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ANTIMICROBIAL CHARACTERIZATION AND THERAPEUTIC APPLICATIONS OF NOVEL SYNTHETIC THIAZOLE COMPOUNDS AGAINST MULTIDRUG-RESISTANT STAPHYLOCOCCI AND ENTEROCOCCI

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ATCC, American Type Culture Collection

BCP, Bacterial Cytological Profiling

BHI, brain heart infusion broth

CA-MRSA, community-associated MRSA

CAMHB, cation-adjusted Mueller-Hinton broth

CCCP, *m*-chlorophenyl carbonyl cyanide phenylhydrazone

CDC, Centers for Disease Control and Prevention

CFU, colony-forming unit

CLint, intrinsic clearance rate

CLSI, Clinical and Laboratory Standards Institute

DMEM, Dulbeco's modified Eagle's medium

DMSO, dimethyl sulfoxide

FBS, fetal bovine serum

FDA, Food and Drug Administration

FIC, fractional inhibitory concentration

FICI, fractional inhibitory concentration index

FPPS, farnesyl diphosphate synthase

HA-MRSA, healthcare-associated MRSA

HAIs, hospital-acquired infections

HPLC, high pressure liquid chromatography

IMV, inverted membrane vesicle

LB, Luria Bertani medium

LC-MS, liquid chromatography-mass spectrometry

MBC, minimum bactericidal concentration

MHB, Mueller-Hinton broth

MIC, minimum inhibitory concentration

mL, milliliter

mM, micro Molar

MOA, mechanism of action

CA-MRSA, community-associated MRSA

CAMHB, cation-adjusted Mueller-Hinton broth

MRSA, methicillin-resistant *Staphylococcus aureus*

MRSP, methicillin-resistant *Staphylococcus pseudintermedius*

MSM, methylsulfonyl methane

MSSP, methicillin-sensitive *S. pseudintermedius*

MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)

NADPH, Dihydronicotinamide-adenine dinucleotide phosphate

NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*

NGM, nematode growth medium

NMR, nuclear magnetic resonance

OM, outer membrane

Papp, apparent permeability coefficient

PAE, post-antibiotic effect

PBS, phosphate-buffered saline

PMF, proton motive force

SSTI, skin and soft tissue infection

T1/2, half-life

TLC, thin layer chromatography

TPSA, topological polar surface area

TSA, Tryptic soy agar

TSB, Tryptic soy broth

µg, microgram

µL, microliter

µM, micro Molar

UPPP, undecaprenyl diphosphate phosphatase

UPPS, undecaprenyl diphosphate synthase

UV, ultraviolet

VISA, vancomycin-intermediate *Staphylococcus aureus*

VRE, vancomycin-resistant Enterococci

VRSA, vancomycin-resistant *Staphylococcus aureus*

ABSTRACT

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Title: Antimicrobial Characterization and Therapeutic Applications of Novel Synthetic Thiazole Compounds against Multidrug-Resistant Staphylococci and Enterococci
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For more than a century, antibiotics have been valuable allies in combating an array of bacterial infections. However, each year nearly 23,000 people in the United States of America and 25,000 people in Europe die due to infections that are recalcitrant to currently available antimicrobials. The emergence of drug-resistant bacterial species, namely methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE), has limited the efficacy of several classes of antibiotics. Compounding this problem further is that many large pharmaceutical companies have left the field of antibacterial drug discovery given the high cost of innovation and low return on investment. Collectively, this highlights an urgent, unmet need to identify and develop new antibacterial agents that attack unique molecular targets in bacterial pathogens. Here, we investigate the antibacterial activity of a new series of phenylthiazole antibiotics against a panel of clinically-relevant 'ESKAPE' pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species). The lead compound **1** was identified through whole-cell screening of libraries of substituted thiazoles and thiadiazoles. Subsequent derivatives were constructed in an attempt to enhance potency, decrease toxicity to host tissues, and improve the lead compound's drug-like properties. Broth microdilution assay results show that the lead **1** and two derivatives (**2** and **3**) possess potent activity against Gram-positive bacterial pathogens including MRSA, methicillin-resistant *Staphylococcus pseudintermedius* (an emerging pathogen of importance in veterinary medicine) and VRE, inhibiting the growth of clinical isolates at concentrations as low as 0.5 μg/mL. The presence of the outer membrane and efflux pumps appears to impede the antibacterial activity of the phenylthiazoles against Gram-negative bacteria. MRSA and VRE mutants resistant to the phenylthiazoles could not be isolated, both via single-step and multi-step resistance

selection analysis. The compounds exerted a rapid bactericidal effect, targeting cell wall synthesis as deduced from Bacterial Cytological Profiling. Transposon mutagenesis suggested three possible targets: YubA, YubB and YubD. YubB is undecaprenyl diphosphate phosphatase (UPPP) and UPPP as well as undecaprenyl diphosphate synthase (UPPS) were inhibited by **1**, as confirmed by traditional enzyme inhibition assays. YubA and YubD are annotated as transporters and may also be targets since **1** collapsed the proton motive force in membrane vesicles. This indicates the phenylthiazole antibacterial agents have a unique mechanism of action that involves inhibition of key enzymes involved in peptidoglycan biosynthesis and potential transporters. This may contribute to the inability to generate bacterial mutants exhibiting resistance to the phenylthiazoles. The compounds were not toxic up to 20-40 μg/mL against different human cell lines including keratinocytes (HaCaT), kidney cells (HEK293), and colorectal cells (HRT-18). Additionally, the compounds were found to be non-toxic (at 20 μg/mL) in a *Caenorhabditis elegans* animal model. Closer inspection of the physicochemical profile and *in silico* pharmacokinetic profile of the lead **1** and more metabolically-stable analogue **3** revealed potential application for use topically (for localized skin infections), intravenously (for systemic infections), and as decolonizing agents. Utilizing a murine skin infection model, **1** and **3** were found to significantly reduce the burden of MRSA in infected lesions by more than 96%. Furthermore, both compounds (at 20 μg/mL) were potent *in vivo*, reducing the burden of VRE in infected *C. elegans*. Taken altogether, the results indicate that phenylthiazoles **1** and **3** are promising novel topical antibacterial agents and decolonizing agents for use in the treatment of drug-resistant staphylococcal and enterococcal infections.

CHAPTER 1. INTRODUCTION

Bacterial resistance to antibiotics is a growing global health epidemic that is impacting every geographic region of the world (1). Reports by the Centers for Disease Control and Prevention in the United States and the European Centre for Disease Control and Prevention indicate more than two million individuals in the United States and nearly 400,000 individuals in Europe are stricken each year with infections caused by multidrug-resistant pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenem-resistant *Klebsiella pneumonia* (KPC) and vancomycin-resistant *Enterococcus faecium* (VRE) (2, 3). Treatment of these infections are often expensive costing residents an estimated $55 billion in the United States and €1.5 billion in the European Union annually (2, 3). Furthermore, the issue of bacterial resistance to antibiotics around the world appears to be getting worse with the emergence of pathogens exhibiting resistance to agents of last resort (including glycopeptides, oxazolidinones, and carbapenems) (4-6). Further compounding the problem is the development and approval of new antimicrobials for use in treating infections caused by multidrug-resistant pathogens has not kept pace with the emergence of bacterial resistance to current antibiotics. Drug development of novel compounds is a time-consuming, costly, and high-risk venture given that few compounds successfully make it through stringent regulatory requirements to reach the marketplace. Though prudent use of effective antimicrobials is a critical step to alleviate complications and costs associated with MRSA infections, new antibacterial agents are urgently needed. The present review briefly highlights key bacterial pathogens of significant concern currently including MRSA and VRE, mechanisms by which these pathogens develop or acquire resistance to antibiotics, strategies to curb and combat antibacterial resistance, and concludes with incentives developed by governmental agencies to entice researchers in industry and academia to reinvest resources to discovering and developing new antibacterial agents.

1.1 Bacterial pathogens of significant concern currently

In a landmark report published in 2013 by the Centers for Disease Control and Prevention (CDC), the agency revealed that each year in the United States infections caused by drug-resistant bacteria result in more than 23,000 deaths (7). A report jointly commissioned by the European Centre for Disease Prevention and Control (ECDA) and European Medicines Agency (EMA) in 2008 determined that nearly 25,000 patients lose their lives to drug-resistant bacterial infections (8). Though all multidrug-resistant bacteria pose a threat to human health, multiple reports published by agencies including the CDC, Infectious Disease Society of America (IDSA), and the ECDA have listed specific pathogens that warrant urgent or serious attention due to the diminishing number of viable therapeutic options remaining to treat infections caused by these particular pathogens. A list of these specific pathogens, the estimated number of drug-resistant infections and deaths they cause each year in the United States (according to the CDC (7)), and examples of classes of antibiotics these pathogens are resistant to are presented in Table 1.1.

In the United States alone, these pathogens negatively impact the lives of over two million people at a cost of $20 billion to the healthcare system and, as highlighted earlier, result in over 23,000 deaths (9). Of these fatalities, nearly half are attributed to a single bacterial pathogen, methicillin-resistant *Staphylococcus aureus*. While once restricted to the healthcare setting (referred to as healthcare-associated MRSA or HA-MRSA), MRSA infections have become a major problem in the community (referred to as community-acquired MRSA or CA-MRSA) affecting a diverse population including healthcare workers, prison inmates, members of the military, athletes, the homeless population, intravenous drug users, newborn babies, and young children (10-19). Furthermore, CA-MRSA infections are typically associated with more severe morbidity and mortality than their HA-MRSA counterparts (20). While CA-MRSA is a leading cause of skin and soft-tissue infections, MRSA has also been associated with more complicated medical diseases including necrotizing pneumonia, osteomyelitis, and sepsis (21-25). However, the emergence of bacterial strains exhibiting resistance to numerous antibiotics has resulted in treatment failure. Indeed, clinical isolates of both CA-MRSA and HA-MRSA have been documented which exhibit resistance to nearly all antibiotic classes including the β-lactams, macrolides, quinolones, tetracyclines, and lincosamides (26-30). Further

exacerbating the problem, are clinical isolates have emerged that exhibit resistance to both first-line antibiotics and drugs deemed agents of last resort (such as linezolid and vancomycin) (5, 31, 32).

A second pathogen of significant concern that is often overlooked, is vancomycin-resistant *Enterococci*. Two species, *E. faecium* and *E. faecalis*, are responsible for the vast majority of enterococcal infections in humans. Unlike staphylococcal infections, enterococcal infections are primarily acquired in the health-care setting. Infections can range from superficial skin infections to more invasive diseases such as urinary tract infections and intra-abdominal infections (particularly problematic in patients undergoing surgery or receiving an organ transplant) (33). Enterococci are commensal organisms of the gastrointestinal tract and have an uncanny ability to acquire resistance to numerous antibiotics. Indeed enterococci are intrinsically resistant (or exhibit reduced susceptibility) to multiple antibiotics including penicillin-based antibiotics, cephalosporins, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole (34). Additionally, though *E. faecium* is typically susceptible to clindamycin and quinupristin-dalfopristin, some strains of *E. faecalis* are resistant to both agents (35). Although vancomycin has been frequently used to treat infections resistant to ampicillin and other antibiotics, more than 80% of ampicillin-resistant *E. faecium* in the United States now exhibit resistance to glycopeptide antibiotics like vancomycin (35). Furthermore, these strains (denoted as vancomycin-resistant enterococci or VRE) exhibit high-level resistance to aminoglycoside antibiotics such as gentamicin and streptomycin which severely limits the number of therapeutic agents available to treat VRE infections.

The multidrug-resistant bacteria highlighted in Table 1.1 utilize a variety of clever methods to both evade the host immune response to infection and neutralize the effect of multiple antimicrobials. For example, several bacteria including *Staphylococcus aureus*, *Acinetobacter baumanii* and, *Klebsiella pneumoniae* produce β-lactamases, enzymes that hydrolyze the β-lactam ring present in the penicillin, cephalosporin, and carbapenem drug classes, thus breaking down and inactivating these antibiotics (36). Other bacterial pathogens, including *Escherichia coli* (AcrAB-TolC) and *Pseudomonas aeruginosa* (MexAB-OprM), express an array of efflux pumps that transport antibiotics out of the bacterial cell before they can exert their effect (37). In addition to expression of efflux

pumps, *P. aeruginosa*'s outer membrane contains an outer membrane porin, OprF, that that prevents substances larger than 500 Daltons (that includes many antimicrobials) from gaining entry into the bacterial cell (36). A question that arises is how did these multidrug-resistant bacteria acquire these different resistance mechanisms to antibiotics?

1.2 Mechanisms by which bacteria become multidrug-resistant

Many present day antibiotics are semisynthetic derivatives of natural products originally isolated from bacteria and fungi (38, 39). For example, penicillin was derived from *Penicillium notatum*, vancomycin was isolated from the bacterium *Amycolatopsis orientalis* via a soil sample, streptomycin was purified from the bacterium *Streptomyces griseus*, and bacitracin was isolated from the bacterium *Bacillus subtilis* (38, 40, 41). These microbes secrete antibiotics as a defense mechanism to protect themselves from attack from other pathogens in their environment (42). A direct consequence of this action is these microorganisms also carry within their genome, genes that encode resistance mechanisms to ensure they are protected from the negative impact of the antibiotics they secrete. For example, *B. subtilis* expresses a transporter (BceAB) responsible for pumping bacitracin out of its cells (43). With time, these resistance mechanisms have been disseminated to other pathogens permitting the rapid spread of resistance to antibiotics.

The consequence of bacterial pathogens' ability to acquire resistance to antibiotics is that the clinical utility of many antimicrobials is relatively short as noted by Richard C. Allen in a 2014 journal article, "It is well established that our current practices of antibiotic use are unsustainable owing to the spread of antibiotic-resistant pathogens…The rapid spread of resistance means that the clinical lifespans of antibiotics are short, which reduces profits, and therefore incentives for the development of novel antibiotics, thus compounding the issue of resistance." (44) This statement is supported by the fact that bacterial resistance to three of the newest antibacterials approved by the FDA within the past decade (linezolid, daptomycin, and tigecycline) has already been observed in the healthcare setting (36). Each of these three antibiotics exerts their antibacterial action via different mechanisms, yet resistance to all three agents has emerged rapidly.

Linezolid, a bacterial protein synthesis inhibitor, received FDA approval in April 2000 and has been used as an agent of last resort for treatment of hard-to-treat infections caused by drug-resistant Gram-positive bacteria (36). However, just over a year after receiving approval, the first clinical isolate of methicillin-resistant *S. aureus* exhibiting resistance to linezolid was found in Boston, Massachusetts (45). Additional *S. aureus* clinical isolates exhibiting resistance to linezolid have been reported in the past decade (46-48). Daptomycin, an antibacterial that directly inserts into the bacterial cell membrane leading to rapid depolarization and cell death, received FDA approval in 2003 for treatment of systemic infections caused by Gram-positive bacteria. Within two years of being available in the clinic, two patients dealing with serious invasive MRSA infections died even after treatment with daptomycin; susceptibility analysis performed on the MRSA clinical isolates found they exhibited resistance to both daptomycin and vancomycin (49). Tigecycline, a broad-spectrum antibiotic that inhibits bacterial protein synthesis, received FDA approval in June 2005. In 2007, a report emerged where researchers isolated a strain of *A. baumanii* exhibiting resistance to tigecycline in Israel (50). In addition to this, resistance to tigecycline has been reported in other Gram-negative bacteria including *E. coli*, *K. pneumoniae*, and *S. enterica* due to the overexpression of a specific efflux pump (AcrAB) (51). Thus the rapid emergence of resistance to three of the newest antibiotics available to clinicians indicates the clinical utility of these antibiotics may be limited in the future.

The reality that our current arsenal of effective antimicrobials is diminishing was captured by a statement made by Dr. Janet Woodcock, Director for the Center for Drug Evaluation and Research at the Food and Drug Administration, in front of the U.S. House of Representatives Subcommittee on Health in 2014 when she stated – "As of today, antimicrobial-resistance mechanisms have been reported for all known antibacterial drugs that are currently available for clinical use in human and veterinary medicine." (52) Bacterial resistance has even been reported for certain agents (such as the antibiotic vancomycin and antimicrobial peptides (AMPs)) for which resistance was thought to be unlikely to occur (44). As it pertains to antimicrobial peptides, researchers initially thought given the rapid bactericidal effect exerted by these agents (by targeted physical disruption of the bacterial cell membrane) and the abundance and effectiveness of numerous AMPs

in nature for many years, bacterial resistance to these agents was unlikely to develop (53). However, this notion was dispelled when two researchers (Michael Zasloff and Graham Bell) identified that resistance to an AMP called pexiganan could be attained both by *E. coli* and *Pseudomonas fluorescens* after repeated subculturing of bacteria with a subinhibitory concentration of the peptide (53).

Glycopeptide antibiotics such as vancomycin play a critical role in the treatment of challenging infections caused by Gram-positive bacteria including staphylococci and enterococci; vancomycin, in particular is considered a drug of last resort for treatment of infections caused by multidrug-resistant Gram-positive bacteria (54). The discovery of vancomycin came at a crucial time as staphylococci had already developed resistance to penicillins, macrolides, and tetracyclines less than 15 years after their discovery and subsequent use in the clinic (41). No clinical isolates were found which exhibited resistance to vancomycin for nearly 30 years after its discovery and use; however in 1988 high-level resistance to vancomycin was discovered in patients impacted by an enterococcal infection (particularly strains of *E. faecium*) (54, 55). This led to the subsequent spread of glycopeptide-resistance around the world and transmission of resistance (encoded in part by the *vanA* gene) to strains of *S. aureus*. Vancomycin-intermediate *S. aureus* (VISA) strains were first identified in the 1990s in Japan but have now been isolated in many parts of the world including Asia, Europe, and North America (36); infections caused by VISA are particularly challenging to treat as these strains are often resistant to almost every class of antibiotics with the exception of agents of last resort (such as linezolid). Just over one decade after strains of VISA were first isolated, a vancomycin-resistant *S. aureus* (VRSA) isolate was identified in the United States in a patient suffering from a foot ulcer in Michigan (56). Though the number of VRSA cases identified to date are limited (at least 13 strains have been reported in the United States since 2002 according to the CDC (7)), the rapid emergence of more strains of *S. aureus* exhibiting resistance to vancomycin presents an ominous sign that vancomycin may not be a viable treatment option for challenging infections caused by multidrug-resistant *S. aureus* and enterococci infections in the near future.

The multidrug-resistant phenotype is not just limited to Gram-positive pathogens such as staphylococci and enterococci, as multidrug-resistant Gram-negative bacteria present a

potentially greater threat to human medicine given the few viable treatment options remaining in our antimicrobial arsenal. The surprising rapid emergence of antibiotic-resistance in Gram-negative pathogens is highlighted by *Acintobacter baumanii*. In the 1970s, strains of *A. baumanii* were sensitive to most traditional antibiotics (57). However, this bacterium has an uncanny ability to acquire resistance elements from other bacteria such that today, many strains are now resistant to nearly all available antibiotics (36). Most multidrug-resistant strains of *A. baumanii*, similar to *P. aeruginosa*, possess a resistance island containing genes encoding for multiple efflux pumps (conferring resistance to numerous antibiotics) (36). Furthermore, *A. baumanii* is very adept at acquiring genes for novel β-lactamases which protect this bacteria from the effect of β-lactam antibiotics (including to agents of last resort such as carbapenems and colistin) (36). The rapid emergence of multidrug-resistance in Gram-negative pathogens is not just limited to *A. baumanii* however. For many years, *E. coli* was highly susceptible to the effect of many antibiotics used to treat Gram-negative bacterial infections. However, this bacterium has utilized horizontal gene transfer to acquire multiple resistance factors such that few antibiotics are currently effective against drug-resistant *E. coli* strains (36). Thus without the development of novel antimicrobial agents, it is likely serious infections caused by multidrug-resistant Gram-negative pathogens including *A. baumanii*, *P. aeruginosa*, and *E. coli* will not have a viable treatment option available in the near future.

The rapid emergence of bacterial resistance to current antibiotics combined with the slow development of new treatment options, has led clinicians to return to using older antibiotics (such as colistin), that have shown effectiveness in treating infections caused by drug-resistant bacteria (particularly Gram-negative bacteria such as *P. aeruginosa*) (36). However, even these older agents are not immune to the issue of bacterial resistance; clinical isolates of *A. baumanii* and *P. aeruginosa* have been found exhibiting resistance to colistin indicating even these older antibiotics may not be useful treatment options in the future (36, 58, 59).

As noted by Pendleton *et al*, in their review of six of the most problematic bacterial pathogens that pose a significant threat to humans worldwide at present, these multidrug-resistant bacteria can acquire resistance to antibiotics, genetically, in three ways (36):

1. Random point mutations in the bacterial chromosome (including in the gene encoding the target protein of a specific antibiotic)
2. Intra- and interspecies transmission/sharing of genetic elements (such as plasmids containing pathogenicity islands with multiple resistance genes) via horizontal gene transfer
3. Introduction of foreign DNA (containing one or more resistance genes) directly into the core bacterial chromosome (genetic recombination)

As it pertains to the first point, a classic example that demonstrates the impact of point mutations conferring antibiotic resistance in pathogens involves the relationship of fluoroquinolones and *P. aeruginosa*. Fluoroquinolones belong to a class of broad-spectrum antibiotics that inhibit DNA gyrase (in Gram-negative bacteria) and topoisomerase IV (in Gram-positive bacteria) (36). This action results in inhibition of cell division in bacteria. However, point mutations in the *gyrA* (encoding DNA gyrase) and *parC* (encoding topoisomerase IV) genes in *P. aeruginosa* have been found to be responsible for the resistance to fluoroquinolones observed in this pathogen (60). Additionally, another study found that resistance to macrolide antibiotics (interfere with bacterial protein synthesis) can arise in *Mycobacterium smegmatis* via a point mutation in one of the two 23S rRNA genes (61). As noted by Pendleton *et al*, random point mutations conferring resistance to an antibiotic can arise when a subinhibitory concentration of the antibiotic is present that would select for resistant strains capable of growing rapidly in this condition (36).

Aside from the emergence of random point mutations in the target gene, bacterial pathogens can acquire resistance genes to antimicrobials by sharing genetic elements (encoding resistance genes) with each other via horizontal gene transfer (via bacterial conjugation for example). The ability of pathogens to acquire resistance genes via horizontal gene transfer was exemplified in 2002 when the first strain of *S. aureus* exhibiting resistance to vancomycin was isolated from a patient's foot ulcer (56). From the same ulcer, physicians also isolated a vancomycin-resistant *Enterococcus* species (VRE). When genetic analysis was performed on both bacterial strains, researchers determined that a plasmid containing the gene encoding for vancomycin resistance in enterococci (*vanA* present on the transposon Tn*1546*) had been transferred to the *S. aureus* strain via bacterial conjugation (56). Vancomycin disrupts cell wall synthesis in bacteria by binding to specific

peptidoglycan precursors (at the C-terminal of the D-alanyl-D-alanine peptide) which interferes with the latter stages of cell wall synthesis. Expression of VanA results in the formation of modified precursors (containing a D-alanyl-D-lactate peptide instead) with reduced binding affinity for vancomycin (and other glycopeptide antibiotics). Horizontal gene transfer has also been implicated in the acquisition of genes encoding a variety of different β-lactamase enzymes (including penicillinases, cephalosporinases, and carbapenemases) exchanged between members of the *Enterobacteriaceae*, specifically in *E. coli* and *K. pneumoniae* (62). Additionally, analysis of the genome of *A. baumanii* strain AYE found this strain contained 52 resistance genes (conferring resistance to numerous antibiotics including several β-lactams, tetracycline, chloramphenicol, fluoroquinolones, and rifampin) (57); by comparing the amino acid sequences of proteins encoded by these resistance genes in *A. baumanii* AYE in relation to other bacteria, these researchers found many of these genes were acquired either via horizontal gene transfer or DNA recombination from *Pseudomonas* species, *Salmonella* species, and *Escherichia* species. Thus horizontal gene transfer and DNA recombination (insertion of foreign DNA containing resistance genes) have played a big role in the acquisition of bacterial resistance to antibiotics.

1.3 Methods to curb the emergence of rapid resistance to antibacterial agents

While it is apparent that bacterial resistance to current antibiotics is a significant challenge, are there any methods that can be employed by researchers and clinicians to slow down or reverse this effect? Several different strategies are currently being employed to address this point. Perhaps the most vital strategy that can be employed globally to slow down the emergence of resistance to antimicrobial agents (as noted by the World Health Organization) is better antibiotic stewardship, as is currently being undertaken in the European Union and the United States. Stewardship entails implementing strategies to monitor the sale of antibiotics (a major problem in underdeveloped nations where antibiotics are sold without a prescription to treat infections that may not be caused by bacteria), ensuring antibiotics are only used to treat bacterial infections (and not viral infections as erroneously prescribed by some physicians (63)), ensuring patients complete the entire course of an antibiotic regimen, and reserving newly approved antibiotics and

agents of last resort (such as vancomycin and linezolid for infections caused by Gram-positive bacteria and carbapenems for Gram-negative bacteria) for dire infections where other antibiotics fail to treat the infection (64). As mentioned by Pendelton *et al*, part of antimicrobial stewardship entails "prescribing the most appropriate antibiotic at the correct dose and time, and for a suitable duration, has been consistently proven to improve patient outcomes and reduce the emergence of antibiotic resistance" (36). Furthermore, the Infectious Disease Society of America motes that effective antibiotic stewardship programs could reduce antibiotic use in hospitals by 22% to 36%, potentially saving these facilities up to $900,000 each year in treatment costs (65). However, less than half of all hospitals in the United States utilize an antibiotic stewardship program (36). Additionally, a recent report by WHO noted that fewer than 40% of countries worldwide had national strategies in place to address the issue of antimicrobial resistance (including adopting an antibiotic stewardship program in their hospitals) (64). Thus the implementation of antimicrobial stewardship programs has the potential to reduce the use of antibiotics in the healthcare setting and potentially slow down the pace of resistance to antimicrobials.

Another strategy to try to address the challenge of antimicrobial resistance is directly targeting the mechanisms in bacteria that confer resistance to antibiotics. Examples include developing small molecule inhibitors of bacterial efflux pumps and inhibitors of β-lactamase (such as clavulante potassium) thus re-sensitizing resistant bacteria to antibiotics (such as penicillin drugs) (36, 39). This particular strategy has already achieved success clinically with the approval and use of the antimicrobial agent amoxicillin/clavulanic acid to treat infections caused by amoxicillin-resistant bacteria (which secrete β-lactamase that is inhibited by clavulanate potassium). This indicates a promising new direction for the development of future antimicrobial agents.

Perhaps the most promising strategy to curb the challenge posed by bacterial resistance to current antibiotics is to invest resources to discover new antibacterial agents with unique mechanisms of action/molecular targets. A significant challenge in antibiotic drug discovery though is many large pharmaceutical companies have left this field due to the high cost of innovation and low return on investment. Discovering new antimicrobial compounds in the laboratory and successfully translating them as drugs in the clinic is both a significant financial ($800 million to over $1 billion in costs) and time-consuming

investment (10-17 years to go from a discovering a compound in the lab and receiving regulatory approval to use in the clinic) (66, 67). The low financial return of antibiotics (one estimate has noted that a net profit may not be earned by a company until 23 years after it initiates the process of early stage discovery of an antibiotic at which time the patent (typically lasts for 20 years from date it was filed) may expire permitting inexpensive generic versions of the drug to be made that further undercut the profit the original discover of the antibiotic can make (68)) in comparison to therapeutic agents used in the treatment of chronic diseases (such as cancer, hypertension/high cholesterol, and diabetes) and the extremely low success rate of receiving regulatory approval for a new antibiotic (estimated in one report to be between 1.5 – 3.5% (68)) has provided the impetus for numerous big pharmaceutical companies both in the United States and abroad to divest in discovering new antimicrobials. Recognizing the need to entice pharmaceutical companies and academic research institutions to reinvest in restocking the antibiotic drug discovery pipeline, governmental agencies have successfully lobbied for incentives to generate new antibacterial agents.

1.4 Current incentives in place for the discovery or new antibacterial agents

On average, 20-30 new drugs receive FDA-approval each year; however few of these new drugs are antibacterial agents (69, 70). This presents a challenging conundrum given the vast majority of drugs currently available in the market were discovered by the pharmaceutical industry. In the US, only 9% of new drugs discovered from 1960 through 1969 were developed by government agencies, universities, and not-for-profit organizations (69). This trend continued to hold true in latter parts of the 20th century as over 93% of new drugs approved in the United States, from 1990 to 1992, were procured from industry; government agencies and academic institutions each accounted for just over 3% of new drugs in this time span (66). Thus industry is a key cog in the identification and development of drugs which are capable of reaching the healthcare setting. However, given the low return on investment for antibiotics, large pharmaceutical companies have moved away from developing new antibiotics. This can be illustrated with a simple example; from 2009-2012, Merck's leading medication for diabetes (Januvia) outsold its top-selling antibiotic (Invanz, a carbapenem antibiotic) by US$11 billion (71). Moreover, a review of

the top 100 best-selling drugs from April 2013 through March 2014 revealed treatments for chronic diseases such as rheumatoid arthritis (Humira, Enbrel, Remicade), depression (Cymbalta, Seroquel XR), asthma (Advair), high-cholesterol (Lipitor, Crestor, Zocor), multiple sclerosis (Copaxone, Tecfidera), Alzheimer's disease (Namenda), diabetes (Lantus Solostar, Januvia), AIDS (Atripla, Truvada, Prezista), high blood pressure (Diovan, Metoprolol), and cancer (Rituxan, Avastin, Gleevec) generated the most sales for pharmaceutical companies; interestingly no antimicrobials were found on this list. Given the associated costs involved with drug discovery, the lack of sales generated by antibiotics (in comparison to drugs developed for chronic diseases such as asthma, diabetes, and high blood pressure), and stringent regulations required for new antibiotics to receive regulatory approval, this significantly reduces the incentive needed by companies to pursue developing novel antimicrobials (72). This has led to several major companies, including Pfizer and Roche, to terminate their antibiotics research & development division; as of 2013, only four major pharmaceutical companies have active antimicrobial drug discovery programs (71, 73). This leaves government agencies, academic institutions, and small companies with the burden of filling this gap to generate new antimicrobials.

Several advocacy groups such as the Infectious Diseases Society of America have successfully lobbied government officials and agencies to develop incentives to encourage pharmaceutical companies and academic research institutions to re-invest resources in the field of antimicrobial drug discovery. One of the successful outcomes of this advocacy effort was the passage of the "Generating Antibiotics Incentives Now Act (GAIN Act)," by the United States Congress in 2012.

The Generating Antibiotic Incentives Now Act of 2011 (GAIN Act also referred to as H.R.2182) focused on providing drug companies several incentives to drive research efforts in developing new antibacterial agents (74). Among the incentives included in this act are:

- Five additional years of exclusive marketing rights granted by the United States Food and Drug Administration (FDA) for new antibiotics approved for treatment of serious and life-threatening infections or that fall under the Qualified Infectious Disease Product (QIDP) designation. As stated in the H.R.2182 bill, QIDP refers to an "antibiotic drug for treating, detecting,

preventing, or identifying a qualifying pathogen (certain pathogens that are resistant to antibiotics)" (74). This incentive grants drug companies more time to generate more profit (to regain money they invested in developing this antibiotic) by preventing other companies from developing inexpensive generic versions of the drug.

- Granting QIDP entities priority, expedited review by the FDA (to fast track the new drug approval process). This helps address the regulatory red tape that can impede progress in attaining approval for new antibiotics which was a point of frustration for pharmaceutical companies.
- Require the FDA to review guidelines associated with clinical trials of antibiotics and clarify requirements that need to be met for a new antibiotic to receive approval.
- The GAIN Act also requires the FDA to provide an up-to-date list of pathogens that do, or have the potential to, pose a threat to the public thus permitting drug companies to develop treatments to target these particular threats.

Since the passage of the GAIN Act, there has been several positive signs that the incentives provided are working to address the need for new antimicrobials, particularly for infections caused by multidrug-resistant pathogens. From May to August 2014, the FDA approved three new antibiotics (Dalvance, Sivextro, and Orbactiv) for use in treating acute bacterial skin and skin structure infections (ABSSSI) caused by bacteria such as *Staphylococcus aureus* (75). All three drugs took advantage of the QIDP provision in the GAIN Act that permitted the sponsors of these drugs to receive a more rapid review and approval of their drug application. These drugs also received five years of marketing exclusivity. In addition to the three drugs noted above, 39 additional antibacterials currently in development have been given the QIDP designation (75), indicating this particular measure of the GAIN Act appears to be working. Furthermore, the author of the GAIN Act (Congressman Phil Gingrey) mentioned in July 2013 that "12 new antibiotics are currently in the final stages of approval process" (76). Also, it appears a limited number of pharmaceutical companies that had abandoned the field of antibiotic drug discovery (such as Roche), are returning in part due to the incentives provided by government legislation encouraging discovery of new antibiotics.

Two additional bills entitled "Antibiotic Development to Advance Patient Treatment Act of 2013" (ADAPT Act also referred to as H.R.3742 introduced to the United States House of Legislation in December 2013) and the "Promise for Antibiotics and Therapeutics for Health Act" (PATH Act also referred to as S.2996) build upon the GAIN Act by targeting the same problem – speeding up the pace at which the FDA approves antibiotics (77, 78). More specifically, these acts of legislation would grant the FDA the ability to fast track approval of a new antibacterial agent (to be used alone or in combination with other agents) to be used in a limited population of patients afflicted with a life-threatening or serious bacterial infection for which few (if any) treatments exist. Part of this legislation limits the amount of data (such as studies pertaining to drug effect on patient safety or side effects) needed to be generated by the drug sponsor in order to receive regulatory approval. This is an incentive that is appealing to drug companies as it potentially curbs or reduces high costs associated with lengthy clinical trials. However, as stated in the bill, this incentive would be limited to antibiotics used for treatment of life-threatening or serious infection for which few treatments currently exist. A question that remains to be addressed is would pharmaceutical companies be willing to invest time and money to develop an antibiotic with a limited clinical application (especially given the limited frequency such an agent may be used in a clinical setting thus limiting the financial gain to a company from the sale of this antibiotic)? Given the limited number of antibiotics in general that have received approval in recent years, the incentive highlighted by the ADAPT and PATH Acts most likely would be insufficient to convince pharmaceutical companies not currently invested in antibiotic discovery to join the effort. However, more studies need to be conducted to address this point and confirm if the incentives highlighted in both the ADAPT and PATH Acts would in fact drive more drug companies to invest in antimicrobial drug development.

Another legislative bill introduced in January 2015 (H.R. 4187, the Development an Innovative Strategy for Antimicrobial Resistant Microorganisms or DISARM Act) focuses on addressing issues pertaining specifically to treating infections caused by antimicrobial resistant microbes. Issues of note addressed by this bill include getting the government's Medicare system to recognize and cover expenses utilizing "DISARM antimicrobial drugs" in hard-to-treat bacterial infections (as normally Medicare supports use of cheaper, older

antibiotics), publishing a list of "DISARM antimicrobial drugs," and the initiation of a study to identify the barriers to and potential solutions to the discovery of new drugs to treat drug-resistant infections (so-called DISARM antimicrobial drugs) (79). While this last point (conducting a study to find solutions to address issues impacting discovery of new drugs for infections caused by antimicrobial resistant microbes) may eventually lead to the enactment of future government incentives to draw pharmaceutical companies into the field of antibiotic drug discovery, by itself, it will not be enough to promote antibiotic discovery by pharmaceutical companies in the near future.

While the GAIN, ADAPT, PATH, and DISARM Acts all address different issues that have impeded antibacterial drug discovery, additional policies that encourage pharmaceutical companies to invest resources in development of new antimicrobials are needed. As highlighted earlier, pharmaceutical companies are the major source of innovation, discovery, and successful translation of promising compounds with antibacterial activity into drugs. More than 90% of all new drugs that received regulatory approval in the early 1990s were discovered by pharmaceutical companies (80). Finding policies that cut down stringent regulatory hurdles and decrease costs associated with clinical trials are crucial to bringing back companies to the field of antimicrobial drug discovery. However, decreasing the length or number of clinical trials required by regulatory agencies most likely would not decrease the total cost associated with drug discovery unless the length of trials was reduced significantly (by 75% according to one study which would be very difficult to achieve in order to ensure a new drug was both safe and effective to use in humans) (81). The infusion of funding from government agencies to support antibiotic drug discovery in pharmaceutical companies (such as the up to $200 million funding approved by the U.S. Department of Health and Human Services to the company GlaxoSmithKline in 2013 to fund its research in antibiotic development (82)) is a needed step in addressing the challenge of restocking the antimicrobial drug discovery pipeline. Such public-private partnerships may be the best method to draw more pharmaceutical companies back into the arena of antimicrobial drug discovery (more so than the passage of government legislation targeting regulatory red tape and increasing marketing exclusivity for antibiotics). The European Union has been investing in public-private partnerships since the late 1990s for the identification and development of novel

antimicrobials; recently a multidisciplinary team of European researchers (from the Novel Approaches to Bacterial Target Identification Validation and Inhibition (NABATIVI) team) working with partners from the company Polyphor discovered a new antibacteial (POL7080) capable of treating infections caused by *Pseudomonas aeruginosa*. NABATIVI (which is funded by the European Union) assisted Polyphor Ltd in completing both pre-clinical research and a phase I clinical trial in 2013 (to confirm the drug was safe to use in human patients) (83). In November 2013, Polyphor licensed their drug to a major drug company (Roche) to perform phase 2 clinical trials to test the effectiveness of POL7080 in patients afflicted with an infection caused by *P. aeruginosa*. This example highlights a potential model for how public-private partnerships can work together to reduce the risk and costs to pharmaceutical companies in developing new antimicrobials. Recently, a similar imitative was started in 2016 the United States entitled CARB-X (Combating Antibiotic-Resistant Bacteria Biopharmaceutical Accelerator) and is anticipated to receive US$350 million in financial support. By smaller research groups (in universities or government agencies) conducting much of the pre-clinical work involved with screening compounds for antibacterial activity and testing for safety and efficacy in suitable animal models then licensing promising lead compounds to pharmaceutical companies to conduct further testing in early stage clinical trials, the risk and costs associated with early stage drug discovery can be reduced. This will permit pharmaceutical companies to invest their resources on latter stages of antibiotic drug discovery (namely in conducting clinical trials and working with regulatory agencies to gain approval for new antimicrobials). Increasing the funding provided by government agencies to support such public-private partnerships to develop new antimicrobials is a key measure that warrants further exploration.

1.5 Conclusion

Antibiotic resistance is a significant global public health challenge that requires urgent attention. Bacterial pathogens, including MRSA and VRE, have acquired unique mechanisms (including expression of efflux pumps and proteins to break down or chemically inactivate antibiotics) that confer resistance to numerous antibiotics including agents of last resort, such as vancomycin. Strategies to combat antibiotic resistance include enacting effective antibiotic stewardship programs in hospitals and clinics and designing

agents to inhibit/reverse resistance mechanisms. However, the single-most effective strategy involves developing new antibacterial agents effective against multidrug-resistant bacterial pathogens. Given the high cost of innovation and low return on investment, many large pharmaceutical companies have left the field of antibiotic drug discovery. However the passage of legislation such as the GAIN Act has provided incentives that are encouraging companies and academic research institutions to reinvest in restocking the antibiotic drug discovery pipeline. The formation of public-private partnerships between companies, governmental agencies, and academic research institutions is necessary in order to collectively address the challenge of bacterial resistance to antibiotics and push promising antibacterial compounds through the preclinical and clinical stages of drug discovery.

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Table 1.1 List of multidrug-resistant bacterial pathogens of significant concern to human health.

Name of Bacterial Pathogen	Gram-positive or Gram-negative	Estimated Number of Multidrug-resistant Infections Annually (United States)	Estimated Number of Deaths Annually (United States)	Antimicrobial Agents/Classes Pathogen is Resistant to
Acinetobacter baumanii	Gram-negative	7,300	500	Cephalosporins, β-lactam antibiotics, aminoglycosides fluoroquinolones, chloramphenicol, and carbapenems
Campylobacter jejuni and Campylobacter coli	Gram-negative	310,000	120	Ciprofloxacin and azithromycin
Carbapenem-resistant (CRE) and Extended spectrum β-lactamase producing (ESBL) Enterobacteriaceae - *Klebsiella pneumonia* - *Escherichia coli*	Gram-negative	35,000	2,300	Nearly all antibiotics including agents of last resort (carbapenems)
Clostridium difficile	Gram-positive	250,000	14,000	Fluoroquinolones
Mycobacterium tuberculosis (in particular extremely drug-resistant tuberculosis or XD-TBR)	Gram-positive	1,042	50	Isoniazid, rifampicin, fluoroquinolone, amikacin, kanamycin, and capreomycin
Neisseria gonorrhoeae	Gram-negative	246,000	N.R.[1]	Cephalosporins (cefixime, ceftriaxone), azithromycin, tetracycline
Pseudomonas aeruginosa	Gram-negative	6,700	440	Certain strains exhibit resistance to nearly all antibiotics including aminoglycosides, cephalosporins, fluoroquinolones, and carbapenems
Non-typhoidal *Salmonella* (serotypes other than Typhi, Paratyphi A, Paratyphi B, and Paratyphi C)	Gram-negative	100,000	450	Ceftriaxone, ciprofloxacin, chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole

Table 7.1 continued

Salmonella serotype Typhi	Gram-negative	3,800	N.R.	Ceftriaxone, azithromycin, and ciprofloxacin
Shigella species (namely *S. flexneri*, *S. sonnei*)	Gram-negative	27,000	40	Ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, and azithromycin
Methicillin-resistant, vancomycin-intermediate, and vancomycin-resistant *Staphylococcus aureus*	Gram-positive	80,461	11,285	β-lactams, cephalosporins, glycopeptides, tetracycline, fluoroquinolones, tetracycline, mupirocin, and linezolid
Streptococcus pneumonia	Gram-positive	1,200,000	7,000	Penicillin and erythromycin-based antibiotics
Vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*	Gram-positive	20,000	1,300	Vancomycin and teicoplanin

[1] N.R. = Not reported

CHAPTER 2. DISCOVERY AND CHARACTERIZATION OF POTENT THIAZOLES VERSUS METHICILLIN- AND VANCOMYCIN-RESISTANT *STAPHYLOCOCCUS AUREUS*

THIS IS A PUBLISHED JOURNAL ARTICLE. Reprinted with permission from **Discovery and Characterization of Potent Thiazoles versus Methicillin- and Vancomycin-Resistant *Staphylococcus aureus*.** Haroon Mohammad, Abdelrahman S. Mayhoub, Adil Ghafoor, Muhammad Soofi, Ruba A. Alajlouni, Mark Cushman, and Mohamed N. Seleem. *Journal of Medicinal Chemistry* **2014** *57* (4), 1609-1615 DOI: 10.1021/jm401905m.

2.1 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a rapidly expanding global health concern. It is currently the most common pathogen linked to patients with skin and soft-tissue infections.(1) Apart from the high mortality and rapid transmission rates, MRSA infections result in an estimated $3 billion to $4 billion of additional health care costs per year.(2) Resistance has also emerged to therapeutic agents once deemed to be the drugs of choice in treating MRSA infections, such as vancomycin (3) and linezolid.(4) Researchers and clinical-care providers are thus facing an increasingly difficult challenge trying to construct novel antimicrobials and new therapeutic options to treat MRSA-related infections.

The thiazole ring is a key structural component for a wide spectrum of therapeutic agents including anticonvulsants,(5) anticancer,(6, 7) and antiviral agents.(8) In this study, whole-cell screening assays of libraries of substituted thiazoles and thiadiazoles identified a novel lead compound that displayed notable antibacterial activity against MRSA. The lead compound **1a** (Figure 2.1) consists of a thiazole central ring connected to two unique structural features – a cationic element at the C5-position and a lipophilic moiety at the C2-position. These two structural components have been hypothesized to contribute to the antibacterial activity of the lead compound. Structural optimizations were focused on the lipophilic side chain at thiazole-C2 of the lead compound in an attempt to enhance the antimicrobial activity of the lead compound against MRSA and VRSA. Chemical

modifications reported here involved building a focused library of phenylthiazoles with different lipophilic moieties at the phenyl para position to define the structure-activity-relationships (SARs) at the thiazole-C2 position in a rigorous way. Our objectives were to investigate the antimicrobial activities of the thiazole derivatives against MRSA and VRSA, ascertain the killing kinetics of MRSA *in vitro* by the lead compound and two derivatives, determine the cytotoxic impact of the derivatives on mammalian cells *in vitro*, and to investigate the physicochemical properties (namely solubility and permeability) of the thiazole compounds.

2.2 Materials and Methods

2.2.1 Chemistry

General. All biologically tested compounds produced HPLC traces in which the major peak accounted for ≥ 95% of the combined total peak area when monitored by a UV detector at 254 nm. ^{1}H NMR spectra were run at 300 MHz and ^{13}C spectra were determined at 75.46 MHz in $CDCl_3$, DMSO-d_6, or CD_3OD. Chemical shifts are given in parts per million (ppm) on the delta (δ) scale. Chemical shifts were calibrated relative to those of the solvents. Flash chromatography was performed on 230-400 mesh silica and preparative TLC separations utilized Analtech Uniplates with glass-supported silica (20 × 20 cm, 2000 micron thickness) and UV indicator (254 nM). The progress of reactions was monitored with Baker-flex silica gel IB2-F plates (0.25 mm thickness). Mass spectra were recorded at 70 eV. High resolution mass spectra for all ionization techniques were obtained from a FinniganMAT XL95. Melting points were determined using capillary tubes with a Mel-Temp apparatus and are uncorrected. HPLC analyses were performed on a Waters binary HPLC system (Model 1525, 20 μL injection loop) equipped with a Waters dual wavelength absorbance UV detector (Model 2487) set for 254 nm, and using a 5 μM C-18 reverse phase column. All reactions were conducted under argon or nitrogen atmosphere, unless otherwise specified. All yields reported refer to isolated yields.

Thiazole ethylketone derivatives **4a-g** were prepared in moderate yields by heating thioamides **3a-g**, obtained by treatment of the corresponding amides with Lawesson's reagent in dry THF, with 3-chloropentane-2,4-dione in absolute ethanol (Figure 2,2). The

methyl ketones **4a-g** were gently heated with aminoguanidine hydrochloride in the presence of lithium chloride as a catalyst to afford hydrazinecarboximidamide derivatives **1a-g** (Figure 2.2). Similarly, the final products **1h**, **7**, **8** and **12** were obtained using a similar synthetic protocol (Figures 2.3-2.5). A more detailed explanation of the synthetic scheme for each compound is described below.

Preparation of Hydrazinecarboximidamides 1a-h, 7, 8, and 12. General Procedure. The ketone derivatives **3a-h**, **5**, **6**, **11** or **16** (1-10 mmol) were dissolved in absolute ethanol (10-50 mL). Aminoguanidine hydrochloride (1 equivalent) and a catalytic amount of LiCl (5-20 mg) were added. The reaction mixtures were heated at reflux for 24 hours. The solvent was evaporated under reduced pressure. The crude product was purified by crystallization from 70% methanol, and then recrystallized from absolute methanol to afford the desired compounds as solids. Compound **1a** (9) is previously reported.

Preparation of Thioamides 3a-g. Thioamides **3a-g** (1-5 mmol), which were obtained by treatment of their corresponding carboxylic acids **2a-g** with thionyl chloride followed by gradual addition to ammonia solution, and Lawesson's reagent (1.2 equiv.) were added to dry THF (15-40 mL). The reaction mixtures were stirred at room temperature for 5-12 hours. The solvent was evaporated under reduced pressure and the residues were partitioned between aqueous NaHCO3 (2 M, 25-50 mL) and ethyl acetate (25-75 mL). The organic solvent was separated and dried over anhydrous MgSO4. After solvent evaporation, the crude products were further purified by silica gel flash chromatography, using hexane-ethyl acetate (4:1), to yield the corresponding thioamides as yellow solids (55-57%) in the desired purity degree. Compounds **3a-g** were characterized previously as follows. 4-*n*-Butylbenzamide,(9) butylthiobenzamide (**3a**),(9) thiobenzamide (**3b**),(10) 4-*n*-propylbenzamide,(9) propylthiobenzamide (**3c**),(9) 4-*n*-pentylbenzamide,(9) pentylthiobenzamide (**3d**),(9) 4-*n*-heptylbenzamide,(11) 4-*n*-heptylthiobenzamide (**3e**)(12, 13) 4-nonylbenzamide,(14) 4-*t*-butylbenzamide,(15) 4-*t*-butylthiobenzamide (**3g**)(16) are reported.

Preparation of Methyl Ketones 4a-i. General Procedure. Thiobenzamides **3a-i** (2-10 mmol) and 3-chloropentane-2,4-dione (1.4 equivalent) were added to absolute ethanol (10-30 mL). The reaction mixtures were heated at reflux for 12-24 hours. After evaporation of solvent under reduced pressure, the brown residues were collected and purified by silica

gel flash chromatography, using hexane-ethyl acetate (9:1), to yield compounds **4a-i** in the desired purity. Compounds **4a**(9) and **4b**(17) are previously reported. Characterizations of compounds **4c-i** are listed below.

4-Nonylthiobenzamide (3f). Yellow solid (550 mg, 76%): mp 57 °C. ^{1}H NMR (DMSO-d_6) δ 9.76 (brs, 1 H), 9.39 (brs, 1 H), 7.82 (d, J = 8.1 Hz, 2 H), 7.21 (d, J = 8.1 Hz, 2 H), 2.58 (t, J = 7.5 Hz, 2 H), 1.55 (m, 2 H), 1.25 (m, 12 H), 0.83 (t, J = 6.9 Hz, 3 H); ^{13}C NMR (DMSO-d_6) δ 200.65, 146.95, 137.71, 128.66, 128.32, 35.78, 32.20, 31.61, 29.88, 29.78, 29.62, 29.56, 23.02, 14.88; ESIMS *m/z* (rel intensity) 264 (MH^+, 100); HREISMS, *m/z* 264.1784 MH^+, calcd for $C_{16}H_{26}NS$ 264.1786.

1-[4-Methyl-2-(4-propylphenyl)thiazol-5-yl]ethanone (4c). White solid (135 mg, 61%): mp 57 ˚C. ^{1}H NMR ($CDCl_3$) δ 8.10 (d, J = 9.0 Hz, 2 H), 7.32 (d, J = 9.0 Hz, 2 H), 2.93 (s, 3 H), 2.6 (m, 5 H), 1.67 (m, 2 H), 0.96 (t, J = 6.3 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 189.43, 171.04, 155.41, 148.87, 131.59, 129.63, 127.87, 126.96, 38.00, 30.36, 24.06, 16.88, 13.67; CIMS *m/z* (rel intensity) 260 (MH^+, 100); HRMS (EI), *m/z* 259.1033 M^+, calcd for $C_{15}H_{17}NOS$ 259.1031.

1-[4-Methyl-2-(4-pentylphenyl)thiazol-5-yl]ethanone (4d). Colorless oil (159 mg, 76%). ^{1}H NMR ($CDCl_3$) δ 7.85 (d, J = 8.7 Hz, 2 H), 7.22 (d, J = 8.7 Hz, 2 H), 2.73 (s, 3 H), 2.60 (t, J = 6.0 Hz, 2 H), 2.50 (s, 3 H), 1.63 (m, 2 H), 1.33 (m, 4 H), 0.87 (t, J = 6.0 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 190.33, 169.56, 159.39, 146.64, 130.71, 130.25, 129.04, 126.77, 35.79, 31.37, 30.79, 30.67, 22.45, 18.41, 13.97; CIMS *m/z* (rel intensity) 288 (MH^+, 100); HRMS (EI), *m/z* 287.1347 M^+, calcd for $C_{17}H_{21}NOS$ 287.1344.

1-[4-Methyl-2-(4-hepylphenyl)thiazol-5-yl]ethanone (4e). Colorless oil (360 mg, 44%). ^{1}H NMR ($CDCl_3$) δ 7.86 (d, J = 8.1 Hz, 2 H), 7.25 (d, J = 8.1 Hz, 2 H), 2.76 (s, 3 H), 2.63 (t, J = 6.0 Hz, 2 H), 2.55 (s, 3 H), 1.62 (m, 2 H), 1.28 (m, 8 H), 0.87 (t, J = 6.0 Hz, 3 H); ^{13}C NMR ($CDCl_3$) δ 190.35, 169.64, 159.42, 146.70, 131.36, 130.73, 130.26, 129.06, 126.80, 35.84, 31.74, 31.13, 30.70, 29.16, 29.09, 22.60, 18.42, 14.05; CIMS *m/z* (rel intensity) 316 (MH^+, 100); HRMS (EI), *m/z* 315.1655 M^+, calcd for $C_{19}H_{25}NOS$ 315.1657.

1-[4-Methyl-2-(4-nonylphenyl)thiazol-5-yl]ethanone (4f). Yellow oil (450 mg, 60%). ^{1}H NMR ($CDCl_3$) δ 7.87 (d, J = 8.1 Hz, 2 H), 7.24 (d, J = 8.1 Hz, 2 H), 2.77 (s, 3 H), 2.63 (t, J = 7.0 Hz, 2 H), 2.55 (s, 3 H), 1.62 (m, 2 H), 1.29 (m, 12 H), 0.87 (t, J = 6.9 Hz, 3 H);

^{13}C NMR ($CDCl_3$) δ 190.42, 169.65, 159.44, 146.71, 130.75, 130.29, 129.07, 126.81, 35.86, 31.83, 31.14, 30.72, 29.48, 29.43, 29.25, 22.63, 18.45, 14.07; ESIMS *m/z* (rel intensity) 344 (MH^+, 100); HRESIMS, *m/z* 344.2052 M^+, calcd for $C_{21}H_{30}NOS$ 344.2048.

1-{2-[4-(*tert*-Butyl)phenyl]-4-methylthiazol-5-yl}ethanone (4g). White solid (557 mg, 62%): mp 53 ˚C. ^{1}H NMR ($CDCl_3$) δ 7.84 (d, *J* = 8.4 Hz, 2 H), 7.43 (d, *J* = 8.4 Hz, 2 H), 2.72 (s, 3 H), 2.49 (s, 3 H), 1.31 (s, 9 H); ^{13}C NMR ($CDCl_3$) δ 190.27, 169.35, 159.36, 154.65, 130.70, 129.97, 126.57, 125.90, 34.85, 31.00, 30.62, 18.37; CIMS *m/z* (rel intensity) 274 (MH^+, 100); HRMS (EI), *m/z* 273.1182 M^+, calcd for $C_{16}H_{19}NOS$ 273.1187.

1-[4-Methyl-2-(naphthalen-2-yl)thiazol-5-yl]ethanone (4h). White solid (110 mg, 67%): mp 109 °C. ^{1}H NMR ($CDCl_3$) δ 8.41 (d, *J* = 0.9 Hz, 1 H), 7.94 (d, *J* = 1.8 Hz, 1 H), 7.84 (m, 3 H), 7.50 (d, *J* = 9.0 Hz, 2 H), 2.76 (s, 3 H), 2.51 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 190.31, 169.28, 159.49, 134.50, 132.98, 131.21, 129.94, 128.79, 127.80, 127.54, 126.93, 126.67, 123.71, 30.69, 18.45; ESIMS *m/z* (rel intensity) 268 (MH^+, 100); HRESIMS, *m/z* 268.0793 MH^+, calcd for $C_{16}H_{14}NOS$ 264.0796.

1-[2-(4-Iodophenyl)-4-methylthiazol-5-yl]ethanone (4i). Brown solid (1050 mg, 58%): mp 123 ˚C. ^{1}H NMR ($CDCl_3$) δ 7.80 (d, *J* = 8.7 Hz, 2 H), 7.68 (d, *J* = 8.7 Hz, 2 H), 2.76 (s, 3 H), 2.56 (s, 3 H); ^{13}C NMR ($CDCl_3$) δ 190.29, 168.05, 159.44, 138.14, 132.11, 131.43, 128.13, 97.66, 30.70, 18.36; CIMS *m/z* (rel intensity) 344 (MH^+, 100); HRMS (EI), *m/z* 342.9535 M^+, calcd for $C_{12}H_{10}INOS$ 342.9528.

1-{2-[4-(1-Cyclohexenyl)phenyl]-4-methylthiazol-5-yl}ethanone (5). A solution of 4-iodophenylthiazole **4i** (100 mg, 0.3 mmol) in dry DMF (5 mL) was charged with $Pd(OAc)_2$ (5 mg), cyclohexene (1 mL) and triethylamine (0.5 mL). The reaction mixture was heated at 80 ˚C for 5 h. The reaction mixture was quenched with distilled water (10 mL) and extracted with ethyl acetate (30 mL). The organic layer was separated and dried over anhydrous Na_2SO_4. After evaporation of solvent under reduced pressure, the oily residue was collected and purified by silica gel flash chromatography, using hexane-ethyl acetate (9:1), to yield faint yellow oil (35 mg, 39%). ^{1}H NMR ($CDCl_3$) δ 7.90 (d, *J* = 8.1 Hz, 2 H), 7.29 (d, *J* = 8.1 Hz, 2 H), 5.76 (m, 2 H), 2.80 (m, 1 H), 2.77 (s, 3 H), 2.55 (s, 3 H), 2.27-1.24 (m, 6 H); ^{13}C NMR ($CDCl_3$) δ 190.45, 169.54, 159.46, 150.94, 130.84, 130.63, 128.42, 127.60, 126.97, 126.33, 40.06, 32.98, 30.73, 29.40, 25.59, 18.45; CIMS

m/z (rel intensity) 298 (MH^+, 100); HRMS (EI), *m/z* 297.1189 M^+, calcd for $C_{18}H_{19}NOS$ 297.1187.

1-[2-(4-Cyclohexylphenyl)-4-methylthiazol-5-yl]ethanone (6). Compound **5** (100 mg, 0.3 mmol) and Pd (50 mg, 10% on activated charcoal) were added to deoxygenated absolute methanol (10 mL). Hydrogen was applied via a balloon. The reaction mixture was stirred at room temperature for 24 h, and then filtered through celite. The filtrate was collected and the solvent was evaporated under reduced pressure to yield compound **7** as a colorless oil (100 mg, 100%). ^1H NMR ($CDCl_3$) δ 7.87 (d, *J* = 8.4 Hz, 2 H), 7.27 (d, *J* = 8.4 Hz, 2 H), 2.75 (s, 3 H), 2.53 (s, 3 H), 2.51 (m, 1 H), 1.85-1.73 (m, 5 H), 1.43-1.24 (m, 5 H); ^{13}C NMR ($CDCl_3$) δ 190.45, 169.65, 159.44, 151.70, 130.74, 130.40, 127.51, 126.88, 44.50, 34.10, 30.70, 26.69, 25.99, 18.44; CIMS *m/z* (rel intensity) 300 (MH^+, 100); HRMS (EI), *m/z* 299.1350 M^+, calcd for $C_{18}H_{21}NOS$ 299.1344.

1-(2-([1,1'-Biphenyl]-4-yl)-4-methylthiazol-5-yl)ethanone (11). The aldehyde **9** (800 mg, 5.2 mmol) was added to a solution of hydroxylamine hydrochloride (725, 10.5 mmol) in DMSO (10 mL), and the reaction mixture was stirred at 100 ˚C for 20 min. The heater was turned off and aqueous NaOH solution (600 mg dissolved in 5 mL distilled water) was slowly added to the reaction mixture over a 2 min period with stirring, and then hydrogen peroxide 50% (5 mL) was slowly and carefully added over a 10 min period. The reaction mixture was further stirred for 12 h and extracted with ethyl acetate (3 x 10 mL), dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to afford the corresponding amide as a white solid. The crude amide (2 mmol) and Lawesson's reagent (980 mg, 2.4 mmol) were added to dry THF (15 mL). The reaction mixture was stirred at room temperature for 12 h. The solvent was evaporated under reduced pressure and the residue was partitioned between aq $NaHCO_3$ (25 mL) and ethyl acetate (25 mL). The organic solvent was separated and dried over anhydrous Na_2SO_4.

The crude product was further purified by silica gel flash chromatography, using hexane-ethyl acetate (4:1), to yield the corresponding thioamide **10** as a yellow solid. The obtained thioamide **10** (215 mg, 1 mmol) and 3-chloro-2,4-pentanedione (0.3 mL, 2.5 mmol) were added to absolute ethanol (10 mL). The reaction mixture was heated at reflux for 24 h. After evaporation of solvent under reduced pressure, the oily residue was collected and

purified by silica gel flash chromatography, using hexane-ethyl acetate (9:1), to yield compound **11** as an off-white solid (290 mg, 49%): mp 124-125 ˚C. ^{1}H NMR ($CDCl_3$) δ 8.36 (d, *J* = 8.4 Hz, 2 H), 7.77 (d, *J* = 8.4 Hz, 2 H), 7.64 (d, *J* = 8.7 Hz, 2 H), 7.46 (m, 3 H), 3.03, 2.64; ^{13}C NMR (CD_3OD) δ 190.45, 153.24, 148.20, 143.78, 140.00, 130.51, 130.28, 128.49, 127.94, 127.14, 126.14, 126.01, 23.90, 17.99; ESIMS *m/z* (rel intensity) 290 (MH^+, 100); HRESIMS, *m/z* 290.1039 MH^+, calcd for $C_{18}H_{27}NS$ 290.1939.

2.2.2 Bacterial Strains, Reagents, and Antibiotics

The complete list and description of bacterial strains presented in this study is located in Table 2.1. MRSA clinical isolates, vancomycin-intermediate *Staphylococcus aureus* (VISA), vancomycin-resistant *Staphylococcus aureus* (VRSA) and linezolid-resistant strains were obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program. In addition, MRSA ATCC 43300 was obtained from the American Type Cultural Collection (Manassas, VA, USA). Lysostaphin (Sigma-Aldrich, St. Louis, MO, USA) at 20 μg/mL was prepared in 50 mM Tris-HCl (pH 8.00) (Sigma-Aldrich, St. Louis, MO, USA). Vancomycin hydrochloride powder was purchased commercially (Gold Biotechnology Inc., St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA).

2.2.3 Determination of Minimum Inhibitory Concentration (MIC)

The MICs of the lead thiazole compound (compound **1a**) and nine derivatives tested against all 18 MRSA strains were determined, in triplicate samples, using the broth microdilution method in accordance with the recommendations contained in the Clinical and Laboratory Standards Institute guidelines. The MIC was categorized as the concentration at which no visible growth of bacteria was observed in a particular well. The average of triplicate MIC determinations is reported along with standard deviation values.

2.2.4 Time-kill Assay

MRSA (USA300) cells, in the logarithmic growth phase, were diluted to 1.0×10^6 colony-forming units (CFU/mL) and exposed to concentrations equivalent to 3.0 × MIC (in triplicate) of compounds **1a**, **1d**, **8**, and vancomycin in tryptic soy broth (TSB) (Becton, Dickinson and Company, Sparks, MD, USA). Viable CFU/mL was determined by serial

dilution and plating on tryptic soy agar (TSA) (Becton, Dickinson and Company, Sparks, MD, USA) plates after 0, 2, 4, 6, 8, 10, and 12 hours of incubation at 37 °C to identify the time required to reduce the bacterial cell count by 3-$\log_{10}$.

2.2.5 *In Vitro* Cytotoxicity Analysis

Compounds were assayed at a concentration of 11 µg/mL in human cervical adenocarcinoma cells (HeLa) to determine the potential toxic effect in vitro. Cells were cultured in Dulbeco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (USA Scientific, Inc.) at 37 °C with 5% CO_2. Controls received DMSO alone at a concentration equal to that in drug-treated cell samples. The cells were incubated with the compounds in a 96-well plate at 37 ºC and 5.0% CO_2 for two hours prior to addition of the assay reagent MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (Promega, Madison, WI, USA). Corrected absorbance readings (actual absorbance readings for each treatment subtracted from background absorbance) were taken using a kinetic ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with each compound was expressed as a percentage of the control, DMSO.

2.2.6 Calculation of Partition Coefficient (log P) and Topological Polar Surface Area (TPSA)

Calculated log P and topological polar surface area (TPSA) values for the thiazole compounds were obtained using Molinspiration Cheminformatics software available on the internet (http://www.molinspiration.com/).

2.2.7 Caco-2 Permeability Assay

Caco-2 cells grown in tissue culture flasks were trypsinized, suspended in medium, and the suspensions were applied to wells of a Millipore 96 well Caco-2 plate. The cells were allowed to grow and differentiate for three weeks, feeding at 2-day intervals. For Apical to Basolateral (A→B) permeability, compound **1a** was added to the apical (A) side and amount of permeation was determined on the basolateral (B) side; for Basolateral to Apical (B→A) permeability, compound **1a** was added to the B side and the amount of permeation was determined on the A side. The A-side buffer contained 100 µM Lucifer yellow dye, in

Transport Buffer (1.98 g/L glucose in 10 mM HEPES, 1.0 × Hank's Balanced Salt Solution) pH 6.5, and the B-side buffer contained Transport Buffer at pH 7.4. Caco-2 cells were incubated with these buffers for two hours, and the receiver side buffer was removed for analysis by LC/MS/MS. To verify the Caco-2 cell monolayers were properly formed, aliquots of the cell buffers were analyzed by fluorescence to determine the transport of the impermeable dye Lucifer Yellow. Any deviations from control values are reported. Data are expressed as permeability ($Papp$) = $(dQ/dt)/C_0A$ where dQ/dt is the rate of permeation, C_0 is the initial concentration of test agent, and A is the area of the monolayer. In bidirectional permeability studies, the efflux ratio (R_E) is also calculated: R_E = ($Papp$(B→A))/($Papp$(A→B)). An $R_E > 2$ indicates a potential substrate for P-glycoprotein or other active efflux transporters.

2.2.8 MDCK-MDR1 Permeability Assay

MDCK-MDR1 cells were grown in tissue culture flasks, trypsinized, suspended in medium, and the suspensions were applied to membranes plate wells (96-well format). The cells were allowed to grow and differentiate for five days. For Apical to Basolateral (A→B) permeability, compound **1a** was added to the apical (A) side and the amount of permeation was determined on the basolateral (B) side; for Basolateral to Apical (B→A) permeability, compound **1a** was added to the B side and the amount of permeation was determined on the A side. The A-side buffer contained 100 µM Lucifer Yellow dye, in Transport Buffer (1.98 g/L glucose, 10 mM HEPES, in 1.0 × Hank's Balanced Salt Solution) pH 7.4. The B-side buffer was Transport Buffer, pH 7.4. MDCK-MDR1 cells were incubated with these buffers for two hours, and the receiver side buffer was removed for analysis by LC/MS-MS. To verify the MDCK-MDR1 cell monolayers were properly formed, aliquots of the cell buffers were analyzed by fluorescence to determine the transport of the impermeable dye Lucifer Yellow. Data are expressed as permeability ($Papp$) = $(dQ/dt)/C_0A$ where dQ/dt is the rate of permeation, C_0 is the initial concentration of compound **1a**, and A is the area of the monolayer. In bidirectional permeability studies, the efflux ratio (R_E) is also calculated: R_E = ($Papp$(B→A))/($Papp$(A→B)). An $R_E > 2$ indicates a potential substrate for P-glycoprotein or other active efflux transporters.

2.2.9 PBS Solubility Screen

Serial dilutions of lead compound **1a**, reserpine, tamoxifen, and verapamil were prepared in phosphate buffered saline (PBS) at 100 × the final concentration. The solutions were diluted 100-fold into PBS in a 96-well plate and mixed. The absorbance of the PBS-containing plate was measured prior to addition of the test agents to determine the background absorbance. After two hours, the presence of precipitate was detected by turbidity (absorbance at 540 nm). An absorbance value of greater than (mean + 3 × standard deviation of the blank), after subtracting the pre-experiment background, is indicative of turbidity. The solubility limit is reported as the highest experimental concentration with no evidence of turbidity.

2.2.10 Microsomal Stability Analysis

Compound **1a** was incubated in duplicate with microsomes at 37 ºC. The reaction contained microsomal protein in 100 mM potassium phosphate, 2 mM NADPH, 3 mM $MgCl_2$, pH 7.4. A control was run for each test agent omitting NADPH to detect NADPH-free degradation. At 0, 10, 20, 40, and 60 minutes, an aliquot was removed from each experimental and control reaction and mixed with an equal volume of ice-cold Stop Solution (methanol containing haloperidol, diclofenac, or other internal standard). Stopped reactions are incubated at least ten minutes at -20 ºC, and an additional volume of water was added. The samples were centrifuged to remove precipitated protein, and the supernatants were analyzed by LC/MS/MS to quantitate the remaining parent. Data are converted to % remaining by dividing by the time zero concentration value. Data are fit to a first-order decay model to determine half-life. Intrinsic clearance is calculated from the half-life and the protein concentrations: $CL_{int} = \ln(2) / (T_{1/2}$ [microsomal protein]).

2.2.11 Statistical Analysis

All statistical analysis was conducted using Kaleida Graph, version 4.03 (Synergy software, Reading, PA). Statistical significance was determined using ANOVA and the Fisher's Least Significant Difference (LSD) test with $\alpha = 0.05$.

2.3 Results and Discussion

2.3.1 Antibacterial activity of thiazole compounds against MRSA and VRSA

The 10 substituted thiazole compounds we synthesized inhibited growth of 18 different strains of MRSA and VRSA at a concentration ranging from 0.4-5.5 μg/mL (Table 2.2). The lead compound **1a** inhibited the growth of MRSA strains at concentrations ranging from 1.4 – 5.5 μg/mL. Subsequently synthesized derivatives demonstrated a two- to five-fold improvement in the MIC values. Initially, the effect of increasing the length of the alkyl side chain, through insertion of methylene units, was explored. As the length of the alkyl side chain increased from two (compound **1c**) to three (compound **1a**) to four (compound **1d**) methylene units, there was a consistent improvement in the MIC values observed against all MRSA strains tested. However, additional lengthening of the alkyl side chain appeared to nullify the improvement observed in the antimicrobial activity, as the MIC for compound **1e** (containing six methylene units) nearly matched or exceeded the values obtained for compound **1d**. This result held true as an increase to eight methylene units (compound **1f**) resulted in an MIC value that nearly matched or exceeded the MIC value attained for compound **1a**. Altogether this indicates that an alkyl side chain with four methylene units exhibits the optimum potency against MRSA and addition of methylene units to the alkyl side beyond four units will not significantly enhance the antimicrobial activity of the lead compound.

Replacement of the linear alkyl side chain with a branched alkane (compound **1g**) produced mixed results. There was a modest improvement in the MIC values against six MRSA strains (1.0 μg/mL for **1g** compared to 1.4 μg/mL for **1a**) and a nearly two- to five-fold enhancement in the MIC for five additional strains. Substitution of the alkyl side chain with a fused ring system (compound **1h**) did not significantly enhance the activity of the derivative against the MRSA strains tested, with the exception of VRSA (a near three-fold reduction in MIC was observed compared to **1a**). However, replacement of the alkyl side chain with conformationally restricted analogues (compounds **7**, **8**, and **12**) demonstrated the most consistent, significant improvement in the MIC value obtained relative to the lead compound (two- to four-fold improvement against 16 MRSA strains tested).

The MIC values obtained for compounds **7**, **8**, and **12** on multiple occasions matched or were lower than the antibiotic vancomycin against the MRSA strains tested. Furthermore, all ten thiazole compounds proved to be more potent than vancomycin in inhibiting growth of VISA and VRSA strains. Compounds **7**, **8**, and **12** also proved more effective at eliminating growth of MRSA NRS119, a strain resistant to linezolid (a drug of last resort in treatment of MRSA infections), and several strains resistant to multiple antibiotic classes including lincosamides, aminoglycosides, fluoroquinolones, and macrolides (USA100, USA200, and USA500). In addition to this, all 10 compounds exhibited excellent activity against MRSA USA300, a strain responsible for most cases of community-acquired MRSA (CA-MRSA) and MRSA skin and soft tissue infections (SSTIs) in the United States.(18, 19).

2.3.2 Time-kill assay of thiazole compounds against MRSA

A drawback of several commercial antimicrobials used to treat MRSA infections, including vancomycin and linezolid, is either they are only capable of inhibiting bacterial growth (but do not kill the bacteria), or they exhibit a very slow bactericidal effect resulting in difficulty in clearing the infection. (20, 21) Thus a compound that demonstrates the ability to rapidly kill MRSA is highly desirable since it limits the possibility of developing bacterial resistance/tolerance. We studied the rate at which the compounds were able to eliminate MRSA (USA300) in vitro in a time-kill assay. The results (Figure 2.6) indicate that at 3.0 × MIC, lead compound **1a**, **1d** (derivative which contains one more methylene unit in the alkyl side chain), and **8** (derivative which replaces the alkyl side chain with a cyclohexane ring) are bactericidal. However, the rate of clearance of MRSA (USA300) varies among the three compounds. Compound **1d** mimics the action of compound **1a**, rapidly eliminating MRSA completely within two hours. This would appear logical as compounds **1a** and **1d** are similar in structure, the major difference resulting from the number of methylene units contained in the alkyl side chain. Compound **8** requires more than double the time (six hours) to logarithmically reduce MRSA colony forming units (CFU) to zero. Though compound **8** appears more potent compared to compounds **1a** and **1d** when comparing MIC values), the latter two appear capable of clearing MRSA colonies (albeit at a higher concentration) more rapidly. Vancomycin was not able to reduce the

number of CFU by 3-$\log_{10}$ within a 12-hour window. Collectively this indicates the thiazole compounds possess a selective advantage over vancomycin in terms of rate of elimination of MRSA cells. This information is clinically significant as it would impact the size and timing of the dose given to patients with an infection caused by MRSA.

In addition to this, combination therapy using multiple antibiotics to treat MRSA infections is commonly used in clinical practice. Antibiotics that are bacteriostatic or exhibit a slow bactericidal effect (such as vancomycin) (20) are often paired with antibiotics exhibiting a rapid bactericidal effect (such as rifampin) in order to limit the emergence of bacterial strains with reduced susceptibility to vancomycin.(20) As the thiazole compounds presented here exhibit a rapid bactericidal effect against MRSA, analysis of synergy between the thiazole compounds and commercial antimicrobials (such as vancomycin and linezolid) for potential use in combination therapy would be an interesting avenue to further explore.

2.3.3 Evaluating toxicity of thiazole compounds against a HeLa cell line

The cytotoxicity assay (Figure 2.7) confirmed that all of the compounds are selective for bacterial cell inhibition over mammalian cells. All compounds tested were not toxic to human cervical adenocarcinoma (HeLa) cells up to 11 μg/mL; this concentration is more than 20-fold higher than the MIC value for the most potent thiazole derivatives (**8** and **12**). Irrespective of the modification made to the alkane side chain of the lead compound (addition of methylene units or substitution with a cyclic moiety), the subsequent derivatives maintained a good toxicity profile when tested against HeLa cells.

2.3.4 Physicochemical properties of the most promising analogues

Physicochemical properties, including solubility and permeability, of potential therapeutic agents are critical factors that need to be explored early in drug development. Though a compound proves potent against a target organism during in vitro studies and exhibits limited toxicity to cultured mammalian cells, the drug-candidate can fail in animal and human studies if the drug is poorly soluble in aqueous solutions or is incapable of passing through cellular barriers. Analysis of the hydrogen bonding potential and lipophilicity of a compound can lend valuable insight into potential solubility and permeability issues. After

documenting the strong antimicrobial activity of the thiazole compounds and determining their limited toxicity against murine macrophage cells, it was critical to establish whether the compounds possess potential solubility and permeability issues. Using Lipinski's Rule of 5 and topological polar surface area (TPSA) as guidelines, the results in Table 2.3 demonstrate all 10 thiazole compounds possess clog P and TPSA values that are associated with good solubility and permeability qualities. Two derivatives (**1e** and **1f**) violate one parameter of the Rule of 5 with each derivative possessing a calculated log P value above 5. These derivatives contain the longest linear alkyl chain (six and eight methylene units for **1e** and **1f**, respectively) connected to the phenylthiazole nucleus. This result supports the notion that an ideal thiazole side chain should have four methylene units, as compounds possessing an alkyl side chain with more than four methylene units exhibit a decrease in the antimicrobial activity against MRSA and pose potential solubility issues.

2.3.5 Examination of the solubility of lead **1a**

To confirm if the thiazole compounds possess good physicochemical properties as predicted, the lead compound was analyzed using the Caco-2 permeability assay, MDCK-MDR1 permeability assay, and a solubility screen utilizing phosphate buffered saline (PBS). The solubility screen was used to determine the highest concentration lead compound **1a** and three control drugs were able to dissolve in PBS before precipitate formed. The PBS solubility screen indicates the lead thiazole compound possesses modest solubility compared to the reference drugs tested. When compared to drugs with poor aqueous solubility, compound **1a** was soluble at twice the concentration of the antihypertensive drug reserpine (31.3 μM) and nearly four times the concentration of the cancer drug tamoxifen (15.6 μM) as presented in Table 2.4. As good solubility is a critical property needed for the development of a drug to be used in an oral formulation, modifications to the lead compound need to be explored to enhance its solubility profile. The solubility profile of the lead thiazole compound can be further enhanced by constructing subsequent derivatives containing ionizable groups (such as a basic amine or carboxylic acid moiety), inclusion of additional hydrogen bond donor groups (OH and NH_2 groups), or incorporating one or more polar groups (such as an ester) into the core structure.

2.3.6 Caco-2 and MDCK-MDR1 bidirectional permeability analysis of compound **1a**

The Caco-2 permeability assay revealed the lead compound (**1a**) surprisingly exhibited poor permeability across the membrane from the apical (A) to basolateral (B) direction as demonstrated in Table 2.5. Compound **1a** exhibits a higher apparent permeability coefficient (P_{app}) in the basolateral to apical direction ($P_{app} = 2.2 \times 10^{-6}$ cm/sec) which mimics the result obtained with the control drug ranitidine ($P_{app} = 1.2 \times 10^{-6}$ cm/sec in the B to A direction compared to 0.2×10^{-6} cm/sec in the A to B direction). Transporters in the membranes can enhance or reduce the permeability of a compound. The presence of efflux transporters on the apical surface of epithelial cells in the intestine may play a role in preventing the absorption of the thiazole compounds and passage through Caco-2 cells. The efflux ratio >2 for the lead thiazole compound supports the notion that the compound may be a substrate for an efflux transporter (possibly P-glycoprotein which is a potential reason for the higher rate of transfer of compound from the B to A direction). To confirm if this was the case, compound **1a** was analyzed using Madin-Darby canine kidney (MDCK) cells transfected with a gene overexpressing multidrug resistance protein 1 (MDR1), also referred to as P-glycoprotein 1 (Pgp). As presented in Table 2.6, a higher rate of transfer of compound **1a** is observed in the B to A direction ($P_{app} = 3.6 \times 10^{-6}$ cm/sec) compared to the A to B direction ($P_{app} = 0.7 \times 10^{-6}$ cm/sec), consistent with what is observed with the Caco-2 permeability assay. The efflux ratio determined from the MDCK-MDR1 permeability assay for compound **1a** is 5.0, indicating the compound may be subject to the effect of Pgp.

An increasing number of hydrogen bond acceptors (oxygen and nitrogen atoms) has been shown to increase the likelihood of Pgp efflux of drugs (22). Thus constructing derivatives of the lead thiazole compound focusing on modifications to the cationic head (where the hydrogen bond acceptor groups are present) is one mechanism to enhance permeability. A delicate balance between addition or substitution of functional groups would need to be achieved to ensure permeability is enhanced without reducing the solubility profile of the thiazole compounds. Another method to enhance passage of the thiazole compounds across the intestinal membrane is to use a higher concentration of the lead compound, especially if the compound is a substrate for efflux transporters. The Caco-2 assay utilized a low concentration (10 μM) of the lead compound. The concentration of

a drug in the gastrointestinal lumen after an oral dose is typically between 50 to 100 μM (22). Thus testing the lead compound at a higher concentration is necessary to confirm if the poor permeability observed is potentially due to the low concentration of compound used in the assay. A higher concentration may help the thiazole compound to effectively cross the intestinal barrier as efflux transporters will eventually become saturated in a concentration-dependent manner permitting compound that has passively diffused across the membrane to remain in the lumen of the intestine. Taken collectively, the permeability profile of the thiazole compounds can be potentially improved by modifying the structure of the lead compound or increasing the concentration of the compound used.

2.3.7 Metabolic stability analysis of compound **1a** in human microsomes

In addition to testing the solubility and permeability characteristics of the lead thiazole compound, the metabolic stability of the compound was investigated using human liver microsomes. As Table 2.7 demonstrates, compound **1a** is subject to metabolism by the liver with a microsomal intrinsic clearance of 80.3 μL/min-mg and a half-life of 28.8 minutes. These values align with the values obtained with verapamil (the metabolized control drug) rather than for warfarin (non-metabolized control drug). Removing the cofactor NADPH significantly reduced the metabolism of compound **1a** indicating that these metabolic processes are NADPH-dependent. The experiment doesn't eliminate other tissues which may play a role in metabolism of the thiazole compounds. The half-life and microsomal intrinsic clearance are important parameters for determining an appropriate dosing regimen for a drug (as drugs which have a short half-life and are rapidly metabolized in the liver will require additional doses for patients in order to clear an infection). To improve metabolic stability of the thiazole compounds, consideration must be given to removing labile groups or altering atoms in the compound involved in its metabolism.

2.4 Conclusion

In conclusion, the identification of novel antimicrobial agents to treat an array of infections caused by methicillin-resistant and vancomycin-resistant *S. aureus* requires a multi-fold approach from whole-cell screening of chemical libraries to rational drug design. We present the exciting discovery of a lead antimicrobial compound, identified from whole-

cell screening of a library of thiazole and thiadiazole compounds, which is capable of inhibiting growth of 18 strains of MRSA and VRSA. The lead compound consists of a thiazole central ring connected to two structural elements critical for activity, namely a cationic element at the C5-position and a lipophilic moiety at the C2-position. A focused library of derivatives containing modifications to the lipophilic moiety was constructed to enhance the antimicrobial activity of the lead compound against MRSA and VRSA. The lead compound and nine derivatives are capable of inhibiting growth of 18 different clinical isolates of MRSA and VRSA at a concentration ranging from 0.5 to 3.0 μg/mL. Furthermore, the lead compound and two derivatives exhibit a rapid bactericidal effect, eliminating MRSA growth in vitro within six hours. In addition to this, six derivatives, including the three most potent compounds against MRSA, are not toxic. The ten thiazole compounds were predicted to have good solubility and permeability characteristics based upon the criterion set forth by Lipinski's Rule of 5. However, analysis of permeability of the lead compound via the Caco-2 and MDCK-MDR1 assays indicated the compound had poor permeability from the apical to basolateral surface of the membrane (possibly due to the effect of the Pgp efflux transporter). We confirmed the lead compound does not target the integrity of the bacterial cell wall or cytoplasmic membrane (data not published); further explanation of the molecular target of the thiazole compounds will be presented in a future study. The characterization of the novel thiazole compounds presents an intriguing step in the development of a novel class of therapeutic agents effective for treating MRSA and VRSA infections.

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Table 2.1 Strains of *Staphylococcus aureus* used in this study.

Strain Name		Isolation		Molecular Typing		Phenotypic Properties
NARSA ID[1]	**Alternate Name**	**Origin**	**Year**	**SCC*mec* type**	***spa* type**	
-	ATCC 43300	United States	-	-	-	Resistant to methicillin
NRS1	ATCC 700699 VISA	Japan	1996	II	TJMBMD MGMK	Resistant to aminoglycosides and tetracycline (minocycline) Glycopeptide-intermediate *S. aureus*
NRS19	VISAHIP07 256	United States	1999	II	TJMBMD MGMK	Glycopeptide-intermediate *S. aureus*
NRS37	VISA; LIM 3	France	1995	I	YHFGFMB QBLO	Glycopeptide-intermediate *S. aureus*
NRS107	RN4220	United States	-	-	YHGGFM BQBLO	Resistant to mupirocin
NRS108	A960649	France	-	I	YHGFMM BQBLO	Resistant to gentamicin
NRS119	SA LinR #12	United States	2001	IV	YHGCMB QBLO	Resistant to linezolid
NRS123	USA400	United States	1998	IV	UJJFKBPE	Resistant to methicillin; susceptible to nonbeta-lactam antibiotics
NRS194	C1999000529	United States	1999	IV	UJFKKPFK PE	Resistant to methicillin
NRS382	USA100	United States	-	II	TJMBMD MGMK	Resistant to ciprofloxacin, clindamycin, erythromycin, and methicillin
NRS383	USA200	United States	-	II	WGKAKA OMQQQ	Resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, and methicillin
NRS384	USA300-0114	United States	-	IV	YHGFMB QBLO	Resistant to erythromycin, methicillin, and tetracycline
NRS385	USA500	United States	-	IV	YHGCMB QBLO	Resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, methicillin, tetracycline, and trimethoprim
NRS386	USA700	United States	-	IV	UJGFMGG M	Resistant to erythromycin and methicillin
NRS387	USA800	United States	-	IV	TJMBMD MGGMK	Resistant to methicillin
NRS483	USA1000	United States	-	IV	-	Resistant to erythromycin and methicillin
NRS484	USA1100	United States	-	IV	-	Resistant to methicillin
VRS10	VRSA	United States	2009	-	-	Resistant to ciprofloxacin, clindamycin, erythromycin, gentamicin, and vancomycin

[1]NARSA = Network on Antimicrobial Resistance in *Staphylococcus aureus*

Table 2.2 The antimicrobial activities (μg/mL) of modified thiazole compounds screened against *Staphylococcus aureus*.

Strains	MIC ± standard deviation of thiazole compounds and vancomycin (VAN) against *S. aureus*										
	1a	**1c**	**1d**	**1e**	**1f**	**1g**	**1h**	**7**	**8**	**12**	VAN
MRSA ATCC 43300	2.8 ± 0	1.2 ± 0	0.8 ± 0	3.0 ± 0	2.2 ± 0	1.9 ± 0	3.8 ± 0	1.2 ± 0	1.5 ± 0	0.6 ± 0	0.7 ± 0
VISA ATCC 700699	1.4 ± 0	1.9 ± 0.7	0.5 ± 0.2	0.7 ± 0	1.1 ± 0	1.6 ± 0.6	3.2 ± 1.1	0.5 ± 0.2	0.7 ± 0	0.6 ± 0	2.9 ± 0
VISA HIP07256	1.4 ± 0	1.9 ± 0.7	0.7 ± 0.3	1.5 ± 0	2.9 ± 1.3	1.9 ± 0	2.6 ± 1.1	0.8 ± 0.3	0.7 ± 0	0.5 ± 0.2	2.9 ± 0
VISA LIM 3	1.4 ± 0	1.6 ± 0.7	0.5 ± 0	0.7 ± 0	1.1 ± 0	1.6 ± 0.6	1.9 ± 0	0.8 ± 0.3	0.5 ± 0.2	0.6 ± 0	2.9 ± 0
MRSA NRS107	1.4 ± 0	1.2 ± 0	0.5 ± 0	1.0 ± 0.4	1.1 ± 0	1.0 ± 0	1.9 ± 0	0.6 ± 0	0.6 ± 0.2	1.2 ± 0	0.7 ± 0
MRSA NRS108	1.4 ± 0	1.2 ± 0	0.9 ± 0.3	3.0 ± 0	4.4 ± 0	1.9 ± 0	1.9 ± 0	1.7 ± 1.0	1.5 ± 0	2.0 ± 0.7	0.7± 0
MRSA NRS119	1.4 ± 0	1.9 ± 0.7	0.9 ± 0.3	1.5 ± 0	2.2 ± 0	1.9 ± 0	3.8 ± 0	0.6 ± 0	0.7 ± 0	0.6 ± 0	1.2 ± 0.4
MRSA USA400	1.4 ± 0	1.2 ± 0	0.9 ± 0.3	1.5 ± 0	4.4 ± 0	1.9 ± 0	1.9 ± 0	0.6 ± 0	0.7 ± 0	0.8 ± 0.3	0.7 ± 0
MRSA NRS194	1.4 ± 0	1.9 ± 0.7	1.1 ± 0	3.0 ± 0	4.4 ± 0	2.6 ± 1.1	1.9 ± 0	0.8 ± 0.3	0.7 ± 0	1.4 ± 0.9	0.7 ± 0
MRSA USA100	5.5 ± 0	1.6 ± 0.7	1.1 ± 0	3.0 ± 2.6	2.2 ± 0	1.3 ± 0.6	1.9 ± 0	1.0 ± 0.3	0.7 ± 0	2.0 ± 0.7	1.2 ± 0.4
MRSA USA200	2.8 ± 0	1.9 ± 0.7	1.1 ± 0	1.2 ± 0.4	2.2 ± 0	2.6± 1.1	2.6 ± 1.1	1.0 ± 0.3	1.2 ± 0.4	0.6 ± 0	0.4 ± 0
MRSA USA300	1.4 ± 0	1.2 ± 0	1.1± 0	2.5 ± 0.9	2.2 ± 0	1.3 ± 0.6	1.9 ± 0	1.2 ± 0	0.7 ± 0	1.0 ± 0.3	0.6 ± 0.2
MRSA USA500	1.4 ± 0	1.9 ± 0.7	1.1 ± 0	3.0 ± 0	3.7 ± 1.3	1.3 ± 0.6	2.6 ± 1.1	1.0 ± 0.3	0.9 ± 0.6	1.6 ± 0.7	1.0 ± 0.4
MRSA USA700	1.8 ± 0.8	1.2 ± 0	0.9 ± 0.3	4.0 ± 1.7	1.8 ± 0.6	1.0 ± 0	2.6 ± 1.1	1.2 ± 0	1.5 ± 0	0.8 ± 0.3	1.0 ± 0.4
MRSA USA800	2.3 ± 0.8	1.9 ± 0.7	0.7 ± 0.3	1.5 ± 0	1.8 ± 0.6	1.3 ± 0.6	1.9 ± 0	0.8 ± 0.3	0.7 ± 0	1.2 ± 0	0.7 ± 0
MRSA USA1000	1.4 ± 0	1.6 ± 0.7	1.1 ± 0	2.5 ± 0.9	2.2 ± 0	1.0 ± 0	1.9 ± 0	0.8 ± 0.3	0.9 ± 0.6	0.8 ± 0.3	0.6 ± 0.2
MRSA USA1100	2.8 ± 0	1.6 ± 0.7	1.1 ± 0	1.5 ± 0	2.2 ± 0	1.9 ± 0	1.9 ± 0	0.6 ± 0	0.7 ± 0	0.6 ± 0	0.7 ± 0
VRSA	1.4 ± 0	1.2 ± 0	1.1 ± 0	1.5 ± 0	2.2 ± 0	1.6 ± 0.6	1.9 ± 0	1.2 ± 0	1.5 ± 0	0.6 ± 0	185.5 ± 0

Table 2.3 Calculation of physicochemical properties of thiazole compounds for Lipinski's Rule of 5.

Compound Number	Acceptable Number→	nViol[1] 1	cLog P[2] < 5	MW[3] < 500	nON[4] < 10	nOHNH[5] < 5	TPSA[6] (Å^2)
1a		0	4.23	346	5	4	87.158
1c		0	3.671	291	5	4	87.158
1d		0	4.735	274	5	4	87.158
1e		1	5.746	373	5	4	87.158
1f		1	6.756	275	5	4	87.158
1g		0	4.072	240	5	4	87.158
1h		0	3.549	240	5	4	87.158
7		0	4.247	288	5	4	87.158
8		0	4.727	366	5	4	87.158
12		0	4.161	303	5	4	87.158

[1]nViol = number of violations
[2]cLog P = Molinspiration calculated Log P
[3]MW = molecular weight
[4]nON = number of hydrogen bond acceptors
[5]nOHNH = number of hydrogen bond donors
[6]TPSA = topological polar surface area

Table 2.4 Evaluation of solubility of lead thiazole compound (**1a**), Reserpine, Tamoxifen, and Verapamil in phosphate buffered saline (PBS).

Compound Tested	Solubility limit (µM)
1a	62.5
Reserpine	31.3
Tamoxifen	15.6
Verapamil	>500

Table 2.5 Evaluation of apparent permeability of lead thiazole compound (**1a**), Ranitidine, Warfarin, and Talinolol via the Caco-2 permeability assay.

Compound Tested	Mean A → B P_{app}[1] (10^{-6} cm/sec)	Mean B → A P_{app}[2] (10^{-6} cm/sec)	Efflux Ratio
1a	0.0	2.2	>2
Ranitidine	0.2	1.2	6.6
Warfarin	42.1	13.8	0.3
Talinolol	0.1	6.8	108.0

[1]Mean A → B P_{app} = mean apparent permeability of test compound from apical to basolateral surface
[2]Mean B → A P_{app} = mean apparent permeability of test compound from basolateral to apical surface

Table 2.6 Evaluation of apparent permeability of lead thiazole compound (**1a**), Ranitidine, Warfarin, and Talinolol via the MDCK/MDR-1 permeability assay.

Compound Tested	**Mean A → B P_{app} (10^{-6} cm/sec)**	**Mean B → A P_{app} (10^{-6} cm/sec)**	**Efflux Ratio**
1a	0.7	3.6	5.0
Ranitidine	0.3	2.2	6.5
Warfarin	4.7	7.2	1.5
Talinolol	0.1	3.1	38.4

[1]Mean A → B P_{app} = mean apparent permeability of test compound from apical to basolateral surface

[2]Mean B → A P_{app} = mean apparent permeability of test compound from basolateral to apical surface

Table 2.7 Evaluation of metabolic stability of lead thiazole compound (**1a**), Verapamil, and Warfarin, in human liver microsomes.

Compound Tested	**NADPH-dependent CL_{int}[1] (µL/min-mg)**	**NADPH-dependent $T_{1/2}$[2] (min)**	**NADPH-free CL_{int} (µL/min-mg)**	**NADPH-free $T_{1/2}$ (min)**
1a	80.3	28.8	0.5	>60
Verapamil	201	11	1	>60
Warfarin	0.3	>60	0.0	>60

[1]CL_{int} = microsomal intrinsic clearance

[2]$T_{1/2}$ = half-life

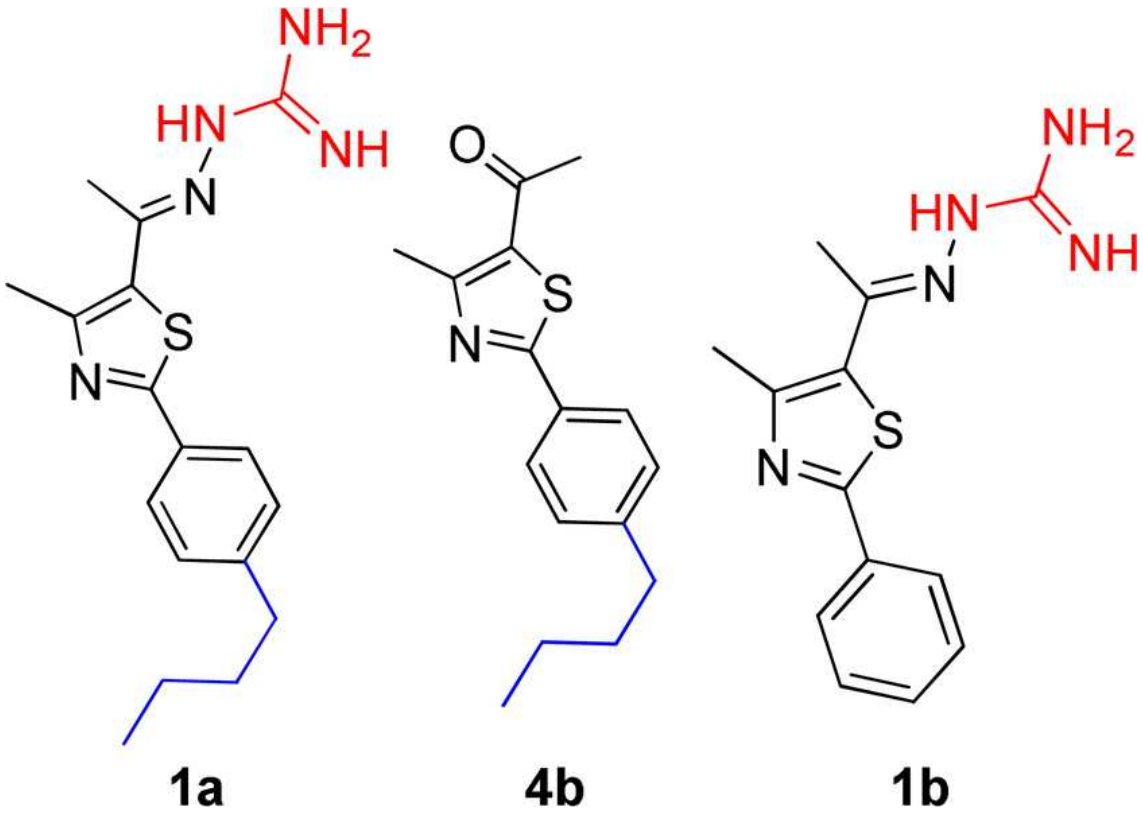

Figure 2.1 Chemical structures of lead **1a** and **4b** (removal of the cationic moiety) and **1b** (removal of the lipophilic alkane side chain).

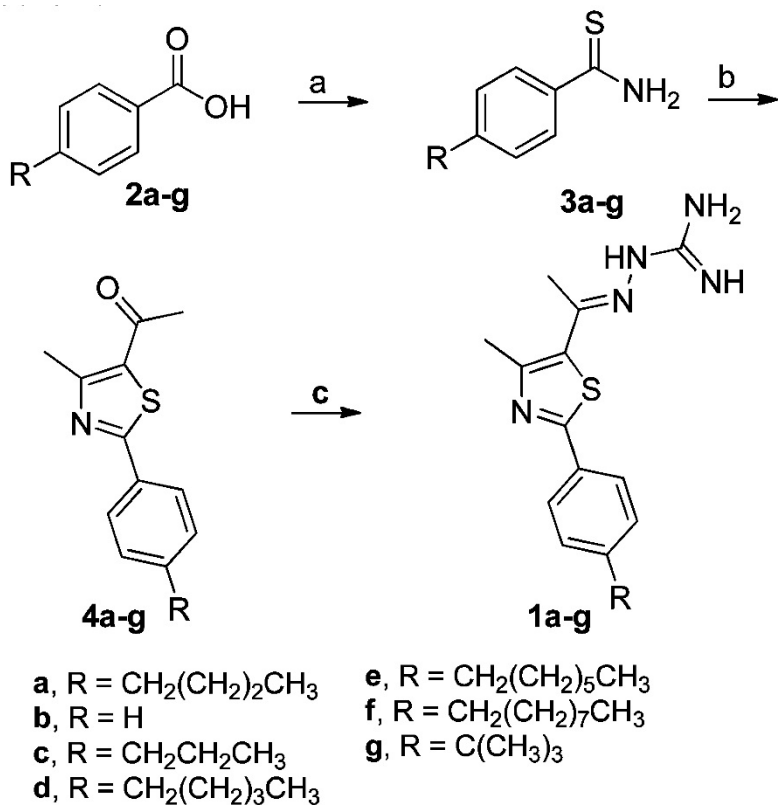

Figure 2.2 Preparation of **1a–g**.

Reagents and conditions: (a) (i) $SOCl_2$, heat to reflux, two hours, (ii) NH_4OH, 0–23 °C, 2–5 hours, (iii) Lawesson's reagent, dry THF, 50–60 °C, 5–24 hours; (b) absolute ethanol, 3-chloropentane-2,4-dione, heat to reflux, 12 hours, 63%; (c) aminoguanidine hydrochloride, absolute EtOH, heat to reflux, 24 hours.

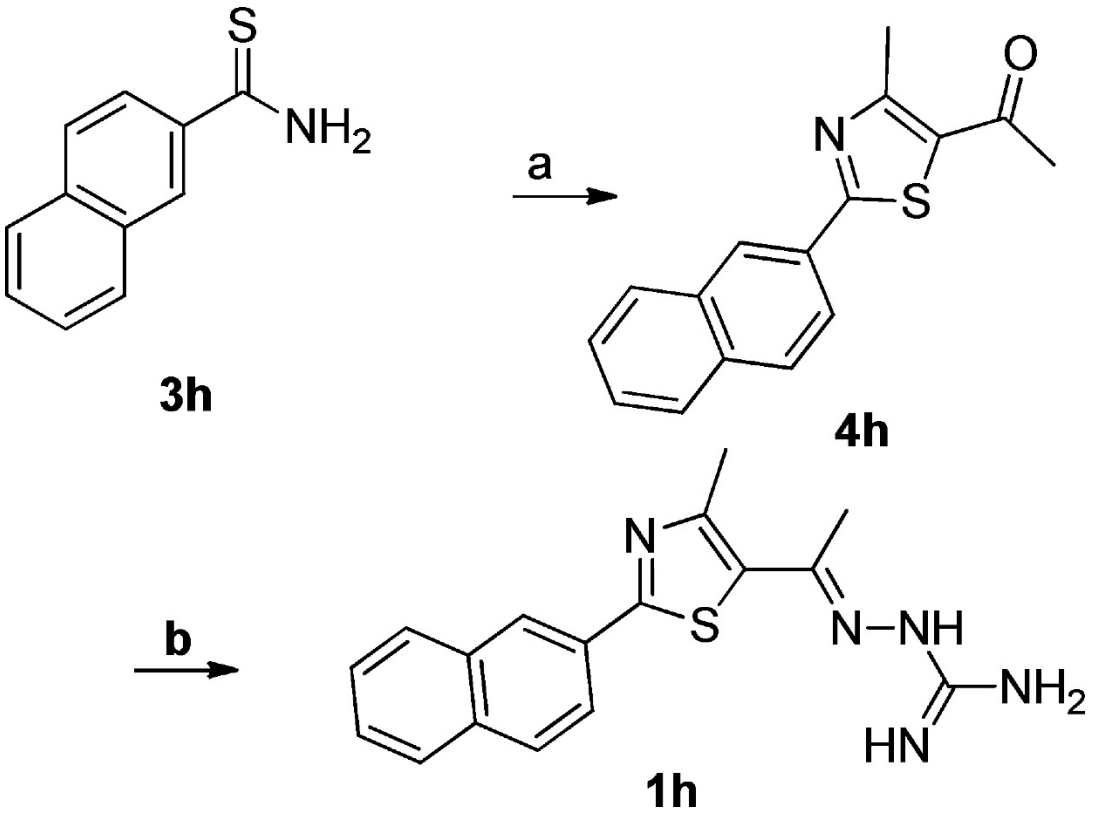

Figure 2.3 Preparation of **1h**.

Reagents and conditions: (a) absolute ethanol, 3-chloropentane-2,4-dione, heat to reflux, 12 hours, 67%; (b) aminoguanidine hydrochloride, absolute ethanol, heat to reflux, 24 hours, 40%.

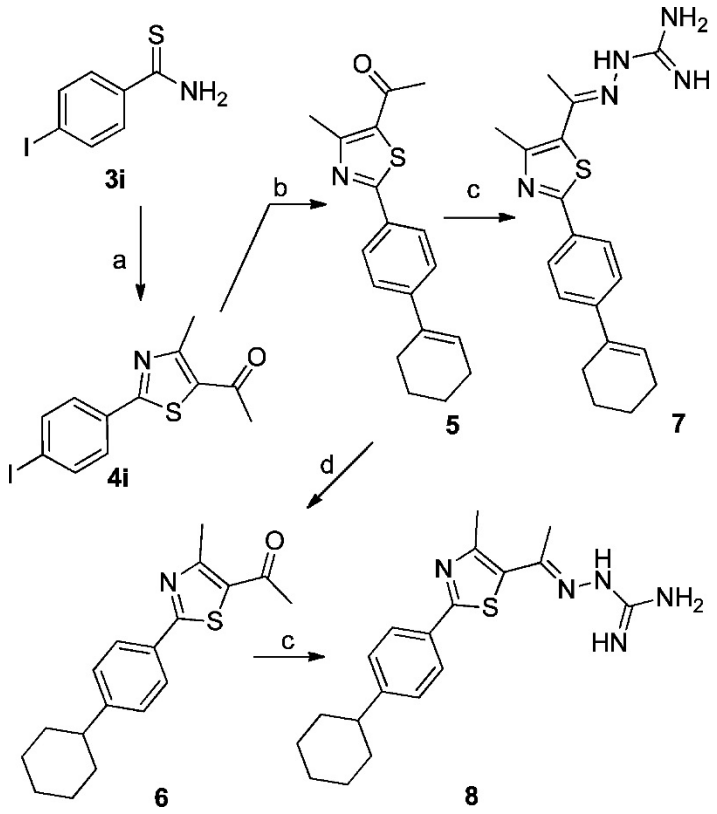

Figure 2.4 Preparation of **7** and **8**.

Reagents and conditions: (a) absolute ethanol, 3-chloropentane-2,4-dione, heat to reflux, 12 hours, 58%; (b) cyclohexene, $PdAcO_2$, Et_3N, DMF, 80 °C, 5 hours, 39%; (c) aminoguanidine hydrochloride, absolute ethanol, heat to reflux, 24 hours; (d) H_2, Pd/C, methanol, 23 °C, 24 hours, 38–42%.

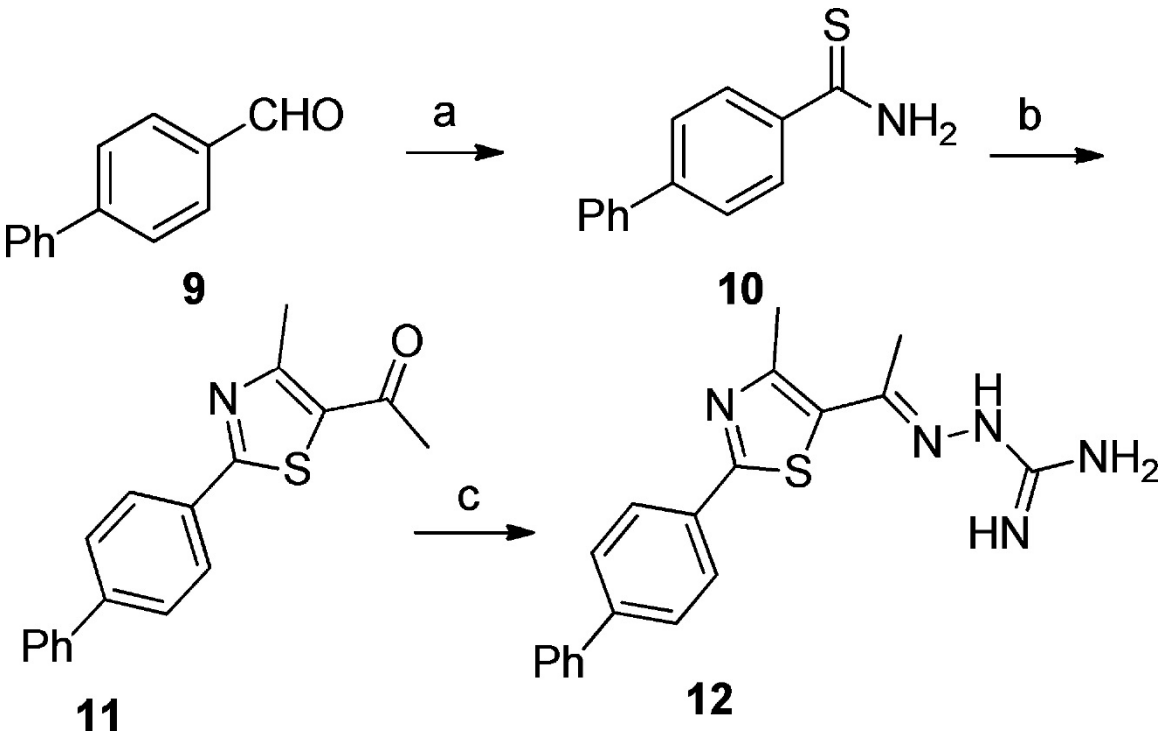

Figure 2.5 Preparation of **12**.

Reagents and conditions: (a) (i) H_2NOH HCl, DMSO, 100 °C, 20 minutes; (ii) NaOH, H_2O_2, 12 hours; (iii) Lawesson's reagent, THF, 23 °C, 12 hours; (b) absolute ethanol, 3-chloropentane-2,4-dione, heat to reflux, 12 hours, 49%; (c) aminoguanidine hydrochloride, absolute ethanol, heat to reflux, 24 hours, 45%.

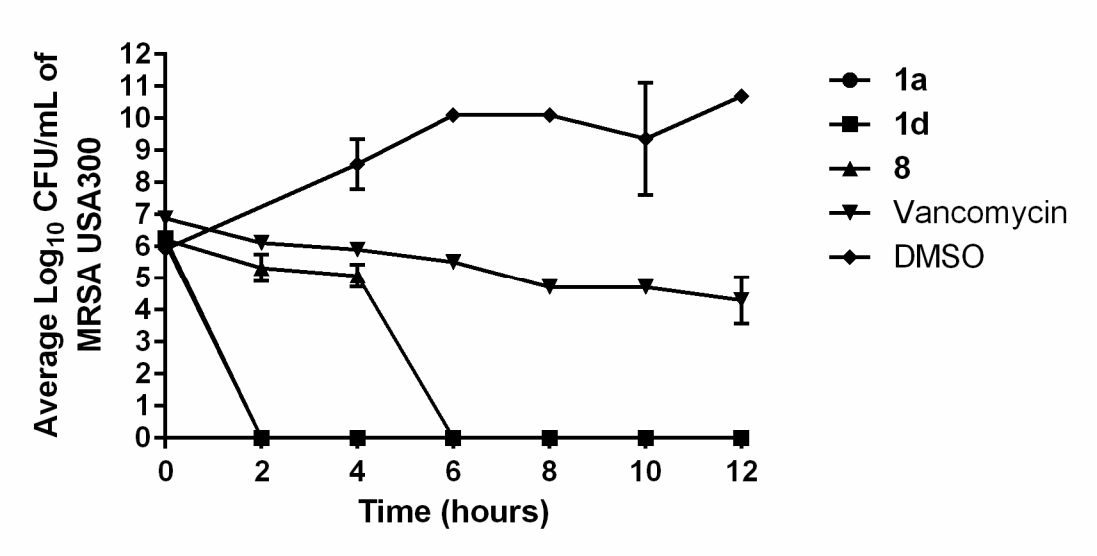

Figure 2.6 Time-kill analysis of the lead compound **1a**, **1d**, **8**, and vancomycin at 3.0 × MIC against methicillin-resistant *Staphylococcus aureus* (MRSA) strain USA300.

Compounds were incubated with bacteria over a 12 hour period at 37 ºC. DMSO served as the negative control. The error bars represent standard deviation values obtained from triplicate samples used for each compound studied.

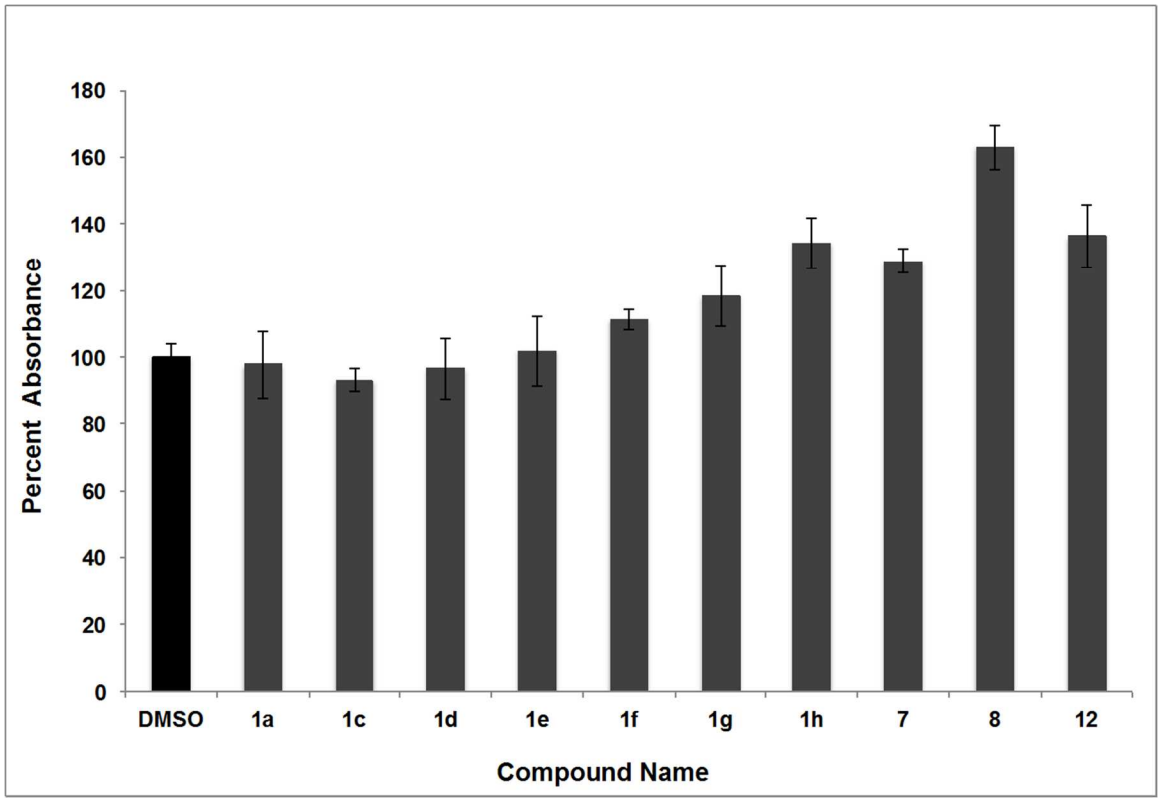

Figure 2.7 Toxicity analysis of thiazole compounds against HeLa cells.

Average absorbance ratio (thiazole compound/DMSO) for cytotoxicity of thiazole compounds at 11 μg/mL against human cervical adenocarcinoma cells (HeLa) using the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay. DMSO was used as a negative control to determine a baseline measurement for the cytotoxic impact of each compound. The corrected absorbance values (actual absorbance value – background absorbance reading) represent an average of a minimum of four samples analyzed for each compound. Error bars represent standard deviation values for the corrected absorbance values.

CHAPTER 3. ANTI-BIOFILM ACTIVITY AND SYNERGISM OF NOVEL THIAZOLE COMPOUNDS WITH GLYCOPEPTIDE ANTIBIOTICS AGAINST MULTIDRUG-RESISTANT STAPHYLOCOCCI

THIS IS A PUBLISHED JOURNAL ARTICLE. Reprinted with permission from **Anti-biofilm activity and synergism of novel thiazole compounds with glycopeptide antibiotics against multidrug-resistant Staphylococci.** Haroon Mohammad, Abdelrahman S. Mayhoub, Mark Cushman, and Mohamed N. Seleem. *The Journal of Antibiotics* (2015) **68,** 259–266; doi:10.1038/ja.2014.142

3.1 Introduction

Antibiotic-resistant bacteria are a major global health concern resulting in 23,000 deaths each year in the United States alone (1). Two species alone, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Staphylococcus epidermidis*, are responsible annually for the majority of skin and soft-tissue infections and infections caused by bacterial biofilms present on indwelling medical devices (2, 3). Biofilms are responsible for 80% of microbial infections which develop in the human body and bacterial biofilms on implanted biomedical devices and tissue surfaces (chronic wound) constitute an ever-increasing threat to human health and place a significant burden on healthcare systems (4). Biofilms consist of a cluster of bacterial cells enclosed within an extracellular matrix which collectively attach to an animate or inanimate surface (4). Cells present within a biofilm pose a key challenge as they demonstrate increased resistance to the effect of antimicrobials (5).

Antibiotics have been key allies in the treatment of bacterial infections for more than 80 years. While several classes of antibiotics were once capable of treating staphylococci-induced infections, strains have emerged which are resistant to an array of antimicrobials once deemed effective including β-lactams (6), macrolides (2), and fluoroquinolones (6, 7). Further exacerbating the issue is the rise of strains (such as vancomycin-resistant *Staphylococcus aureus* (VRSA)), which are resistant to antibiotics deemed drugs of last resort for treatment of staphylococcal infections, including

glycopeptide antibiotics like vancomycin (8). Conventional antibiotics face an added challenge in the treatment of biofilm infections as bacteria present within a biofilm may be 1000-fold more resistant to antibiotics compared to their planktonic equivalents (5). Thus there is a critical need for the discovery of novel antimicrobials and treatment strategies to circumvent this growing public health concern.

Several thiazole compounds have been shown to be effective anticonvulsant(9), anticancer (10, 11), and antiviral agents (12). However, limited studies have been performed to characterize their abilities as antimicrobial agents, particularly against MRSA. Darwish *et al*, synthesized a series of thiadiazole analogues incorporating a sulfonamide group and found they possessed activity against *Streptococcus pneumoniae* and *Bacillus subtilis* (13). Additionally, Desai *et al*, constructed a series of novel hybrid compounds which combined the thiazole and 1,3,4-oxadiazole pharmacophores but found they had limited activity against *S. aureus* (minimum inhibitory concentration (MIC) of six of 12 analogues constructed was 500 μg/mL or higher) (14). Furthermore, a third study assessing a series of disubstituted 1,3-thiazole derivatives found the most potent analogue possessed modest activity against a single strain of *S. aureus* tested (MIC of 50 μg/mL) (15). None of these studies assessed broader therapeutic applications of thiazole compounds beyond use as single agents to inhibit bacterial growth *in vitro*.

We recently discovered a novel lead thiazole compound **1** which exhibited potent antimicrobial activity against MRSA (Figure 3.1) (16). The lead compound is composed of a thiazole nucleus connected to a cationic amino head at the C5-position and a lipophilic phenylalkyl tail at the C2-position. The aims of the present study are to identify if the lead compound **1** and the most potent synthesized derivative **2** have potential to be used in combination with glycopeptide antibiotics commonly used to treat MRSA infections, to analyze the ability of **1** and **2** to re-sensitize VRSA strains to glycopeptide antibiotics, and to assess if these compounds are capable of disrupting staphylococcal biofilms using an *in vitro* model of *S. epidermidis*. Results garnered from this study will provide valuable insight into potential therapeutic applications of thiazole compounds for use as antibacterial agents.

3.2 Materials and Methods

3.2.1 Bacterial Strains and Reagents

The bacterial strains of methicillin-resistant and vancomycin-intermediate *Staphylococcus aureus* utilized were obtained from the Network on Antimicrobial Resistance in Staphylococcus aureus (NARSA). *Staphylococcus epidermidis* ATCC 35984 was obtained from the American Tissue Culture Collection. Antibiotics were purchased commercially from Gold Biotechnology (St. Louis, MO, USA) (vancomycin hydrochloride) and Biotang Inc. (Waltham, MA, USA) (teicoplanin). Both antibiotics were dissolved in dimethyl sulfoxide to obtain a stock 10 mM solution.

3.2.2 Synthesis of Thiazole Compounds **1** and **2**

The detailed synthetic protocols and spectral data of final products **1** and **2** as well as all intermediates have been previously reported (16),(17).

3.2.3 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Against MRSA, VISA, VRSA, and *S. epidermidis*

The MICs of the thiazole compounds, vancomycin, and teicoplanin against MRSA, VISA, VRSA, and *S. epidermidis* were determined using the broth microdilution method, in accordance with the recommendations contained in the CLSI guidelines (with the exception that Mueller-Hinton broth (MHB) was used instead of cation-adjusted MHB) (18). Bacteria were prepared in phosphate buffered saline (PBS) until a McFarland standard of 0.5 was achieved. The solution was diluted 1:300 in MHB to reach a starting inoculum of 1×10^5 colony-forming units (CFU/mL). Bacteria were transferred to a 96-well microtiter plate. Thiazole compounds and antibiotics were added (in triplicate) to wells in the first row of the microtitier plate and then serially diluted along the ordinate. The plate was incubated at 37 °C for 20-24 hours before the MIC was determined. The MIC was categorized as the concentration at which no visible growth of bacteria was observed in a particular well.

The MBC was determined by plating 5 µL from wells on the 96-well microtiter plate (where the MIC was determined) were no growth was observed onto tryptic soy agar (TSA) plates. The TSA plates were then incubated at 37 °C for 20-24 hours before the MBC was

determined. The MBC was categorized as the concentration where ≥99% reduction in bacterial cell count was observed.

3.2.4 Time-kill Analysis of Thiazole Compounds and Glycopeptide Antibiotics Against MRSA

MRSA NRS123 (USA400) cells in the logarithmic growth phase were diluted to ~1×10^8 colony-forming units (CFU/mL) and exposed to concentrations equivalent to 2, 4, and 8 × MIC (in triplicate) of thiazole compounds **1** and **2**, teicoplanin, and vancomycin in Mueller-Hinton broth (MHB). 20 µL samples were collected after 0, 2, 4, 6, 8, 10, 12, and 24 hours of incubation at 37 °C and subsequently serially diluted in PBS. Bacteria were then transferred to TSA plates and incubated at 37 °C for 18-20 hours before viable CFU/mL was determined. The test agent was deemed bactericidal if it successfully produced a 3-log10 reduction in the bacterial count within 24 hours, as reported elsewhere (19).

3.2.5 Single-step Resistance Selection

The frequency of spontaneous single-step resistance of the thiazole compounds and glycopeptide antibiotics to five MRSA strains was determined as reported elsewhere (20, 21). Briefly, bacterial cultures (>1×10^9 CFU/mL) were spread onto Mueller-Hinton agar plates (10-mm diameter) containing each compound/antibiotic at 4 × MIC. Plates were incubated aerobically at 37 °C for 48 hours. The frequency of resistance was calculated as the number of resistant colonies per inoculum (21).

3.2.6 Combination Therapy Analysis of Thiazole Compounds with Glycopeptide Antibiotics

The relationship between the thiazole compounds and glycopeptide antibiotics (vancomycin and teicoplanin) was assessed via a standard checkerboard assay (22). Bacteria equivalent to a McFarland standard of 0.5 were prepared in PBS. The bacteria were then diluted in MHB to achieve a starting cell density of 1×10^5 CFU/mL. MHB was transferred to all wells of a 96-well microtiter plate. The thiazole compounds and glycopeptide antibiotics were diluted in MHB to achieve a starting concentration equivalent to 2 × or 4 × MIC, respectively. The glycopeptide antibiotic was serially diluted along the abscissa of the microtiter plate while the thiazole compound was serially diluted

along the ordinate. The plate was incubated for 20-24 hours at 37 °C. The MIC of the test compound in combination with each glycopeptide antibiotic studied was determined as the lowest concentration of each compound/antibiotic where no visible growth of bacteria was observed. The fractional inhibitory concentration index (ΣFIC) was calculated for each combination as follows:

$$\Sigma FIC = \left(\frac{MIC\,thiazole\ compound\ in\ combination\ with\ glycopeptide\ antibiotic}{MIC\,thiazole\ compound\ alone}\right) + \left(\frac{MIC\,glycopeptide\ antibiotic\ in\ combination\ with\ thiazole\ compound}{MIC\,glycopeptide\ antibiotic}\right)$$

A synergistic relationship was classified as an FIC index less than or equal to 0.5. FIC values above 0.5 but less than 4.0 were characterized as indifference while FIC values above 4.0 were classified as antagonistic.

3.2.7 Re-sensitization of VRSA Strains to Vancomycin Using Broth Microdilution Method

MHB was inoculated with VRSA (5×10^5 CFU/mL), as described elsewhere (23). 5-ml aliquots of the bacterial suspension were divided into microcentrifuge tubes. Compound **1** or **2** (at ½ × MIC) was introduced into each tube. After sitting at room temperature for 30 minutes, 1 ml samples from each tube were transferred to a new centrifuge tube prior to addition of the antibiotic (either vancomycin or teicoplanin at a concentration equivalent to their MIC). Using a 96-well microtiter plate, rows 2-12 were filled with the remaining 4 ml bacterial suspension (containing either compound 1 or 2). 200-µl aliquots from tubes containing both the thiazole compound and glycopeptide antibiotic were transferred to row 1 of the 96-well plate. After aspirating contents in the first row 4-6 times, 100 µL was transferred from wells in row 1 to row 2. This process was repeated to dilute the remaining wells containing no antibiotic. Untreated bacteria served as a control. The plate was incubated at 37 °C for 20 hours before the MIC was recorded. The MIC was categorized as the concentration at which no visible growth of bacteria was observed in a particular well. A fold reduction was calculated by comparing the MIC of the antibiotic alone compared to the MIC of the antibiotic given in combination with **1** or **2**.

3.2.8 Staphylococcus Biofilm Mass Reduction Determination

The microtiter dish biofilm formation assay (24) was utilized to assess the ability of the thiazole compounds to disrupt an adherent staphylococcal biofilm, similar to what has been described elsewhere (25). *S. epidermidis* ATCC 35984 was transferred to tryptic soy broth and incubated at 37 °C for 24 hours before being diluted 1:200 in tryptic soy broth + 1% glucose. This solution was transferred to each well of a 96-well microtiter plate and incubated at 37 °C for 24 hours to permit biofilm formation on the well surface. Bacteria were removed and wells were washed twice with PBS. Compounds **1**, **2**, or vancomycin were added (in triplicate) to wells and serially diluted. The microtiter plate was then incubated at 37 °C for 24 hours. The plate was washed twice by submerging in deionized water. 0.1% (w/v) crystal violet was added to each well and allowed to stain the biofilm for 20 minutes before addition of 95% ethanol to decolorize. Using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA), the optical density of each well at 595 nm was measured. Percent biofilm mass reduction was calculated for each treatment regimen as compared to the control (wells receiving no treatment).

3.2.9 Kinetic Solubility Determination of Compound **2**

Serial dilutions of compound **2** were prepared in DMSO at 100× the final concentration. Compound **2** was then diluted 100-fold into PBS in a 96-well plate and mixed. The absorbance of the PBS-containing plate was measured prior to addition of the test agents to determine the background absorbance. After two hours, the presence of precipitate was detected by turbidity (absorbance at 540 nm). An absorbance value of greater than (mean + 3× standard deviation of the blank), after subtracting the pre-experiment background, was indicative of turbidity. The solubility limit is reported as the highest experimental concentration for compound **2** with no evidence of turbidity.

3.2.10 Caco-2 Bidirectional Permeability Assessment of Compound **2**

To assess the ability of compound **2** to passively permeate through epithelial tissue, a Caco-2 permeability assay was performed as described previously (16). Caco-2 cells grown in tissue culture flasks were trypsinized, suspended in medium, and the suspensions were applied to wells of a Millipore 96 well Caco-2 plate. The cells were allowed to grow and

differentiate for three weeks, feeding at 2-day intervals. For Apical to Basolateral (A→B) permeability, compound **2** was added to the apical (A) side and amount of permeation was determined on the basolateral (B) side; for Basolateral to Apical (B→A) permeability, compound **2** was added to the B side and the amount of permeation was determined on the A side. The A-side buffer contained 100 μM Lucifer yellow dye, in Transport Buffer (1.98 g/L glucose in 10 mM HEPES, 1.0 × Hank's Balanced Salt Solution) at pH 6.5, and the B-side buffer contained Transport Buffer at pH 7.4. Caco-2 cells were incubated with these buffers for two hours, and the receiver side buffer was removed for analysis by LC/MS/MS. To verify the Caco-2 cell monolayers were properly formed, aliquots of the cell buffers were analyzed by fluorescence to determine the transport of the impermeable dye Lucifer Yellow. Any deviations from control values are reported. Data are expressed as permeability $Papp = \frac{-(\frac{dQ}{dt})}{C0A}$ where $\frac{dQ}{dt}$ is the rate of permeation, C_0 is the initial concentration of test agent, and A is the area of the monolayer. In bidirectional permeability studies, the efflux ratio (R_E) is also calculated: $R_E = \frac{Papp(B\rightarrow A)}{Papp(A\rightarrow B)}$. An $R_E > 2$ indicates a potential substrate for P-glycoprotein or other active efflux transporters.

3.2.11 Statistical Analysis

All statistical analysis was performed using the two-tailed Student's t-test ($P < 0.05$) utilizing Microsoft Excel software.

3.3 Results and Discussion

3.3.1 Determination of the Antimicrobial Activity of the Thiazole Compounds and Glycopeptide Antibiotics

We have designed and synthesized a series of thiazole derivatives containing modifications to the lipophilic alkyl side chain of **1** (16). Antimicrobial susceptibility analysis of these derivatives, using the standard broth microdilution assay (26), revealed compound **2** exhibited the most potent antibacterial activity against multidrug-resistant staphylococci. As Table 3.1 demonstrates, the minimum inhibitory concentration (MIC) for **1** was 1.38 μg/mL; compound **2** showed similar activity inhibiting growth of the same strains at a

concentration of 1.40 μg/mL. At these concentrations, the compounds are not toxic to mammalian cells as confirmed in a previous study (16).

The thiazole compounds exhibited activity against MRSA strains resistant to several different classes of antibiotics including macrolides (NRS384), fluoroquinolones (NRS385), aminoglycosides (NRS385), tetracyclines (NRS384), and oxazolidinones (NRS119). Additionally, both **1** (MIC from 1.38-2.77 μg/mL) and **2** (MIC from 0.70-1.40 μg/mL), unlike vancomycin (MIC from 2.97-760.68 μg/mL), retained their antimicrobial activity against strains of vancomycin-intermediate *S. aureus* (VISA) and VRSA strains. Furthermore, both thiazole compounds were more potent than teicoplanin against two VISA strains ($MIC_{Teicoplanin}$ from 0.94-7.52 μg/mL) and all three VRSA strains tested ($MIC_{Teicoplanin}$ from 60.51-120.30 μg/mL[1]). Thus, **1** and **2** exhibit a selective advantage over vancomycin and teicoplanin in their antibacterial activity against both VISA and VRSA.

Antimicrobial agents that exhibit bactericidal activity are hypothesized to contribute to a more rapid recovery from infection and a better clinical outcome, compared to their bacteriostatic counterparts (27). To ascertain whether the thiazole compounds were bacteriostatic or bactericidal, the minimum bactericidal concentration (MBC) was determined. The MBC was calculated as the lowest concentration of compound/drug that produced a ≥99.9% reduction in the bacterial cell count as compared to the initial inoculum (28). As Table 3.1 demonstrates, both thiazole compounds are bactericidal. Against five MRSA strains (NRS107, NRS119, NRS123, NRS385, and ATCC 43300), all VISA strains, and two VRSA strains (VRS1 and VRS4), **1** and **2** possess MBC values equivalent to their MICs or one-fold higher than the MICs. This is similar to what is observed with vancomycin, a known bactericidal antibiotic, with MBC values equal to or one-fold higher than the MICs for all MRSA and VISA strains tested. Teicoplanin exhibits MBC values equivalent to its MIC against two MRSA strains, four-fold higher than its MIC against two additional MRSA strains, and MBC values 16-fold higher than the MIC values against three strains of MRSA (NRS194, USA300, and USA400).

3.3.2 Time-kill Analysis of Thiazole Compounds and Glycopeptide Antibiotics

In order to confirm that **1** and **2** were bactericidal agents, we next examined how rapidly the thiazole compounds were able to kill a high inoculum of MRSA. Using a standard time-

kill assay, MRSA USA400 (NRS123), a predominant strain linked to many community-acquired MRSA infections (29), was exposed to 2, 4, and 8 × MIC of **1**, **2**, teicoplanin, or vancomycin. Samples were collected at specific time points and transferred to tryptic soy agar (TSA) plates to determine the number of viable bacteria remaining post-treatment. As depicted in Figure 3.2, both **1** and **2** exhibit bactericidal activity at all concentrations tested; however the time to achieve a 3-log10 reduction in CFU/mL differs depending on the concentration of the test agent. For compound **1**, MRSA is completely eliminated after 24 hours at 2 × MIC, after 10 hours at 4 × MIC, and after only two hours at 8 × MIC. Analogue **2** produces a 3-log10 reduction in CFU/mL after 10 hours at 2 × MIC; however, it is not able to completely eradicate MRSA similar to the parent compound. At higher concentrations, **2** successfully eliminates MRSA completely after 24 hours (at 4 × MIC); at the highest concentration tested (8 × MIC), analogue **2** proves superior to both antibiotics tested as it rapidly eliminates MRSA within 10 hours. Vancomycin required 24 hours to completely eradicate MRSA at both 4 and 8 × MIC; at 2 × MIC, vancomycin produced a 3-log10 reduction in CFU/mL within 24 hours but was not able to eliminate all bacteria completely (similar to analogue **2**). These results are similar to what has been previously published regarding vancomycin's slow bactericidal activity (30). Teicoplanin required 24 hours to completely eliminate MRSA at all three concentrations tested. Thus, in addition to retaining antimicrobial activity against VISA and VRSA strains, **1** and **2** possess an additional advantage over vancomycin and teicoplanin in their ability to rapidly kill MRSA, particularly at higher concentrations.

Rapid bactericidal activity is an important factor in reducing the emergence of bacterial resistance to an antimicrobial agent and is important clinically in preventing an infection from spreading (27). Additionally, bactericidal agents have been shown both clinically and in *in vivo* studies to be superior to bacteriostatic agents for the treatment of certain invasive diseases such as endocarditis (31). Furthermore, rapid bactericidal activity is an important quality for consideration in using a particular agent in combination with other antibiotics, such as vancomycin (30). The results from the time-kill assay provided valuable insight into the possibility that the thiazole compounds could be potentially paired with other antibiotics against MRSA, given **1** and **2** possess rapid bactericidal activity.

3.3.3 Assessment of Single-step Resistance

After confirming compounds **1** and **2** possessed rapid bactericidal activity against MRSA, we next turned our attention to assessing the likelihood MRSA would develop resistance quickly to these thiazole compounds. A single-step resistance selection experiment was performed by subculturing a high inoculum of MRSA (>1 × 10^9 CFU/mL) onto TSA plates containing **1**, **2**, vancomycin, or teicoplanin at a concentration equivalent to 4 × MIC. The likelihood of bacterial resistance arising (via spontaneous mutations in the bacterial genome) to these compounds/antibiotics was examined using five MRSA strains. Table 3.2 presents the mutation frequencies generated against each tested agent: for **1**, 1.19×10^{-8} to $>1.73\times10^{-10}$; for **2**, $>1.73\times10^{-10}$ to $>2.33\times10^{-10}$; for teicoplanin, 2.73×10^{-7} to 3.03×10^{-9}; and for vancomycin, 3.03×10^{-10} to $>8.47\times10^{-10}$. The values obtained for teicoplanin and vancomycin are similar to what has been reported elsewhere (32).

The thiazole compounds produce a similar mutation frequency as both teicoplanin and vancomycin. Interestingly, **2** demonstrates a mutation frequency similar to or better than vancomycin against the five MRSA strains tested. Even at lower (2 × MIC) concentrations, resistant mutants are difficult to isolate against this particular compound (data not published). It took 30 years to isolate a strain of *S. aureus* exhibiting resistance to vancomycin (1). Thus the results presented here support the notion that MRSA is unlikely to develop rapid resistance to the thiazole compounds, in particular compound **2**. The data obtained from both the time-kill and single-step resistance selection experiments demonstrate the thiazole compounds possess two important characteristics necessary for an ideal antibiotic for MRSA, rapid bactericidal activity and low potential for bacterial resistance development (33).

3.3.4 Combination Testing of Thiazole Compounds with Glycopeptide Antibiotics

Glycopeptide antibiotics, chiefly vancomycin, have been a principal source of treatment of MRSA infections for many years (33). However, extensive use of these antibiotics opens the door for the emergence of strains with reduced susceptibility to these antibiotics (30). Combination therapy, pairing vancomycin with another antimicrobial, has been used in the healthcare setting both to reduce the likelihood of resistant strains to vancomycin from rapidly emerging and to improve the morbidity associated with MRSA infections. For

example, vancomycin has been combined with subinhibitory concentrations of clindamycin and linezolid to reduce toxins generated by *S. aureus* during infection (34, 35). Identifying other antimicrobial partners capable of being paired with vancomycin can potentially prolong the clinical utility of this antibiotic.

To ascertain whether **1** and **2** have potential to be combined with vancomycin against MRSA, the checkerboard assay was utilized (22). In this assay, one antibiotic/compound is serially diluted along the abscissa followed by diluting the second antibiotic/compound along the ordinate in a 96-well plate. The fractional inhibitory concentration (FIC) is then calculated as a ratio of the MIC of each antibiotic/compound when given in combination relative to the MIC of each antibiotic/compound given alone. The FIC index (ΣFIC) is a summation of the FICs for each antibiotic/compound tested in combination. $\Sigma FIC \leq 0.50$ is indicative of synergism between the antibiotic and compound. Results from the checkerboard assay experiment are presented in Table 3.3. Both thiazole compounds were found to exhibit a synergistic relationship with vancomycin against six of the seven MRSA strains tested with ΣFIC values ranging from 0.07 to 0.50 for **1** and ΣFIC values ranging from 0.13 to 0.50 for **2**. At ¼ × MIC for **2**, a 16-fold reduction in the MIC for vancomycin (when combined with **2**) was observed for all six MRSA strains where synergy was detected (data not presented). As vancomycin is known to be a nephrotoxic agent, using a lower concentration of this drug in MRSA infections is highly desirable as it has the potential benefit of reducing this side effect in patients (33). When tested against VISA, **1** failed to exhibit synergy with vancomycin while **2** demonstrated a synergistic relationship with vancomycin against one strain (NRS19).

We were curious to explore if the synergistic relationship observed was limited just to vancomycin or could be observed with other glycopeptide antibiotics as well. Teicoplanin was used to further explore the partnership between thiazole compounds and glycopeptide antibiotics. Interestingly, the checkerboard assay revealed that neither **1** nor **2** exhibited a synergistic relationship with teicoplanin against MRSA. This suggests that combination therapy involving the thiazole compounds may be limited to only being paired with vancomycin though further studies with other glycopeptide antibiotics are needed to confirm this observation. Additionally, as vancomycin targets cell wall biosynthesis in *S. aureus*, it would be worthwhile to explore if a synergistic relationship would be observed

between these thiazole compounds and other cell wall biosynthesis inhibitors (such as β-lactam antibiotics). Collectively, the results shed valuable insight into thiazole compounds serving as potential future partners with vancomycin against MRSA. This discovery can potentially prolong the usage of vancomycin as a therapeutic agent for MRSA infections by reducing the likelihood of strains developing resistance to vancomycin used in monotherapy.

3.3.5 Re-sensitization of VRSA to Glycopeptide Antibiotics

The emergence of *S. aureus* strains resistant to vancomycin presents an additional challenge to clinical care providers dealing with the growing epidemic of multidrug-resistant bacterial infections. Identifying clever strategies to prolong the use of current antibiotics against multidrug-resistant bacteria is necessary. One strategy that has been explored recently is suppressing antibiotic resistance by re-sensitizing resistant bacteria using a secondary compound (23). As the thiazole compounds were found to possess a synergistic relationship with vancomycin against MRSA, we postulated that the thiazole compounds may be capable of re-sensitizing VRSA strains to vancomycin. Initially, the MIC of **1** and **2** was determined using the broth microdilution assay. Next, Mueller-Hinton broth was inoculated with either compound **1** or **2** (at ½ × MIC). Vancomycin was then serially diluted in both the inoculated media alone and media supplemented with the thiazole compounds. The MICs of vancomycin in the presence of the thiazole compounds was compared to vancomycin used alone. A fold-reduction was calculated by dividing the MIC of vancomycin alone by the MIC of vancomycin + the thiazole compound.

As Table 3.4 presents, both thiazole compounds were capable of re-sensitizing VRSA to vancomycin. Compound **1** was able to produce a four-fold reduction in the MIC of vancomycin when the two agents were combined against VRSA. Amazingly, compound **2** proved to be superior to **1** as it produced a 512-fold reduction in the MIC of vancomycin against two VRSA strains tested. Furthermore, compound **2** produced a 32-fold reduction in the MIC of teicoplanin against two VRSA strains (VRS4 and VRS5) and a 64-fold reduction against strain VRS1. Thus compound **2** was capable of re-sensitizing VRSA to both vancomycin and teicoplanin. Substitution of the alkane side chain (in **1**) with a phenyl group (in **2**) produced a dramatic improvement in the thiazole compounds' ability to re-

sensitize VRSA to the effect of glycopeptide antibiotics. Using the checkerboard assay, we found that compound **2** exhibited a synergistic relationship with both vancomycin and teicoplanin against two VRSA strains (VRS4 and VRS5) with ΣFIC = 0.50. Thus, compound **2** holds promise for future use to suppress vancomycin-resistance in VRSA strains, prolonging the utility of glycopeptide antibiotics against these strains.

3.3.6 *S. epidermidis* Biofilm Mass Reduction

Bacterial biofilms which form on the surface of indwelling medical devices, such as intravascular catheters, are a major problem in hospitals. These biofilms can lead to life-threatening bloodstream infections associated with high mortality and treatment costs (36). Staphylococci, primarily *S. epidermidis* and *S. aureus*, are responsible for many invasive infections which develop from bacterial biofilms that form on the surface of medical devices (3, 37). Further exacerbating this problem, traditional antibiotics are not effective at disrupting these biofilms as cells present within the biofilm exhibit increased resistance to antibiotics (5). Identifying antimicrobials capable of disrupting these biofilms is necessary to combat this growing problem.

Recent studies have demonstrated that thiazole and thiazolidinone compounds possess the ability to disrupt bacterial biofilms (38, 39). To examine if the potential therapeutic application of **1** and **2** could be expanded beyond just inhibition of planktonic bacteria, the ability of both thiazole compounds to disrupt staphylococcal biofilm was analyzed. First, to confirm the thiazole compounds were capable of inhibiting planktonic bacteria, the MIC of each compound and vancomycin against a biofilm-forming clinical isolate of methicillin-resistant *S. epidermidis* was assessed using the broth microdilution technique. Compounds **1** and **2** were found to inhibit bacterial growth at 2.77 and 0.61 μg/mL, respectively (Table 3.1). Vancomycin inhibited growth of planktonic *S. epidermidis* at a concentration of 0.74 μg/mL. Next, to determine if compounds **1** and **2** had the potential to disrupt staphylococcal biofilm, the crystal violet reporter assay was used against a mature *S. epidermidis* biofilm (24). As Figure 3.3 demonstrates, **1** (at 8 × MIC) and **2** (at 32 × MIC) significantly disrupted *S. epidermidis* biofilm, reducing the biofilm mass by 56.7% and 65.2% respectively. These compounds proved to be far superior to vancomycin; even at 128 × MIC, vancomycin was only able to reduce *S. epidermidis*

biofilm mass by 21.5%. The thiazole compounds thus possess anti-biofilm activity and are capable of disrupting adherent staphylococci biofilm much better than a traditional antibiotic, vancomycin.

3.3.7 *In vitro* Pharmacokinetic Analysis of Compound **2**

Assessment of a compound's drug-like properties is important early in drug development to identify and address potential issues, especially those associated with aqueous solubility and permeability. Previously we reported the lead thiazole compound **1** possessed moderate aqueous solubility (21.6 μg/mL) but poor permeability across a biological membrane (Caco-2 apparent permeability, P_{app} (A → B) = 0.0 × 10^{-6} cm/sec) (16). We were interested to examine if compound **2**, containing a phenyl ring substitution in place of the linear alkane side chain present in **1**, would exhibit an improved pharmacokinetic profile. Initially, a turbidometric solubility screen was used to assess the maximum concentration compound **2** was able to dissolve in an aqueous buffer (phosphate-buffered saline). Table 3.5 demonstrates, the phenyl ring substitution resulted in a significant decrease in the aqueous solubility of compound **2** (2.70 μg/mL) relative to **1**. After determining compound **2** exhibited poor aqueous solubility, this compound's ability to passively diffuse across a biological membrane was assessed.

The Caco-2 permeability assay was utilized to determine if compound **2** was more permeable than compound **1**. 10 μM of **2** was added to the apical (A) surface of a Caco-2 bilayer and the rate of transfer of the compound to the basolateral (B) surface was measured. The process was then repeated in reverse to assess the rate of transfer from the B to A direction. As Table 3.6 demonstrates, the rate of transfer of compound **2** from the apical to basolateral surface was not measurable (P_{app} (A → B) = 0.0 × 10^{-6} cm/sec). However, the rate of transfer from the B to A surface was measured to be 1.2 × 10^{-6} cm/sec. This is similar to what is observed with the poorly permeable drug control ranitidine (P_{app} (B → A) = 1.7 × 10^{-6} cm/sec). Thus the results indicate compound **2** does not exhibit improved permeability relative to **1**. The discrepancy between the rate of transfer of compound **2** across the basolateral and apical surfaces results in an efflux ratio >2; this suggests that **2** may be a substrate for an efflux transporter (such as P-glycoprotein). One method to overcome the effect of efflux transporters is to saturate the transporters, by using

a higher concentration than 10 μM of compound **2** used for the assay, thus permitting passive transfer of the compound across the apical surface of the membrane.

While limited solubility and permeability characteristics are not encouraging to consider biologically-active compounds as drug-candidates for subsequent clinical steps, recent formulation technology has been able to overcome such limitations to propel valuable compounds with similar kinetic profiles into the market. For instance, the orally administrated protease inhibitor telaprevir possesses an aqueous solubility profile similar to compound **2**. By using a spray drying dispersion technique, telaprevir's water solubility, permeability and the consequent bioavailability were dramatically improved (40). Moreover, formulators have more techniques to handle poor water solubility such as using the solvent/antisolvent method (41). By shedding light on the limited pharmacokinetic profile of compound **2**, we are opening a gate for formulators to investigate their time and effort improving the pharmacokinetic profile of this very promising antimicrobial agent.

3.4 Conclusion

We have successfully developed an approach to synthesize phenylthiazole compounds with potent antibacterial activity against methicillin-resistant (MRSA), vancomycin-intermediate (VISA), and vancomycin-resistant *Staphylococcus aureus* (VRSA). The most potent derivative **2** exhibited MIC values ranging from 0.70 to 1.40 μg/mL and MBC values ranging from 1.40 to 11.17 μg/mL against MRSA, VISA, and VRSA. Both compounds **1** and **2** rapidly eliminated MRSA within 10 hours, at 8 × MIC, while vancomycin required 24 hours; additionally both thiazole compounds exhibited low resistance frequencies, similar to vancomycin. Lead **1** behaved synergistically when combined with vancomycin exhibiting ΣFIC ranging from 0.07 to 0.50 against six MRSA strains while derivative **2** behaved synergistically with vancomycin exhibiting ΣFIC ranging from 0.09 to 0.50 against six MRSA strains. Interestingly, compound **2** demonstrated the ability to re-sensitize two VRSA strains to vancomycin and teicoplanin reducing their MIC by 512-fold and 32-fold, respectively. Additionally, both compounds **1** and **2** exhibited strong anti-biofilm activity reducing adherent *S. epidermidis* biofilm by 56.7% and 65.2%, respectively. As compound **2** did not demonstrate good solubility or permeability properties, incorporating advanced formulation techniques are a must to

improve its pharmacokinetic profile. In addition, further derivatives will be constructed with the aim of improving the thiazole compounds' drug-like properties while maintaining their strong antibacterial properties. Collectively, the thiazole compounds prepared here have the versatility to potentially be used for multiple therapeutic applications including being used alone or in combination with vancomycin against multidrug-resistant staphylococci, to re-sensitize VRSA to vancomycin, or to disrupt mature staphylococcal biofilms.

3.4.1 References

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Table 3.1 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of thiazole compounds **1** and **2**, teicoplanin, and vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) strains.

	Strain	MIC and MBC (μg/mL) of thiazole compounds, teicoplanin, and vancomycin against *S. aureus*							
		1		**2**		Teicoplanin		Vancomycin	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
MRSA	NRS107	1.38	2.77	1.40	1.40	0.94	0.94	0.74	1.49
	NRS119	1.38	1.38	1.40	1.40	0.94	0.94	0.74	1.49
	NRS123 (USA400)	1.38	11.07	1.40	5.58	0.94	15.04	0.37	0.37
	NRS194	1.38	5.54	1.40	11.17	0.94	15.04	0.74	0.74
	NRS384 (USA300)	1.38	1.38	1.40	2.80	0.94	15.04	0.74	0.74
	NRS385 (USA500)	1.38	1.38	1.40	1.40	0.94	3.76	0.74	0.74
	ATCC 43300	1.38	2.77	1.40	2.80	0.94	3.76	0.74	0.74
VISA	NRS1	1.38	1.38	0.70	1.40	3.76	7.52	2.97	2.97
	NRS19	1.38	1.38	1.40	1.40	0.94	0.94	2.97	2.97
	NRS37	1.38	1.38	0.70	1.40	7.52	7.52	2.97	2.97
VRSA	VRS1	2.77	2.77	1.40	1.40	120.30	>240.60	760.68	760.68
	VRS4	2.77	2.77	0.70	2.80	60.15	60.15	760.68	760.68
	VRS5	2.77	5.54	1.40	5.58	60.15	60.15	760.68	760.68
S. epidermidis	ATCC 35984	2.77	N.D.[1]	0.70	N.D.	N.D.	N.D.	0.74	N.D.

[1]Abbreviation: N.D. = Not Determined

Table 3.2 Single-step frequency of resistance determination for compounds **1** and **2**, teicoplanin, and vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA).

MRSA Strain	**Compound/Antibiotic Name**			
	1	**2**	**Teicoplanin**	**Vancomycin**
NRS107	$>1.73\times10^{-10}$	$>1.73\times10^{-10}$	1.91×10^{-8}	$>1.73\times10^{-10}$
NRS119	1.99×10^{-8}	$>8.47\times10^{-10}$	2.73×10^{-7}	$>8.47\times10^{-10}$
NRS123 (USA400)	5.93×10^{-9}	$>2.33\times10^{-10}$	2.21×10^{-9}	$>2.33\times10^{-10}$
NRS384 (USA300)	1.35×10^{-8}	$>3.03\times10^{-10}$	3.03×10^{-9}	3.03×10^{-10}
NRS385 (USA500)	1.19×10^{-8}	$>3.31\times10^{-10}$	1.79×10^{-8}	$>3.31\times10^{-10}$

Table 3.3 Fractional inhibitory concentration index (ΣFIC) range of thiazole compounds **1** and **2** in combination with teicoplanin and vancomycin against methicillin-resistant and vancomycin-intermediate *Staphylococcus aureus* (MRSA and VISA).

Strain	Vancomycin				Teicoplanin			
	ΣFIC[1] (+**1**)	Result	ΣFIC (+**2**)	Result	ΣFIC (+**1**)	Result	ΣFIC (+**2**)	Result
MRSA NRS107	0.50-0.56	S/I	0.31-0.50	S	0.53-1.00	I	0.53-0.53	I
MRSA NRS119	0.07-0.31	S	0.28-0.31	S	0.50-0.53	S/I	0.53-0.75	I
MRSA NRS123	0.19-0.50	S	0.19-0.31	S	0.53-1.00	I	0.53-1.00	I
MRSA NRS194	0.13-0.50	S	0.13-0.56	S/I	0.28-1.00	S/I	0.28-1.00	S/I
MRSA NRS384	0.16-0.50	S	0.13-0.31	S	0.53-1.00	I	0.53-0.75	I
MRSA NRS385	0.13-0.50	S	0.13-0.31	S	0.53-1.00	I	0.53-1.00	I
MRSA ATCC 43300	0.09-0.50	S	0.09-0.31	S	0.50-0.52	I	0.50-0.56	S/I
VISA NRS1	0.26-0.56	S/I	0.63	I	0.16-1.00	S/I	0.19-2.00	S/I
VISA NRS19	0.53-0.56	I	0.50	S	0.53- 1.03	I	0.53-1.03	I
VISA NRS37	0.53-0.56	I	0.75	I	0.09-1.01	S/I	0.13-2.00	S/I

[1]Results for the FIC index are as follows: ≤ 0.5, synergistic (S); >0.5 to ≤4.0, indifference (I); >4.0, antagonistic (A). Results are reported from two independent experiments.

Table 3.4 Re-sensitization of vancomycin-resistant *Staphylococcus aureus* (VRSA) to vancomycin and teicoplanin using a subinhibitory concentration (½ × MIC) of compound **1** or **2**.

Strain	**1** + Vancomycin		**2** + Vancomycin		**1** + Teicoplanin		**2** + Teicoplanin	
	Re-sensitization	ΣFIC[1]	Re-sensitization	ΣFIC	Re-sensitization	ΣFIC	Re-sensitization	ΣFIC
VRS1	<4-fold	>1.50	<4-fold	1.00	0-fold	>2.00	64-fold	0.63
VRS4	<4-fold	1.13	512-fold	0.50	0-fold	2.00	32-fold	0.50
VRS5	4-fold	1.25	512-fold	0.50	2-fold	1.50	32-fold	0.50

[1] Results for the FIC index (ΣFIC) are as follows: ≤ 0.5, synergistic (S); >0.5 to ≤4.0, indifference (I); >4.0, antagonistic (A).

Table 3.5 Evaluation of solubility of thiazole compound **2**, Reserpine, Tamoxifen, and Verapamil in phosphate-buffered saline (PBS).

Compound Tested	**Solubility Limit (μg/mL)[1]**
2	2.70
Reserpine	19.05
Tamoxifen	5.80
Verapamil	>227.30

[1] Solubility limit corresponds to the highest concentration of test compound where no precipitate was detected.

Table 3.6 Evaluation of physicochemical properties (apparent permeability) of thiazole compound **2**, Ranitidine, Warfarin, and Talinolol via the Caco-2 permeability assay.

Compound Tested	**Mean A → B[1] P_{app} (10^{-6} cm/sec)**	**Mean B → A[2] P_{app} (10^{-6} cm/sec)**	**Efflux Ratio[3]**
2	0.0[4]	1.2	>2
Ranitidine	0.2	1.7	8.5
Warfarin	27.6	11.1	0.4
Talinolol	0.1	8.3	83

[1] Mean A → B P_{app} = mean apparent permeability of test compound from apical to basolateral surface

[2] Mean B → A P_{app} = mean apparent permeability of test compound from basolateral to apical surface

[3] Efflux ratio = $\frac{Papp(B \rightarrow A)}{Papp(A \rightarrow B)}$

[4] Compound not detected in receiver compartment (peak below limit of detection); permeability may be underestimated

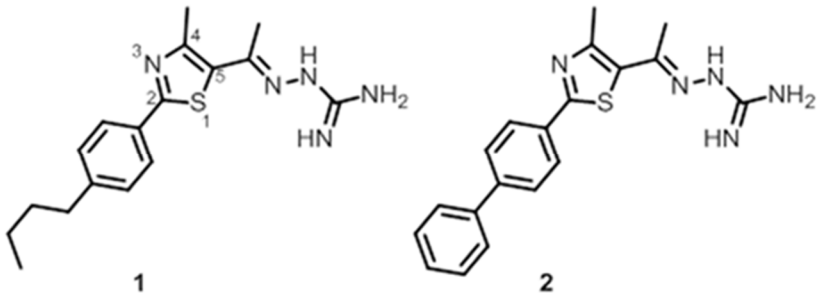

Figure 3.1 Chemical structure of thiazole compounds **1** and **2**.

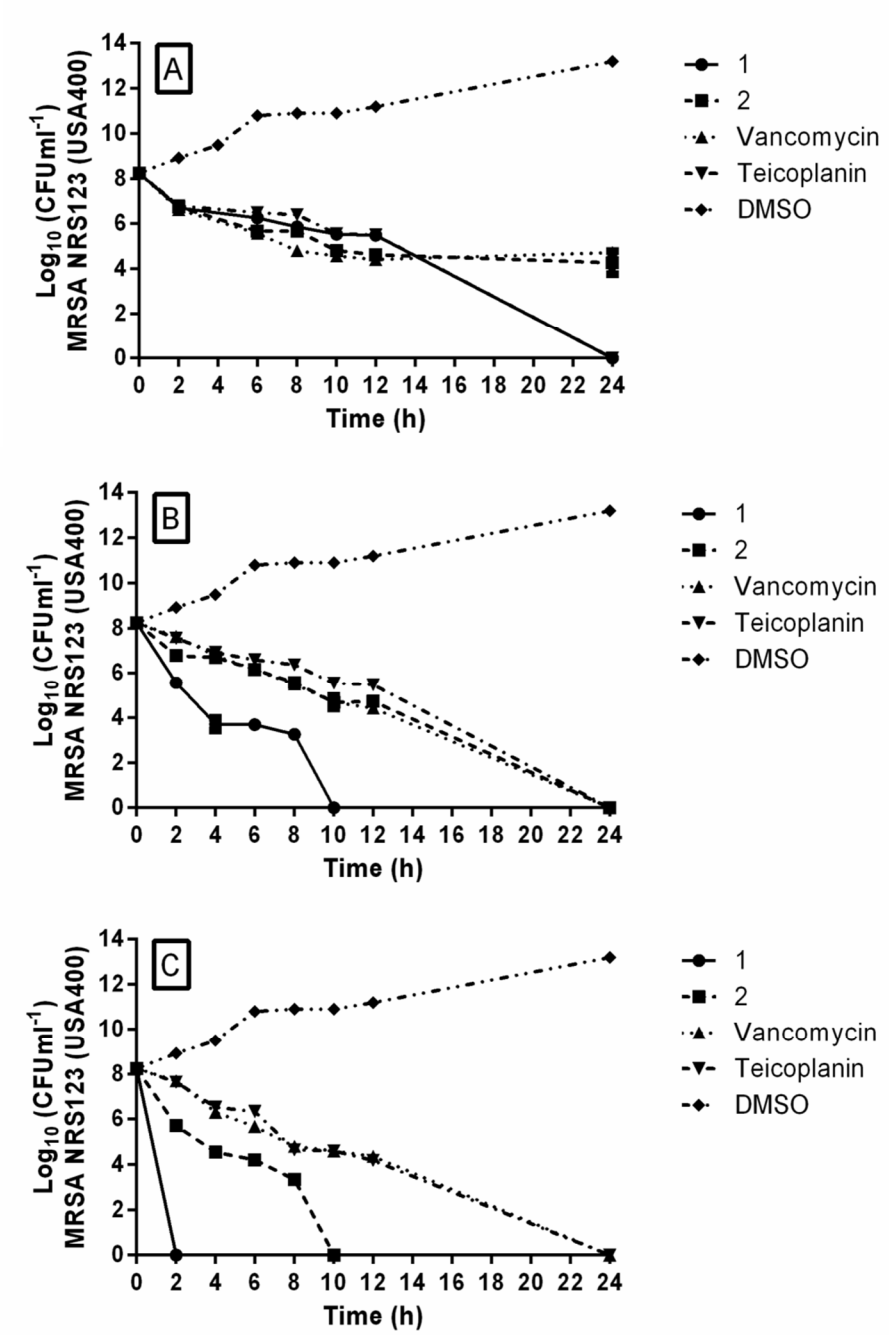

Figure 3.2 Time-kill analysis of the lead compound **1**, derivative **2**, teicoplanin, and vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA) strain NRS123 (USA400) at A) 2 × MIC, B) 4 × MIC, and C) 8 × MIC.

Error bars represent standard deviation values.

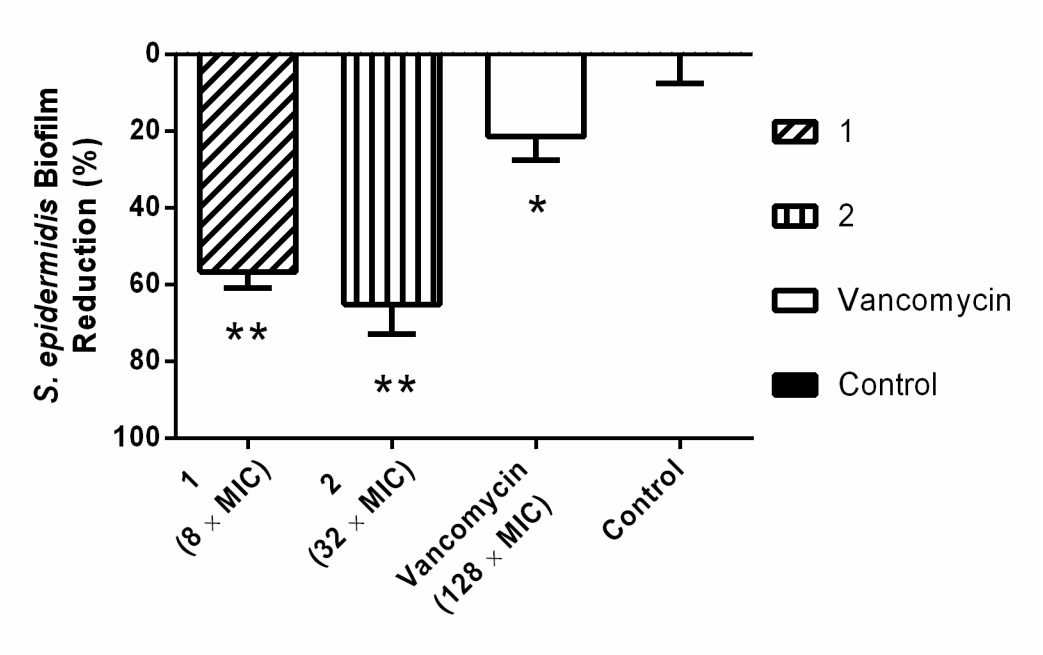

Figure 3.3 Efficacy of thiazole compounds **1** and **2** and vancomycin (all at 64 μM) in disrupting an established methicillin-resistant *S. epidermidis* ATCC 35984 biofilm.

Bacteria were incubated at 37 °C in MHB medium supplemented with glucose for 24 hours to allow biofilm formation. Wells were subsequently rinsed with PBS before MHB containing different concentrations of each test agent was added. Following incubation for 24 hours, wells were washed again and left to dry. The adherent biofilm was stained with crystal violet and then the dye was extracted with ethanol before turbidity was measured at 595 nm. Data are presented as percentage of biofilm mass reduction compared to untreated wells (control). All experiments were done in triplicate. One asterisk (*) indicates data are statistically different when compared to the control ($P < 0.05$). Two asterisks (**) indicate the data are statistically different from the vancomycin-treated wells ($P < 0.05$).

CHAPTER 4. SYNTHESIS AND ANTIBACTERIAL EVALUATION OF A NOVEL SERIES OF SYNTHETIC PHENYLTHIAZOLE COMPOUNDS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA)

4.1 Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) infections remain a significant public health challenge globally. Though reports have indicated the incidence of healthcare-associated MRSA (HA-MRSA) infections have diminished (1, 2), transmission of community-associated MRSA (CA-MRSA) infections, primarily strains USA300 and USA400 (3), has continued to present major problems amongst a diverse population including healthcare workers (4), prison inmates (5, 6), military service personnel (6), contact sport athletes (7, 8), homeless individuals (9), intravenous drug users (9, 10), tattoo recipients (11), neonates (12), and young children (13, 14). Moreover, CA-MRSA infections are typically associated with more severe morbidity and mortality than their HA-MRSA counterparts (15). While CA-MRSA is a leading cause of skin and soft-tissue infections (16, 17), MRSA has also been associated with more complicated medical diseases including necrotizing pneumonia (18), osteomyelitis (19), and sepsis (20), leading to over 11,000 deaths annually (21).

A recent study has estimated the total annual burden upon society for treatment of CA-MRSA infections alone may exceed US$13 billion (22). Part of the associated cost is due to failure of current antimicrobials to treat certain clinical isolates of MRSA that have developed resistance to these therapeutic agents. Indeed, clinical isolates of both CA-

MRSA and HA-MRSA have been documented that exhibit resistance to an array of different antibiotic classes including the β-lactams (23), macrolides (24), quinolones (25, 26), tetracyclines (27), and lincosamides (27). Further exacerbating the problem, strains have emerged which exhibit resistance to first-line antibiotics (such as mupirocin (27, 28) for the treatment of MRSA skin infections) and drugs deemed agents of last resort (such as linezolid (29, 30) and vancomycin (31)). Prudent use and development of effective antimicrobials is a critical step to alleviate complications and costs associated with MRSA infections. Therefore there is an urgent need for the development of novel therapeutic agents and treatment strategies to circumvent this significant global health issue.

Utilizing whole-cell screening of a library of substituted thiazoles, our research group identified a novel lead thiazole compound that possesses potent antimicrobial activity against clinically relevant isolates of MRSA, vancomycin-intermediate *S. aureus* (VISA), and vancomycin-resistant *S. aureus* (VRSA) (32). The basic structure of the lead **1** consists of a central thiazole ring connected to two distinct moieties – a lipophilic side chain at C2 and a cationic amino group at C5. The objectives of the present study were to construct a series of analogues to the lead **1** (Table 4.1) with modifications to the functional groups at both the thiazole-C2 and C5 positions to more rigorously ascertain the structure-activity relationship of these compounds against a diverse array of HA-MRSA and CA-MRSA isolates, identify new derivatives exhibiting an improved toxicity profile against mammalian cells, and to enhance the metabolic stability profile of the lead **1**.

4.2 Materials and Methods

4.2.1 Chemistry

The detailed synthetic protocols and spectral data of the lead **1** (Figure 4.1) in addition to all intermediates have been reported elsewhere (32, 33). All thiazole compounds were dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to achieve a stock 10 mM solution.

The (4-iodophenyl)thiazole derivative **3** was prepared by heating a mixture of the commercially available 4-iodothiobenzamide **2** and 3-chloro-2,4-pentanedione in absolute ethanol, as illustrated in Figure 4.2. The phenylthiazolyl methyl ketone derivatives **4** and **6**

were prepared via the Sonogashira cross coupling of the (4-iodophenyl)thiazole derivative **3** with commercially available 1-hexyne and 1-nonyne, respectively, in DMF using a bis(triphenylphosphine)palladium(II) dichloride catalyst, copper(I) iodide co-catalyst, and cesium carbonate base (Figure 4.2). The hydrazinecarboximidamide derivatives **5** and **7** were synthesized by treatment of the phenylthiazolyl methyl ketone derivatives **4** and **6**, respectively, with aminoguanidine hydrochloride in the presence of a catalytic amount of lithium chloride in absolute ethanol (Figure 4.2).

The amide derivatives **10-13** were prepared in quantitative yields by reacting the 4-butylphenylthiazole acid chloride intermediate **8** (34) with the appropriate amines in THF, as illustrated in Figure 4.3. Compound **16** was synthesized in three steps, starting with the formation of the amide derivative **9** by way of reacting the acid chloride intermediate **8** with ammonium hydroxide in THF at room temperature. The amide intermediate **9** was then heated in thionyl chloride to give the nitrile intermediate **14**, which upon subsequent treatment with NaN_3 in the presence of iodine gave the tetrazole-containing thiazole derivative **16** as shown in Figure 4.3. The nitrile intermediate **14** was also treated with hydroxylamine hydrochloride in absolute ethanol with a catalytic amount of potassium carbonate to afford the thiazole derivative **15**. The phenylthiazolyl methyl ketone derivative **18** was prepared by treatment of the commercially available 4-aminothiobenzamide **17** with 3-chloro-2,4-pentanedione in absolute ethanol.

Synthesis of the hydrazinecarboximidamide derivative **19** was achieved by treatment of the phenylthiazolyl methyl ketone derivative **18** with aminoguanidine hydrochloride in the presence of a catalytic amount of lithium chloride (Figure 4.4).

Phenylthiazole methylketone derivatives **21a-d** and **24** were prepared via the Suzuki-Miyaura cross coupling of the (4-iodophenyl)thiazole derivative **3** with the commercially available phenylboronic acid derivatives **20a-d** and **23**, respectively, in the presence of a catalytic quantity of palladium(II) acetate and (2-biphenyl)dicyclohexylphosphine ligand, as shown in Figure 4.5. Synthesis of the hydrazinecarboximidamide derivatives **22a-d** and **25** was achieved by treatment of phenylthiazole methylketone derivatives **21a-d** and **24**, respectively, with aminoguanidine hydrochloride in the presence of lithium chloride as catalyst (Figure 4.5).

^{1}H NMR spectra were recorded in $CDCl_3$ or DMSO-d_6 using a 300 MHz spectrometer. Chemical shifts are reported in units of ppm on the delta (δ) scale and coupling constants (*J*) are reported in units of Hz. The following splitting abbreviations are used: s = singlet, d = doublet, t = triplet and m = multiplet. All melting points were recorded using capillary tubes on a Mel-Temp apparatus and are not corrected. Mass spectral analyses were performed at the Purdue University Campus-Wide Mass Spectrometry Center. Reagents and solvents were purchased from commercial vendors and were used as received without further purification, unless otherwise stated.

1-(2-(4-Iodophenyl)-4-methylthiazol-5-yl)ethanone (3).

4-Iodothiobenzamide (**2**, 3.80 mmol) and α-chloropentanedione (0.611 mg, 4.56 mmol) were added to absolute ethanol (50 mL). The reaction mixture was heated at reflux for 24 hours. After removal of solvent under reduced pressure, the residue was purified by silica gel chromatography using hexanes–ethyl acetate (7:3) to provide the desired compound as light orange solid (0.800 g, 62%): mp 105-106 ^{o}C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.81 (d, *J* = 6.6 Hz, 2 H), 7.70 (d, *J* = 6.6 Hz, 2 H), 2.77 (s, 3 H), 2.56 (s, 3 H).

1-(2-(4-(Hex-1-yn-1-yl)phenyl)-4-methylthiazol-5-yl)ethanone (4).

1-(2-(4-Iodophenyl)-4-methylthiazol-5-yl)ethanone (**3**, 0.5 g, 1.45 mmol), 1-hexyne (0.373 g, 7.73 mmol), cesium carbonate (0.947 g, 2.91 mmol), dichloro-*bis*(triphenylphosphine)palladium(II) (0.051 g, 0.072 mmol) and CuI (0.027 g, 0.145 mmol) were dissolved in DMF (6 mL). The reaction mixture was purged with argon for 20 minutes. The sealed tube was closed, placed in an oil bath and stirred at 65 ^{o}C for 15 hours. The reaction mixture was filtered through celite, and the celite was washed with chloroform (50 mL). The organic phase was washed with 1% hydrochloric acid (30 mL), water (3 x 40 mL) and brine (30 mL). The organic layer was dried over Na_2SO_4, concentrated and purified by silica gel flash column chromatography using hexanes–ethyl acetate (8:2) to provide the desired compound as yellow syrup (0.400 g, 92.5%): IR (film) 1945, 1675, 1111, 819, 666 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.90 (d, *J* = 8.5 Hz, 2 H), 7.46 (d, *J* = 8.3 Hz, 2 H), 2.76 (s, 3 H), 2.56 (s, 3 H), 2.45 (t, *J* = 6.9 Hz, 2 H), 1.59 (m, 4 H), 0.97 (t, *J* = 7.3 Hz, 3 H); ESIMS *m/z* (rel intensity) 298 (MH^+, 100).

(*Z*)-2-(1-(2-(4-(Hex-1-yn-1-yl)phenyl)-4-methylthiazol-5-yl)ethylidene) hydrazinecarboximidamide (5).

The thiazole derivative **4** (200 mg, 0.673 mmol) was dissolved in absolute ethanol (10 mL), and aminoguanidine hydrochloride (0.088 mg, 0.808 mmol) and a catalytic amount of LiCl (5 mg) were added. The reaction mixture was heated at reflux for 24 hours. The solvent was evaporated under reduced pressure. The crude product was purified by crystallization from 70% methanol and then recrystallized from methanol to afford the desired compound as a yellow solid (80 mg, 46%): mp 253-254 ºC. IR (KBr) 3329, 2227, 1678, 1143, 836, 657 cm^{-1}; ^1H NMR (DMSO-d_6) δ 11.35 (br s, 1 H), 7.88 (d, J = 8.1 Hz, 2 H), 7.69 (br s, 3 H), 7. 49 (d, J = 8.1 Hz, 2 H), 2.60 (s, 3 H), 2.44 (s, 3 H), 2.41 (t, J = 7.1 Hz, 2 H), 1.48 (m, 4 H), 0.93 (t, J = 7.1 Hz, 3 H); ESIMS *m/z* (rel intensity) 354 (MH^+, 100); HRESIMS calcd for $C_{19}H_{24}N_5S$ 354.1509 (MH^+), found 354.1514; HPLC purity 98.07% (1% TFA in MeOH:H_2O – 85:15).

1-(4-Methyl-2-(4-(non-1-yn-1-yl)phenyl)thiazol-5-yl)ethan-1-one (6).

The thiazole derivative **3** (750 mg, 2.19 mmol), 1-nonyne (1.44 mL, 8.76 mmol), $PdCl_2(PPh_3)_2$ (76.6 mg, 0.11mmol), copper(I) iodide (41.6 mg, 0.22 mmol) and Cs_2CO_3 (1.42 g, 4.38 mmol) were added to a sealed tube under argon for 10 minutes, and then DMF (7.5 mL) was added. The tube was once again evacuated and purged with argon for 5 minutes and heated to 70 °C for 15 hours. The tube was allowed to cool to room temperature, and the solids were removed by filtration and the filter cake was extracted with additional $CHCl_3$ (50 mL). The combined filtrate and extracts were concentrated under vacuum and extracted with EtOAc (2 × 50 mL) and washed with water (2 × 50 mL). After evaporation of the solvent under reduced pressure, the residue was collected and purified by flash chromatography (SiO_2, hexanes-EtOAc, 8.8:1.2) to yield the desired compound **6** as a dark green oil (772 mg, 100%); ^1H NMR (300 MHz, $CDCl_3$) δ 7.9 (t, J = 6.8 Hz, 2 H), 7.46 (d, J = 8.4 Hz, 2 H), 2.76 (s, 3 H), 2.55 (s, 3 H), 2.44 (t, J = 7.1 Hz, 3 H), 1.63 (t, J = 7.7 Hz, 2 H), 1.30 (m, J = 3.3 Hz, 9 H), 0.9 (q, J = 6.2 Hz, 4 H).

(*E*)-2-(1-(4-Methyl-2-[4-(non-1-yn-1-yl)phenyl]thiazol-5-yl)ethylidene)hydrazinecarboximidamide (7).

The thiazole derivative **6** (140 mg, 0.41 mmol), aminoguanidine hydrochloride (90.85 mg, 0.83 mmol), and a catalytic amount of LiCl (5 mg) were added to absolute ethanol (10 mL).

The reaction mixture was heated at reflux for 24 hours. After evaporation of the solvent under reduced pressure, the crude residue was extracted with $CHCl_3$/MeOH (90:10, 2 × 40 mL) and washed with water (2 × 50 mL) and brine (40 mL). The extracts were pooled together, dried over Na_2SO_4 and stripped of solvents under reduced pressure. The residue was suspended in $CHCl_3$/hexanes (50:50, 50 mL) and filtered through Whatman filter paper to afford the desired product (**7**) as a yellow-white solid (200 mg, 100%): mp 255-260 °C dec, ^{1}H NMR (300 MHz, DMSO-d_6) δ 7.88 (d, J = 8.3 Hz, 3 H), 7.49 (d, J = 8.4 Hz, 3 H), 2.60 (s, 3 H), 2.49 (q, J = 1.7 Hz, 5 H), 1.26 (s, 12 H), 0.85 (s, 4 H); ESIMS *m/z* (rel intensity) 395 (M^+, 57).

2-(4-Butylphenyl)-4-methylthiazole-5-carboxamide (9).

Acid chloride **8** (34) (0.200 g, 0.682 mmol) was dissolved in THF (20 mL) and then 30% aq NH_4OH (10 mL) was added. The reaction mixture was stirred at room temperature for 24 hours. The THF was removed on a rotary evaporator and the crude product was extracted with EtOAc (2 × 25 mL) and washed with water (2 × 20 mL) and brine (20 mL). The combined organic layer was dried over Na_2SO_4 and concentrated to afford the amide **9** (0.185 g) in quantitative yield: mp 160-161 ºC. IR (KBr) 3245, 1691, 1611, 1121, 846, 665 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.84 (d, J = 8.2 Hz, 2 H), 7.26 (d, J = 8.2 Hz, 2 H), 5.79 (br s, 2 H), 2.74 (s, 3 H), 2.66 (t, J = 7.5 Hz, 2 H), 1.63 (m, 2 H), 1.39 (m, 2 H), 0.94 (t, J = 7.3 Hz, 3 H); APCIMS *m/z* (rel intensity) 275 (MH^+, 100); HPLC purity 97.89% (1% TFA in MeOH:H_2O – 90:10).

2-(4-Butylphenyl)-*N*-(*N*-carbamimidoylcarbamimidoyl)-4-methylthiazole-5-carboxamide (10).

Acid chloride **8** (0.200 g, 0.682 mmol) was dissolved in THF (20 mL) and then biguanidine hydrochloride (0.467 g, 3.41 mmol) followed by triethylamine (0.344 g, 3.41 mmol) were added. The reaction mixture was stirred at room temperature for 24 hours. The THF was removed on a rotary evaporator and the crude product was extracted with EtOAc (2 × 30 mL) and washed with water (2 × 20 mL) and brine (20 mL). The combined organic layer was dried over Na_2SO_4, concentrated and purified by silica gel flash column chromatography using chloroform-methanol (9.5:0.5) to provide the desired compound as a yellow solid (0.070 g, 30%): mp 150-151 ºC. IR (KBr) 3312, 1694, 1655, 1148, 823, 666 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.89 (br s, 2 H), 7.25 (br s, 2 H), 2.80 (s, 3 H), 2.67 (t,

J = 7.2 Hz, 2 H), 1.60 (m, 2 H), 1.38 (m, 2 H), 0.94 (t, *J* = 7.1 Hz, 3 H); ESIMS *m/z* (rel intensity) 359 (MH^+, 65), 341 (MH^+-NH_3, 68); HRESIMS calcd for $C_{17}H_{23}N_6OS$ *m/z* 359.1248 (MH^+), found 359.1251; HPLC purity 95.16% (1% TFA in MeOH:H_2O – 90:10).

2-(2-(4-Butylphenyl)-4-methylthiazole-5-carbonyl)hydrazinecarboximidamide (11).

Acid chloride **8** (0.200 g, 0.682 mmol) was dissolved in THF (20 mL) and then amino guanidine hydrochloride (0.377 g, 3.41 mmol) followed by triethylamine (0.344 g, 3.41 mmol) were added. The reaction mixture was stirred at room temperature for 24 hours. The THF was removed on a rotary evaporator and the crude product was extracted with EtOAc (2 × 30 mL) and washed with water (2 × 20 mL) and brine (20 mL). The combined organic layer was dried over Na_2SO_4, concentrated and purified by silica gel flash column chromatography using hexanes–ethyl acetate (4:6) to provide the desired compound as a yellow solid. (0.090 g, 40%): mp 174-175 ^oC. IR (KBr) 3322, 1698, 1658, 1462, 1155, 856, 665 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.87 (d, *J* = 8.0 Hz, 2 H), 7.24 (d, *J* = 8.0 Hz, 2 H), 2.78 (s, 3 H), 2.67 (t, *J* = 7.6 Hz, 2 H), 1.63 (m, 2 H), 1.39 (m, 2 H), 0.94 (t, *J* = 7.3 Hz, 3 H); ESIMS *m/z* (rel intensity) 332 (MH^+, 100); HRESIMS calcd for $C_{16}H_{22}N_5OS$ *m/z* 332.1112 (MH^+), found 332.1210; HPLC purity 96.57% (1% TFA in MeOH:H_2O – 90:10).

(*R*)-2-(4-Butylphenyl)-*N*-(2,3-dihydroxypropyl)-4methylthiazole-5-carboxamide (12).

A mixture of the acid chloride derivative **8** (0.25 g, 0.9 mmol) and (R)-(-)-3-amino-1,2-propanediol (0.154 g, 1.7 mmol) in THF (15 mL) were stirred at room temperature for 48 hours. The THF was removed under reduced pressure and the resulting crude oil was extracted with chloroform (2 × 50 mL) and the extract was washed with water (2 × 50 mL) and brine (50 mL). The combined extracts were dried over Na_2SO_4 (10 g), filtered and concentrated under vacuum. The residue was purified by flash column chromatography (SiO_2, $CHCl_3$-MeOH, 9.4:0.6) to afford the product **12** as dark red crystals (0.17 g, 57%): mp 93-95 °C. ^1H NMR (300 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.2 Hz, 2 H), 7.24 (d, *J* = 7.2 Hz, 4 H), 6.32 (s, 1 H), 3.89 (d, *J* = 5.0 Hz, 1 H), 3.65 (m, *J* = 5.2 Hz, 4 H), 2.73 (s, 3 H), 2.63 (t, *J* = 7.6 Hz, 2 H), 1.61 (t, *J* = 7.9 Hz, 2 H), 1.35 (q, *J* = 7.5 Hz, 2 H), 0.92 (t, *J* = 6.1 Hz, 3 H); ESIMS *m/z* (rel intensity) 349 (MH^+, 100).

2-(4-Butylphenyl)-*N*-[(dimethylamino)methyl]-4-methylthiazole-5-carboxamide (13).

A mixture of acid chloride derivative **8** (0.4 g, 1.44 mmol) and *N,N*-dimethylethylenediamine (0.63 mL, 5.7 mmol) in THF (15 mL) were stirred at room temperature for 48 hours. The THF was removed under reduced pressure and the resulting residue was purified by flash chromatography (SiO_2, $CHCl_3$-MeOH, 9.3:0.7) to afford the product **13** as a pink solid (0.123 g, 24%): mp 79-81 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83 (d, J = 8.2 Hz, 2 H), 7.25 (d, J = 9.7 Hz, 4 H), 3.49 (t, J = 5.8 Hz, 2 H), 2.72 (s, 3 H), 2.63 (t, J = 7.6 Hz, 2 H), 2.55 (t, J = 5.9 Hz, 2 H), 2.3 (s, 6 H), 1.61 (t, J = 7.8, 2 H), 1.36 (t, J = 7.4, 2 H), 0.92 (t, J = 7.3 Hz, 3 H); ESIMS *m/z* (rel intensity) 346 (MH^+, 100).

2-(4-Butylphenyl)-4-methylthiazole-5-carbonitrile (14).

Amide **9** (0.400 g, 1.45 mmol) was dissolved in thionyl chloride (20 mL) and the solution was heated to reflux for seven hours. Thionyl chloride was removed under reduced pressure, EtOAc (30 mL) was added and the mixture was washed with saturated aqueous $NaHCO_3$ (2 × 15 mL) and water (2 × 15 mL). The organic layer was dried over Na_2SO_4, concentrated and purified by silica gel flash column chromatography using hexanes–ethyl acetate (9:1) to provide the desired compound as yellow syrup (0.300 g, 81%): IR (KBr) 2246, 1456, 1122, 841, 665 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.83 (d, J = 8.1 Hz, 2 H), 7.27 (d, J = 8.1 Hz, 2 H), 2.67 (s, 3 H), 2.64 (m, 2 H), 1.63 (m, 2 H), 1.38 (m, 2 H), 0.94 (t, J = 7.3 Hz, 3 H); ESIMS *m/z* (rel intensity) 256 (M^+, 33), 213 (M^+-C_3H_7, 100).

(*Z*)-2-(4-Butylphenyl)-*N*'-hydroxy-4-methylthiazole-5-carboximidamide (15).

A mixture of the thiazole derivative **14** (0.19 g, 0.74 mmol), hydroxylamine hydrochloride (0.07 g, 1 mmol) and K_2CO_3 (0.102 g, 0.74 mmol) in absolute EtOH (15 mL) was stirred at room temperature for one hour and then heated at reflux overnight. The EtOH was removed under reduced pressure and the resulting crude residue was purified by flash column chromatography (SiO_2, hexanes-EtOAc, 9:1) to afford the product **15** as an off white to light yellow solid (35.5 mg, 17%): mp 135-137 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.81 (d, J = 8.05 Hz, 2 H), 7.25 (t, J = 5.7 Hz, 2 H), 2.63 (t, J = 7.6 Hz, 5 H), 1.59 (q, J = 7.4 Hz, 2 H), 1.34 (p, J = 11.2 Hz, 2 H), 0.92 (t, J = 7.28 Hz, 3 H); ESIMS *m/z* (rel intensity) 290 (M^+, 100).

2-(4-Butylphenyl)-4-methyl-5-(1*H*-tetrazol-5-yl)thiazole (16).

I_2 (20 mg) was added to a mixture of nitrile (**14**, 0.2 g, 0.781 mmol) and NaN_3 (0.076 g, 1.17 mmol) and the mixture was stirred at 120 °C for 15 hours. After completion of the reaction, EtOAc (15 mL) and 4 M HCl (10 mL) were added and the mixture was stirred vigorously for 10 minutes. The organic layer was separated and the aqueous layer was extracted with EtOAc (2 × 10 mL). The combined organic layer was washed with brine (4 × 15 mL), dried over Na_2SO_4, concentrated and purified by silica gel flash column chromatography using hexane-ethyl acetate (5:5) to provide the desired compound as a light yellow solid (0.085 g, 37%): mp 170-171 °C. IR (KBr) 1825, 1415, 1098, 844, 664 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.84 (d, *J* = 8.1 Hz, 2 H), 7.22 (d, *J* = 8.1 Hz, 2 H), 2.85 (s, 3 H), 2.64 (t, *J* = 7.5 Hz, 2 H), 1.63 (m, 2 H), 1.35 (m, 2 H), 0.93 (t, *J* = 7.3 Hz, 3 H); ESIMS *m/z* (rel intensity) 300 (MH^+, 100); HRESIMS calcd for $C_{15}H_{18}N_5S$ *m/z* 300.1267 (MH^+), found 300.1270; HPLC purity 98.25% (1% TFA in MeOH:H_2O – 90:10).

1-(4-Methyl-2-(4-aminophenyl)thiazol-5-yl)ethanone (18).

4-Aminothiobenzamide (0.6 g, 3.80 mmol) and α-chloropentanedione (0.611 mg, 4.56 mmol) were added to absolute ethanol (50 mL). The reaction mixture was heated at reflux for 24 hours. After removal of solvent under reduced pressure, the residue was purified by silica gel chromatography using hexanes–ethyl acetate (6:4) to provide the desired compound as light brown solid (0.920 g, 97%): mp 204-205 °C. IR (KBr) 3334, 1745, 1637, 1145, 865, 666 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 7.89 (d, *J* = 8.5 Hz, 2 H), 7.07 (d, *J* = 8.5 Hz, 2 H), 2.66 (s, 3 H), 2.53 (s, 3 H).

(E)-2-(1-(4-Methyl-2-(4-aminophenyl)thiazol-5-yl)ethylidene) hydrazinecarboximidamide (19).

The thiazole derivative **18** (0.250 g, 0.954 mmol) was dissolved in absolute ethanol (50 mL), and aminoguanidine hydrochloride (0.125 g, 1.14 mmol) and a catalytic amount of LiCl (15 mg) were added. The reaction mixture was heated at reflux for 24 hours. The solvent was evaporated under reduced pressure. The crude product was purified by crystallization from 70% methanol and then recrystallized from methanol to afford the desired compound as a yellow solid (0.175 g, 58%): mp > 280 °C. IR (KBr) 3402, 1705, 1665, 1156, 826, 665 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 11.44 (br s, 1 H), 7.81 (d, *J* = 8.4 Hz, 2 H), 7.75 (br s, 3 H), 7.00 (d, *J* = 8.4 Hz, 2 H), 2.58 (s, 3 H), 2.41 (s, 3 H);

ESIMS *m/z* (rel intensity) 289 (MH^+, 100); HRESIMS calcd for $C_{13}H_{17}N_6S$ *m/z* 289.1123 (MH^+), found 289.1120; HPLC purity 96.58% (1% TFA in MeOH:H_2O – 90:10).

1-(2-(4'-Hydroxy-[1,1'-biphenyl]-4-yl)-4-methylthiazol-5-yl)ethanone (21a). Iodide **3** (0.172 g, 0.5 mmol), the 4-hydroxyphenyl boronic acid (**20a**, 0.205 g, 1.5 mmol), tripotassium monophosphate (0.424 g, 2 mmol) and (2-biphenyl)dicylohexylphosphine (18 mg) were dissolved in dry toluene (15 mL) and the solution was purged with argon for 20 minutes. $Pd(OAc)_2$ (25 mg, 0.24 mmol) was added to the reaction mixture and the reaction mixture was heated at 90 ºC under argon for 24 hours. Ethyl acetate (40 mL) was added to reaction mixture, which was then washed with water (2 × 25 mL). The combined organic layer was dried over Na_2SO_4, concentrated and purified by flash column chromatography (EtOAc:hexanes 3:7 to 0.9:9.1) to afford the desired compound as yellow solid (0.150 g, 93%): mp 212-214 ºC. IR (KBr) 3356, 1745, 1123, 856, 665 cm^{-1}; ^1H NMR (300 MHz , $CDCl_3$) δ 7.86 (d, *J* = 8.3 Hz, 2 H), 7.51 (d, *J* = 8.3 Hz, 2 H), 7.38 (d, *J* = 8.5 Hz, 2 H), 6.79 (d, *J* = 8.6 Hz, 2 H), 2.64 (s, 3 H), 2.45 (s, 3 H); ESIMS *m/z* (rel intensity) 309 (M^+, 100).

1-(2-(4'-Fluoro-[1,1'-biphenyl]-4-yl)-4-methylthiazol-5-yl)ethanone (21b). Iodide **3** (0.172 g, 0.5 mmol), 4-fluorophenyl boronic acid (**20b**, 0.209 1.5 mmol), tripotassium monophosphate (0.424 g, 2 mmol) and (2-biphenyl)dicylohexylphosphine (18 mg) were dissolved in dry toluene (15 mL) and the solution was purged with argon for 20 minutes. $Pd(OAc)_2$ (25 mg, 0.24 mmol) was added to the reaction mixture and the reaction mixture was heated at 90 ºC under argon for 24 hours. Ethyl acetate (40 mL) was added to reaction mixture, which was then washed with water (2 × 25 mL). The combined organic layer was dried over Na_2SO_4, concentrated and purified by flash column chromatography (EtOAc:hexanes 3:7 to 0.9:9.1) to afford the desired compound as an off-white solid (0.140 g, 90%): mp 127-128 ºC. IR (KBr) 2956, 1689, 1123, 819, 659 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.05 (d, *J* = 7.6 Hz, 2 H), 7.64 (m, 4 H), 7.23 (d, *J* = 7.5 Hz, 2 H), 2.79 (s, 3 H), 2.58 (s, 3 H); ESIMS *m/z* (rel intensity) 312 (M^+, 100); HPLC purity 98.50% (1% TFA in MeOH:H_2O – 90:10).

1-(4-Methyl-2-(4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)thiazol-5-yl)ethanone (21c).

Iodide **3** (0.172 g, 0.5 mmol), 4-trifluromethylphenyl boronic acid (**20c**, 0.228 g, 1.5 mmol), tripotassium monophosphate (0.424 g, 2 mmol) and (2-biphenyl)dicylohexylphosphine (18 mg) were dissolved in dry toluene (15 mL) and the solution was purged with argon for 20 minutes. $Pd(OAc)_2$ (25 mg, 0.24 mmol) was added to the reaction mixture and the reaction mixture was heated at 90 ºC under argon for 24 hours. Ethyl acetate (40 mL) was added to reaction mixture, which was then washed with water (2 × 25 mL). The combined organic layer was dried over Na_2SO_4, concentrated and purified by flash column chromatography (EtOAc:hexanes 3:7 to 0.9:9.1) to afford the desired compound as an off-white solid (0.150 g, 80%): mp 116-117 ºC. IR (KBr) 2959, 1938, 1650, 1111, 819, 659 cm^{-1}; ^1H NMR (300 MHz , $CDCl_3$) δ 8.09 (d, J = 8.3 Hz, 2 H), 7.72 (m, 6 H), 2.80 (s, 3 H), 2.58 (s, 3 H); ESIMS *m/z* (rel intensity) 361 (M^+, 100); HPLC purity 98.75% (1% TFA in MeOH:H_2O – 90:10).

(E)-2-(1-(2-(4'-Hydroxy-[1,1'-biphenyl]-4-yl)-4-methylthiazol-5-yl)ethylidene)hydrazinecarboximidamide (22a).

Compound **21a** (0.150 g, 0.485 mmol) was dissolved in absolute ethanol (50 mL) and aminoguanidine hydrochloride (0.064 g, 0.588 mmol) and a catalytic amount of LiCl (10 mg) were added. The reaction mixture was heated at reflux for 24 hours. The solvent was evaporated under reduced pressure. The crude product was purified by crystallization from 70% methanol, and then recrystallized from methanol to afford the desired compound as a light yellow solid (0.115 g, 66%): mp 252-254 ºC. IR (KBr) 3398, 1695, 1655, 1142, 855, 665 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 9.73 (s, 1 H), 8.02 (d, J = 8.3 Hz, 2 H), 7.74 (d, J = 8.4 Hz, 2 H), 7.59 (d, J = 8.6 Hz, 2 H), 6.88 (d, J = 8.6 Hz, 2 H), 2.70 (s, 3 H), 2.56 (s, 3 H); ESIMS *m/z* (rel intensity) 366 (MH^+, 100); HRESIMS calcd for $C_{19}H_{20}N_5OS$ 366.1045 (MH^+), found 366.1048; HPLC purity 96.11% (1% TFA in MeOH:H_2O – 90:10).

(*E*)-2-(1-(2-(4'-Fluoro-[1,1'-biphenyl]-4-yl)-4-methylthiazol-5-yl)ethylidene)hydrazinecarboximidamide (22b).

Compound **21b** (0.1 g, 0.321 mmol) was dissolved in absolute ethanol (50 mL) and aminoguanidine hydrochloride (0.064 g, 0.588 mmol) and a catalytic amount of LiCl (10 mg) were added. The reaction mixture was heated at reflux for 24 hours.

The solvent was evaporated under reduced pressure. The crude product was purified by crystallization from 70% methanol, and then recrystallized from methanol to afford the desired compound as a light yellow solid (0.077 g, 65%): mp 273-274 ºC. IR (KBr) 3308, 1687, 1645, 1146, 823, 666 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 11.73 (br s, 1 H), 8.00 (d, J = 8.3 Hz, 2 H) 7.81 (m, 8 H), 7.32 (m, 2 H), 2.62 (s, 3 H), 2.44 (s, 3 H); ESIMS *m/z* (rel intensity) 368 (MH^+, 100); HRESIMS calcd for $C_{19}H_{19}FN_5S$ *m/z* 368.1245 (MH^+), found 368.1251; HPLC purity 95.78% (1% TFA in MeOH:H_2O – 90:10).

(*E*)-2-(1-(4-Methyl-2-(4'-(trifluoromethyl)-[1,1'-biphenyl]-4-yl)thiazol-5-yl)ethylidene)hydrazinecarboximidamide (22c).

Compound **21c** (0.1 g, 0.277 mmol) was dissolved in absolute ethanol (50 mL) and aminoguanidine hydrochloride (0.064 g, 0.588 mmol) and a catalytic amount of LiCl (10 mg) were added. The reaction mixture was heated at reflux for 24 hours. The solvent was evaporated under reduced pressure. The crude product was purified by crystallization from 70% methanol, and then recrystallized from methanol to afford the desired compounds as yellow solid (0.088 g, 76%): mp 269-270 ºC. IR (KBr) 3583, 3307, 1678, 1145, 821, 665 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 11.78 (br s, 1 H), 8.05 (d, J = 8.4 Hz, 2 H), 7.98 (d, J = 8.2 Hz, 2 H), 7.90 (m, 8 H), 2.62 (s, 3 H), 2.45 (s, 3 H); ESIMS *m/z* (rel intensity) 418 (MH^+, 100); HRESIMS calcd for $C_{20}H_{19}F_3N_5S$ *m/z* 418.1423 (MH^+), found 418.1420; HPLC purity 96.10% (1% TFA in MeOH:H_2O – 90:10).

(*E*)-2-(1-(2-(4'-Acetyl-[1,1'-biphenyl]-4-yl)-4-methylthiazol-5-yl)ethylidene)hydrazinecarboximidamide (22d).

Compound **11d** (0.150 g, 0.472 mmol) was dissolved in absolute ethanol (50 mL) and aminoguanidine hydrochloride (0.064 g, 0.588 mmol) and a catalytic amount of LiCl (10 mg) were added. The reaction mixture was heated at reflux for 24 hours. The solvent was evaporated under reduced pressure. The crude product was purified by crystallization from 70% methanol, and then recrystallized from methanol to afford the desired compound as a light yellow solid (0.062, 35%): mp >280 ºC. IR (KBr) 3402, 1715, 1655, 1446 1125, 856, 665 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 11.68 (br s, 1 H), 8.10 (d, J = 8.2 Hz, 4 H), 7.91 (m, 6 H), 7.80 (d, J = 8.2 Hz, 2 H), 2.72 (s, 3 H), 2.58 (s, 3 H), 2.40 (s, 3 H); ESIMS *m/z* (rel intensity) 392 (MH^+, 100); HRESIMS calcd for $C_{21}H_{22}N_5OS$ *m/z* 392.1165 (MH^+), found 392.1169.

1-(4-Methyl-2-(4-(naphthalen-1-yl)phenyl)thiazol-5-yl)ethanone (24)

Iodide **3** (0.172 g, 0.5 mmol), 1-naphalene boronic acid (**23**, 258 g, 1.5 mmol), tripotassium monophosphate (0.424 g, 2 mmol) and (2-biphenyl)dicylohexylphosphine (18 mg) were dissolved in dry toluene (15 mL) and the solution was purged with argon for 20 minutes. $Pd(OAc)_2$ (25 mg, 0.24 mmol) was added to the reaction mixture and the reaction mixture was heated at 90 ºC under argon for 24 hours. Ethyl acetate (40 mL) was added to reaction mixture, which was then washed with water (2 × 25 mL). The combined organic layer was dried over Na_2SO_4, concentrated and purified by flash column chromatography (EtOAc: hexane 3:7 to 0.9:9.1) to afford the desired compound as a light brown solid (0.170 g, 99%): mp 141-142 ºC. IR (KBr) 2287, 1670, 1006, 801, 663 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 8.11 (d, *J* = 6.5 Hz, 2 H), 7.90 (m, 3 H), 7.61 (m, 7 H), 2.82 (s, 3 H), 2.60 (s, 3 H); ESIMS *m/z* (rel intensity) 344 (MH^+, 100); HRESIMS calcd for $C_{22}H_{18}NOS$ *m/z* 344.1123 (MH^+), found 344.1125.

(*E*)-2-(1-(4-Methyl-2-(4-(naphthalen-1-yl)phenyl)thiazol-5-yl)ethylidene)hydrazinecarboximidamide (25).

Compound **24** (0.1 g, 0.291 mmol) was dissolved in absolute ethanol (50 mL) and aminoguanidine hydrochloride (0.064 g, 0.588 mmol) and a catalytic amount of LiCl (10 mg) were added. The reaction mixture was heated at reflux for 24 hours. The solvent was evaporated under reduced pressure. The crude product was purified by crystallization from 70% methanol, and then recrystallized from methanol to afford the desired compound as a yellow solid (0.057, 49 %): mp 241-242 ºC. IR (KBr) 3299, 2300, 1675, 1618, 1142, 800, 664 cm^{-1}; ^1H NMR (300 MHz, DMSO-d_6) δ 11.63 (br s, 1 H), 8.08 (m, 5 H), 7.65 (d, *J* = 5.1 Hz, 1 H); 7.61 (m, 9 H), 2.64 (s, 3 H), 2.44 (s, 3 H); ESIMS *m/z* (rel intensity) 400 (MH^+, 100); HRESIMS calcd for $C_{23}H_{22}N_5S$ *m/z* 400.1323 (MH^+), found 400.1327; HPLC purity 95.55% (1% TFA in MeOH:H_2O – 85:15).

4.2.2 Bacterial strains and reagents

Clinical isolates of MRSA, VISA, and VRSA were obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program. In addition, MRSA ATCC 43300 was obtained from the American Type Cultural Collection (Manassas, VA, USA). Vancomycin hydrochloride powder was purchased commercially (Gold

Biotechnology Inc., St. Louis, MO, USA) and dissolved in DMSO to prepare a 10 mM stock solution.

4.2.3 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against MRSA, VISA, and VRSA strains

The MICs of the thiazole compounds and vancomycin against seven clinical isolates of MRSA, three clinical isolates of VISA, and three clinical isolates of VRSA were determined using the broth microdilution method in accordance with the recommendations contained in the CLSI guidelines (35). Bacteria were prepared in phosphate-buffered saline (PBS) to achieve a McFarland standard of 0.5. The solution was subsequently diluted 1:300 in Mueller-Hinton broth (MHB) to reach a starting inoculum of 1×10^5 colony-forming units (CFU/mL). Bacteria were then transferred to a 96-well microtiter plate. Thiazole compounds and vancomycin were added (in triplicate) to wells in the first row of the microtiter plate and then serially diluted along the vertical axis. The plate was incubated at 37 °C for 18-20 hours before the MIC was determined as the lowest concentration where visible growth of bacteria was not observed.

The MBC was determined by plating 5 μL from wells on the 96-well microtiter plate (where the MIC was determined), where no growth was observed, onto tryptic soy agar (TSA) plates. The TSA plates were then incubated at 37 °C for 18-20 hours before the MBC was determined. The MBC was classified as the concentration where ≥99% reduction in bacterial cell count was observed.

4.2.4 Time-kill analysis of thiazole compounds 1, 5, and 25 and vancomycin against MRSA

MRSA USA300 cells in late logarithmic growth phase were diluted to ~1×10^8 colony-forming units (CFU/mL) and exposed to concentrations equivalent to 3 × MIC (in triplicate) of thiazole compounds **1**, **5**, and **25** and vancomycin in MHB. 20 μL samples were collected after 0, 2, 4, 6, 8, 10, 12, and 24 hours of incubation at 37 °C and subsequently serially diluted in PBS. Bacteria were then transferred to TSA plates and incubated at 37 °C for 18-20 hours before viable CFU/mL was determined.

4.2.5 *In vitro* cytotoxicity analysis

Compounds **1**, **5**, **22b-d** and **25** were assayed at concentrations of 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL against a human embryonic kidney (HEK293) cell line to determine the potential toxic effect to mammalian cells *in vitro*. Cells were cultured in Dulbeco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (USA Scientific, Inc.) at 37 °C with 5% CO_2. Controls received DMSO alone at a concentration equal to that in drug-treated cell samples. The cells were incubated with the compounds in a 96-well plate at 37 ºC and 5% CO_2 for two hours prior to addition of the assay reagent MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (Promega, Madison, WI, USA). Absorbance readings (at OD_{490}) were taken using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with each compound was expressed as a percentage of the viability of DMSO-treated control cells.

4.2.6 Microsomal stability analysis

The metabolic stability analysis of analogue **5** was performed as described previously (32). Compound **5** was incubated in duplicate with human liver microsomes at 37 ºC. The reaction contained microsomal protein in 100 mM potassium phosphate, 2 mM NADPH, 3 mM $MgCl_2$, pH 7.4. A control was run for each test agent omitting NADPH to detect NADPH-free degradation. At 0, 10, 20, 40, and 60 minutes, an aliquot was removed from each experimental and control reaction and mixed with an equal volume of ice-cold Stop Solution (methanol containing haloperidol, diclofenac, or other internal standard). Stopped reactions were incubated at least ten minutes at -20 ºC, and an additional volume of water was added. The samples were centrifuged to remove precipitated protein, and the supernatants were analyzed by LC/MS/MS to quantitate the remaining parent. Data were converted to % remaining by dividing by the time zero concentration value. Data were fit to a first-order decay model to determine half-life. Intrinsic clearance was calculated from the half-life and the protein concentrations as follows:

$$CL_{int} = \ln(2) / (T_{1/2} [\text{microsomal protein}])$$

4.2.7 Statistical analysis

All statistical analysis was performed using the unpaired t-test ($P < 0.05$) utilizing GraphPad Prism 6 software. Data for both the time-kill assay and toxicity analysis of the tested compounds are presented as mean ± standard deviation (as depicted by the error bars).

4.3 Results and Discussion

4.3.1 Antibacterial activity of thiazole compounds and vancomycin against MRSA, VISA, and VRSA

To ascertain the structure-activity relationships of the lead thiazole compound more thoroughly, derivatives were initially constructed with modifications to the thiazole-C5 cationic moiety (keeping the lipophilic alkane side chain at thiazole-C2 intact). Substitution of the ethylidenehydrazine-1-carboximidamide of the lead **1** with moieties such as a tetrazole (**16**), an amide derivative (**9-13**), or a hydroxamidine (**15**) results in complete abolishment of antimicrobial activity against MRSA (minimum inhibitory concentration (MIC) > 35.1 μg/mL (Table 4.1)). This trend continues when the amino moiety is replaced with a ketone in derivatives **21b-c** and **24**. Interestingly, derivatives **22b-c** and **25** (consisting of the same cationic head group as the lead **1** but with substitutions to the linear alkane side chain at thiazole-C2 identical to those in compounds **21b-c** and **24**) retain antimicrobial activity; among the groups studied thus far, the ethylidenehydrazine-1-carboximidamide is the only one to retain potency at this position of the structural series and we therefore retained it in all future analogs.

Modifications made to the linear alkane side chain at thiazole-C2 revealed hydrophobic, nonpolar moieties at this position are preferred for the compound to retain potent antimicrobial activity. The presence of a hydrophilic, polar group, such as an amine (**19**) or alcohol (**22a**) at this position, results in complete loss of antimicrobial activity, with both compounds possessing a MIC > 36.9 μg/mL (Table 4.1). Replacement of the alkane side chain with hydrophobic, polar substituents such as an acetyl group (**22d**, MIC = 6.3 μg/mL), a fluoride (**22b**), or a trifluoromethyl group (**22c**) results in the compounds possessing antimicrobial activity, but with a MIC higher than the parent compound. On the other hand, substitution of the alkane side chain with a nonpolar, hydrophobic moiety, such

as an alkyne (**5** with MIC of 1.4 μg/mL) or naphthalene (**25** with MIC of 1.6 μg/mL) functional group, results in derivatives with potent antimicrobial activity (nearly identical MIC to the lead **1**). Once again this confirms that a more nonpolar, hydrophobic functional group is needed at the C5 position for the thiazole compounds to possess potent antibacterial activity. This is in agreement with previously reported findings where alkane, cycloalkane, cycloalkene, and arene substitutions at thiazole-C2 resulted in compounds with stronger activity against MRSA (32). Interestingly, extending the alkyne length from a hexyne (**5**) to a nonyne (**7**) group results in diminished anti-MRSA activity with the MIC increasing nine-fold from 1.4 μg/mL to 12.6 μg/mL. This is similar to what was previously found with lengthening of the alkane side chain at thiazole-C2; increasing the alkane side chain beyond four methylene units resulted in a drastic reduction in antimicrobial activity of the compounds. Future studies examining decreasing the alkyne side chain length at thiazole-C5 and its effect on anti-MRSA activity warrant further exploration. Additionally, repositioning the nonpolar moiety (at the ortho and meta positions of the phenyl substituent connected to C2 on the thiazole ring) would be of interest to assess if the para position plays a crucial role in the antimicrobial activity of the compounds.

After confirming that five derivatives (**5**, **22b-d** and **25**) possessed strong antimicrobial activity against a single strain of MRSA, we next assessed their activity against an array of clinically relevant multidrug-resistant HA-MRSA and CA-MRSA strains as well as vancomycin-intermediate (VISA) and vancomycin-resistant (VRSA) *S. aureus* isolates. All five compounds maintained their activity (with MICs identical or two-fold higher than those reported against MRSA ATCC43300) against MRSA isolates exhibiting resistance to mupirocin (NRS107), linezolid (NRS119), erythromycin (USA300), tetracycline (USA300), ciprofloxacin (USA500), clindamycin (USA500), and gentamicin (USA500) (Table 4.2); this indicates cross-resistance between these antibiotics and the thiazole compounds is unlikely to occur. The thiazole derivatives also exhibited potent activity against strains of MRSA (USA300 and USA400) responsible for the majority of MRSA-related skin and soft tissue infections in North America (3, 36). Additionally, analogues **5** (MIC between 1.3-2.6 μg/mL), **22b** (MIC between 2.9-5.9 μg/mL), and **25** (MIC of 1.6 μg/mL) proved to be similar in activity or better than vancomycin (MIC of 3.0 μg/mL) against two VISA isolates tested. Furthermore, while all

three VRSA strains exhibited resistance to vancomycin (MIC > 190.2 μg/mL), the lead thiazole (**1**) and the five most potent derivatives retained their antimicrobial activity with MIC values ranging from 0.7 μg/mL (for **1**) to 6.7 μg/mL (for **22c**). Finding alternative therapeutic options (such as these thiazole compounds) to vancomycin and linezolid, agents of last resort for treatment of severe MRSA infections, is critical to address the burden of these challenging infections.

Subsequent to establishing that the lead compound and the five most active analogues exhibited potent antimicrobial activity against a diverse spectrum of CA-MRSA, HA-MRSA, VISA, and VRSA isolates, we next turned our attention to assessing whether these compounds were bacteriostatic or bactericidal. Antimicrobial agents that are bactericidal, as opposed to their bacteriostatic counterparts, are thought to help patients recover more rapidly from infections, resulting in a better clinical outcome (37). To assess if the thiazole compounds were bacteriostatic or bactericidal, the minimum bactericidal concentration (MBC) was determined. As Table 4.2 presents, all six thiazole compounds tested exhibited MBC values that were identical to or two-fold higher than their MIC values. The results mimic those of vancomycin, a known bactericidal antibiotic, indicating the thiazole compounds are bactericidal.

4.3.2 Time-kill analysis of most potent thiazole analogues against MRSA USA300

To confirm the thiazole compounds are in fact bactericidal agents against MRSA, a time-kill analysis was performed. MRSA USA300 cells in late logarithmic growth were treated with 3 × MIC of the lead thiazole (**1**), the two most potent derivatives (**5** and **25**), or vancomycin. Interestingly, a simple substitution at thiazole-C2 from an alkane/alkyne (**1**/**5**) to the more conformationally-restricted naphthalene analogue (**25**), results in a dramatic shift in the rate of bacterial killing by the thiazole compounds. As Figure 4.6 demonstrates, compounds **1** and **5** completely eradicate MRSA growth within four hours while compound **25** requires 10 hours to achieve the same effect. Though all three compounds possess nearly identical MIC values, the structural modifications made at thiazole-C2 significantly affect the rate of bacterial killing observed for each compound against MRSA.

While all three thiazole compounds exhibit the ability to eliminate MRSA growth completely within 10 hours, vancomycin requires 24 hours to achieve the same result. This

is similar to what has been reported elsewhere regarding vancomycin's slow bactericidal activity (38). Rapid bactericidal activity is considered to be a critical factor in slowing the emergence of bacterial resistance to an antimicrobial agent and is important clinically in preventing an infection from spreading (37). Additionally, bactericidal agents have been shown both clinically and through *in vivo* studies to be superior to bacteriostatic agents for the treatment of certain invasive diseases such as endocarditis (39). Thus these thiazole compounds may have the potential to be utilized in a wide array of clinically important MRSA diseases from skin and soft tissue infections to systemic infections such as endocarditis.

4.3.3 Toxicity analysis of potent thiazole derivatives against mammalian cells

Selective toxicity is an important property that both approved antibiotics and novel antimicrobial compounds must possess. The ability for antimicrobial agents to exhibit their activity on the target microorganism while not causing harm to host (mammalian) tissues is important to ascertain early in the drug discovery process. Previously, the lead thiazole (**1**) was found to be nontoxic to human cervical (HeLa) cells at a concentration of 11 μg/mL (32). A principal objective of the present study was to develop new analogues of the lead that exhibited an improved/more selective toxicity profile. To assess this, the lead compound and five most potent derivatives against MRSA (**5**, **22b-d** and **25**) were screened against a human embryonic kidney (HEK293) cell line using the MTS assay. Figure 4.7 presents the results garnered. At a concentration of 40 μg/mL, the lead **1** and compounds **22b** and **22c** proved to be toxic to mammalian cells. However, three of the novel analogues – compounds **5** (alkynyl side chain), **22d** (*p*-acetylbenzyl), and **25** [*p*-(1-naphthyl)] – exhibit an improved toxicity profile compared to the lead **1** at the tested concentration. This concentration (40 μg/mL) represents a 25- (for **25**) to 28-fold (for **5**) difference between the MIC values determined against MRSA for these compounds. Thus there is a significant improvement in the toxicity profile of these novel analogues when compared to the lead compound.

4.3.4 Metabolic stability analysis of compound 5

Previously, microsomal stability analysis of the lead **1** revealed this compound was metabolized fairly rapidly (intrinsic clearance rate of 80.3 μL/min-mg and half-life of 28.8 minutes) via a NADPH-mediated process (such as via the cytochrome P450 system) (32). As compound **5** demonstrated nearly identical antimicrobial activity to the lead compound, we were curious to assess if the substitution of an alkane side chain with an alkyne at thiazole-C2 would enhance the metabolic stability of the compound, preventing its conversion to potentially inactive metabolites. Using pooled human liver microsomes, **5**, similar to the parent compound, was found to be metabolized via a NADPH-mediated process (intrinsic clearance rate of 3.7 μL/min-mg as compared to 0.0 μL/min-mg in the absence of the cofactor, NADPH) (Table 4.3). Interestingly, the slower clearance rate correlates with an improved half-life for compound **5** (as compared to the lead compound) that exceeds four hours. This marked improvement in the metabolic stability of the thiazole compound is important as it has the potential to positively impact the pharmacokinetic profile of this compound, reduce the frequency of doses needed to be administered for treatment (fewer doses leads to improved patient compliance), while also ensuring the active drug circulates within the patient's system to assist with treating and clearing an infection. Additionally, compounds that are metabolically stable are less susceptible to experiencing issues pertaining to toxicity and drug-drug interactions caused by metabolites (40). The metabolic stability analysis combined with the enhanced toxicity profile of compound **5** (as compared to the lead) warrants further analysis of this compound as a potential novel antibiotic for the treatment of MRSA infections.

4.4 Conclusion

We present herein a novel series of 2,5-disubstituted thiazole compounds exhibiting potent activity against clinically relevant isolates of MRSA, VISA, and VRSA. A rigorous analysis of the structure-activity relationship of these analogues reveals the ethylidenehydrazine-1-carboximidamide head group (at thiazole-C5) and a nonpolar, hydrophobic moiety (at thiazole-C2) are critical for the thiazole compound's antibacterial action. Three derivatives with substitutions at thiazole-C2 (an alkyne, *p*-acetylbenzene, and

p-naphthalene) demonstrate an improved toxicity profile against mammalian cells compared to the lead compound. Furthermore, the alkyne substitution results in a compound that is more stable to metabolism as assessed via human liver microsomes. Collectively, the results present critical information necessary for further analysis and development of these thiazole compounds as novel antimicrobial agents for use in treatment of infections caused by multidrug-resistant *S. aureus*.

4.5 References

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Table 4.1 Minimum inhibitory concentration (MIC) of thiazole compounds against methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300.

Analogue	MIC (μg/mL)
1 (lead)	1.3
5	1.4
7	12.6
9	>35.1
10	>45.8
11	>42.4
12	44.5
13	44.2
15	>37.0
16	>38.3
19	>36.9
21b	>39.8
21c	>46.2
22a	>46.7
22b	5.9
22c	3.3
22d	6.3
24	>43.9
25	1.6

Table 4.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of thiazole compounds **1**, **5**, **22b**-**22d**, **25** and vancomycin against seven methicillin-resistant (MRSA), three vancomycin-intermediate (VISA), and three vancomycin-resistant *Staphylococcus aureus* (VRSA) strains.

	1		**5**		**22b**		**22c**		**22d**		**25**		**Vancomycin**	
***S. aureus* strain**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
NRS107 (MRSA)	2.6	2.6	1.4	1.4	2.9	5.9	3.3	6.7	6.3	12.5	1.6	1.6	<1.5	<1.5
NRS119 (MRSA)	2.6	2.6	1.4	1.4	5.9	11.7	6.7	6.7	6.3	12.5	1.6	1.6	<1.5	<1.5
NRS194 (MRSA)	1.3	1.3	1.4	1.4	5.9	11.7	6.7	13.3	6.3	6.3	1.6	1.6	0.7	0.7
USA300 (MRSA)	1.3	1.3	1.4	1.4	5.9	5.9	6.7	6.7	6.3	6.3	1.6	1.6	0.7	0.7
USA 400 (MRSA)	1.3	1.3	1.4	1.4	5.9	11.7	6.7	13.3	6.3	6.3	1.6	1.6	0.7	0.7
USA500 (MRSA)	1.3	1.3	1.4	1.4	5.9	5.9	3.3	3.3	6.3	6.3	1.6	3.2	0.7	0.7
ATCC 43300 (MRSA)	1.3	1.3	1.4	1.4	5.9	5.9	3.3	3.3	6.3	6.3	1.6	1.6	0.7	0.7
NRS1 (VISA)	1.3	2.6	0.7	0.7	2.9	2.9	3.3	3.3	6.3	6.3	1.6	1.6	3.0	3.0
NRS19 (VISA)	1.3	2.6	1.4	1.4	5.9	5.9	3.3	3.3	12.5	12.5	1.6	1.6	<1.5	<1.5
NRS37 (VISA)	2.6	2.6	1.4	1.4	2.9	5.9	3.3	3.3	3.1	6.3	1.6	1.6	3.0	3.0
VRS1 (VRSA)	0.7	0.7	1.4	1.4	2.9	2.9	6.7	6.7	3.1	3.1	3.2	3.2	>190.2	>190.2
VRS4 (VRSA)	0.7	1.3	1.4	2.8	2.9	2.9	6.7	6.7	1.6	3.1	3.2	3.2	>190.2	>190.2
VRS5 (VRSA)	0.7	1.3	2.8	2.8	2.9	2.9	6.7	6.7	6.3	6.3	3.2	3.2	>190.2	>190.2

Table 4.3 Evaluation of metabolic stability of thiazole compound **5**, Verapamil, and Warfarin in human liver microsomes.

Compound/Drug Tested	**NADPH-dependent CL_{int}[1] (µL/min/mg)**	**NADPH-dependent $T_{1/2}$[2] (min)**	**NADPH-free CL_{int} (µL/min/mg)**	**NADPH-free $T_{1/2}$ (min)**
5	3.7	>240	0.0	>240
Verapamil	213	10.8	0.0	>240
Warfarin	0.0	>240	0.0	>240

[1] CL_{int}= microsomal intrinsic clearance
[2] $T_{1/2}$ = half-life

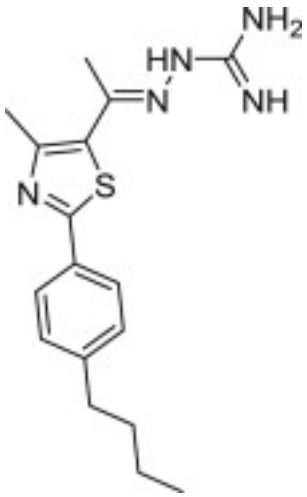

Figure 4.1 Chemical structure of the lead compound **1**.

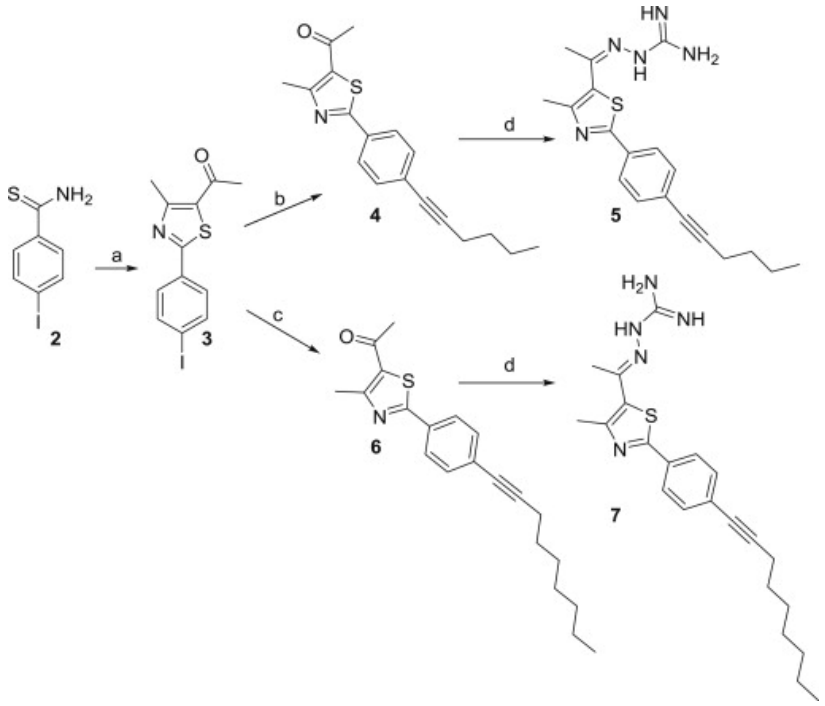

Figure 4.2 Synthetic scheme for compounds **2**-**7**.

Reagents and conditions: (a) 3-chloro-2,4-pentanedione, EtOH, reflux, 24 hours; (b) 1-hexyne, $PdCl_2(PPh_3)_2$, CuI, Cs_2CO_3, DMF, sealed tube, 65 °C, 15 hours; (c) 1-nonyne, $PdCl_2(PPh_3)_2$, CuI, Cs_2CO_3, DMF, sealed tube, 70 °C, 15 hours; (d) aminoguanidine HCl, LiCl, EtOH, reflux, 24 hours.

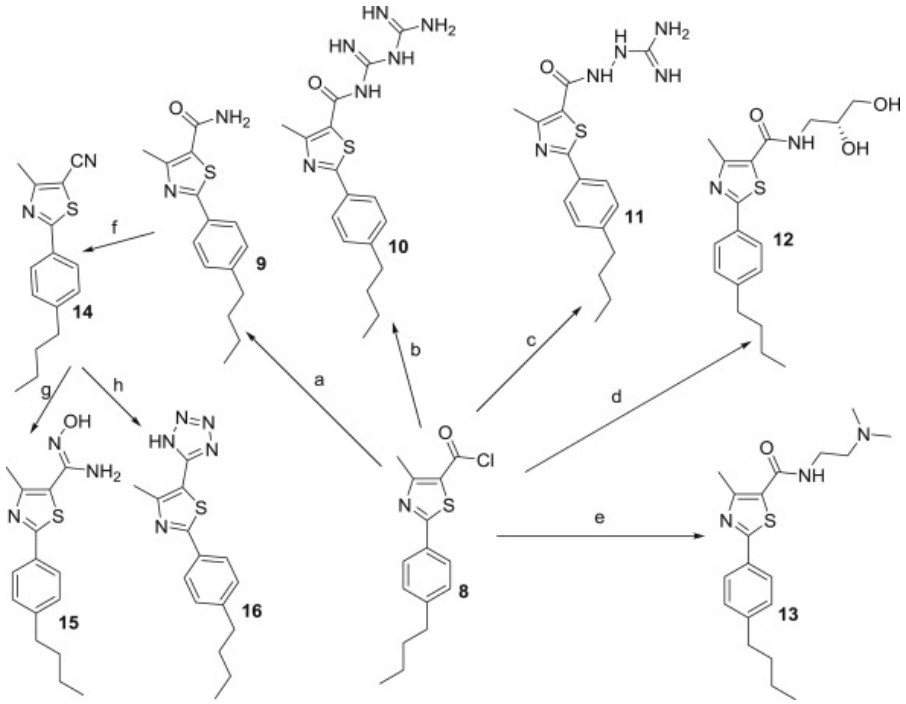

Figure 4.3 Synthetic scheme for compounds **8** – **16**.

Reagents and conditions: (a) 30% aq NH_4OH, THF, rt, 24 hours; (b) biguanidine hydrochloride, Et_3N, THF, 24 hours; (c) aminoguanidine hydrochloride, Et_3N, THF, 24 hours; (d) (R)-(−)-3-amino-1,2-propanediol, THF, rt, 24 hours; (e) N,N-dimethylethylenediamine, THF, rt, 48 hours; (f) thionyl chloride, reflux, 7 hours; (g) NH_2OH HCl, K_2CO_3, EtOH, 78 °C, 24 hours; (h) NaN_3, I_2, DMF, 120 °C, 15 hours.

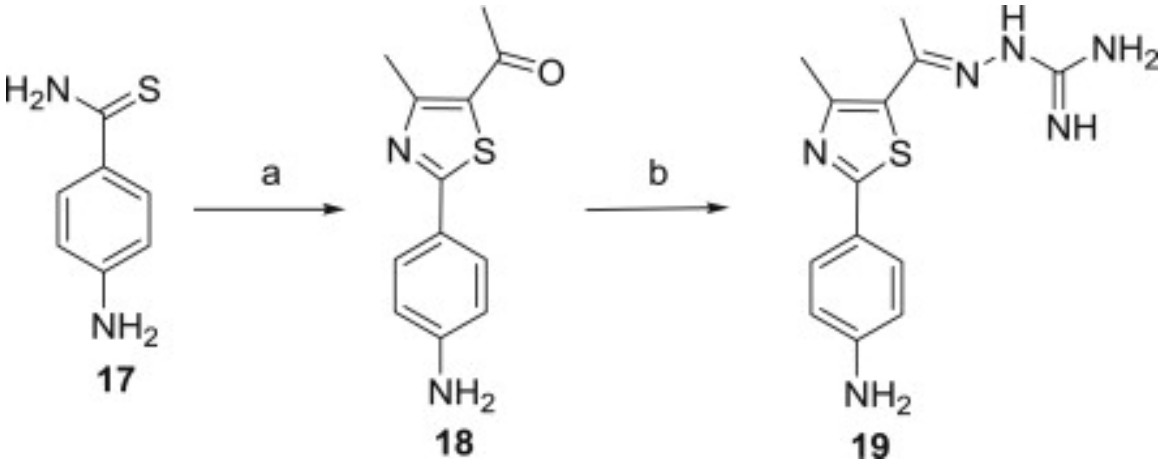

Figure 4.4 Synthetic scheme for compounds **17**-**19**.

Reagents and conditions: (a) 3-chloro-2,4-pentanedione, EtOH, reflux, 20 hours; (b) aminogaunidine hydrochloride, LiCl, EtOH, reflux, 24 hours.

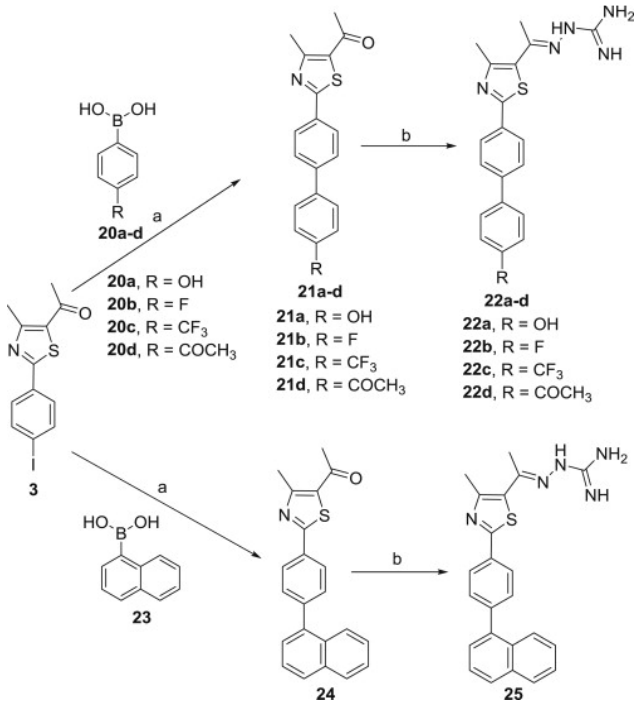

Figure 4.5 Synthetic schemes for compounds **20a-d**, **21a-d**, **22a-d**, **23**, **24**, and **25**.

Reagents and conditions: a) Pd(OAc)2, (2-biphenyl)dicyclohexylphosphine, K_3PO_4, toluene, 90 °C, 24 hours; b) aminoguanidine hydrochloride, LiCl, EtOH, reflux, 24 hours.

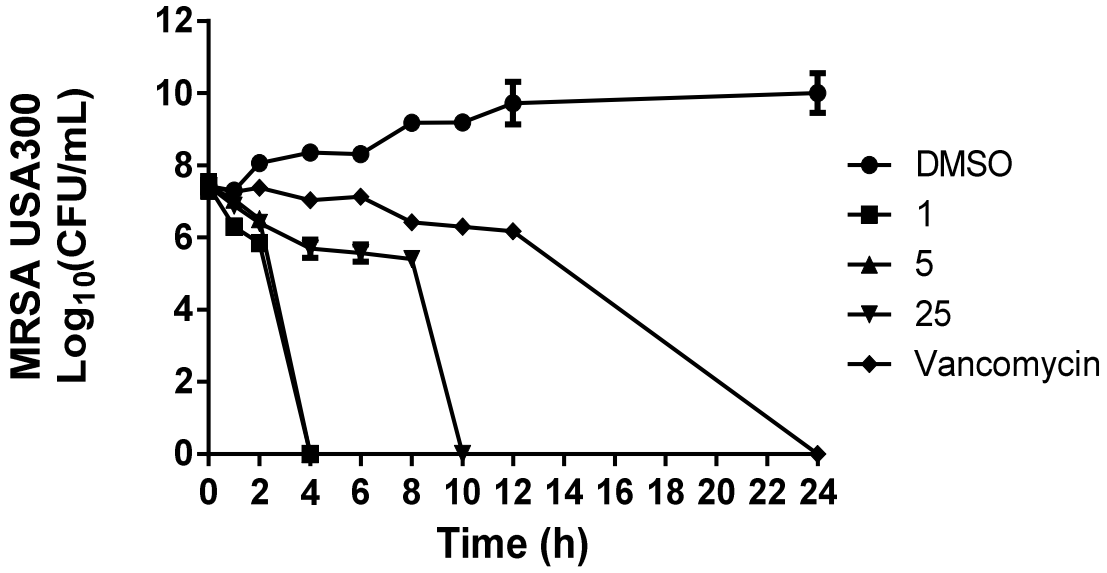

Figure 4.6 Time-kill analysis of thiazole compounds **1**, **5**, **25**, and vancomycin against methicillin-resistant *Staphylococcus aureus* (MRSA USA300).

Bacteria were incubated with test agents over a 24 hour period at 37 °C. DMSO served as a negative control. The error bars represent standard deviation values obtained from triplicate samples used for each compound/antibiotic studied.

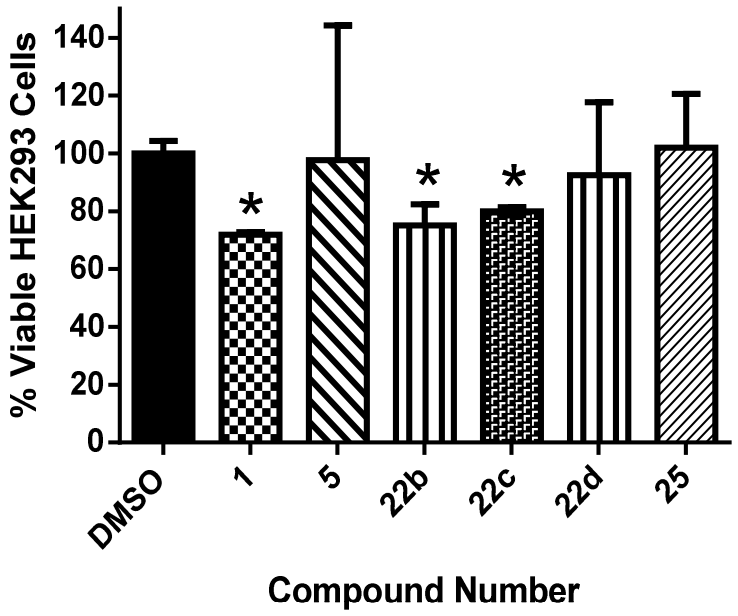

Figure 4.7 Percent viable HEK293 cells after exposure to thiazole compounds. Percent viable mammalian cells (measured as average absorbance ratio (test agent relative to DMSO)) for cytotoxicity analysis of thiazole compounds **1**, **5**, **22b-d**, and **25** at 40 µg/mL against HEK293 cells using the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay. DMSO was used as a negative control to determine a baseline measurement for the cytotoxic impact of each compound. The absorbance values represent an average of a minimum of three samples analyzed for each compound. Error bars represent standard deviation values for the absorbance values. An unpaired t-test, $P \leq 0.05$, demonstrated statistical difference between the values obtained for compounds **1**, **22b** and **22c** relative to the cells treated with DMSO (denoted with asterisks).

CHAPTER 5. ANTIBACTERIAL EVALUATION OF SYNTHETIC THIAZOLE COMPOUNDS *IN VITRO* AND *IN VIVO* IN A METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) SKIN INFECTION MOUSE MODEL

THIS IS A PUBLISHED JOURNAL ARTICLE. Reprinted with permission from **Antibacterial Evaluation of Synthetic Thiazole Compounds *in vitro* and *in vivo* in a Methicillin-resistant *Staphylococcus aureus* (MRSA) Skin Infection Mouse Model.** Haroon Mohammad, Mark Cushman, and Mohamed N. Seleem. *PLoS ONE* (2015) **10,** e0142321; doi: 10.1371/journal.pone.0142321

5.1 Introduction

Ten percent of all hospital admissions in the United States each year are due to patients suffering from skin and soft tissue infections (SSTIs) (1). SSTIs can range from simple abscesses, cellulitis, and traumatic wound infections to complicated infections (infected burns, diabetic foot ulcers, and major abscesses) (1). SSTIs are often caused by the bacterial pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) (2, 3). Indeed, 58% of all SSTIs treated in the United States alone were caused by MRSA, according to an epidemiological study of one national health care system (4). This agrees with a 2004 study conducted in emergency room departments in 11 cities where MRSA was responsible for 59% of patients presenting with a SSTI (5). The large number of *S. aureus*-based SSTIs has placed a significant economic burden on the healthcare system. A recent report examining the increase in *S. aureus*-SSTI hospitalizations in the United States documented a dramatic rise in the annual cost of treating infected patients from $3.36 billion to $4.50 billion (from the years 2001 through 2009) (6). A recent increase in skin abscesses has been observed and has been associated with a rise in strains of community-associated MRSA (CA-MRSA) (7). Of these strains, MRSA USA300 has been linked most frequently to skin infections in the United States (5, 8).

According to guidelines provided by the Infectious Diseases Society of America, treatment of moderate to severe skin infections caused by MRSA involves incision and

drainage of the affected region combined with administration of empirical antibiotics (such as clindamycin, vancomycin, linezolid, and mupirocin) (9, 10). However, strains of MRSA exhibiting resistance to several of these antibiotics including vancomycin (11, 12), clindamycin (13, 14), and topical ointments like mupirocin (14-16), indicate that such therapies may be rendered ineffective in the future. Therefore, development of novel antimicrobials capable of treating MRSA-induced SSTIs is an important step necessary to circumvent the burden of this public health issue.

Previous research by our group has identified a lead disubstituted phenylthiazole compound (compound **1**, Figure 5.1) that exhibited potent antimicrobial activity *in vitro* against a diverse array of clinically-significant strains of MRSA (17). Derivatives of the lead **1** were synthesized to elucidate the structure-activity relationships of this compound. These derivatives revealed that the aminoguanidine moiety at thiazole-C5 is critical for antibacterial activity (18). Furthermore, a nonpolar, hydrophobic group is favored at thiazole-C2. Analogues to the lipophilic alkyl tail of the lead **1** were subsequently constructed in order to enhance the antimicrobial activity of these thiazole compounds, to improve their toxicity profile, and to refine their physicochemical properties (18). These particular phenylthiazole compounds possess several excellent characteristics *in vitro* including rapid bactericidal activity against MRSA, a low frequency of bacterial resistance developing, and they have been shown to possess the ability to be used in combination with currently approved antibiotics, such as vancomycin, against MRSA (19). Studies performed to date indicate these compounds have potential to be used as topical antimicrobials for treatment of MRSA skin infections. The objectives of the current study were to assess the antibacterial activity of the lead thiazole compound and four analogues (Fig. 1) *in vitro* against antibiotic-resistant *S. aureus* strains isolated from/responsible for skin infections, to assess the ability of these compounds to be paired with mupirocin as a treatment option against MRSA, to confirm the compounds have limited toxicity to human keratinocytes, and to verify the thiazole compounds can retain their antimicrobial activity *in vivo* in an established murine MRSA skin infection model. Confirmation of the ability of these compounds to successfully treat mice infected with a MRSA skin infection will lay the foundation for further assessment of these compounds as novel antimicrobials for treatment of MRSA skin infections.

5.2 Materials and Methods

5.2.1 Synthesis of thiazole compounds **1-5**

Synthetic schemes, spectral data, and purity (>95%, determined by HPLC) of thiazole compounds **1-5** (Fig. 1), in addition to all intermediates, have been reported elsewhere (17, 18, 20).

5.2.2 Bacterial strains and reagents used in this study

Clinical isolates of *S. aureus* were obtained through the Network of Antimicrobial Resistance in *Staphylococcus aureus* (NARSA) program (Table 5.1). Clindamycin hydrochloride monohydrate (TCI America, Portland, OR, USA, >98.0% purity) and mupirocin (pure USP) (AppliChem, St. Louis, MO, USA) powders were purchased commercially and dissolved in dimethyl sulfoxide (DMSO) (for clindamycin) or ethanol (for mupirocin) to prepare a stock solution (10 μg/mL). Lipoderm was purchased from the Professional Compounding Centers of America (Houston, TX, USA). Cation-adjusted Mueller-Hinton broth (CAMHB) (Sigma-Aldrich, St. Louis, MO, USA), tryptic soy broth (TSB) (Becton, Dickinson and Company, Franklin Lakes, NJ, USA), mannitol salt agar (Hardy Diagnostics, Santa Maria, CA, USA), phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA), Dulbeco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO, USA), fetal bovine serum (FBS) (USA Scientific, Inc., Orlando, FL, USA), petroleum jelly (Equate [Walmart, Inc.], Bentonville, AR, USA), and 96-well plates (CellTreat Scientific Products, Shirley, MA, USA) were all purchased from commercial vendors.

5.2.3 Determination of minimum inhibitory concentration (MIC) against drug-resistant *S. aureus* strains

The MIC of thiazole compounds **1-5**, clindamycin, and mupirocin was determined against five different MRSA strains and one highly mupirocin-resistant *S. aureus* strain isolated from skin wounds, using the broth microdilution method, following the guidelines outlined by the Clinical and Laboratory Standards Institute (21). A bacterial suspension equivalent to a McFarland standard of 0.5 was prepared and subsequently diluted 1:300 in CAMHB. This bacterial suspension (~1×10^5 colony forming unit (CFU/mL)) was then added to

each well of a 96-well microtiter plate. Compounds **1-5**, clindamycin, or mupirocin were added (in triplicate) to the first row of the plate and then serially diluted down the ordinate. Plates were incubated at 37 °C for 18-20 hours and then the MIC was ascertained. The MIC was classified as the lowest concentration of each test agent where bacterial growth could not be visualized.

5.2.4 Assessment of synergistic relationship between thiazole compounds and mupirocin against MRSA

The checkerboard assay was utilized to asses if the most potent thiazole compounds (**1-3**) have potential to be combined with mupirocin for treatment of MRSA infections (22). Briefly, a bacterial suspension (1×10^5 CFU/mL) in CAMHB was added to each well of a 96-well microtiter plate. Compounds **1-3** and mupirocin were diluted in CAMHB in order to reach the desired starting concentration (2 × or 4 × MIC). Mupirocin was serially diluted along the horizontal axis of the plate while compound **1**, **2**, or **3** was diluted along the vertical axis. Plates were incubated for at least 18 hours at 37 °C and the MIC of each compound was recorded. The fractional inhibitory concentration index (ΣFIC) was computed for each combination using the following equation:

$$\Sigma\text{FIC} = \left(\frac{MIC\,thiazole\ compound\ in\ combination\ with\ mupirocin}{MIC\,thiazole\ compound\ alone}\right) + \left(\frac{MIC\,mupirocin\ in\ combination\ with\ thiazole\ compound}{MIC\,mupirocin\ alone}\right)$$

A FIC index less than or equal to 0.50 was classified as synergism, as described previously (19). FIC values above 0.50 but less than 4.00 were classified as indifference, while FIC values greater than 4.00 were indicative of antagonism.

5.2.5 *In vitro* cytotoxicity analysis of thiazole compounds against HaCaT cells

Compounds **1-5** were assayed (at concentrations of 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL) against a human keratinocyte (HaCaT) cell line (Catalogue Number: T0020001, AddexBio, San Diego, CA, USA) to determine the potential toxic effect to mammalian skin cells *in vitro* as described before (18). Briefly, cells were cultured in DMEM supplemented with 10% FBS at 37 °C with CO_2 (5%). Control cells received DMSO alone at a concentration equal to that in drug-treated cell samples. The cells were incubated with

the compounds (in triplicate) in a 96-well plate at 37 ºC with CO_2 (5%) for two hours prior to addition of the assay reagent MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (Promega, Madison, WI, USA). Absorbance readings (at OD_{490}) were taken using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with each compound was expressed as a percentage of the viability of DMSO-treated control cells (average of triplicate wells ± standard deviation). The toxicity data was analyzed via a one-way ANOVA, with post hoc Dunnet's multiple comparisons test ($P < 0.05$), utilizing GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA).

5.2.6 *In vivo* assessment of antimicrobial activity of thiazole compounds **1-5** and mupirocin in a MRSA skin infection mouse model

The MRSA murine skin infection study was reviewed, approved, and performed under the guidelines of the Purdue University Animal Care and Use Committee (PACUC) (protocol number: 1207000676) and carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. To initiate the formation of a skin wound, eight groups (n = 5) of eight-week old female BALB/c mice (obtained from Harlan Laboratories, Indianapolis, IN, USA) were disinfected with ethanol (70%) and shaved on the middle of the back (approximately a one-inch by one-inch square region around the injection site) one day prior to infection, similar to what has been described elsewhere (23, 24). To prepare the bacterial inoculum, an aliquot of overnight culture of MRSA USA300 was transferred to fresh TSB and shaken at 37 ºC until an OD_{600} value of ~1.0 was achieved. The cells were centrifuged, washed once with PBS, re-centrifuged, and then re-suspended in PBS. Mice then received an intradermal injection (20 µL) containing ~2.76×10^8 CFU/mL MRSA USA300. An open wound formed at the site of injection, 48 hours post-infection. Topical treatment was initiated subsequently with each group of mice receiving the following: compound **1-5** (2%, using petroleum jelly as the vehicle), mupirocin (2%, using petroleum jelly as the vehicle), compound **1** (2%, using Lipoderm as an alternative vehicle), and a control group receiving the control vehicle (20 mg, petroleum jelly) alone. Each group of mice receiving a particular treatment regimen was housed separately in a ventilated cage with appropriate bedding, food, and water. Mice were checked twice daily during infection and treatment to

ensure no adverse reactions were observed. In the event a mouse was observed to become severely ill, the subject was euthanized per the IRB protocol. Mice were treated twice daily for three days. Mice were humanely euthanized via CO_2 asphyxiation 24 hours after the last dose was administered. The region around the skin wound was lightly swabbed with ethanol (70%) and excised. The tissue was subsequently homogenized in TSB (1 mL). The homogenized tissue was then serially diluted in PBS before plating onto mannitol salt agar plates. The plates were incubated for 20-22 hours at 37 ºC before viable CFU were counted and MRSA reduction in the skin wound post-treatment was determined for each group. Data were analyzed using a one-way ANOVA, with post hoc Holm-Sidak's multiple comparisons test ($P < 0.05$), utilizing GraphPad Prism 6.0.

5.3 Results and Discussion

5.3.1 Antimicrobial activity of thiazole compounds **1**-**5** against MRSA strains isolated from skin wounds

Previous work has established thiazole compounds **1**-**5** possess potent antimicrobial activity against MRSA (particularly isolates derived from healthcare-associated MRSA cases). To confirm these compounds maintain their antibacterial activity against CA-MRSA strains and MRSA isolates derived from patients presenting with infected wounds (Table 5.1), the broth microdilution assay was utilized to determine the lowest concentration each compound was able to inhibit the growth of these strains (denoted as the minimum inhibitory concentration or MIC).

When tested against these important clinical isolates of drug-resistant *S. aureus*, the thiazole compounds exhibited strong antimicrobial activity similar to (and in several cases better than) mupirocin. As presented in Table 5.2, the lead thiazole **1** exhibits the most potent activity with a MIC value of 1.3 μg/mL against all six drug-resistant staphylococcal strains tested. The biphenyl and butyne analogues (**2** and **3,** respectively) possess MIC values ranging from 2.8 to 5.6 μg/mL. All five thiazole compounds possess antimicrobial activity against MRSA strains exhibiting resistance to an array of antibiotics including β-lactams, fluoroquinolones (USA800), tetracycline (USA300), and erythromycin (USA300 and USA1000). This indicates cross-resistance between these antibiotics and the thiazole compounds is unlikely to occur. Additionally, the compounds exhibit potent antimicrobial

activity against *S. aureus* NRS107 (MIC values range from 1.3 to 13.3 μg/mL), a strain exhibiting a high-level of resistance to mupirocin (MIC of 1024.0 μg/mL). Furthermore, compounds **1** and **2** (MIC of 1.3 and 2.8 μg/mL, respectively) are more active than mupirocin (MIC of 4.0 μg/mL) against three additional MRSA strains (USA800, USA1000, and USA1100). Clindamycin, when tested against four of the five MRSA strains, was found to have a MIC of 0.1 μg/mL. This MIC value is similar to what has been reported elsewhere for clindamycin (25). Collectively, the results confirm that the thiazole compounds do possess potent antimicrobial activity against important CA-MRSA strains and MRSA isolates responsible for infected wounds in patients.

5.3.2 Combination therapy using thiazole compounds with mupirocin against MRSA

The susceptibility analysis performed with the thiazole compounds indicated they have potential to be used alone for the treatment of MRSA skin/wound infections. While the use of a single agent to treat such infections is often used in the clinical setting, combination therapy using two or more antibiotics is favorable for multiple reasons. Among these reasons include that combination therapy has the potential to slow down the emergence of resistant bacterial strains to antibiotics, to reduce potential negative side effects to patients (by using lower concentrations/doses of each drug), and to alleviate the morbidity related to bacterial infections (26, 27). Given that multiple topical treatments for skin infections involve a combination of more than one antibiotic, such as Neosporin (consisting of bacitracin, neomycin, and polymyxin B sulfate) and Polysporin ointment (consisting of bacitracin, polymyxin B sulfate, and gramicidin) (28), the identification of compounds to pair with known antibiotics has good potential to expand the available treatment options. Mupirocin has been a key ally in the treatment of MRSA skin infections; however, isolates exhibiting moderate to high-level of resistance to mupirocin (MIC $\geq$ 512 μg/mL) have emerged, particularly in environments where this antibiotic has been extensively utilized (15, 29, 30). Identifying agents that can be partnered with mupirocin has the potential to extend the usage of this particular antimicrobial in the clinical setting.

In an earlier study, Alou *et al*, demonstrated that mupirocin forms a synergistic relationship with amoxicillin-clavulanate against MRSA isolates tested *in vitro* via the checkerboard assay (31). Amoxicllin is a β-lactam antibiotic that interferes with bacterial

cell wall synthesis by inhibiting crosslinking of peptidoglycan subunits in the bacterial cell wall (32). Preliminary studies conducted with our thiazole compounds indicate they also interfere with cell wall synthesis in bacteria; thus we were curious to assess if the thiazole compounds could be used in combination with mupirocin against MRSA, similar to what was found with amoxicillin-clavulanate. Using the checkerboard assay, it was discovered that the most potent thiazole compounds (**1**-**3**) exhibited a strong degree of synergy (FIC index ≤ 0.50) with mupirocin against two of the most prevalent MRSA strains responsible for skin infections (Table 5.3). Against MRSA USA300, all three compounds exhibited a fractional inhibitory concentration (FIC) index ranging from 0.09 to 0.13 when combined with mupirocin. A similar trend was observed when this combination was tested against MRSA USA400, with FIC values ranging from 0.05 to 0.13. The data provide evidence that supports the prospect that these particular thiazole compounds can be successfully paired with mupirocin to treat MRSA infections (and potentially prolong the utility of mupirocin in the clinical setting).

5.3.3 Toxicity analysis of thiazole compounds to human keratinocytes

Selective toxicity is important to ensure compounds with promising antimicrobial activity don't possess negative side effects to mammalian tissues. Certain regimens (in particular antiseptics) used for treatment of skin infections and wounds have been found to exhibit toxicity to human keratinocytes and impair wound healing, thus limiting their use as therapeutic options (33-36). Prior to validating the antimicrobial activity of the thiazole compounds in a MRSA skin infection model, it was critical to confirm the thiazole compounds were not toxic to human keratinocytes. Using the MTS assay with a human keratinocyte (HaCaT) cell line, it was confirmed that thiazole compounds **1**-**5** were not toxic at a concentration of 10 μg/mL (Figure 5.2). Interestingly, the four analogues constructed from compound **1** demonstrated an improved toxicity profile, as they were found to be non-toxic to HaCaT cells up to a concentration of 20 μg/mL. Taken altogether, the data indicate the most potent thiazole compounds *in vitro* (**1**-**3**) are not toxic to human keratinocytes at concentrations up to seven-fold higher than the compounds' MIC values determined against MRSA.

5.3.4 Assessment of topical application of thiazole compounds *in vivo* via a murine MRSA skin infection model

As thiazole compounds **1**-**5** exhibited excellent activity against MRSA *in vitro* and displayed no toxicity to human keratinocytes at the compounds' MIC, we moved to confirm that these compounds could maintain their antimicrobial activity *in vivo*, using an established MRSA murine skin infection model. After the formation of an open wound (infected with MRSA) in the dorsal region of infected mice, each group of mice was treated with a suspension of compounds **1**-**5** (2%), mupirocin (2% suspension), or petroleum jelly (20 mg, used as a vehicle for topical delivery of the compounds/antibiotic) twice daily for three days. The reduction in bacterial burden present in the wounds of infected mice was determined after cessation of treatment. Reduction of bacterial burden in infected wounds is critical to promote proper wound repair and to prevent a severe inflammatory response from being triggered that may negatively impact healing of wounded tissues (37).

As presented in Figure 5.3, four thiazole compounds mimic mupirocin's ability to drastically reduce the burden of MRSA present in skin wounds. Compounds **3**-**5** produce a 1.47 to 1.62 $\log_{10}$ reduction in MRSA CFU; this corresponds to a greater than 96% reduction in the bacterial burden, as compared to mice receiving only the vehicle alone (petroleum jelly) for treatment. The lead **1** exceeds the effect of mupirocin, producing a 2.27 $\log_{10}$ reduction in MRSA CFU in the skin wound (relative to the 2.07 $\log_{10}$ reduction observed with mupirocin). The emergence of increasing resistance to mupirocin, a drug of choice, amongst MRSA strains makes it extremely important to find alternative options for treatment (particularly for skin infections), such as these thiazole compounds. Interestingly, one of the most potent compounds against MRSA USA300 *in vitro* (the biphenyl analogue **2**, MIC of 2.8 μg/mL) is the least effective compound *in vivo* (produces a 0.47 $\log_{10}$ reduction in MRSA CFU, that was found to not be statistically significant); this provides a stark reminder that the behavior of compounds *in vitro* needs to be validated with *in vivo* studies to confirm their viability as novel treatment options.

Antimicrobial compounds that can be administered topically (such as thiazole compounds **1**, **3**, **4**, and **5**) for treatment of localized skin lesions have certain advantages over their systemic counterparts. These advantages include the ability to avoid adverse systemic side effects, the ability to localize/concentrate the drug at the target site of

infection (providing increased concentration of the drug), lower treatment costs, and a reduced likelihood of inducing bacterial resistance to the treatment agent (36, 38). Overall, the results garnered from the present study indicate the thiazole compounds (in particular the lead **1**) do warrant further investigation as a topical treatment option for MRSA-infected skin wounds.

5.3.5 Impact of changing vehicles in reduction of MRSA burden present *in vivo* in infected skin wounds

After confirming four thiazole compounds (**1**, **3**-**5**) have potential for use as novel topical antimicrobials against MRSA, we examined if changing the vehicle used for delivery may further enhance the reduction in bacterial burden present in infected wounds. To assess this, a 2% suspension of the most potent compound (**1**), using Lipoderm as an alternative vehicle, was tested using the murine MRSA skin infection model described above. Lipoderm has been used commercially as a transdermal delivery vehicle to enhance permeation of active pharmaceutical compounds through the skin (39). It was hypothesized that switching vehicles (from petroleum jelly) to Lipoderm would enhance penetration of the thiazole compounds into the skin wound, thus permitting a greater reduction in the bacterial burden present. As Figure 5.3 demonstrates, changing vehicles from petroleum jelly to Lipoderm does enhance the reduction in the bacterial load in the skin wound of mice that is achieved by compound **1**. A 0.4-$\log_{10}$ improvement in the reduction of MRSA CFU for compound **1** is observed when Lipoderm is used. This corresponds to a > 99.6% reduction in MRSA present in the skin wound after treatment. Thus switching vehicles from petroleum jelly to Lipoderm appears to permit enhanced penetration of the thiazole compounds into skin wounds, leading to an increased reduction in MRSA burden.

5.4 Conclusion

In this study, we demonstrate that five novel synthetic phenylthiazole compounds exhibit potent antimicrobial activity *in vitro* against clinically-relevant strains of MRSA responsible for skin and wound infections. Additionally, compounds **1**-**3** exhibit a strong synergistic relationship when combined with mupirocin against two highly prevalent strains of CA-MRSA. Furthermore, three compounds are not toxic to human keratinocytes

at a concentration seven times higher than their MIC against MRSA. The antimicrobial activity of compounds **1**, **3**, **4**, and **5** is confirmed *in vivo* in a murine MRSA skin infection model (> 96% reduction in bacterial load observed, post-treatment). Substitution of the vehicle from petroleum jelly to Lipoderm permits a nearly 0.4-$\log_{10}$ additional reduction in bacterial load achieved by compound **1**, indicating this vehicle may be more suitable for enhanced penetration of the compound into infected tissues. Collectively, the results provide valuable information to further develop these thiazole compounds as topical antimicrobial agents for treatment of skin infections and wounds infected by MRSA. Future work with these thiazole compounds includes constructing additional analogues of the lead compound **1** in an effort to improve its potency against MRSA and enhance its toxicity profile with human keratinocytes. Additionally, addressing the limited physicochemical properties of these compounds (through structural modifications of lead compound **1**) is an important next step in order to expand the therapeutic potential of these compounds so they can be administered orally/intravenously for treatment of invasive MRSA infections (both complicated skin infections and systemic infections).

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Table 5.1 Drug-resistant clinical isolates of *Staphylococcus aureus* used in this study.

NARSA[1] Strain ID	Alternate Strain Designation	US State Isolated From	Year Isolated	Antimicrobial Resistance Phenotype
NRS107	RN4220	Connecticut	1991	Highly mupirocin-resistant and rifampicin
NRS123 (MRSA)	USA400	North Dakota	1998	β-lactams
NRS384 (MRSA)	USA300-0114	Mississippi	-	Erythromycin, β-lactams, and tetracycline
NRS387 (MRSA)	USA800	Washington	-	β-lactams and fluoroquinolones
NRS483 (MRSA)	USA1000	Vermont	1993-1994	Erythromycin and methicillin
NRS484 (MRSA)	USA1100	Alaska	1996	β-lactams

[1]NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*.

Table 5.2 Minimum inhibitory concentration (MIC in μg/mL) of thiazole compounds **1**-**5**, clindamycin, and mupirocin (tested in triplicate) against five methicillin-resistant *Staphylococcus aureus* (MRSA) and one mupirocin-resistant *S. aureus* (NRS107) strain isolated from skin wounds.

	***S. aureus* Strain Number**					
Compound Number/Name	**NRS107**	**USA300**	**USA400**	**USA800**	**USA1000**	**USA1100**
1	1.3	1.3	1.3	1.3	1.3	1.3
2	2.8	2.8	2.8	2.8	2.8	2.8
3	2.8	5.6	5.6	5.6	2.8	5.6
4	13.3	13.3	13.3	13.3	13.3	13.3
5	6.4	6.4	12.8	6.4	12.8	6.4
Clindamycin	0.1	1.8	0.1	0.1	0.1	0.1
Mupirocin	1024.0	1.0	1.0	4.0	4.0	4.0

Table 5.3 Combination testing of thiazole compounds **1-3** with mupirocin against clinically-prevalent strains of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA).

Compound Number	ΣFIC range [1] MRSA USA300	ΣFIC range MRSA USA400
1	0.09 – 0.13	0.13
2	0.09	0.09 – 0.13
3	0.09 – 0.13	0.05 – 0.13

[1] ΣFIC, fractional inhibitory concentration index. Results for the FIC index (ΣFIC) are as follows: ≤ 0.50, synergistic; >0.50 to ≤4.00, indifference; >4.00, antagonistic. ΣFIC range provided is from two independent experiments.

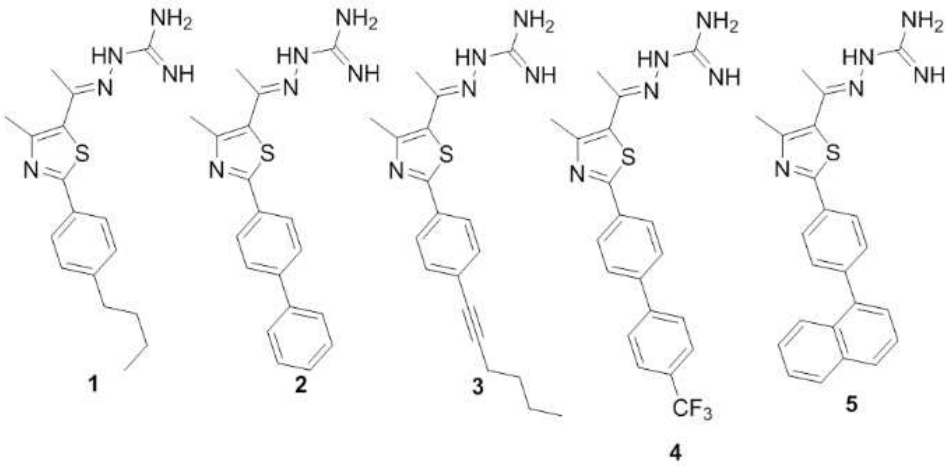

Figure 5.1 Chemical structures of thiazole compounds **1-5** presented in this study.

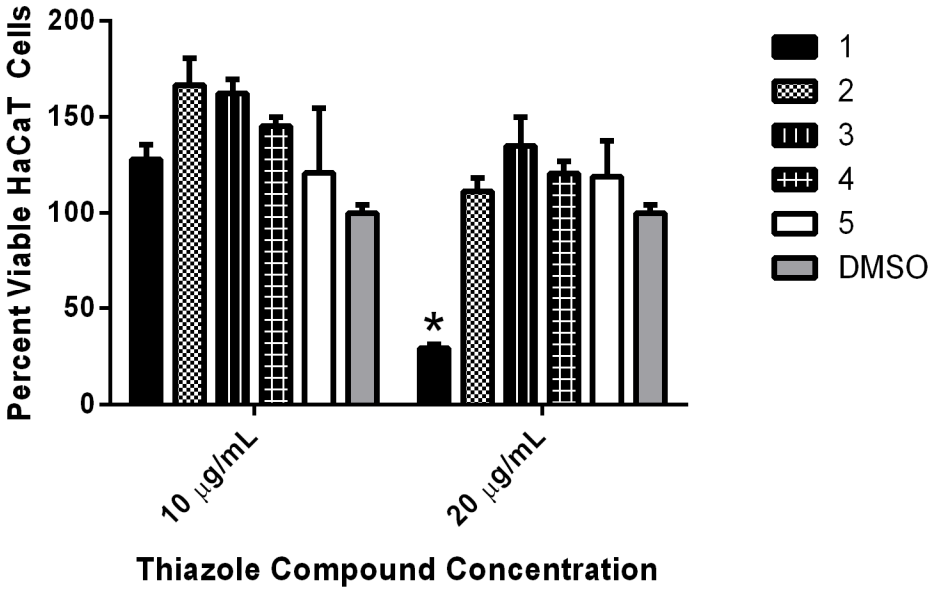

Figure 5.2 Toxicity analysis of thiazole compounds against human keratinocytes (HaCaT).

Percent viable mammalian cells (measured as average absorbance ratio (test agent relative to DMSO)) for cytotoxicity analysis of thiazole compounds **1**, **2**, **3**, **4**, and **5** (tested in triplicate) at 10 and 20 μg/mL against HaCaT cells using the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Dimethyl sulfoxide (DMSO) was used as a negative control to determine a baseline measurement for the cytotoxic impact of each compound. The absorbance values represent an average of a minimum of three samples analyzed for each compound. Error bars represent standard deviation values for the absorbance values. A one-way ANOVA, with post hoc Dunnet's multiple comparisons test, determined statistical difference between the values obtained for compound **1** and DMSO (denoted by the asterisk) ($P < 0.05$).

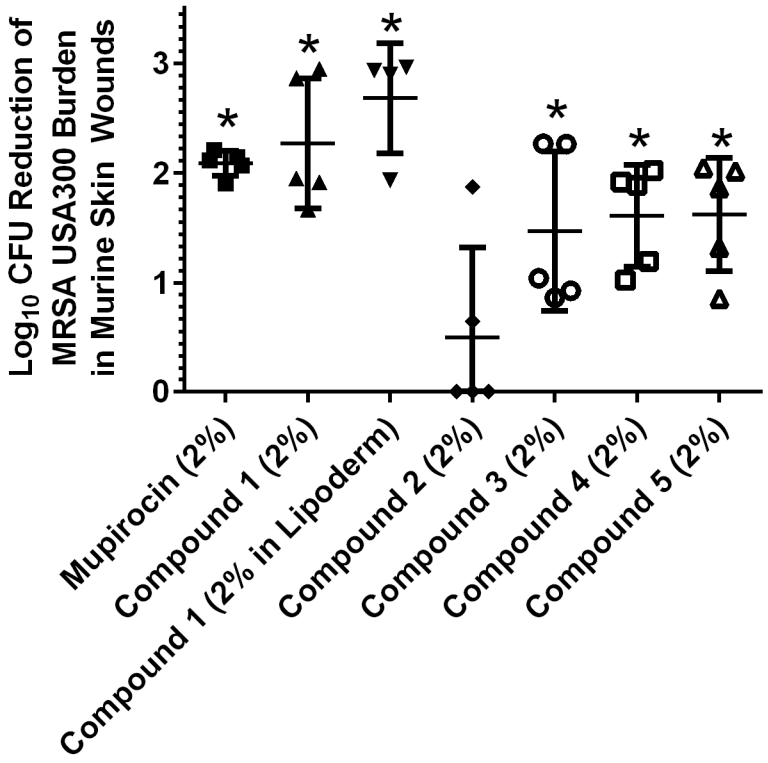

Figure 5.3 Average log10-reduction in MRSA USA300 burden in infected murine skin wounds.

Evaluating the effectiveness of treatment of MRSA skin lesions in mice with mupirocin (2%), thiazole compounds **1**-**5** (2%), and compound **1** (2%, using Lipoderm as the vehicle) twice daily for three days. The average $\log_{10}$-reduction in bacterial burden (relative to the negative control group (petroleum jelly)) was calculated and presented in the figure. Error bars represent standard deviation values. A one-way ANOVA, with post hoc Holm-Sidak's multiple comparisons test revealed statistical difference (denoted by asterisk) between compounds **1**, **3**, **4**, **5**, **1** (using Lipoderm as the vehicle), and mupirocin relative to the negative control ($P < 0.05$).

CHAPTER 6. ANTIBACTERIAL CHARACTERIZATION OF NOVEL SYNTHETIC THIAZOLE COMPOUNDS AGAINST METHICILLIN-RESISTANT *STAPHYLOCOCCUS PSEUDINTERMEDIUS*

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6.1 Introduction

Staphylococcus pseudintermedius is a significant problem in veterinary medicine as it is a major source of opportunistic infections in companion animals and the leading causative agent of canine pyoderma (1). It has also been linked to other severe infections in companion animals including urinary tract infections, skin wounds, surgical site infections, and otitis (2-4). The challenge to combat *S. pseudintermedius* infections has become more difficult with the emergence of clinical isolates (primarily methicillin-resistant *S. pseudintermedius*) exhibiting resistance to multiple antibiotic classes including β-lactams, lincosamides, fluoroquinolones, macrolides, sulfonamides, aminoglycosides, tetracyclines, and chloramphenicol (1, 5, 6). In several cases, patients that contracted an infection caused by *S. pseudintermedius*, in particular newborn puppies, have died or been euthanized due to the lack of effective treatment to remedy the medical condition (4, 7, 8). Thus there is a critical need for the discovery and characterization of novel antimicrobials to treat infections caused by methicillin-sensitive and methicillin-resistant *S. pseudintermedius*.

Thiazole compounds have been shown to be useful in multiple therapeutic applications including as anticancer, antiviral, and anticonvulsant agents (9-11) but their potential use as antibacterials has not been fully examined. Previous investigation into thiazole compounds synthesized by our research group has revealed these compounds exhibit potent antimicrobial activity against multidrug-resistant strains of *Staphylococcus*

aureus, including methicillin-resistant *S. aureus* (MRSA) (12, 13). MRSP bears similar genetic and phenotypic traits to MRSA, including expression of the *mecA* gene that encodes a modified penicillin-binding protein that confers resistance to β-lactam antibiotics (14). Additionally, *S. pseudintermedius* has been shown to express surface proteins similar to *S. aureus* that play an important role in bacterial colonization of host tissues (15). Furthermore, both staphylococcal species secrete similar virulence factors, including exfoliative toxins and leukocidins, that may play an important role in promoting pathogenesis of disease in infected hosts (16-18).

Given the genetic and phenotypic similarities between *S. pseudintermedius* and *S. aureus*, we suspected that the thiazole compounds we have found to be potent inhibitors of MRSA would also be active against MRSP. The objectives of the present study were to characterize the antibacterial activity of six of the most potent thiazole compounds (against MRSA) (Fig. 1) against clinical isolates of MSSP and MRSP, to ascertain the likelihood of MRSP acquiring rapid resistance to these novel compounds, and to determine if the compounds could be used to re-sensitize MRSP to the effect of β-lactam antibiotics. Additionally, we assessed the physicochemical profile of the most promising analogue and examined the ability of MRSP to recover after exposure to the thiazole antibiotics, via a post-antibiotic effect assay. The results garnered lend valuable insight into the pharmacological utility of thiazole compounds as a possible future therapeutic option for the treatment of *S. pseudintermedius* infections.

6.2 Materials and Methods

6.2.1 Bacterial isolates and chemical reagents

Fifteen isolates of *S. pseudintermedius* (eight MSSP and seven MRSP), identified at the Indiana Animal Disease Diagnostic Laboratory from specimens collected from dogs admitted to the small animal teaching hospital at Purdue University, were included in the study. The specimens were not collected specifically for this research study but were obtained from patients admitted to the hospital for treatment. Clinical specimens were inoculated onto 5% sheep blood agar and incubated at 35 °C for 18-24 hours. Standard methods including examination of colony morphology and hemolysis and biochemical

tests such as tube coagulase, Voges-Proskauer (VP) and fermentation tests for maltose, trehalose and lactose, were used in addition to matrix-assisted laser desorption ionization time of flight mass spectrometry (19) to identify isolates (19, 20). Antimicrobial susceptibility was determined by broth microdilution using the SensiTitre (Thermofisher Scientific). Isolates demonstrating resistance to oxacillin, a surrogate for methicillin, with a minimum inhibitory concentration (MIC) value greater than or equal to 0.5 μg/mL were tested for the presence of *mecA* by PCR as previously described (21). Bacterial isolates used in this study are presented in Table 6.1.

Clindamycin hydrochloride monohydrate (Tokyo Chemical Industry, Portland, OR, USA), oxacillin sodium salt monohydrate (Tokyo Chemical Industry, Portland, OR, USA), rifampicin (Sigma-Aldrich, St. Louis, MO, USA), and vancomycin hydrochloride (Gold Biotechnology, St. Louis, MO, USA) were purchased commercially. All antibiotics were dissolved in dimethyl sulfoxide (DMSO) to obtain a stock 10 mM solution.

6.2.2 Synthesis of thiazole compounds **1-6**

The detailed synthetic protocols and spectral data of final products **1-6** as well as all intermediates have been previously reported (12, 22, 23). Chemical structures of compounds **1-6** are presented in Figure 6.1. All compounds were dissolved in DMSO to obtain a stock 10 mM solution.

6.2.3 Determination of minimum inhibitory concentration and minimum bactericidal concentration (MBC) against *S. pseudintermedius*

The MICs of the thiazole compounds, clindamycin, and rifampicin against eight clinical isolates of MSSP and seven clinical isolates of MRSP were determined using the broth microdilution method, in accordance with the recommendations contained in the CLSI guidelines (24). Bacteria were prepared in phosphate-buffered saline (PBS) until a McFarland standard of 0.5 was achieved. The solution was subsequently diluted 1:300 in Mueller-Hinton broth (MHB) to reach a starting inoculum of 1×10^5 colony-forming units (CFU/mL). Bacteria were then transferred to a 96-well microtiter plate. Thiazole compounds and antibiotics were added (in triplicate) to wells in the first row of the microtiter plate and then serially diluted along the vertical axis. The plate was incubated at

37 °C for 22-24 hours before the MIC was determined. The MIC was categorized as the concentration where there was no visible growth of bacteria observed.

The MBC was determined by plating 5 µL from wells on the 96-well microtiter plate (where the MIC was determined) where no growth was observed onto tryptic soy agar (TSA) plates. The TSA plates were then incubated at 37 °C for 22-24 hours before the MBC was determined. The MBC was categorized as the concentration where ≥99% reduction in bacterial cell count was observed.

6.2.4 Time-kill analysis of thiazole compounds and antibiotics against MRSP

MRSP cells in late logarithmic growth phase were diluted to ~1×10^7CFU/mL and exposed to concentrations equivalent to 4 × MIC (in triplicate) of thiazole compounds **1**-**6** and rifampicin in MHB. Samples (20 µL) were collected after 0, 2, 4, 6, 8, 10, 12, and 24 hours of incubation at 37 °C and subsequently serially diluted in PBS. Bacteria were then transferred to TSA plates and incubated at 37 °C for 18-20 hours before viable CFU/mL values were determined.

6.2.5 Cell membrane disruption analysis

In order to investigate the antimicrobial effect of the thiazole compounds on the integrity of the bacterial cell membrane, the release of 260 and 280 nm absorbing components was determined spectrophotometrically (25). The cell suspension of 1.2×10^9 CFU/mL MRSP was incubated with 4 × MIC of compound **2** at 37 °C for 30 minutes. Untreated MRSP cells or cells treated with vancomycin (inhibits cell wall synthesis in bacterial cells) served as negative controls. For the release of 260 and 280 nm absorbing material, the bacterial suspension (control) was treated with lysostaphin (in 50 mM Tris-HCl, pH 8.00) for 30 minutes. Lysostaphin was used as a positive control due to its mode of action being the disruption of the cross-linking of the pentaglycin bridges in the cell wall of staphylococci bacteria (26). The absorbance of cell supernatant at 260 and 280 nm was determined using a spectrophotometer (Jenway 6305). The average OD_{260} and OD_{280} values of duplicates of each treatment option were calculated and expressed as the proportion of average OD_{260} (or OD_{280}) for each treatment option compared to the average OD_{260} (or OD_{280}) for the positive control (lysostaphin).

6.2.6 *In vitro* cytotoxicity analysis

Compounds were assayed at concentrations of 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL against a murine macrophage cell line (J774.A1) (ATCC® TIB-67™, American Type Culture Collection (ATCC), Manassas, VA, USA) to determine the potential toxic effect *in vitro*. Cells were cultured in Dulbeco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) with 10% fetal bovine serum (USA Scientific, Inc.) at 37 °C with 5% CO_2. Controls received DMSO alone at a concentration equal to that in drug-treated cell samples. The cells were incubated with the compounds in a 96-well plate at 37 ºC and 5.0% CO_2 for two hours prior to addition of the assay reagent MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) (Promega, Madison, WI, USA). Absorbance readings were taken using a kinetic ELISA microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with each compound was expressed as a percentage of the control, DMSO.

6.2.7 Multi-step resistance selection

To assess the ability of MRSP to develop resistance to the thiazole compounds, a multi-step resistance selection experiment was performed, as described elsewhere (27). The broth microdilution method for MIC determination against a clinical isolate of MRSP was repeated for ten passages over a period of ten days. The initial inoculum was prepared to a McFarland standard of 0.5. The solution was subsequently diluted 1:300 in MHB to reach a starting inoculum of 1×10^5 CFU/mL. For each subsequent passage, the inoculum for the MIC determination was adjusted to a final density of approximately 5×10^5 CFU/mL using the contents of a well containing a subinhibitory concentration of the compound (where bacterial growth was observed from the previous passage). Bacteria were then transferred to a new 96-well microtiter plate. Thiazole compounds **3-6** and clindamycin were added (in triplicate) to wells in the first row of the microtiter plate and then serially diluted along the ordinate. The plate was incubated at 37 °C for 22 hours before the MIC was determined by visual inspection. Resistance was classified as a greater than four-fold increase in the initial MIC, as reported elsewhere (28).

6.2.8 Combination therapy assessment of thiazole compounds with oxacillin

The relationship between the thiazole compounds and oxacillin was assessed via a standard checkerboard assay (29). Bacteria equivalent to a McFarland standard of 0.5 were prepared in PBS. The bacteria were next diluted in MHB to achieve a starting cell density of 1×10^5 CFU/mL. MHB was transferred to all wells of a 96-well microtiter plate. The thiazole compounds and oxacillin were diluted in MHB to achieve a starting concentration equivalent to 2 × or 4 × MIC, respectively. Oxacillin was serially diluted along the abscissa of the microtiter plate while the thiazole compound was serially diluted along the ordinate. The plate was incubated for 22-24 hours at 37 °C. The MIC of the test compound in combination with oxacillin was determined as the lowest concentration of each compound/antibiotic where no visible growth of bacteria was observed. The fractional inhibitory concentration index (ΣFIC) was calculated for each combination as described previously (13). A synergistic relationship was classified as an FIC index less than or equal to 0.5. FIC values above 0.5 but less than 4.0 were characterized as indifference while FIC values above 4.0 were classified as antagonistic.

6.2.9 Re-sensitization of MRSP to oxacillin using broth microdilution method

MHB was inoculated with MRSP (5×10^5 CFU/mL), as has been previously described (30). Aliquots (5 mL) of the bacterial suspension were divided into microcentrifuge tubes. The thiazole compounds tested (at ½ × MIC) were introduced into each tube. After sitting at room temperature for 30 minutes, 1 mL samples from each tube were transferred to a new centrifuge tube prior to addition of oxacillin (at a concentration equivalent to its MIC). Using a 96-well microtiter plate, rows 2-12 were filled with the remaining 4 mL bacterial suspension (containing the thiazole compound). Aliquots (200 µL) from tubes containing both the thiazole compound and oxacillin were transferred to row 1 of the 96-well plate. After aspirating contents in the first row 4-6 times, 100 µL was transferred from wells in row 1 to row 2. This process was repeated to dilute the remaining wells containing no antibiotic. Untreated bacteria served as a control. The plate was incubated at 37 °C for 22 hours before the MIC was recorded. The MIC was categorized as the concentration at which no visible growth of bacteria was observed in a particular well. A fold reduction was

calculated by comparing the MIC of the antibiotic alone compared to the MIC of the antibiotic given in combination with the thiazole compounds.

6.2.10 Kinetic solubility determination of compound **3**

Serial dilutions of compound **3** were prepared in DMSO at 100× the final concentration. Compound **3** was then diluted 100-fold into PBS in a 96-well plate and mixed. The absorbance of the PBS-containing plate was measured prior to addition of the test agents to determine the background absorbance. After two hours, the presence of precipitate was detected by turbidity (absorbance at 540 nm). An absorbance value of greater than (mean + 3× standard deviation of the blank), after subtracting the pre-experiment background, was indicative of turbidity. The solubility limit is reported as the highest experimental concentration for compound **3** with no evidence of turbidity as described previously (12).

6.2.11 Microsomal stability analysis

Compound **3** was incubated in duplicate with dog liver microsomes at 37 ºC. The reaction contained microsomal protein in 100 mM potassium phosphate, 2 mM NADPH, 3 mM $MgCl_2$, pH 7.4. A control was run for each test agent omitting NADPH to detect NADPH-free degradation. At 0, 10, 20, 40, and 60 minutes, an aliquot was removed from each experimental and control reaction and mixed with an equal volume of ice-cold Stop Solution (methanol containing haloperidol, diclofenac, or other internal standard). Stopped reactions were incubated at least 10 minutes at -20 ºC, and an additional volume of water was subsequently added. The samples were centrifuged to remove precipitated protein, and the supernatants were analyzed by LC/MS/MS to quantitate the remaining parent. Data were converted to % remaining by dividing by the time zero concentration value. Data were then fitted to a first-order decay model to determine half-life. Intrinsic clearance was calculated from the half-life and the protein concentrations, as has been described elsewhere (12).

6.2.12 Post-antibiotic effect

To assess if the thiazole compounds exhibit a post-antibiotic effect (PAE) against MRSP, MRSP cells in late logarithmic growth phase (~1×10^8 CFU/mL) were incubated with 4 × MIC of thiazole compounds **1**-**6**, clindamycin, or rifampicin for one hour at 37 °C. A tube

containing untreated bacterial cells served as a control. Afterward, the compound/antibiotic was washed out by diluting bacteria 1:1000 in MHB. Counts of CFU for all cultures were obtained after washing. Aliquots (100 µL) of bacteria were removed every hour (for 10 hours), serially diluted in PBS, and plated on TSA plates. TSA plates were incubated for 20 hours at 37 °C before CFU values were determined. The PAE was calculated using the same formula described elsewhere (31): PAE = $T - C$, where T is the time required for the count of CFU values in the test culture to increase one $\log_{10}$ above the count observed immediately after removal of the test agent and C is the time required for the count of CFU in the untreated control culture to increase one $\log_{10}$ above the count observed immediately after completion of the same procedure used on the test culture for removal of test agent.

6.2.13 Statistical analysis

All statistical analyses were performed with GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Data generated from cytotoxicity analysis of the thiazole compounds against J774.A1 cells and the 260 and 280 nm cell leakage analysis were analyzed using one-way ANOVA, with post hoc Tukey's multiple comparisons test ($P < 0.05$).

6.3 Results

6.3.1 MICs and MBCs of thiazole compounds and antibiotics against *S. pseudintermedius*

All six thiazole compounds exhibited potent antimicrobial activity against all *S. pseudintermedius* isolates tested (Table 6.2). The MIC_{50} values obtained for the compounds against methicillin-sensitive *S. pseudintermedius* were in close proximity to one another ranging from 0.30 µg/mL for compound **2** to 0.80 µg/mL for compound **6**. These values mimicked the results obtained for clindamycin (MIC_{50} of 0.48 µg/mL), a first-line antibiotic recommended for use in the treatment of pyoderma infections (32). The MBC_{50} values matched or were up to three-fold higher than the MIC_{50} values determined for the thiazole compounds; this indicates that these compounds exhibit bactericidal activity against methicillin-sensitive *S. pseudintermedius*. The compounds retained their antimicrobial activity against the isolates of methicillin-resistant *S. pseudintermedius* tested. Interestingly, compounds **3** and **6** showed a nearly two-fold improvement in the

MIC_{50} value obtained against MRSP isolates as compared to the MSSA isolates. The thiazoles retained their bactericidal activity against MRSP isolates with MBC_{50} values ranging from 0.42 μg/mL for compound **5** to 1.47 μg/mL for compound **4**.

6.3.2 Time-kill analysis of thiazole compounds and rifampicin

To confirm the thiazole compounds were bactericidal against MRSP, a time-kill assay was performed using 4 × MIC of each compound and rifampicin. As Figure 6.2 demonstrates, the thiazole compounds are bactericidal but the rate of killing varies. The lead compound (**1**) required four hours to completely eliminate MRSP. Derivatives **3** and **6** showed improved killing kinetics and mimic rifampicin's rapid bactericidal activity, completely eliminating MRSP within two hours. Compounds **2** and **5** require eight hours to achieve the same result while compound **4** exhibits the slowest rate of bacterial killing, requiring 12 hours to completely eliminate MRSP.

6.3.3 MRSP cell membrane disruption assessment

Disruption of the physical integrity of the bacterial cell membrane (such as formation of pores in the membrane) has been associated with antimicrobials that exhibit rapid bactericidal activity. To assess if the thiazole compounds' mode of action is disruption of the integrity of the MRSP cell membrane, the leakage of intracellular contents at 260 and 280 nm was analyzed after exposure of bacterial cells to a high concentration of compound **2** (4 × MIC) for 30 minutes. Figure 6.3 demonstrates that the thiazole compounds do not match the action of lysostaphin (a known membrane-disrupting agent). Less than 20% of the intracellular content (at 260 nm) is released after treatment with the thiazole compound as compared to cells treated with lysostaphin. This result confirms that the thiazole compounds do not act in a manner that involves disruption of the physical integrity of the MRSP cell membrane.

6.3.4 Toxicity analysis of thiazole compounds

Toxicity to host tissues is an important characteristic to assess with new compounds early in the drug discovery process. To determine if the thiazole compounds were toxic to eukaryotic cells, the viability of murine macrophage cells (J774.A1) exposed to each thiazole compound was assessed using the MTS assay. The lead **1** and compounds **2** and **6**

proved to be toxic at a concentration of 10 μg/mL (Figure 6.4). However, derivatives **3**, **4**, and **5** exhibited an improved toxicity profile over the lead compound (matching the results obtained with clindamycin), demonstrating they were not toxic to mammalian cells at 10 μg/mL. This is more than 20-fold higher than the MIC_{50} values obtained for these three compounds against clinical isolates of MRSP.

6.3.5 Multi-step resistance selection of MRSP to thiazole compounds

To assess the potential for rapid emergence of resistance of MRSP to the thiazole compounds, a multi-step resistance selection experiment was performed. The initial MICs of compounds **3**, **4**, **5**, and **6** were determined via the broth microdilution method and were found to be 1.41 μg/mL (compound **3**), 1.47 μg/mL (compound **4**), 1.67 μg/mL (compound **5**), and 1.60 μg/mL (compound **6**). Bacteria were then subcultured for ten serial passages to determine if a shift in the MIC of each compound tested would be observed against MRSP. After the second serial passage of compound **5**, there was a two-fold shift in the MIC; the MIC remained stable at 3.34 μg/mL until the seventh passage where a second increase in the MIC was observed to 6.68 μg/mL (Figure 6.5). Compounds **4** and **5** followed a similar course to one another; the MICs of both compounds remained stable for three passages before a two-fold shift was observed in both compounds after the fourth passage. The MIC did not increase again for both compounds after six additional passages. MRSP was not able to develop resistance to compound **3** even after ten passages.

6.3.6 Combination therapy and re-sensitization of MRSP to oxacillin in the presence of the thiazole compounds

As MRSP strains exhibit resistance to the effect of β-lactam antibiotics, such as oxacillin, we assessed whether exposure of MRSP to a subinhibitory concentration of the thiazole compounds could re-sensitize the bacteria to the effect of these antibiotics. After initial exposure of MRSP to a subinhibitory concentration (½ × MIC) of the thiazole compound, the bacteria were next treated with oxacillin. Using the broth microdilution assay, the MIC of oxacillin needed to inhibit MRSP growth in the presence and absence of each thiazole compound was determined. As Table 6.3 presents, MRSP was re-sensitized to the effect of oxacillin in the presence of a subinhibitory concentration of all six thiazole compounds. There was a 128-fold reduction in the MIC of oxacillin observed in the presence of ½ ×

MIC of compounds **1**, **2**, **3**, **4**, and **6** and a 64-fold reduction in the MIC observed with compound **5**. The checkerboard assay was used to assess if these compounds could be used in combination with oxacillin against MRSP. The assay revealed four compounds (**1**, **3**, **5**, and **6**) exhibited a synergistic relationship with oxacillin with ΣFIC values ranging from 0.19 to 0.38.

6.3.7 Solubility and metabolic stability assessment of compound **3**

The solubility of novel compounds in aqueous solutions and stability to host metabolic processes are important to analyze to determine if promising compounds possess suitable drug-like properties. To assess the ability of the thiazole compounds to dissolve in aqueous solutions, a turbidimetric solubility analysis was performed with compound **3** in phosphate-buffered saline. As Table 6.4 demonstrates, the compound is soluble in PBS up to a concentration of 5.51 μg/mL. This resembles the result obtained for the poorly aqueous drug tamoxifen, indicating compound **3** possesses limited aqueous solubility.

To analyze the stability of compound **3** to metabolic processes present in the liver, the compound was incubated with dog liver microsomes. As Table 6.5 shows, this compound is metabolized very slowly (similar to the drug warfarin) with a NADPH-dependent intrinsic clearance rate of 18.7 μL/min-mg and a half-life of over two hours. The data from Table 6.5 indicate that this compound is a substrate of a NADPH-dependent metabolic process. There is a nearly three-fold reduction in the intrinsic clearance rate (down to 6.6 μL/min-mg) and a marked increase in the half-life (nearly six hours) of compound **3** in the absence of the co-factor NADPH.

6.3.8 Post-antibiotic effect of thiazole compounds and antibiotics

In vitro pharmacodynamic analysis can provide valuable information regarding establishing a proper dosing regimen for drug candidates. One method to obtain this information is to determine if a compound/drug exhibits a post-antibiotic effect. The PAE for the thiazole compounds, clindamycin, and rifampicin was determined against a clinical isolate of MRSP. Table 6.6 reveals that all six thiazoles exhibit a long PAE ranging from 8 hours (for compounds **2** and **5**) to > 9 hours (for the remaining four compounds). This is

similar to what was observed with rifampicin (PAE > 9 hours) and superior to what was observed with clindamycin (PAE of only two hours).

6.4 Discussion

S. pseudintermedius infections have become a growing problem in veterinary medicine; until fairly recently, the vast majority of infections observed in small animal veterinary facilities could be treated with an array of efficacious antimicrobials (32, 33). However, the rapid emergence and global spread of multidrug-resistant *S. pseudintermedius* (namely MRSP) in the past ten years has presented a significant challenge to veterinary practitioners (3, 34). Clinical isolates have been identified that exhibit resistance to numerous antibiotic classes, limiting the treatment options available for veterinarians. This underscores the critical need to identify and develop new antibiotics and unique therapeutic strategies to combat this growing medical challenge.

The present study examines the antibacterial potential of novel synthetic thiazole compounds against clinical isolates of MSSP and MRSP. We have previously demonstrated the lead compound and derivative **2** possess potent antimicrobial activity against important strains of multidrug-resistant *S aureus* (primarily MRSA) of concern to both humans and animals (12). Four additional derivatives (compounds **3**-**6**) of the lead compound were subsequently constructed in an attempt to enhance the antibacterial activity of the lead while mitigating potential toxicity to host tissues. Structural variation constructed focused on modification of the lipophilic alkane side chain of the lead compound, resulting in the butyne analogue **3**, fluorobiphenyl derivative **4**, trifluoromethyl analogue **5**, and the naphthyl derivative **6**. All four were previously found to exhibit potent activity against MRSA so they were also included in this study. As *S. pseudintermedius* and *S. aureus* share similar genetic and phenotypic characteristics, we hypothesized that these thiazole compounds would possess potent antibacterial activity against MSSP and MRSP. This conjecture was confirmed via the broth microdilution method; all six thiazole compounds exhibited potent antibacterial activity against clinical isolates of both MSSP (MIC_{50} ranged from 0.30-0.80 μg/mL) and MRSP (MIC_{50} ranged from 0.40-1.47 μg/mL). These results proved similar to what was found with clindamycin (MIC_{50} of 0.48 μg/mL against both MSSP and MRSP), an antibiotic of choice for treatment of pyoderma

infections (32). Interestingly, the thiazole compounds retained their antibacterial activity against nine MSSP and MRSP isolates that were found to be resistant to clindamycin and other antibiotics; this indicates there is no cross-resistance present between these antibiotics and the thiazole compounds. This further supports the notion that these thiazole compounds have potential to be used as novel antibacterial agents, particularly against *S. pseudintermedius* infections resistant to treatment with other antibiotics.

We were curious to find out if the thiazole compounds possessed bacteriostatic or bactericidal activity. It has been suggested bactericidal antimicrobials have several advantages over their bacteriostatic counterparts, including helping patients recover more rapidly from infection, improving the clinical outcome of disease, reducing the potential emergence of bacterial resistance to the antibiotic, and limiting the spread of infection (35). Preliminary analysis indicated the thiazole compounds were bactericidal as they possessed MBC_{50} values identical to or two- to three-fold higher than their MIC_{50} values against both MSSP and MRSP isolates. While structural modifications made to the lead thiazole compound did not significantly impact the MIC_{50} and MBC_{50} values found for the subsequently constructed derivatives, there was a significant difference observed in the bacterial killing kinetics against MRSP. A time-kill assay revealed that the alkyne derivative **3** and the naphthyl derivative **6** exhibited superior activity to the lead **1**, rapidly eliminating MRSP within two hours (the lead compound required double the time to achieve the same effect). This was similar to the result obtained with rifampicin, an antibiotic of last resort for pyoderma infections (32).

Rapid bactericidal activity has been shown to be important in the treatment of diseases caused by staphylococci such as endocarditis, meningitis, and osteomyelitis (35, 36). Thus these thiazole compounds possess a selective advantage over bacteriostatic agents in their ability to be used for treatment of more severe clinical diseases. However, one pitfall of antimicrobials that are rapidly bactericidal is many tend to work as membrane-disrupting agents (35, 37). Such agents have limited therapeutic applications, almost exclusively being restricted to use as topical ointments (37). As the thiazole compounds were found to exhibit rapid bactericidal activity, we examined if the mode of action of the thiazole compounds was via disruption of the MRSP cell membrane. A cell leakage analysis confirmed that the thiazole compounds do not physically disrupt the

integrity of the bacterial membrane similar to the positive control lysostaphin. The exact mechanism of action of these thiazole compounds against staphylococci is being investigated and will be the subject of the next chapter.

After confirming the thiazole compounds do in fact possess potent antibacterial activity and are capable of rapidly eliminating MRSP (in a mechanism that does not involve physical disruption of the bacterial cell membrane), we next focused our attention to assessing potential toxicity concerns with these compounds against mammalian cells. Structural modifications made to the lead thiazole compound played an important role in enhancing the toxicity profile of the thiazoles. The lead compound and biphenyl derivative **2** were found to be toxic to murine macrophage cells at a concentration of 10 μg/mL. Surprisingly, replacement of the alkyl moiety in the lead with an alkyne, monofluoro, or trifluoromethyl group (as in compounds **3**-**5**, respectively) significantly improved the toxicity profiles of the compounds. These three derivatives were not toxic to murine macrophage cells at 10 μg/mL which represents a greater than 20-fold difference over the MIC_{50} values determined against MRSP.

The ability of bacteria to develop resistance rapidly to antimicrobial compounds is important to assess early in drug discovery. Previously, we have reported results of a single-step resistance selection experiment that demonstrated MRSA is unlikely to develop rapid resistance to thiazole compounds **1** and **2** (13). We decided to extend this analysis to the newest thiazole derivatives (compounds **3**-**6**) against MRSP but with an additional twist – testing if bacterial resistance could be induced after repeated exposure to each compound over 10 serial passages. There was no change observed in the MIC for compound **3**, a two-fold increase in the MIC for compounds **4** and **5**, and a four-fold increase in the MIC of compound **6** after 10 passages. Collectively, the results provide data supporting a low probability of MRSP-resistance developing rapidly to these thiazole compounds (as a greater than four-fold increase, as compared to the initial MIC, was not observed for any of the compounds tested).

While discovery of novel antimicrobials for use in monotherapy is one important avenue to address the burden of multidrug-resistant bacterial infections, other therapeutic strategies must be explored. Recently, suppression of MRSA resistance to β-lactam antibiotics by using these agents in combination with other antimicrobial compounds has

been explored as an alternative therapeutic strategy (38, 39). This has the potential to prolong the usage of β-lactam antibiotics (particularly those that are less susceptible to degradation by β-lactamase such as first-generation cephalosporins) in the clinical setting. As first-generation cephalosporins are frequently used as first-line agents to treat staphylococcal infections present in small animal veterinary practices, β-lactam antibiotics still play a very integral role in the clinic (40). Prolonging the ability to use these antibiotics against resistant strains of staphylococci, such as MRSP, is extremely important. No studies have been reported thus far testing the ability of antimicrobial compounds to suppress MRSP resistance to β-lactam antibiotics. In an earlier study, we demonstrated that thiazole compound **2** can re-sensitize vancomycin-resistant *Staphylococcus aureus* (VRSA) to the effect of vancomycin (13). As glycopeptide antibiotics (such as vancomycin) and β-lactam antibiotics both target cell wall synthesis in bacteria, we hypothesized that the thiazole compounds would be able to re-sensitize MRSP to the effect of β-lactam antibiotics. Bacterial susceptibility to oxacillin is used as a standard to determine if bacteria are sensitive or resistant to β-lactam antibiotics (as resistant strains can appear sensitive to other β-lactam antibiotics *in vitro* but exhibit resistance to these antibiotics *in vivo*) (40). Using a clinical isolate identified as MRSP, we used the broth microdilution assay to first confirm that the isolate was resistant to oxacillin (MIC = 128 μg/mL). Next, the isolate was exposed to a subinhibitory concentration (½ × MIC) of each thiazole compound for 30 minutes; afterward, the broth microdilution assay was used to determine the sensitivity of the isolate to oxacillin. All six compounds demonstrated the ability to re-sensitize MRSP to oxacillin (a 64 to 128-fold reduction in the MIC of oxacillin was observed after pre-treatment with the thiazole compounds). Furthermore, the checkerboard assay confirmed that compounds **1**, **3**, **5**, and **6** exhibited a synergistic relationship with oxacillin with ΣFIC values ranging from 0.19 to 0.38. This analysis confirmed that in addition to being used as antimicrobial agents alone in the treatment of *S. pseudintermedius* infections, the thiazole compounds have the potential to be used i.) in combination with β-lactam antibiotics against MRSP or ii.) to suppress resistance of MRSP to β-lactam antibiotics. This expands the potential therapeutic applications of these compounds beyond just use in monotherapy. Additionally, the finding that thiazole compounds can be effectively combined with oxacillin, an inhibitor of cell wall biosynthesis, against MRSP paves the way for further

investigation of combination therapy of thiazole compounds with other cell wall synthesis inhibitors.

After confirming the thiazole compounds have potential use as antibacterial agents for the treatment of *S. pseudintermedius* infections, it was important to assess if the newly constructed derivatives exhibited suitable drug-like properties (such as aqueous solubility for drug absorption and metabolic stability). As compound **3** appeared the most promising drug candidate (due to its rapid bactericidal activity, improved toxicity profile, low induction of MRSP resistance, and ability to suppress MRSP resistance to β-lactam antibiotics), it was selected for further analysis. Previously, it was found that the lead compound has moderate aqueous solubility in phosphate-buffered saline (solubility limit of 20.56 μg/mL) (12). Substitution of the alkane in the lead compound with an alkyne (as in compound **3**) resulted in a reduction in the aqueous solubility observed. However, this substitution significantly enhanced the metabolic stability of compound **3**, when compared to the lead compound. Previously the lead compound was cleared by human liver microsomes at a rate of 80.3 μL/min-mg and had a half-life just under 30 minutes (12). When the same analysis in human liver microsomes was performed for the modified derivative **3**, a significant improvement in the metabolic stability profile of this compound was observed (clearance rate decreased to 3.7 μL/min-mg and half-life was more than 240 minutes (as detailed in chapter 5). In this study, compound **3** was analyzed using dog liver microsomes (to compare if the results found in human liver microsomes could be confirmed, given that metabolic processes in dogs and humans differ). Compound **3** was found to have a metabolic clearance rate of 18.7 μL/min-mg (> four-fold improvement in how rapidly the compound is metabolized and cleared from liver cells compared to the lead compound) in dog liver microsomes. Additionally, the half-life of 123 minutes for compound **3** is a significant improvement over the result found for the lead compound. This result is important as it ensures this compound is unlikely to be rapidly metabolized and excreted from the patient's body, thus decreasing the size and frequency of doses needed to be administered to treat a patient afflicted with a bacterial infection attributed to *S. pseudintermedius*. Though compound **3** possesses poor aqueous solubility, formulation technology has been shown to be an effective strategy to employ to overcome this limitation and advance promising compounds to the market (41). Identification of this

limitation early in the drug discovery process provides an area for formulation scientists and medicinal chemists to address to propel compound **3** into further drug discovery stages.

The metabolic stability analysis performed with compound **3** provided valuable evidence that fewer doses of this compound would need to be administered to treat a patient dealing with an infection. The post-antibiotic effect analysis performed further validated this observation. PAE analysis has been shown to be an important parameter to establish an optimal dosing regimen (size and frequency of doses given to patients) (42). As compound **3** exhibits a long PAE (> 9 hours) against MRSP, this indicates bacteria are very slow to recover after exposure to this compound. Thus, patients would need to be subjected to fewer doses of this particular compound (as compared to clindamycin, for example, where the PAE against MRSP was found to be only two hours). This is clinically significant as antimicrobials that demonstrate a PAE (in particular an extended PAE as is observed with the thiazole compounds) possess several advantages including reduced costs (fewer doses needed for treatment), limited toxicity to host tissues, and greater patient cooperation in sticking to the prescribed treatment regimen (43).

6.5 Conclusion

In this study we have demonstrated novel thiazole compounds synthesized by our research group do in fact possess potent antibacterial activity against clinical isolates of both methicillin-sensitive and methicillin-resistant *S. pseudintermedius*. The lead compound and five derivatives are capable of inhibiting bacterial growth at concentrations similar to clindamycin, a drug of choice in canine pyoderma infections. Though all six compounds are bactericidal, two derivatives (**3** and **6**) exhibit superior killing kinetics by completely eliminating MRSP within two hours (similar to rifampicin). Compound **3** appears to be the most suitable derivative to continue with further studies involving *S. pseudintermedius* as it is not toxic to mammalian cells at a concentration 20-fold higher than its MIC_{50} value against MRSP. Additionally, MRSP is predicted not to develop rapid resistance to this compound even after multiple exposures/doses. Furthermore, this compound exhibits a markedly improved metabolic stability profile compared to the lead compound. While the thiazole compounds show promise for use alone to treat *S. pseudintermedius* infections, these compounds also demonstrate the ability to re-sensitize MRSP to the effect of oxacillin;

this opens the door for the potential use of these compounds to prolong the utility of β-lactam antibiotics for treatment of infections caused by MRSP.

6.6 References

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Table 6.1 Clinical isolates of *Staphylococcus pseudintermedius* used in this study.

Methicillin-sensitive *Staphylococcus pseudintermedius* isolates					
Isolate Name	**Species**	**Breed**	**Age**	**Origin**	**Resistance Phenotype[1]**
SP1	Canine	Mixed	9 years	Urine	PEN, AMP
SP2	Canine	Cocker Spaniel	10.5 years	Urine	PEN, AMP, CLIN, ENRO, ERYTH, GEN, MARBO, TMP-SMX
SP6	Canine	Labrador Retriever	6 years	Ear	None
SP7	Canine	Cocker Spaniel	10 years	Ear	AMK, PEN, AMP, CLIN, ENR, ERM, GEN, MARB, TMP-SMX
SP11	Canine	Mixed	9.5 years	Ear	PEN, AMP, CHL, CLIN
SP12	Canine	West Highland White	15.5 years	Urine	PEN, AMP, AMK, ENR, GEN, MARB, TMP-SMX
SP15	Canine	Mixed	9.5 years	Urine	None
SP23	Canine	Boxer	9.5 years	Wound	PEN, AMP
Methicillin-resistant *Staphylococcus pseudintermedius* isolates					
SP3	Canine	English Bulldog	8 months	Orthopedic implant	AMP, PEN, AMO, CEF, ERM, CLIN, IMI,OXA, TIC
SP5	Canine	Mixed	10.5 years	Urine	AMK, AMP, PEN, AMO, CEF, CHL, CLIN, ENR, ERM, GEN, IMI, MARB, OXA, TIC, TMP-SMX
SP8	Canine	Maltese	10 years	Urine	AMP, PEN, AMO, CEF, ERM, CLIN, IMI, OXA, TIC, CHL
SP9	Canine	Mixed	4 years	Skin	AMK, AMP, PEN, AMO, CEF, CHL, CLIN, ENR, ERM, GEN, IMI, MARB, OXA, TIC, TMP-SMX
SP14	Canine	Golden Retriever	4.5 years	Ear	PEN, CHL, CLIN, ERM
SP25	Canine	Mixed	11.5 years	Urine	AMK, AMP, PEN, AMO, CEF, CHL, CLIN, ENR, ERM, GEN, IMI, MARB, OXA, TIC, TMP-SMX
SP28	Canine	West Highland White	15 years	Urine	AMK, AMP, PEN, AMO, CEF, CHL, CLIN, ENR, ERM, GEN, IMI, MARB, OXA, TIC, TMP-SMX

[1]Abbreviations: PEN: penicillin, AMP, ampicillin, AMK: amikacin, CEF: cefpodoxime, CLIN: clindamycin, GEN: gentamycin, CHL: chloramphenicol, ENR: enrofloxacin, MARB: marbofloxacin, ERM: erythromycin, TMP-SMX: trimethoprim/sulfamethoxazole, TIC: ticarcillin, IMI: imipenem, AMO: amoxicillin, OXA: oxacillin.

Table 6.2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of thiazole compounds **1**-**6**, clindamycin, and rifampicin (triplicate samples) against eight methicillin-sensitive *Staphylococcus pseudintermedius* and seven methicillin-resistant *Staphylococcus pseudintermedius* isolates.

	Methicillin-sensitive *Staphylococcus pseudintermedius*				**Methicillin-resistant *Staphylococcus pseudintermedius***			
Compound Number/ Antibiotic	**MIC_{50}[1] (µg/mL)**	**MIC Range (µg/mL)**	**MBC_{50}[2] (µg/mL)**	**MBC Range (µg/mL)**	**MIC_{50} (µg/mL)**	**MIC Range (µg/mL)**	**MBC_{50} (µg/mL)**	**MBC Range (µg/mL)**
1	0.35	0.17-1.38	0.46	0.17-2.77	0.69	0.35-1.38	0.92	0.35-1.38
2	0.30	0.15-0.61	0.61	0.15-2.42	0.46	0.15-0.61	0.61	0.30-0.81
3	0.71	0.18-0.94	0.71	0.18-1.41	0.48	0.18-1.41	0.71	0.18-1.41
4	0.43	0.18-2.94	1.10	0.18-2.94	1.47	0.37-1.47	1.47	0.31-2.94
5	0.42	0.21-1.67	0.42	0.21-6.67	0.42	0.21-1.67	0.42	0.21-3.34
6	0.80	0.20-1.60	1.06	0.20-3.19	0.40	0.20-1.60	0.80	0.20-1.60
Clindamycin	0.48	0.24-61.37	0.48	0.24->61.37	0.48	0.24->30.69	30.69	0.24->61.37
Rifampicin	<0.41	<0.41	<0.41	<0.41	<0.41	<0.41	<0.41	<0.41

[1]MIC_{50} corresponds to the lowest concentration of each test agent that inhibited growth in 50% of bacterial isolates screened.

[2]MBC_{50} corresponds to the lowest concentration of each test agent that killed 50% of bacterial isolates screened.

Table 6.3 Combination testing of thiazole compounds with oxacillin and re-sensitization of methicillin-resistant *Staphylococcus pseudintermedius* SP3 to oxacillin using a subinhibitory concentration (½ × MIC) of thiazole compounds **1**-**6**.

Compound Number	**Re-sensitization**	**ΣFIC[1]**
1	128-fold	0.19
2	128-fold	0.56
3	128-fold	0.38
4	128-fold	0.63
5	64-fold	0.38
6	128-fold	0.38

[1]Results for the FIC index (ΣFIC) are as follows: ≤ 0.5, synergistic (S); > 0.5 to ≤ 4.0, indifference (I); > 4, antagonistic (A).

Table 6.4 Evaluation of solubility of thiazole compound **3**, Reserpine, Tamoxifen, and Verapamil in phosphate-buffered saline.

Compound Tested	**Solubility Limit (µg/mL)[1]**
3	5.51
Reserpine	19.05
Tamoxifen	5.80
Verapamil	>227.30

[1]The solubility limit corresponds to the highest concentration of test compound where no precipitate was detected.

Table 6.5 Evaluation of metabolic stability of thiazole compound **3**, Verapamil, and Warfarin (in duplicate) in dog liver microsomes.

Compound/Drug Tested	**NADPH-dependent CL_{int}[1] (µL/min/mg)**	**NADPH-dependent $T_{1/2}$[2] (min)**	**NADPH-free CL_{int} (µL/min/mg)**	**NADPH-free $T_{1/2}$ (min)**
3	18.7	123	6.6	351
Verapamil	244	9	0.0	>240
Warfarin	0.0	18.7	0.0	>240

[1]CL_{int}= microsomal intrinsic clearance rate

[2]$T_{1/2}$ = half-life

Table 6.6 *In vitro* post-antibiotic effect (PAE) of thiazole compounds **1**-**6**, clindamycin, and rifampicin against methicillin-resistant *Staphylococcus pseudintermedius*.

Compound Tested	**Post-antibiotic Effect (hours)**
1	>9
2	8
3	>9
4	>9
5	8
6	>9
Clindamycin	2
Rifampicin	>9

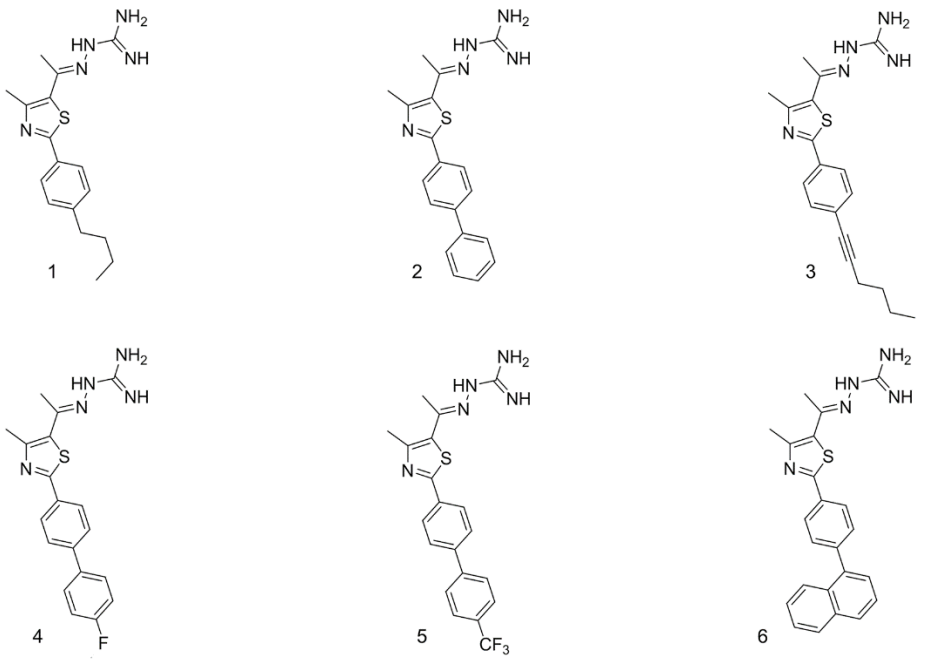

Figure 6.1 Chemical structures of thiazole compounds **1**-**6** utilized in this study.

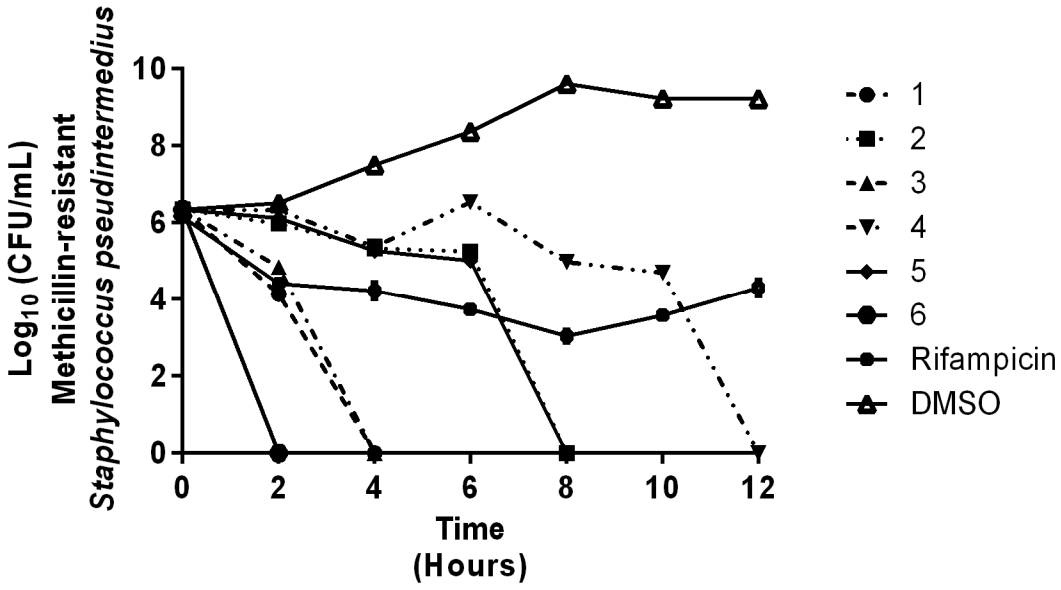

Figure 6.2 Time-kill analysis of thiazole compounds and rifampicin against methicillin-resistant *Staphylococcus pseudintermedius* (MRSP).

Bacteria were incubated with test agents over a 24 hour incubation period at 37 °C. DMSO served as a control. The error bars represent standard deviation values obtained from triplicate samples used for each compound studied.

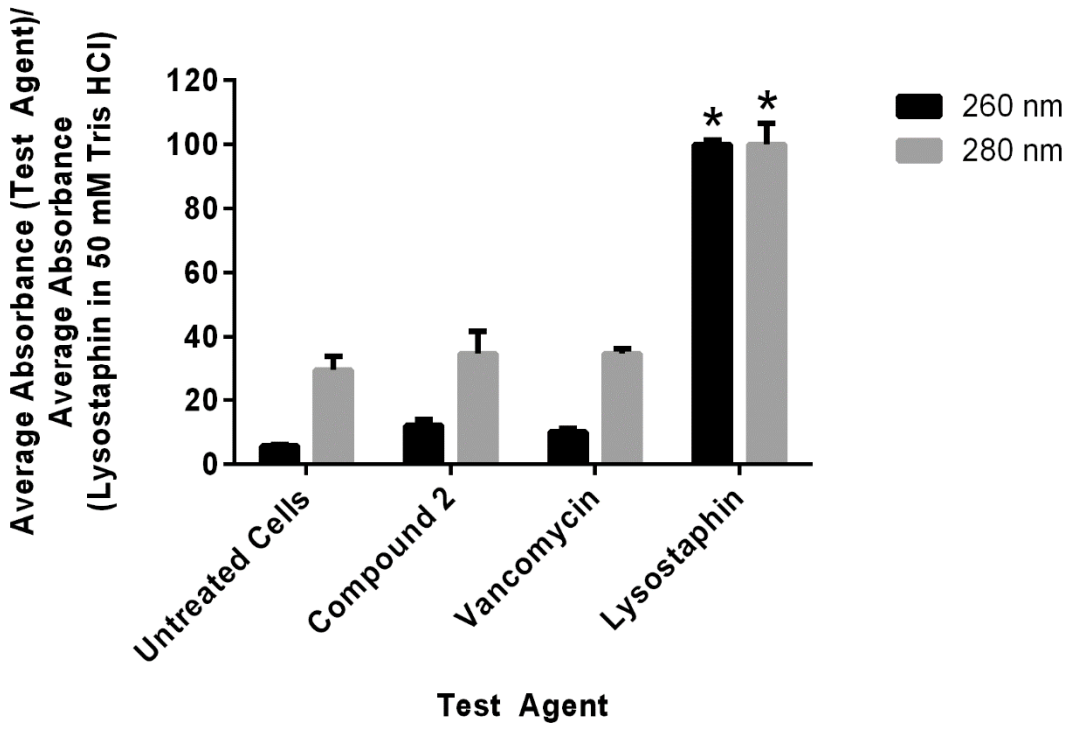

Figure 6.3 Loss of 260 and 280 nm cellular absorbing material for thiazole compound **2**, vancomycin, and lysostaphin against methicillin-resistant *Staphylococcus pseudintermedius* (MRSP).

Untreated cells represent the negative control while lysostaphin (in 50 mM Tris-HCl, pH 8.00) served as the positive control. The figure represents the ratio of the average absorbance value obtained for each treatment against the average absorbance value obtained for the positive control. The error bars represent standard deviation values of two experiments where triplicate samples were used for each treatment option. A paired t-test, $P \leq 0.05$, demonstrated no statistical difference between the values obtained for compound **2** and vancomycin relative to the untreated cells but significant difference in the values absorbance values obtained for lysostaphin as compared to both untreated cells and compound **2**.

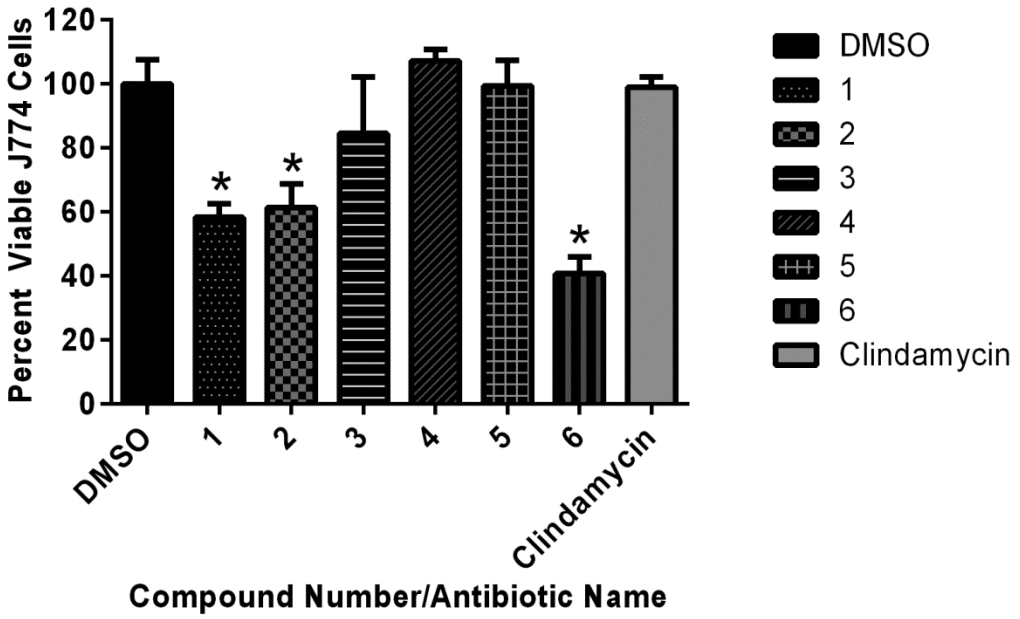

Figure 6.4 Toxicity analysis of compounds **1**-**6** against a murine macrophage cell line (J774).

Average absorbance ratio (test agent/DMSO) for cytotoxicity of thiazole compounds at 10 µg/mL against murine macrophage cells (J774.A1) using the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay. DMSO was used as a negative control to determine a baseline measurement for the cytotoxic impact of each compound. The absorbance values represent an average of a minimum of three samples analyzed for each compound. Error bars represent standard deviation values for the corrected absorbance values. A paired t-test, $P \leq 0.05$, demonstrated statistical difference between the values obtained for compounds **1**, **2**, and **6** relative to the cells treated with DMSO.

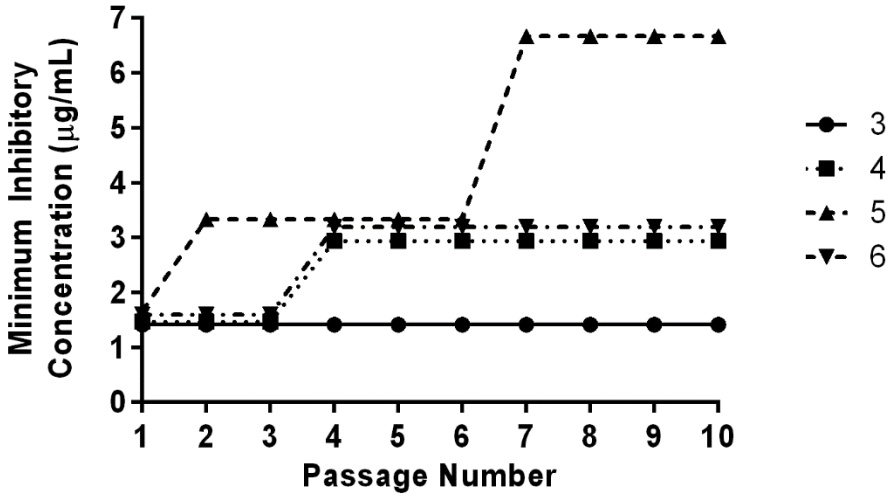

Figure 6.5 Multi-step resistance selection of thiazole compounds **3** - **6** against methicillin-resistant *Staphylococcus pseudintermedius* (MRSP).

Bacteria were serially passaged over a ten-day period and the broth microdilution assay was used to determine the minimum inhibitory concentration of each compound against MRSP after each successive passage.

CHAPTER 7. PHENYLTHIAZOLE ANTIBACTERIAL AGENTS TARGETING CELL WALL SYNTHESIS EXHIBIT POTENT ACTIVITY *IN VITRO* AND *IN VIVO* AGAINST VANCOMYCIN-RESISTANT ENTEROCOCCI

7.1 Introduction

According to the US CDC, there are approximately 1.7 million hospital-acquired infections (HAIs) in the US each year resulting in nearly 100,000 deaths and an estimated $20 billion in healthcare costs (1). Many of the organisms responsible have become resistant to most antibiotics, contributing to prolonged illness, high treatment costs, increased treatment failure, and death (2, 3). Bacterial pathogens such as vancomycin-resistant enterococci (VRE), in particular isolates of *Enterococcus faecalis* and *Enterococcus faecium*, are of particular concern and have been identified as leading sources of nosocomial infections (4). These range from skin infections to intra-abdominal infections to urinary tract infections, with immunocompromised individuals—including the elderly and patients undergoing organ transplants and cancer chemotherapy—being at particular risk (4).

Enterococcal infections were initially susceptible to many therapeutic agents including β-lactams (in particular ampicillin), glycopeptides (vancomycin), fluoroquinolones, and aminoglycosides (5). However, the ability of enterococci to colonize the gastrointestinal tract of patients hospitalized for long periods has permitted these organisms to acquire resistance, particularly after repeated drug exposure (4), severely limiting the number of effective therapeutic options available. Moreover, VRE strains have been isolated that exhibit resistance to newer antibacterial agents, including linezolid and daptomycin (6, 7). The problem of antibiotic resistance is compounded by the diminishing number of new antibiotics being approved. From 1980–1984, 19 new antibiotics were approved by the US FDA and this number plummeted to one new approval from 2010–2012 (8). The approval of three new antibiotics in 2014 indicates regulatory agencies understand the urgent need for new treatment options (9) but the ever-present nature of resistance development necessitates the continuous search for new drugs, and new drug leads.

Our group recently explored the antimicrobial activity of a broad range of phenylthiazoles against drug-resistant staphylococci (10-14), three of which (**1**-**3,** Figure

1) had potent activity against both *E. faecalis* and *E. faecium*. We examine here the activity of these compounds against clinical isolates of VRE; their mechanisms of action; potential synergies with other antibiotics; toxicity, and activity in an *in vivo* model of VRE infection, in *Caenorhabditis elegans*.

7.2 Materials and Methods

7.2.1 Synthesis of Thiazole Compounds **1-3**

Synthetic schemes, spectral data, and purity (>95%, determined by HPLC) of compounds **1-3** (Figure 1), in addition to all intermediates, have been reported elsewhere (12, 14).

7.2.2 Bacterial Strains and Reagents Used

Clinical isolates of *E. faecalis* and *E. faecium* were obtained through BEI Resources (Table 7.1). *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter cloacae* were obtained from the American Type Culture Collection (Manassas, VA, USA). *Escherichia coli* OP50, *E. coli* 1411 and *E. coli* ΔAcrAB were described before (15, 16). The human colorectal cell line (HRT-18) was obtained from the American Type Culture Collection (Manassas, VA, USA). Ampicillin (IBI Scientific, Peosta, IA), chloramphenicol (Sigma-Aldrich, St. Louis, MO, USA), ciprofloxacin (Enzo Life Sciences, Farmingdale, NY, USA), doxycycline monohydrate (Alfa Aesar, Ward Hill, MO, USA), linezolid (Chem-Impex International Inc., Wood Dale, IL, USA), and vancomycin hydrochloride (Gold Biotechnology Inc., St. Louis, MO, USA) were from the vendors noted above. Compounds were dissolved in dimethyl sulfoxide (DMSO) (for ampicillin, doxycycline, linezolid, and vancomycin), ethanol (for chloramphenicol), or 0.1 N HCl (for ciprofloxacin), in order to prepare stock solutions (10 mg/mL). Tryptic soy broth (TSB), tryptic soy agar (TSA), and brain heart infusion broth (BHI) were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Phosphate-buffered saline (PBS) (Sigma-Aldrich, St. Louis, MO, USA), RPMI-1640 medium (American Type Culture Collection, Manassas, VA, USA), fetal horse serum (American Type Culture Collection, Manassas, VA, USA), and 96-well plates (CellTreat Scientific Products, Shirley, MA, USA) were all purchased from the vendors listed above. Nematode growth medium (NGM) and M9 medium were prepared as described in the literature (17).

7.2.3 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MICs of thiazole compounds **1**-**3** and control antibiotics (linezolid, vancomycin) were determined against all bacterial strains tested using a broth microdilution method, following the guidelines outlined by the Clinical and Laboratory Standards Institute, as described elsewhere (14, 18). Strains of *E. faecalis*, *A. baumannii*, *E. cloacae*, *E. coli* and *P. aeruginosa* were cultured in TSB, while *E. faecium* strains were cultured in BHI. For gram-negative bacterial strains, the MIC was also tested in the presence of a sub-inhibitory concentration (¼ × MIC) of colistin (to permeabilize the outer membrane). Concentrations of colistin used were 0.25 μM for *A. baumanii* and *E. cloacae*, 0.0625 μM for *E. coli* and *K. pneumoniae*, and 1 μM for *P. aeruginosa*. Plates containing test agents and bacteria were incubated at 37 °C for 18-22 hours prior to determining the MIC. The MIC was taken to be the lowest concentration of each test agent where bacterial growth could not be visualized.

The MBC for each test agent against *E. faecalis* and *E. faecium* was determined using basically the methods described previously (14), with the following modifications. Aliquots (5 μL) of *E. faecalis* were transferred to TSA plates while aliquots of *E. faecium* were transferred onto BHI agar plates. Plates were incubated at incubated at 37 °C for 18-22 hours before the MBC, the concentration where >99% reduction in bacterial cell count was observed, was determined.

7.2.4 Time-Kill Analysis of Compounds **1**-**3** and Linezolid against VRE

Vancomycin-resistant *E. faecalis* ATCC HM-201 and vancomycin-resistant *E. faecium* ATCC 700221 cells in late logarithmic growth phase were diluted to ~5×10^6 colony-forming units (CFU)/mL and (in triplicate) exposed to concentrations equivalent to 4 × MIC of **1**-**3** or linezolid in either TSB (for *E. faecalis*) or BHI (for *E. faecium*). 100 μL samples were collected after 0, 2, 4, 6, 8, 10, 12, and 24 hours of incubation at 37 ºC, and subsequently serially diluted in PBS. Bacteria were then transferred to either TSA (for *E. faecalis*) or BHI agar plates (for *E. faecium*) and were incubated at 37 °C for 18-22 hours before viable CFU/mL values were determined.

7.2.5 Cytotoxicity Analysis of Thiazole Compounds in Cell Culture

Compounds **1**-**3** were assayed at concentrations of 5 µg/mL, 10 µg/mL, 20 µg/mL, and 40 µg/mL against a human colorectal (HRT-18) cell line to determine their effects to mammalian cells *in vitro*, as described elsewhere (19). Cells were cultured in RPMI-1640 medium with 10% fetal horse serum at 37 °C with 5% CO_2. Cells were incubated with compounds in 96-well plates at 37 ºC and 5% CO_2 for either 2 or 24 hours prior to addition of the assay reagent MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (Promega, Madison, WI, USA). Absorbance readings (at OD_{490}) were taken using a kinetic microplate reader (Molecular Devices, Sunnyvale, CA, USA). The quantity of viable cells after treatment with each compound are expressed as a percentage of the viability of untreated cells.

7.2.6 Single-Step Resistance Selection

The frequency of spontaneous single-step resistance of *E. faecalis* ATCC 31975 to the phenylthiazole compounds was carried out as described elsewhere (20, 21). Briefly, bacterial cultures (ranging from 5×10^8 CFU/mL to 5×10^{10} CFU/mL) were spread onto TSA plates (10-mm diameter) containing compound 1, 2, or 3 at either 2 ×, 4 ×, or 8 × MIC. Plates were incubated aerobically at 37 °C for 48 hours. The MIC of colonies present on each plate was checked (to determine any shift relative to the wild-type strain) using the broth microdilution method outlined above. The frequency of resistance was calculated as the number of resistant colonies per inoculum (21).

7.2.7 Multi-Step Resistance Selection of VRE to Thiazole Compounds

To assess the ability of VRE to develop resistance to the thiazole compounds after repeated exposure, a multi-step resistance selection experiment was performed, as described elsewhere (22). The broth microdilution method for MIC determination against a clinical isolate of vancomycin-resistant *E. faecalis* (ATCC 51299) and an isolate of vancomycin-resistant *E. faecium* (ATCC HM-968) was repeated for 14 passages over a period of two weeks. The initial inoculum was prepared to a McFarland standard of 0.5. The solution was subsequently diluted 1:300 in either TSB (for *E. faecalis*) or BHI (for *E. faecium*) to reach a starting inoculum of 1×10^5 CFU/mL. For each subsequent passage, the inoculum for the

MIC determination was adjusted to a final density of approximately 5×10^5 CFU/mL using the contents of a well containing a sub-inhibitory concentration of the compound (where bacterial growth was observed from the previous passage). Bacteria were then transferred to a new 96-well microtiter plate. Phenylthiazole compounds **1**-**3**, ampicillin, daptomycin, and linezolid were added (in triplicate) to wells in the first row of the microtiter plate, and then serially diluted along the vertical axis. The plate was incubated at 37 °C for a minimum of 18 hours before the MIC was determined, by visual inspection. Resistance was classified as a greater than four-fold increase in the initial MIC, as reported elsewhere (23).

7.2.8 Bacterial Cytological Profiling of Thiazole Compounds against *Bacillus subtilis* and *E. coli*

Cells were grown in Luria Bertani (LB) medium at 30 °C (*E. coli*) or 37 °C (*B. subtilis*) until the optical density at 600 nm (OD_{600}) was ~0.2. Cells were then left untreated, treated with compounds, or treated with compounds in the presence of 0.5 M sucrose (*E. coli*) or MSM (*B. subtilis*) as described previously (24-26). After 30 minutes or two hours, cells were stained with FM 4−64 (1 μg/mL) to visualize the membranes; DAPI (2 μg/mL *E. coli*, 1 μg/ml *B. subtilis*) to visualize the DNA, and SYTOX Green (1 μg/mL), a vital stain which is normally excluded from cells with an intact membrane but brightly stains cells that are lysed (26). Images were collected using a Delta Vision Spectris Deconvolution microscope, as described previously (26).

7.2.9 Inhibition of Cell Wall Synthesis in Enterococci by Compound **1** via UDP-N-acetylmuramyl-pentapeptide Accumulation

To investigate whether the phenylthiazole compounds exhibit their effects on enterococci by inhibiting cell wall synthesis, as suggested by previous work in which we found synergistic activity of thiazoles with known cell-wall biosynthesis inhibitors, we determined the accumulation of the final soluble cell wall precursor (UDP-N-acetylmuramyl-pentapeptide) inside bacterial cells. We used the procedure described previously (27), with the following modifications: *E. faecalis* NR-31975, in early logarithmic growth stage ($OD_{600} \sim 0.5$), was incubated with 130 μg/mL chloramphenicol for 15 minutes at 37 ºC. Bacteria were subsequently incubated with either 10 × MIC of compound **1** or vancomycin (positive control) for 30 minutes at 37 ºC. Untreated samples

served as a negative control. Samples were then centrifuged at 10,000 rpm, the supernatant discarded, and the pellet re-suspended in 1 mL of sterile deionized water. The cell pellet was boiled at 100 ºC for 30 minutes before samples were chilled on ice for 10 minutes. UDP-N-acetylmuramyl-pentapeptide levels were measured using an Agilent High Performance Liquid Chromatograph coupled to a time-of-flight mass spectrometer (HPLC-MS). A Waters XBridge Phenyl (2.1 x 100 mm, 3.5 um) column was used, with mobile phases of water, 0.1% formic acid (Buffer A) and acetonitrile, 0.1% formic acid (Buffer B). A gradient of 5-20% Buffer B over 14 minutes was then employed at a flow rate of 0.3 mL/min, with an electrospray source in positive ionization mode. Extracted ion chromatograms (EICs) were generated at a *m/z* of 1150.3588 (20 ppm window). The mass error for UDP-N-acetylmuramyl-pentapeptide was less than 1 ppm.

7.2.10 Molecular Target Identification Using Genomic Insertion of a Transposon with a Strong Outward-Oriented Promoter

Overexpression of the target/resistance mechanism was carried out using a transposon with a strong outward-oriented promoter for the random overexpression of neighboring genes, in *Bacillus subtilis*. The pEP26 delivery vector carrying the transposon with the promoter (TnHyJump) was transformed into *B. subtilis*, as described (28). For transposon integration into bacterial DNA, cells were grown for 10 hours at 25 ºC, serially diluted, sub-cultured in dual-selection TSA plates containing 5 µg/ml chloramphenicol (for transposon selection) and 3 × MIC compound **1** (for selection of compound resistance), then incubated overnight at 42 ºC. Growth at 42 ºC is non-permissive for the maintenance of the delivery vector, so chloramphenicol/compound **1** resistance arises mainly from the chromosomal insertion of the transposon. 12 colonies out of 142 colonies on 3 × MIC compound **1** were screened for MIC shift (resistance) against compound **1** using the broth microdilution method to confirm resistance. Genomic DNA was extracted from resistant colonies (recombinants that were capable of growth at concentrations that were inhibitory to the control) and were sent to the Purdue Genomics Core Facility for sequencing. Insertion sites were identified by sequencing. Transposon location within the resistant *B. subtilis* genome, orientation, and flanking genes were determined by performing a BLASTN search on the NCBI public BLAST server.

7.2.11 HsFPPS, EcUPPS and EcUPPP Inhibition Assays

Human FPPS, EcUPPS and EcUPPP were purified as described previously (29-31). Compound **1** was prepared as a 20 mM stock solution in DMSO and then serially diluted from 200 μM to 0.2 μM. 0.8 mg EcUPPS was incubated with compound **1** at room temperature for 30 minutes in HEPES buffer (100 mM HEPES, 50 mM NaCl, 0.5 mM $MgCl_2$, 0.02% DDM (w/v), pH 7.5) before adding the reaction mixture with 10 μM IPP and FPP, 0.375 U/mL inorganic phosphatase. The 100 μL reaction was quenched by the same volume of the malachite green mixture from a malachite green phosphate assay kit (BioAssay Systems). For the EcUPPP inhibition assay, the 20 mM stock solution of compound **1** was serially diluted from 300 μM to 0.8 μM. Compound **1** was incubated with 0.125 μM EcUPPP at room temperature for 15 minutes in HEPES buffer (50 mM HEPES, 150 mM NaCl, 10 mM $MgCl_2$, 0.02% DDM (w/v), pH 7.5), followed by the addition of 35 μM FPP and incubation at 37 °C for 20 minutes. The 100 μL reaction was then mixed with the same volume of the malachite green mixture. The released phosphate in EcUPPS and EcUPPP assays was monitored by absorbance at 620 nm after 30 minutes development. Dose response curves were constructed using GraphPad PRISM (Graphpad Software, San Diego, CA).

7.2.12 Uncoupler Assays

Proton translocation out of EcIMVs was measured by the fluorescence increase of ACMA. The excitation and emission wavelengths were 410 and 480 nm, respectively. IMVs (0.1 mg/mL membrane protein), 2 μM ACMA and 0.5 mM ATP/succinate were added in HEPES buffer (10 mM HEPES-KOH, 5 mM $MgSO_4$, 100 mM KCl, pH 7.5). The uncoupler CCCP and compound **1** were serially diluted in the reaction mixture. Dose response curves were constructed using GraphPad PRISM (Graphpad Software, San Diego, CA).

7.2.13 Re-sensitization of VRE to Vancomycin and Aminoglycoside Antibiotics

TSB (for *E. faecalis* ATCC HM-201 and *E. faecalis* ATCC 51299) or BHI (for *E. faecium* ATCC 700221 and *E. faecium* HM-968) were inoculated with VRE (5×10^5 CFU/mL), as described previously (13), with the following modifications. Aliquots (5 mL) of the

bacterial suspensions were divided into micro-centrifuge tubes and compounds **1**, **2**, or **3** (at ½ × MIC) were introduced into each tube. After sitting at room temperature for 30 minutes, 1 mL samples from each tube were transferred to a new micro-centrifuge tube, prior to addition of the antibiotic (either vancomycin or gentamicin at concentrations equivalent to their MIC). Plates containing the test agents and bacteria were then incubated at 37 °C for 18-21 hours after which the MIC values were measured. A fold-reduction was calculated by comparing the MIC of the antibiotic alone compared to the MIC of the antibiotic given in combination with the phenylthiazole compound.

7.2.14 Combination Therapy of Phenylhiazole Compounds With Conventional Antibiotics

Possible synergistic interactions between the thiazole compounds and ampicillin, ciprofloxacin, doxycycline, and linezolid were determined via checkerboard assay (32). Initially, compound **1** was examined in combination with the four antibiotics against a single strain of *E. faecalis* (ATCC 51299) prior to investigating compounds **1-3** in combination with ciprofloxacin against *E. faecalis* ATCC 49532 and *E. faecalis* ATCC 49533. Bacteria equivalent to a McFarland standard of 0.5 were prepared in PBS. The bacteria were then diluted in TSB to achieve a starting cell density of 1×10^5 CFU/mL. TSB was transferred to all wells of a 96-well micro-titer plate. The phenylthiazole compounds and antibiotics were diluted in TSB to achieve a starting concentration equivalent to 2 × or 4 × the MIC. Compounds were serially diluted along the horizontal axis of the microtiter plate while the antibiotics were serially diluted along the vertical axis. Plates were incubated for 20 hours at 37 °C. The MIC of the test compound in combination with each antibiotic studied was taken to be the lowest concentration of each compound/antibiotic where no visible growth of bacteria was observed. The fractional inhibitory concentration index (ΣFIC) was calculated for each combination, as described previously (11). A synergistic relationship was classified as an FIC index less than or equal to 0.5. FIC values above 0.5 but less than 2.0 were characterized as additive, values between 2.0 and 4.0 characterized as indifference, while FIC values above 4.0 were classified as antagonistic.

7.2.15 *In vivo* Analysis of Toxicity and Efficacy of Phenylthiazole Compounds

To examine the toxicity of the thiazole compounds *in vivo* and to examine their efficacy in treating a VRE infection *in vivo*, we used the *C. elegans* animal model. The temperature-sensitive sterile mutant strain *C. elegans* AU37 [sek-1(km4); glp-4(bn2) I] was used because this strain is sterile at room temperature and capable of laying eggs only at 15 °C. Additionally, the strain is quite susceptible to infection, due to a mutation in the *sek-1* gene of the p38 mitogen-activated protein kinase pathway (33, 34). Briefly, worms were grown for 5 days at 15 °C (permitting worms to lay eggs) on NGM agar plates seeded with a lawn of *E. coli* OP50. Eggs were harvested by bleaching (35) and maintained for 24 hours at room temperature with gentle agitation, for hatching. Hatched larvae were transferred to a new NGM plate seeded with *E. coli* OP50 and were kept at room temperature for 4-5 days until the worms reached their adult growth stage. Adult worms were then collected and washed three times with PBS in a 1:10 (worm:PBS) ratio to remove *E. coli*.

In order to examine the toxicity of the thiazole compounds to *C. elegans*, 15-20 adult worms were transferred to wells of a 96-well microtiter plate. Worms were incubated with either 10 or 20 µg/mL of compounds **1**, **3**, linezolid (positive control), or sterile water (negative control) (in triplicate). After 24, 48, 72, 96, and 120 hours, worms were examined microscopically to examine viability. The number of worms that survived each treatment regimen were counted and results are presented as percent viable worms.

To test the antibacterial activity of the thiazole compounds against VRE *in vivo*, adult worms were transferred to TSA agar plates seeded with a lawn of *E. faecalis* HM-201, for infection (36). After two hours of infection, worms were collected and washed with M9 buffer, three times, before transferring 20-25 worms to wells in a 96-well microtiter plate. Worms were incubated with 20 µg/mL of compounds **1**, **3**, linezolid (positive control), or sterile water (negative control) (in triplicate). After treatment for 20 hours, worms were washed three times with M9 buffer and then examined microscopically to examine morphological changes, and viability. They were subsequently lysed in micro-centrifuge tubes containing 200 mg of 1.0-mm silicon carbide particles (Biospec Products, Bartlesville, OK) that were vortexed for one minute. Samples were serially diluted and plated onto TSA plates containing 50 µg/mL gentamicin to select for VRE. Plates were incubated at 37 °C for 18 hours before viable CFUs were determined.

7.2.16 *In silico* Pharmacokinetic Analysis

The pharmacokinetic profile (oral and intravenous dose of 600 mg) was simulated using chemPK version 2.0 (Cyprotex Inc., Cheshire, United Kingdom), for compounds **1, 3,** and linezolid.

7.3 Results

7.3.1 Antibacterial Activity of Compounds **1-3** Against the ESKAPE Pathogens *Enterococcus faecium, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp

We first investigated the antibacterial activity of phenylthiazole compounds **1-3** against a panel of ESKAPE pathogens using the broth micro-dilution method. Examination of the spectrum of activity of **2-3** revealed that they were inactive against most Gram-negative pathogens (minimum inhibitory concentration, MIC > 128 μg/mL), Table 7.2, the exception being with *A. baumannii* where the MIC was 8 μg/mL, similar to that found with erythromycin. Compound **1** also exhibited limited activity against the same pathogens (MIC ranges from 8-64 μg/mL), indicating that all three compounds are generally ineffective against Gram-negative bacteria. These results are in contrast to our earlier results on *S. aureus*, differences that could be due to the presence of the outer membrane (OM) in Gram-negative bacteria, and/or efflux pumps. We thus next investigated whether the presence of the OM and/or efflux pumps did in fact contribute to the lack of antibacterial activity observed for **1-3** against Gram-negative bacteria.

In the presence of a sub-inhibitory concentration of the membrane-disrupting antibiotic colistin, which permeabilizes the outer membrane, the MIC of compounds **1-3** against Gram-negative bacteria decreased dramatically. For example, **2** and **3** were inactive against *K. pneumoniae*, *P. aeruginosa* and *E. coli* when tested alone (MIC > 128 μg/mL), but in the presence of sub-inhibitory levels of colistin, both compounds exhibited potent antibacterial activity (MIC from 1 to 4 μg/mL). This behavior is similar to that seen with the antibiotic erythromycin, whose activity is known to be impeded by the presence of the OM. For example, with *K. pneumoniae*, *P. aeruginosa*, and *E. coli*, we find that erythromycin alone lacks activity (MIC > 128 μg/mL), but in the presence of colistin, erythromycin has potent activity against each organism (MIC from 0.5 to 1 μg/mL). It thus

appears likely that the OM impedes both phenylthiazole as well as erythromycin entry into Gram-negative bacteria.

When the antibacterial activity of compounds **1-3** was examined against *E. coli* 1411 and a mutant strain containing a deletion of the gene encoding the AcrAB efflux pump, there was a major decrease in the MIC in the mutant. Against *E. coli* 1411, compound **1** inhibited growth at 64 μg/mL, and compounds **2** and **3** were inactive (MIC > 128 μg/mL). However, against the mutant strain (*Escherichia coli* 1411 Δ*acrAB*), all three compounds were potent inhibitors of bacterial growth (MIC of 4 μg/mL). This behavior is similar to that observed with linezolid, a known substrate of the AcrAB efflux pump (37). It thus appears that the lack of activity of the phenylthiazoles against Gram-negative bacterial pathogens is due *both* to the presence of the OM as well as drug efflux pumps. Fortunately, in earlier work we found that compounds **1-3** exhibited potent antibacterial activity against the Gram-positive pathogen, methicillin-resistant *S. aureus* (MIC values ranged from 1.3 to 5.6 μg/mL) (10), and as shown in Table 7.2, all three compounds are potent inhibitors of the growth of another important Gram-positive pathogen, vancomycin resistant *E. faecium* (MIC = 0.5 μg/mL). Thus, the phenylthiazole compounds appear to be potent leads against clinically-relevant Gram-positive pathogens, including MRSA and VRE. Plus, the fact that they synergize with cell wall biosynthesis inhibitors in *S. aureus* suggests, perhaps, a similar target area in enterococci.

7.3.2 Phenylthiazole Compounds Retain Their Potent Activity against Clinical Isolates of Drug-Resistant Enterococci

To further evaluate the antibacterial activity of compounds **1-3** against enterococci, we determined MIC values against 24 strains of *E. faecalis* and *E. faecium* (including 16 strains resistant to vancomycin), isolated from diverse sources including blood, urine, peritoneal fluid, sputum, and feces (Table 7.1), from infected patients. All three compounds exhibited potent antibacterial activity against all isolates tested (Table 7.2). Interestingly, **1-3** were most active against isolates of vancomycin-resistant *E. faecium*, inhibiting growth at concentrations ranging from 0.5 to 4 μg/mL (Table 7.3), with MIC50 values of 1 (for compounds **2** and **3**) to 4 μg/mL (for compound **1**). These values are similar to those we find with the antibiotic linezolid (MIC range from 0.5 to 2 μg/mL). Against *E. faecalis*, **1** and **3** retained their potent antibacterial activity (MIC range from 1-8 μg/mL), but there

was an increase in the MIC of compound **2** (MIC = 16 μg/mL, against multiple strains). However, all compounds proved quite effective at inhibiting the growth of VRE. The compounds also retained activity against isolates exhibiting resistance to ampicillin, ciprofloxacin, gentamicin, streptomycin, erythromycin, and doxycycline. This indicates that cross-resistance between these antibiotics and **1**-**3,** against enterococci, is unlikely to occur.

We next investigated whether the antibacterial effects were bacteriostatic, or bactericidal. To address this, we determined the minimum bactericidal concentrations, MBCs. Against most strains of *E. faecalis* and *E. faecium*, the MBC values were equal to or higher than the MIC values for compounds **1** and **3**, indicating that the two compounds are bactericidal. A similar trend was observed for compound **2** against *E. faecalis*. However, against *E. faecium*, the MBC for **2** was more than four-fold higher than the MIC, indicating **2** may be bacteriostatic, particularly against vancomycin-resistant isolates. As expected, ampicillin exhibited bactericidal activity against enterococci strains sensitive to this antibiotic, while linezolid exhibited bacteriostatic activity against both *E. faecalis* and *E. faecium* (MBC was more than four-fold higher than the MIC).

7.3.3 Compounds **1** and **3** Rapidly Eradicate Vancomycin-Resistant Enterococci as Determined by Time-Kill Analysis

In order to confirm the bactericidal activity of the phenylthiazole compounds against VRE, we carried out time-kill assays. As shown in Figure 7.2, compounds **1** and **3** exhibited rapid bactericidal activity against both vancomycin-resistant *E. faecium* as well as vancomycin-resistant *E. faecalis*. Compound **1** (at 4 × MIC) was capable of completely eradicating both strains of VRE within two hours, and no bacterial re-growth was observed over the following 22 hours. Compound **3** matched compound **1** in completely eliminating vancomycin-resistant *E. faecalis* within two hours (Figure 7.2B), but required four hours to achieve the same effect against vancomycin-resistant *E. faecium* (Figure 7.2A). Compound **2** also exhibited rapid bactericidal activity against *E. faecalis*, completely eradicating bacterial growth within two hours. However, although **2** produced a gradual reduction in CFU against vancomycin-resistant *E. faecium* after 24 hours, the compound was not capable of completely eradicating the bacteria. This supports the MBC results for **2** against vancomycin-resistant *E. faecium*, indicating that **2** is bacteriostatic. The

bacteriostatic activity of linezolid against both E. *faecium* and *E. faecalis* is also confirmed since the antibiotic was not able to generate a 10^3 × reduction in VRE CFU over 24 hours.

7.3.4 Compounds **1-3** Exhibit Limited Toxicity to Human Colorectal Cells

Enterococci are commensal organisms of the human gastrointestinal tract. We thus next examined the toxicity of all three compounds against a human colorectal (HRT-18) cell line. When the compounds were incubated with cells for a short period (two hours), compound **1** was non-toxic up to 20 μg/mL (Figure 7.3A). Compounds **2** and **3** exhibited an improved toxicity profile since both were not toxic up to 40 μg/mL. When compounds were incubated with HRT-18 cells for 24 hours (Figure 7.3B), the toxicity profile of **1** remained the same (not toxic up to 20 μg/mL), while **2** and **3** were toxic at 40 μg/mL, but non-toxic at 20 μg/mL.

7.3.5 Single-Step and Multi-Step Resistance Selection of Enterococci to Compounds **1-3**

Given the promising results described above, we next sought to examine the likelihood that enterococci will develop resistance to **1-3,** using two different methods. First, we attempted to isolate spontaneous mutants exhibiting resistance using a single-step resistance selection experiment. In the presence of a high inoculum of *E. faecalis* NR-31975 (5×10^8 CFU/mL), no resistant mutants to **1-3** were isolated at concentrations of 2 ×, 4 ×, or 8 × MIC. When the inoculum size was increased (to 5×10^{10} CFU/mL), the same result was obtained, indicating a frequency of resistance $> 2 \times 10^{-11}$. We next attempted to isolate resistant mutants to **1-3** via a multi-step resistance selection experiment using two strains of VRE: *E. faecalis* ATCC 51299 and *E. faecium* ATCC HM-968. A four-fold increase in MIC was categorized as resistance. Against *E. faecium* (Figure 7.4A), there was no shift in the MIC observed for compounds **1** and **3** over 14 serial passages (similar to results obtained with linezolid). A one-fold increase (that is, the MIC increases from $x \rightarrow 2x$) in MIC was observed for linezolid after the third passage and for compound **2**, after the eleventh passage. However, no additional increase in the MIC of either agent was observed thereafter. Against *E. faecalis* (Figure 7.4B), a one-fold increase in MIC for compounds **1** and **3** was observed after the sixth passage; no further increase in MIC was observed until the last passage. For compound **2**, a one-fold increase in MIC was observed after the sixth

passage and an additional two-fold increase was observed after the eleventh passage. No additional increase was observed thereafter. No increase in MIC was observed for linezolid over the 14 serial passages. These results indicate a low likelihood for enterococci to develop rapid resistance to, in particular, **1** and **3**.

7.3.6 Compound **1** Exerts its Antibacterial Activity by Inhibiting Cell Wall Synthesis

In order to investigate the mechanism of action of the phenylthiazoles, compound **1** was subjected to Bacterial Cytological Profiling (BCP) in representative Gram-positive (*Bacillus subtilis*) and Gram-negative (*E. coli*) bacteria. BCP identifies the likely pathway targeted by novel antibiotics by comparing their cytological effects with those found using a library of cytological profiles generated by using antibacterials with known mechanisms of action (MOAs), or by the rapid proteolytic depletion of essential proteins (24-26). When *E. coli* Δ*tolC* (which lacks an effective efflux pump) was treated with compound **1**, cells lysed and formed spheroplasts after two hours (Figure 7.5). Spheroplasts and misshapen cells were very prevalent in the presence of 0.5 M sucrose, which osmotically stabilizes cells lacking a functional cell wall. Lysis and cell shape defects were observed as early as 30 minutes after addition of compound **1** to the medium. In contrast, cells incubated with the cell wall biosynthesis inhibitor D-cycloserine formed misshapen cells and spheroplasts after 30 minutes (Figure 7.5), and cells were completely lysed after two hours (data not shown). These results suggest that compound **1** inhibits cell wall biosynthesis in *E. coli* Δ*tolC*.

To determine whether **1** had the same effect in a Gram-positive bacterium, we examined the effects of **1** in *B. subtilis*, again using BCP. *B. subtilis* incubated with **1** at 5 × MIC rapidly lysed, with 95% (n = 131) of cells being permeable to Sytox Green (a nucleic acid stain that is impermeable to live cells) within 30 minutes of treatment. Since disruption of either the cell wall or the cell membrane can result in permeabilized cells (Figure 7.6), we next investigated the effects of **1** with control agents in the presence of dimethylsulfone (a.k.a. methylsulfonyl methane, MSM), which osmotically stabilizes cells for better observation of cell shape defects. Cells treated with **1** for two hours in the presence of MSM were slightly misshapen or bent and contained pools of membrane (Figures 7.7 and Figure 7.8). Figure 7.8 shows four examples of cells containing a small bulge at the site of

the bend that could be visualized with phase contrast microscopy. These cells appeared very similar to vancomycin-treated cells, which also show subtle cell shape-defects, forming bends, bulges, and pools of membrane (Figure 7.7 and Figure 7.4A). Unlike compound **1** and vancomycin, D-cycloserine generated very obvious cell shape defects (Figure 7.7), and Triton X-100 detergent-treated cells were lysed without affecting overall cell shape. MSM suppresses cell lysis and permeability defects for cell wall active antibiotics, but not for membrane active compounds (Figure 7.8 & Figure 7.9) (24). We found MSM dramatically suppressed the permeability phenotypes of compound **1**, as well as vancomycin and D-cycloserine, but had no effect on Triton X-100 treated cells, suggesting that **1** inhibits cell wall/peptidoglycan synthesis (Figure 7.8) in both *B. subtilis* and *E. coli* Δ*tolC*, implicating broadly conserved target(s) and MOA. But what are the actual targets of **1**?

7.3.7 Target Identification

Peptidoglycan biosynthesis involves numerous enzymes and a simplified metabolic pathway is shown in Figure 7.10. The first step involves the sequential addition of two molecules of isopentenyl diphosphate (IPP, **4**) to dimethylallyl diphosphate (DMAPP, **5**) to form the (C15) isoprenoid farnesyl diphosphate (FPP, **6**) in a reaction catalyzed by farnesyl diphosphate synthase (FPPS), with IPP/DMAPP being produced by the mevalonate pathway in *S. aureus* and the non-mevalonate (methylerythritol phosphate, MEP) pathway in *B. subtilis* and *E. coli*. FPP then reacts with 8 additional IPP molecules to form the (C55) isoprenoid undecaprenyl diphosphate (UPP, **7**) in a reaction catalyzed by undecaprenyl diphosphate synthase (UPPS). UPP is converted to undecaprenyl monophosphate (UP, **8**) by undecaprenyl diphosphate phosphatase (UPPP), then UP reacts with UDP-N-acetylmuramyl pentapeptide (**9**) to form Lipid I (**10**) in a reaction catalyzed by MraY, followed by conversion to Lipid II, and after several more steps, peptidoglycan (11) is formed. Drugs such as ampicillin and vancomycin inhibit these later stages in cell wall synthesis as they interfere with peptidoglycan crosslinking.

We first sought to see if **1** resulted in changes in the concentration of compound **9**, the final soluble cell wall precursor in peptidoglycan biosynthesis, in *E. faecalis* NR-31975 cells. We found that treatment of cells with compound **1** resulted in a similar LC-MS result

to treatment of cells with vancomycin, a known inhibitor of bacterial cell wall synthesis. This can be seen in the results shown in Figure 7.11 in which there are large increases in compound **9** accumulation with **1** or vancomycin treatment, implicating inhibition of peptidoglycan biosynthesis. A peak was present in the chromatograms at the same retention time (~8.76 minutes) for both **1** and vancomycin-treated samples, and had the correct m/z for the pentapeptide, *m/z* = 1150.3588, a <1 ppm error. These results support inhibition of a target in the peptidoglycan biosynthesis pathway, but they do not suggest a specific molecular target.

We next tried to identify the molecular target of the phenylthiazole **1** by using a target overexpression experiment, in *Bacillus subtilis*. The metabolic function(s) in *B. subtilis* inhibited by the compound should in principle be restored by over-expression of the targeted protein(s) via genomic insertion of a transposon with a strong outward-oriented promoter. That is, resistance to compound **1** should be achieved by over-expression of the drug-resistance gene—which could be a molecular target in peptidoglycan biosynthesis—but also perhaps an efflux pump (28). In the presence of a high concentration of compound **1**, only bacterial colonies where the transposon successfully inserts adjacent to the biological target/resistance mechanism survive, due to overexpression of the target/resistance mechanism by the bacterium. Using this approach we identified three possible targets: 1) *yubA*, locus tag BSU31160, a putative inner membrane AI-2E (autoinducer-2-exporter) family protein; 2) *yubB*, locus tag BSU31150, undecaprenyl diphosphate phosphatase (UPPP) and 3) *yubD*, locus tag BSU31130, a putative major facilitator superfamily transporter. Clearly, these results strongly suggest UPPP as a likely target since UPPP is in the peptidoglycan biosynthesis pathway, Figure 7.10. If UPPP (YubB) is inhibited by **1**, there would be a decrease in UPP and UP levels and this would be expected to lead to accumulation of the MraY substrate **9** (since it would not have a substrate to react with).

To test this hypothesis we used the *E. coli* UPPP expression system described previously (30, 38) and determined the IC_{50} for inhibition by **1**. We found a 6 μM IC_{50} (corresponding to 2 μg/mL), consistent with a UPPP target, Figure 7.12A. We also tested for HsFPPS and EcUPPS inhibition. There was no inhibition of FPPS (data not shown), but UPPS was inhibited with a 19 μM IC_{50} (corresponding to 6.3 μg/mL), Figure 7.12B.

What is interesting here is that UPPS as well as UPPP are both inhibited at low μM levels and since these two enzymes are adjacent to each other in the biosynthetic pathway, this multi-target inhibition is expected to contribute to their activity, in cells, and is very similar to the dual UPPS/UPPP inhibition we have reported with other inhibitors (38).

What, then—if anything—is the involvement of YubA and YubD in the activity of **1** in cells? At present, these proteins have not been characterized in detail but both are annotated as transporters, raising the question: do the phenythiazoles also target membrane transporters? Upon inspection of the structure of **1** (as well as **2**, **3**) it is clear that each compound has a polar aminoguanidine "headgroup" (pKa ~7) and a lipophilic "tail". In other work, we and others have shown that many such compounds—lipophilic bases—can act as protonophore uncouplers, collapsing the proton motive force (PMF) in cells, as determined by using fluorescence probes as well as by ^{31}P NMR spectroscopy (39, 40). That work led to a re-appraisal of the mechanism of action (MOA) of the tuberculosis (TB) drug lead SQ109, as well as the MOA of TB drugs in clinical use such as bedaquiline and clofazimine (40). More importantly, many TB drug leads that had been thought to target the trehalose monomycolate transporter MmpL3 (mycobacterial membrane protein large 3) in a direct fashion are now thought to actually function by collapsing the PMF, inhibiting the function of PMF-driven transporters. If **1** were to also collapse the PMF, this could indirectly inhibit the transporters YubA and YubD, identified in the transposon mutagenesis experiments.

To determine whether **1** is a protonophore uncoupler, we used the *E. coli* inverted membrane vesicle (IMV) system described previously (41). Results with **1** and the potent, known uncoupler CCCP (m-chlorophenyl carbonyl cyanide phenylhydrazone) are shown in Figure 7.12C and Figure 7.12D with both ATP-powered PMF generation as well as succinate/O_2-powered PMF generation. The IMVs have their ATPase on the outside of the vesicle so ATP hydrolysis through the ATPase, or succinate/O_2, drives H^+ into the vesicles, the fluorophore ACMA (9-amino-6-chloro-2-methoxyacridine) accumulates and its fluorescence is self-quenched (the signal goes down). Addition of CCCP or **1** collapses the PMF and fluorescence increases (back to normal). For CCCP, the IC_{50} is 0.4 μM in ATP and 0.2 μM in succinate; for **1**, the EC_{50} for PMF collapse is 3.8 μg/mL in ATP and 6.6

μg/mL in succinate, Figure 7.12C and Figure 7.12D. This is relatively weak uncoupling but could inhibit some transporters, including drug efflux pumps.

At present, the three-dimensional structures of YubA, YubB (UPPP) and YubD have not been reported. However, a structural model for UPPP (together with site-directed mutagenesis results) for UPPP has been reported (30) and is shown in Figure 7.13, together with RaptorX predicted structural models for YubA and YubD, and an X-ray structure for UPPS (42) (PDB ID code 1X06 (43)). YubA and YubD are both membrane proteins and proteins with known structures were used to create these homology models are all transporters, including PMF-driven multi-drug efflux pumps. We thus propose that **1** inhibits both UPPS and UPPP by directly binding to these proteins, in addition to potentially affecting YubA and/or YubD function, by acting as a protonophore uncoupler, reducing ΔpH, with such multi-targeting contributing to the very low rate of resistance that we observe.

7.3.8 Resensitization of Enterococci to the Effects of Other Antibiotics

The discovery that the phenylthiazole **1** inhibits peptidoglycan synthesis led us to investigate its ability to resensitize VRE to the effects of known antibiotics. Compounds **1**-**3** have thus far been shown to be potent single-agent inhibitors of the growth of drug-resistant strains of *S. aureus*, *E. faecium* and *E. faecalis*. However, combination therapy using two or more antibiotics has multiple potential advantages over monotherapy including reducing the size and frequency of doses needed to resolve infection while mitigating toxicity issues associated with single agents (such as vancomycin). Additionally, pairing a bactericidal agent (such as cell wall synthesis inhibitors) with an aminoglycoside is already known to be necessary for treating certain enterococcal infections, such as endocarditis.

Previously, we demonstrated that **1** resensitizes vancomycin-resistant *S. aureus* (VRSA) to vancomycin (11). With aminoglycoside antibiotics, resistance is due to an inability to cross the cell wall to reach the bacterial ribosome (5), and increased accumulation of such antibiotics has been observed in enterococci in the presence of a cell wall synthesis inhibitor (5). We therefore next examined whether VRE exposed to a sub-inhibitory concentration (½ × MIC) of compound **1** were re-sensitized to the effects of

vancomycin and gentamicin. Phenylthiazole **1** was able to re-sensitize *E. faecium* ATCC 700221 to the effect of vancomycin —a 256-fold decrease in the MIC, Table 7.5. However, against *E. faecium* HM-968 and two strains of E. *faecalis* (HM-201 and HM-934), no resensitization to vancomycin was observed. Likewise, a sub-inhibitory concentration of **1** was unable to resensitize either *E. faecium* (ATCC 700221) or *E. faecalis* (ATCC 51299) to the effect of gentamicin (data not shown). Nevertheless, the results found with vancomycin were of interest, so we next examined whether the thiazole compounds would exhibit synergistic relationships with antibiotics frequently used to treat enterococcal infections. Using checkerboard assays, we first tested compound **1** in combination with ampicillin, ciprofloxacin, doxycycline, or linezolid against *E. faecalis* ATCC 51299. While ampicillin (FIC index = 3.00), doxycycline (FIC index = 2.00), and linezolid (FIC index = 2.00) exhibited indifferent or additive relationships, ciprofloxacin demonstrated synergy with compound 1 (FIC index = 0.50). We then examined if synergisms between compounds **1-3** and ciprofloxacin would be observed when tested against additional strains of *E. faecalis* (ATCC 49532 and ATCC 49533), but all three compounds exhibited an additive relationship against both strains (FIC index ranging from 0.63 to 1.00, Table 7.6).

7.3.9 Compounds **1** and **3** Retain Their Potent Antibacterial Activity *in vivo* Against VRE

The finding that the phenylthiazole compounds possess good *in vitro* activity against many VRE strains and exert their antibacterial effect by targeting cell wall synthesis prompted us to investigate the efficacy of these compounds *in vivo* in a *C. elegans* animal model. Vancomycin-resistant *E. faecalis* HM-201, a highly pathogenic strain, was examined with compounds **1** and **3** since these compounds exhibited rapid bactericidal activity, *in vitro*. Based upon the results from the HRT-18 cell growth inhibition experiments, we chose a dose of 20 μg/mL. To verify that this concentration was not toxic to *C. elegans*, worms were exposed to compounds **1**, **3**, and a control antibiotic (linezolid), and viability was observed over a 24-hour period. All worms survived when exposed to compound **1** (Figure 7.14A) as well as linezolid, and 90% survived after exposure to compound **3**. *C. elegans* AU37 were then infected with VRE HM-201 and subsequently treated for 18 hours with 20 μg/mL **1**, **3**, linezolid, or PBS. After treatment, worms were lysed and the VRE burden inside infected worms determined. Compounds **1** (89% decrease) and **3** (94% decrease)

produced significant decreases in VRE burden in infected *C. elegans* (Figure 7.14B). In contrast, the bacteriostatic antibiotic linezolid was unable to reduce the burden of VRE in infected worms at this concentration.

7.3.10 *In silico* Examination of the Pharmacokinetic Profile of Compounds **1** and **3**

The promising results obtained in the *C. elegans* infection experiment led us to examine the pharmacokinetic profiles of compounds **1** and **3** in order to identify appropriate routes of administration. Utilizing computer modeling, the pharmacokinetic profiles of both compounds, in addition to linezolid, were simulated utilizing a dose of 600 mg (as is commonly administered for linezolid in human patients for the treatment of enterococcal infections). As shown in Table 7.7, the results indicate that neither compound **1** nor compound **3** would be suitable for oral use for the treatment of systemic enterococcal infections since neither is predicted to reach a concentration in plasma/blood sufficient to inhibit bacterial growth. The maximum plasma concentration (C_{max}) predicted for compound **1** is 1.02 μg/mL, whereas the MIC_{50} ranges from 2 to 4 μg/mL. Similarly for compound **3**, the C_{max} is predicted to be 1.83 μg/mL, while the MIC_{50} ranges from 1 to 8 μg/mL. Intravenous administration of compounds **1** and **3** is predicted to result in slow rates of clearance (8.22 mL/min-kg and 8.71 mL/min-kg, respectively) and moderate half-lives (6.42 and 8.38 hours, respectively) which could alleviate the need for multiple daily dosing. The low values obtained for the volume of distribution at steady-state (2.10 L/kg for compound **1** and 2.55 L/kg for compound **3**) are similar to the value obtained for linezolid (1.12 L/kg), indicating that **1** and **3** are not expected to distribute extensively into tissues. These pharmacokinetic simulations clearly indicate, then, that intravenous administration of **1**, **3** would be required for treatment of systemic enterococcal infections.

7.4 Discussion

The burden of resistance to currently available antibiotics necessitates the development of new antibacterial agents, targeting in particular the ESKAPE microorganisms, a significant threat given their ability to evade many antibiotics. One member of this group are the vancomycin-resistant Enterococci, which are commensal microorganisms of the human gastrointestinal tract. Their intrinsic resistance (or reduced susceptibility) towards multiple

antibiotics (including penicillin-based antibiotics, cephalosporins, aminoglycosides, fluoroquinolones, and trimethoprim-sulfamethoxazole) limits the number of therapeutic agents available (44). In addition, some species exhibit differing levels of sensitivity to specific antibiotics, which further complicates treatment options. For example, although *E. faecium* is typically susceptible to clindamycin and quinupristin-dalfopristin, some strains of *E. faecalis* are resistant to both agents (45).

The present study identifies three phenylthiazole compounds that exhibit potent activity against both drug-resistant *E. faecalis* and *E. faecium*. When examined against a clinically-relevant panel of *E. faecalis*, the MIC_{50} of compounds **1-3** ranged from 4 to 8 μg/mL. Against a panel of *E. faecium*, the MIC_{50} of compounds **1-3** ranged from 1 to 8 μg/mL. The compounds maintain their activity against strains exhibiting resistance to numerous antibiotics including ampicillin, linezolid, and vancomycin, an important finding given the emergence of ampicillin-resistant strains of *E. faecium*. Although vancomycin has been frequently used to treat infections caused by these strains, more than 80% of ampicillin-resistant *E. faecium* in the United States now exhibit resistance to glycopeptide antibiotics like vancomycin. Additionally, these strains exhibit high-level resistance to aminoglycoside antibiotics such as gentamicin and streptomycin (45). Given that compounds **1-3** exhibit potent activity against both ampicillin-resistant and vancomycin-resistant enterococci, they represent potentially important leads for the treatment of drug-resistant infections caused by both *E. faecium* and *E. faecalis*.

As noted above, enterococci are intrinsically resistant to many antibiotics. However, enterococci also have the ability to acquire genetic material via horizontal gene transfer. Indeed, more than 25% of the genome of *E. faecalis* is composed of DNA acquired externally (46). This ability to acquire exogenous genomic material has contributed in part to the rapid development of resistance to newer antibacterial agents, such as daptomycin and tigecycline (45). We thus examined if we could isolate enterococcal mutants exhibiting resistance to the phenylthiazoles. Initially, a single-step resistance selection experiment using *E. faecalis* NR-39175 was conducted in order to isolate mutants exhibiting resistance to compounds **1-3**. Even at an inoculum size > 10^{10} CFU/mL, no resistant mutants were isolated, which corresponds to a low frequency of mutation, > 2×10^{-11}. Next, we serially passaged compounds **1-3** against two vancomycin-resistant strains (one *E. faecalis* and one

E. faecium strain) over a 14-day period. Once again, no resistant mutants (categorized as a >four-fold increase in MIC) were isolated, indicating that rapid resistance to these thiazole agents is unlikely to occur.

To further understand the antibacterial activity of these phenylthiazoles, Bacterial Cytological Profiling (BCP) was employed to narrow down the compounds' mechanism of action. BCP suggests the mechanism of action of an antibiotic by comparing changes in cytological parameters for new compounds to those observed using a library of known drugs. Since BCP has not yet been developed for *E. faecalis* or *E. faecium*, we examined the effect of compound **1** in *B. subtilis*, as well as in an efflux pump-defective mutant of *E. coli* that is sensitive to many antibiotics that are typically ineffective against Gram-negative bacteria. In both organisms, we obtained results that suggested that inhibition of cell wall synthesis was a likely target. In *E. coli*, cells lysed or formed spheroplasts as early as 30 minutes after exposure to compound **1**. In *B. subtilis*, notable cell shape defects were observed, similar to the effects seen with vancomycin in osmotically buffered media. We then found an increased accumulation of the final soluble precursor of peptidoglycan synthesis in the bacterial cytoplasm (**9**, UDP-N-acetylmuramyl pentapeptide) both in the presence of compound **1** and vancomycin, again implicating inhibition of peptidoglycan (cell wall) biosynthesis.

We next utilized a transposon mutagenesis assay that suggested three possible targets: the isoprenoid biosynthesis protein undecaprenyl diphosphate phosphatase (UPPP, a.k.a. *yubB*) as well as two transporters, *yubA* and *yubD*. We found that **1** inhibited an expressed UPPP (YubB) at low μg/mL concentrations, in addition to inhibiting the previous enzyme in the pathway, UPPS (at 6.3 μg/mL). The roles of YubA and YubD are currently unknown, but based on bioinformatics and computational modeling they are both predicted to be transporters (with some template models being PMF-driven multi-drug efflux pumps). This suggested the possibility that **1** might be a protonophore uncoupler, which (using *E. coli* inverted membrane vesicles) was found to be the case.

Taken together, these results suggested that the phenylthiazoles might resensitize VRE to the effects of vancomycin and the aminoglycoside antibiotic gentamicin since in earlier work we reported that compounds **1** and **2** were capable of resensitizing vancomycin-resistant *S. aureus* to the effects of vancomycin. When similar experiments

were conducted with compound **1** against VRE, only one strain of vancomycin-resistant *E. faecium* (ATCC 700221) was resensitized with a large improvement in the MIC being observed in the presence of a sub-inhibitory concentration of **1**. Sensitivity to vancomycin was not restored in the remaining VRE strains. Closer inspection of the susceptibility data of these strains to vancomycin suggested one reason for this behavior: the MIC of vancomycin against *E. faecium* ATCC 700221 was 256 μg/mL, but in the remaining strains, the MIC of vancomycin was 512 μg/mL or higher. So, strains of VRE that exhibit high-level resistance to vancomycin may not be amenable to resensitization, and a similar pattern has been observed with high-level resistance to aminoglycoside antibiotics (47).

As noted above, recent reports have demonstrated that antibacterial agents that inhibit peptidoglycan biosynthesis can lead to increased uptake of aminoglycoside antibiotics, by enterococci (5). Thus, enterococci become susceptible to aminoglycoside antibiotics in the presence of a sub-inhibitory concentrations of cell wall biosynthesis inhibitors. However, we did not observe this when we examined our compounds, at sub-inhibitory concentrations, with gentamicin. This is likely due to the fact that the isolates tested exhibited high level resistance to aminoglycosides (MIC > 128 mg/L), which has been shown to nullify the effectiveness of pairing a cell wall biosynthesis inhibitor with an aminoglycoside (45). High level resistance to aminoglycosides occurs through enterococci acquiring mutations in the target (ribosome) or enzymes (such as aminoglycoside acetyltransferases, AACs) that transfer acetyl groups to the amino groups of aminoglycoside antibiotics, rendering them ineffective (48). Thus, the ability of the phenylthiazole compounds to resensitize VRE to the effects of vancomycin (and potentially, aminoglycosides) may be strain-specific, and be limited to isolates exhibiting low-to-moderate resistance.

The final step in our study was to determine if the thiazole compounds retained antibacterial activity *in vivo* against VRE in an animal model of infection. We used *C. elegans*, a well-established *in vivo* model for early-stage drug discovery (33, 49, 50). Both compounds **1** and **3** (at 20 μg/mL) proved superior to the bacteriostatic antibiotic, linezolid, in reducing the burden of VRE in infected worms. However, *in silico* pharmacokinetic analysis revealed that oral administration of these compounds (simulating a dose of 600 mg) would not achieve plasma concentrations sufficient to inhibit VRE growth, due to

limited permeability across the gastrointestinal tract. Two reasons behind the expected limited oral bioavailability are that the aminoguanidine is charged at physiological pH and might be a substrate for the P-gp efflux system (51) and previously, a Caco-2 bidirectional permeability analysis of compounds **1** and **3** revealed limited ability to cross from the apical to basolateral surface of a polarized monolayer of cells. While this may limit the utility of these compounds for oral treatment of systemic enterococcal infections, it may open the door for use intravenously (for systemic infections) or for use as decolonizing agents. That is, an alternative approach to quelling infection is to reduce or eliminate VRE from the gastrointestinal tract of colonized patients at high risk of infection, including organ transplant recipients and patients undergoing chemotherapy (4, 52).

For example, VRE colonization in liver transplant patients has been linked to an increased risk of infection and death (53). However, decolonization of VRE is very difficult given that enterococci in the gastrointestinal tract can range from 1–10 million CFU/g of stool (53). Bacteriostatic antibiotics such as linezolid are unable to significantly reduce this burden. Other antibacterial agents examined for use as decolonizing agents including bacitracin and gentamicin are problematic since many patients are unable to tolerate the side-effects associated with treatment (53). The ability of the phenylthiazole compounds to rapidly eradicate a high inoculum of VRE within two hours (as shown with the time-kill assay) suggests that they warrant further investigation as decolonizing agents. Specifically, their inability to cross the GI tract and reach the bloodstream may prove beneficial. Ramoplanin, an orally administered lipoglycodepsipeptide antibiotic that inhibits cell wall synthesis was recently approved for the treatment of *Clostridium difficile* infection. As with our compounds, ramoplanin is not absorbed systemically, but when administered orally, was shown to suppress gastrointestinal colonization of VRE in up to 90% of patients in a phase II clinical trial. A phase III clinical trial validated the efficacy of ramoplanin as a decolonizing agent to prevent bloodstream infections caused by VRE (53).

An alternative application for the phenylthiazoles would of course be to examine their ability to treat skin infections caused by enterococci. Though not as frequent as infections caused by *S. aureus*, enterococci have been associated with 8% of all complicated skin and soft tissue infections, particularly in the lower extremities, and in polymicrobial infections (54, 55). Polymicrobial skin infections caused by *S. aureus*,

enterococci, and other bacterial species were present in nearly one half of all affected patients, in one study (55). We previously demonstrated that compounds **1** and **3** were potent topical antibacterial agents in a murine model of MRSA skin infection with both compounds reducing the burden of MRSA in infected skin wounds by 96% (10). Future studies with these phenylthiazole compounds will aim to address their potential application as decolonizing agents, as well as topical antibacterial agents for the treatment of enterococcal skin infections in animal models.

7.5 Conclusion

In this study, we demonstrate that three phenylthiazole compounds exhibit potent antimicrobial activity *in vitro* against clinically-relevant strains of vancomycin-resistant enterococci. These agents are rapidly bactericidal against both *E. faecalis* and *E. faecium* and are not toxic to mammalian tissues, at 20 μg/mL, both in cell culture (HRT-18) and in an animal model (*C. elegans*). We were not able to isolate VRE mutants exhibiting resistance to these compounds. The phenylthiazoles exert their antibacterial effect at least in part by inhibiting isoprenoid biosynthesis, targeting undecaprenyl diphosphate phosphatase (UPPP) and undecaprenyl diphosphate synthase (UPPS). The results of transposon mutagenesis identified UPPP (YubB) as well as YubA and YubD as possible targets. The latter two are putative transporters and we find that in addition to inhibiting UPPP and UPPS, **1** collapses the PMF (in membrane vesicles), suggesting it may also block YubA and/or YubD function. The antimicrobial activity of **3** against VRE was confirmed *in vivo* in a *C. elegans* whole animal model (the compounds reduced the bacterial load of infected worms by ~ 90%). Collectively, the results provide valuable information to be used in the development of this class of compounds as antimicrobial and decolonizing agents for infections caused by vancomycin-resistant enterococci.

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Table 7.1 Strains of *E. faecium* and *E. faecalis* utilized in this study.

Strain ID	Alternate Strain ID	Isolated From	Year	Source	Antimicrobial Resistance Phenotype
E. faecalis ATCC 49532	UWH 1921	Wisconsin, USA[1]	-	Blood	Gentamicin
E. faecalis ATCC 49533	UWH 1936	Wisconsin, USA	-	Blood	Streptomycin
E. faecalis NR-31887	B3336	-	1987	Blood	Gentamicin
E. faecalis NR-31975	MMH594	Wisconsin, USA	1985	Blood	Erythromycin and gentamicin
E. faecalis ATCC 29212	-	-	-	Urine	-
E. faecalis ATCC 51299 (VRE)[2]	NJ-3	Missouri, USA	-	Peritoneal fluid	Vancomycin
E. faecalis HM-201 (VRE)	TX0104	Connecticut, USA	2002	Blood of endocarditis patient	Ciprofloxacin and vancomycin
E. faecalis HM-934 (VRE)	ERV103	Bogota, Columbia	2006	Human secretion	Ciprofloxacin and vancomycin
E. faecalis NR-31972 (VRE)	SF28073	Michigan, USA	2003	Urine	Erythromycin, gentamicin, and vancomycin
E. faecalis HM-334 (VRE)	S613	-	2004	Blood	Vancomycin
E. faecalis HM-335 (VRE)	R712	-	2004	Blood	Vancomycin
E. faecium HM-204	TX1330	Texas, USA	1994	Feces	
E. faecium HM-463	TX0133a04	Texas, USA	2006	Blood of diabetic patient with endocarditis	Ampicillin and ciprofloxacin
E. faecium HM-959	513	-	-	-	Ampicillin, ciprofloxacin, and doxycycline
E. faecium NR-28979 (VRE)	E1162	France	1997	Blood	Ampicillin and vancomycin
E. faecium ATCC 700221 (VRE)	-	Connecticut, USA	-	Feces	Teicoplanin and vancomycin
E. faecium HM-968 (VRE)	ERV102	Colombia	2006	Oral sputum	Vancomycin
E. faecium NR-31914 (VRE)	E0120	Netherlands	1995	Ascites fluid	Vancomycin

Table 7.1 continued

E. faecium NR-31912 (VRE)	Patient #3-1	-	-	Stool	Vancomycin
E. faecium NR-31909 (VRE)	Patient #2-1	-	-	Stool	Vancomycin
E. faecium NR-31903 (VRE)	Patient #1-1	-	-	Stool	Linezolid and vancomycin
E. faecium NR-31915 (VRE)	E0164	Netherlands	1996	Turkey feces	Gentamicin and vancomycin
E. faecium E1071 (VRE)	-	Netherlands	2000	-	Vancomycin
E. faecium NR-31916 (VRE)	E0269	Netherlands	1996	Turkey feces	Gentamicin and vancomycin

[1]USA = United States of America
[2]VRE = vancomycin-resistant enterococci

Table 7.2 Minimum inhibitory concentration (MIC) of thiazole compounds **1-3** and control antibiotics against the ESKAPE pathogens (excluding *S. aureus*). For Gram-negative bacteria, the MIC in the presence of ¼ × MIC of colistin (COL) was used to examine the impact of the outer membrane on negating the antibacterial activity of the compounds.

	1		**2**		**3**		**Erythromycin**		**Linezolid**
Bacterial strain	**(-) COL**	**(+) COL**	**(-) COL**	**(+) COL**	**(-) COL**	**(+) COL**	**(-) COL**	**(+) COL**	
Acinetobacter baumanii ATCC 19606	8	1	8	1	8	1	8	0.5	-
Escherichia coli O157:H7 ATCC 35150	16	4	>128	4	>128	1	64	1	-
Enterobacter cloacae BAA-1154	64	1	>128	1	>128	1	128	1	-
Klebsiella pneumoniae BAA-1706	16	1	>128	2	>128	1	128	0.5	-
Pseudomonas aeruginosa ATCC 15442	64	1	>128	1	>128	1	128	1	-
Escherichia coli 1411	64	-	>128	-	>128	-	-	-	>128
Escherichia coli 1411 Δ*acrAB*	4	-	4	-	8	-	-	-	8
Enterococcus faecium (VRE) ATCC 700221	0.5	-	0.5	-	0.5	-	-	-	0.5

Table 7.3 Minimum inhibitory concentration (MIC_{50}) (in μg/mL) and minimum bactericidal concentration (MBC_{50}) of thiazole compounds **1-3**, vancomycin, and linezolid against 50% of vancomycin-sensitive (VSE) and vancomycin-resistant *Enterococcus faecalis* or *E. faecium* (VRE) strains.

Bacterial species (number of isolates)	**1**		**2**		**3**		**Vancomycin**		**Linezolid**	
	MIC_{50}	MBC_{50}	MIC_{50}	MBC_{50}	MIC_{50}	MBC_{50}	MIC_{50}	MBC_{50}	MIC_{50}	MBC_{50}
Enterococcus faecium (3)	4	4	8	16	4	4	1	-	2	16
Vancomycin-resistant *Enterococcus faecium* (10)	2	4	1	16	1	4	>64	>64	1	128
Enterococcus faecalis (5)	4	4	8	16	8	8	1	-	1	64
Vancomycin-resistant *Enterococcus faecalis* (6)	4	4	2	4	4	4	>64	>64	1	64

Table 7.4 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of thiazole compounds **1**-**3**, ampicillin, ciprofloxacin, doxycycline, vancomycin, and linezolid against six vancomycin-sensitive (VSE) and eight vancomycin-resistant *Enterococcus faecalis* or *E. faecium* (VRE) strains.

	1		**2**		**3**		**Ampicillin**		**Ciprofloxacin**		**Doxycycline**		**Vancomycin**		**Linezolid**	
Strain	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. faecalis ATCC 49532	1	4	2	4	2	4	0.5	1	1	1	0.5	32	1	-	1	64
E. faecalis ATCC 49533	8	8	8	16	8	16	0.5	0.5	1	1	>64	>64	1	32	1	64
E. faecalis NR-31887	8	8	16	16	8	8	0.5	0.5	0.5	0.5	32	>64	1	8	1	16
E. faecalis NR-31975	4	4	2	2	2	4	-	-	-	-	-	-	1	64	1	128
E. faecalis ATCC 29212	4	-	16	-	8	-	-	-	-	-	-	-	4	-	4	-
E. faecalis ATCC 51299 (VRE)	2	2	2	4	2	4	-	-	-	-	-	-	16	-	1	128
E. faecalis HM-201 (VRE)	8	8	16	16	4	4	0.5	0.5	64	>64	1	32	64	64	1	16
E. faecalis HM-934 (VRE)	8	8	16	16	8	8	1	1	>64	>64	1	64	>64	>64	1	64

Table 7.4 continued

E. faecalis NR-31972 (VRE)	2	4	2	16	4	4	-	-	-	-	-	-	>128	ND[1]	1	64
E. faecalis HM-334 (VRE)	4	4	2	2	2	2	-	-	-	-	-	-	>64	>64	1	32
E. faecalis HM-335 (VRE)	2	2	1	1	1	1	-	-	-	-	-	-	>64	>64	1	32
E. faecium HM-204	4	16	8	32	4	8	1	1	1	1	1	32	1	16	2	32
E. faecium HM-463	4	4	8	16	4	4	16	32	>64	>64	0.5	16	0.5	0.5	0.5	8
E. faecium HM-959	4	4	8	8	4	4	>64	>64	>64	>64	>64	>64	1	16	2	16
E. faecium NR-28979 (VRE)	2	4	1	16	1	4	0.5	0.5	2	2	32	>64	>64	>64	1	16
E. faecium ATCC 700221 (VRE)	0.5	2	0.5	2	0.5	0.5	-	-	-	-	-	-	>64	ND	0.5	>128
E. faecium HM-968 (VRE)	2	4	1	16	1	4	-	-	-	-	-	-	>64	ND	0.5	128
E. faecium NR-31914 (VRE)	2	4	1	32	2	4	-	-	-	-	-	-	>128	ND	1	>128

Table 7.4 continued

E. faecium NR-31912 (VRE)	2	4	2	16	2	4	-	-	-	-	-	-	>64	>64	2	32
E. faecium NR-31909 (VRE)	2	2	1	1	1	1	-	-	-	-	-	-	64	-	1	8
E. faecium NR-31903 (VRE)	2	4	2	16	1	4	-	-	-	-	-	-	>64	>64	32	>64
E. faecium NR-31915 (VRE)	4	4	1	1	1	8	-	-	-	-	-	-	4	ND	2	64
E. faecium E1071 (VRE)	4	4	4	16	4	4	-	-	-	-	-	-	>64	>64	2	32
E. faecium NR-31916 (VRE)	4	4	2	2	2	2	-	-	-	-	-	-	>64	>64	1	32

[1]ND = Not determined

Table 7.5 Minimum inhibitory concentration (MIC) (μg/mL) of vancomycin alone and in the presence of a subinhibitory concentration of compound **1** against vancomycin-resistant enterococci.

Strain	**Vancomycin**	**Vancomycin + ½ × MIC Compound 1**	**Fold-improvement in MIC of vancomycin**
E. faecium ATCC 700221	256	1	256-fold
E. faecium HM-968	1024	>128	No change
E. faecalis HM-201	512	>128	No change
E. faecalis HM-934	>512	>128	No change

Table 7.6 Fractional inhibitory concentration (FIC) index of thiazole compounds **1**-**3** tested in combination with ciprofloxacin against enterococci via the checkerboard assay.

Test Combination	***E. faecalis* ATCC 51299**	***E. faecalis* ATCC 49532**	***E. faecalis* ATCC 49533**
1 + Ciprofloxacin	0.50	0.75	0.63
2 + Ciprofloxacin	-	1.00	0.75
3 + Ciprofloxacin	-	1.00	1.00

Table 7.7 *In silico* pharmacokinetic analysis for compounds **1**, **3**, and linezolid (simulated at 600 mg).

	Oral			**Intravenous**		
	Compound **1**	Compound **3**	Linezolid	Compound **1**	Compound **3**	Linezolid
C_{max}[1] (μg/mL)	1.02	0.82	5.33	-	-	-
t_{max}[2] (hours)	1.67	1.83	2.75	-	-	-
AUC_{last}[3] (μg -hour/mL)	7.32	6.31	104.18	17.33	16.29	122.21
Fraction absorbed (FA_{last})	0.86	0.84	0.92	-	-	-
Bioavailability, F (%)	42.1	38.48	79.77	-	-	-
CL[4] (mL/min-kg)	-	-	-	8.22	8.71	1.09
$t_{1/2}$[5] (hours)	-	-	-	6.42	8.38	12.31
MRT[6] (hours)	-	-	-	4.25	4.87	17.04
V_d[7] (L/kg)	-	-	-	4.57	6.32	1.11
V_{ss}[8] (L/kg)	-	-	-	2.10	2.55	1.12
AUC (μg -hour/L)	-	-	-	17.38	16.40	130.60

[1]C_{max} = maximum concentration of drug in plasma/blood
[2]t_{max} = time required to reach C_{max}
[3]AUC = area under the curve
[4]CL = rate of clearance
[5]$t_{1/2}$ = half-life
[6]MRT = mean residence time
[7]V_d = volume of distribution
[8]V_{ss} = volume of distribution at steady-state

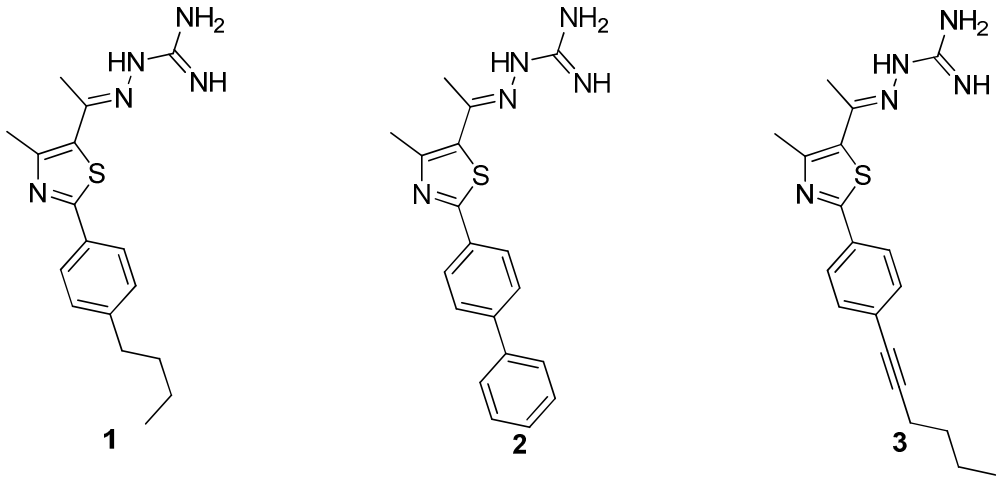

Figure 7.1 Chemical structures of thiazole compounds presented in this study.

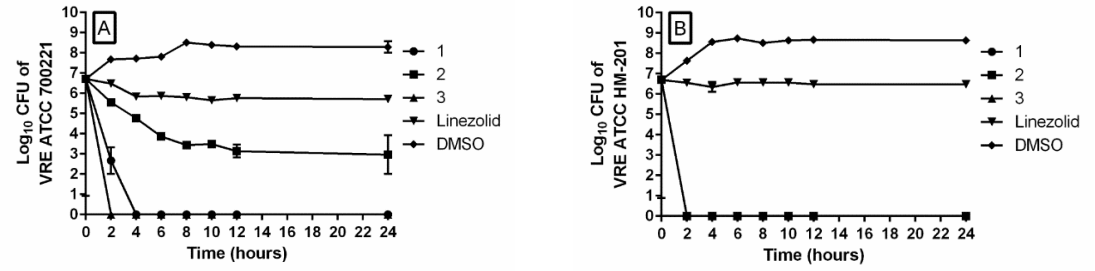

Figure 7.2 Time-kill analysis of thiazole compounds **1**, **2**, **3**, and linezolid.

Test agents (all tested at 4 × MIC) were incubated with bacteria over a 24 hour period at 37 °C against A) vancomycin-resistant *Enterococcus faecium* ATCC 700221 and B) vancomycin-resistant *Enterococcus faecalis* HM-201. DMSO served as a negative control. The error bars represent standard deviation values obtained from triplicate samples used for each compound/antibiotic studied.

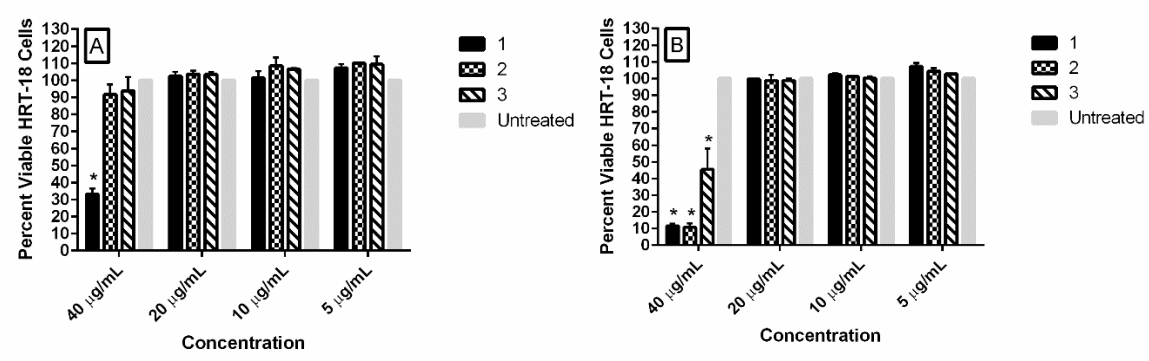

Figure 7.3 Toxicity analysis of compounds **1**-**3** against human colorectal (HRT-18) cells.

Percent viable mammalian cells (measured as average absorbance ratio (test agent relative to untreated cells)) for cytotoxicity analysis of thiazole compounds **1**, **2**, and **3** at 5, 10, 20, and 40 µg/mL against human colorectal (HRT-18) cells using the MTS 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium) assay. Untreated cells served as a negative control to determine a baseline measurement for the cytotoxic impact of each compound. Test agents were incubated with cells for either A) two hours or B) 24 hours. The absorbance values represent an average of a minimum of three samples analyzed for each compound. Error bars represent standard deviation values for the absorbance values. A one-way ANOVA (with post-hoc Dunnett's multiple comparisons test), $P \leq 0.05$, demonstrated statistical difference between the values obtained for compounds **1**, **2**, and **3** relative to untreated cells at 40 µg/mL (denoted by an asterisk, *).

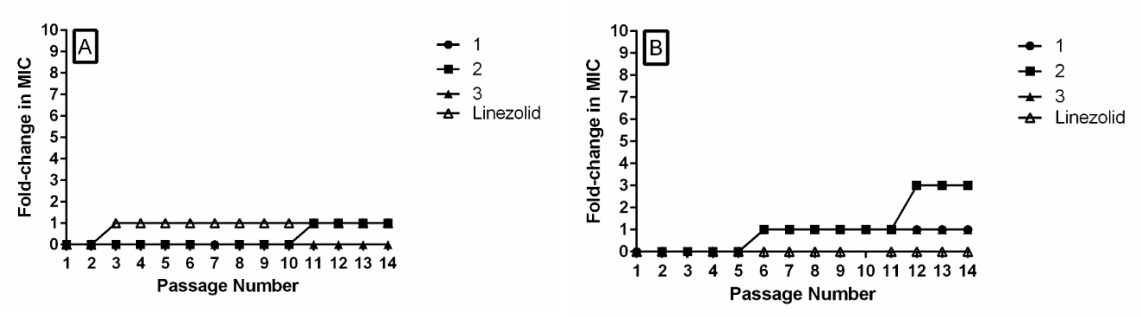

Figure 7.4 Multi-step resistance selection of thiazole compounds **1**, **2**, **3**, and linezolid against vancomycin-resistant Enterococci.

A) vancomycin-resistant *Enterococcus faecium* HM-968 and B) vancomycin-resistant *Enterococcus faecalis* ATCC 51299. Bacteria were serially passaged over a fourteen-day period and the broth microdilution assay was used to determine the minimum inhibitory concentration of each compound against VRE after each successive passage. A four-fold shift in MIC would be indicative of bacterial resistance to test agent.

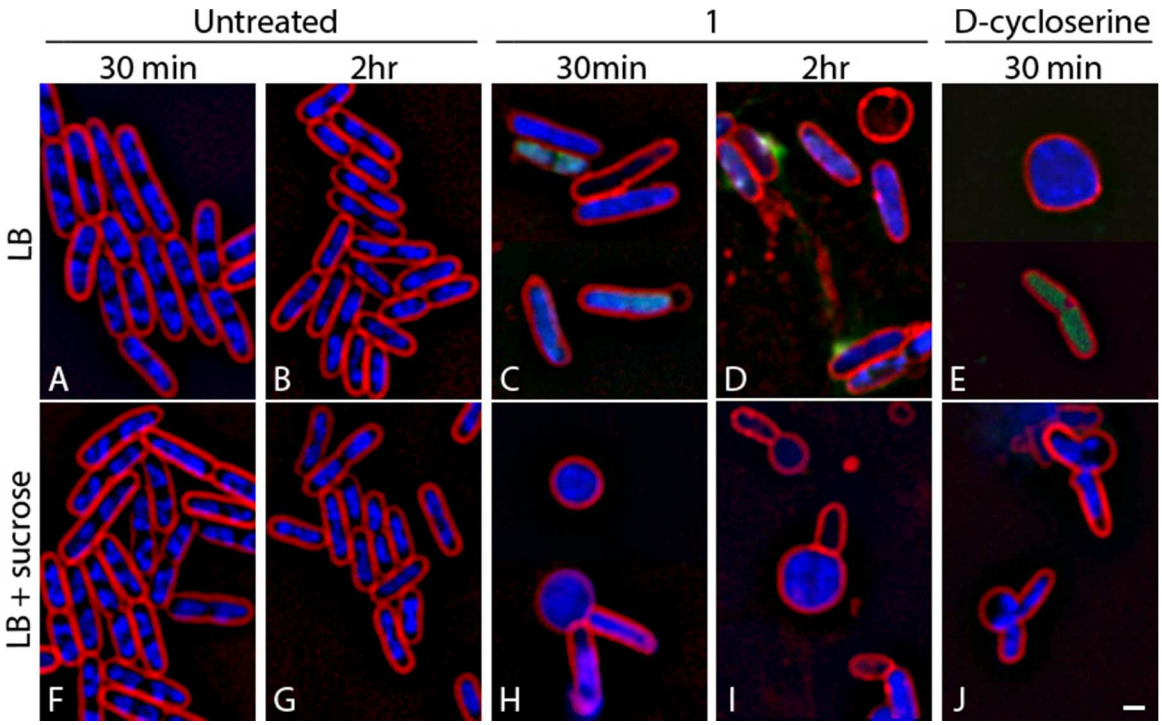

Figure 7.5 Compound **1** inhibits cell wall biosynthesis in *E. coli* Δ*tolC*.

(A, B, F, G) Untreated cells. (C, D, H, I) Cells treated with compound **1** for either 30 minutes or two hours at 5 × MIC (25 μg/mL). (E, J) Cells treated with D-cycloserine at 5 × MIC (125 μg/mL) for 30 minutes. Cells (F-J) were treated in the presence of 0.5 M sucrose to facilitate visualization of cell shape defects. Cells were stained with FM 4−64 (red), DAPI (blue), and SYTOX Green (green). Scale bar is 1 μm.

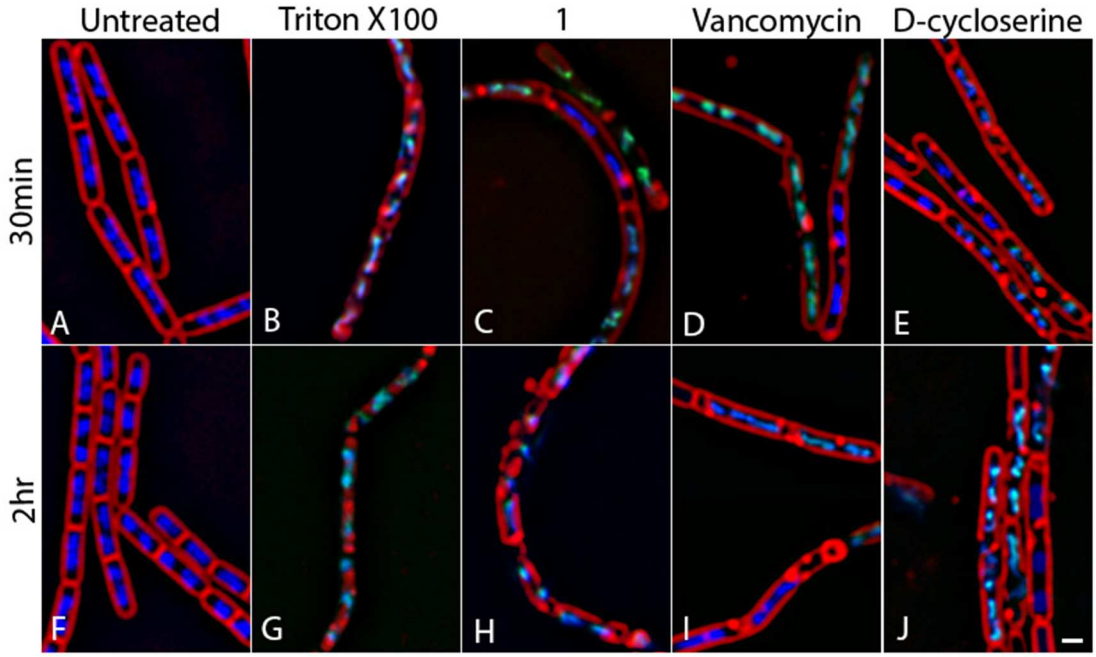

Figure 7.6 Profiles of membrane and cell wall active compounds in *B. subtilis* grown in LB at 37 °C.

(A, F) Untreated *B. subtilis* cells show no lysis. (B, G) Cells treated with 0.1% Triton-X-100, a membrane active compound. (C, H) Cells treated with compound **1** at 5 × MIC (12.5 µg/mL). (D, I) Cells treated with Vancomycin at 5 × MIC (0.78 µg/mL). (E, J) Cells treated with D-cycloserine at 1 × MIC (37.5 µg/mL). Both cell wall inhibitors and membrane active compounds cause lysis, which is observed by the increase in staining by SYTOX. Cells were stained with FM 4−64 (red), DAPI (blue), and SYTOX Green (green).

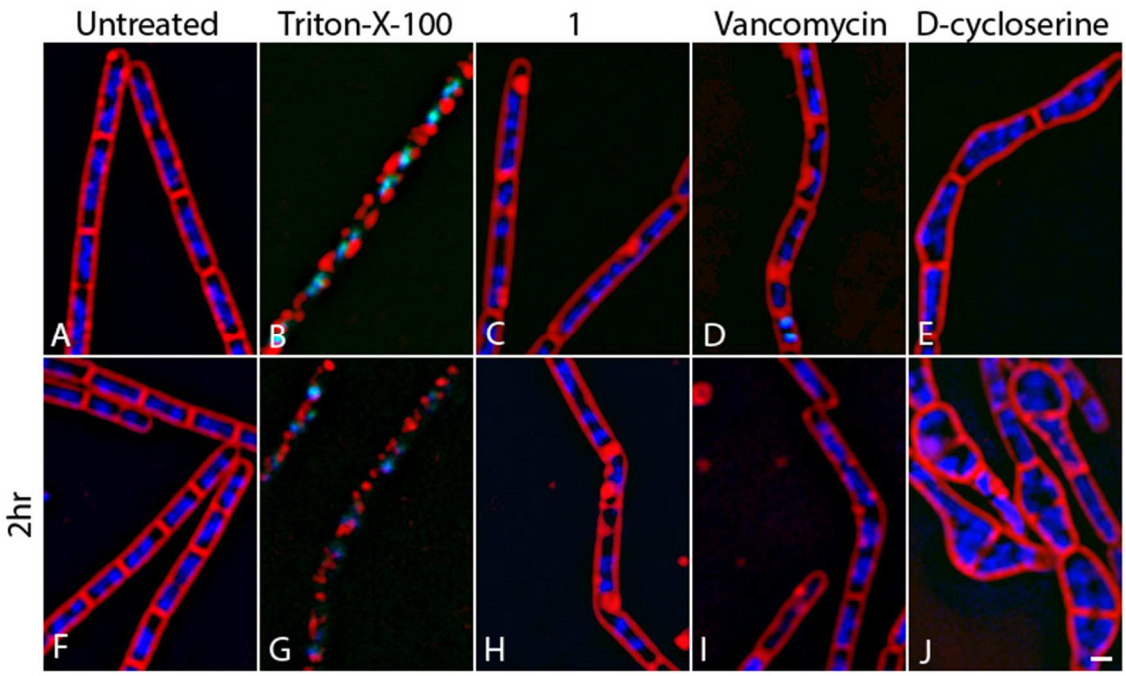

Figure 7.7 Profiles of membrane and cell wall active compounds in *B. subtilis* grown in LB in the presence of MSM at 37 °C.

(A, F) Untreated cells show no cell shape defects or lysis. (B, G) Cells treated with 0.1% Triton-X-100. (C, H) Cells treated with compound **1** at 5 × MIC (12.5 µg/mL), show subtle cell shape defects consistent with cell wall inhibition. (D, I) Cells treated with vancomycin at 5 × MIC (0.78125 µg/mL). (E, J) Cells treated with D-cycloserine at 1 × MIC (37.5 µg/mL).

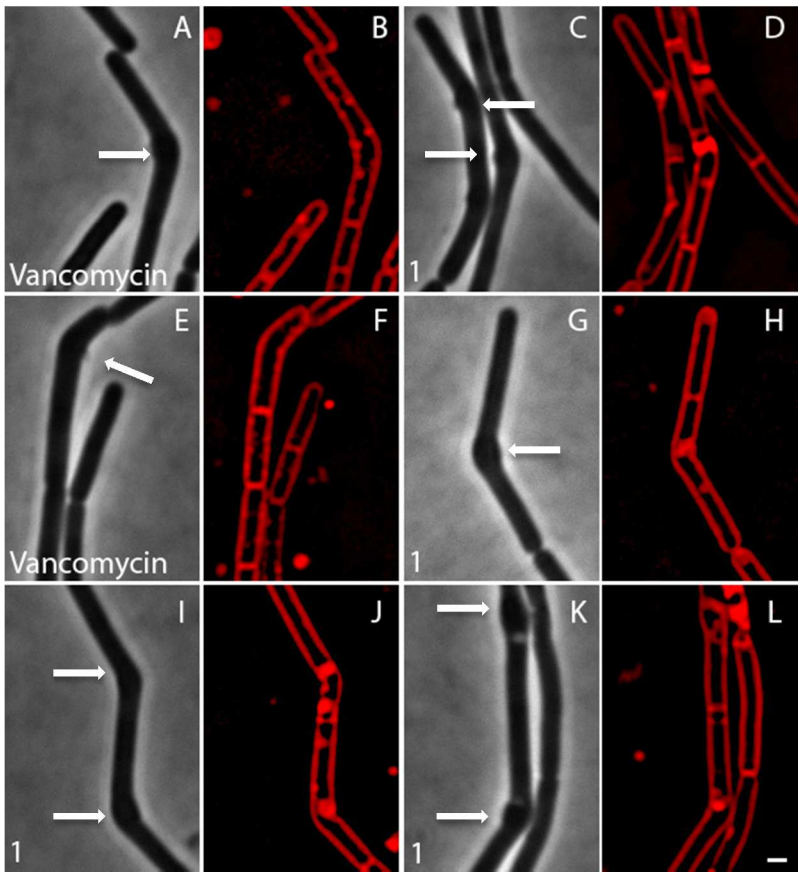

Figure 7.8 Comparison of cell shape defects in *Bacillus subtilis* cells treated with compound **1** or vancomycin.

All cells are grown in LB in the presence of MSM at 37°C and are shown at two hours. Both compound **1** and vancomycin lead to slight bending of the cells and bulges, as observed by the arrows.

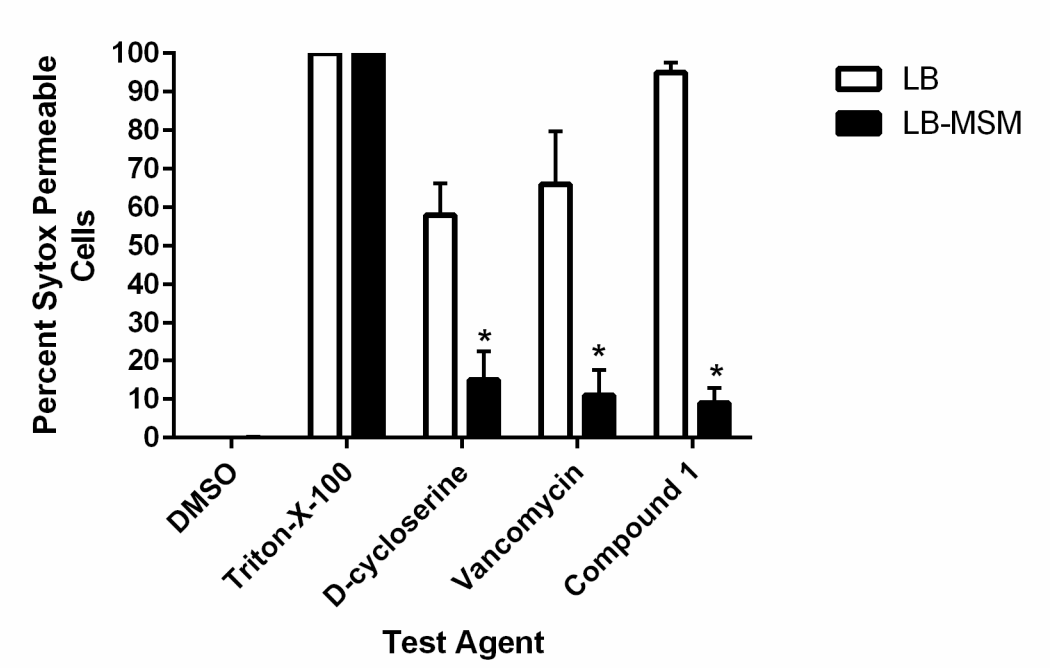

Figure 7.9 Percent Sytox permeable *Bacillus subtilis* cells.

Sytox permeable cells were counted at two hours. Untreated cells have no Sytox permeable cells in either LB or LBMSM, which osmotically stabilizes spheproplasts. Lysis caused by treatment with Triton X-100, a membrane active compound, is unaffected by LBMSM whereas lysis caused by cell wall inhibitors D-cycloserine and vancomycin, and by compound **1** is greatly reduced by growth in LBMSM. A two-way ANOVA with post-hoc Sidak's multiple comparisons test found statistical significance ($P < 0.05$) between LB and LB-MSM groups for cells receiving treatment with D-cycloserine, vancomycin, and phenylthiazole compound **1**.

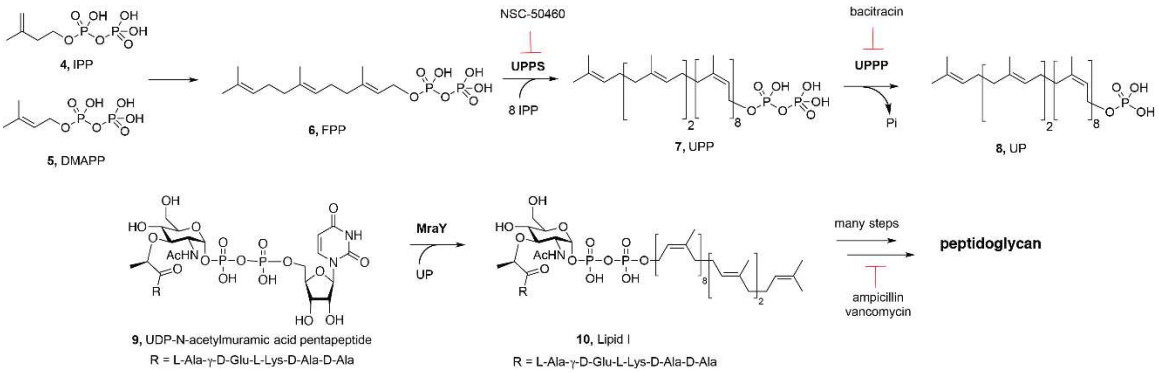

Figure 7.10 Schematic illustration of key steps in peptidoglycan biosynthesis in most bacteria and sites of action of known drugs and inhibitors.

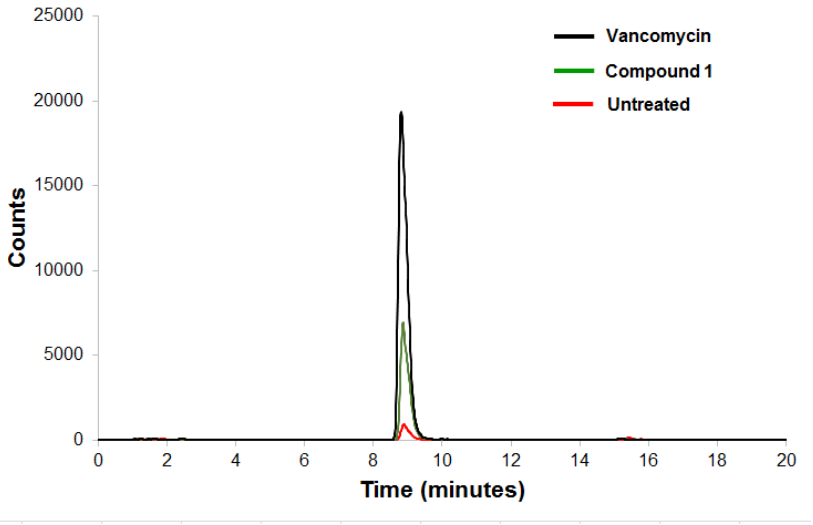

Figure 7.11 Detection of final soluble cell wall precursor (UDP-N-acetylmuramyl pentapeptide) inside bacterial cytoplasm.

HPLC chromatogram of *E. faecalis* NR-31975 treated with 10 × MIC of compound **1** or vancomycin for 30 minutes. After centrifugation, the bacterial pellet was boiled for 30 minutes to release contents present in the bacterial cytoplasm. The lysate was analyzed using HPLC/MS to determine the accumulation of the final soluble precursor in cell wall synthesis, UDP-N-acetylmuramyl pentapeptide (designated by the black arrows).

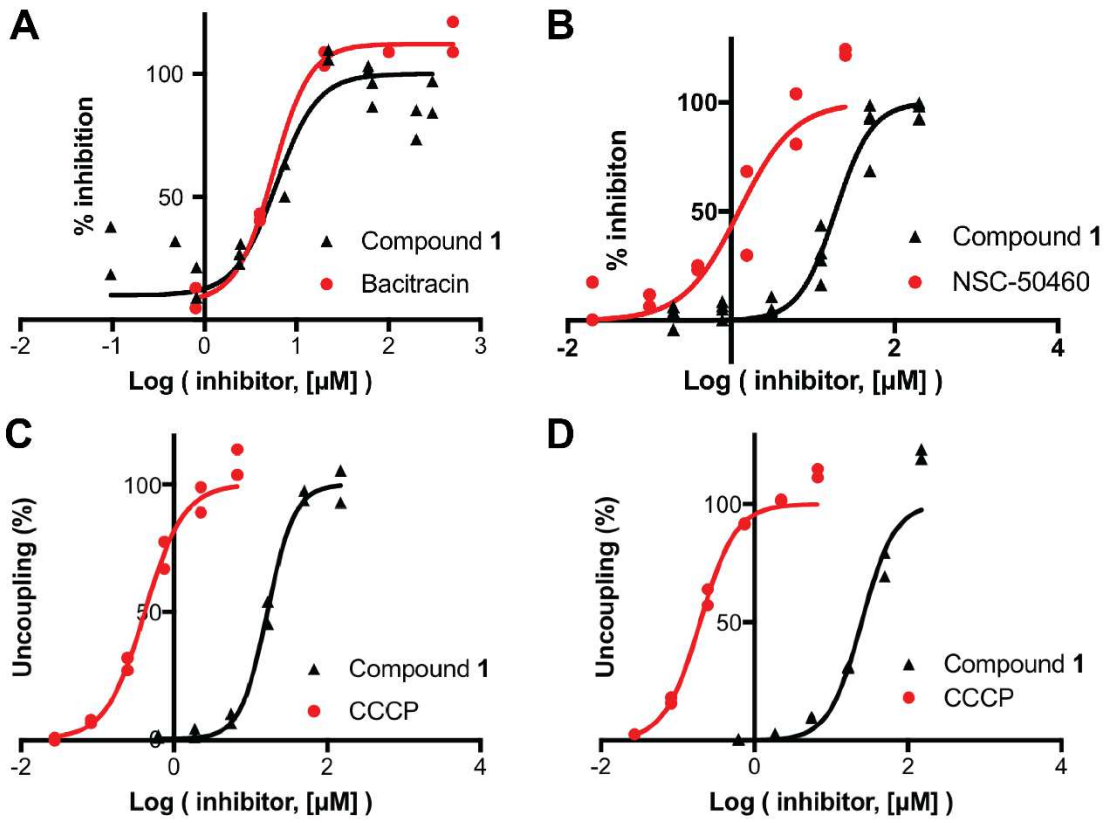

Figure 7.12 Dose response curves for enzyme inhibition by **1**, and its effects on the PMF.

A) UPPP (YubB) inhibition; bacitracin control. B) UPPS inhibition, bisamidine NSC-50460 control. C) PMF collapse in *E. coli* IMVs, ATP driven PMF, CCCP control. D) As C but succinate/O_2-driven PMF generation.

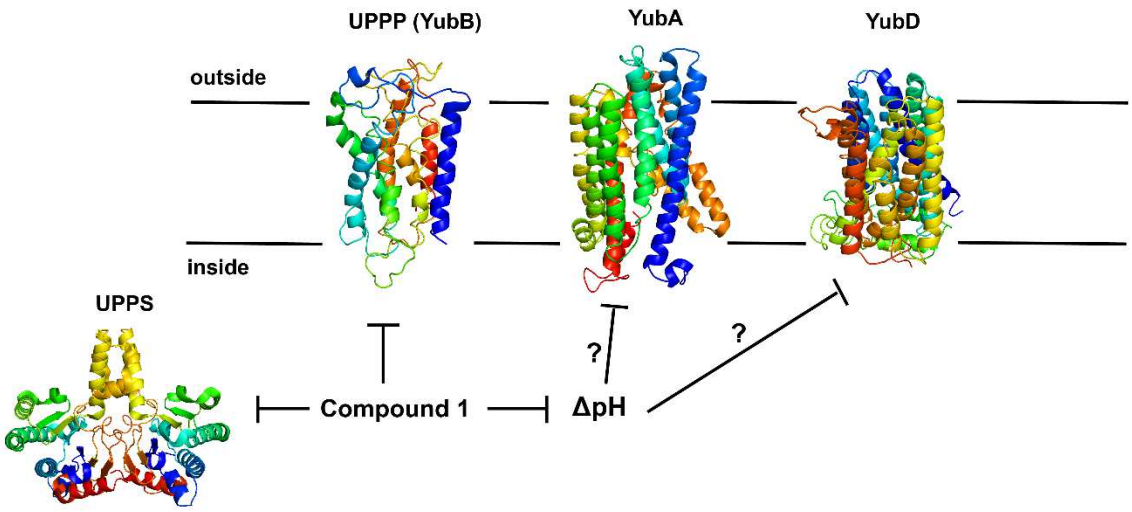

Figure 7.13 Structures of proposed targets of compound **1**.

The UPPS structure is the X-ray structure of EcUPPS, PDB ID code 1X06. The membrane protein structures are models for UPPP (YubB), YubA and YubD. Compound **1** inhibits UPPS and UPPP at low μM levels and collapses the PMF (in EcIMVs), suggested to affect the activity of YubA or YubD, putative transporters identified as targets in the transposon mutagenesis experiment (together with UPPP/YubB).

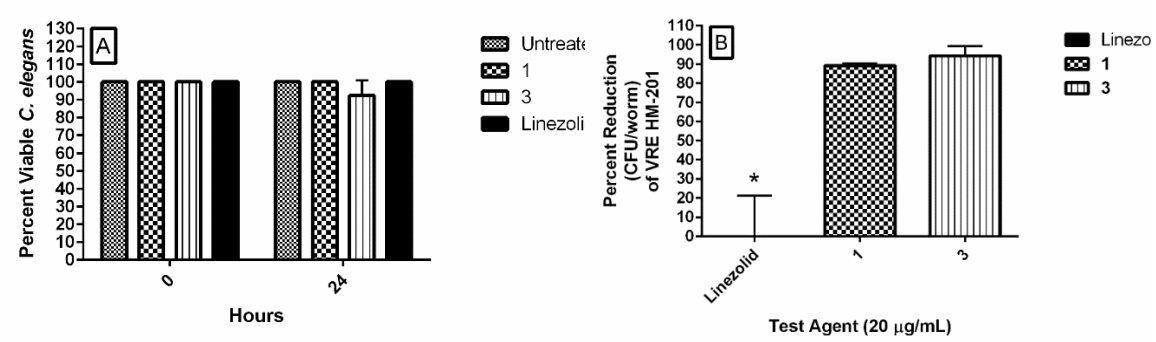

Figure 7.14 *In vivo* examination of toxicity and antibacterial activity of thiazole compounds **1** and **3** (tested at 20 μg/mL) of *C. elegans* AU37 infected with vancomycin-resistant *Enterococcus faecalis* HM-201.

Linezolid served as a positive control. A) Worms (in L4 stage of growth) were treated with 20 μg/mL of compound **1**, **3**, or linezolid and percent viable worms was determined after 24 hours of exposure. B) Worms (in L4 stage of growth) infected with vancomycin-resistant *E. faecalis* HM-201 for two hours before transferring 20-25 worms to wells of a 96-well plate. Test agents were added and incubated with worms for 18 hours. Worms were sacrificed and the number of viable colony-forming units of *E. faecalis* HM-201 in infected worms was determined for each treatment regimen. The figure presents the percent reduction of *E. faecalis* HM-201 (relative to untreated worms). Asterisks (*) denote statistical significance ($P < 0.05$) in bacterial reduction relative to the positive control (linezolid) using a two-tailed Student's t-test (with post-hoc Holm-Sidak test for multiple comparisons).

VITA

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Education

Purdue University, West Lafayette, Indiana
Ph.D. Candidate in Microbiology (December 2016)
B.S. in Biological and Food Process Engineering (December 2008)
B.S. in Pharmaceutical Sciences (December 2008)

Publications

Refereed Journal Articles

1.) Thangamani, S., Eldesouky, H., **Mohammad, H.**, Pascuzzi, P., Avramova, L., Hazbun, T., Seleem, M.N. Just Accepted (September 28, 2016). Ebselen exerts antifungal activity by regulating glutathione (GSH) and reactive oxygen species (ROS) production in fungal cells. BBA-General Subjects. Manuscript ID: BBAGEN-16-748R1.

2.) Abushahba, M.F.*, **Mohammad**, **H.***, and Seleem, M.N. Targeting Multidrug-resistant Staphylococci with an anti-*rpoA* Peptide Nucleic Acid Conjugated to the HIV-1 TAT Cell Penetrating Peptide. June 2016. Molecular Therapy – Nucleic Acids. 5(7):e339. doi: 10.1038/mtna.2016.53. (***Co-first authors**)

3.) Seleem, M.A., Disouky, A.M., **Mohammad, H.**, Abdelghany, T.M., Mancy, A. S., Bayoumi, S.A., Elshafeey, El-Morsy, A., A., Seleem, M. N., and Mayhoub, A. Second Generation Phenylthiazole Antibiotics with Enhanced Pharmacokinetic Properties. May 2016. Journal of Medicinal Chemistry. 59 (10): 4900-4912. doi: 10.1021/acs.jmedchem.6b00233

4.) Thangamani, S., **Mohammad**, **H.**, Abushahba, M.F., Sobreira, T.J., Hedrick, V.E., Paul, L.N., and Seleem, M.N. Antibacterial activity and mechanism of action of auranofin against multi-drug resistant bacterial pathogens. March 2016. Scientific Reports. 6:22571. doi: 10.1038/srep22571.

5.) Thangamani, S., **Mohammad**, **H.**, Sobreira, T.J., Abushahba, M.F., and Seleem, M.N. Repurposing auranofin for the treatment of cutaneous staphylococcal infections. March 2016. International Journal of Antimicrobial Agents. 47(3):195-201. doi: 10.1016/j.ijantimicag.2015.12.016.

6.) Abushahba, M.F., **Mohammad**, **H.**, Thangamani, S., Hussein, A., and Seleem, M.N. Impact of different cell penetrating peptides on the efficacy of antisense therapeutics for targeting intracellular pathogens. February 2016. Scientific Reports. 6:20832. doi: 10.1038/srep20832.

7.) **Mohammad, H**., Cushman, M., and Seleem, M.N. Antibacterial Evaluation of Novel Synthetic Thiazole Compounds *in vitro* and *in vivo* Against A Methicillin-resistant *Staphylococcus aureus* (MRSA) Murine Skin Infection Model. November 2015. PLoS ONE. 10(11):e0142321. doi: 10.1371/journal.pone.0142321.

8.) Thangamani, S., **Mohammad**, **H.**, Sobreira T.J., Paul, L.N., Hedrick, V.E., and Seleem, M.N. Exploring simvastatin, an antihyperlipidemic drug, as a potential topical antibacterial agent. November 2015. Scientific Reports. 5:16407. doi: 10.1038/srep16407.

9.) Davis, D.C.*, **Mohammad, H**.*, Younis, W., Creemer, C.N., Seleem, M.N., Dai, M. Discovery and Characterization of Aryl Isonitriles as A New Class of Compounds versus Methicillin- and Vancomycin-resistant *Staphylococcus aureus*. August 2015. European Journal of Medicinal Chemistry.101: 384-390. doi: 10.1016/j.ejmech.2015.06.031. (***Co-first authors**)

10.) **Mohammad, H**., Reddy, P.V.N., Monteleone, D., Mayhoub, A.S., Cushman, M., Hammac, G.K., and Seleem, M.N. Antibacterial Characterization of Novel Synthetic Thiazole Compounds Against Methicillin-resistant *Staphylococcus pseudintermedius*. June 2015. PLoS ONE. 18:10(6):e0130385. doi: 10.1371/journal.pone.0130385.

11.) **Mohammad, H**., Reddy, P.V.N., Monteleone, D., Mayhoub, A.S., Cushman, M., and Seleem, M.N. Synthesis and Antibacterial Evaluation of A Novel Series of Synthetic Phenylthiazole Compounds Against Methicillin-resistant *Staphylococcus aureus* (MRSA). March 2015. European Journal of Medicinal Chemistry. 94: 306-316. doi: 10.1016/j.ejmech.2015.03.015.

12.) **Mohammad, H**., Mayhoub, A.S., Cushman, M., and Seleem, M. N. Anti-Biofilm Activity and Synergism of Novel Thiazole Compounds with Glycopeptide Antibiotics Against Multidrug-Resistant Staphylococci. April 2015. Journal of Antibiotics. 68 (4):259-266. doi: 10.1038/ja.2014.142.

13.) **Mohammad, H**., Thangamani, S., and Seleem, M. N. Antimicrobial Peptides and Peptidomimetics – Potent Therapeutic Allies for Staphylococcal Infections. 2015. Current Pharmaceutical Design. 21(16):2073-88.

14.) Thangamani, S., **Mohammad, H**., Younis, W., and Seleem, M.N. Repurposing Non-antimicrobial Drugs for Treatment of Staphylococcal Infections. 2015. Current Pharmaceutical Design. 21(16):2089-100.

15.) **Mohammad, H**., Mayhoub, A.S., Ghafoor, A., Soofi, M., Alajlouni, R.A., Cushman, M. and Seleem, M.N. Discovery and Characterization of Potent Thiazoles Versus Methicillin- and Vancomycin-Resistant *Staphylococcus aureus*. February 2014. Journal of Medicinal Chemistry. 57(4): 1609-1615. doi: 10.1021/jm401905m.

Journal Articles Under Review

1.) Eassa, I.,* **Mohammad, H.,*** Qassem, O., Younis, W., Abdelghany, T., Elshafeey, A., Moustafa, M., Seleem, M. N., Mayhoub, A. S. Diphenylurea: a Novel Discovery for Combating Methicillin- and Vancomycin-Resistant *Staphylococcus aureus*. Journal of Medicinal Chemistry. Manuscript ID: jm-2016-01664f. (***Co-first authors**)

2.) Yahia, E., **Mohammad, H.**, Abdelghany, T. M., Fayed, I., Seleem, M. N., and Mayhoub1, A. S. Phenylthiazole Antibiotics: A Metabolism-Guided Approach to Overcome Short Duration of Action. European Journal of Medicinal Chemistry. Submitted August 2016.

3.) Mohamed, M., Brezden, A., **Mohammad, H**., Chmielewski, J., Seleem, M. N. A short D-enantiomeric antimicrobial peptide with potent immunomodulatory and antibiofilm activity against multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Antimicrobial Agents and Chemotherapy. Manuscript ID: AAC01840-16R1.

4.) Ghosh, C., Yadav, V., Younis, W., **Mohammad, H.**, Hegazy, Y., Seleem, M. N., Sanyal, K., Haldar, J. Aryl-alkyl-lysines: Membrane-active fungicides that act against biofilms of *Candida albicans*. ACS Infectious Diseases. Manuscript ID: id-2016-00192v.

5.) Thangamani, S., Hazbun, T., Maland, M., **Mohammad, H.**, Pascuzzi, P., Avramova, L., Koehler, C., Seleem, M. N. Auranofin exhibits antifungal activity by targeting mitochondrial protein(s). Frontiers in Cellular and Infection Microbiology. Submitted August 2016.

Selected Presentations

Invited Presentations

1.) **H. Mohammad**. Antibacterial Characterization of Novel Synthetic Thiazole Compounds Against Methicillin-Resistant *Staphylococcus pseudintermedius*. 29th Annual Phi Zeta Research Day. West Lafayette, IN. April 11, 2016.

2.) **H. Mohammad**. New Drugs for Bad Bugs - Development of Novel Synthetic Antimicrobials for Treatment of MRSA Infections. Purdue University Office of Interdisciplinary Graduate Programs (OIGP) Spring Reception, Keynote Address. West Lafayette, IN, April 1, 2015.

3.) **H. Mohammad**. Bad Bugs Need Good Drugs. Purdue MSA RADx Talks, West Lafayette, IN, November 19, 2014.

Oral Presentations

1.) **H. Mohammad**. Repurposing Auranofin and Ebselen for Treatment of Invasive Fungal Infections. Purdue Institute for Drug Discovery Postdoctoral Award Presentation, West Lafayette, IN, November 8, 2016.

2.) **H. Mohammad**. From Contamination to the Discovery of a Novel Broad-spectrum Antibacterial Agent. Purdue Comparative Pathobiology Department Seminar, West Lafayette, IN, September 29, 2016.

3.) **H. Mohammad** & Mohamed N. Seleem. Phenylthiazole Antibiotics for Multidrug-resistant *Staphylococcus aureus* (MRSA) Infections. Purdue Institute for Immunology, Inflammation and Infectious Disease Charting a Roadmap for Pre-clinical Drug Development and Beyond Workshop, West Lafayette, IN, September 2, 2016.

4.) **H. Mohammad**. Gut Check: Discovery of Phenylthiazole Antibiotics Effective Against Vancomycin-resistant Enterococci. Hitchhiker's Guide to the Biomolecular Galaxy Symposium, West Lafayette, IN, May 11, 2016.

5.) **H. Mohammad**. Saving Fido – Unearthing a novel topical antimicrobial for treatment of multidrug-resistant staphylococcal skin infections in companion animals. Conference of Research Workers in Animal Diseases, Chicago, IL, December 7, 2015.

6.) **H. Mohammad**. Saving Fido – Unearthing a novel topical antimicrobial for treatment of multidrug-resistant staphylococcal skin infections in companion animals. Purdue Comparative Pathobiology Department Seminar, West Lafayette, IN, November 5, 2015.

7.) **H. Mohammad**. Bad Bugs Need Good Drugs, 3MT Thesis Competition, Purdue University Graduate School, West Lafayette, IN, April 15, 2015.

8.) **H. Mohammad**. MRSA on the Rise, Therapeutics in Demise, Thiazoles Emerge as Future Allies. Purdue Comparative Pathobiology Department Seminar, West Lafayette, IN, October 30, 2014.

9.) **H. Mohammad**. Bad Bugs Need Good Drugs: Characterization of Substituted Thiazole Compounds for Treatment of Methicillin-resistant *Staphylococcus aureus* (MRSA) Infections. Purdue Comparative Pathobiology Department Seminar, West Lafayette, IN, November 14, 2013.

10.) **H. Mohammad**. A Super Drug for A Superbug. 5th Annual Regional Population, Health, and Environmental Economics Graduate Student Summit, West Lafayette, IN, April 14, 2012.

11.) **H. Mohammad**. Old Bug vs. New Drug: Novel Substituted Thiazoles for Treatment of Methicillin-resistant *Staphylococcus aureus* (MRSA) Infections. Purdue Comparative Pathobiology Department Seminar, West Lafayette, IN, November 29, 2012.

International Conference Poster Presentations

1.) E. Mostafa, **H. Mohammad**, M.N. Seleem & A.S. Mayhoub. Diphenylureas: a Novel Discovery for Beating Methicillin and Vancomycin Resistant *Staphylococcus aureus* (MRSA & VRSA). FUE 3rd International Conference of Pharmaceutical Sciences, Cairo, Egypt, February 9-11, 2015.

2.) M. A. Seleem, **H. Mohammad**, M.N. Seleem & A.S. Mayhoub. Design & Synthesis of New Phenylthiazole Derivatives with Enhanced Pharmacokinetic Profile for Treatment of Methicillin and Vancomycin Resistant *Staphylococcus aureus* (MRSA & VRSA). FUE 3rd International Conference of Pharmaceutical Sciences, Cairo, Egypt, February 9-11, 2015.

3.) W.M. Townsend, **H. Mohammad**, and M.N. Seleem. Equine Ulcerative Keratitis: Comparison of Histology, Cytology, and Mycotic Culture with Results of Polymerase Chain Reaction using Universal Fungal Primers, 2013 IEOC/Acrivet Symposium, Jackson Hole, WY, June 8, 2013.

4.) **H. Mohammad**, A.S., Mayhoub, A. Ghafoor, M. Soofi, R.A. Alajlouni, M. Cushman, and M.N. Seleem. A New Drug for an Old Bug: Antimicrobial Activity of Novel Substituted Thiazoles Against Methicillin-Resistant *Staphylococcus aureus*, Conference of Research Workers in Animal Diseases, Chicago, IL, December 2, 2012.

National Conference Poster Presentations

1.) **H. Mohammad**, M. Cushman, and M.N. Seleem. Skin Deep - Novel Thiazole Compounds Exhibit Potent Antibacterial Activity *in vitro* and *in vivo* in a Methicillin-resistant *Staphylococcus aureus* (MRSA) Skin Infection Mouse Model. ASM Microbe & Interscience Conference on Antimicrobial Agents and Chemotherapy 2016, Boston, MA, June 16 – 20, 2016.

2.) **H. Mohammad**, M. Sedlak, N.W.Y. Ho, and N.S. Mosier. Effect of Na^+, K^+, NH_4^+, and Glycerol on the Glucose and Xylose Co-fermentation of Glucose and Xylose by *S. cerevisiae*424A(LNH-ST), 31st Symposium on Biotechnology for Fuels and Chemicals, San Francisco, CA, May 3 – 6, 2009.

3.) M. Sedlak, **H. Mohammad**, N.W.Y. Ho, and N.S. Mosier. Combined Effect of Acetic Acid and pH on the Co-fermentation of Glucose and Xylose by Recombinant Yeast, 29th Symposium on Biotechnology for Fuels and Chemicals, Denver, CO, April 29 – May 2, 2007.

Regional Conference Poster Presentations

1.) **H. Mohammad**, M. Cushman, and M.N. Seleem. Attacking the Multidrug-resistance Puzzle with Phenylthiazole Antibiotics. Purdue Institute for Immunology, Inflammation and Infectious Disease Graduate Student/Postdoc Symposium, West Lafayette, IN, August 10, 2016.

2.) **H. Mohammad**, W. Younis, M. Cushman, and M.N. Seleem. Discovery of Thiazole Compounds with Potent Antimicrobial Activity Against Drug-resistant Enterococci. The 8th Annual Interdisciplinary Graduate Program Spring Reception, Purdue Office of Interdisciplinary Graduate Programs, West Lafayette, IN, May 2, 2016.

3.) **H. Mohammad**, W. Younis, M. Cushman, and M.N. Seleem. Evaluation of Phenylthiazole Antibacterial Agents Targeting Drug-resistant Enterococci. The 33rd Herbert C. Brown Lectures in Organic Chemistry, West Lafayette, IN, April 15, 2016.

4.) **H. Mohammad**, W. Younis, M. Cushman, and M.N. Seleem. Evaluation of Phenylthiazole Antibacterial Agents Targeting Drug-resistant Enterococci. The 29th Annual Phi Zeta Research Day, Purdue College of Veterinary Medicine, West Lafayette, IN, April 11, 2016.

5.) **H. Mohammad***, W. Younis, M. Cushman, and M.N. Seleem. Gut Check: Identification of Phenylthiazole Antibacterial Agents Effective Against Vancomycin-resistant Enterococci (VRE). Indiana Branch of the American Society for Microbiology (ASM) Annual Meeting 2016, Fort Wayne, IN, April 2, 2016.
***Awarded second-place in PhD Division**

6.) **H. Mohammad***, W. Younis, M. Cushman, and M.N. Seleem. *In vitro* and *in vivo* examination of novel phenylthiazole antibacterial compounds against vancomycin-resistant enterococci (VRE). 2016 Health and Disease: Science, Culture, Policy Research Poster Session, West Lafayette, IN, March 28, 2016.
***Awarded first-place in Inflammation, Immunology and Infectious Diseases Division**

7.) **H. Mohammad***, W. Younis, M. Cushman, and M.N. Seleem. *In vitro* and *in vivo* examination of novel phenylthiazole antibacterial compounds against vancomycin-resistant enterococci (VRE). 2016 Sigma Xi Graduate Student Research Poster Award Competition, West Lafayette, IN, March 2, 2016.
***Awarded first-place in Life Sciences Division**

8.) **H. Mohammad***, M. Cushman, and M.N. Seleem. Antibacterial Effect of Synthetic Thiazole Compounds *in vitro* and *in vivo* in a Methicillin-resistant *Staphylococcus aureus* (MRSA) Skin Infection Mouse Model. The 28th Annual Phi Zeta Research Day, Purdue College of Veterinary Medicine. West Lafayette, IN, April 14, 2015.
***Awarded first-place in Basic Research Division**

9.) **H. Mohammad**, A.S. Mayhoub, P.V.N. Reddy, D. Monteleone, M. Cushman, G.K. Hammac, and M.N. Seleem. Good Drugs for Bad Bugs –Thiazole Compounds As New Partners Against Methicillin-resistant *Staphylococcus pseudintermedius* (MRSP) Infections. The 7th Annual Interdisciplinary Graduate Program Spring Reception, Purdue Office of Interdisciplinary Graduate Programs, West Lafayette, IN, April 1, 2015.

10.) **H. Mohammad**, A.S. Mayhoub, P.V.N. Reddy, M. Cushman, and M.N. Seleem. Skin Deep – Synthetic Thiazole Compounds Emerge as New Allies for Drug-resistant Staphylococcal Skin Infections. 2015 Health and Disease: Science, Culture, Policy Research Poster Session, West Lafayette, IN, March 5, 2015.

11.) **H. Mohammad**, A.S. Mayhoub, P.V.N. Reddy, M. Cushman, and M.N. Seleem. Skinning Staph: Therapeutic Applications for Novel Thiazole Compounds Against Drug-resistant Staphylococcal Skin Infections. 2015 Sigma Xi Graduate Student Research Poster Award Competition, West Lafayette, IN, February 18, 2015.

12.) **H. Mohammad**, A.S. Mayhoub, P.V.N. Reddy, M. Cushman, and M.N. Seleem. Therapeutic Applications for Novel Thiazole Compounds Against Multidrug-resistant Staphylococci. ESE Symposium. West Lafayette, IN, October 20, 2014.

13.) **H. Mohammad**, A.S. Mayhoub, P.V.N. Reddy, M. Cushman, and M.N. Seleem. New Drugs for Bad Bugs: Antimicrobial Versatility of Novel Synthetic Thiazole Compounds Against Multidrug-resistant Staphylococci. Purdue/Lilly Tech Day. West Lafayette, IN, May 8, 2014.

14.) **H. Mohammad**, A.S. Mayhoub, P.V.N. Reddy, M. Cushman, and M.N. Seleem. Antimicrobial Versatility of Novel Thiazole Compounds Against Multidrug-Resistant Staphylococci. The 27th Annual Phi Zeta Research Day, Purdue College of Veterinary Medicine. West Lafayette, IN, April 14, 2014.

15.) **H. Mohammad**, A.S. Mayhoub, M. Cushman, and M.N. Seleem. Antimicrobial Versatility and Anti-Biofilm Activity of Novel Synthetic Thiazole Compounds Against Multidrug-Resistant Staphylococci. The 6th Annual Interdisciplinary Graduate Program Spring Reception, Purdue Office of Interdisciplinary Graduate Programs, West Lafayette, IN, April 2, 2014.

16.) **H. Mohammad**, A.S. Mayhoub, P.V.N. Reddy, M. Cushman, and M.N. Seleem. Antimicrobial Versatility of Novel Thiazole Compounds Against Multidrug-Resistant Staphylococci. 2014 Health and Disease: Science, Culture, Policy Research Poster Session, West Lafayette, IN, March 31, 2014.

17.) **H. Mohammad**, A.S. Mayhoub, M. Cushman, and M.N. Seleem. Antimicrobial Versatility and Anti-Biofilm Activity of Novel Synthetic Thiazole Compounds Against Multidrug-Resistant Staphylococci. 2014 Sigma Xi Graduate Student Research Poster Award Competition, West Lafayette, IN, February 12, 2014.

18.) M. Ali, **H. Mohammad**, A.S. Mayhoub, A. Shehata, M. Cushman, and M.N. Seleem. *In vitro* Activity and Cell Toxicity of Novel Modified Thiazole Compounds. 2014 Sigma Xi Graduate Student Research Poster Award Competition, West Lafayette, IN, February 12, 2014.

19.) **H. Mohammad**, A.S. Mayhoub, R.A. Alajlouni, M. Cushman, and M.N. Seleem. Bad Bugs Need New Drugs – Antimicrobial Activity of Novel Substituted Thiazoles Against Methicillin-Resistant *Staphylococcus aureus* (MRSA). The 26th Annual Phi Zeta Research Day, Purdue College of Veterinary Medicine. West Lafayette, IN, April 15, 2013.

20.) **H. Mohammad**, A.S. Mayhoub, R.A. Alajlouni, M. Cushman, and M.N. Seleem. MRSA on the Rise, Therapeutics in Demise, Thiazoles Emerge As Future Allies. The 5th Annual Interdisciplinary Graduate Program Spring Reception, Purdue Office of Interdisciplinary Graduate Programs, West Lafayette, IN, April 1, 2013.

21.) **H. Mohammad**, A.S. Mayhoub, A. Ghafoor, M. Soofi, R.A. Alajlouni, M. Cushman, and M.N. Seleem. A Super Drug for A Superbug – Modified Thiazole Compounds Show Promise In Battle Against Methicillin-Resistant *Staphylococcus aureus* (MRSA). The 25th Annual Phi Zeta Research Day, Purdue College of Veterinary Medicine, West Lafayette, IN, April 16, 2012.

22.) **H. Mohammad**, A.S. Mayhoub, A. Ghafoor, M. Soofi, R.A. Alajlouni, M. Cushman, and M.N. Seleem. Has MRSA Met Its Match? – Modified Thiazole Compounds Show Promise Against MRSA, 5th Annual Regional Population, Health, and Environmental Economics Graduate Student Summit, West Lafayette, IN, April 14, 2012.

23.) **H. Mohammad**, A.S. Mayhoub, A. Ghafoor, M. Soofi, R. A. Alajlouni, M. Cushman, and M.N. Seleem. Modified Thiazole Compounds Show Promise As A New Therapeutic Tool Against MRSA, 2012 Sigma Xi Graduate Student Research Poster Award Competition, West Lafayette, IN, February 15, 2012.

Awards

Purdue Institute for Drug Discovery Post-Doctoral Fellowship (January – December 2017)
Bilsland Dissertation Fellowship (August – December 2016)
American Society for Microbiology Travel Award (ASM Microbe 2016) (June 2016)
Purdue Graduate Student Government Travel Grant (ASM Microbe 2016) (April 2016)
Phi Zeta Omicron Chapter Graduate Student Research Award (April 2016)
First-place (Life Sciences Division), Purdue Society of Sigma Xi Poster Competition (April 2016)
Second-place (PhD Division), Indiana Branch of American Society for Microbiology Annual Meeting Poster Presentation (April 2016)
First-place (Inflammation, Immunology and Infectious Diseases Division), Purdue Health & Disease Research Symposium (March 2016)
First-place (Basic Research), Phi Zeta (Omicron Chapter) Poster Competition (April 2015)
Purdue University Office of Interdisciplinary Graduate Programs (OIGP) Most Outstanding Interdisciplinary Graduate Project Award (April 2015)
Purdue Graduate School 3-Minute Thesis Competition, Audience Choice Award (April 2015)
Purdue Graduate Student Government (PGSG) Graduate Student Excellence Award (April 2014)

Patent

"Aryl Isonitriles as A New Class of Antimicrobial Compounds" Dai, M. J., Seleem, M., Davis, D. C., **Mohammad, H. T.** *US Provisional Application* filed (US, 62/143,031).

Professional Memberships

American Association for the Advancement of Science (AAAS) (2015 – Present)
American Society for Microbiology (ASM) (2015 – Present)

Ad-hoc Reviewer for Journals

Current Pharmaceutical Design	(Special edition, November to December 2014)
PLoS ONE	(August 2015 – current)
Scientific Reports	(November 2015 – current)

Research Experience

Laboratory of Molecular Microbiology, Purdue University
Graduate Research Assistant/*Lab Manager*/*Lab Organization Officer* (August 2011-Present)
PhD Advisor: Dr. Mohamed N. Seleem, Department of Comparative Pathobiology

- Identified four new series of synthetic chemical compounds with potent antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) using whole-cell screening
- Investigated the antibacterial mechanism of action of synthetic compounds utilizing target overexpression in *Bacillus subtilis* (via transposon mutagenesis) and biochemical assays
- Evaluated toxicity of promising antibacterial compounds against multiple human cell lines (HRT-18, HaCaT, HeLa, HEK-293)
- Examined ability of synthetic compounds to inhibit biofilm formation and toxin production
- Verified the antibacterial activity of phenylthiazole compounds in a murine model of MRSA skin infection and *Caenhorhabditis elegans* model of enterococcal infection
- Trained new graduate, undergraduate, and professional students proper safety procedures and protocols for conducting experiments with biosafety level 2 microorganisms
- Reviewed grant proposals and journal articles of lab members prior to submission

Laboratory of Renewable Resources Engineering (LORRE), Purdue University
Undergraduate Engineering Research Assistant (May 2006 – May 2009)
Research Advisor: Dr. Nathan Mosier, Department of Agricultural & Biological Engineering

- Discovered effect of various inhibitors (acetic acid, excess sugar, excess ethanol, salts) on production of fuel-grade ethanol from pure sugars (glucose and xylose)
- Designed process to produce liquefied corn mash and remove antimicrobial agent added to corn mash prior to fermentation

Summer Undergraduate Research Fellowship (SURF), Purdue University
Undergraduate Research Assistant (May 2005 – August 2005)
Research Advisor: Dr. Arif Ghafoor, Department of Electrical & Computer Engineering

Leadership & Community Outreach

Briarwood Apartments HEADS UP Program
Youth mentor and homework tutor (December 2015 – current)
*Co-organizer, "Yes We Can! Making the College Dream A Reality" Spring Fest Tour**
(April 2016)
*Awarded $1200 grant from Purdue University Office of Engagement

Indiana University School of Medicine – Lafayette Briarwood Health Fair
Co-organizer, Microbial Infection and Prevention Information Table (August 2016)

Purdue Graduate Student Government
Academic and Professional Development Committee (August 2015 – May 2016)
*Advancement Committee** (August 2015 – May 2016)
*Awarded $500 for organization of "Restaurant Hope" service project
Career Fair Committee (August 2015 – December 2015)

Purdue Veterinary Medicine Family Program Series
Youth career presentation "Microbes here, microbes there, microbes everywhere"
(March 2016)

Science Fair Judge
Lafayette Regional Science & Engineering Fair (March 2016)
Lafayette Catholic Schools Education Fair (February 2013)