pneumoniae*, *P. aeruginosa*, or *E. cloacae* isolates in the presence of subinhibitory concentrations of the known efflux inhibitor, reserpine.³¹ We determined that, while 100 μM of reserpine or 2 μM of compound **1** individually had no effect on the growth of *P. aeruginosa* isolate 1420, the two combined strongly inhibited growth of this strain (Supporting Information, Figure S1A). Similarly, when using *P. aeruginosa* strain 1414 and frontrunner **19**, we observed complete inhibition of growth when this agent was paired with reserpine (Supporting Information, Figure S1B). The effects observed appear to be universal regardless of strain or compound tested. For example, *K. pneumoniae* strain 1441, when used with compound **16** (Supporting Information, Figure S1C), or *E. cloacae* strain 1446 when tested with compound **7** (Supporting Information, Figure S1D), resulted in complete inhibition of growth in combination with 100 μM of reserpine. It should be noted that the data presented herein represents a worst-case scenario. For example, compound **1** was the least active of any agent against *P. aeruginosa* strain 1420; the same is true for all other pairings presented. Similar data was returned for all front runner agents against all 10 isolates of the three Gram-negative organisms (data not shown). These findings support the hypothesis that inherent efflux mechanisms of certain Gram-negative organisms result in MIC variations for the lead bis-cyclic guanidines between clinical isolates. As such, we suggest that any minor decrease in activity for these agents can be restored by the use of a known efflux pump inhibitor.

Assessing Bactericidal Characteristics. We next set out to perform a thorough *in vitro* and *in vivo* characterization of these five lead agents to assess their antimicrobial activities. To do this, we first used a minimal bactericidal concentration (MBC) assay to distinguish whether these compounds were bactericidal or bacteriostatic in nature. Upon analysis, all compounds were found to be bactericidal at concentrations close to their MICs (Table 1). Compounds **1** and **2** proved to be the most bactericidal, with the former agent having MBC₉₀ values ranging from 1.9 μM (against *E. faecium*) to 4.0 μM (against *P. aeruginosa*), while the

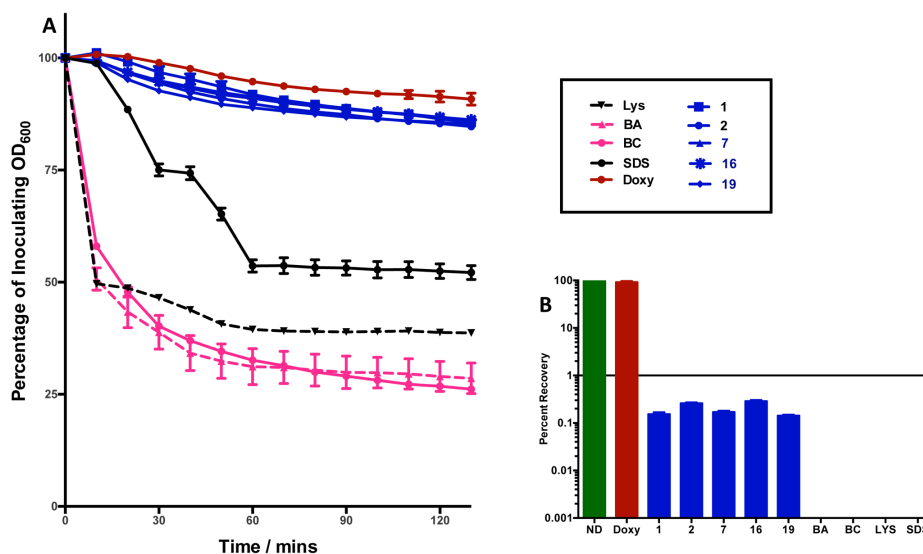


Figure 6. Bis-cyclic guanidines are bactericidal but not bacteriolytic. (A) Time kill studies were performed using MRSA and the front runner agents (at MIC concentrations), alongside positive ($4 \mu\text{M}$ lysostaphin, 0.001% benzalkonium chloride (BA), 0.001% benzethonium chloride (BC), and 2.0% sodium dodecyl sulfate (SDS)), and negative ($200 \mu\text{M}$ doxycycline (Doxy)) control agents. Shown is the optical density of cells relative to starting values from three independent experiments. Error bars are shown \pm SEM. (B) Cell viability of all samples after the 120 min experiment. Compounds were removed by centrifugation and washing of cells, followed by serial dilution and enumeration. Percent recovery was determined by comparison to no drug (ND) controls.

latter had MBC_{90} values ranging from $1.7 \mu\text{M}$ (against *E. faecium*) to $4.2 \mu\text{M}$ (against *E. cloacae*). Similarly, compound 19 was also strongly bactericidal, with MBC_{90} values ranging from $2.6 \mu\text{M}$ (against *K. pneumoniae*) to $4.7 \mu\text{M}$ (against *A. baumannii*). For the most part, compound 16 was significantly bactericidal in effects, with MBC_{90} values ranging from $2.8 \mu\text{M}$ (against *A. baumannii*) to $4.5 \mu\text{M}$ (against *K. pneumoniae*); however, it is MBC_{90} against *E. cloacae* was slightly higher at $9.6 \mu\text{M}$. Finally, compound 7 was strongly bactericidal against the first four ESKAPE pathogens, with MBC_{90} values ranging from 2.2 to $3.3 \mu\text{M}$; however, this number rose to $6.4 \mu\text{M}$ against *E. cloacae* and $14 \mu\text{M}$ against *P. aeruginosa*. As such, all compounds displayed effective bactericidal activity, with many proving so even at very low concentrations.

Considering the strong bactericidal nature of the bis-cyclic guanidines, we next assessed the ability of front runner agents to lyse bacterial cells. As such, a time kill assay was performed using all five lead agents against exponentially growing MRSA cells (Figure 6a). Alongside we also used positive controls agents, including sodium dodecyl sulfate (SDS), lysostaphin (Lys) (a *S. aureus* specific lytic agent), benzalkonium chloride (BA), and benzethonium chloride (BC). These latter two agents are cationic detergents and were included because the bis-cyclic guanidines have the potential to be cationic in nature at physiological pH. For our lead agents at MIC, we observed limited change in bacterial density over the 2 h period. Such findings were similar to our negative control, doxycycline (a translation inhibitor). By way of comparison, we recovered only 52% of cells upon exposure SDS. More profoundly, we achieved >50% lysis of MRSA cells within only 10 min of exposure to the positive control agent lysostaphin,

with viability continuing to decrease over time. Finally, both cationic detergents proved highly lytic toward MRSA, with only 29% and 33% of cells surviving exposure to benzalkonium chloride or benzethonium chloride, respectively. At 120 min, cultures from these tests were serially diluted and cell viability assessed by CFU mL^{-1} . The bacteriostatic control antibiotic doxycycline displayed a 92% recovery of cells once the antibiotic was washed out. Incubation with the lytic control agents (BA, BC, SDS, and Lys) resulted in 0% cell recovery after the 120 min assay. With regard to the bis-cyclic guanidines, we observed a 2.5-log reduction in viability after the 2 h period (0.2% recovery). As such, it would appear that although our front runner compounds result in significant bacterial death during initial incubation, this is not the result of bacterial lysis. Accordingly, these data effectively demonstrate that although the bis-cyclic guanidines are strongly bactericidal, their mode of action does not appear to be via bacterial cell lysis, unlike that of simple cationic detergents.

Determining the Antibiofilm Capacity of Lead Agents. Biofilm formation is a common feature for all of the ESKAPE pathogens and has profound influence of disease severity and mortality.³² Biofilms form on implanted devices, as well as on bone and in the heart, and are innately resistant to antimicrobial intervention.³³ As such, we next set out to assess whether our front runner compounds displayed antibacterial activity. These were performed using minimum biofilm eradication assays (MBEC), as described previously.³⁴ While the MBEC_{50} values for the lead compounds were found to be in excess of MIC and MBC_{90} data, we did observe some highly promising antibiofilm effects with each agent (Table 1). Compound 19 proved to be our most

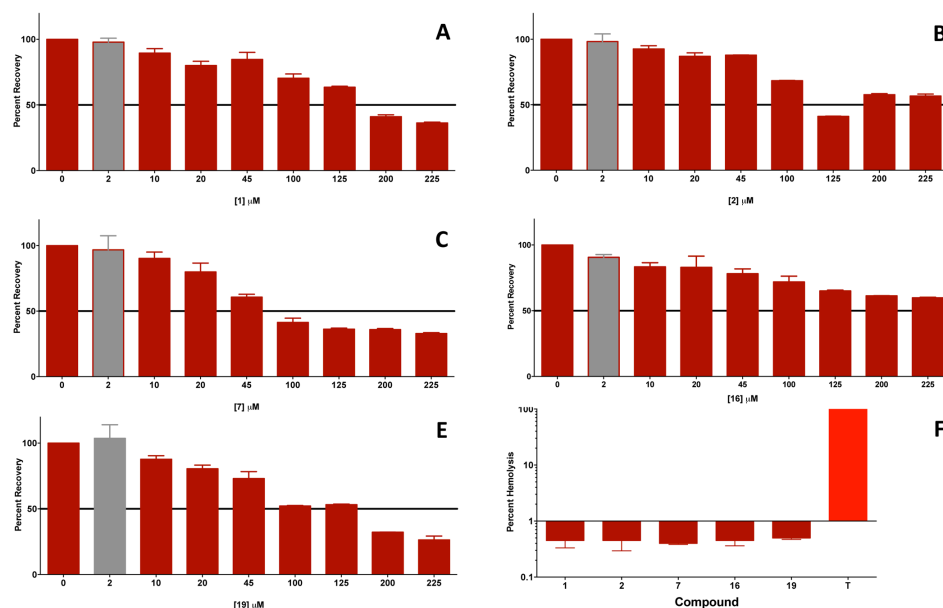


Figure 7. Cytotoxicity of lead agents. Shown is the survival of A549 cells measured using an MTT assay with all five lead agents (A–E). Data is presented as percent recovery compared to vehicle only controls. Error bars are shown \pm SEM from at least three independent experiments; MICs are denoted by gray coloring. A solid black line is shown for IC_{50} value determination. Hemolytic capacity toward human erythrocytes was also measured using the lead agents (F). Data is shown as percent hemolysis compared to positive (1% Triton-X100 (T), 100% hemolysis) controls. Lead agents were added at a concentration of 10 μM . Error bars are shown \pm SEM from at least three independent experiments. A solid black line is shown at 1% hemolysis.

effective in this regard, having MBEC_{50} values $\leq 8.6 \mu\text{M}$ against *S. aureus*, *K. pneumoniae*, and *P. aeruginosa*, and between 13 and 24 μM for the remaining organisms. For 16, we determined MBEC_{50} values of 6.3 and 7.6 μM for *K. pneumoniae* and *S. aureus*, respectively, and 13 to 34 μM against the other four species. The remaining three agents (1, 2, and 7) had MBEC_{50} values that were typically higher than this; however, 2 and 7 were strongly active against *K. pneumoniae* (4.7 and 4.8 μM) and *S. aureus* (2.2 and 4.4 μM) biofilms. As such, it appears that bis-cyclic guanidines not only have strong potential as broad spectrum antibacterial agents but also have the capacity to limit biofilms formed by each of the ESKAPE pathogens.

Exploring the Potential for Front-Runner Toxicity toward Human Cells. Ensuring selectivity for prokaryotic over eukaryotic cells is of primary importance during the development of antimicrobial agents. As such, we next performed cytotoxicity testing for the five lead bis-cyclic guanidines using human A549 adenocarcinomic alveolar basal epithelial cells. The screening of these five lead agents revealed remarkably low toxicity (Figure 7 and Table 1). Specifically, compounds 2 (Figure 7B) and 16 (Figure 7B) allowed for $>65\%$ recovery at concentrations up to 100 μM and $>50\%$ at 225 μM . Compound 1 (Figure 7A) and 19 (Figure 7E) allowed for $>63\%$ and 53% recovery at concentrations up to 125 μM , respectively, with a slight decline to around 40% and 35% at 225 μM . Compound 7 (Figure 7C) yielded less favorable results but still displayed limited toxicity, with $>60\%$ recovery at 45 μM , a concentration

that is 25 \times the MIC. After this concentration, A549 recovery was consistently $\geq 33\%$ at concentrations up to 225 μM . The cytotoxicity data was used to determine IC_{50} values where possible, as well as activity indices ($\text{AI} = \text{IC}_{50}/\text{MIC}$), to gain a sense of therapeutic window and selectivity (Table 1). Importantly, compound 16 never resulted in 50% human cell toxicity, meaning that it has an AI value far in excess of 139. For compounds 1, 19, and 2, we obtained IC_{50} s of 164, 146, and 125 μM , which resulted in selectivity windows of $\text{AI} = 100$, $\text{AI} = 88$, and $\text{AI} = 82$, respectively. Finally, even compound 7, which had slightly more toxic effects, had an IC_{50} of 66 μM and an $\text{AI} = 43$. As such, each of our front-runner compounds appears to have excellent specificity for bacterial cells over their eukaryotic counterparts.

To ensure that these findings were not specific to the cell line used, we next assessed the tendency of lead bis-cyclic guanidines to lyse human red blood cells (hRBCs). In agreement with data from A549 cells, hemolysis assays reveal that bis-cyclic guanidine have little to no apparent toxicity toward human cells, demonstrating no effective capacity to lyse hRBCs (Figure 7F). Using all lead agents at 10 μM ($>5 \times$ MIC for each molecule), we observed hemolysis levels ranging from 0.34% to 0.5%, which clearly demonstrates that lead agents have limited ability to lyse red blood cells. This is placed in context when one compares these values to that of the positive control (Triton-X100, 100% hemolysis). The inability to lyse hRBCs in addition to the lack of toxicity toward A549 cells reveal a high selectivity of bis-cyclic guanidines toward bacterial cells over human counterparts and

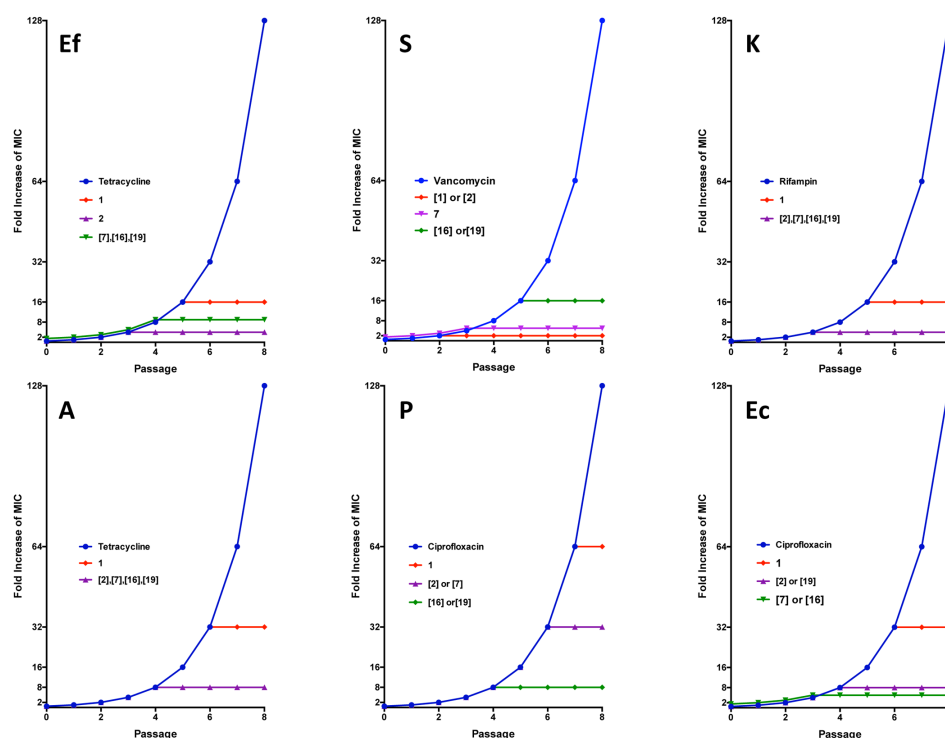


Figure 8. Exploring adaptive tolerance by ESKAPE pathogens to front runner agents. ESKAPE pathogens were serially passaged for 8 days in fresh liquid media (changed every 24 h), with the concentration of compound increased 2-fold each day. Shown are the increases in MIC observed over time. Ef = *E. faecium*, Ec = *E. cloacae*.

therefore suggests that bis-cyclic guanidines have very strong potential for development as new antibacterial agents.

Exploring the Potential for ESKAPE Pathogen Resistance to Front Runner Bis-cyclic Guanidines. An important attribute of potential antimicrobial agents is that the development of resistance to their action is not readily attained. Thus, we determined the spontaneous mutation frequencies for each of our five frontrunner agents. Despite numerous attempts using agar containing compounds at concentrations ranging from 2 to $10 \times$ MIC, we could not generate spontaneous mutants for any of the ESKAPE pathogens ($>1 \times 10^{11}$ CFU collectively tested for each organism). This is in good agreement with work by Rideout et al.^{13a} and their study of agents chemically related to the bis-cyclic guanidines, where spontaneous mutants could also not be generated. In the absence of spontaneous mutants, we next performed stepwise resistance assays by serially passaging of ESKAPE organisms in liquid media over eight separate cycles (1 per day). For each passage, the concentration of front-runner compound was increased 2-fold, alongside a control agent (*E. faecium* and *A. baumannii* = tetracycline, *P. aeruginosa* and *E. cloacae* = ciprofloxacin, *S. aureus* = vancomycin, *K. pneumoniae* = rifampin). Against *S. aureus*, lead compounds 1 and 2 displayed the smallest increase in MIC, with only a 2-fold decrease in

sensitivity observed (Figure 8). We also observed limited resistance for 7, where a 4-fold increase in MIC was noted after eight passages. Finally, 16 and 19 both led to a 16-fold reduction in susceptibility, which, while higher than our other compounds, was significantly less than that of the control, vancomycin. For this latter agent, we noted a continued doubling of the MIC for every passage up to 128-fold increase in MIC. The control agents for each of the other five pathogens behaved similarly, with continued doubling up to 128-fold of the original MIC. However, in each case, the bis-cyclic guanidines outperformed the existing, approved, control agents. Lead agents 2, 7, 16, and 19 were remarkably effective at limiting resistance development in the Gram negative species *K. pneumoniae*, *A. baumannii*, and *E. cloacae*. Testing with these agents revealed a sensitivity limit of ≤ 8 -fold, with concentrations higher, resulting in complete inhibition of bacterial growth. Against *P. aeruginosa*, lead agents 16 and 19 had the smallest increase in sensitivity at 8-fold, a promising observation for a pathogen known to readily develop resistance to antimicrobial agents. As such, there appears to be very limited potential for resistance to our front-runner agents, with no spontaneous mutation seemingly apparent and limited room for adaptive tolerance to their affects.

Lead Bis-cyclic Guanidines Are Efficacious during in Vivo Infection. As a final measure of the suitability of our lead compounds to serve as antibacterial agents, we studied the in vivo efficacy in mice. Using MRSA as a representative ESKAPE organism, we infected mice with 1×10^8 bacterial cells in 5% mucin via intraperitoneal injection. At 1 h postinfection, mice were then IV injected with either vancomycin (positive control) or IM with our front-runner compounds. Each group of mice was compared to a negative control group receiving only vehicle (45% w/v (2-hydroxypropyl)- β -cyclodextrin in water). At $2 \times$ MIC for compound 1, all mice survived the five-day infection period (Figure 9). Similarly, compounds 2, 16, and 19 also proved highly

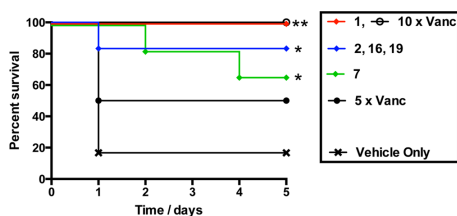


Figure 9. Front runner bis-cyclic guanidines are efficacious during in vivo infection. Mice were IP infected with a lethal dose of *S. aureus*. After 1 h, they were then injected with either front-runner bis-cyclic guanidines (at $2 \times$ MIC), vancomycin (positive control, at $5 \times$ MIC and $10 \times$ MIC) or vehicle alone (negative control). Mice were then monitored for 5 days, and the significance of mortality measured using a log rank and chi square test with 1-degree of freedom. * = $p > 0.05$, ** = $p > 0.01$.

efficacious, with only a single mouse succumbing to infection after the first day and the rest surviving through day 5. Finally, compound 7 was only marginally less effective, with one mouse lost on each of days 2 and 4, which still resulted in statistically significant protection compared to vehicle only controls. When using our control agent vancomycin, we observed 50% protectivity at $5 \times$ MIC (not significant) and 100% protectivity at $10 \times$ MIC. On the basis of these encouraging results, we suggest that our lead bis-cyclic guanidines have excellent in vivo activity, even at very low doses.

CONCLUSIONS

The combinatorial scaffold libraries in this study allowed for the assessment of >6 million compounds for antibacterial activity against the ESKAPE pathogens. The screening ultimately identified a bis-cyclic guanidine scaffold with broad spectrum activity toward each of these organisms. The utilization of a positional scanning library (PSL) was crucial in identifying the most effective functional groups at each of the three variant positions of the core scaffold. The PSL data guided synthesis of 27 individual compounds with significantly increased activity toward all six ESKAPE pathogens. The five most promising individual compounds were chosen as lead agents for further characterization of antibacterial activity (1, 2, 7, 16, and 19). These lead agents proved to be strongly bactericidal (but not bacteriolytic), had promising abilities to eradicate biofilms created by each of the ESKAPE pathogens, and demonstrated little capacity for the development of resistance. Moreover, the bis-cyclic guanidines proved to be highly selective toward bacteria, revealed by low toxicity toward human lung epithelial cells and erythrocytes. Finally, using a murine model of lethal peritonitis, we observed in

vivo efficacy of the bis-cyclic guanidines. Taken together, we present the discovery of a novel class of bis-cyclic guanidines that have high specificity toward the ESKAPE pathogens in vitro and in vivo and which display significant promise for development as antibacterial agents.

MATERIALS AND METHODS

Synthesis of Library 2157 and Individual Compounds and Construction of Scaffold Ranking Plate. *General Synthesis of Bis-cyclic Guanidines (Scheme 1).* Library 2157 as well as the individual compounds reported herein (1–54) were synthesized following the same synthetic scheme (Scheme 1).^{17,35,36} Utilizing the “tea-bag” methodology,³⁷ 100 mg of *p*-methylbenzhydrylamine (MBHA) resin (1.1 mmol/g, 100–200 mesh) was sealed in a mesh “tea-bag,” neutralized with 5% diisopropylethylamine (DIEA) in dichloromethane (DCM), and subsequently swelled with additional DCM washes. Fmoc-L-Lys(Boc)-OH was coupled in dimethylformamide (0.1 M DMF) for 120 min in the presence of diisopropylcarbodiimide (DIC, 6 equiv) and 1-hydroxybenzotriazole hydrate (HOBt, 6 equiv) (1, Scheme 1). The Fmoc protecting group was removed with 20% piperidine in DMF for 20 min, and the R_1 carboxylic acids was coupled (10 equiv) in the presence of DIC (10 equiv) and HOBt (10 equiv) in DMF (0.1M) for 120 min (2, Scheme 1). The Boc protecting group was then removed with trifluoroacetic acid (TFA) in DCM for 30 min and subsequently neutralized with 5% DIEA/DCM (3x). Boc-amino acids (R_2) were coupled utilizing standard coupling procedures (6 equiv) with DIC (6 equiv) and HOBt (6 equiv) in DMF (0.1 M) for 120 min. The Boc group was removed with 55% TFA/DCM for 30 min and subsequently neutralized with 5% DIEA/DCM (3x). Carboxylic acids (R_3) were coupled (10 equiv) in the presence of DIC (10 equiv) and HOBt (10 equiv) in DMF (0.1M) for 120 min (3, Scheme 1). All coupling reactions were monitored for completion by the ninhydrin test. Reductions were performed in a 4000 mL Wilmad LabGlass vessel under nitrogen. Tetrahydrofuran (THF, 1.0 M) borane complex solution was used in 40-fold excess for each amide bond. The vessel was heated to 65 °C and maintained at this temperature for 96 h. The solution was then removed and the bags washed with THF and methanol (MeOH). Once completely dry, bags were treated overnight with piperidine at 65 °C and washed several times with DMF, DCM, and methanol (4, Scheme 1). Before proceeding, the completion of reduction was monitored by LCMS analysis of a control compound (4, Scheme 1) that was cleaved from solid support (HF, anisole, 0 °C 7 h). Cyclization (5, Scheme 1) was performed with a 5-fold excess (for each cyclization) of cyanogen bromide (CNBr) in a 0.1 M anhydrous DCM solution overnight. Following the cyclization, the bags were rinsed with DMF and DCM. The resin was cleaved with HF in the presence of anisole in an ice bath at 0 °C for 90 min (6, Scheme 1). After removal of the HF by gaseous N_2 , the products were then extracted from the vessels with 95% acetic acid in water, transferred to scintillation vials, frozen, and lyophilized. Compounds were then reconstituted in 50% acetonitrile and water, frozen, and lyophilized three more times. For initial screening (data shown in section “Screening of Individual Compounds”), the individual compounds were tested as crude material in case the activity was driven by a side reaction that was also present in the original positional scanning library. After initial screening, the five front runner compounds, 1, 2, 7, 16, and 19, were selected for purification and all data reported in section “Exploring the Antibacterial Activity of Front-Runner Agents Using a Library of ESKAPE Pathogen Isolates” and beyond is from the purified stock of these five compounds. All chirality was generated from the corresponding amino acids. As previously reported by our group and others, the reduction of polyamides with borane is free of racemization.^{38,38b,39} For those compounds with multiple chiral centers, a single diastereomer was obtained.

LCMS Analysis of Crude Material. Purity and identity of initial crude compounds was verified using a Shimadzu 2010 LCMS system, consisting of a LC-20AD binary solvent pumps, a DGU-20A degasser unit, a CTO-20A column oven, and a SIL-20A HT auto sampler. A Shimadzu SPD-M20A diode array detector was used for detections. A full spectra range of 190–600 nm was obtained during analysis. Chromato-

graphic separations were obtained using a Phenomenex Luna C18 analytical column (5 μ m, 50 mm \times 4.6 mm i.d.). The column was protected by a Phenomenex C18 column guard (5 μ m, 4 \times 3.0 mm i.d.). All equipment was controlled and integrated by Shimadzu LCMS solutions software version 3. Mobile phases for LCMS analysis were HPLC grade or LCMS grade obtained from Sigma-Aldrich and Fisher Scientific. The mobile phases consisted of a mixture of LCMS grade acetonitrile/water (both with 0.1% formic acid for a pH of 2.7). The initial setting for analysis was 5% acetonitrile (v/v), then linearly increased to 95% acetonitrile over 6 min. The gradient was then held at 95% acetonitrile for 2 min before being linearly decreased to 5% over 0.1 min and held until stop for an additional 1.9 min. The total run time was equal to 12 min, and the total flow rate was 0.5 mL/min. The column oven and flow cell temperature for the diode array detector was 30 $^{\circ}$ C. The auto sampler temperature was held at 15 $^{\circ}$ C, and 5 μ L was injected for analysis.

HPLC Purification (Compounds 1, 2, 7, 16, and 19). All purifications were performed on a Shimadzu Prominence preparative HPLC system, consisting of LC-8A binary solvent pumps, a SCL-10A system controller, a SIL-10AP auto sampler, and a FRC-10A fraction collector. A Shimadzu SPD-20A UV detector was used for detection. The wavelength was set at 214 nm during analysis. Chromatographic separations were obtained using a Phenomenex Luna C18 preparative column (5 μ m, 150 mm \times 21.5 mm i.d.). The column was protected by a Phenomenex C18 column guard (5 μ m, 15 mm \times 21.2 mm i.d.). Prominence prep software was used to set all detection and collection parameters. The mobile phases for HPLC purification were HPLC grade obtained from Sigma-Aldrich and Fisher Scientific. The mobile phase consisted of a mixture of acetonitrile/water (both with 0.1% formic acid). The initial setting for separation was 2% acetonitrile, which was held for 2 min, then the gradient was linearly increased to 20% acetonitrile over 4 min. The gradient was then linearly increased to 55% acetonitrile over 36 min. The HPLC system was set to automatically flush and re-equilibrate the column after each run for a total of four column volumes. The total flow rate was set to 12 mL/min, and the total injection volume was set to 3900 μ L. The fraction collector was set to collect from 6 to 40 min. The corresponding fractions were then combined and lyophilized.

LCMS Analysis of Purified Compounds. The purity and identity of purified compounds 1, 2, 7, 16, and 19 (all data reported from section "Exploring the Antibacterial Activity of Front-Runner Agents Using a Library of ESKAPE Pathogen Isolates" through to the end of the manuscript was generated with purified compounds) were carried out using a Shimadzu 2020 LCMS system, consisting of a LC-20AD binary solvent pumps, a DGU-20A degasser unit, a CTO-20A column oven, and a SIL-20A HT auto sampler. A Shimadzu SPD-M20A diode array detector was used for detections. A full spectra range of 190–460 nm was obtained during analysis. Chromatographic separations were obtained using a Phenomenex Gemini C18 analytical column (5 μ m, 250 mm \times 2 mm i.d.). The column was protected by a Phenomenex C18 column guard (5 μ m, 4 mm \times 2 mm i.d.). All equipment was controlled and integrated by Shimadzu Lab Solutions software version 5.53 SP3. Three different sets of conditions were used for analysis. Condition 1 (acetonitrile/water pH 2.7): The mobile phase consisted of a mixture of LCMS grade acetonitrile/water (both with 0.1% formic acid for a pH of 2.7) with initial settings for analysis of 5% organic mobile phase (v/v), which was linearly increased to 95% organic mobile phase over 38 min. The gradient was then held at 95% organic mobile phase for 4 min, then linearly decreased to 5% over 2 min and held until stop for an additional 1 min. The total run time was equal to 46 min. Condition 2 (methanol/water pH 7.4): The mobile phase consisted of LCMS grade methanol/water containing 10 mM ammonium bicarbonate (adjusted pH 7.4 with formic acid). The initial setting for analysis was 5% organic mobile phase (v/v), which was linearly increased to 95% organic mobile phase over 38 min. The gradient was then held at 95% organic mobile phase for 4 min, then linearly decreased to 5% over 2 min and held until stop for an additional 1 min. The total run time was equal to 46 min. Condition 3 (methanol/water pH 5.14): The mobile phase consisted of LCMS grade methanol/water containing 50 mM ammonium formate (adjusted pH 5.14 with formic acid). The initial setting for analysis was 60% methanol (v/v), which was linearly increased to 80% methanol over 10 min, before

the gradient was linearly increased to 83% methanol over 25 min. The gradient was again linearly increased to 95% methanol over 3 min and held at 95% for an additional 4 min. Then the gradient was linearly decreased to 60% methanol over 2 min and held until stop for a total run time of 46 min.

NMR Analysis of Purified Compounds. 1 H and 13 C NMR spectra were obtained utilizing the Bruker 400 Ascend (400 and 100 MHz, respectively). NMR chemical shifts were reported in δ (ppm) using the δ 7.26 signal of CDCl₃ (1 H NMR) and the δ 77.16 signal of CDCl₃ (13 C NMR) as internal standards.

(5)-4-Butyl-3-hexyl-1-(4-((S)-2-imino-3-(3-(trifluoromethyl)phenethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (1). Using the synthetic approach described in Scheme 1 for the synthesis of compound 1 was synthesized using the following reagents: (α - α -trifluoro-*m*-tolyl) acetic acid (R₁), Boc-L-norleucine (R₂), and heptanoic acid (R₃). Final crude product was purified by HPLC as described above. 1 H NMR (400 MHz, chloroform-*d*): δ 8.7 (br s, 2 H) 7.5–7.6 (m, 2 H) 7.4–7.5 (m, 2 H) 4.0–4.1 (m, 1 H) 3.8–3.9 (m, 1 H) 3.5–3.7 (m, 5 H) 3.3–3.5 (m, 2 H) 3.1–3.3 (m, 3 H) 2.9–3.1 (m, 2 H) 1.7–1.9 (m, 1 H) 1.5–1.6 (m, 5 H) 1.2 (d, *J* = 8.1 Hz, 2 H) 1.2–1.4 (m, 13 H) 0.9–1.0 (m, 6 H). 13 C NMR (100 MHz, chloroform-*d*) δ 169.0, 159.5, 157.2, 139.4, 132.7, 129.2, 56.4, 51.0, 46.1, 45.1, 43.0, 42.8, 33.5, 31.6, 31.5, 31.4, 29.0, 26.8, 26.5, 26.4, 22.5, 22.4, 20.5, 14.0, 13.9. LCMS (ESI⁺) calcd for C₃₀H₄₉F₃N₆, 551.40; found [M + H]⁺, 551.35. LCMS retention time (214 nm): condition 1 (acetonitrile/water pH 2.7), 17.168 min; condition 2 (methanol/water pH 7.4), 33.528 min; condition 3 (methanol/water pH 5.14), 9.254 min. All three conditions showed the desired compound accounting for 100% peak area and peak height.

(5)-4-Butyl-3-(4-cyclohexylbutyl)-1-(4-((S)-2-imino-3-(3-(trifluoromethyl)phenethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (2). Using the synthetic approach described in Scheme 1 for the synthesis of compound 2 was synthesized using the following reagents: (α - α -trifluoro-*m*-tolyl) acetic acid (R₁), Boc-L-norleucine (R₂), cyclohexanecarboxylic acid (R₃). Final crude product was purified by HPLC as described above. 1 H NMR (400 MHz, chloroform-*d*): δ 8.7 (br s, 2 H) 7.5–7.6 (m, 2 H) 7.4–7.5 (m, 2 H) 4.0–4.1 (m, 1 H) 3.8 (d, *J* = 9.9 Hz, 1 H) 3.5–3.7 (m, 3 H) 3.5 (br s, 1 H) 3.3–3.5 (m, 2 H) 3.1–3.30 (m, 3 H) 2.9–3.0 (m, 2 H) 2.2 (br s, 3 H) 2.0 (s, 1 H) 1.6–1.9 (m, 6 H) 1.5–1.60 (m, 3 H) 1.4–1.5 (m, 2 H) 1.2–1.4 (m, 12 H) 0.8–1.0 (m, 4 H). 13 C NMR (100 MHz, chloroform-*d*) δ 159.5, 157.2, 139.4, 132.7, 129.2, 123.5, 58.6, 56.4, 50.1, 46.1, 45.1, 43.0, 42.8, 37.5, 37.1, 33.5, 33.4, 33.3, 31.5, 31.4, 27.1, 26.6, 26.4, 26.3, 23.9, 22.5, 20.5, 13.9. LCMS (ESI⁺) calcd for C₃₃H₄₃F₃N₆, 591.43; found [M + H]⁺, 591.45. LCMS retention time (214 nm): condition 1 (acetonitrile/water pH 2.7), 18.363 min; condition 2 (methanol/water pH 7.4), 34.487 min; condition 3 (methanol/water pH 5.14), 12.048 min. All three conditions showed the desired compound accounting for 100% peak area and peak height.

(5)-4-(Cyclohexylmethyl)-3-hexyl-1-(4-((S)-2-imino-3-(3-(trifluoromethyl)phenethyl)imidazolidin-4-yl)butyl)imidazolidin-2-imine (7). Using the synthetic approach described in Scheme 1 for the synthesis of compound 7 was synthesized using the following reagents: (α - α -trifluoro-*m*-tolyl) acetic acid (R₁), Boc-L-cyclohexylalanine (R₂), and heptanoic acid (R₃). Final crude product was purified by HPLC as described above. 1 H NMR (400 MHz, chloroform-*d*): δ 8.7 (br s, 2 H) 7.5–7.6 (m, 2 H) 7.4–7.5 (m, 2 H) 4.0–4.1 (m, 1 H) 3.8–3.9 (m, 1 H) 3.5–3.7 (m, 4 H) 3.3–3.5 (m, 3 H) 3.1–3.3 (m, 3 H) 2.8–3.1 (m, 3 H) 1.6–1.8 (m, 8 H) 1.5–1.6 (m, 4 H) 1.2–1.4 (m, 14 H) 0.9–1.1 (m, 5 H). 13 C NMR (100 MHz, chloroform-*d*) δ 159.5, 157.2, 139.4, 132.7, 129.2, 77.2, 58.6, 54.7, 51.8, 46.2, 45.2, 43.0, 42.8, 39.8, 34.3, 33.5, 32.5, 31.6, 31.4, 28.9, 26.7, 26.5, 26.3, 26.2, 26.1, 25.9, 22.5, 20.5, 14.0. LCMS (ESI⁺) calcd for C₃₃H₄₃F₃N₆, 591.43; found [M + H]⁺, 591.45. LCMS retention time (214 nm): condition 1 (acetonitrile/water pH 2.7), 18.380 min; condition 2 (methanol/water pH 7.4), 35.664 min; condition 3 (methanol/water pH 5.14), 12.373 min. All three conditions showed the desired compound accounting for 100% peak area and peak height.

(5)-1-(4-((S)-3-(4-Cyclohexylbutyl)-2-iminoimidazolidin-4-yl)butyl)-4-(cyclohexylmethyl)-3-hexylimidazolidin-2-imine (16). Using the synthetic approach described Scheme 1 for the synthesis of compound 16 was synthesized using the following reagents: cyclohexanecarboxylic acid (R₁), Boc-L-cyclohexylalanine (R₂), and heptanoic

acid (R_2). Final crude product was purified by HPLC as described above. $^1\text{H NMR}$ (400 MHz, chloroform- d): δ 8.7 (br s, 2 H) 3.8–3.9 (m, 2 H) 3.6–3.8 (m, 5 H) 3.4–3.5 (m, 1 H) 3.3 (dd, $J = 9.72, 5.81$ Hz, 1 H) 3.1–3.2 (m, 3 H) 1.5–1.8 (m, 17 H) 1.2–1.4 (m, 22 H) 1.0–1.2 (m, 2 H) 0.8–1.0 (m, 6 H). $^{13}\text{C NMR}$ (100 MHz, chloroform- d) δ 169.1, 159.5, 157.2, 57.8, 54.7, 51.8, 46.1, 45.3, 42.8, 41.9, 39.8, 37.5, 37.2, 34.3, 33.4, 33.3, 32.5, 31.6, 31.4, 28.9, 27.5, 26.8, 26.7, 26.6, 26.5, 26.4, 26.2, 26.1, 25.9, 23.9, 22.6, 20.5, 14.0. LCMS (ESI $^+$) calcd for $\text{C}_{33}\text{H}_{46}\text{N}_6$, 557.52; found $[\text{M} + \text{H}]^+$, 557.50. LCMS retention time (214 nm): condition 1 (acetonitrile/water pH 2.7), 19.706 min; condition 2 (methanol/water pH 7.4), 37.568 min; condition 3 (methanol/water pH 5.14), 14.248 min. All three conditions showed the desired compound accounting for 100% peak area and peak height.

(*S*)-1-(4-((*S*)-3-(2-((3*S*,5*S*,7*S*)-Adamantan-1-yl)ethyl)-2-iminoimidazolidin-4-yl)butyl)-4-butyl-3-hexylimidazolidin-2-imine (**19**). Using the synthetic approach described in Scheme 1 for the synthesis of compound **19** was synthesized using the following reagents: 1-adamantaneacetic acid (R_1), Boc-*n*-norleucine (R_2), and heptanoic acid (R_3). Final crude product was purified by HPLC as described above. $^1\text{H NMR}$ (400 MHz, chloroform- d): δ 8.7 (br s, 2 H), 3.9 (dd, $J = 9.6, 4.7$ Hz, 1 H), 3.8–3.9 (m, 1 H), 3.6–3.8 (m, 5 H), 3.4–3.5 (m, 1 H), 3.3 (dd, $J = 9.7, 5.6$ Hz, 1 H), 3.0–3.3 (m, 3 H), 2.0 (br s, 4 H), 1.5–1.8 (m, 18 H), 1.2–1.4 (m, 15 H), 0.9–1.0 (m, 6 H). $^{13}\text{C NMR}$ (100 MHz, chloroform- d) δ 169.2, 159.5, 157.3, 57.4, 56.4, 51.1, 46.0, 45.4, 42.9, 42.1, 40.5, 36.9, 31.7, 31.6, 31.5, 29.0, 28.5, 26.8, 26.7, 26.6, 26.4, 22.6, 22.5, 20.5, 14.0, 13.9. LCMS (ESI $^+$) calcd for $\text{C}_{33}\text{H}_{46}\text{N}_6$, 541.49; found $[\text{M} + \text{H}]^+$, 541.45. LCMS retention time (214 nm): condition 1 (acetonitrile/water pH 2.7), 18.270 min; condition 2 (methanol/water pH 7.4), 36.038 min; condition 3 (methanol/water pH 5.14), 12.692 min. All three conditions showed the desired compound accounting for 100% peak area and peak height.

Positional Scanning Library 2157. Positional scanning library 2157 was synthesized using the general Scheme 1. The positional scanning library incorporates both individual and mixtures of amino acids (R_2) and carboxylic acids (R_1 and R_3). The synthetic technique facilitates the generation of information regarding the likely activity of individual compounds from screening of the library.^{14,40,41} Equimolar isokinetic ratios have previously been determined and calculated for each of the amino and carboxylic acids utilized for the respective mixtures.^{42,43} The bis-cyclic guanidine library 2157 has a total diversity of 45864 compounds ($42 \times 26 \times 42 = 45864$). The R_1 and R_3 positions as shown in Scheme 1 (6) each consist of 42 carboxylic acids and the R_2 contains 26 amino acids.

Scaffold Ranking Library. The scaffold ranking library contains one sample for each of the 37 positional scanning libraries tested. Each of these samples contains an approximate equal molar amount of each compound in that library. So, for example, the sample 2157 in the scaffold ranking library contains 45864 compounds in approximately equal molar amounts. These samples can be prepared by mixing the cleaved products of the complete positional scanning library, as was the case for sample 2157, or they can be synthesized directly as a single mixture.^{6,14}

Bacterial Strains and Growth Conditions. For this study we used a representative panel of multidrug resistant clinical ESKAPE pathogen isolates (Supporting Information Table S6).^{34,44,45,46,47} Gram-positive organisms were grown in tryptic soy broth media (TSB), while Gram-negative organisms were grown in lysogeny Broth (LB), as described by us previously.³⁵

MIC and MBC Determination Assays. The minimum inhibitory concentration (MIC) for the combinatorial libraries, deconvolved 2157 library, and individual compounds were determined as follows. Broth cultures of ESKAPE strains were grown overnight before being diluted 1 in 1000 in fresh media. Sterile 96-well plates were loaded with culture, and compounds (in DMF) were added at decreasing concentrations to equal a total volume of 200 μL per well. Care was taken to not add more than 2.0% DMF to any well. Plates were then incubated at 37 $^\circ\text{C}$, and MICs determined after 24 h by visual inspection for a lack of turbidity in wells. All assays were performed in triplicate with identical results obtained. For both the scaffold ranking and positional scanning samples, relative broad-spectrum activity was determined via stacked scores:

$$\text{stacked score} = \sum_{i=\text{E, S, K, A, P, E}} \frac{100}{\text{MIC of } i\text{th pathogen}}$$

Minimal bactericidal concentrations (MBC) were determined for 1, 2, 7, 16, and 19 using MIC cultures. Briefly, compound was washed by centrifugation and serial dilution before plating on tryptic soy agar (TSA). Plates were incubated for 24 h at 37 $^\circ\text{C}$ and cell viability assessed by determining CFU/ml at each concentration for every compound. Percent recovery was then determined compared to CFU/mL from no drug controls. All concentrations and controls were tested using three biological replicates, alongside two technical replicates for each data point.

Time Kill Assay. Time kill assays were performed in a 96-well microtiter plate using a BioTek Synergy2 plate reader. To prepare bacterial cultures, stationary phase MRSA cells were inoculated into fresh TSB and grown for 3 h. After this time, cultures were inoculated into a 96-well microtiter plate at an OD₆₀₀ of 0.5, followed by the addition of test agent at MIC concentrations. In parallel, 2.0% sodium dodecyl sulfate (SDS), 0.001% benzalkonium chloride, 0.001% benzethonium chloride, and 4 μM lysostaphin were used as positive controls. Doxycycline (200 μM), a bacteriostatic translation inhibitor that does not result in cell lysis, was used as a negative control. Assays were performed in triplicate over the span of 130 min, with OD₆₀₀ readings taken every 10 min.

MBEC Determination Assays. The minimum biofilm eradication concentration (MBEC) was determined in 96-well microtiter plates as follows. Broth cultures of ESKAPE strains were grown using the conditions described above. Biofilms for each of the ESKAPE pathogens were generated from these as we have previously described for *S. aureus*, however, human serum was not used for nonstaphylococcal organisms.^{46,48} For all organisms, biofilms were developed by standardizing an overnight culture into fresh media to an OD₆₀₀ of 0.5 and adding 150 μL into each well of a 96-well microtiter plate. Biofilms were allowed to develop for 24 h before the media was carefully removed and 200 μL of fresh media added containing a range of front-runner agent (above and below MIC). These cultures were incubated at 37 $^\circ\text{C}$ overnight alongside no drug controls. After 24 h, the media was removed from wells and the biofilm resuspended in phosphate buffered saline (PBS). Cultures were mixed by vigorous pipetting before being serially diluted in PBS and plated in duplicate on relevant agar. Plates were incubated at 37 $^\circ\text{C}$ for 24 h and CFUs determined by enumeration. Each analysis was performed using three technical replicates, and antibiofilm activity was determined by comparing treated to untreated samples.

Cytotoxicity Assay. Cytotoxicity assays were performed using human A549 cells (adenocarcinoma human alveolar epithelial cells) as described by us previously.⁴⁶ Briefly, cells were cultured in F-12K nutrient mixture (Kaighn's Modification) media containing L-glutamine, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin for 3 days at 37 $^\circ\text{C}$ and 5% CO₂. Cells were then diluted to $1.0 \times 10^5 \text{ mL}^{-1}$ using F-12K supplemented media and added to 96-well tissue culture plates at a volume of 100 μL . Plates were incubated for 24 h at 37 $^\circ\text{C}$ and 5% CO₂, allowing the cells to adhere to the plastic. After this time, media was carefully removed and 200 μL of fresh F-12 added with test compounds at concentrations ranging from 1 to 125 μM . Plates were then incubated for 48 h at 37 $^\circ\text{C}$ and 5% CO₂. After 48 h, the media was removed and new media added containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), followed by incubation for 4 h at 37 $^\circ\text{C}$ and 5% CO₂. After 4 h, 50 μL of media was removed, replaced with DMSO, and incubated for 10 min at 37 $^\circ\text{C}$ in order to solubilize any formazan produced. A Biotek plate reader was used to measure the absorbance of formazan production at 540 nm, and IC₅₀ values were determined for each of the five compounds. Front runners were solvated in 45% w/v (2-hydroxypropyl)- β -cyclodextrin in water for these studies, which was also used alone as a negative control. IC₅₀ values were determined for each compound by comparison to vehicle only controls to assess toxicity to human cells.

Hemolysis Assay. A hemolysis assay was performed using whole human blood (Bioreclamation) as described previously,⁴⁹ with the following modifications. Human red blood cells (hRBCs) were resuspended 20% v/v in 1 \times HA buffer (4.25 mL 10% NaCl; 1 mL CaCl₂ in 50 mL sterile water), and lead agents were added at a

concentration of 10 μM to a final volume of 100 μL . Cells were incubated for 15 min at 37 $^{\circ}\text{C}$ before being centrifuged at 5500g for 1 min to pellet nonlysed hRBCs. The supernatant was removed, added to a 96-well microtiter plate, and the OD₅₄₃ read using a Biotek synergy2 plate reader. The negative control was vehicle only (DMF), and the positive control was 1.0% triton X-100. Assays were performed in triplicate, with data displayed as percent hemolysis compared to controls, defined as percent hemolysis = (OD₅₄₃ test sample – OD₅₄₃ no drug control)/(OD₅₄₃ triton X-100 – OD₅₄₃ no drug control) \times 100.

Resistance Assays. To test potential resistance toward the lead agents, a serial passage assay was performed alongside control compounds (*E. faecium* and *A. baumannii* = tetracycline, *P. aeruginosa* and *E. cloacae* = ciprofloxacin, *S. aureus* = vancomycin, *K. pneumoniae* = rifampin). ESKAPE pathogens were grown overnight in liquid media at 37 $^{\circ}\text{C}$. These cultures were then diluted 1:100 in fresh media and seeded into a 96-well plate. Lead bis-cyclic guanidines or control agents were added to respective wells at half MIC concentrations. Plates were then incubated for 24 h at 37 $^{\circ}\text{C}$, with bacteria removed from these cultures on the following day, to inoculate fresh media (1:100 dilution) containing compounds at a 2-fold higher concentrations. These were then grown overnight, and the procedure repeated for a total of eight days. The cultures were observed for a lack of growth, indicating strains were no longer able to resist the action of a given compound. Each experiment was performed in triplicate, yielding identical results.

Assessing Efficacy during Bacterial Infection in Vivo. A murine model of lethal peritonitis was used to demonstrate the effectiveness of the bis-cyclic guanidines to clear bacterial infections, as described by us previously.⁴⁸ All animal studies received written approval after review by the Institutional Animal Care and Use Committee in the Division of Comparative Medicine and Division of Research Integrity and Compliance at the University of South Florida. Six mice per group were injected with 1×10^8 CFU mL⁻¹ of *Staphylococcus aureus* (USA300 strain FPR3757) in PBS containing 5% mucin. After 1 h, mice were inoculated with either 5 \times MIC (4 nM) or 10 \times MIC (8 nM) of vancomycin (IV, positive control), 2 \times MIC (2 μM) of front runner agents (IM, test group), or vehicle alone (IV; 45% w/v (2-hydroxypropyl)- β -cyclodextrin in water; negative control). Mice were monitored twice daily for five days to assess mortality. The clinical end point of this study was when the mice reached a pre-moribund state. Characteristics of pre-moribund state include: hunched posture, rapid, shallow, and/or labored breathing, ruffled fur, lethargy, failure to respond to stimuli, soiled anogenital area, paralysis, paresis, head tilt, circling, vocalizations, nonpurposeful movements, and/or were unable to eat or drink. Those mice reaching this state prior to the completion of the five-day infection period were euthanized. The number of mice surviving between control and treatment groups was compared and analyzed for statistical significance using a log rank test.

SAS Maps. SAS maps were generated following a standard and well-validated protocol.^{7,20} Briefly, for each pair of compounds *i*th and *j*th, potency differences were determined as the absolute difference between their pIC₅₀ activity values. On a relative scale, the potency similarity (PS_{*ij*}) was measured with the expression:

$$PS_{i,j} = 1 - \frac{|A_i - A_j|}{\max - \min}$$

where *A_i* and *A_j* are the activity values of the *i*th and *j*th molecules, and max–min indicates the range of activities in the data set. Pairwise structural similarities were computed using the Tanimoto coefficient⁵⁰ with radial fingerprints as implemented in Canvas.²⁸ Property similarities were computed with 6 continuous coordinates: MW, PSA, AlogP, RB, HBA, and HBD.

Properties were autoscaled with mean centering using the equation:⁵¹

$$p_k = \frac{P_k - \bar{P}_k}{\sigma_{P_k}}$$

where *p_k* denotes the scaled version of the *k*th property for the *i*th molecule, *P_k* denotes the unscaled value, and \bar{P}_k and σ_{P_k} denote, respectively, the mean and standard deviation of the *k*th property over all

molecules in the study. The Euclidean distance between a pair of molecules in the property space was then computed with the expression:

$$d_{ij} = \left[\sum_{k=1}^K (p_{ki} - p_{kj})^2 \right]^{1/2}$$

where *d_{ij}* denotes the Euclidean distance between the *i*th and *j*th molecules, and *P_{ki}* and *P_{kj}* denote the value of the scaled property *k* of the *i*th and *j*th molecules, respectively. In this work *K* = 6 for the four physicochemical properties. Then, Euclidean distances were scaled from 0 to 1 as follows:

$$sd_{ij} = \frac{d_{ij} - \min d_{ij}}{\max d_{ij} - \min d_{ij}}$$

where *sd_{ij}* is the scaled distance and max_{*d_{ij}*} and min_{*d_{ij}*} indicate the range of distances in the data set. Pairwise property similarities were measured with the expression:

$$PS_{ij} = 1 - sd_{ij}$$

where *PS_{ij}* represents the molecular similarity using four continuous descriptors of the *i*th and *j*th molecules and *sd_{ij}* is the scaled distance.

■ ASSOCIATED CONTENT

Supporting Information

Chemical composition of the synthetic scaffold ranking library; deconvolving the 2157 library; minimal inhibitory concentrations of the deconvolved 2157 libraries against the ESKAPE pathogens; chemical composition of individually synthesized bis-cyclic guanidines; MIC data for individual compounds; structures of individually synthesized bis-cyclic guanidines; minimal inhibitory concentrations of lead bis-cyclic guanidine against the entire panel of ESKAPE pathogens; clinical ESKAPE strains used in this study; bacterial efflux accounts for variations in MIC of front runner agents between individual isolates. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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APPENDIX 2:

Identification of a Novel Polyamine Scaffold with Potent Efflux Pump Inhibition Activity Towards Multi-Drug Resistant Bacterial Pathogens

Authors Contributions:

RF: Performed experiments, collected data, analyzed data, drafting and revision of manuscript

GD: Chemical synthesis

KA: Performed experiments

RS: Mathematical data analysis

GW: Acquisition of funding, data interpretation

MG: Concept and design, data interpretation, drafting of manuscript

RH: Acquisition of funding, concept and design, final approval of manuscript

LS: Acquisition of funding, concept and design, data interpretation and analysis, drafting, revision, and final approval of manuscript

1 **Identification of a novel polyamine scaffold with potent**
2 **efflux pump inhibition activity towards multi-drug resistant**
3 **bacterial pathogens**

4

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15 **Running Title:** Polyamine Efflux Inhibitors

16 **Keywords:** Efflux Pumps; Efflux Pump Inhibitors; Polyamines; Bacterial Resistance.

17

18 **Abstract**

19 We have previously reported the use of combinatorial chemistry to identify broad-spectrum
20 antibacterial agents. Herein, we extend our analysis of this technology towards the discovery of
21 anti-resistance molecules, focusing on efflux pump inhibitors. Using high throughput screening
22 against multi-drug resistant *P. aeruginosa*, we identified a polyamine scaffold that demonstrated
23 strong efflux pump inhibition without possessing any antibacterial effects. We determined that
24 these molecules were most effective with an amine functionality at the R1 position and benzene
25 functionalities at the R2 and R3 positions. From a library of 188 compounds, we studied the
26 properties of 5 lead agents in detail, observing a 5 - 8-fold decrease in the 90% effective
27 concentration of both tetracycline and chloramphenicol towards *P. aeruginosa* isolates. Using
28 ethidium bromide accumulation assays we determined that our molecules were not only active
29 against *P. aeruginosa* efflux pumps, but against those from *A. baumannii* and *S. aureus* as well.
30 When studying off target effects we observed no disruption of bacterial membrane polarity, no
31 general toxicity towards mammalian cells, and no inhibition of calcium channel activity in
32 human embryonic kidney cells. Finally, combination treatment with our lead agents engendered
33 a marked increase in the bactericidal capacity of tetracycline, and significantly decreased
34 viability within *P. aeruginosa* biofilms. As such, we report a novel polyamine scaffold that has
35 broad and specific efflux pump inhibiting activity, whilst at the same time lacking problematic
36 off-target effects that have been a major hallmark of other such molecules developed in the past.

37

38

39

40 **Introduction**

41 The continued increase of antimicrobial resistant bacterial infections is an ongoing public health
42 crisis in the U.S., with mortality rates increasing yearly.[1] This problem can be directly linked
43 to ever growing demands for antibiotics, coupled with a diminishing therapeutic arsenal that has
44 been exacerbated by a continual decline in antibiotic discovery over the past 30 years.[2] This
45 presents the scenario of a much talked about post-antibiotic era, where conventional antibiotics
46 will no longer be effective and common infections may once again become fatal.[3] A primary
47 issue is that typical drug discovery efforts often result in the development of therapeutics with
48 known mechanisms of action, thus allowing bacteria to rapidly evolve resistance to these new
49 agents.[4] Consequently, new strategies are urgently needed for the discovery of novel
50 therapeutics targeting multi-drug resistant organisms.[2, 3]

51 The selective pressure antibiotics place on heterogeneous bacterial communities often directly
52 leads to resistant clones becoming dominant within infectious populations.[2] Novel therapeutics
53 targeting resistant bacterial strains would be therapeutically advantageous, specifically focusing
54 on those isolates that prove the most difficult to eradicate. A unique way to do this is to interfere
55 with bacterial resistance mechanisms, rather than focusing on bacterial viability. Such treatment
56 options could restore the effectiveness of numerous obsolete clinical antibiotics, reclaiming
57 many important therapeutics. Co-administration of such anti-resistance agents alongside existing
58 antibiotics may also lead to decreased resistance, as multiple targets within the cell are impacted
59 simultaneously.[5] Hence, anti-resistance approaches may exponentially increase the number of
60 available therapeutic options, whereas conventional antibiotic development commonly yield only
61 a single new drug.[6, 7]

62 A primary method by which bacteria resist the action of antibacterial agents is via efflux pump
63 extrusion. Efflux pumps (EPs) are complexes within the bacterial cell envelope used to export
64 toxic substances such as antibiotics from the intracellular environment before damage to the cell
65 occurs.[8] Efflux pumps are found in most multi-drug resistant nosocomial pathogens, with
66 many EPs having similar and overlapping substrate specificities. As such, targeting bacterial
67 efflux pumps via therapeutic intervention could effectively resensitize cells to a broad spectrum
68 of antibacterial agents. [6, 7, 9] Indeed, recent studies have shown that strains overexpressing
69 EPs commonly display an average >2-fold increased minimal inhibitory concentrations (MIC)
70 towards multiple antibiotics. [9, 10]

71 Efflux mediated resistance was first described almost 40 years ago in a study demonstrating that
72 tetracycline insensitivity could result from plasmid-encoded transport systems.[11] Following
73 this, Nelson et al. observed that polyamine tetracycline derivatives could increase the
74 effectiveness of tetracycline when administered concomitantly.[12-14] Early inhibitors targeting
75 EPs, such as reserpine, were discovered from existing drugs, however their use was limited by
76 the need for administration at very high doses in order to be effective.[15] They also suffered
77 from off-target effects, with compounds such as verapamil, reserpine, and thioridazine not only
78 inhibiting bacterial EPs, but eukaryotic transporters as well.[16, 17] Specifically, calcium
79 channel inhibitors like verapamil, when administered at higher doses have been shown to cause
80 cardiac arrest.[18] More recent agents, such as MC-207,110 (phenylalanine arginine beta
81 naphthalamide, or PaβN), have been shown to have increased specificity towards bacterial efflux
82 systems,[6, 7, 19, 20]; however, the advancement of this scaffold has been abandoned as it has
83 been shown to generally depolarize prokaryotic membranes and cause significant
84 nephrotoxicity.[20-22] Although a number of efflux pump inhibitors (EPIs) with improved

85 activity have been identified in recent years,[23-35] the only advancement into clinical trials to
86 date has been for the proton pump inhibitor omeprazole, used in combination with amoxicillin
87 and clarithromycin to treat *Helicobacter pylori* infections.[36, 37] Therefore, there is a clear need
88 to identify new EPs with enhanced properties, and limited toxicity. This is particularly true for
89 Gram negative species, such as *Pseudomonas aeruginosa*, which have impermeable outer
90 membranes and commonly overexpress efflux systems.[38, 39] Indeed, this organism has 10
91 Resistance nodulation division (RND) efflux pumps that collectively extrude β -lactams,
92 fluoroquinolones, SDS, tetracycline, erythromycin, ethidium bromide, crystal violet, and
93 homoserine lactones.[40] Moreover, given the broad substrate range of *P. aeruginosa* EPs, the
94 inhibition of one pump can be alleviated by the upregulation of additional EPs with parallel
95 targets.[41]

96 In previous work by our group we used combinatorial chemistry to identify broad spectrum
97 antibacterial agents.[42, 43] In the present study, we extend our analysis of this technology
98 towards the discovery of anti-resistance agents, specifically focusing on efflux pump inhibitors.
99 Using high throughput combinatorial scaffold library screening against multi-drug resistant *P.*
100 *aeruginosa* isolates we identified a polyamine scaffold derived from a reduced acyl peptide that
101 demonstrated strong efflux pump inhibition and limited cytotoxicity towards eukaryotic cells.
102 We suggest that these molecules possess excellent potential for future development as anti-
103 resistance agents targeting bacterial EPs.

104

105 **Materials and Methods**

106 **Combinatorial chemistry.** The design and synthesis of the Torrey Pines scaffold
107 ranking library has previously been described.[44-47] The library is comprised of 84 different
108 scaffolds, each with 10,000-750,000 compounds, in an approximately equal molar amount. The
109 polyamine library chosen for analysis contains 399,766 analogs; from this, 188 individual
110 compounds were chosen for analysis. Detailed chemical characterization for scaffold libraries
111 and individual compounds can be found in the general chemistry method section in **S1 Text**.
112 Individual compounds were synthesized as described in Scheme A in **S1 Text**.

113 **Bacterial strains and growth conditions.** The bacterial strains used in this study
114 are multi-drug resistant clinical isolates that have previously been described (**S1 Table**).[42]
115 Organisms were grown in tryptic soy broth (TSB) for overnight cultures and cation adjusted
116 Mueller Hinton broth (CA-MH II) was used for experimental procedures. All incubations were
117 performed at 37 °C.

118 **Checkerboard potentiation assays.** Scaffold ranking library samples and individual
119 polyamines were screened using checkerboard inhibitory assays to assess the potentiation of
120 tetracycline and chloramphenicol. The test utilized a 96-well plate microtiter assay where the
121 concentration of the scaffold or individual polyamine was decreased from 25 $\mu\text{g mL}^{-1}$ to 0.8 μg
122 mL^{-1} (average molarity of 65 μM to 4 μM) along the rows, and the concentration of tetracycline
123 or chloramphenicol was increased from 0.4 μM to 100 μM across the columns. Plates were
124 incubated statically at 37 °C for 20 hours, and the optical density (OD_{600}) was determined using a
125 Synergy 2 plate reader (Biotek). Potentiation modeling (detailed below) was performed to

126 determine fold change in the 50% and 90% effective concentration of tetracycline or
127 chloramphenicol.

128 **Statistical analysis of checkerboard assays.** Potentiation was quantified using a
129 mathematical model developed by our group to assess the ability of library samples and
130 individual compounds to effectively enhance the activity of tetracycline or chloramphenicol.[48]
131 This was used to differentiate libraries or compounds that possessed only antibacterial activity
132 from those that had synergistic activity with tetracycline or chloramphenicol. In this way, only
133 libraries and compounds that potentiated tetracycline or chloramphenicol activity were pursued.
134 The model is based on the following equation for modeling the percentage activity of a mixture
135 of two agents with independent action[48]:

$$136 \quad \%_{Antibiotic \& \textit{Comp}}(\mathbf{x}_1, \mathbf{x}_2) = \%_{Antibiotic}(\mathbf{x}_1) + \%_{Comp}(\mathbf{x}_2) - \%_{Antibiotic}(\mathbf{x}_1) \cdot \%_{Comp}(\mathbf{x}_2)$$

137 Here, \mathbf{x}_1 and \mathbf{x}_2 are the concentrations of antibiotic (tetracycline or chloramphenicol) and
138 library/compound (Comp) tested, respectively. This equation can be rearranged to model the
139 effective percent activity of the antibiotic alone, after accounting for compound activity:

$$140 \quad Eff\%_{Antibiotic}(\mathbf{x}_1) = \frac{\%_{Antibiotic \& \textit{Compound}}(\mathbf{x}_1, \mathbf{x}_2) - \%_{Compound}(\mathbf{x}_2)}{1 - \%_{Compound}(\mathbf{x}_2)}$$

141 Thus, the model-adjusted checkerboards show the antibiotic activity post-potentiation, and from
142 that one can determine the true change in MIC.

143 **Ethidium bromide efflux inhibition assay.** Ethidium bromide efflux assessment
144 was performed by following the fluorescence of ethidium bromide in a 96 well plate assay, as
145 described previously.[6, 7, 21, 49, 50] Bacterial cells were grown overnight at 37 °C in TSB,

146 before being synchronized for three hours in fresh media to exponential phase. Cultures were
147 pelleted at 900 x g for 20 mins and the supernatant removed. The resulting pellet was thrice
148 washed, and resuspended in 20 mM sodium phosphate buffer to an OD₆₀₀ of 0.2. Ethidium
149 bromide was next added at a sub inhibitory concentration of 25 μM and incubated at room
150 temperature for 15 minutes to equilibrate. After equilibration, cells were inoculated into a black
151 walled 96-well plate to a density of 1 x 10⁶ CFU. Using a Biotek plate reader, the fluorescence of
152 cells was monitored for 2 minutes with 530nm excitation and 600nm emission. When baseline
153 readings were complete, polyamines **247**, **250**, **266**, **271**, and **314** were added at 25 μg mL⁻¹
154 alongside the positive control PaβN at the same concentration (all EPI concentrations were
155 maximum potentiating concentrations (MPC)). The solvent DMF was used as a no treatment
156 control alongside tetracycline alone. After addition of all compounds, fluorescence was
157 monitored every five minutes for a total of 90 minutes. After this time, cells were serially diluted
158 and plated to ensure that treatment with ethidium bromide did not affect viability. For graphical
159 representation, the final maximum relative fluorescence at 90 minutes was used for comparison
160 of lead agents to controls.

161 **Bacterial membrane depolarization.** To determine the level of membrane
162 depolarization by polyamine compounds a 3, 3'-dipropylthiadicarbocyanine Iodide (DiSC₃)
163 fluorescence dye was used. Exponentially growing cultures were prepared as described above,
164 before being harvested by centrifugation. Cells were next washed in buffer A (5 mM HEPES (pH
165 7.2), 5mM glucose) and resuspended to an OD₆₀₀ of 0.2 in the same buffer containing 100 mM
166 KCl and 2μM DiSC₃. Samples were allowed to equilibrate for 15 minutes at room temperature to
167 ensure uptake and quenching of the dye in bacterial membranes. Cells were aliquoted into 96-
168 well plates and polyamines were added alongside the known efflux inhibitor PAβN (all at 25 μg

169 mL⁻¹). Nisin (25 µg mL⁻¹) was used as a positive control to display depolarization effects. A
170 Biotek plate reader was used to monitor fluorescence of wells, with a 622nm excitation and
171 670nm emission. For graphical representation, the maximum relative fluorescence at 2 minutes
172 was used for comparison of lead agents to controls.

173 **Eukaryotic cell cytotoxicity.** To assess toxicity of polyamine compounds we used
174 HepG2 human liver carcinoma cells and Hek293T human embryonic kidney epithelial cells. The
175 viability of cell lines was determined using an MTT molecular probe as previously described by
176 our group.[42] Briefly, **247, 266, 271** (Hek293T), or **250, 271, 314** (HepG2), alongside control
177 EPI PaßN, were diluted in 10% DMF from 25 µg mL⁻¹ to 3 µg mL⁻¹ using 2-fold dilutions,
178 before being added to cells in DMEM with 10% FBS and 1% Penicillin/Streptomycin. Cells
179 were then incubated for 48 hours at 37 °C with 5% CO₂. Following this, viability was assessed by
180 the addition of MTT and measuring the OD₅₇₀ in a Biotek Synergy 2 plate reader. Percent
181 recovery was determined compared to no drug controls.

182 **Eukaryotic calcium channel activity assay.** The effects of polyamine efflux
183 inhibitors on eukaryotic ion channels was performed using a calcium channel assay kit (Life
184 Technologies) and the Hek293T kidney cell line. Cells (5 x 10⁴) were inoculated into a black
185 walled 96-well plate and allowed to attach overnight at 37 °C with 5% CO₂. After this, the Fluo-
186 4 dye supplemented with Probenecid (5mM) was added and allowed to equilibrate for one hour
187 at 37 °C with 5% CO₂. After this time, fluorescence was measured for 120 seconds using a
188 Biotek Synergy plate reader with a 495 nm excitation and 516 nm emission. Cells were then
189 treated with solvent only controls (10% DMF), as well as polyamine compounds **250, 266, 271**,
190 and the known calcium channel inhibitor verapamil (all at 25 µg mL⁻¹). Fluorescence was

191 monitored for 120 seconds, before calcium channels were stimulated with carbamylcholine
192 chloride (137 μM). Readings were then taken at 6 second intervals, with peak relative
193 fluorescence at 18 seconds used graphically for comparison of lead agents to controls.

194 **Bactericidal and biofilm assessment.** Lead agents were screened for bactericidal
195 activity as described by us previously[42, 51], with the following modifications. Varying
196 concentrations of tetracycline (0, 12, 25, and 50 μM) were used in combination with the MPC of
197 lead agents **247**, **250**, **266**, **271**, and **314** against *P. aeruginosa*. Data is shown as percent
198 recovery by dividing the CFU mL^{-1} of treatment groups by the CFU mL^{-1} recovered from a no
199 treatment control that did not have tetracycline or efflux inhibitors. Biofilm experiments were
200 performed similar to those described by us previously[42, 52], with the following modifications.
201 Polyamine agents **247**, **250**, **266**, **271**, and **314** were added at MPC into biofilm containing wells
202 alongside varying concentrations of tetracycline (0, 12, 25, and 50 μM). Cellular viability was
203 determined by serial dilution after a 20-hour incubation at 37 °C. Values were converted to
204 percent recovery using no treatment controls. All data was generated from three biological
205 replicates and two technical replicates.

206

207 **Results**

208 **Scaffold ranking library.** We have previously described the synthesis and antibacterial
209 activity of the Torrey Pines scaffold ranking library towards the ESKAPE pathogens. With the
210 success of this screening, we decided to expand our approaches towards the development of anti-
211 resistance agents, specifically targeting efflux mechanisms[42]. As such, the 81 Torrey Pines
212 scaffold samples were screened for their ability to decrease the 90% effective concentration
213 (EC₉₀) of the known efflux pump substrate, tetracycline, towards a tetracycline resistant strain of
214 *P. aeruginosa* (tetracycline alone EC₉₀ = 82.5 μM). Upon analysis, 17 libraries resulted in a
215 potentiated tetracycline EC₉₀ of ≥ 2-fold, whilst 6 resulted in a fold potentiation of ≥ 4 (**Fig 1, S2**
216 **Table**). A consideration with these studies is that we wish to identify efflux pump inhibitors,
217 rather than compounds that have bacterial killing activity themselves. Upon testing the top 17
218 libraries we determined that 7 of them, including the 5 most active scaffolds, had individual
219 antibacterial activity. Of the remaining 10 libraries, 2229 (polyamines derived from reduced acyl
220 peptides) had the best potentiating effects (> 4-fold, tetracycline EC₉₀ lowered to 21.03 μM),
221 without itself having antibacterial activity. For this reason, we prioritized the 2229 polyamine
222 scaffold for further assessment.

223

224 **Figure 1. Screening of combinatorial libraries to identify scaffolds that inhibit bacterial**
225 **efflux pumps.** The Torrey Pines scaffold libraries (TPL) were screened for potentiation of
226 tetracycline activity against a clinical tetracycline-resistant *P. aeruginosa* isolate. Data is
227 represented as fold potentiation, which is defined as the EC₉₀ tetracycline concentration + TPL /

228 the EC₉₀ tetracycline concentration (no TPL). In each assay, the TPL concentration used was 25
229 µg ml⁻¹. The libraries represented by a red bar displayed inhibition of bacterial growth
230 themselves, in the absence of tetracycline, whilst blue bars represent libraries that display no
231 inhibition of bacterial growth. Positive (PAβN) and negative (10% DMF) control compounds
232 were used, and are denoted by black bars. Note that only libraries displaying 2-fold or greater
233 potentiation are shown.

234

235 **Lead efflux inhibitor screening.** To explore suitability of polyamine derivatives as
236 efflux pump inhibitors, a library of 188 individual compounds contained within the Torrey Pines
237 chemical collection were screened for their ability to decrease the 50% and 90% effective
238 concentration of tetracycline towards *P. aeruginosa* (**S3 Table**), however these studies were
239 expanded to include EC₅₀ determinations as well as EC₉₀ to provide depth to our structure
240 activity relationship analysis.

241 Upon analysis, 37 individual polyamines were found to inhibit bacterial growth alone at or below
242 the maximum concentration tested 25 µg mL⁻¹. Of the 151 remaining polyamines, 72 reduced the
243 tetracycline EC₅₀ by < 2-fold, 30 decreased the tetracycline EC₅₀ between 2 - 5-fold, and 49
244 decreased the tetracycline EC₅₀ by ≥ 5-fold. From this latter group, 10 were also successful at
245 decreasing the 90% effective concentration by ≥ 5-fold. Four of the 10 most effective
246 polyamines (**247**, **250**, **266**, **271**) had an amine functionality at the R1 position, *S*-
247 methylbenzene at the R2 position, and ethylbenzene at the R3 position. Interestingly, both
248 stereoisomers of methylbutylamine (**247**= *S*-*N*-methylbutylamine: **266**= *R*-*N*-methylbutylamine)
249 were found to create strong potentiation at the R1 position. From the remaining six polyamines,

250 three (**314**, **338**, and **348**) had *S*-methylbenzene at the R1 position, an amine functionality at the
251 R2 position, and ethylbenzene at the R3 position; while three (**393**, **414**, and **453**) had *S*-
252 methylbenzene at the R1 and R2, and varied aromatic groups at the R3 position; thus lacking an
253 amine functionality at the R1 or R2 position. Although polyamines **393**, **414**, and **453** displayed
254 promising fold-potential values, these agents were not selected as lead agents when
255 considering that a large portion (24%) of polyamines with the R1 and R2 functionality defined
256 by *S*-methylbenzene displayed antibacterial activity themselves. In contrast however, the
257 majority (52%) of polyamines with *S*-methylbenzene at the R2 and R3 positions displayed ≥ 2 -
258 fold potentiation of tetracycline activity without displaying inhibition alone. Therefore, we
259 prioritized polyamines with amine functionalities at the R1 position (**247**, **250**, **266**, **271**), as this
260 was the most promising orientation for the positive charge. In addition, while the 10 most
261 potentiating polyamines were shown to decrease the EC₅₀ of tetracycline from 47.8 μ M to ≤ 9
262 μ M, there were a subset of four agents (**247**, **250**, **271**, **314**) that were more effective at
263 decreasing the EC₉₀ than the EC₅₀ revealing their activity does not plateau before 90% bacterial
264 inhibition is achieved. Therefore, we chose polyamines **247**, **250**, **266**, **271**, **314** from the 10 most
265 potentiating polyamines to undergo secondary validation assessment to explore their EPI-like
266 properties (**Fig 2**).

267

268 **Figure 2. Structure of core polyamine scaffold (2229) and individual polyamine lead**
269 **molecules.**

270

271 Using dose response studies (**Fig 3A and S1 Fig**), we determined that the most effective lead
272 was compound **271**, potentiating the tetracycline EC₉₀ by 8.5-fold and its EC₅₀ by 8.2-fold (**Table**
273 **1**). With regards to the remaining four compounds, we determined that **247** resulted in an 8.3-
274 fold decrease of the EC₉₀ and a 5-fold decrease of the EC₅₀. Of note, these two compounds are
275 similar with *S*-methylbenzene at the R2 position, and ethylbenzene at the R3 position, however,
276 they differ slightly at the R1 position (**247**=*S*-*N*-methylbutylamine; **271**=*R*-*N*-propylamine).
277 Additionally, compounds **250** and **266** both display a 7.8-fold and 5.8-fold potentiation of the
278 tetracycline EC₉₀ respectively, with strong EC₅₀ values of 7.0 and 6.8-fold potentiation.
279 Interestingly, **266** displayed more promising EC₅₀ fold-potentiation than EC₉₀, however this is a
280 common feature of competitive EPIs;[40] indeed our studies reveal a similar effect for the well
281 described EPIs reserpine and PAβN (**Table 1**). Both **250** and **266** also have *S*-methylbenzene at
282 the R2 position and ethylbenzene at the R3 position similar to compounds **247** and **271**, but again
283 vary at the R1 position (**250** = *S*-ethylamine; **266** = *R*-*N*-methylbutylamine). Compound **314** was
284 found to have EC₅₀ and EC₉₀, fold potentiation values of 5 and 7.5-fold, respectively. Of note,
285 **314** has an *S*-methylbenzene at the R1 position and an amine functionality (this time
286 propylamine) at the R2.

287

288 **Figure 3. Polyamine lead agents potentiate the activity of unrelated antibiotic efflux**
289 **substrates.** *P. aeruginosa* cells were treated with polyamine molecules at increasing
290 concentrations, alongside tetracycline (A) or chloramphenicol (B). Shown is the fold potentiation
291 of each antibiotic (EC₉₀ values) as the EPI concentration was increased.

292

293 **Table 1. Potentiation assessment of lead polyamine compounds.**

	EPI* ^a	TET+EPI* ^b	FP* ^c	TET+EPI* ^b	FP* ^c ₂₉₄
247	>25	9	5.0	12	8.3
250	>25	9	7.0	12	7.8
266	>25	9	6.8	16	5.8 ₂₉₅
271	>25	5	8.2	10	8.5
314	>25	9	5.0	12	7.5
Reserpine	>25	4	11.2	>50	1 ₂₉₆
PAβN	>25	0.7	65.5	11.63	8 ₂₉₇

298 All assays were performed in triplicate alongside no drug controls, known EPIs Reserpine and PAβN, and
 299 tetracycline alone controls (data not shown due to repetitive values, average concentration for EC₅₀ across
 300 all replicates = 47.8 μM; EC₉₀ = 82.5 μM).

301 *50% effective concentrations (EC₅₀)

302 [#]90% effective concentrations (EC₉₀)

303 ^aEPI is the inhibitory concentration of the individual Efflux Pump Inhibitor (EPI) alone (μg mL⁻¹).

304 ^bTET+EPI is the respective EC tetracycline (μM) in the presence of each compound.

305 ^cFP is Fold Potentiation = EC tetracycline + EPI / EC tetracycline (no EPI).

306

307 To confirm that the activity of our lead agents was not merely confined to tetracycline, we next
 308 explored the potentiation of an unrelated antibiotic efflux substrate, chloramphenicol (**Fig 3B**
 309 **and S1 Fig**). Each of the polyamine agents again displayed an increase in the potentiation of
 310 chloramphenicol EC₉₀ in a dose responsive manner. Agent **266** displayed the highest potentiation
 311 values, although all compounds performed in a markedly similar, and effective manner. As such,
 312 it would appear that our polyamines are capable of potentiating the activity of multiple antibiotic
 313 substrates in *P. aeruginosa* strains, which speaks to their utility for further development.

314 **Polyamines function via the inhibition of bacterial efflux**
315 **mechanisms.** Following these promising results, we sought to validate our findings using
316 more direct means. Accordingly, the polyamines were assessed using an ethidium bromide
317 fluorescence assay that has been widely used to identify efflux inhibitors.[6, 7, 21, 49, 50]
318 Fluorescence of ethidium bromide occurs during intercalation with DNA; thus active efflux
319 mechanisms decrease such fluorescence by extruding ethidium bromide before it can interact
320 with its target. Thus, disruption of efflux pump activity leads to the accumulation of intracellular
321 ethidium bromide and a subsequent increase in fluorescence over time compared to efflux
322 proficient cells. Importantly, when we treated *P. aeruginosa* with lead polyamines, followed by a
323 sub-lethal concentration of ethidium bromide, we observed an increase in fluorescence (**Fig 4A**)
324 compared to no drug controls; indicating inhibition of efflux systems. To determine if these
325 effects are solely limited to *P. aeruginosa*, we next tested other Gram negative pathogens. When
326 these assays were repeated with *A. baumannii*, we observed similar results (**Fig 4B**), indicating
327 the broad-spectrum nature of these agents. Furthermore, when we assayed the Gram-positive
328 pathogen *S. aureus*, we again derived similar findings (**S2 Fig**). Collectively, these data suggest
329 that our triamine molecules are not only effective inhibitors of bacterial efflux mechanisms, but
330 that these effects appear to be broad-spectrum in range.

331

332 **Figure 4. Polyamine molecules have broad spectrum EPI activity.** *P. aeruginosa* (A) or *A*
333 *baumannii* (B) cells were treated with a sub-lethal concentration of ethidium bromide (25 μ M) in
334 combination with tetracycline, the known efflux inhibitor PA β N, lead polyamine agents at 25 μ g

335 mL⁻¹, or vehicle (10% DMF) (ND). Graphs demonstrate fluorescence after 90-minute exposure
336 displayed as relative fluorescent units. Error bars are shown ±SEM.

337

338 **Polyamines act competitively with PAβN to potentiate the**
339 **tetracycline effective concentration.** To further confirm the EPI activity of
340 polyamine agents, we performed a checkerboard assessment to determine the relationship
341 between our front runner molecules and the control compound PAβN. If our polyamine agents
342 inhibit a target other than efflux pumps, then combination treatment would produce a synergistic
343 action. However, if the agents are both inhibiting efflux pumps, the result of combination
344 treatment would be antagonistic.[53, 54] Upon analysis we observed a clear competitive
345 interaction between PAβN and polyamine agents in the presence of tetracycline (**S3 Fig**). This
346 further confirms that the polyamine agents are inhibiting efflux pumps of bacterial species.

347 **Polyamine molecules do not randomly depolarize prokaryotic or**
348 **eukaryotic membranes.** A number of EPIs discovered to date have been shown to non-
349 specifically inhibit efflux mechanisms through the general depolarization of charge across
350 bacterial membranes.[21, 40] To determine if such effects were true of our polyamines, we
351 assessed membrane depolarization using the molecular probe DiSC₃. In cells with normal
352 membrane polarity, the bacterial membrane will quench fluorescence of the DiSC₃ dye.
353 However, if the membrane is depolarized, the dye is released and fluorescence increases over
354 time. Our results reveal that the polyamines had no effect on bacterial membranes, behaving
355 similar to negative controls (**Fig 5A**). Whilst the positive control nisin and PAβN treated cell

356 membranes displayed a strong increase in fluorescence, indicating membrane destabilization,
357 cells treated with chloramphenicol or solvent only (10% DMF) decreased in fluorescence.
358 Polyamines treated cells displayed essentially no change in fluorescence, revealing no
359 depolarization when compared to the known efflux inhibitor PA β N.

360

361 **Figure 5. Polyamines do not destabilize prokaryotic or eukaryotic membranes.** (A) Shown
362 is the change in fluorescence of *P. aeruginosa* cells using a Disc3 dye assay. Data is presented as
363 change in fluorescence before and after addition of lead compounds, PA β N, and nisin at 25 μ g
364 mL⁻¹. (B): Calcium channel activity assays to assess inhibition of calcium channel pumps in
365 HEK 293 cells after the addition of polyamines 250, 266, 271 or the positive control verapamil at
366 25 μ g mL⁻¹. Data is presented as change in fluorescence of cells before and after addition of the
367 Fluo-4 dye. No Drug (ND) and/or Chloramphenicol (CM) were used as negative controls.

368

369 Another key consideration when developing EPIs is their impact on eukaryotic efflux systems, as
370 many such molecules identified to date have non-specific effects on mammalian ion transport
371 systems as well.[15] As such, we tested the effects of the polyamines in this regard against
372 human embryonic kidney epithelial cells (Hek293T), alongside the known, and toxic EPI,
373 verapamil. In these studies, we determined that our polyamine efflux inhibitors mirrored no drug
374 controls when assessed for their ability to interfere with eukaryotic calcium channel activity (**Fig**
375 **5B**). Specifically, lead compound treated cells exhibited increased fluorescence in the presence
376 of the Fluoro-4 dye, whilst verapamil decreased fluorescence, representing the inhibition of
377 calcium channel activity. Thus it would appear that our polyamines are not only specific EPIs,

378 but that their effects are selective for prokaryotic membrane pumps, over their eukaryotic
379 counterparts.

380 **Lead polyamine EPIs lack general toxicity towards eukaryotic cells.**

381 Given the lack of effect of polyamines towards eukaryotic ion channels, we next assessed
382 general cytotoxicity towards human cells. As such, polyamine lead compounds were tested
383 against both HepG2 and Hek293T cell lines using MTT assays (**Fig 6**). In so doing, we
384 determined that front runners **247**, **266**, and **271** had extremely low toxicity towards Hek293T
385 cells. Specifically, when treated with 25 $\mu\text{g mL}^{-1}$ of these compounds, cells displayed 84%, 72%,
386 and 75% recovery compared to solvent only controls, whilst the known EPI PA β N returned only
387 63% cell viability. In support of this, HepG2 cell recovery after treatment with **250**, **314** or **217**
388 generated similar results; even at the highest concentration tested (again 25 $\mu\text{g mL}^{-1}$) we
389 observed 80%, 77%, and 74% cell viability. In comparison, the known efflux inhibitor PA β N
390 tested at the same concentration allowed for 68% recovery of HepG2 cells. The higher toxicity of
391 PA β N was perhaps unsurprising considering that this agent has been shown to depolarize
392 membranes at higher concentrations.[21]

393

394 **Figure 6. Lead polyamines lack general toxicity towards eukaryotic cells.** Shown is the
395 percent recovery of Hek293T cells (A) and HepG2 cells (B) when tested using a MTT
396 cytotoxicity assay following treatment with polyamine leads. Conversion of MTT to formazan
397 was assessed and converted to percent recovery using 0.01 % Triton 100X as 100% death, and
398 no treatment (DMF) as 100% survival. These controls were used to calculate percent recovery,

399 and to determine LD_{50s} (dotted line). Data is from at least three biological replicates, with error
400 bars shown \pm SEM.

401

402 **Polyamine EPIs strongly enhance the bactericidal activity of**

403 **tetracycline.** We next set out to explore the impact of polyamines on the bactericidal effects

404 of tetracycline. The rationale for this was that, although tetracycline is a bacteriostatic antibiotic,

405 it is known to be bactericidal at high concentrations. [55] Treatment with our polyamines alone at

406 25 $\mu\text{g mL}^{-1}$ resulted in minimal impact to bacterial viability, with $\geq 95\%$ of cells recovered for

407 all compounds, in contrast to PA β N which returned only 76% viability at the same concentration.

408 Tetracycline treatment alone at 12, 25, and 50 μM allowed for 53%, 35%, and 1% respective

409 bacterial recovery. However, combination treatment with tetracycline and the MPC of all lead

410 agents resulted in decreased bacterial viability. For example, combination treatment with 12 μM

411 of tetracycline and polyamine **266** displayed the greatest decrease in bacterial viability, similar to

412 the control PA β N. Specifically, the percent recovery decreased to 0.79% and 0.76% when treated

413 with PA β N or polyamine **266** respectively (**Fig 7**). Although not as impressive as **266** and PA β N,

414 combination treatment with polyamines **247**, **250**, **271**, and **314** resulted in 2.9%, 2.2%, 2%, and

415 5.3% recovery, respectively. Interestingly, we found that increasing tetracycline alone from 12

416 μM to 25 μM resulted in 17.8% less recovery, however combination treatment revealed a

417 significant decrease in bacterial viability. In combination with 25 μM tetracycline, our

418 polyamines appeared to outperform PA β N as they allowed for $\leq 0.08\%$ recovery, whilst

419 combination treatment with PA β N allowed for 0.2% recovery. Furthermore, 50 μM treatment

420 with tetracycline decreased bacterial viability to 1% alone, however this was drastically

421 decreased with combination EPI treatment. Specifically, polyamines **250**, **266**, and **314** resulted
422 in the greatest decrease in bacterial recovery, allowing for 0.01% recovery. This was marginally
423 less recovery than that of the control PAβN and polyamine **271**, which allowed for 0.02%
424 recovery. Polyamine **247** displayed the least decrease in viability with combination treatment,
425 although it still decreased bacterial recovery to 0.04%. Given that bactericidal activity is often
426 preferred to bacteriostatic effects, particularly for immunocompromised patients, these findings
427 are considered encouraging.

428

429 **Figure 7. Polyamine EPIs strongly enhance the bactericidal activity of tetracycline.** *P.*
430 *aeruginosa* cells were treated with tetracycline at 0, 12, 25, and 50 μM in combination with no
431 drug (ND), or 247, 250, 266, 271, 314 and PAβN at 25 μg mL⁻¹. The dotted line on the graph
432 denotes 90% bactericidal activity. Data is from at least three biological replicates, with error bars
433 shown ±SEM.

434

435 **Polyamine potentiation of tetracycline activity limits biofilm**
436 **formation by *P. aeruginosa*.** Biofilm formation is responsible for chronic, drug-
437 resistant bacterial infections by a number of pathogens, and particularly *P. aeruginosa*.
438 Considering the strong potentiation of tetracycline activity engendered by our lead agents, we
439 next chose to determine if they had significant impact on the viability of cells within biofilms.
440 Treatment with EPIs **247**, **250**, **266**, **271**, and **314** alone at 25 μg mL⁻¹ (maximum potentiating
441 concentrations (MPC)) respectively, resulted in negligible impact on biofilm viability, with

442 99.99% of cells recovered for all compounds other than **266** which allowed for 92% recovery.
443 Similarly, tetracycline treatment alone at 12 and 25 μM had little impact, allowing for 88% and
444 84% biofilm recovery, respectively (**Fig 8**); only at 50 μM were more pronounced effects
445 observed, with only 9% cells recovered. Combination treatment with 12 μM tetracycline and the
446 MPC of all lead agents resulted in a significant decrease in biofilm recovery, however, with
447 viability of 9.1-11% observed for **271**, **247**, **266**, and **314** respectively. Combination treatment
448 with lead agent **250** resulted in a slightly higher biofilm recovery of 21%, however still
449 improving tetracycline alone biofilm eradication by 67%. Increasing tetracycline concentration
450 by itself from 12 μM to 25 μM only resulted in 4% more eradication, however in combination
451 with our EPs, recovery decreased to 0.5% and 0.6% for **271** and **247** respectively. Similarly,
452 treatment with agents **266**, **250**, and **314** resulted in 1% biofilm recovery. This biofilm
453 eradication was particularly impressive when compared to the activity of the positive control
454 PA β N (7% recovery at 25 μM tetracycline). Furthermore, at the highest tetracycline
455 concentration (50 μM) combination treatment with PA β N produced a 1% biofilm recovery while
456 agents **271** and **247** allowed for only 0.2% recovery. Treatment with agent **250**, **266**, and **314**
457 resulted in similar recovery of 0.5%, 0.6% and 0.6% respectively. These results suggest a
458 potential benefit of combination treatment with our polyamine molecules and known efflux
459 antibiotics to inhibit biofilm formation.

460

461 **Figure 8. Polyamine potentiation of tetracycline activity limits biofilm formation by *P.***
462 ***aeruginosa*.** The lead polyamine agents were tested for their ability to impact viability of a pre-
463 formed biofilm. *P. aeruginosa* cells were treated with tetracycline at 0, 12, 25, and 50 μM in
464 combination with no drug (ND), or **247**, **250**, **266**, **271**, **314** and PA β N at 25 $\mu\text{g mL}^{-1}$. The dotted

465 line on the graph denotes 90% reduction in cell viability within biofilms. Data is from at least
466 three biological replicates, with error bars shown \pm SEM.

467

468

469 **Discussion**

470 Antibiotic resistance is a developing crisis in clinical settings, with an increasing number of
471 bacterial isolates discovered each year that are resistant to our therapeutic arsenal.[56, 57] Efflux
472 pumps are a major contributor to multi-drug resistance because they help circumvent the action
473 of a broad range of substrates that includes multiple antibiotics classes. [37, 40, 58] This is
474 compounded by the observation that most bacterial species utilizes multiple efflux pumps with
475 an overlapping range of substrates.[8, 9, 15] With this considered, the development of broadly
476 active efflux pump inhibitors is considered desirable, so as to focus on the reclamation or
477 reactivation of a wide swath of existing therapeutics. [6, 49, 59, 60]

478 The potentiating modeling utilized in this study allowed for the identification of polyamines that
479 increased the effectiveness of tetracycline without displaying any toxic effects themselves. This
480 highlights the importance of potentiation modeling for the identification of anti-resistance agents,
481 as opposed to synergistic agents that display antimicrobial properties as well. Potentiation
482 modeling is a more advantageous approach to identifying adjuvant agents because synergy
483 assessment is reliant on the therapeutic agent having antibacterial activity.[48] EPIs identified
484 using synergistic activities, i.e. PaβN, have been unsuccessful due to off-target effects, causing
485 bacterial growth inhibition.[6, 7, 21] Importantly, in support of our approach, it has been shown
486 that the concomitant treatment with an antibiotic and adjuvant agent that blocks the mechanism
487 of resistance towards that antibiotic, but that has no antimicrobial properties itself, can lead to
488 decreased resistance development.[61, 62] Although a subset of polyamines was discovered
489 during our screening with antimicrobial effect themselves, their structure activity relationship

490 was taken into consideration during lead polyamine selection as discussed in detail below, and
491 these molecules were eliminated from further consideration.

492 Efflux pump discovery began with the finding that efflux of host antimicrobial polyamines by
493 the *S. aureus* Tet38 efflux pump facilitated skin colonization, and the ability to survive within an
494 abscess environment.[63] Beginning with the initial development of polyamine tetracycline
495 derivatives to increase the effectiveness of tetracycline,[12-14], polyamine molecules have been
496 shown to increase the therapeutic potential of common antibiotics.[64-66] Kwon et. al. revealed
497 the endogenous polyamines spermidine and spermine, found within all living cells, when
498 administered exogenously were effective at increasing the therapeutic potential of β -lactams
499 towards Gram negative organisms by blocking the outer membrane porin OprD.[64] They also
500 found these natural polyamines were shown to potentiate activity of chloramphenicol and β -
501 lactams towards *Escherichia coli* and *S. aureus*.[64] Similarly, in *Bacillus subtilis*, the efflux
502 pump BIt is dedicated to the extrusion of spermidine, however it has also been shown to
503 opportunistically bind to other polyamine molecules.[66] Furthermore, in *Mycobacterium*
504 *tuberculosis*, there is a significant increase in the effectiveness of fluoroquinolones administered
505 alongside polyamine treatment.[65] However, it has been shown that natural polyamines and
506 Pa β N have toxicity associated with the amine substituents, therefore much care must be taken
507 when developing therapeutics of this nature.[67, 68] Even though this toxicity is known, amine
508 molecules continue to be pursued to target multiple disease states.[69-73] Moreover, there are
509 many therapeutics in used in clinics today that contain amines, ie. aminoglycosides, and despite
510 their toxicities approaches have been successful to decrease these effects.[74] In fact, a recent
511 antibiotic adjuvant has been approved by the FDA in 2014 to target beta lactamase producing
512 enteric species ceftolozane/tazobactam (Zerbaxa) and the ceftolozane counterpart of this

513 therapeutic combination contains multiple amine substituents.[75] These studies collectively
514 reveal the therapeutic potential of polyamine molecules as broad spectrum adjuvant agents.

515 The polyamine agents discovered in this study were successful in not only returning the
516 effectiveness of tetracycline but also an unrelated antibiotic efflux substrate, chloramphenicol.
517 Importantly, this finding reveals that our polyamines are not just allowing for the increased
518 effectiveness of one, but multiple commercial antibiotics from a broad range of different classes.
519 This finding suggests that the polyamines discovered in our study inhibit through direct
520 competitive inhibition.[76] This mechanism of efflux inhibition capitalizes on the broad-
521 spectrum binding affinity of efflux pumps by blocking the substrate binding extrusion protomer
522 on the distal binding site.[20, 77] Interestingly, tetracycline binds to the “groove” region of the
523 binding pocket in the binding protomer, while chloramphenicol binds to the deeper “cave”
524 region. Covalent binding to this “cave” region is the ultimate in efflux inhibition as it causes the
525 binding pocket to collapse and become non-functional, therefore inhibiting multiple substrates
526 from binding.[77]

527 Notably, the direct measurement of ethidium bromide fluorescence revealed our polyamines
528 inhibit the efflux pumps of Gram negative species *P. aeruginosa* and *A. baumannii*, as well as
529 the Gram-positive organism *S. aureus*. There are five families of efflux pumps: MFS, ABC,
530 SMR, and MATE are used by all bacterial species, whilst RND efflux pumps are found only in
531 Gram negative species.[8] RND is the main Gram negative efflux system, and its absence in
532 Gram positive organisms reveals that if the polyamine agents are active in both Gram positive
533 and negative organisms, they are likely inhibiting more than one family of efflux pumps. This
534 can be attributed to the competitive inhibition nature of efflux pumps inhibitors that harnesses
535 the broad substrate recognition of efflux pumps for more effective efflux inhibition in multiple

536 organisms. For example, the efflux pump families of RND, ABC, SMR, and MFS all recognize
537 substrates with polycationic properties.[78] Further to this, RND pumps found in *P. aeruginosa*
538 and *E. coli* recognize and extrude tetracycline, while ABC and SMR pumps in *S. aureus* also
539 expel this same antibiotic.[78] This would explain the activity of the previously identified efflux
540 pump inhibitor Baicalein, which is derived from the plant *Thymus vulgaris*, and has been found
541 to potentiate tetracycline activity by blocking the MFS family TetK efflux pumps of *E. coli* and
542 *S. aureus*. [78-80] Moreover, our polyamine EPIs appear to be acting in a competitive manner
543 with the positive control PA β N, potentially competing for the same substrate binding pocket.[21,
544 81] In fact, our polyamines resemble the known efflux pump inhibitor PA β N more so than the
545 other well-known efflux pump inhibitor 1-(1-naphthylmethyl)-piperazine (NMP), which is
546 shown not to potentiate antibiotic activity towards *P. aeruginosa*. [82] This would explain the
547 activity of the recent identification of a pyranopyridine inhibitor, MBX2319 that was designed
548 based on NMP and found to have potent activity towards Enterobacteriaceae but little activity
549 towards *P. aeruginosa*. [83] However, a pyridopyrimidine scaffold discovered by Nakashima et
550 al., with more similarities to PA β N was found to potentiate chloramphenicol and tetracycline and
551 further revealed to bind the distal pocket of both *P. aeruginosa* MexAB and *E. coli* AcrB,
552 whereas MBX2319 was specific to AcrB. [81, 84]

553 Importantly, we observed our polyamines did not have deleterious effects on bacterial cell
554 membranes, as is seen for the known EPI PA β N. [21] Many efflux pump inhibitors discovered to
555 date have been found to cause disruption of the bacterial cell membrane leading to their
556 ineffectiveness as therapeutic agents. [19, 85] Disruption of the bacterial cell membrane causes
557 inhibition of efflux through a secondary effect of membrane depolarization, leading to inhibitory
558 activity alone, and the common identification of false positive, non-specific EPIs. [16, 21] It is,

559 however, perhaps unsurprising that our polyamines do not disturb bacterial membranes, as the
560 potentiation modeling used herein specifically sought to eliminate the selection of false positive
561 EPIs. The identification of 37 polyamines that inhibited bacterial viability themselves, although
562 less than ideal, lead to knowledge of a structure activity relationship for efflux pump specificity.
563 Those polyamines found to inhibit bacterial viability themselves may be disturbing the cellular
564 membrane based on the position of positive charges within their structure. Our analysis revealed
565 24% of the polyamines (26 out of 103) with R1 and R2 both defined by *S*-methylbenzene lead
566 to antibacterial activity, while only 7% (4 out of 60) that have *S*-methylbenzene at R2 and R3,
567 and 12% (7 out of 60) that have *S*-methylbenzene at R1 and R3, had antibacterial activity. With
568 this knowledge, we focused our attention on compounds that had no antibacterial activity
569 themselves to avoid selecting membrane depolarizing agents.

570 To increase our confidence in the specificity of the polyamines discovered herein, we
571 demonstrate that they have limited toxicity towards two human cell lines and no inhibitory
572 effects on the eukaryotic Ca^{2+} channel activity of human kidney cells. To date, many efflux
573 inhibitors (such as verapamil) have been shown to have non-specific inhibition of eukaryotic
574 transporters, and therefore create unwanted side effects as therapeutic agents.[6, 86-88]
575 Verapamil is a non-specific inhibitor of calcium channels, found to also inhibit the function of P-
576 glycoprotein ABC transporters in mammalian cells. [89] This inhibitor was found to have *in*
577 *vitro* function as a bacterial efflux inhibitor, however due to its general toxicity, the use of this
578 compound is limited to angina, hypertension, and cardiac arrhythmia.[89, 90] With limited
579 toxicity and secondary effects, the polyamine EPIs discovered in our study appear to have more
580 favorable characteristics than others previously discovered, suggesting promising potential as
581 adjuvant agents.

582 Taken together, the polyamines discovered in this study have potential as therapeutic adjuvants
583 to rescue the effects of multiple antibiotics towards both Gram positive and Gram negative
584 species. They appear to be acting in a specific manner, and have none of the undesirable
585 membrane targeting characteristics of previously developed EPIs. As such, we suggest that the
586 polyamines developed herein are a promising scaffold for further development of anti-resistance
587 agents to help alleviate the burden of multi-drug resistant bacterial pathogens.

588

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805

806 **Supporting information**

807 **S1 Text. General chemistry methods.**

808 **S1 Table. Clinical isolates used in this study.**

809 **S2 Table. Potentiation assessment of combinatorial scaffold libraries.**

810 **S3 Table. Potentiation assessment of a polyamine-derived library of compounds.**

- 811 **S1 Fig. Front runner polyamines potentiate the activity of unrelated antibiotic efflux**
812 **substrates.**
- 813 **S2 Fig. Polyamine Molecules Have Broad Spectrum EPI Activity.**
- 814 **S3 Fig. Polyamine molecules are antagonistic to the activity of PA β N.**

APPENDIX 3:

Characterizing the Antimicrobial Activity of N_2, N_4 -Disubstituted Quinazoline-2,4-Diamines toward Multidrug-Resistant *Acinetobacter baumannii*

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Author: Renee Fleeman, Kurt S. Van Horn, Megan M. Barber et al.

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Authors Contributions:

RF: Performed experiments, collected data, analyzed data, drafting and revision of manuscript

KVH: Chemical synthesis, analyzed data, drafting and revision of manuscript

MB: Chemical synthesis

WB: Performed experiments

DF: Chemical synthesis

RM: Acquisition of funding, concept and design, drafting, revision, and final approval of manuscript

LS: Acquisition of funding, concept and design, data interpretation and analysis, drafting, revision, and final approval of manuscript



Characterizing the Antimicrobial Activity of N^2,N^4 -Disubstituted Quinazoline-2,4-Diamines toward Multidrug-Resistant *Acinetobacter baumannii*

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ABSTRACT We previously reported a series of N^2,N^4 -disubstituted quinazoline-2,4-diamines as dihydrofolate reductase inhibitors with potent *in vitro* and *in vivo* antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) strains. In this work, we extended our previous study to the Gram-negative pathogen *Acinetobacter baumannii*. We determined that optimized N^2,N^4 -disubstituted quinazoline-2,4-diamines are strongly antibacterial against multidrug-resistant *A. baumannii* strains when the 6-position is replaced with a halide or an alkyl substituent. Such agents display potent antibacterial activity, with MICs as low as 0.5 μ M, while proving to be strongly bactericidal. Interestingly, these compounds also possess the potential for antibiofilm activity, eradicating 90% of cells within a biofilm at or near MICs. Using serial passage assays, we observed a limited capacity for the development of resistance toward these molecules (4-fold increase in MIC) compared to existing folic acid synthesis inhibitors, such as trimethoprim (64-fold increase) and sulfamethoxazole (128-fold increase). We also identified limited toxicity toward human cells, with 50% lethal doses (LD_{50} s) of ≤ 23 μ M for lead agents 4 and 5. Finally, we demonstrated that our lead agents have excellent *in vivo* efficacy, with lead agent 5 proving more efficacious than tigecycline in a murine model of *A. baumannii* infection (90% survival versus 66%), despite being used at a lower dose (2 versus 30 mg kg^{-1}). Together, our results demonstrate that N^2,N^4 -disubstituted quinazoline-2,4-diamines have strong antimicrobial and antibiofilm activities against both Gram-positive organisms and Gram-negative pathogens, suggesting strong potential for their development as antibacterial agents.

KEYWORDS *Acinetobacter baumannii*, quinazoline, biofilm, dihydrofolate reductase inhibitors, Gram negative antibacterial

Acinetobacter baumannii is one of the most successful nosocomial pathogens, causing infections that have over the past few decades become increasingly difficult to treat. The ability of *A. baumannii* to survive for prolonged periods on abiotic surfaces, alongside broad antimicrobial resistance, allows it not only to survive but also to thrive in hospital settings (1). Consequently, there has been an alarming increase in mortality associated with infections caused by this difficult-to-treat organism (2). In addition to eliciting fatal nosocomial infections, this pathogen is a primary agent of infections in military personnel, often resulting from combat trauma or burns (3, 4). These often result in chronic wound infections and biofilm-mediated disease, with the latter resulting from surgery and implanted devices (4). Such chronic *A. baumannii*

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infections lead to complications, extended rehabilitation, increased use of hospital resources, and considerably increased mortality (4).

Drug resistance in *A. baumannii* has resulted in few antibiotics left to eradicate the infections it causes, with clinicians often turning to last-resort, toxic treatment options (1, 5, 6). The worldwide incidence of pan-drug-resistant (PDR) *A. baumannii* has spread quickly, at least in part due to its naturally transformable nature, leading to an increased capacity to acquire new determinants of resistance (1, 6). The occurrence of PDR isolates, with no effective treatment options, seemingly marks the beginning of a postantibiotic era for *A. baumannii*; thus, measures must be taken to identify effective therapeutic options (7).

Quinazolines are an emerging class of compounds that have a broad range of biological activities ranging from anticancer, anti-inflammatory, antipsychotic, antidiabetic, antileishmanial (8, 9), and antibacterial (10–15). Kung et al. discovered a series of 2-substituted quinazolines with broad-spectrum antibacterial activity, inhibiting RNA synthesis and translation in a number of bacterial species (16). More relevant to this study, Harris et al. revealed 5-substituted 2,4-diaminoquinazolines that inhibited the dihydrofolate reductase (DHFR) enzyme of *Escherichia coli* and *Staphylococcus aureus* (17). In so doing, they determined that the 5-substituted position of the 2,4-diaminoquinazolines was not as important for enzyme binding affinity as the general structural type of the group. Unfortunately, these molecules were not specific toward the bacterial DHFR enzyme but also inhibited the bovine liver DHFR enzyme (17). Further analysis revealed that smaller substituents created greater activity in bacterial cells, while larger substituents were more active toward the bovine enzyme. However, unlike the quinazolines identified in this study, the 5-substituted 2,4-diaminoquinazolines proved ineffective in animal models of infection (17).

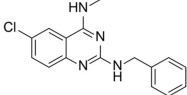
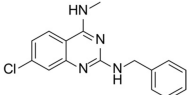
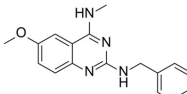
Our group has recently shown the utility of N^2,N^4 -disubstituted quinazoline-2,4-diamines for the treatment of *S. aureus* infections (18). Specifically, we have shown them to be active against a library of methicillin-resistant *S. aureus* (MRSA) isolates, displaying strong bactericidal activities, with limited cytotoxic and hemolytic capacities toward human cells. Mechanism-of-action profiling reveals that much like other quinazoline compounds, they appear to function by targeting bacterial dihydrofolate reductase (18–21). We have also shown their potential for antibiofilm activity, low frequencies of mutation, and *in vivo* efficacy using murine models of infection (18).

In this study, we have further explored the impact of N^2,N^4 -disubstituted quinazoline-2,4-diamines as antibacterial agents, focusing specifically on the Gram-negative species *A. baumannii*. Using a library of multidrug-resistant isolates, we revealed that these compounds are broadly bactericidal dihydrofolate reductase inhibitors. In addition, we observed that these compounds have low incidences of resistance and possess the potential for antibiofilm activity. Finally, we showed that the compounds are efficacious *in vivo* using a murine model of *A. baumannii* infection. Together, our results demonstrate for the first time the very real potential of quinazoline-derived compounds as antibacterial agents against the important human pathogen *A. baumannii*.

RESULTS AND DISCUSSION

N^2,N^4 -Disubstituted quinazoline-2,4-diamines are active against multidrug-resistant *A. baumannii* isolates. We have previously reported the activity of N^2,N^4 -disubstituted quinazoline-2,4-diamines against MRSA strains (18). To determine if our compounds have activity against any other bacterial species, we screened them against the other ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae*) pathogens. In so doing, we identified a number of analogues that were effective against *A. baumannii* but lacked activity toward other members of the ESKAPE pathogen set. To explore these findings more broadly, we expanded our studies to include a clonally diverse collection of *A. baumannii* isolates (Table 1). Strong activity was found against

TABLE 1 SAR focusing on benzenoid ring substitution of various quinazolin-2,4-diamines^a

Compound	Structure	MIC (μ M) for indicated strain					
		1403	1646	1649	1650	1651	1652
1		50	2	25	15	6	20
2		>50	6	30	>50	10	50
3		>50	2	25	50	10	30

^aSulfamethoxazole (SMX) and trimethoprim (TMP) were internal controls for each *in vitro* MIC assay: SMX, 138 μ M (1403), 118 μ M (1646), 118 μ M (1649), 118 μ M (1650), 118 μ M (1651), and 118 μ M (1652); TMP, 103 μ M (1403), 34 μ M (1646), 517 μ M (1649), 120 μ M (1650), 103 μ M (1651), and 103 μ M (1652).

a number of strains, with single-digit micromolar MICs noted for three benzenoid-substituted *N*²-benzyl-*N*⁴-methylquinazolin-2,4-diamines 1, 2, and 3 against strain 1646.

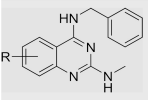
Following the identification of active quinazolines 1, 2, and 3, additional *N*²-benzyl-*N*⁴-methylquinazolin-2,4-diamines were made with either 6- or 7-substitutions (Table 2). Substitution at the 6-position with a bromo or a methyl group was found to be more beneficial for activity than substitution at the 7-position when comparing 6-bromoquinazolin-2,4-diamine 4 with its 7-substituted counterpart 6 or the 6-methyl-substituted quinazolin-2,4-diamine 5 with its 7-substituted analogue 7. Importantly, *N*²-benzyl-*N*⁴-methylquinazolin-2,4-diamine analogue 8, which lacks any substitution at the benzenoid ring, was inactive, with an MIC of \geq 50 μ M, and therefore demonstrated the importance of a 6- or 7-substituent on the benzenoid ring.

A similar trend was observed with *N*⁴-benzyl-*N*²-methylquinazolin-2,4-diamine analogues when comparing 6-substituted compounds 10 and 11 with the 7-substituted analogues 14 and 15 (Table 3). Furthermore, substitution in 6- or 7-position with an electron withdrawing chloro or a bromo moiety yielded quinazolines 9, 10, 13, and 14, which were more potent than corresponding methyl- or methoxy-substituted analogues 11, 12, and 15. Of all the quinazolines of the first two subseries tested, only

TABLE 2 Probing benzenoid substitution of *N*²-benzyl-*N*⁴-methylquinazolin-2,4-diamines^a

Compound	R	MIC (μ M) for indicated strain					
		1403	1646	1649	1650	1651	1652
4	6-Br	50	2	12	15	8	20
5	6-Me	10	2	25	50	10	20
6	7-Br	>50	6	25	>50	10	>50
7	7-Me	50	12	25	50	12	35
8	6-H, 7-H	>50	50	>50	>50	>50	>50

^aSulfamethoxazole and trimethoprim were internal controls for each *in vitro* MIC assay: SMX, 138 μ M (1403), 118 μ M (1646), 118 μ M (1649), 118 μ M (1650), 118 μ M (1651), and 118 μ M (1652); TMP, 103 μ M (1403), 34 μ M (1646), 517 μ M (1649), 120 μ M (1650), 103 μ M (1651), and 103 μ M (1652).

TABLE 3 Probing benzenoid ring substitution of *N*⁴-benzyl-*N*²-methylquinazolin-2,4-diamines^a


Compound	R	MIC (μM) for indicated strain					
		1403	1646	1649	1650	1651	1652
9	6-Cl	>50	4	50	50	6	>50
10	6-Br	>50	2	50	>50	>50	>50
11	6-Me	>50	6	>50	>50	12	>50
12	6-OMe	>50	6	>50	>50	8	>50
13	7-Cl	>50	4	>50	>50	>50	>50
14	7-Br	>50	4	>50	>50	>50	>50
15	7-Me	>50	12	>50	>50	>50	>50

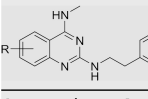
^aSulfamethoxazole and trimethoprim were internal controls for each *in vitro* MIC assay: SMX, 138 μM (1403), 118 μM (1646), 118 μM (1649), 118 μM (1650), 118 μM (1651), and 118 μM (1652); TMP, 103 μM (1403), 34 μM (1646), 517 μM (1649), 120 μM (1650), 103 μM (1651), and 103 μM (1652).

compound 5 was active against the clinically important strain 1403, with an MIC of 10 μM, leading us to believe that continued work on the benzenoid ring would be highly beneficial.

Extending the *N*²-benzyl chain to an *N*²-phenethyl was investigated to see if an increase in activity would be found (Table 4). Compound 16, with no benzenoid substitution, was 4-fold more active than the benzyl analogue 8 (Table 1). Compounds 17 to 20 were also found to be slightly more potent than the benzyl analogues 9 to 12 (Table 3), with MICs of 2 or 4 μM.

With the importance of substitution at the 6-position identified, new analogues were evaluated with vinyl, alkyl, or aryl substitutions (Table 5; see also Fig. S1 in the supplemental material). While the MIC barrier of 2 μM against the most susceptible strain (1646) was not broken, major advances were seen in activity against the most resistant isolate (1403). In particular, *n*-pentyl-, cyclohexenyl-, and cyclohexyl-substituted quinazolines 27, 29, and 30 had MICs of 2 μM against most isolates besides strain 1652, for which they had MICs of 10 μM and 30 μM. These three compounds revealed that large, bulky, and lipophilic groups at the 6-position are not only tolerated but also beneficial for inhibiting the growth of *A. baumannii*. Phenyl- and furanyl-substituted quinazolines 31 and 32 were less active, as were the vinyl and ethyl analogues 22 and 23, the isopropenyl and isopropyl analogues 24 and 25, and the cyclopentenyl-quinazoline 28.












Lead quinazolines have bactericidal activity. Lead quinazolines 4, 5, 26, 29, 27, and 30 were selected to be further evaluated for antimicrobial effects. The first assay utilized was a minimal bactericidal concentration (MBC) assay, to assess whether leads compounds were bacteriostatic or bactericidal. The six lead agents were screened to

TABLE 4 Benzenoid ring substitutions of *N*⁴-methyl-*N*²-phenethylquinazolin-2,4-diamines^a


Compound	R	MIC (μM) for indicated strain					
		1403	1646	1649	1650	1651	1652
16	6-H	>50	12	>50	>50	>50	>50
17	6-Cl	>50	2	>50	>50	>50	>50
18	6-OMe	>50	4	>50	>50	>50	>50
19	6-Br	>50	2	>50	>50	10	>50
20	6-Me	>50	2	>50	>50	10	>50

^aSulfamethoxazole and trimethoprim were internal controls for each *in vitro* MIC assay: SMX, 138 μM (1403), 118 μM (1646), 118 μM (1649), 118 μM (1650), 118 μM (1651), and 118 μM (1652); TMP, 103 μM (1403), 34 μM (1646), 517 μM (1649), 120 μM (1650), 103 μM (1651), and 103 μM (1652).

TABLE 5 Extension of the 6-position of *N*²-benzyl-*N*⁴-methylquinazolin-2,4-diamines^a

Compound	R	MIC (μ M) for indicated strain					
		1403	1646	1649	1650	1651	1652
21	6-Me	10	2	25	50	10	20
22		25	2	10	20	25	25
23		30	2	10	30	2	30
24		>50	2	25	>50	10	>50
25		>50	25	35	>50	35	>50
26		10	2	10	25	2	30
27		2	2	2	2	2	10
28		>50	2	50	>50	30	>50
29		2	2	10	10	2	30
30		2	2	2	2	2	10
31		30	10	20	20	2	50
32		40	25	>50	>50	15	>50

^aSulfamethoxazole and trimethoprim were internal controls for each *in vitro* MIC assay: SMX, 138 μ M (1403), 118 μ M (1646), 118 μ M (1649), 118 μ M (1650), 118 μ M (1651), and 118 μ M (1652); TMP, 103 μ M (1403), 34 μ M (1646), 517 μ M (1649), 120 μ M (1650), 103 μ M (1651), and 103 μ M (1652).

identify their MBC₉₀ toward each of the six *A. baumannii* isolates used in the SAR studies (Table S2), with data for isolate 1646 detailed in Table 6. Lead agents were all found to be broadly bactericidal, with MBC₉₀ values ranging from 0.8 μ M to 1.8 μ M. Compounds 4 and 5 were found to be the most efficacious at eliminating bacterial

TABLE 6 *In vitro* antibacterial assessment of front-runner quinazoline compounds against multidrug-resistant *A. baumannii*

Compound	MIC (μM)	MBC ₉₀ (μM)	MBEC ₉₀ (μM)	LD ₅₀ (HepG2) (μM)	Activity index ^a
4	0.5	0.8	3.3	23	46
5	1	0.8	2.8	22	22
26	1	1.8	8.9	16	22
29	2	1.5	8.9	23	12
27	1	1.1	11.5	12	12
30	2	1.1	41.2	11	6

^aActivity index = LD₅₀/MIC.

growth, with an MBC₉₀ value of 0.8 μM . Further to this, we were able to obtain complete eradication of bacterial growth for these two compounds at 1 μM for 4 and 5 μM for 5. Although marginally less effective, compounds 26, 29, 30, and 27 all still efficiently reduced bacterial viability, with MBC₉₀ values of 1.8 μM , 1.5 μM , 1.1 μM , and 1.1 μM , respectively. Moreover, compound 26 resulted in complete bacterial eradication at 5 μM , which is only 5 times its MIC.

Front-runner agents impact the viability of cells within a biofilm. *A. baumannii*, like many nosocomial pathogens, utilizes biofilm formation to increase persistence and decrease sensitivity to the action of antibiotics. Accordingly, the ability to impact cell viability within a biofilm is an important attribute for novel antimicrobial compounds. Therefore, we next tested our isolates for this activity, again using our library of multidrug-resistant strains (Table S2), with data from strain 1646 shown in Table 6. As with our bactericidal profiling, lead quinazolines 4 and 5 again had the most promising activity, with 90% biofilm eradication (MBEC₉₀) seen at 3.3 μM and 2.8 μM , respectively (Table 6). Further to this, analogue 5 was the most effective lead agent, with a 3-log reduction in biofilm viability observed at 10 μM . Compound 4 reduced biofilm viability by 3.6 logs, but not until a concentration of 50 μM . Lead quinazoline 30 had biofilm eradication potential similar to 4, reducing viability by 4.2 logs at 50 μM , although its MBEC₉₀ (1-log reduction) was found to be close to this value at 41 μM . Compounds 26 and 29 also had promising activity, with both displaying MBEC₉₀ at a concentration of 8.9 μM . Extended testing with these two quinazolines revealed that compound 29 reduced biofilm viability by 1.6 logs at 50 μM , while compound 26 resulted in a 1.4-log reduction in biofilm viability at the same concentration.

Lead quinazoline derivatives appear to function by targeting DHFR. To determine if lead quinazolines inhibit the *A. baumannii* dihydrofolate reductase (DHFR) enzyme, similar to that seen for sister compounds in our work with MRSA, an *in vitro* rescue assay was performed (18). Accordingly, the viability of *A. baumannii* cells was tested using lead quinazoline 5 in the presence or absence of tetrahydrofolic acid (THF; 0 μM to 225 μM), the end product produced by DHFR. After 24 h of incubation, we determined that only 10 μM THF was sufficient to rescue bacterial growth from the inhibitory effects of lead agent 5. These data suggest that the potential mechanism of action for our compounds is perhaps via inhibition of tetrahydrofolic acid production. While this finding supports data generated by us and others regarding the impact of quinazoline molecules on bacterial cells (18–21), we cannot discount the possibility of other potential targets within for these compounds within *A. baumannii*.

Quinazoline-derived compounds induce limited capacity for resistance. An important attribute of novel antibiotics is the ability to fend off the development of resistance toward their effects. To assess this capacity, *A. baumannii* strains were incubated overnight with 0.5 \times MIC of each of the front runners. The next day, cells were washed and used to inoculate fresh medium that contained a 2-fold increase in drug. This was repeated for a total of 8 days, alongside sulfamethoxazole (SMX) and trimethoprim (TMP) controls, both of which target the same pathway as our lead agents (Fig. 1), as well as an unrelated agent, tetracycline (TET) (22). Upon analysis, we determined that all of our front-runner compounds outperformed SMX, TMP, and TET,

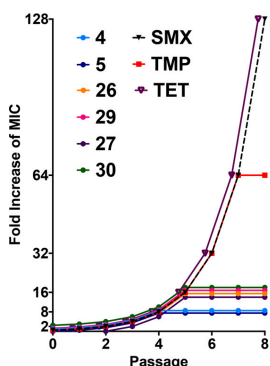


FIG 1 Lead quinazolines generate limited resistance by *A. baumannii* isolates. The six lead quinazolines were tested in a stepwise serial passage assay, alongside sulfamethoxazole (SMX), trimethoprim (TMP), and tetracycline (TET) controls. The first passage began with 0.5× the MIC, and with each passage, the concentrations of all compounds were increased 2-fold. The graph displays the fold increase in the MIC over the course of 8 days. Representative data generated using strain 1403 are shown.

generating much lower incidences of resistance. Specifically, lead agents 4 and 5 had the most striking effects, with MICs increasing over the 8-day test period only 4-fold, compared to 64-fold (TMP) and 128-fold (SMX and TET) for the control agents. All of the other 4 agents were similarly impressive in their ability to limit resistance development, resulting in an increased MIC of only 16-fold, which, while not as promising as the results for agents 4 and 5, is still profoundly reduced compared to our controls.

Front-runner quinazolines have limited toxicity toward human cells. In order to gain a sense of the toxicity of lead quinazolines toward eukaryotic cells, we determined 50% lethal doses (LD_{50}) for human HepG2 cells (Table 6; Fig. 2A). Importantly, we observed >50% cell viability for all compounds at concentrations up to 6 μ M. Furthermore, 4 of our 6 lead agents returned >50% viability at 12 μ M, while compounds 26 and 29 were only marginally less promising, returning HepG2 cell viabilities of 49% and 42%, respectively, at this concentration. When we used a 25 μ M concentration of each lead quinazoline or control antibiotic, we observed only fractionally less than 50% recovery. Importantly, lead agents 4 and 5 at 25 μ M performed the best, with 43% and 44% viability observed, respectively. Similarly, lead agents 26 and 30 allowed for 41%

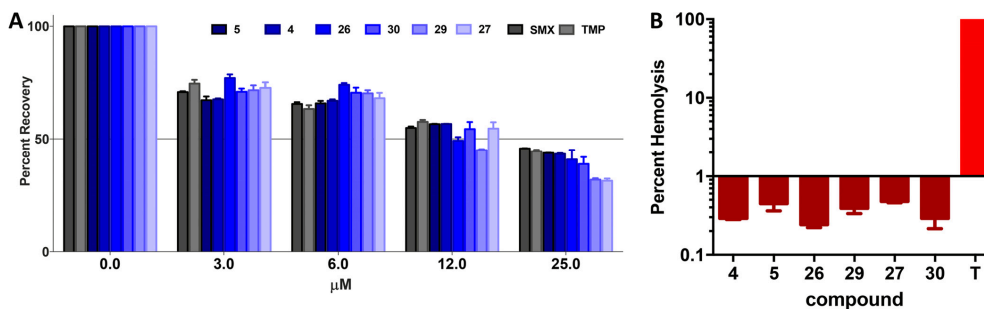


FIG 2 Cytotoxicity toward human cells. (A) Lead quinazolines were tested at 25 μ M, 12 μ M, 6 μ M, and 3 μ M against human HepG2 cells, compared to solvent-only controls. The known antibiotics sulfamethoxazole and trimethoprim, which target the same pathway as our quinazolines, were also tested in parallel. (B) The six lead quinazolines were tested at 10 μ M against whole human blood for the ability to lyse RBCs. Shown is the percent lysis of human red blood cells compared to a Triton X-100 (T) control at a concentration of 1%.

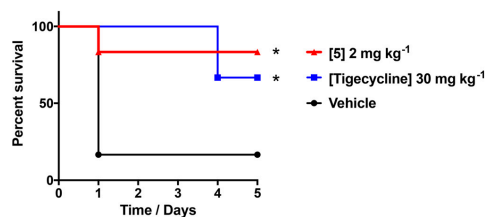


FIG 3 Lead quinazolines are efficacious *in vivo*. Mice were administered a lethal dose of *A. baumannii* 1646. They were then given lead quinazoline 5 at 2 mg kg⁻¹, tigecycline at 30 mg kg⁻¹, or a vehicle. Survival was monitored over a 5-day period. Statistical significance was determined using a log rank (Mantel-Cox) test. *, *P* < 0.05.

and 39% respective viability at this concentration, while treatment with lead agents 27 and 29 resulted in 31% and 32% viability, respectively. The control compounds sulfamethoxazole and trimethoprim returned 46% and 45% viability at the highest concentration tested, which is in line with data generated from our front-runners. To place lead compound data in context, lead agents 4 and 5 have the greatest therapeutic window for infection treatment. Specifically, lead agent 4 possesses a 46-fold preference in specificity toward bacteria, with an MIC (0.5 μM) much lower than the LD₅₀ (23 μM) toward human liver cells. Similarly, lead agent 5 displayed a 22-fold activity index (AI; LD₅₀/MIC), which is a measure of specificity toward bacterial cells (Table 6). As an additional measure of toxicity, we next tested the hemolytic capacity of the front-runners using whole human blood (Fig. 2B). Importantly, we observed negligible capacity of our lead quinazolines to lyse human red blood cells (hRBCs) when incubated for 1 h at a concentration of 10 μM. Specifically, we observed average hemolysis well below 1% (range = 0.24% to 0.47%), while the positive control (1% Triton X-100) produced 100% lysis during a similar time frame.

N²,N⁴-Disubstituted quinazoline-2,4-diamines are efficacious *in vivo*. As a final assessment, we used a murine model of lethal *A. baumannii* infection to determine the efficacy of quinazolines *in vivo*. This was performed using front-runner 5, which had the most promising properties from all of our biological testing. Accordingly, mice were inoculated with a lethal dose of *A. baumannii* via intraperitoneal (i.p.) injection on the right side of the abdomen. One hour postchallenge, mice were treated with an i.p. injection of 2 mg kg⁻¹ of front-runner 5 on the left side of the abdomen. As a control, we also performed similar testing using 30 mg kg⁻¹ of tigecycline, which we already know our test strain to be susceptible to *in vitro*. In so doing, we determined that quinazoline 5 resulted in a statistically significant survival rate of 83% of infected animals, compared to only 17% for vehicle-only controls (Fig. 3). We also saw significant survival of animals injected with tigecycline, although this was at a rate of 66%, which is inferior to that of our front-runner agent. Consequently, this would suggest that our class of N²,N⁴-disubstitutedquinazoline-2,4-diamines have excellent potential for development as antibacterial agents targeting multidrug-resistant *A. baumannii* infections.

Concluding remarks. A library of N²,N⁴-disubstituted quinazoline-2,4-diamines, which was previously shown to have antibacterial activity against MRSA (18), was also found to have potent effects toward the multidrug-resistant Gram-negative species *A. baumannii*. We assessed 73 N²,N⁴-disubstituted quinazoline-2,4-diamines and found that 6- or 7-substituted N²-benzyl-N⁴-methylquinazoline-2,4-diamines displayed promising activity, with MICs ranging from 0.5 to 30 μM against the six strains of *A. baumannii* tested. Over 30 molecules were designed and synthesized to conduct a structure-activity relationship study to systematically probe the substituents in the N²-, N⁴-, 6-, and 7-positions. The most potent *in vitro* activities were obtained with quinazoline-2,4-diamines bearing an N²-benzyl moiety and an N⁴-methyl group. Furthermore, quinazolines with substitutions in the 6-position with a halide or alkyl group

were more potent than analogues with substitutions at the 7-position. 6-*n*-Pentyl- and 6-cyclohexyl-substituted quinazolines 27 and 30 were among the most effective agents, since they were equipotent with single-digit micromolar MICs against the six tested *A. baumannii* strains. Following this, front-runner compounds 4, 5, 26, 29, 27, and 30 were tested for bactericidal activities and biofilm eradication. We found that the lead quinazolines 4 and 5 displayed the strongest bactericidal and biofilm activity toward *A. baumannii*, with MBC₉₀ values of <1 μM and MBEC₉₀ values of <4 μM. These compounds also allowed for limited resistance development, displaying only a 4-fold increase in MIC against *A. baumannii* over an 8-day period, which was only a fraction of that observed for control compounds. Using a murine model of infection, we determined that lead agent 5 was more effective, and at lower concentrations, in rescuing mice from a lethal dose of *A. baumannii* than our control agent tigecycline. Our results reveal the potent antibacterial activities of *N*²-benzyl-*N*⁴-methylquinazoline-2,4-diamines against *A. baumannii* and show their potential for development to treat both Gram-positive and Gram-negative multidrug-resistant infections.

MATERIALS AND METHODS

General. All strains used in this study are listed in Table S1 in the supplemental material.

Synthetic protocols and compound characterization. Full details of compound synthesis and characterization can be found in the supplemental material.

Antibacterial activity assessment. MIC and minimal bactericidal concentration (MBC) assays were performed in this study as documented by us previously (18, 23–25). Briefly, *A. baumannii* strains were grown in tryptic soy broth overnight cultures at 37°C with shaking. MIC determination was performed in a 96-well plate by diluting overnight cultures 1:1,000 in Mueller-Hinton broth (MHB) and adding 195 μl to each well. Subsequently, 5 μl of quinazoline (or control compound) was added before incubation for 24 h at 37°C. Following this, MICs were determined as the lowest concentration to produce a complete absence of growth. All compounds were diluted prior to testing in dimethyl sulfoxide (DMSO) to assess multiple concentrations with the addition of the same volume of solvent. MBC assays were performed in an identical manner to MIC experiments; however, after 24 h of incubation, bacterial cells were serially diluted in phosphate-buffered saline (PBS) and recovered on antibiotic-free tryptic soy agar (TSA) for 24 h at 37°C. MBC₉₀ values were calculated using linear regression of the percent recovery compared to no-treatment controls.

Biofilm eradication determination assay. Biofilm eradication determination assays were performed as described by us previously (25, 26), as follows. Each of the *A. baumannii* strains was grown overnight in MHB. The next day, these were used to seed fresh MHB to an optical density at 600 nm (OD₆₀₀) of 0.5, with 150 μl then added to the wells of a 96-well plate and grown for 24 h at 37°C. After 24 h, the planktonic bacteria were carefully removed and fresh MHB was added with increasing concentrations of lead quinazolines. After incubation at 37°C for 24 h, planktonic cells were removed and biofilms were washed three times with PBS. Biofilms were then resuspended in PBS and plated for cell viability on TSA. Biofilm recovery was assessed compared to that with no-drug controls and determined as percent eradication. This was used to determine MBEC₉₀ values (minimal biofilm eradication concentration), where the viability of cells within the biofilm was reduced by 90%.

Investigating the mechanism of action of quinazoline-based compounds. To evaluate the effect quinazolines have on DHFR reduction of dihydrofolic acid, a tetrahydrofolic acid rescue assay was performed as described by us previously (18). *A. baumannii* strain 1403 was grown overnight in LB and then diluted 1:1,000 into fresh medium. These cultures were then seeded into a sterile 96-well plate with tetrahydrofolic acid added at concentrations ranging from 0 to 225 μM. Lead quinazoline 25 was then added at 1×, 2×, and 5× the MIC, and cultures were incubated at 37°C for 18 h. MICs were determined and used to assess whether the addition of tetrahydrofolic acid rescued *A. baumannii* growth from quinazoline inhibition. Assays were repeated in triplicate, alongside trimethoprim and sulfamethoxazole controls.

Serial passage assay. In order to test potential resistance toward the quinazolines, a serial passage assay was performed alongside control compounds (sulfamethoxazole and trimethoprim), as described by us previously (25). *A. baumannii* strain 1403 was grown overnight in LB medium at 37°C. The next day, cultures were diluted 1:100 in fresh medium and seeded into a 96-well plate. Lead quinazolines or control agents were added to respective wells at half MICs. Plates were then incubated for 24 h at 37°C, followed by the removal of aliquots from these cultures to inoculate fresh medium (1:100 dilution) containing compounds at 2-fold higher concentrations. These were then grown overnight and the procedure was repeated for a total of 8 days. The cultures were observed for a lack of growth, indicating that strains were no longer able to resist the action of a given compound. Each experiment was performed in triplicate, yielding identical results.

HepG2 cytotoxicity. Cytotoxicity assays were performed using human HepG2 cells (human liver epithelial with hepatocellular carcinoma), as described by us previously (18, 25). Cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, for 3 days at 37°C and 5% CO₂. Cells were then diluted to 1 × 10⁵ ml⁻¹ using fresh DMEM and added to 96-well tissue culture plates at a volume of 100 μl. Plates were incubated for

24 h at 37°C and 5% CO₂, allowing the cells to adhere to the plastic. After this time, medium was carefully removed and 200 μ l of fresh DMEM was added with test compounds at concentrations of 0, 1, 2, 5, 10, 15, 30, and 50 μ M. Plates were then incubated for 48 h at 37°C and 5% CO₂. After 48 h, the DMEM was removed and 100 μ l of new medium containing 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) was added, followed by incubation for 4 h at 37°C and 5% CO₂. After 4 h, 75 μ l of medium was removed and replaced with 50 μ l of 16% (wt/vol) SDS and DMSO, followed by incubation for 10 min at 37°C to solubilize any formazan produced. A BioTek plate reader was used to measure the absorbance of formazan production at 540 nm. Lead compounds were solvated in 100% DMSO for these studies, which served as the negative control. LD₅₀s were determined for each compound by comparison to vehicle-only controls.

Hemolysis assay. A hemolysis assay was performed using whole human blood (Bioreclamation), as described previously (25). Briefly, human red blood cells (hRBCs) were resuspended in 20% vol/vol 1 × HA buffer (4.25 ml of 10% NaCl and 1 ml of CaCl₂ in 50 ml of sterile water), before lead compounds were added at 2 μ M, 10 μ M, and 20 μ M, in a final volume of 100 μ l. Cells were incubated for 15 min at 37°C before being centrifuged at 5,500 × g for 1 min to pellet nonlysed hRBCs. The supernatant was removed and added to a 96-well microtiter plate, and the OD₅₄₃ was read using a BioTek Synergy2 plate reader. The negative control was vehicle only (DMSO), and the positive control was 1% Triton X-100. Assays were performed in triplicate, with data displayed as percent hemolysis compared to controls, defined as percent hemolysis = (OD₅₄₃ of test sample – OD₅₄₃ of no-drug control)/(OD₅₄₃ of Triton X-100 – OD₅₄₃ of no-drug control) × 100.

In vivo efficacy testing using a murine model of lethal peritonitis. A murine model of lethal peritonitis was used to demonstrate the effectiveness of the lead quinazolines to clear bacterial infections, as described by us previously (25). Six mice per group were infected via intraperitoneal (i.p.) injection (right side) with 7.5 × 10⁸ CFU ml⁻¹ of *A. baumannii* 1646 in PBS containing 5% mucin. After 1 h, mice were inoculated by i.p. injection to the left side of the abdomen with either 2 mg/kg of lead agent 25 (test group), 30 mg kg⁻¹ of tigeicycline (positive control), or a vehicle alone [45% (wt/vol) (2-hydroxypropyl)- β -cyclodextrin in water (negative control)]. Mice were monitored twice daily for 5 days to assess mortality. All animal studies received written approval after review by the Institutional Animal Care and Use Committee in the Division of Comparative Medicine and Division of Research Integrity and Compliance at the University of South Florida. The clinical endpoint was reached for this study when the mice reached a premonitory state. The numbers of mice surviving in control and treatment groups were compared and analyzed for statistical significance using a log rank (Mantel-Cox) test.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00059-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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