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Reclaiming the Activity of Lost Therapeutics

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Reclaiming the Activity of Lost Therapeutics

by

Rallya Telussa

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Public Health
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Abstract

ESKAPE pathogens are notorious in causing nosocomial infections and escaping current antibiotic treatments. There has been a dramatic increase in nosocomial infections accompanied with a decrease in the number of antibiotics developed, leading to significant increase in morbidity and mortality among patients. In an attempt to combat this problem, derivatives of ciprofloxacin, rifabutin and beta-lactam antibiotics were synthesized and tested against the ESKAPE pathogens. From minimum inhibitory concentration assays, 4 ciprofloxacin analogs and 8 beta-lactam analogs were found to be effective against multiple bacterial species. Additionally, 12 rifabutin analogs and 23 beta-lactam analogs were potent against single bacterial species, primarily toward methicillin-resistant Staphylococcus aureus (MRSA) at a concentration of ≤ 25 µg mL⁻¹. Based on the effectiveness against methicillin-resistant Staphylococcus aureus (MRSA), three rifabutin analogs were selected for further testing. Two rifabutin analogs (DU644 and DU645) were found to possess between a one to twofold mean increase of inhibitory activities, while the other rifabutin analogs (DU650) demonstrated up to a twofold decrease of inhibitory activity when compared to the parent drug. These compounds were then examined for their bactericidal and antibiofilm activities against MRSA. From these assays, we found that DU644 and DU645 were 4 times more bactericidal and antibiofilm against MRSA when compared to the parent drug. In addition, rpoB mutation validation results confirmed that modification of these rifabutin derivatives at the C-3 and C-4 positions, and bearing an imidazolyl ring carrying substituted spiropiperidyl ring, did not change their mechanism of action towards the beta-subunit of RNA polymerase. Cytotoxicity
testing performed using human hepatocellular carcinoma epithelial cells (hepG2) showed that at concentrations ranged from 1.25 µg mL$^{-1}$ to 25 µg mL$^{-1}$, DU644 and DU645 showed very low toxicity. Collectively, structural drugs modifications of these obsolete drugs are able to restore their antibacterial activities against MRSA, which is notable as the most infectious nosocomial pathogen. Therefore, further development and application of rifabutin analogs might be beneficial for medical use to combat MRSA infections.
Chapter One: Introduction

Nosocomial infections play a pivotal role in increasing large numbers of illnesses and death among patients (Hsueh, 2002). About 2 million people in US hospitals become ill as a result of these infections, resulting in nearly 100,000 deaths every year (Kleven et al., 2007). Therefore, these infections have become a prominent public health problem that needs to be addressed.

The acronym of ESKAPE pathogens (coined by the Infectious Disease Society of America), represents six drug-resistant bacterial species, *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species. The members of this group are capable of resisting current antibiotics and have become the leading causes of nosocomial infections in United States hospitals (Rice, 2008). The number of patients that die from methicillin-resistant *Staphylococcus aureus* infections (MRSA), for example, exceeds those who die of HIV/AIDS in US hospitals (Boucher and Corey, 2008). In addition to this, as cited from the National Nosocomial Infections Surveillance System Report CDC 2004, extremely resistant *Acinetobacter* strains, multi-drug resistant *P. aeruginosa*, and carbapenem-resistant *Klebsiella* species are rapidly emerging, and are a major concern not only in United States, but also in other parts of the world (Boucher et al., 2009). Furthermore, resistance toward the antibiotic of last resort, colistin, was reported among Enterobacteriaceae, primarily among *Escherichia coli*, and *K. pneumoniae* (Liu et al., 2016; Malhotra-Kumar et al., 2016; McGann et al., 2016). Most recently, in the United States, *E. coli* MRSN 388634 with plasmid carrying *mcr-1* gene resistance to colistin was reported, presaging the emergence of
extremely multidrug resistant bacteria (McGann et al. 2016). These findings indicate that we are close to post antibiotic era, in which minor injuries and mild infections become deadly (WHO, 2015).

The discovery of penicillin unlocked the door of the golden era of antibiotic research from the years 1940 to 1962, leading to the discovery of streptomycin, chloramphenicol, and tetracycline (Singh and Barrett, 2006). These antibiotics vastly decreased the number of bacterial diseases at that time, making this a triumph that was thought to be the end of bacterial disease (Penesyan, Gillings, and Paulsen, 2015). However, soon after penicillin was prescribed as a wonder drug that saved lives from threatening infections, bacterial resistance toward penicillin emerged and therefore allowed bacteria to expand resistance toward other developed antibiotics. In an effort to overcome these problems, scientists and pharmaceutical industries worked on discovering and disseminating new classes of antibiotics (Ventola, 2015). Nevertheless, the belief that the bacterial infection crisis was resolved, was abolished by emerging antibiotic resistance cases reported afterward, such as Staphylococcus aureus resistant strains that were identified in England in 1961; these strains were resistant to penicillin G, streptomycin, and tetracycline (Jevons, 1961).

One of the reasons that bacteria can resist the antibacterial actions of these drugs is related to their extraordinary adaptability to survive in all possible climates and conditions, even in very harsh environments that cannot be occupied by human beings (Spellberg et al., 2008). Furthermore, the replication process of bacteria can occur within 20-30 minutes, which allows them to have a more rapid reproduction compared to other organisms. These abilities grant bacteria the capacity to survive antibiotic treatment, and pass on resistance to their next generation. Therefore, the problem of misusing antibiotics may not be the cornerstone of resistance
development, but rather it affects the speed in which bacteria develop resistance. Indeed, the genetic analysis of microbial pathways revealed that bacteria acquired beta-lactamase enzymes to withstand the effects of beta-lactam antibiotics more than 2 billion years ago, long before humans discovered penicillin (Hall, Salipante, and Barlow, 2004). Obviously, the proper use of antibiotics is very important to reduce selective pressures that could escalate the pace of resistance. This necessitates the stable and rapid development of new antibiotics to answer the threats of drug resistant bacteria.

Bacterial resistance toward antibiotics is mainly obtained either by genetic mutation or by horizontal gene transfer. Genetic mutations result in reduced penetration of drugs (porins) (Low et al., 2001), increased extrusion of drugs (efflux pumps) (Yan et al., 2006), target modification (Nagai et al., 2002), and hydrolytic enzyme production (Linstrom, Boman, and Steele, 1970). In addition to these, bacterial resistance is also acquired through mobile genetic elements, plasmids and transposons, which can transfer new resistance genes to other bacteria and can cause multiple resistance towards several antibiotics (Normark and Normark, 2002). The following is an explanation of each bacterial resistance mechanism and genetic resistance transfer pathway:

**Porin Mutations and Active Efflux Pumps**

As is widely known, the bacterial outer membrane is the first protection against antibiotics. The outer membrane of Gram-negative bacteria consists of a lipid bilayer that is not permeable to large positively or negatively charged molecules. The first transport system for molecules entering the membrane is that of influx, regulated by porins. The shift of porin expression during antibiotic treatment points to bacterial adaptability towards drugs, which limits membrane permeability and decreases the concentration of antibiotic that enters the cell (Low et al., 2001). The second
transport system is that of bacterial protein export, used to extrude harmful substances such as antibiotics out of the cell. This system is known as efflux, and can be overexpressed due to genetic mutations within efflux genes and their regulatory elements (Yan et al., 2006). These extrusion mechanisms can specifically export a particular substance or expel various substances such as multiple antibiotics, leading to multidrug resistance (Webber, 2002). Broadly, multidrug resistance mechanisms result from the decreased permeability of the membrane related to porin alteration combined with efflux mechanisms (Hancock, 1998). Therefore, both influx and efflux, transport systems of bacteria, are notable as conjoint factors that contribute to bacterial resistance against antimicrobials.

**Antibiotic Alteration and Target Modification**

Antibiotic alteration and inactivation by bacterial enzymes are other examples of resistance mechanisms. A classic example of alteration is the inactivation of beta-lactam molecules through enzymatic hydrolysis by beta-lactamase enzymes, produced by resistant bacteria (D'Agata et al., 1998), resulting in beta-lactam antibiotics losing their effectiveness. Another example is the function of chloramphenicol acetyltransferases (CATs) that catalyze acetylation of chloramphenicol, thereby inactivating antibacterial effects (Schwarz et al., 2004). Another resistance mechanism performed by bacteria is target modification, where each antibiotic has a specific target to bind, and the modification of the target abrogates the antibiotic’s activity (Nagai et al., 2002).
Horizontal Gene Transfer

In addition to the resistance mechanisms occurring as a result of genomic mutations, horizontal gene transfer also contributes in generating bacterial resistance. This occurs through transformation, transduction or conjugation. Transformation is the process by which the DNA of one bacteria is taken up by another, while transduction is DNA transfer via phages (Davison, 1999). Conjugation, is the transfer of DNA through direct contact; in some bacteria this process involves plasmids that play role in delivering multidrug resistance genes and increasing the numbers of multidrug resistant bacteria (Poulin-Laprade, Carraro, and Burrus, 2015).

In the face of the swift increase of resistance among the ESKAPE pathogens, the decrease in the pace of antibiotic development has also become problematic. The Infectious Diseases Society of America (IDSA) discovered an abrupt 75% decline of FDA antibiotic approval between 1983 and 2007 (Boucher et al., 2009). In an effort to overcome these problems, a national and global scale antibacterial research and development initiative was issued by the IDSA with the aim to develop 10 new or modified antibiotics by the year 2020; this is known as the 10 x 20 initiative (IDSA, 2010). To accelerate this commitment, USF scientists work together, discovering novel antibacterials and modifying obsolete ones. Accordingly, structural drug modification of ciprofloxacin, rifabutin, and beta-lactam antibiotics were performed in the course of our research to increase their potential against the ESKAPE pathogens. Studies on structurally modified ciprofloxacin, rifabutin, and beta-lactams that were previously performed become the foundation of our studies and therefore will be discussed in this chapter.
N-Acylated Ciprofloxacin

Fluoroquinolones are the most routinely prescribed drugs for adults in US, forming a large and narrow spectrum of antibacterial agents (Linder et al., 2005). This class of antibiotics function by binding to the type II topoisomerase, DNA gyrase, as well as topoisomerase IV; the enzymes that mediate re-ligation and separation of double stranded DNA (Mustaev et al., 2014). Thus fluoroquinolones inhibits DNA replication of bacteria (Becnel Boyd et al., 2008). The most widely used fluoroquinolone is ciprofloxacin (Linder et al., 2005). However, its frequent use has led to bacterial resistance, which occurs through many mechanisms, including trans-conjugated plasmids (Cheung, 2005) or from chromosomal mutations that occur in the Quinolone Resistance Determining Region of target genes (Yoshida et al., 1991).

In order to increase the potential antibacterial activity of ciprofloxacin, and reduce resistance, N-acylated ciprofloxacin have been synthesized by the addition of lipophilic acyl residues toward the nitrogen atom of the piperazinyl ring (Cormier et al., 2012) Figure 1 displays one of our N-acylated ciprofloxacin analogs that was tested against the ESKAPE pathogens.

![Figure 1. Structural modification of ciprofloxacin.](image)

This figure displays a regular ciprofloxacin (i), and its analog that was modified through the addition of acyl group at the nitrogen atom of the piperazinyl ring (ii).

A previous study conducted by Cormier et al. (2012) confirms that N-acylated ciprofloxacin have more potent antibacterial activity than regular ciprofloxacin against Staphylococcus aureus, Bacillus anthracis, Enterococcus faecalis, Bartonella species, and
*Escherichia coli*. The results of this investigation show that from eighteen derivatives tested, six compounds were as active as ciprofloxacin in inhibiting methicillin-susceptible *Staphylococcus aureus* (MSSA) at a concentration of 10 µg mL\(^{-1}\) and three derivatives inhibited MSSA at a concentrations of < 10 µg mL\(^{-1}\). Moreover, the MIC of ciprofloxacin analogs toward *B. henselae* was achieved at 0.2 µg mL\(^{-1}\), whilst that of the parental drug was 0.5 µg mL\(^{-1}\). In addition to this, bacterial frequency mutations toward these derivatives was assessed through spontaneous mutation assays, showing that at 2.5 x MIC, the three selected compounds produced no bacterial growth recovery. Nevertheless, at a concentration of 1 x MIC, bacterial colonies were obtained. Conversely, resistant colonies were found at 2.5 x MIC of ciprofloxacin. Biochemical examination was performed to investigate the ability of these ciprofloxacin analogs to interfere with DNA gyrase, revealing a stable action of the drug with this enzyme that obstructed its activity. Furthermore, toxicity assays for all analogs using *Saccharomyces cerevisiae* showed no detrimental effects on eukaryotic cells. Therefore, based on the results mentioned above, N-acylated ciprofloxacins have more effective antibacterial activity toward Gram-positive and Gram-negative bacteria than regular ciprofloxacin.

**Rifabutin Analogs**

Another widely used antibacterial agent, rifamycin, works by inhibiting DNA-dependent RNA polymerase, hence preventing the initiation of transcription. One member of the rifamycin family, rifabutin, possesses broad spectrum activity against Gram-positive and Gram-negative bacteria (Kunin, 1996). In addition, rifabutin has potent activity as an anti-mycobacterial (Barrow *et al*., 2015), and anti-staphylococcal agent in HIV/AIDS patients (Styrt, Chaisson, and Moore, 1997). Acquired resistance to rifamycins results from a mutation that occurs within the *rpoB* gene,
which encodes the beta-subunit of the DNA-dependent RNA polymerase (Bodmer et al., 1995; Jin & Gross, 1988). Some mutations present resistance to rifabutin alone, to other rifamycins, or to all rifamycin family members (Williams et al., 1998). In an effort to restore antibacterial activity of rifamycins, semisynthetic analogs were synthesized via the modification of C-3 and C-4 positions into an imidazolyl ring, carrying a spiro-piperidyl group (Sanfilippo et al., 1980; Barluenga et al., 2006). **Figure 2** shows two of our rifabutin analogs that were modified at C-3 and C-4 positions, carrying aromatic rings substituted with halogen elements.

![Structural modification of rifabutin](image)

**Figure 2. Structural modification of rifabutin.** This figure displays a regular rifabutin (i), and its analogs that were modified at C-3 and C-4 positions into an imidazolyl ring carrying a spiro-piperidyl group (ii and iii).

Previous synthesized rifabutin derivatives were examined for their anti-mycobacterial activity against susceptible and multidrug resistant strains of mycobacterium species (Garcia et al. 2010). It was found that at a concentration of ≤ 0.02 µg mL⁻¹, two rifabutin analogs inhibited the growth of rifabutin susceptible strains of *M. tuberculosis*, and at a concentration of ≤ 0.5 µg mL⁻¹, these analogs were able to inhibit the growth of multidrug-resistant strains. In contrast, the susceptibility of these strains towards regular rifampin and rifabutin occurs at 50 µg mL⁻¹ and 10
μg mL⁻¹, respectively. To find a rationale behind this significant deviation between bioactivity of the rifabutin analogs and the parent drug toward multi-drug resistant tuberculosis (MDR-TB), molecular dynamics calculations were performed. In doing so, it was determined that the newer compounds have a stronger binding affinity to RNA polymerase in resistant strains. In addition, toxicity tests conducted on bovine endothelial cells showed no harmful effects yielded by these derivatives. In conclusion, rifabutin analogs have more potent anti-mycobacterial activity compared to regular rifabutin, due to a stronger mode of action.

**Beta-lactam Analogs**

In the last 70 years, since the discovery of penicillin in 1940, beta-lactam antibiotics have become the most common drugs used to treat bacterial infections. The mechanism of action of beta-lactam antibiotics is to bind with penicillin binding proteins (PBPs) that function in bacterial cell wall synthesis (Beadle, Nicholas, and Shoichet, 2001). The broad use of beta-lactams in the treatment of bacterial infections has led to increased resistance of Gram-positive and Gram-negative bacteria toward these drugs; including *Staphylococcus aureus* (Zhang, 2001), *Pseudomonas aeruginosa* (Van der Bij et al., 2011), *Acinetobacter baumannii* (Mera et al., 2010) and *Klebsiella pneumoniae* (Palasubramaniam, Muniandy, and Navaratnam, 2009). Bacterial resistance toward beta-lactams can be obtained through several ways: porin mutations (Fang et al., 2014) and the activity of specific genes encoding multidrug efflux activity (Li, Nikaido, and Poole, 1995) that affect intrusion and extrusion of antibiotics, respectively. Moreover, bacterial beta-lactamase enzymes production can inactivate the drugs and cause multidrug resistance (Van der Bij et al., 2011; Mahdian et al., 2015; Pollini et al., 2013; Caltagirone et al., 2015), which can be
mediated by plasmids that transfer beta-lactamase resistance from one bacteria to another (Caltagirone et al., 2015).

![Diagram of beta-lactam structures](image)

**Figure 3. Structural modification of beta-lactam.** This figure displays a regular beta-lactam (i), and its analog that was modified at C-3, C-4, and N-1 positions of the beta-lactam ring (ii).

Attempts to restore the effectiveness of beta-lactam antibiotics have been performed through structural drug modifications. Turos et al. (2002) developed N-thiolated beta-lactams and assessed their bioactivity against Gram-positive and Gram-negative bacteria. One of the N-thiolated beta-lactam derivatives developed by Dr. Turos, depicted in Figure 3, was screened against the ESKAPE pathogens. Previous studies on the antibacterial activity of N-thiolated beta-lactams revealed their specificity against the growth of Gram-positive bacteria, especially staphylococci (including MRSA), with lower MICs compared to that of penicillin. Specifically, the most active beta-lactam derivatives inhibited the growth of MSSA at a concentration of 15 µg mL⁻¹, and MRSA strains at a concentration of 5-10 µg mL⁻¹. These results are in contrast to penicillin that was able to inhibit the growth of MRSA at a concentration exceeding 64 µg mL⁻¹. In addition, N-thiolated beta-lactams seemingly have a different mechanism of action compared to regular beta-lactam, based on differential morphology of bacterial cells after treatment with the analogues. Moreover, the effectiveness of N-thiolated derivatives examined by Turos et al. (2002)
against MRSA indicated that they were able to escape the activity of beta-lactamase enzymes produced by resistant bacteria. To continue exploring the mechanism of action of N-thiolated beta-lactams at a molecular level, further work by the Turos group demonstrated a direct interaction between beta-lactam derivatives and co-enzyme A. Furthermore, these compounds significantly disrupted fatty acid synthesis in bacteria, inhibiting lipid metabolism and impeding the growth of bacteria (Revell et al., 2007). In addition to these findings, Prosen et al. (2011) also confirmed the potent antibacterial activity of N-thiolated beta-lactams on Gram-positive bacteria, demonstrating growth inhibition of MRSA at a concentration of 5 µg mL\(^{-1}\). This suggests that N-thiolated beta-lactam derivatives are a potent class of antibiotics, though their specific target pathway has yet to be identified.

**Project Aim**

Literature reviews concerning the enhanced bioactivity of ciprofloxacin, rifabutin, and beta-lactam analogs against resistant bacteria, and the urgent need to find treatments to solve the problem of nosocomial infections, has inspired our research against the ESKAPE pathogens. Specifically, this study aimed to determine if structural modifications of ciprofloxacin, rifabutin, and beta-lactam antibiotics resulted in more potent antibacterial activities against the ESKAPE pathogens compared to parent drugs. Assessments of antibacterial activities of these derivatives performed to achieve this goal included minimum inhibitory concentration, bactericidal, antibiofilm, spontaneous mutation, and target mutation validation.
Chapter Two: Materials and Methods

Setting

This research was conducted from August 2015 to May 2016 at Shaw lab at the University of South Florida. Here in Shaw lab, we focus on performing drug discovery to combat the ESKAPE pathogens, and exploring disease-causing mechanisms of MRSA at the molecular level. My research was to determine the antibacterial activity of novel variants of ciprofloxacin, rifabutin and beta-lactams against the ESKAPE pathogens. Antibacterial assessments that I performed were minimum inhibitory concentration, bactericidal, antibiofilm, spontaneous mutation, and target mutation validation. Moreover, cytotoxicity assessment was also conducted to determine toxicity effect of our compounds toward human hepatocellular carcinoma epithelial cell (HepG2). Ciprofloxacin and beta-lactam analogs were provided by our collaborators from Dr. Turos laboratory at the University of South Florida, whilst rifabutin analogs came from Dr. Maria-Paz Cabal at Instituto Universitario de Química, Spain.

Materials

Bacterial Strains:

*Enterococcus faecium* strain 1449

Methicillin-resistant *Staphylococcus aureus* strain 635

*Klebsiella pneumoniae* strain 1433

*Acinetobacter baumannii* strain 1403
Pseudomonas aeruginosa strain 1419

Enterobacter cloacae strain 1454

**Primer Sequences**

OL1524 R *rpoB* (TAA TAG CCG CAC CAG AAT CA)

OL1525 F *rpoB* (GTG TAA AAG TGC GTC TAA TC)

**Tryptic Soy Broth (TSB)**

3% tryptic soy

**Tryptic Soy Agar (TSA)**

3% tryptic soy

1.5% agar

**Mueller Hinton Broth (MHBI)**

2.2% Mueller Hinton

**Biofilm Media**

3% sodium chloride (NaCl)

3% tryptic soy

0.5% dextrose (C\textsubscript{6}H\textsubscript{12}O\textsubscript{6})
**Bacterial Growth Media**

All media for culturing bacteria were prepared using deionized water and sterilized using an autoclave. In particular, Gram-positive bacteria were grown in 5 mL of TSB, whilst Gram-negative bacteria were grown in MHBI. The overnight culture of bacteria were performed in 5 mL of TSB or MHBI for 24 hours at 37 °C in a shaking incubator. TSA was prepared to allow for bacterial recovery after being exposed to compounds, thus the number of bacteria colonies could be counted. Biofilm media was utilized to perform biofilm assay.

**Phosphate Buffered Saline (PBS)**

- 0.14 % sodium phosphate dibasic anhydrous (Na₂HPO₄)
- 0.02% potassium chloride (KCl)
- 0.02% potassium phosphate monobasic anhydrous (KH₂PO₄)
- 0.8% sodium chloride (NaCl)

**Buffer**

Phosphate buffered saline (PBS) was prepared using deionized water, adjusted with 1 M hydrochloric acid (HCl) to pH 7.4, and sterilized using an autoclave.

**Methods**

A key assessment of an antibacterial agent is to determine whether the compound inhibits bacterial growth (bacteriostatic), or if it works by killing the bacteria (bactericidal). This is first determined through a minimum inhibitory concentration (MIC) assay, the gold standard to test the susceptibility of bacteria towards a compound (Andrews, 2001). In this assay, ranges of
concentrations for a given compound were examined to find the lowest concentration required to inhibit the growth of bacteria. Subsequently, minimum bactericidal concentration (MBC) assays were conducted to determine the ability of lead compounds to completely terminate bacterial viability. MIC and MBC protocols were carried as performed by Van Horn et al. (2014).

**Minimum Inhibitory Concentration Assay (MIC)**

Bacteria were grown in 5 mL of tryptic soy broth (TSB) for 24 hours in order to obtain a large enough bacterial sample for MIC testing. After 24 hours at 37°C, Gram-positive and Gram-negative bacteria were diluted 1:1000 in fresh TSB or Mueller Hinton Broth (MHBI), respectively. Following this, bacteria were transferred into 96 well plates and the compounds were added for a total volume of 200 µL per well. Assays were conducted in triplicate for each concentration tested. The plates were then incubated under static conditions for 24 hours at 37°C. The MIC value of each compound was determined based on visual inspection (lack of turbidity) of the lowest concentration that completely inhibited bacterial growth.

**Minimum Bactericidal Concentration (MBC)**

Following overnight incubation with compounds, samples were serially diluted from 10^{-1} to 10^{-7} in PBS and thereafter spot plated in duplicate onto TSA to obtain a countable number of bacteria. Nutrient agar plates were incubated at 37°C for 24 hours. Following incubation, the colony forming units (CFU) recovered on plates was calculated and converted to CFU mL^{-1} using the dilution factor of the plate where the CFU were obtained. Subsequently, recovery of the bacteria was determined by comparing the CFU mL^{-1} of the treatment group to the CFU mL^{-1} of
the no drug control. The MBC of the lead agents were determined as the lowest concentration of the agent that killed 99% of bacteria.

**Antibiofilm Assay**

The protocol for this assay was performed based on the experiment conducted by Fleeman *et al.* (2015). Twenty percent human plasma was added to 96-well plates and incubated at 4°C overnight. Next, overnight cultures of MRSA were grown in 5 mL of TSB at 37°C for 24 hours. Following this, overnight cultures were standardized in fresh biofilm media to reach an optical density (OD<sub>600</sub>) of 0.5. Thereafter, the human plasma was removed and 150 µL of the diluted culture was added to the wells and incubated at 37°C for 24 hours. After incubation, the media was carefully removed from the 96 well plate and replaced with fresh biofilm media combined with the lead compounds and/or controls to a total volume of 200 µL per well. Each assay was conducted in triplicate. Samples were incubated for 24 hours at 37°C before being processed. The media was carefully removed from the wells without disturbing the biofilm and gently washed three times with PBS to remove planktonic bacteria. After washing, the biofilms were resuspended in 100 µL of sterile PBS by vigorous pipetting. The resuspended biofilm was then transferred into a new 96-well plate and serially diluted as described above. Aliquots were plated onto nutrient agar plates, and incubated at 37°C overnight. Bacterial colonies were counted, and the colony forming unit CFU mL<sup>-1</sup> was converted to percent recovery by comparison to no drug controls.

**Bacterial Resistance Assay**

Bacterial resistance assessment was performed using spontaneous mutations assays described by Van Horn *et al.* (2014). MRSA overnight cultures were prepared as described above.
Following this, TSA was prepared and mixed together with the selected compounds to generate plates with 5 x MIC, 10 x MIC, 20 x MIC, or 50 x MIC. 1 mL of overnight cultures were pelleted through centrifugation and resuspended in 100 µL of TSB media. The resuspended samples were plated directly onto nutrient agar containing our lead compounds, and incubated overnight at 37°C. The mutation frequency of bacteria for each compound was measured by dividing the number of colonies on the plates with the total inoculum of bacteria (CFU mL⁻¹ on no drug plates). The concentration of the compounds was increased from the lower concentration (5 x MIC), to the higher concentration (10 x, 20 x, or 50 x MIC) when the bacterial recovery was not enumerable due to mutation rates that were too high. Each experiment was performed in triplicate.

**rpoB Mutation Validation Assay**

In order to ascertain whether or not our lead compounds employ the same mechanism of action as the parent compound, mutant colonies from spontaneous mutation assays were randomly selected, and their *rpoB* gene was sequenced and analyzed. Prior to the sequencing process, DNA of the mutants were extracted based on Shaw lab’s *S. aureus* chromosomal DNA extraction protocols. PCR product purification was performed using QIAquick PCR purification kit (Qiagen) as described by the manufacturer’s protocols with the following modification.

**DNA Extraction**

Mutants from the spontaneous mutation assays were randomly selected, and grown in 5 mL TSB at 37°C, overnight. The overnight samples were centrifuged for 10 minutes and resuspended in 600 µL TE buffer. Bacterial cultures were lysed using glass beads for 1 minute using a Minibeadbeter Biospec. Samples were then centrifuged at full speed for 1 minute to remove
precipitants, and the supernatants were transferred into new eppendorf tubes. To isolate the DNA from protein contaminant, 200 µL of sarkosyl (1.6%) and 5 µL proteinase K were added into the eppendorf tubes. The reactions were incubated at 60°C for 1 hour. Following incubation, 700 µL phenol-chloroform was added into reactions and the samples were centrifuged at full speed for 1 minute; this was done to separate the remaining protein from DNA. The upper liquid layer of each sample, containing DNA, was carefully transferred to a new eppendorf tube. Following this, the DNA was precipitated by mixing the samples with 500 µL isopropanol and 100 µL of 3M sodium acetate prior to overnight incubation at -80°C. Thereafter, samples were centrifuged at 13,000 x g for 10 minutes and the supernatant was removed. To wash the DNA, 500 µL of 70% ethanol was added to the pellet, and then samples were centrifuged at full speed for 5 minutes. After centrifugation, the ethanol was discarded and the tubes were air dried at room temperature for 4 minutes. After the ethanol was completely removed from the tubes, 200 µL of RNA free water was added into the tube and mixed with samples; the concentration of DNA was measured using a Nanodrop ND-1000 spectrophotometer. DNA was then stored at 4°C for 24 hours.

**PCR Purification**

After storage, the concentrated DNA was diluted to 60 ng µL⁻¹, while the master mix for PCR reaction was prepared by mixing 20 µL of 2x phusion master mix with 20 µL RNA free water. Subsequently, 1 µL of the diluted DNA, 1 µL of reverse (5′-GTG TAA AAG TGC GTC TAA TC-3′) and forward primer (5′-TAA TAG CCG CAC CA G AAT CA-3′), and 17 µL of master mix was mixed together in new PCR tubes. The samples were run on a Bio-Rad Thermal Cycler for 2 hours. After that, gel electrophoresis was performed to confirm the size of the amplicon. Furthermore, 100 µL phosphate buffer (PB) was added into the each PCR tube containing 20 µL
of PCR product, samples were then transferred into spin columns and spun at 13,000 x g for 1 minute; for a total of three times. Subsequently, the liquid contained on the bottom of the tubes was discarded, and the DNA retained on the columns was washed with 700 µL washing buffer (PE buffer). The columns were centrifuged at full speed for 1 minute, and the liquid on the bottom of the tubes was removed; to completely remove the ethanol from the column, they were re-spun at full speed for 3 minutes. The columns were placed into a fresh eppendorf tube, and 30 µL of free RNA water was pipetted onto the membrane of the columns. The tubes were incubated at room temperature for 1 minute, and centrifuged at full speed for 1 minute. The liquid from the bottom of the tubes was transferred back onto the membrane of the column and spun for 1 minute; this step was repeated once. Next, the column was discarded, and the concentration of the DNA was measured using a nanodrop spectrophotometer. Following the PCR purification process, the PCR products were sent to Eurofins MWG Operon LLC, a Eurofins Genomic company for sequencing. Nucleotides of the mutants were compared with those of the reference strain through NCBI blast nucleotide and protein database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Protein sequencing analysis was further conducted utilizing Pfam database (http://pfam.xfam.org/) to reveal the location and type of amino acid change within the RpoB protein of the mutants.

**Cytotoxicity Assay**

The protocol for this toxicity assay was performed based on the method of Fleeman et al. (2015), with the following modifications. Human HepG2 cells were cultured in Dulbecco Modified Eagle Medium (DMEM) containing L-glutamine, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, and incubated at 37°C incubator with 5% CO₂ for 72 hours. Cells were then diluted to 1.0 x 10⁵ mL⁻¹ in 96 well tissue culture plates (CellStar) at a total volume
of 100 µL per well. After this, the diluted cells were incubated for 24 hours in a 37°C incubator with 5% CO₂. Following incubation, media was gently removed and replaced with fresh DMEM supplemented with test compounds at a concentration of 1.5 to 50 µg mL⁻¹. The cells were incubated with compound for 2 days in a 37°C incubator with 5% CO₂. Next, the media containing compound was removed and substituted with media containing 3-(4,5- dimethylthiazol-2-yl)- 2,5- diphenyltetrazolium bromide (MTT). The plates were then incubated for 4 hours, followed by the removal and replacement of 50 µL of media with 100% dimethyl-sulfoxide (DMSO). Subsequently, plates were incubated for 10 minutes at 37°C in order to dissolve the formazan crystals produced. Formazan quantification was performed using a Synergy2 Biotek plate reader, recording at 540 nm. The lethal dose (LD₅₀) for each compound was determined by calculating the compound concentration at which 50% of the cells were killed. The calculation for LD₅₀ was based on the derived linear regression equation as described by (Houard et al. 2013).
Chapter Three: Results

Minimum Inhibitory Concentration Determination Assay (MIC)

In this study the antibacterial activity of the analogues of three classes of structurally modified antibiotics, ciprofloxacin, rifabutin, and beta-lactams, were determined using a high throughput phenotypic assay. The first approach employed as a preliminary drug assessment was determination of the minimum inhibitory concentration (MIC) for 1,248 compounds against the ESKAPE pathogens after 24 hours of incubation. From this high throughput screening, 4 ciprofloxacin analogs and 8 beta-lactam analogs were found to be active against multiple bacterial species, whereas 12 rifabutin analogs and 23 beta-lactam analogs were found to be active against a single bacterial species at a concentration of ≤ 25 µg mL\(^{-1}\).

MICs of Ciprofloxacin Analogs toward the ESKAPE Pathogens

<table>
<thead>
<tr>
<th>Compounds</th>
<th>E. faecium</th>
<th>S. aureus</th>
<th>K. pneumoniae</th>
<th>A. baumannii</th>
<th>P. aeruginosa</th>
<th>E. cloacae</th>
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<tbody>
<tr>
<td>DU008</td>
<td>25 µg mL(^{-1})</td>
<td>25 µg mL(^{-1})</td>
<td>1 µg mL(^{-1})</td>
<td>1 µg mL(^{-1})</td>
<td>5 µg mL(^{-1})</td>
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<tr>
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<td>25 µg mL(^{-1})</td>
<td>1 µg mL(^{-1})</td>
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<tr>
<td>DU632</td>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ciprofloxacin (positive control)</td>
<td>100 µg mL(^{-1})</td>
<td>-</td>
<td>100 µg mL(^{-1})</td>
<td>100 µg mL(^{-1})</td>
<td>100 µg mL(^{-1})</td>
<td>100 µg mL(^{-1})</td>
</tr>
</tbody>
</table>

Table 1. MIC values of ciprofloxacin analogs. This table displays the MIC of ciprofloxacin analogs and ciprofloxacin toward the growth of the ESKAPE pathogens. MIC was performed to determine the lowest concentration of the drugs in inhibiting the reproduction of bacteria. The compounds were tested at the highest concentration of 100 µg mL\(^{-1}\) to the lower concentration where the MIC was found.

The MIC values of ciprofloxacin analogs were determined for the four derivatives (Table 1), demonstrating activity against both Gram-positive and Gram-negative bacteria. Amongst these,
DU008 displayed the best activity, resulting in complete inhibition of all Gram-positive species, *E. faecium* and *S. aureus* (MRSA CBD-635), with an MIC achieved at 25 µg mL\(^{-1}\). In addition, DU008 was also active against all Gram-negative bacteria, with MICs of 1 µg mL\(^{-1}\) for *K. pneumoniae, A. baumannii, and E. cloacae*, and 5 µg mL\(^{-1}\) for *P. aeruginosa*. Likewise, compound DU667 was active against *E. faecium* at a concentration of 5 µg mL\(^{-1}\), yet showed no inhibition toward MRSA at a concentration of 100 µg mL\(^{-1}\). In addition, DU667 inhibited all Gram negative species, with an MIC of 1 µg mL\(^{-1}\) for *K. pneumoniae, A. baumannii, and E. cloacae*, and 10 µg mL\(^{-1}\) for *P. aeruginosa*. Similar to DU667, DU165 also possessed very low activity against MRSA; nevertheless, it was active against *E. faecium* and all four Gram-negative bacteria. The inhibitory activity of DU165 toward *K. pneumoniae* and *E. cloacae* was achieved at a concentration of ≤ 5 µg mL\(^{-1}\), and for *E. faecium, A. baumannii and P. aeruginosa* at a concentration of ≤ 25 µg mL\(^{-1}\). In contrast, DU632 was the least active compound amongst all ciprofloxacin analogs, with inhibitory activity achieved at 100 µg mL\(^{-1}\) for *E. faecium* and *A. baumannii*; however, DU632 displayed no activity against MRSA, *K. pneumoniae, P. aeruginosa*, and *E. cloacae* at a concentration of 100 µg mL\(^{-1}\). Moreover, the regular ciprofloxacin was not able to inhibit MRSA at a concentration of 100 µg mL\(^{-1}\); nevertheless, it was able to inhibit the other ESKAPE pathogens with MIC values achieved at 100 µg mL\(^{-1}\). Figure 4 represents an overview of bioactivity for all ciprofloxacin analogs. The scaled score for each compounds showed that each of this ciprofloxacin analog has broad-spectrum activities toward Gram-positive bacteria, primarily toward *E. faecium*, and toward all Gram-negative bacteria; however, DU632 showed no inhibition toward *K. pneumoniae, P. aeruginosa, and E. cloacae*. 
Twelve rifabutin analogs were tested against the ESKAPE pathogens in this study and displayed narrow inhibitory activity against MRSA (Table 2, Figure 5); four rifabutin analogs selectively inhibited...
MRSA at a concentration of $\leq 0.08 \, \mu g \, mL^{-1}$ (DU645, DU644, DU649, and DU650), while the other eight analogs showed lower inhibitory activity with MICs achieved exceeding $0.16 \, \mu g \, mL^{-1}$. DU645 was the most active MRSA inhibitor with MIC achieved at 0.02 $\mu g \, mL^{-1}$. The other two active analogs, DU644 and DU649, inhibited the growth of MRSA at a concentration of 0.04 $\mu g \, mL^{-1}$; whilst the inhibitory activity of DU650 was achieved at 0.08 $\mu g \, mL^{-1}$. In contrast, rifabutin, displayed higher MIC value toward MRSA than those of DU645, DU644, and DU649; rifabutin inhibited MRSA at a concentration of 0.05 $\mu g \, mL^{-1}$.

Moreover, rifabutin was also active against another Gram-positive bacteria, E. faecium, and all Gran negative species: K. pneumoniae, A. baumannii, P. aeruginosa, and E. cloacae with MIC achieved at 0.4 $\mu g \, mL^{-1}$, 12.5 $\mu g \, mL^{-1}$, 3.125 $\mu g \, mL^{-1}$, 12.500 $\mu g \, mL^{-1}$, and 25 $\mu g \, mL^{-1}$, respectively.

**Figure 5. Inhibitory activity of rifabutin analogs.** This graph displays the bioactivity of rifabutin analogs in inhibiting the growth of methicillin-resistant S. aureus (MRSA) and was made based on their MIC values that had been converted into a scaled score. The scaled score was created by the equation; Scaled score = $100/\text{MIC (}\mu g \, mL^{-1}).$

**MICs of Beta-lactam Analogs toward the ESKAPE Pathogens**

The MIC values of beta-lactam analogs against the ESKAPE pathogens demonstrated that many of these analogs possessed a narrow spectrum of activity against Gram-positive bacteria,
primarily MRSA, and some had wide antibacterial activities against *E. faecium*, MRSA, and Gram-negative bacteria, particularly *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa* (Table 3, Table 4, Figure 6, Figure 7). Thirty-seven of 66 beta-lactam analogs showed narrow inhibition spectrum toward MRSA with MIC of ≤ 100 µg mL⁻¹. DU074 and DU100, for example, were active against MRSA with an MIC was achieved at 1 µg mL⁻¹ and 100 µg mL⁻¹, respectively. Similarly, those with broad-spectrum inhibition also had MICs of ≤ 100 µg mL⁻¹ toward multiple pathogens. DU1060, for example, inhibited the growth of *E. faecium*, MRSA, and *A. baumannii* at a concentration of 25 µg mL⁻¹, *K. pneumoniae* and *P. aeruginosa* at a concentration of 50 µg mL⁻¹, and *E. cloacae* at a concentration of 100 µg mL⁻¹. DU1036, however, had a MIC of 5 µg mL⁻¹ for *E. faecium* and MRSA, and 25 µg mL⁻¹ for *A. baumannii*. Conversely, the MICs of ampicillin, a regular beta-lactam antibiotic, were achieved at higher concentrations than those of beta-lactam analogs. Specifically, ampicillin had an MIC of 100 µg mL⁻¹ for *E. faecium*, and showed no discernable inhibitory activity for MRSA, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii*, and *E. cloacae* at a concentration of 100 µg mL⁻¹.

![Figure 6. Inhibitory activity of beta-lactam analogs. This graph displays the bioactivity of beta-lactam analogs in inhibiting the growth of the ESKAPE pathogens, and was created based on their MIC values that had been converted into a scaled score. The scaled score was composed by the equation; Scaled score = 100/MIC (µg mL⁻¹).](image-url)
Figure 7. Inhibitory activity of beta-lactam analogs. This graph displays the bioactivity of beta-lactam analogs in inhibiting the growth of the ESKAPE pathogens, and was created based on their MIC values that had been converted into a scaled score. The scaled score was composed by the equation; Scaled score = 100/MIC (µg mL\(^{-1}\)).

<table>
<thead>
<tr>
<th>Beta-lactam analogs</th>
<th>E. faecium</th>
<th>S. aureus</th>
<th>K. pneumoniae</th>
<th>A. baumannii</th>
<th>P. aeruginosa</th>
<th>E. cloacae</th>
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<td>-</td>
<td>-</td>
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<td>-</td>
<td>100 µg mL(^{-1})</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>25 µg mL(^{-1})</td>
<td>50 µg mL(^{-1})</td>
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<td>DU124</td>
<td>-</td>
<td>25 µg mL(^{-1})</td>
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<td>100 µg mL(^{-1})</td>
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<td>Ampicillin (positive control)</td>
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Table 3. MIC values of beta-lactam analogs. This table displays the MIC of beta-lactam analogs and ampicillin toward the growth of the ESKAPE pathogens. MIC assessment was performed to determine the lowest concentration of the compounds in inhibiting the growth of bacteria. The compounds were tested at the highest concentration of 100 µg mL\(^{-1}\) to the lower concentration where the MIC was found.
<table>
<thead>
<tr>
<th>β-lactam derivatives</th>
<th><em>E. faecium</em></th>
<th><em>S. aureus</em></th>
<th><em>K. pneumoniae</em></th>
<th><em>A. baumannii</em></th>
<th><em>P. aeruginosa</em></th>
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<td>25 µg mL⁻¹</td>
<td>25 µg mL⁻¹</td>
<td>-</td>
<td>25 µg mL⁻¹</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DU1041</td>
<td>-</td>
<td>5 µg mL⁻¹</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DU1034</td>
<td>-</td>
<td>5 µg mL⁻¹</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DU1042</td>
<td>-</td>
<td>5 µg mL⁻¹</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DU1036</td>
<td>5 µg mL⁻¹</td>
<td>5 µg mL⁻¹</td>
<td>-</td>
<td>25 µg mL⁻¹</td>
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<td>-</td>
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<tr>
<td>DU947</td>
<td>-</td>
<td>1 µg mL⁻¹</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DU948</td>
<td>-</td>
<td>1 µg mL⁻¹</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DU949</td>
<td>-</td>
<td>1 µg mL⁻¹</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DU527</td>
<td>100 µg mL⁻¹</td>
<td>100 µg mL⁻¹</td>
<td>-</td>
<td>100 µg mL⁻¹</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DU404</td>
<td>100 µg mL⁻¹</td>
<td>10 µg mL⁻¹</td>
<td>-</td>
<td>100 µg mL⁻¹</td>
<td>-</td>
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<tr>
<td>DU364</td>
<td>100 µg mL⁻¹</td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DU475</td>
<td>1 µg mL⁻¹</td>
<td>1 µg mL⁻¹</td>
<td>100 µg mL⁻¹</td>
<td>25 µg mL⁻¹</td>
<td>100 µg mL⁻¹</td>
<td>-</td>
</tr>
<tr>
<td>DU901</td>
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<td>-</td>
<td>25 µg mL⁻¹</td>
<td>25 µg mL⁻¹</td>
<td>25 µg mL⁻¹</td>
<td>25 µg mL⁻¹</td>
</tr>
<tr>
<td>DU1059</td>
<td>10 µg mL⁻¹</td>
<td>25 µg mL⁻¹</td>
<td>100 µg mL⁻¹</td>
<td>25 µg mL⁻¹</td>
<td>100 µg mL⁻¹</td>
<td>-</td>
</tr>
<tr>
<td>DU1014</td>
<td>100 µg mL⁻¹</td>
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<td>-</td>
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<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4. MIC values of beta-lactam analogs. This table displays the MIC of beta-lactam analogs and ampicillin toward the growth of the ESKAPE pathogens. MIC assessment was performed to determine the lowest concentration of the compounds in inhibiting the growth of bacteria. The compounds were tested at the highest concentration of 100 µg mL⁻¹ to the lower concentration where the MIC was found.
From these high throughput screening results, we selected the three most active rifabutin analogs that possessed activity against MRSA to further perform drug characterization through minimum bactericidal concentration (MBC) determination, spontaneous mutation and *rpoB* mutation validation, and eukaryotic cytotoxicity assessment.

<table>
<thead>
<tr>
<th>β-lactam derivatives</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>E. faecium</em></td>
</tr>
<tr>
<td>DU1007</td>
<td>100 µg mL⁻¹</td>
</tr>
<tr>
<td>DU1012</td>
<td>100 µg mL⁻¹</td>
</tr>
<tr>
<td>DU1004</td>
<td>-</td>
</tr>
<tr>
<td>DU1060</td>
<td>25 µg mL⁻¹</td>
</tr>
<tr>
<td>DU1080</td>
<td>100 µg mL⁻¹</td>
</tr>
<tr>
<td>DU1068</td>
<td>25 µg mL⁻¹</td>
</tr>
<tr>
<td>DU932</td>
<td>100 µg mL⁻¹</td>
</tr>
<tr>
<td>DU2121</td>
<td>-</td>
</tr>
<tr>
<td>DU2069</td>
<td>-</td>
</tr>
<tr>
<td>DU530</td>
<td>100 µg mL⁻¹</td>
</tr>
<tr>
<td>DU2141</td>
<td>25 µg mL⁻¹</td>
</tr>
<tr>
<td>DU516</td>
<td>100 µg mL⁻¹</td>
</tr>
<tr>
<td>DU2163</td>
<td>50 µg mL⁻¹</td>
</tr>
<tr>
<td>DU957</td>
<td>-</td>
</tr>
<tr>
<td>DU983</td>
<td>-</td>
</tr>
<tr>
<td>DU378</td>
<td>100 µg mL⁻¹</td>
</tr>
<tr>
<td>Ampicillin (positive control)</td>
<td>100 µg mL⁻¹</td>
</tr>
</tbody>
</table>

**Table 5. MIC values of β-lactam analogs.** This table displays the MIC of β-lactam analogs and ampicillin toward the growth of the ESKAPE pathogens. MIC assessment was performed to determine the lowest concentration of the compounds in inhibiting the growth of bacteria. The compounds were tested at the highest concentration of 100 µg mL⁻¹ to the lower concentration where the MIC was found.

From these high throughput screening results, we selected the three most active rifabutin analogs that possessed activity against MRSA to further perform drug characterization through minimum bactericidal concentration (MBC) determination, spontaneous mutation and *rpoB* mutation validation, and eukaryotic cytotoxicity assessment.

**Minimum Bactericidal Concentration Determination Assay (MBC<sub>90</sub>)**

Determining the ability of the compounds in inhibiting bacterial growth (bacteriostatic), or terminating bacterial viability (bactericidal) are necessary in drug characterization. Therefore, after performing MIC testing, MBC assays were conducted to assess bactericidal activities of our frontrunner compounds. MBC assay was performed for the most active rifabutin analogs, **DU644**, **DU645**, and **DU650**, toward our clinical MRSA isolate. The MBC results on **Figure 8** and **Table**
suggest that all lead compounds possess potent bactericidal activity against MRSA; **DU644** and **DU645** are the most effective analogs with MBC$_{99}$ achieved at 0.04 μg mL$^{-1}$, showing very strong bactericidal effects. The MBC$_{99}$ of **DU644** was equal to its MIC value toward MRSA, whilst that of **DU645** was twofold higher than its MIC. On the other hand, **DU650** possess the weakest bactericidal activity with MBC$_{99}$ achieved at 0.16 μg mL$^{-1}$. In contrast, 99% bactericidal activity of the regular rifabutin was achieved at 0.1 μg mL$^{-1}$. Based on these results, **DU644** and **DU645** showed stronger bactericidal capacity toward MRSA with their MBC values about two times lower than that of the parent drug.

<table>
<thead>
<tr>
<th>Rifabutin analogs</th>
<th>MBC$_{99}$ (μg mL$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU644</td>
<td>0.04</td>
</tr>
<tr>
<td>DU645</td>
<td>0.04</td>
</tr>
<tr>
<td>DU650</td>
<td>0.16</td>
</tr>
<tr>
<td>Rifabutin (positive control)</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**Table 6. MBC values of the lead compounds.** This table display the MBC of DU644, DU645, DU650, includes rifabutin in achieving 99% bactericidal effect toward MRSA.

**Figure 8. Bactericidal activity of the lead compounds and the parent drug.** This figure displays the bactericidal activity of DU644 (i), DU645 (ii), DU650 (iii), and rifabutin (iv) in terminating 99% of MRSA.
Minimum Biofilm Eradication Concentration Assay (MBEC<sub>50</sub>)

<table>
<thead>
<tr>
<th>Rifabutin analogs</th>
<th>MBEC&lt;sub&gt;50&lt;/sub&gt; (µg mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU644</td>
<td>0.02</td>
</tr>
<tr>
<td>DU645</td>
<td>0.02</td>
</tr>
<tr>
<td>DU650</td>
<td>0.16</td>
</tr>
<tr>
<td>Rifabutin (positive control)</td>
<td>0.08</td>
</tr>
</tbody>
</table>

*Table 7. MBEC values of the lead compounds.* This table displays minimum biofilm eradication concentration of DU644, DU645, and DU650, includes rifabutin in eradicating 50% of MRSA biofilms.

The MBEC<sub>50</sub> of our lead rifabutin analogs displayed on Figure 9 and Table 7 represent their promising antibiofilm activities against MRSA biofilms. **DU644** and **DU645** are the most active antibiofilm analogs with MBEC<sub>50</sub> achieved at 0.02 µg mL<sup>-1</sup>; the MBEC<sub>50</sub> of **DU644** was twofold lower than its MBC<sub>99</sub>, whilst that of **DU645** was found to be equal its MIC value against MRSA. In contrast, the MBEC<sub>50</sub> of the regular rifabutin was four times higher than those of **DU644** and **DU645**, and was two times lower than that of **DU650**. The MBEC<sub>50</sub> of **DU650** achieved at 0.16 µg mL<sup>-1</sup> that was equal to its MBC value.

*Figure 9. Antibiofilm activity of the lead compounds and the parent drug.* This figure displays the antibiofilm activity of DU644 (i), DU645 (ii), DU650 (iii), and rifabutin (iv) in terminating 50% of MRSA biofilms.
Spontaneous Mutation Frequency

<table>
<thead>
<tr>
<th>Compound</th>
<th>Number of mutants colony and their mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 x MIC</td>
</tr>
<tr>
<td>DU644</td>
<td>Lawn</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>DU645</td>
<td>Lawn</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>DU650</td>
<td>Lawn</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifabutin</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 8. Spontaneous mutation frequency results.** This table displays the number of mutants colony and their resistance frequency toward DU644, DU645, DU650, and the rifamycin members (rifabutin and rifampicin).

The results on Table 8 show that at 5 x MIC, MRSA attained resistance towards all the lead compounds; however, as the concentration increases, the number of colony decreases. Among the frontrunner compounds, we found that at 10 x MIC, **DU650** was able to significantly reduce the number of bacterial colonies that grew on plates, producing 238 colonies with a mutation frequency of 5 x 10^{-8}. Likewise, rifabutin and rifampicin were capable of reducing the number of bacterial colonies at the concentration of 10 x MIC and yielded 580 and 639 mutant colonies with mutation rates of 6 x 10^{-8} and 7 x 10^{-8}, respectively. **DU645**, on the other hand, was active in reducing the number of MRSA colonies to an enumerable number of 196 and possessed a mutation rate of 6 x 10^{-8} at the concentration of 20 x MIC. In contrast, we noticed that MRSA was swiftly developed resistance toward **DU644**, since the compound was not able to reduce the number of bacterial colonies grown on plates at three lower concentrations (5 x, 10 x and 20 x MIC); nevertheless, at 50 x MIC we obtained 190 colonies with mutation rate of 7 x 10^{-8}.
**rpoB Mutation Validation**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Genotype</th>
<th>Phenotype MIC (µg mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mutation position</td>
<td>Amino acids substitution</td>
</tr>
<tr>
<td>Rifabutin #1</td>
<td>His 481</td>
<td>Asn</td>
</tr>
<tr>
<td>Rifabutin #2</td>
<td>Glu 465</td>
<td>His</td>
</tr>
<tr>
<td></td>
<td>His 481</td>
<td>Tyr</td>
</tr>
<tr>
<td>Rifampicin #1</td>
<td>His 481</td>
<td>Asp</td>
</tr>
<tr>
<td>Rifampicin #2</td>
<td>Gly 471</td>
<td>Cys</td>
</tr>
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<td></td>
<td>Gly 364</td>
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</tr>
<tr>
<td>645</td>
<td>Phe 367</td>
<td>Val</td>
</tr>
<tr>
<td></td>
<td>Lys 373</td>
<td>Asn</td>
</tr>
<tr>
<td>650 #1</td>
<td>Pro 352</td>
<td>Ser</td>
</tr>
<tr>
<td></td>
<td>Leu 408</td>
<td>Phe</td>
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<tr>
<td></td>
<td>Asn 417</td>
<td>Thr</td>
</tr>
<tr>
<td></td>
<td>Iso 442</td>
<td>Thr</td>
</tr>
<tr>
<td>650 #2</td>
<td>Thr 443</td>
<td>Pro</td>
</tr>
</tbody>
</table>

*Table 9. rpoB mutation validation results.* This table displays the position and the type of amino acid changes on the RpoB protein of the mutants of the lead compounds and the rifamycin members (rifabutin and rifampicin); these mutations grants high resistance for the mutants against DU645, DU650, rifabutin, and rifampicin.

The results of *rpoB* mutation displayed in Table 9 demonstrate that mutations toward rifabutin and rifampicin were primarily found at amino acid Histidine-481 of the *rpoB* gene, granting high resistance toward these antibiotics and yielding an MIC of > 200 µg mL\(^{-1}\). Moreover, amino acid substitutions of glutamine at position 465 with a histidine, and of glycine at position 471 and 364 by a cysteine, also conferred high resistance to rifabutin and rifampicin (MIC > 200 µg mL\(^{-1}\)), respectively. In contrast, mutational changes toward rifabutin analogs were not located at amino acid position 481, however, these different mutational locations were also primarily correlated to high MIC value of > 200 µg mL\(^{-1}\). Point mutational changes of mutant 645 in response to 20 x MIC of DU645 occurred at two different positions; amino acid substitution of phenylalanine at position 367 by a valine, and lysine 373 by an asparagine, yielding a remarkable MIC change from 0.04 µg mL\(^{-1}\) to > 200 µg mL\(^{-1}\). Similarly, point mutational changes displayed
by mutants of DU650 from 10 x MIC, granted them high resistance toward this compound; mutant 650 #1 possessed an amino acid change at position 352, 408, 417, whilst mutant 650 #2 had one amino acid change of a tyrosine 443 by a proline, yielding high resistance with MIC of > 200 µg mL⁻¹ toward DU650.

Cytotoxicity

![Graph showing cytotoxicity](image)

Figure 10. *In vitro* cytotoxicity of the lead compounds and the parent drug. This figure displays *in vitro* cytotoxicity of DU644, DU645, DU650, and rifabutin at a concentration ranges from 1.50 µg mL⁻¹ to 50 µg mL⁻¹ toward human hepatocellular carcinoma epithelial cell (HepG2).

It is highly important for an antimicrobial agent to have high selectivity toward prokaryotic cells and therefore will not produce health risk effects in humans. Accordingly, cytotoxicity of lead rifabutin analogs was performed against HepG2 cells. The potential toxicity screening of these three lead agents displayed on Figure 10 shows low toxicity. Specifically, DU644 and DU645 demonstrated very low toxicity, allowing for > 75% HepG2 cell recovery at the concentration ranging from 1.5 µg mL⁻¹ to 25 µg mL⁻¹. The percentage of cells recovered by DU644, DU645, and rifabutin (positive control) at the concentrations ranging from 1.5 µg mL⁻¹ to 6 µg mL⁻¹ were quite similar: at 1.5 µg mL⁻¹ to 3 µg mL⁻¹, DU644 and rifabutin produced about 82% cell recovery,
except for DU644 that produced a slightly increased percent recovery of around 90% at 3 μg mL\(^{-1}\). HepG2 cell recovery decreased as the concentration of the compounds increased; At 6 μg mL\(^{-1}\), DU645 and rifabutin recovered 78% and 79% cell viability; whilst DU644 allowed for 88% cell recovery. For DU644 and DU645, we discerned > 76% cell recovery at 12 μg mL\(^{-1}\) and 25 μg mL\(^{-1}\); in contrast, rifabutin was only able to recover < 70% cell viability. However, when the concentration was increased to 50 μg mL\(^{-1}\), the percent recovery for DU644 and DU645 declined to 36% and 34%, respectively. In contrast, rifabutin was able to recover 57% of the Hepg2 cell at the concentration of 50 μg mL\(^{-1}\). Among all the lead agents, DU650 was the least favorable one, since it recovered < 65% of the HepG2 cells at the concentration ranging from 1.5 to 25 μg mL\(^{-1}\), and < 30% cell viability at the concentration of 50 μg mL\(^{-1}\). The data from this experiment were further used to assess the lethal dose (LD\(_{50}\)), the concentration of the compound that kills 50% of the cell lines. The LD\(_{50}\) of DU644, DU645, and DU650 were discerned at the concentration of 43 μg mL\(^{-1}\), 40 μg mL\(^{-1}\), and 32 μg mL\(^{-1}\), respectively.
Chapter Four: Discussion

The results of antibacterial screening on rifabutin analogs against the ESKAPE pathogens observed through the MIC assessment revealed their high specificity toward MRSA. Typically, modification at the spiropiperidyl ring increases the activity of rifamycins antibiotic toward Gram positive bacteria, especially, *S. aureus* and *M. tuberculosis* more than toward Gram negative species (Sanfilippo et al., 1980). Moreover, two of our lead analogs (DU644 and DU645) possess increased inhibitory, bactericidal and antibiofilm activities against MRSA when compared to the parent rifabutin. The increased antibacterial activities of rifabutin analogs occur as a result of drug modifications at C-3 and C-4 positions of spiropiperidyl ring carrying aromatic rings substituted with halogens (Figure 11) with which DU644 and DU645 possess bromine (Br) substituent, whilst

![Figure 11. Chemical structures of the lead compounds.](image)
DU650 has iodine (I). The role of halogen substituents in increasing antibacterial activities of a compound has been well documented. A study by Lu et al. (2001) informs the role of bromine substituent in enhancing the inhibitory and bactericidal activities of fluoroquinolone antibiotic against resistant *Mycobacterium smegmatis* and *S. aureus*; whilst that of Bahrin et al. (2016) shows the effectiveness of halogen substituents such as bromine and iodine in increasing bioactivity of flavonoid compound against *Escherichia coli* and *S. aureus*.

From our study, the highest inhibitory activity of rifabutin analogs (DU644 and DU645) was observed at 0.02 µg mL⁻¹ and 0.04 µg mL⁻¹, respectively; the highest inhibitory activity of the parent drug toward MRSA was observed at 0.05 µg mL⁻¹. Moreover, our rifabutin analogs possess a high degree of specificity toward MRSA, compared to the parental drugs that showed inhibition against Gram-negatives of the ESKAPE pathogens at a concentration exceeding 3 µg mL⁻¹. The increased antibacterial effectiveness of rifamycin analogs toward MRSA confirmed findings of Sanfilippo et al. (1980), where linear aliphatic chains substituents at N position of the spiropiperidyl ring increased the activity of rifabutin analogs against *S. aureus* and *M. tuberculosis*, yet decreased their effectiveness against *Escherichia coli*. This presumably related to the increase of lipophilicity of the compounds (Sanfilippo et al., 1980). Therefore, structural drug modifications of rifabutin enhance the antibacterial activity of the drug toward MRSA.

In addition to their increased inhibitory activities against MRSA, our lead agents DU644 and DU645 are also more bactericidal toward MRSA than is the parental drug. During the course of MBC assay, 99% bactericidal activities of DU644 and DU645 were achieved at 0.04 µg mL⁻¹. However, the highest bactericidal activity of rifabutin was achieved at 0.1 µg mL⁻¹. This indicates structural drug modification on rifabutin is beneficial in increasing its bactericidal activity against MRSA.
Biofilm has been characterized as a virulence factor of *Staphylococcus aureus*, contributing to its ability to resist therapeutic intervention (Ohadian *et al*. 2014). During the course of our research in assessing antibiofilm activity of the lead rifabutin analogs, we found their potential antibiofilm abilities in eradicating MRSA biofilms. Importantly, two of our three lead compounds, **DU644**, and **DU645**, were able to clear 50% of MRSA biofilm at a concentration of 0.02 µg mL⁻¹, whilst the 50% biofilm activity of the regular rifabutin occurred at 0.08 µg mL⁻¹. This suggests that **DU644** and **DU645** possess more antibiofilm activity than the parental drug. In addition to this, **DU644** and **DU645** possess similar bactericidal and antibiofilm activities against MRSA; this occurred as a result of the same halogen substituent, bromine, they have on the aromatic rings that attached to the spiropiperidyl ring. Moreover, it is well known that rifabutin possesses good tissue penetration, is able to penetrate into polymorphonuclear leukocytes, lymphocytes, and macrophages (Kunin, 1996), and is also active against *M. tuberculosis* that is difficult to treat with other antibiotics (Moraes *et al*., 2015). Therefore, structural drug modifications on rifabutin increase its activity in penetrating and clearing MRSA biofilms. These results propose rifabutin analogs as potent antibiofilm drug candidates against MRSA biofilm formation.

During the course of spontaneous mutation assessment we determined that MRSA spontaneously mutated toward our lead agents and the rifamycin members (rifabutin, and rifampicin). From this assay, we found MRSA developed resistance toward our lead compounds at their lowest tested concentration. However, as the concentration of the compounds increased, the number of colonies arising on plates decreased. At the concentrations higher than 5 x MIC, the lead agents were able to reduce the number of MRSA that grew on plates and to yield resistance frequencies of 10⁻⁸ per nucleotide per reproduction cycle. This result was in line with previous
studies, with which the mutation rate of *S. aureus* toward rifamycins usually are obtained at $10^{-7}$ to $10^{-8}$ (Aubry-Damon, Soussy, and Courvalin, 1998; Morrow and Harmon, 1979).

The survival of each mutant against high concentrations of the lead agents and of the rifamycin members (rifabutin and rifampicin) indicated the occurrence of the mutations within the *rpoB* gene that encodes the target of rifamycin. The results of *rpoB* mutation validation assay affirm that the nucleotide changes led to the amino acid replacement within the RpoB protein of the rifabutin analogs and rifamycin mutants, and conferred high resistance toward these compounds. The RpoB protein point mutational changes of the rifabutin and rifampicin mutants were primarily found at the amino acid histidine position 481; of these mutations, three histidine 481 were replaced by an asparagine, a tyrosine, and an aspartic acid. These amino acid substitutions granted MRSA a robust resistance against rifabutin and rifampicin, increasing their MICs from $0.05 \mu g \text{ mL}^{-1}$ and $0.06 \mu g \text{ mL}^{-1}$ to $> 200 \mu g \text{ mL}^{-1}$. There is mounting evidence suggesting the pivotal role of Histidine 481 in conferring high resistance of *Staphylococcus aureus* toward rifamycin members (Yu, 2005; Wichelhaus *et al.*, 1999; Aubry-Damon, Soussy, and Courvalin, 1998). In addition, the other different positions and types of amino acid alterations were also found in Rifabutin #2 and Rifampicin #2 mutants: the replacement of glutamine 465 by a histidine and of glycine 471 and 364 by a cysteine had conferred high resistance for the mutants against rifabutin and rifampicin, respectively. Moreover, protein sequencing analysis utilizing Pfam data base revealed the location of amino acid substitution of position 465, 471, and 481 lie on the catalytic region; whilst that at position 364 was located at the protrusion region. In contrast, seven point mutational changes of the rifabutin analog mutants were not located at the catalytic region of the *rpoB* protein, but were all found at the protrusion region. The amino acids substitution at position 367 and at 373 of mutant 645, at positions 352, 408, 417, and 442 of mutant 650 #1,
and at position 443 of mutant 650 #2, had also granted MRSA a robust resistance against DU645 and DU650, yielding to an MIC of > 200 µg mL\(^{-1}\). Point mutational changes at the catalytic and the protrusion regions of the rpoB protein potentially alter the conformation of the RNA polymerase binding site and weaken its affinity toward rifampicin (Alifano et al., 2015). From these results we confirmed that our lead agents performed the same mechanism of action as rifabutin and rifampicin through interference with the beta-subunit of RNA polymerase.

Toxicity potential of rifabutin is worrisome, since some pharmacokinetin studies reported its adverse health effects in humans when not used in a proper dosage or when combined with other drugs. Studies by Zhang et al. (2011) revealed rifabutin administration led to skin rash when used as a single treatment, caused neutropenia and increased liver enzyme when used in combination with antiretroviral drugs for TB treatment in HIV/AIDS patients. Therefore, it is important to assess the potential cytotoxicity of our lead agents. The results of the cytotoxicity testing we performed suggest rifabutin analogs possess a negligible toxicity when their concentration was increased from 25 µg mL\(^{-1}\) to 50 µg mL\(^{-1}\). Moreover the 50% killing activities (LD\(_{50}\)) of DU644, DU645, and DU650 were achieved at 43 µg mL\(^{-1}\), 40 µg mL\(^{-1}\), and 32 µg mL\(^{-1}\), respectively. In contrast, the LD\(_{50}\) of the regular rifabutin was not determined during the assay; however at the highest tested concentration (50 µg mL\(^{-1}\)), rifabutin allowed for 57% cell recovery. From in vitro cytotoxicity results, we revealed our rifabutin analogs possessed a low level of toxicity when compared to the parent drug.
Chapter Five: Conclusions

Strengths and Limitations

Strengths of this study are as follows. The antibacterial activities, target mutation validation, spontaneous mutation and cytotoxicity assessments performed in our study were straightforward, easy to prepare, and reproducible. The study was performed in an established laboratory under the direction of a seasoned scientist and researchers. Moreover, these assessments were based off well-established techniques for drug discovery. In addition, the findings of this study are consistent with the findings of the earlier studies performed on ciprofloxacin (Cormier et al., 2012), rifabutin analogs (Turos et al., 2002), and beta lactam analogs (Sanfilippo et al., 1980) toward resistant bacteria.

Limitations of this study include incomplete drug characterizations for ciprofloxacin analogs and beta-lactam analogs due to shortcomings of time and amount of compounds we had during the course of this research. I have to graduate on summer 2016 and this does not give me enough time to complete my research on these two class of analogs. Moreover, we ran out of the active ciprofloxacin and beta lactam analogs after we finished performing the MIC assessments for these analogs.

Future Directions

As a potential drug for MRSA infections, it is important to perform in vivo assessments for rifabutin analogs and to optimize their antibacterial capacity through the investigation of
substituents at C-3, C-4, or N position of the spiropiperidyl ring that may help to decrease MRSA resistance towards these drugs. In addition, it is also important to perform bactericidal and antibiofilm assessments for ciprofloxacin and beta-lactam analogs; therefore, the aim of determining the efficacy of these analogs toward the ESKAPE pathogens can be fulfilled.

**Implications**

Infections by the ESKAPE pathogens are still problematic for their growing resistance towards a wide range of antibiotics and for the decline of antibiotic approval to solve these problems (Boucher *et al.*, 2009). Among these resistant pathogens, MRSA is the leading cause of nosocomial infections and has led to higher mortality rate compared to those led by HIV/AIDS and tuberculosis (Boucher and Corey, 2008). MRSA causes up to 80,461 of nosocomial infections and kills up to 11,285 of hospitalized patients in the USA every year (CDC, 2013). Moreover, this bacterium is medically important in causing prosthetic device-related infection due to its ability to form biofilms that are almost impossible to clear by immune system and antibiotics (Song *et al.*, 2013). In addition, it also strikes individuals with healthy immune system, primarily causing skin and soft tissue infections (SSTIs) (Stryjewski and Chambers, 2008). Accordingly, MRSA becomes a pivotal public health problem to be resolved. In conclusion, the results of anti-MRSA activities of our rifabutin analogs, particularly their potent antibiofilm activities, are very promising. Therefore, as a potent antibacterial agent against MRSA, further development and application of these compounds might be beneficial for medical use to combat MRSA infections.
List of References


