5-2015

Structure-Activity Relationship Studies of Small Molecules Directed Against the T-Box Specifier Loop

Spencer Weintraub
University at Albany, State University of New York

“Structure-activity Relationship Studies of Small Molecules Directed Against the T-box Specifier Loop”

An honors thesis presented to the
Department of Biological Sciences
University at Albany
State University at New York
Fulfillment of the Honors Program Requirements

By: Spencer Weintraub
2015

The Honors College
University at Albany

BIOLOGICAL SCIENCES
Certification of the Completion of a Senior Honors Thesis

- Successful completion of a senior honors thesis is required for graduation from The Honors College.
- The determination of whether a student's work satisfies the criteria for a senior honors thesis is left to each department.
- Each honors student must return this completed certification, along with an electronic copy of his or her senior honors thesis, to the honors college office.
- This certification must be signed by the student's thesis advisor and by either the Director of the department's honors program or the Chair of the department.

This Honors Thesis has been read and approved by the signatures below and, is hereby recommended for acceptance.

Student Name: Spencer Weintraub **Student ID:** 001074048

Thesis Title: Structure-activity Relationship Studies of Small Molecules Directed Against the T-box Specifier Loop

Department in which Thesis was completed: BIOLOGICAL SCIENCES

Honors Thesis Presentation Date April 24th, 2015

	Print name	Signature	Date
***Senior Honors Thesis Research Advisor:**	Paul Agris		4/20/15
***Honors Thesis Committee Member:**	KATHLEEN McDONOUGH		4/28/15
Honors Thesis Committee Member (optional):			

***Required**

CHOOSE ONE: ☒ **Department Chair** OR ☐ Director of Department Honors Committee

	Print name	Signature	Date
Biology Honors Committee Director & Department Chair	RICHARD S. ZITOMER		

Original – Dr. Jeffrey Haugaard, Associate Vice Provost and Honors College Director LC-31
Copy – Department of Biological Sciences
Copy – Registrar's Office

Abstract:

High rates of antibiotic use have resulted in the pervasiveness of multi-drug resistant organisms. With rising drug-resistance the development of antibiotics with new targets against these pathogens is imperative. The T-box regulatory mechanism is a process specific to Gram-positive bacteria and controls many essential genes including aaRS genes, which are code for the aminoacyl-tRNA synthetases required to charge tRNA. The T-box Specifier Loop is a novel target for antibacterial drug discovery as we hypothesize that a small compound bound to the Specifier Loop will inhibit transcription of essential bacterial genes resulting in bacterial cell death or growth arrest. We used an *in silico* drug discovery approach combined with structure-activity relationship studies (SAR) to evaluate small molecules as potential putative antibiotics directed against Gram-positive bacteria. SAR studies have been used to determine important structural elements of the initial hit compounds so that a compound with optimal activity and Gram-positive selectivity can be developed into an antimicrobial agent.

Acknowledgements:

Committee:

I would like to thank Drs. Agris and Mcdonough for being my Committee members for my undergraduate honors thesis and for their critical reading of this work. Their efforts and roles were essential to me in all of the work that I have conducted and presented here.

Department Guidance:

I would like to thank Dr. Zitomer for his guidance throughout my thesis writing process so that I was able to complete this task.

Critical Readers:

I would like to thank Drs. Kyla Frohlich and Gabrielle Todd for critical reading of this paper and helpful discussion.

Computer Simulations:

I would like to thank Srivathsan Ranganathan from the RNA Institute for conducting the second *in silico* that was essential for me to continue my SAR studies efficiently.

Funding:

This work was supported by the Interdisciplinary Pilot Research Program (IPRP) award from the RNA Institute at the University at Albany to Dr. Paul F. Agris, a Technology Accelerator Fund from the University at Albany to Dr. Paul F. Agris and Dr. Kathleen A. McDonough, the SUNY Networks of Excellence Health Now award to Dr. Paul F. Agris and Dr. Kathleen A. McDonough, Enhancing STEM Research Experiences for SUNY Undergraduates awarded to Spencer F. Weintraub and University at Albany Foundation, Undergraduate Research Endowed Fellowship to Spencer F. Weintraub.

Table of Contents:

Introduction:

Many drug-resistant pathogens have risen in frequency and lethality. High rates of antibiotic use have resulted in the pervasiveness of advantageous mutations against antibiotics.[1] This epidemic of resistance has developed from increased frequency of prescription, which is supported by studies showing a positive correlation between the amounts of regional antibiotic consumption with the rate of prevalence of antibiotic resistant bacterial infections. The US Centers for Disease Control and Prevention conservatively approximate 2 million illnesses and 23,000 deaths annually as a consequence of resistance (Zhang et al)[2] An increase in resistance of bacterial infections in healthcare facilities is beginning to reduce the amount of available antimicrobial agents. Thus, the development of antibiotics with new targets against multi-drug resistant organisms is imperative. Addressing the issue of resistance has brought a focus onto nosocomial infections that are developing strong resistance to current antibiotic treatments. [1]

Gram-positive bacterial infections have demonstrated increasing resistance despite many attempts to preclude the evolution of these microorganisms. Gram-positive cocci bacteria specifically demonstrate dangers as strains such as, Methicillin Resistant *Staphylococcus aureus* (MRSA), Penicillin Resistant *Streptococcus pneumoniae* (PRSP), Vancomycin Resistant *Enterococci* (VRE), and many others have broadened resistance to many of their current treatments. A surveillance study, conducted on nosocomial infections and their transmission throughout 463 hospitals in China, demonstrated that the prevalence of MRSA was much higher in ICU patients (77.6%) than in both inpatients (50.2%) and out patients (26.1%). Evaluation of the prevalence of resistant strains conducted during 2006-2007 demonstrated that across the 463 hospitals all had at least 1 hospital associated infection (HAI). Further data showed that 28,502 HAIs were reported across 25,384 patients, which reflects the frequency of nosocomial

infections. On the basis of this study, the 10 most common HAIs were determined in these hospitals and the 3 most prevalent of these infections were Gram-positive organisms.[3]

The excessive use of antibiotics in a variety of circumstances, which may be deemed inappropriate or unnecessary, have resulted in the acceleration of resistance. Estimates approximate that about 50% of prescribed antibiotics are inappropriate. The development of new antibiotics with decreased opportunity for resistance is integral as research must be cost effective in searching for new antimicrobial treatments.[4] There have only been three new classes of antibiotics that have been introduced since the year 2000. Thus, the need for non-toxic antibiotics against multi-drug resistant Gram-positive organisms is crucial.[5] Comprehension of current antibiotic mechanisms is an essential aspect of understanding the development of resistance and how to find future antibacterial agents to combat these evolving organisms. There are 5 major target mechanisms of which current antimicrobials interfere or inhibit, including (1) cell wall synthesis; (2) protein synthesis; (3) nucleic acid synthesis; (4) a metabolic pathway; or (5) the bacterial membrane structure.[4]

Evolutionary adaptations have allowed bacterial infections to evolve several mechanisms of resistance. Intrinsic resistance is the use of a natural mechanism that is innate to a bacterium. Acquired resistance, on the other hand, is the development of spontaneous mutations or acquisition of genes through methods such as horizontal gene transfer. Four major mechanisms of resistance have arisen including alteration of antibiotic binding site, creation of enzymes that inactivate or destroy the antibiotic, development of an efflux pump, and development of a porin or channel that prevents entry of an antibiotic into the cell.[4]

There are many modifications that can occur in the binding site of the antibiotic and can prevent it from acting on its target.[4] This mechanism is one of the ways Gram-positive organisms

have evolved against Beta Lactams, which are some of the oldest currently used antibiotics. This class of antibiotics includes many molecules, all of which contain a B-Lactam ring, including Penicillins, Cephalosporins, and Monobactams. The mechanism of action for these antibiotics is to interfere with enzymes such as transpeptidases, carboxypeptidases, and endopeptidases, which are necessary for the building and maintaining peptidoglycan of the cell wall. These enzymes are often referred to as Penicillin Binding Proteins (PBPs). Many Gram-positive organisms have developed resistance that interferes with these antibiotics binding site. *S. pneumoniae* became resistant to Penicillins and Cephalosporins as a result of a mutation in the *PBP* gene, causing the antibiotic target site to be modified. These modifications rendered these drugs ineffective because the organism developed PBPs that no longer could bind the Beta Lactam ring. Both *S. aureus* and *Enterococcus faecium* developed resistance to antibiotics in the Beta Lactam class via target site modification. The development of this type of resistance is dangerous, as they are genetically transferrable across species allowing other bacterial strains to create mutant PBPs. [6]

Another extremely common method of resistance is the evolution of enzymes that have developed the capability to inactivate drugs. This method of resistance has caused great devastation to current treatments.[4] The Gram-positive bacteria *Mycobacterium tuberculosis* has developed resistance to the aminoglycoside (AG), Kanamycin A (KAN), which targets the 30s ribosomal subunit. *M. tuberculosis* was able to adapt through the development of mutations on the promotor region of the *eis* (enhanced intracellular survival) gene resulting in a gain of function of AG acetyltransferases, which acetylates the antibiotic destroying its activity. In this case, *M. tuberculosis* developed AG acetyltransferases that are capable of acetylating the amino group of KAN causing inactivation. This gene has been seen in other organisms and can pose a great danger to the ability to treat this multi-drug resistant Gram-positive pathogen that has

shown incredible resilience.[7] Resistance such as this has occurred in Gram-positive organisms against other antibiotics such as, Beta Lactams, which are hydrolyzed on the beta lactam ring by Beta Lactamases resulting in inactivity.[4]

The development of efflux pumps is an example of intrinsic resistance that can potentially eradicate currently used antibiotics. Some estimates predict that 5-10% of bacterial genes contribute to this function.[8] In many cases, if this mechanism develops against a non-specific antibiotic, then it will likely prevent the effectivity of the other antibiotics with a similar mode of action, making this mechanism highly dangerous. Gram-positive pathogens utilize this resistance mechanism for a variety of antimicrobial agents. Organisms have developed efflux mechanisms against several antibiotics from the fluoroquinolone class including ciprofloxacin, norfloxacin, and ofloxacin. These antibiotics target DNA synthesis machinery, including DNA gyrase and topoisomerase.[4] Efflux pumps are more precarious than other methods of resistance, in that they may be a bridge as a single step mutant can result in a multi-drug resistant pathogen. This occurs when low level resistance created by efflux pumps to one antibiotic in the class eventually results in resistance to several antibiotics within the class.[8]

The final mechanism is the altering of porin channels that prevent drug entry, which lowers the antibiotic concentration in the cell. This mechanism is more common in Gram-negative organisms because they have an additional outer membrane that consists of a lipid bilayer.[4] Porins are open pores in the membrane and antibiotics are able to pass through these pores to gain easy access into the cell. This is especially effective as many of the antibiotics that are currently used have intracellular targets. Bacterial cells can develop resistance to this through alteration to the voltage and conductance of these porins putting them into a closed or inactivated state that will not allow entry of the antibiotic into the cell. This mechanism is utilized by many

Gram-negative pathogens such as, *Pseudomonas aeroginos, Neisseria gonorrhoeae, Enterobacter aerogenes, klebsiella pneumonia* and many others.[9] However, this can occur in Gram-positive pathogens, such as Vancomycin-*intermediate S. aureus* (VISA), which displays phenotypic passage-selection against the antibiotic Vancomycin resulting from the development a thickened peptidoglycan wall to prevent Vancomycin access into the cell.[10]

With resistance on the rise and Gram-positive infections utilizing many mechanisms to combat all current treatments, there is a desperate need for the development of new antibiotics. In 2013, Gram-positive organisms appeared in all three categories of CDC's levels of concern including Urgent Threats, Serious Threats, and Concerning Threats. The economic effects of these infections have been devastating due to the expense of hospitalization and loss of work force. Approximations for the year 2008 are 20 billion dollars in healthcare expenses exacerbated by an additional estimated cost of 35 billion dollars from loss of work productivity.[11]

Currently, the most dangerous Gram-positive organism is *Clostridium difficile*, which has had catastrophic effects on American society causing 250,000 infections and 14,000 deaths per year. This organism was classified as an Urgent Hazard Level due to its potential to become widespread across the country. *C. difficile* occurs on a nosocomial and non-nosocomial basis costing 1 billion dollars in excess medical costs per year. This microorganism has had a drastic impact on the senior citizen population; killing 90% of the senior citizens that it infects. Other dangers include, Drug-Resistant *S. pneumoniae*, which is currently considered at Serious Threat Level and causes 1.2 million infections annually, 7000 deaths, and costs 19,000 dollars in excess hospitalizations. Approximately, 160,000 children under the age of 5 will be admitted into a hospital as a result of this Gram-positive infection. Several other resistant organisms have also bombarded the healthcare system. *S. aureus* has expanded its resistance against many of the past

successful antibiotics. With new strains evolving against current antibiotics, including methicillin resistant *S. aureus* (MRSA) and Vancomycin-resistant *S. aureus* (VRSA), which are Serious Threat and Concerning Threat levels respectively. It is essential that new antibiotic targets are discovered against Gram-positive bacteria.[11,12]

All of these infections are evolving resistance via aforementioned mechanisms and resulting in drastic health care costs, morbidity and mortality. The resistance rate has drastically increased as treatments of patients in chemotherapy, complex surgery, afflicted with rheumatoid arthritis, dialysis for renal disease or receiving organ and bone marrow transplants all pose high risk for infections. The use of antibiotics in these patients breeds resistance because of decreased ability to fight infection as a result of immunodeficiency. In many cases certain antibiotics cannot be used or are dose limited because of the toxicity in patients with some of these conditions, posing a great threat for the development of resistance. This is due to the fact that low doses expose many bacteria to the antibiotic, but may not effectively kill every viable cell. This may result in spontaneous mutations that create resistance to an antibiotic. Ultimately, even limiting the over-prescription of antibiotics, preventing infection, and tracking infections will only slow and not eliminate the problem of resistance.[11]

RNA has increasingly become recognized as an advantageous target for antibiotic drug development in the fight against antibiotic resistance. The integral nature of RNA in the genetic and functional processes of a cell makes it a desirable target for drugs. Riboswitches are RNA regulatory elements that change structural conformation upon metabolite or ligand binding to alter gene expression. These metabolite sensing regulators are energetically conservative as they almost exclusively occur at the 5'UTR preventing unnecessary transcription of the operon. The region with the most conservation is the four nucleotide binding domain that is often referred to

as the “aptamer” domain. Interaction at this aptamer domain results in a second binding event at the “expression platform”, which determines whether or not downstream gene expression will occur.[13]

The T-box regulatory mechanism is not a riboswitch in a conventional sense, but it functions very similarly in its method of regulation. This regulatory element’s is specific to Gram positive bacteria and is used by many operons encoding essential genes. One essential gene that is regulated through this mechanism is the aaRS gene, which is responsible for making aminoacyl-tRNA synthetases. These enzymes are essential for catalyzing reactions that charge the tRNA with the proper amino acids. [14] Regulating at the transcription attenuation level, the T-box mechanism efficiently uses the binding of a charged and uncharged tRNA species to regulate translation of amino-acid related genes, such as amino-tRNA synthetases and amino acid transporters. The tRNA species anticodon specificity is essential to regulation as deviation in a single nucleotide can result in a significant decrease in expression of genes.[15]

T-box regulation consists of a specific tRNA binding to two distinct regions in the 5’ UTR. Figure 1 illustrates these binding events and the formation of both the attenuation and downstream reading events. The first interaction occurs in stem I at the Specifier Loop where a base pair binding event between the anticodon of a specific tRNA and the Specifier Loop on Stem I of the 5’UTR occurs. After the first binding event, a second binding event will occur at the second Loop taking the form of one of two potential structures, a terminator or antiterminator helix. If the binding tRNA is charged, the amino acid present prevents stabilization of the antiterminator helix and a terminator Loop is formed, resulting in no downstream reading of the operon. The T-box mechanism is predisposed to termination as the terminator helix is a more thermodynamically stable structure then the anti-terminator helix. However, if an uncharged

tRNA is bound to the Stem I Specifier Loop, then the interaction will promote a second binding event between the NCCA sequence at the acceptor end of the tRNA and the UGGN residues of anti-terminator bulge region in Stem II of the mRNA 5'UTR. This interaction prevents the formation of the terminator helix allowing for expression of the downstream coding sequences.[15] Specific tRNA recognition by the nascent transcript will result in an increased expression of the genes imperative to tRNA aminoacylation.[14]

Figure 1

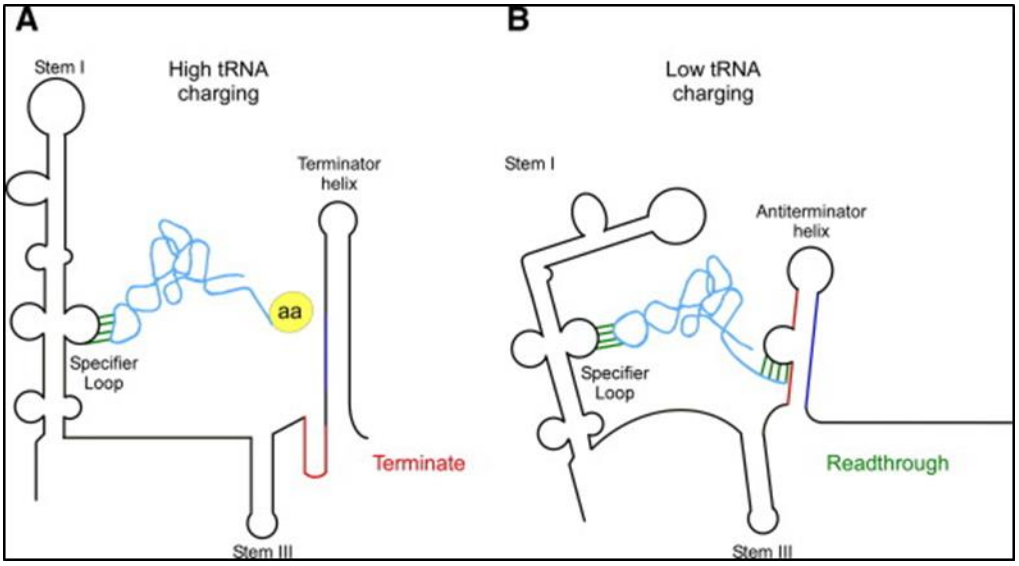

Figure 1:

"The T box mechanism. Expression of genes in the T box family is regulated by the ratio of charged to uncharged tRNA in the cell. (A) Aminoacylated tRNA binds only to the Specifier Loop; the presence of the amino acid prevents interaction of the acceptor end of the tRNA with the antiterminator. The more stable terminator helix (blue-black) forms and transcription terminates. (B) Uncharged tRNA interacts at both the Specifier Loop and the antiterminator; this stabilizes the antiterminator (red-blue) which sequesters sequences (blue) that otherwise participate in formation of the terminator helix, and transcription reads through the termination site and into the downstream coding sequence. Binding of uncharged tRNA results in structural changes throughout the leader RNA. The tRNA is shown in cyan; the amino acid (aa) is shown as a yellow circle attached to the 3′ end of the charged tRNA. Positions of base-pairing between the leader RNA and the tRNA (Specifier Loop–tRNA anticodon, antiterminator bulge–tRNA acceptor end) are shown as green lines."

Description and Image Adapted from:
Nicholas J. Green , Frank J. Grundy , Tina M. Henkin
"The T box mechanism: tRNA as a regulatory molecule"
FEBS Letters, Volume 584, Issue 2, 2010, 318 – 324
http://dx.doi.org/10.1016/j.febslet.2009.11.056

As aforementioned, this process requires Specifier sequence recognition of the tRNA anticodon and the anti-terminator bulge that interacts with the acceptor end of the tRNA. The tRNA recognition site has an apex region with an adenosine-guanine bulge that is referred to as the distal Loop. The Loop to Loop structural region serves as a docking platform for the tRNA to bind for gene expression. This marks some of the major similarities to the structural events that occur in a conventional riboswitch.[14] Further evidence to support the riboswitch nature of this mechanism is that *in vivo* and *in vitro* studies demonstrate that a single tRNA in its entirety is sufficient to promote the regulatory response.[16]

The genes encoded by the T-box mechanism are essential genes for bacterial growth and proliferation. Multiple T-boxes are present in a single organism. Many Gram-positive bacteria such as, *Bacillus Subtilis* and *S. pneumonia*e, which have been shown to contain T-boxes upstream of valS, pheS, pheT, glyQ, glyS, thrS, and ileS genes as well as for other genes as well.[17] The T-box Specifier Loop is a novel target for antibacterial drug discovery as we hypothesize that a small compound bound to the Specifier Loop will inhibit transcription of essential bacterial genes resulting in bacterial cell death or growth arrest. Due to the conserved nature of the Specifier Loop there is potential for a broad spectrum of antibacterial activity against Gram-positive pathogens. Since there are multiple T-boxes within each bacterium, there is a decreased chance of resistance emerging, as a single small molecule antibiotic could shut off multiple T-boxes limiting the bacteria's ability to evolve by altering the antibiotics binding site. The high conservation of this region suggests that any major alteration to this binding site may result in a loss of function and bacterial cell death. The T-box regulatory mechanism is not present in eukaryotic cells, which reduces the potential for cytotoxicity. With these factors in

mind, a small molecule that can bind to T-boxes and disrupt gene regulation is a strong candidate for development of new antibiotics.[16]

Using *in silico* analysis of the T-box Specifier Loop, small compounds that are likely to disrupt T-box function were identified. Bacterial growth arrest studies identified three initial hit compounds, one of which demonstrated Gram-positive specificity and displayed moderate-to-low cytotoxicity in human cells. Based on this data, structure activity relationship studies (SAR) were conducted with disk diffusion assays against *S. aureus* with structural analogs of our initial hits. Minimum Inhibitory Concentration (MIC) assays and Minimum Bactericidal Concentration (MBC) assays were conducted against *S. aureus* and the Gram-negative bacterium *Escherichia coli* for analogs that displayed antibacterial activity with disc diffusion assays. Using the successful compound structures as a template, an additional *in silico* docking study was conducted on selected analogs with structures identified as essential to antibiotic activity. Top candidates of this docking simulation as well as other carefully selected analogs will be tested using both MIC and MBC assays to further assess and refine structural determinants required for antibacterial activity. These SAR studies have allowed for the determination of important biochemical features for Gram-positive activity including, the heterocyclic thiazole, norbornane carboxylic acid, and benzene. Evidence suggests that a meta orientation on the benzene ring between the thiazole and a long carbon chain may produce optimal antimicrobial activity.

Methods:

Chemical Compound Re-suspension:

Compounds were commercially available and purchased from Ambinter, Asinex, Enamine, Akos, Molport, Prinston Biomoleular and Chembridge. All compounds that were used for the experiments were suspended in 100% DMSO at a concentration of 15 mg/mL for disk

diffusion assays and 10mg/mL for MIC assays. DMSO was chosen because many of the compounds displayed poor solubility in the presence of standard media or water. All compounds were aliquoted and stored at -80°C until the day of the experiment. Individual aliquots of compound, gentamicin and DMSO were thawed on ice on the day of an experiment. Control antibiotics were prepared in 100% H_2O, and stored at -20°C until experimental use.

Bacterial Culturing:

Staphylococcus aureus, subsp. *aureus* Rosenbach ATCC 29213 (ATCC) was used for all anti-microbial activity experiments. This strain was used as it is laboratory strain of *S. aureus* commonly used for anti-microbial activity testing. For each experiment, different types of media's were used, Tryptic Soy Broth (TSB) and Luria Bertani agar (LBA). The TSB (Sigma) was used for growth of liquid cultures and prepared water as per manufacturer's instructions, and supplemented with 2.5 g of dextrose per liter of media as a preferred carbon source. Media was sterilized by autoclave. An overnight culture was set up prior to each experiment by placing a 3-4 morphologically similar colonies of *S. aureus* into a sterile culture tube with 3 mL of TSB. The culture was placed in a shaking incubator at 37 °C for 18 hours at 225 rpm. After 18 hours a new culture was prepared so that the bacteria would be in the logarithmic stage of growth for each experiment. Using the overnight culture, 60 µL of bacterial culture was pipetted into a new tube of 2.94 mL of TSB. This culture provided a 1:50 ratio of bacteria to media and was placed in the shaking incubator at 37°C for 3-4 hours prior to the experiment so that bacterial culture would be in logarithmic stage of growth. An OD_{600} reading between 0.4-0.8 indicates exponential growth stage. LB agar was chosen as solid media for antibiotic susceptibility testing. The media was made with a ratio 35 g of media per liter of DI water as per manufactures instructions. Media was sterilized by autoclave and poured into sterile petri dishes with a volume of 25 mL in each

plastic petri dish to ensure reproducibility. All media was made and poured under sterile conditions to prevent contamination. Plates were stored at 4°C.

Disk Diffusion Assay:

The antimicrobial susceptibility testing was done using the Kirby-Bauer disc diffusion method. Disk diffusion assay protocol was modeled after Bauer et al.[18] Under sterile conditions disks were placed on a prepared sterile sheet of aluminum foil and compounds were spotted onto each disk at a concentration of 150 µg/mL. A sterile cotton swab was dipped into the bacterial solution of *S. aureus* and then used to completely cover the surface of a pre-warmed LB agar plates. Plates were dried in an incubator for 10 minutes and then the spotted disks were placed onto the inoculated petri dishes antibiotic side down to maximize compound diffusion through the agar. Following incubation for 18 hours at 37 °C, the zones of inhibition were measured and photographed. After all compounds were tested in triplicate, those with ambiguous results were retested in triplicate at a higher concentration of 300 µg/mL using the same procedure stated above. This was done so that all compounds that successfully inhibited bacterially growth could be determined accurately.

Minimum Inhibitory Concentration (MIC)/Minimum Bactericidal Assays (MBC):

The minimum inhibitory concentration assay is used to determine the lowest concentration that an antimicrobial can inhibit visible growth of bacteria. The results of the MIC assay can be used to conduct a minimum bactericidal assay, which is used to determine the lowest effective concentration that an antimicrobial kills all viable bacteria. Prior to the experiment all The MIC assay was set up using a 96 well plate. All MICs and MBC antimicrobial testing was conducted based on the NCCLS standard with modifications.[19]

Compounds were serially diluted 2-fold from 481 µg/mL to 0.23 µg/mL. There was a sterility control well containing 100 µL TSB where no inoculum was added. A growth control well was implemented with 80µL TSB to which 20 µL standardized inoculum was added. A DMSO control was used to ascertain the growth level in the presence of solvent without compound, which was used to demonstrate any impact of the presence of the final 4.81% DMSO. Additionally, Gentamicin, a clinical antibiotic, was used as a control and was serially diluted two fold from 48.1 µg/mL to 0.092 µg/mL to confirm expected MIC against laboratory strain of use. A culture in exponential growth was diluted as per the McFarland Standard to OD_{600} of 0.08 to 0.12 which contains 1-2x10^8 CFU/mL. After an additional 1:2.5 dilution was carried out. All wells were inoculated within 15 minutes of dilution with 20 µL containing 8x10^5 CFU/mL. After all necessary wells were inoculated and thoroughly mixed, a micro plate reader was used to measure the OD_{600} of each well. The plate was then allowed to shake at 100 rpm at 37°C in the incubator for 20 hours. After this time period, another OD_{600} reading was taken to determine a change in optic density. This provided a quantitative determination to whether or not there is bacterial growth. The MIC was determined as the lowest compound concentration at which no visible growth was observed. Criteria for MIC was assessed using graphed data assigning a MIC value based on the concentration that showed close to a 100% inhibition just prior to a large increase in growth.

To determine the MBC, all wells from the MIC experiment with a concentration equal or greater than the MIC were individually spread onto separate dishes of LB agar and incubated at 37°C for 24 hours. The MBC was recorded as the lowest compound concentration where there were no viable bacterial colonies. If the MBC was is no more then 4 fold of the MIC of a specific

compound then this compound was determined to be bactericidal and if it was not then it was deemed bacteriostatic.

In silico screening:

Using *in silico* analysis, 250,000 small molecules were screened to determine compounds likely to bind to the T-box Specifier Loop of the Gram-positive bacterium, *Bacillus subtilis*. Of this large small molecule database, 200 molecules showed potential to disrupt T-box function. This work was conducted by Albany Molecular Research Institute using sophisticated computer technology that assessed the binding energy of small molecules to the target. From this list of 200 compounds, 28 of these were selected for bacterial growth arrest studies. Figure 2 demonstrates the progression of compounds from the initial *in silico* to the analogs currently being tested. Biological testing resulted in the identification of two initial hit compounds PKZ 1800, and PKZ 0600, which became the focus for structure-activity relationship studies based on their greater antimicrobial activity and Gram-positive selectivity. To narrow necessary structural elements for antimicrobial activity, we began SAR studies of analogs of these two initial hit compounds. SAR round 1 was conducted with disk diffusion assays against *S. aureus* using 20 structural analogs of parent compound PKZ 1800 and 15 analogs of PKZ 0600. An additional 15 compounds were tested from the original list of 200 in search of more effective small molecule families then current initial hits under SAR evaluation.

Based on the data provided by both the disk diffusion and liquid screening assays, structural searches were conducted in small molecule libraries that are publicly available. These searches were based on modifications to parent compound PKZ 1800 in a chemical draw program so that analogs with specific desired structures could be found. An additional *in silico* analysis was done by The RNA Institute of 22 compounds chosen from these chemical libraries.

This additional *in silico* was conducted to determine binding energies of these new potential compounds for future testing in search of a PKZ 1800 analog that may have improved biological activity. Binding energies of each compound for the second *in silico* were ascertained and compared to prior successful parent compound PKZ 1800 as well as ethanol as a negative control.

Figure 2:

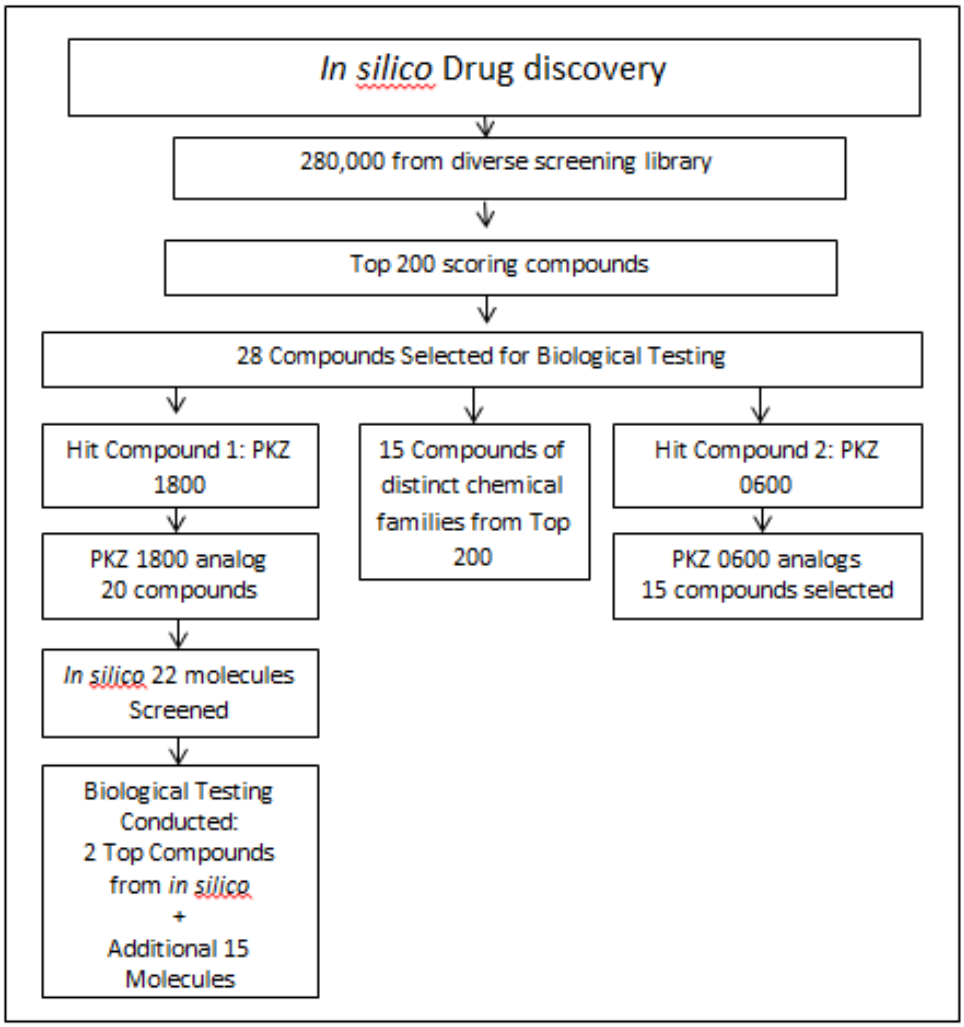

Figure 2
This figure demonstrates the chronological of compound testing and selection. It reflects the initial testing done that identified the first 2 hit compounds all the way until the current PKZ 1800 analogs being tested in the second round of SAR studies.

Liquid-Culture Screen:

Structural analogs PKZ 1821-1834 were tested in an initial screening at a concentration of 500µg/mL or 250 µg/mL, depending on solubility restraints. This initial testing was to determine which compounds demonstrate antimicrobial activity so that a full MIC assay could be conducted against both *S. aureus* and *E. coli* for the successful compound. To a 96-well plate we added 170 µL of media, 20 µL of media with inoculum, and 10 µL of 10 mg/mL compound for 500 µg/mL final concentration or 5 µl of 10 mg/ml compound for 250 µg/mL final. Controls included a sterility control (TSB only), a positive growth control (TSB+bacteria), and DMSO control (5%DMSO+TSB+bacteria). Additionally, a titration of Gentamicin from 50 µg/mL to 0.1 µg/mL was used to confirm there was no contamination by ensuring that the anticipated MIC of Gentamicin against the lab strain used was seen. Compound inhibition was determined quantitatively by measuring OD_{600} reading immediately after well inoculation and re-measuring following a 20 hour incubation period with plate shaking at 100 rpm at 37°C. Inhibition was determined by the change in OD_{600} after the incubation period. If there is increase in optical density over the incubation period it demonstrates that bacterial growth is present.

Results:

Antibacterial testing of 15 Distinct Chemical Families:

The first *in silico* yielded 200 compounds likely to disrupt T-box function and 28 of these compounds had been screened for activity. With 2 initial hits being pursued for SAR studies the search for potential more effective chemically distinct families continues. There 15 molecules of chemically distinct families screened during SAR studies. First disk diffusion assays were conducted at 150µg/mL against *S. aureus* to see if there was antibacterial activity. Of these 2 compounds there were 2 that showed potential inhibition, but the results were ambiguous. To further ascertain there potential these compounds were screened at 300µg/mL, where they showed effectivity. These compounds were then tested in micro-dilution assays to determine the MIC and MBC concentrations. The results of these compounds screens are reflected by Table I. Both of these compounds, PKZ 0024 and PKZ 0034 showed no Gram-positive activity. PKZ 0034 demonstrated no activity against *E.coli* or *S.aureus* and PKZ 0024 showed activity against only *E.coli* at 481 µg/mL.

Table I: Distinct Chemical Families from AMRI Docking

AMRI docking Top 200 molecules	Zone of Inhibition (cm) Concentration 150µg/mL	Zone of Inhibition (cm) Concentration 300µg/mL
0021	NI	NT
0022	NI	NT
0023	NI	NT
0024	1.05 *	1.31*
0025	NI	NT
0026	NI	NT
0027	NI	NT
0028	NI	NT
0029	NI	NT
0030	NI	NT
0031	NI	NT
0032	NI	NT
0033	NI	NT
0034	NI	0.956*
0035	NI	NT
Compound	*S. aureus* MIC (MBC)	*E. coli* MIC (MBC)
0024	N/O (N/O)	481 (N/O)
0034	N/O (N/O)	N/O (N/O)

Table I: Legend

NI= No Inhibition

NT= Not Tested

MIC = Minimum Inhibitory Concentration Assay

MBC =Minimum Bactericidal Concentration *Assay*

N/O= Not observed

IND= Indeterminable

*All concentrations above are in µg/mL

SAR of Initial Hit Compounds PKZ 1800/PKZ 0600:

Previous work has shown that initial hit compound PKZ 0600 demonstrated activity against various Gram-positive organisms and some Gram-negative organisms. All PKZ 0600 analogs were screened using disk diffusion assays against *S. aureus* to determine if they had any activity. Those compounds with antimicrobial activity were selected for MIC and MBC assays. Table II shows the number of analogs tested and the number that showed activity in disk diffusion assays. PKZ 0600 had several components to its chemical structure, including a honey comb of three fused cyclic rings with an amide linkage followed by a carbon chain, leading to a piperzine moidy. This honey comb includes a fused benzene ring, pyrolidine ring, and partially unsaturated pyridine ring, which was substituted with an amide, alcohol, and carbonyl group. PKZ 0600 analogs varied in number and structure of fused rings with alternative structures cyclic and non-cylic in nature replacing the piperzine moidy.

Compounds PKZ 0601, 0602, 0603, 0606, 0609, 0612, 0613 and 0615 all maintained an identical honey comb structure to PKZ 0600 including all three substituents attached to the pyrolidine ring. Variation between these analogs occurs in the alternate structures replacing the piperizine moidy. Analogs PKZ 0603, 0609, 0613 and 0615 showed antibacterial activity, but demonstrated no Gram-positive selectivity. Compounds PKZ 0603, 0609, and 0615 showed antibacterial activity against *S. aureus* in disk diffusion assays. Table III shows the results of MIC assays of these compounds against both *S. aureus* and *E. coli*. All three of these compounds demonstrated higher activity against the Gram-negative bacterium *E. coli* with MIC values of 235µg/mL. These 3 compounds PKZ 0603 0609 and 0615 all had MIC values >235µg/mL against *S. aureus* demonstrating less activity against Gram-positive pathogens.

Compounds PKZ 0604, 0605, and 0614 retained the honey comb structure of the parent compound, but contained an oxygen substituent instead of an alcohol on the partially unsaturated pyrolidine. Additionally, these compounds varied in there replacement of the piperizine moidy. Of these compounds only PKZ 0605 was effective and demonstrated minimal activity in disk diffusion assays, but it was not commercially available so no further testing was conducted. PKZ 0600 analogs showed no specificity to Gram-positive bacteria in micro-dilution assays suggesting non-specific binding. As seen on Table I there was one exception to this, which was PKZ 0611. PKZ 0611 demonstrated high Gram-positive antimicrobial activity and no activity against the Gram-negative bacteria. This compound was unique containing 4 fused cyclic rings including, 2 piperidine rings with an oxygen substituent fused to pyran ring containing multiple oxygen substituents and an unsubstituted benzene ring. No obvious structural similarities were present between this PKZ 0600 analog and the effective PKZ 1800 analogs that showed Gram-positive selectivity. There were 8 successful compounds amongst the PKZ 0600 analogs. Of these compounds, 5 contained a honey comb structure with two fused benzene rings and piperizine ring, but 6 unsuccessful compounds also had these chemical elements. There was no single element or combination of elements that were exclusively present in successful molecules making definitive conclusions difficult to draw.

Prior testing conducted on initial hit PKZ 1800 showed Gram-positive selectivity. It was proven effective in both solid and liquid media culture screenings and was subject to testing with a variety of bacterial strains. Disk diffusion assays were conducted to ascertain analogs that had activity against *S.* aureus. This was done so that only those compounds with sufficient activity were tested in MIC and MBC assays. PKZ 1800 analogs revealed many significant findings and showed three chemical structures that were prominent in all of the compounds with the highest

antibiotic activity. Disk diffusion assays were used to narrow in on compounds with activity levels of interest so that full MICs were only done on those compounds. Full MIC values were determined for compounds that showed activity in disk diffusion assays.

There are 3 major components of the parent compound including a Norbornane carboxylic acid with an amide linkage in a meta orientation to the benzene ring substituent on the heterocyclic thiazole. Additionally, there was a methyl group ortho to the benzene ring. Of the 20 analogs of PKZ 1800 tested, 13 of these analogs contained the heterocyclic thiazole. All effective compounds retained this integral feature including compounds 1805, 1808, 1810, 1813, 1819 and 1820. Compounds PKZ 1819 and PKZ 1820 contained the norbornane carboxylic acid, the heterocyclic thiazole, and the benzene ring identical to the parent compound. PKZ 1820 and PKZ 1819 showed inhibitory activity at concentrations of 117µg/mL and 481µg/mL respectfully, but had no activity against *E. coli*. These compounds differed from the parent compound in their substituents of the benzene ring. The parent compound has an isopropyl and the thiazole in a meta orientation on the benzene ring. PKZ 1819 has identical structure to the parent compound, but has two fluorine's one ortho and one meta in orientation to the thiazole substituent on the benzene ring. PKZ 1820 has two chlorines one in ortho position and one in para position. Table III shows these results demonstrating compounds PKZ 1819 and 1820 have antibacterial activity and Gram-positive selectivity. PKZ 1813 contained both the norbornane carboxylic acid and the heterocyclic thiazole, but in replacement of the benzene ring the molecule had a thietane ring with no substituents. PKZ 1813 was effective in disk diffusion assays, but showed no activity in MIC assays against *S. aureus* or *E. coli.*

Analogs PKZ 1805, 1808, and 1810 contained both the heterocyclic thiazole and the benzene ring with a cyclohexanoic acid with partial unsaturation in place of a norbornane

carboxylic acid. The benzene ring substituents vary with these compounds with longer and shorter carbon chains instead of the isopropyl group present on the parent compound. PKZ 1805 showed minimal success in disk diffusion assays and was not available commercially so no future testing was conducted with this molecule. As seen on Table III, compound PKZ 1808 showed antibacterial activity at the 235µg/mL against *S. aureus* and 481 µg/mL against *E. coli*. PKZ 1808 had some Gram-positive selectivity. PKZ 1810 demonstrated antibacterial activity with no Gram-positive selectivity having MIC's of 481µg/mL against both *S. aureus* and *E. coli*.

Table II:

Parent Compound	Compounds Tested	Compounds Successful
PKZ 1800	20	6
PKZ 0600	15	8

Table II: Description

This table demonstrates the amount of the structural analogs that showed activity against that showed activity at ≤300 µg/mL. Disk Diffusion assays were used to screen out compounds with activity of potential interest.

Table III:

Compound	*S. aureus* MIC (MBC)	*E. coli* MIC (MBC)
1808	235 (N/O)	481 (N/O)
1810	481 (N/O)	481 (N/O)
1813	N/O (N/O)	N/O (N/O)
1819	481 (N/O)	N/O(N/O)
1820	117 (N/O)	N/O(N/O)
0603	481 (N/O)	235 (N/O)
0607	481 (N/O)	235 (N/O)
0609	N/O (N/O)	235 (N/O)
0610	235 (481)	235 (481)
0611	58.8 (N/O)	N/O (N/O)
0613	N/O (N/O)	N/O (N/O)
0614	N/O (N/O)	N/O (N/O)
0615	481 (N/O)	235 (481)

Table III: Legend

MIC = Minimum Inhibitory Concentration Assay

MBC =Minimum Bactericidal Concentration *Assay*

N/O= Not observed

ND= Indeterminable

SAR round 2 of PKZ 1800:

For the next round of SAR PKZ 1800 analogs became the main compound of focus. This was due to the fact that amongst the PKZ 0600 analogs there was no Gram-positive selectivity and structural determinations were ambiguous. Additionally, cytotoxicity data conducted by the Wadsworth center demonstrated that the PKZ 0600 parent compound had far higher cytotoxicity to mammalian cells then PKZ 1800. With all of these factors considered, efforts were invested in compounds similar in structure to parent compound PKZ 1800. PKZ 1800 analogs displaying antibacterial activity and Gram-positive selectivity in SAR round 1 were used as a structural template for future compounds of testing. Compounds were chosen based on the presence of specific combinations of structures that might further enhance antimicrobial activity or provide valuable information about the importance of certain structures. This included compounds both containing and lacking the benzene ring, methyl group, isopropyl group or norbornane carboxylic acid. All molecules contained the heterocyclic thiazole. Unique molecules with new structural elements replacing the norbornane carxboxylic acid were tested in search of a possibly better replacement for the norbornane structure.

The 22 potential molecules selected were put into an *in silico* analysis to ascertain whether these new compounds were likely to bind the Specifier Loop. Top candidates of this docking simulation as well as other carefully selected analogs were tested at 500 µg/mL to determine if they were compounds of interest. Using solubility for guidelines, all compounds that were not soluble enough to determine an accurate determination of growth were used at lower testable concentrations. This included concentrations that would have an initial OD_{600} less than 0.7. Table IV shows the results from this initial screen, which yielded 5 compounds with 100% inhibition at 500 µg/mL. The results of these experiments can be seen on Table IV.

As seen on Table IV, Analogs PKZ 1821, 1822, 1823, 1825 and 1833 showed 100% inhibition at 500 µg/mL. PKZ 1821, 1822, 1823, 1825, and 1833 have been tested with full MIC and MBC assays against *S. aureus* and *E. coli* (Table V). Analog PKZ 1821 was identical to the parent compound with the exception of the removal of a single methyl group bound to the heterocyclic thiazole ortho in relation to the benzene ring. PKZ 1821 demonstrated a MIC of 58µg/mL against *S. aureus* and no MIC against *E. coli*. PKZ 1822's structure includes the removal of the methyl group and the addition of a carbon to the isopropyl group bound to the benzene ring making it an isobutyl group. As reflected in Table V, PKZ 1822 had the highest antibacterial activity and Gram-positive selectivity of any compound tested with a MIC of 29 µg/mL against *S. aureus* and no MIC against *E. coli*.

Chemical compound PKZ 1823 lacked the methyl group on the heterocyclic thiazole, and contained 2 methyl groups on the benzene ring instead of the isopropyl group. This compound only showed activity at 250 µg/mL in initial screen (Table IV), but did not show any activity in the full MIC assay testing at $\leq$ 235 µg/mL (Table V). PKZ 1825 maintains the exact same structure as the parent compound, but removes the entire isopropyl group from the benzene ring. Additionally, PKZ 1825 lacks the carboxylic acid. As seen on Table III, PKZ 1825 showed activity at 235µg/mL against both *S. aureus* and *E. coli* suggesting that the carboxlic acid on the Norbornane is essential for Gram-positive selectivity. The higher activity of PKZ 1821 and PKZ 1822 may suggest the importance of the carboxylic acid present on the norbornane. It also supports that the methyl substituent on the thiazole rings is not necessary for optimal activity. Lastly, PKZ 1833 was a unique compound containing an alternative structure to the norbornane. In addition, this compound lacks the methyl group on the heterocyclic thiazole and has an amide in replacement of the benzene ring. This is the only compound that demonstrated activity without

the benzene ring. This compound was tested at 500 µg/mL and showed activity (Table IV), but showed no activity at ≤ 235 µg/mL in the full MIC assay (Table V).

Table IV:

Concentration (µg/mL)	Compound	%Inhibition	Solubility
500	1821	100*	NCS
	1822	100*	NCS
	1823	100*	NCS
	1824	47	S
	1825	100*	NCS
	1826	53*	NCS
	1827	46	S
	1828	57	S
	1829	44	S
	1830	36	NCS
	1831	0	S
	1832	31	NCS
	1833	100	NCS
	1834	0	NCS
250	1821	100	NCS
	1822	100	NCS
	1823	100	NCS
	1825	100	NCS
	1826	47	NCS

Table IV: Legend

NCS = Not completely soluble

S = Soluble

* = Background OD above 0.7

Table V:

Compound	*S. aureus* MIC (MBC)	*E. coli* MIC (MBC)
PKZ 1821	58.8 (N/O)	N/O (N/O)
PKZ 1822	29.4 (IND)	N/O (N/O)
PKZ 1823	250 (N/O)	N/O (N/O)
PKZ 1825	235 (N/O)	235 (N/O)
PKZ 1833	N/O (N/O)	N/O (N/O)

Table V: Legend

MIC = Minimum Inhibitory Concentration Assay

MBC =Minimum Bactericidal Concentration *Assay*

N/O= Not observed MIC at concentrations ≤235 µg/mL

IND= Indeterminable

*All concentrations above are in µg/mL

Discussion:

Thorough evaluation of the chemical structures of the first round SAR compounds suggests that the heterocyclic thiazole is an essential component for antibacterial activity amongst PKZ 1800 analogs. All successful PKZ 1800 analogs contained this integral structural feature. Due to the fact that some compounds that were not effective contained the heterocyclic thiazole, this suggests that the structure is necessary, but may not be sufficient for optimal activity. The presence of a benzene ring seems to act synergistically with the thiazole ring as all

compounds that depicted significant antimicrobial activity in micro-dilution assays contained the two connected aromatic structures. Interestingly, neither of these components would ensure the desired Gram-positive specificity from these antimicrobial agents. Further evaluation of PKZ 1800 analogs revealed that all compounds containing the norbornane carboxylic acid structure showed specificity towards inhibition of the Gram-positive organisms. PKZ 1800 analogs, PKZ 1808 and PKZ 1810 lacked the selectivity that was seen in PKZ 1819 and 1820. The major distinguishing chemical element between these pairs of analogs was the presence of a norbornane carboxylic acid. PKZ 0600 analogs demonstrated no commonalities amongst successful compounds making structural assertions very difficult. Based on the Gram-positive selectivity, increased antimicrobial activity, and cytotoxicity, PKZ 1800 analogs became the main focus of second round SAR studies.

Our structure-activity relationship studies reflected the integral nature of the heterocyclic thiazole present in PKZ 1800. It was present in all effective PKZ 1800 analogs. Thiazoles have pharmaceutical significance and have been shown to have a variety of biological functions. They have led to a variety of antifungals, antioxidants, antibacterial, antitubercular, antidiuretic, anti-inflammatory and anticancer activities.[20]

Currently used thiazole antibiotics have been shown to act on bacterial RNA targets. A study conducted by Lentzen et al[21] showed that thiazole antibiotics thiostrepton, micrococcin, siomycin, and nosiheptide have a 100 fold higher selectivity for inhibition of bacterial translation over eukaryotic. Results from this study suggest that thiazole antibiotics nosiheptide, thiostrepton, and siomycin had a high affinity for the L11 binding domain (L11BD) of 23S ribosomal RNA and interact to alter its confirmation. 3D modeling revealed stacking interactions between all four antibiotic thiazole moieties and the quinaldic acid (QA) residue of the rRNA.[21]

The benzene ring appears to be a significant component as all compounds with antibacterial activity in second round SAR had this structure. Several compounds tested in the liquid culture screen assay were almost identical to parent compound PKZ 1800, except without a present benzene ring and yet these compounds showed no activity. Pi stacking is a potential mechanism of interaction between a benzene ring and RNA target. Variation in effective structures may be a result of the diversity of substituents of this element. A recent computation study by Marcey L Waters[22] discusses the strong interactions that can occur between biological elements and aromatic rings as a result of their hydrophobic nature. This includes interactions with water where a quadrupole moment may form resulting in an increase in electron density towards the face the aromatic ring making it more reactive with negatively charged RNA.[22] A study by Cammers-Goodwin et al[23] suggested that water can act with the hydrogens of aromatic rings to facilitate pi stacking.[23] Under normal conditions the large presence of water in a cell may facilitate this type of interaction between a small putative antibiotic and Specifier Loop of the T-box element. Both aromatic rings including the heterocyclic thiazole and the benzene ring appear essential for significant activity. Their importance may be because of a hydrophobic stacking mechanism that promotes interaction between these aromatic rings and the Specifier Loop of a nascent mRNA.

Antibacterial activity of aromatic rings can be altered drastically by the position and identity of functional groups substituents. In an antimycobacterial study conducted by Makam et alREF against *M. tuberculosis* thiazole compounds linked to benzene rings through amines were tested and the effectivity of varying substituents and positions were evaluated. In one case, the presence of an alcohol attached at 2nd, 3rd and 4th positions of the benzene ring resulted in

significant differences in bacterial activity. At the 3rd and 4th position a MIC >200 μM was seen, but at the 2nd position activity significantly increased, resulting in a MIC of 25 μM.[24]

Our results are reflective of this as there as changes in activity were observed depending on the substituents present on the aromatic rings. PKZ 1822 was the most effective compound in SAR studies. It differed from PKZ 1800 by removal of a methyl group on the heterocyclic thiazole and presence of an isobutyl group instead of an isopropyl group on the benzene ring. These alterations may be significant as lengthening the carbon chain on the benzene ring increases surface area and van der waals forces, which may allow for more efficient binding to the Specifier Loop. PKZ 1821 had increased activity from past analogs and also lacked the methyl group on the heterocyclic thiazole. PKZ 1825 demonstrated the least activity and Gram-positive selectivity of the three compounds, and still contained the methyl group on the thiazole. This evidence supports that the methyl group has no contribution to bacterial inhibition and possibly hinders it. Once main structural elements are revised, a future direction for consideration would be to conduct thorough evaluation of the effectiveness of various substituents at different positions on the aromatic rings to further optimize activity.

Determining structural components important for antibacterial activity allows for a faster and more efficient approach to drug discovery, which has been aided by modern technology. Resistance is on the rise and the process to discover new and novel antibiotics is extensive and costly. Utilizing sophisticated computer software is an essential tool in expediting the process to creating new antibiotics.[25] Ultimately, as stated in the CDC threat report the resistance process can only be "slowed" not stopped.[11] Computer-aided drug design (CADD) provides bio-chemical prediction models such as, *in silico*, which conducts large scale predictions on chemical molecules binding energies, poor activity, poor absorption, distribution, metabolism, excretion,

and toxicity preventing wasted time and resources. Newer developments in these technologies have allowed for efficient discovery with expedited refining of physicochemical and ADMET/PK (pharmacokinetic) properties.[25]

With new and efficient models to test molecules there is great need for novel targets. RNA has a diverse and integral function to transcription regulation, gene expression, and translation of protein. The diversity and specificity of RNA coupled with its lack of the extensive repair mechanisms of DNA make it an ideal target for drug discovery. Prior RNA drug targeting has been directed towards rRNA with drug classes such as aminoglycosides. mRNA and riboswitch regulator elements on nascent transcripts are novel drug targets for a variety of reasons. These regulatory elements monitor essential processes for bacterial survival and remain very distinct from eukaryotic mRNA.[26]

The combination of rising resistance, decrease in antibiotic innovation, strict US Food and Drug Administration (FDA) regulation, and lack of economic incentive for pharma companies, demonstrates the need for efficient methods to drug discovery. RNA targets such as riboswitches are ideal drug targets because their integral nature in bacterial survival decreases possibility of resistance and their elucidated structure allows for efficient computerized approach.[27] Using *in silico* screening and bacterial susceptibility to narrow integral structural elements allows for the expedited discovery of new novel antibiotic classes, which are desperately in demand.

References:

1. Ronald N. Jones, The emergent needs for basic research, education, and surveillance of antimicrobial resistance: Problems facing the report from the American Society for Microbiology task force on antibiotic resistance, Diagnostic Microbiology and Infectious Disease, Volume 25, Issue 4, August 1996, Pages 153-161, ISSN 0732-8893
2. Zhang, Ruifang, et al. "Antibiotic Resistance As A Global Threat: Evidence From China, Kuwait And The United States." Globalization & Health 2.(2006): 6-14. Academic Search Complete. Web. 18 Feb. 2015
3. Chunjiang Zhao, Hongli Sun, Hui Wang, Yudong Liu, Bijie Hu, Yunsong Yu, Ziyong Sun, Yunzhuo Chu, Bin Cao, Kang Liao, Jin'e Lei, Zhidong Hu, Liyan Zhang, Xiaobing Zhang, Yingchun Xu, Zhanwei Wang, Minjun Chen, Antimicrobial resistance trends among 5608 clinical Gram-positive isolates in China: results from the Gram-Positive Cocci Resistance Surveillance program (2005–2010), Diagnostic Microbiology and Infectious Disease, Volume 73, Issue 2, June 2012, Pages 174-181, ISSN 0732-8893
4. Catherine M. Oliphant, Kathryn Eroschenko, Antibiotic Resistance, Part 1: Gram-positive Pathogens, The Journal for Nurse Practitioners, Volume 11, Issue 1, January 2015, Pages 70-78, ISSN 1555-4155
5. Bassetti M., Merelli M., Temperoni C., Astilean A. (2013). New antibiotics for bad bugs: where are we? Ann. Clin. Microbiol. Antimicrob. 12, 22.10.1186/1476-0711-12-22
6. Murray, Patrick R, Rosenthal Ken S., Kobayashi George S. , Pfaller Kobayshi Michael A. "Antibacterial Agents." Medical Microbiology. 4th ed. St. Louis: Mosby, 2002. Print
7. Tsodikov, Oleg V., Keith D. Green, and Sylvie Garneau-Tsodikova. "A Random Sequential Mechanism Of Aminoglycoside Acetylation By Mycobacterium Tuberculosis Eis Protein." Plos ONE 9.4 (2014): 1-5. Academic Search Complete. Web. 4 Feb. 2015.
8. George G. Zhanel, Daryl J. Hoban, Kristen Schurek, James A. Karlowsky, Role of efflux mechanisms on fluoroquinolone resistance in Streptococcus pneumoniae and Pseudomonas aeruginosa, International Journal of Antimicrobial Agents, Volume 24, Issue 6, December 2004, Pages 529-535, ISSN 0924-8579
9. Delcour A. H. Outer membrane permeability and antibiotic resistance. Biochimica et Biophysica Acta—Proteins and Proteomics. 2009;1794(5):808–816.
10. Lambert, P.A. (2002), Cellular impermeability and uptake of biocides and antibiotics in Gram-positive bacteria and mycobacteria. Journal of Applied Microbiology, 92: 46S–54S. doi: 10.1046/j.1365-2672.92.5s1.7.x
11. Centers for Disease Control and Prevention (CDC). Antibiotic resistance threats in the United States, 2013. Atlanta: CDC; 2013. Available from: http://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf
12. Spellberg B, Guidos R, Gilbert D, Bradley J, Boucher HW, Scheld WM, Bartlett JG, Edwards J Jr. Infectious diseases society of america; the epidemic of antibiotic-resistant infections: a call to action for the medical community from the infectious diseases society of America. Clin Infect Dis.

13. Andrew D. Garst, Robert T. Batey, A switch in time: Detailing the life of a riboswitch, Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, Volume 1789, Issues 9–10, September–October 2009, Pages 584-591, ISSN 1874-9399
14. Grigg J.C., Chen Y., Grundy F.J., Henkin T.M., Pollack L., Ke A. T box RNA decodes both the information content and geometry of tRNA to affect gene expression, Proceedings of the National Academy of Sciences of the United States of America, Volume 110, Issue 18, Pages 7240-7245, 2013
15. Nicholas J. Green, Frank J. Grundy, Tina M. Henkin, The T box mechanism: tRNA as a regulatory molecule, FEBS Letters, Volume 584, Issue 2, 21 January 2010, Pages 318-324, ISSN 0014-5793, http://dx.doi.org/10.1016/j.febslet.2009.11.056.
16. Tina M. Henkin, The T box riboswitch: A novel regulatory RNA that utilizes tRNA as its ligand, Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms, Volume 1839, Issue 10, October 2014, Pages 959-963, ISSN 1874-9399.
17. Wai-Leung N, Kazmierczak K, Robertson G, Gilmour R, Winkler M. Transcriptional Regulation and Signature Patterns Revealed by Microarray Analyses of Streptococcus pneumoniae R6 Challenged with Sublethal Concentrations of Translation Inhibitors. Journal Of Bacteriology [serial online]. January 2003
18. Biemer JJ. (1973) Antimicrobial susceptibility testing by the Kirby-Bauer disc diffusion method. Ann Clin Lab Sci. 3:135-40; Andrews JM. (2001) The development of the BSAC standardized method of disc diffusion testing. J Antimicrob Chemother. 2001 Jul;48 Suppl 1:29-42.
19. Andrews, Jennifer M. "Determination of Minimum Inhibitory Concentration." Journal of Antimicrobial Chemotherapy 48 (2001): 1. Web.
20. Abdul Rouf, Cihangir Tanyeli, Bioactive thiazole and benzothiazole derivatives, European Journal of Medicinal Chemistry, Available online 22 October 2014, ISSN 0223-5234
21. Georg Lentzen, Roscoe Klinck, Natalia Matassova, Fareed Aboul-ela, Alastair I.H. Murchie, Structural Basis for Contrasting Activities of Ribosome Binding Thiazole Antibiotics, Chemistry & Biology, Volume 10, Issue 8, August 2003, Pages 769-778, ISSN 1074-5521
22. Marcey L Waters, Aromatic interactions in model systems, Current Opinion in Chemical Biology, Volume 6, Issue 6, 1 December 2002, Pages 736-741, ISSN 1367-5931
23. M.D. Sindkhedkar, H.R. Mulla, A. Cammers-Goodwin, Three-state, conformational probe for hydrophobic, pi-stacking interactions in aqueous and mixed aqueous solvent systems: anisotropic solvation of aromatic rings, J Am Chem Soc, 122 (2000), pp. 9271–9277
24. Parameshwar Makam, Ramakrishna Kankanala, Amresh Prakash, Tharanikkarasu Kannan, 2-(2-Hydrazinyl)thiazole derivatives: Design, synthesis and in vitro antimycobacterial studies, European Journal of Medicinal Chemistry, Volume 69, November 2013, Pages 564-576, ISSN 0223-5234
25. I.M. Kapetanovic, Computer-aided drug discovery and development (CADDD): In silico-chemico-biological approach, Chemico-Biological Interactions, Volume 171, Issue 2, 30 January 2008, Pages 165-176, ISSN 0009-2797

26. Weiling Hong, Jie Zeng, Jianping Xie, Antibiotic drugs targeting bacterial RNAs, Acta Pharmaceutica Sinica B, Volume 4, Issue 4, August 2014, Pages 258-265, ISSN 2211-3835
27. Attilio Fabbretti, Claudio O. Gualerzi, Letizia Brandi, How to cope with the quest for new antibiotics, FEBS Letters, Volume 585, Issue 11, 6 June 2011, Pages 1673-1681, ISSN 0014-5793