DISCOVERY OF RESISTANCE-REVERSING AGENTS IN ANTIBIOTIC RESISTANT STRAINS OF *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* FROM NATURAL PRODUCT LIBRARIES

by
Andrew Braxton Watkins

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Approved by

____
Advisor: Dr. Alice Clark

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Reader: Dr. Melissa Jacob

____
Reader: Dr. Colin Jackson

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From my first breath to the completion of my thesis, I have been blessed by the wonderful love and support of my family. This thesis is dedicated to my parents, Richard and Angie Watkins, as well as to my grandmother, Shirley Pace. Without their loving guidance, I would not be here today.

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ABSTRACT

ANDREW B. WATKINS: Discovery of Resistance-Reversing Agents in Antibiotic Resistant Strains of *Escherichia coli* and *Klebsiella pneumoniae* from Natural Product Libraries
(Under the direction of Dr. Melissa Jacob)

Objective: The objective of this exploratory research is to discover compounds, particularly from natural products, that inhibit ESBL, KPC, and NDM-1 mechanisms of antibiotic resistance in the Gram negative bacteria *Escherichia coli* and *Klebsiella pneumoniae*.

Methods: This objective will be accomplished utilizing a high-volume bioassay testing natural product samples from the National Center for Natural Products Research. This assay tests samples against 6 different strains of bacteria known to express β-lactamases in the presence and absence of sub-inhibitory concentrations of the test antibiotic. By structuring the assay in this way, differentiation may be made between the inherent antibacterial activity of samples and the synergistic effects between the sample and the antibiotic. Optical density (OD) readings will be used to determine bacterial growth or the lack thereof. Samples showing pronounced activity only in the presence of the antibiotics will be considered active and will be tested in a checkerboard assay to confirm activity.

Results: Of over 5,000 samples tested, 35 samples showed synergistic activity, giving a hit rate of 0.7%. Of these 35, the most promising three hits were tested in follow-up checkerboard assays. These three samples all demonstrated synergistic effects with fractional inhibitory concentrations (FIC) of <0.5. Of the samples tested in the

checkerboard assays, one was a plant extract, one was a pure compound, and one was a fungal soil isolate obtained from the National Cancer Institute.

Conclusions: This study showed the benefits of using a high-volume screen to test samples against resistant bacterial strains. Continued research in this field could prove to be beneficial to the discovery of new drugs for clinically relevant therapeutic applications.

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List of Abbreviations

APB	3-Aminophenylboronic Acid
ATCC	American Type Culture Collection
BA	Boronic Acid
CEFO	Cefotaxime
CFU	Colony-Forming Unit
CLAV	Clavulanic Acid (Clavulanate)
CLSI	Clinical and Laboratory Standards Institute
CRE	Carbapenem-Resistant *Enterobacteriaceae*
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediamine-N,N,N',N'-Tetraacetic Acid
ESBL	Extended-Spectrum β-Lactamase
FIC	Fractional Inhibitory Concentration
HPLC	High-Performance Liquid Chromatography
IC_{50}	Half Maximal Inhibitory Concentration
KPC	*Klebsiella pneumoniae* Carbapenemase
MBL	Metallo-β-Lactamase
MIC	Minimum Inhibitory Concentration
NCNPR	National Center for Natural Products Research
NDM	New Delhi Metallo-β-Lactamase
OD	Optical Density
ULF	Ultra Low Freezer

CHAPTER I

INTRODUCTION

History of Antibiotics

Antibiotics are arguably one of the most important and impactful discoveries in the field of medicine; however, due to repeated and prolonged antibiotic misuse and complex genetic mechanisms within bacteria, resistance to these antibiotics has emerged, spread, and become a serious problem worldwide in many different settings.[1] According to the Center for Disease Control and Prevention's (CDC) *Antibiotic Resistance Threats in the United States, 2013*, over 2 million people per year develop serious infections with bacteria that are resistant to the antibiotics usually used to treat such infections. At least 23,000 people die directly from these infections, and many more die from other conditions or complications brought about by these infections. It is estimated that infections with resistant bacteria cost the United States as much as $20 billion per year in direct costs, as well as $35 billion per year (2008 economy dollars) in productivity loss and additional costs.[2] It is possible that these numbers have risen even more in the years since this report. Therefore, there is an urgent need for the discovery and development of new antibiotics to address the threat of resistant bacteria.[1]

Although antibiotics and their uses are viewed predominately as a "recent" occurrence, evidence has suggested that they have been used in some capacity for thousands of years. Researchers actually found traces of tetracycline, a 20th century

antibiotic produced by *Streptomyces* bacteria, in the soil and in bones and enamel of skeletons from ancient Sudanese Nudia from the years 350-550 AD as well as in skeletons from Dakhleh Oasis, Egypt, dating back to the late Roman period. These findings indicate that it is possible that tetracycline may have been consumed in its natural form from soil. Moreover, it is interesting to note that there was a low rate of infectious disease documentation in the Sudanese Nubian records, and there were no signs of bone infection in the remains from Dakhleh Oasis, which points to the possibility of a protective effect of tetracycline, or other natural remedies, on these ancient peoples. More evidence of the ancient use of antibiotics is the discovery of antibiotic substances found in soils and herbs from folk stories and from Traditional Chinese Medicine therapies.[3]

Ancient peoples may have used plants and other natural substances as a type of antibiotic, but the actual "antibiotic era" did not begin until the early to mid 1900's. Crucial discoveries such as Paul Ehrlich's method of testing numerous compounds against a common microbe or target in a screen or bioassay laid the foundation for antibiotic development, including the discovery of Salvarsan used to treat syphilis.[3]

Subsequent screening programs by chemists of the Bayer company, leading to the discovery of Pronotsil, and the quintessential serendipitous discovery of penicillin in 1928 by Royal Army Medical Corps veteran Alexander Fleming ushered in the age of antibiotics.[4] Fleming was one of the first people to warn about the dangers of antibiotic resistance in penicillin if the drug was used in quantities too small or for time periods too short.[3]

Emergence of Antibiotic Resistance

Although the aforementioned antibiotics may be different and carry different stories of their discoveries, there lies an underlying factor that connects them to each other as well as to all other used antibiotics: resistance. According to the World Health Organization (WHO), antimicrobial resistance refers to the resistance to antimicrobial drugs by the common microorganisms that would usually be treated with the drug. Antibiotic resistance is simply a more specific subtype of antimicrobial resistance in which bacteria become resistant to antibiotics.[5] Although antibiotic resistance has been hastened in recent years due to widespread misuse, it has been found that it is a naturally occurring phenomenon present even before the commercial use of penicillin.[1,5] In a study in 2006, a group of scientists sampled bacteria from different soils in different environments, and found that many of the collected strains were resistant to multiple classes of antibiotics, both synthetic and natural, including new antibiotic drugs. This study showed the wide variety of antibiotic resistance mechanisms present naturally in soil microbes. These resistance mechanisms most likely evolved as competitive defense mechanisms against naturally produced antibiotics, but now they could further develop into clinically significant resistance problems.[6]

A factor that makes antibiotic resistance even more of a problem is the ability of bacteria to exchange genes that code for mechanisms that cause resistance. Resistance is usually present in certain bacterial strains in the form of an endogenous protein able to inhibit the mechanism of action of an antibiotic compound. This protein is produced via translation of an mRNA transcript transcribed from a resistance gene. Resistance genes such as these are usually brought into the cell from the outside environment or from

another bacterium in the form of a plasmid.[7] A plasmid is a circular piece of DNA that exists separately from normal chromosomal DNA, is replicated separately, and can be passed horizontally to other bacteria through the process of conjugation. In this process of conjugation, a projection called a sex pilus extends from one bacterium to another and connects the two cells. A DNA pore is then formed, and a plasmid is replicated and transferred to the new cell, giving each cell a copy of the plasmid. When such a plasmid contains a gene that encodes resistance, it is called a "resistance plasmid," and the transfer of such a plasmid can be an efficient way to spread antibiotic resistance to different strains of bacteria. Another mechanism for the acquisition of resistance is through random genetic mutations in the host bacterium. These random insertions, deletions, or inversions of DNA nitrogenous bases can result in serious mutations in genes, and if these mutations happen to impart resistance and an advantage in survival, they will be passed to all subsequent generations. This method of resistance acquisition follows Darwin's theory of natural selection, and is a major driving force behind antibiotic resistance.[8]

Factors Affecting Resistance in Today's Society

The occurrence of natural selection in resistant microbes can be problematic even when it is left to act alone, but it can become even more of a problem with the addition of man-made selective pressures such as antibiotic misuse and abuse. In cases like these, the incorrect use of antibiotic drugs can increase the spread of resistance. When an antibiotic attacks a group of bacteria, the susceptible bacteria are killed, leaving only those bacteria that are resistant to the drug. These resistant bacteria, with no competitors present, are

able to thrive and reproduce to form a new colony consisting solely of resistant bacteria. This problem is even more prevalent when antibiotics are administered in too low of a dose or when patients do not take a full course of antibiotic treatment, as correctly predicted by Fleming.[9] According to a large meta analysis conducted by Kardas et al., the overall antibiotic therapy compliance rate of patients was 62.2%. The same study found that 28.6% of the included patients used "leftover" antibiotics from previous infections.[10] Low compliance rates such as these provide ample opportunities for resistant bacterial communities to develop and thrive by the mechanism previously discussed. The problem of resistance can also be worsened by the incorrect prescription of antibiotic drugs by physicians. The CDC estimates that 50-150 million antibiotic prescriptions per year are unneeded, and a seminar conducted by Levy found that over 80% of physicians had prescribed antibiotics on demand against better judgment.[9] This flooding of the population with antibiotics also helps develop and spread resistance.

Two other factors that contribute to resistance are agricultural applications of antibiotics and overuse of antibacterial cleaning supplies. Antibiotics are used extensively in agriculture to promote the growth and development of livestock that are to be used for food. The use of these antibiotics, even in low quantities, puts selective pressure on populations of bacteria, leading to the development of resistance. Through the excretions of livestock, these bacteria can enter the water source where they can be spread directly or indirectly to humans through ingestion or irrigation of other edible plants.[11] The spread of resistant strains combined with the capabilities of bacteria to transfer resistance plasmids creates a situation that can lead to clinically relevant types of resistance. As the desire to keep surfaces and bodies clean and free from bacteria has risen, so too has the

use of antibiotic-containing cleaning supplies.[12] Many of these supplies include triclosan, an antibacterial compound with broad spectrum activity that many believe may contribute to antibacterial resistance. Triclosan was found to be one of the most common pharmaceutical chemicals present in water samples from 139 rivers in the United States in 1999-2000, which testifies to the extent of its use.[13] Research has suggested that it has some non-specific activity, but also works on a specific target, giving it the possibility of promoting resistance to other antibiotics. The potential for this cross-resistance in clinically relevant strains of bacteria is rather small, but it is still a possibility and should be taken seriously. Because of triclosan's widespread use and its possibility of conferring cross-resistance, research is being conducted to determine the benefits and detriments of its use, and caution should be taken when using large quantities of substances containing triclosan.[12]

Chapter II

Literature Review

Clinically Relevant Mechanisms of Resistance

There are many different bacterial mechanisms that confer antibiotic resistance; because of this, the CDC organized the known mechanisms into a hierarchy of clinical significance in a report from 2013. In this report, they listed resistant strains of bacteria as "urgent," "serious," or "concerning" threats.[2] Mechanisms relevant both clinically and to this project include carbapenem-resistant *Enterobacteriaceae* [CRE] (urgent threat) and extended-spectrum β-lactamase producing *Enterobacteriaceae* [ESBL's] (serious threat). A subtype of the CRE's, called NDM-1, is emerging as a dangerous and clinically significant mechanism. All of these mechanisms are present in *Enterobacteriaceae*, a family of gram-negative bacteria including *Escherichia coli*, *Klebsiella pneumonia*, and many other strains of bacteria.[14] Strains from this family are able to cause pneumonia, blood and wound infections, and meningitis.[15] All of these mechanisms also involve the production of an enzyme known as β-lactamase. β-lactamase is an enzyme that is able to disable β-lactam antibiotics (e.g. penicillins) by hydrolyzing the β-lactam ring within the compound and is responsible for a large number of resistance mechanisms. These β-lactamase enzymes are classified in different ways, the simplest of which is the Ambler system, established in 1980. This system categorizes enzymes into four classes (A, B, C, D) based on their amino acid sequences. Class A β-lactamases include broad spectra,

extended-spectra, and carbapenemase enzymes from both plasmids and chromosomal DNA; Class B enzymes are metallo-β-lactamases such as NDM-1; Class C enzymes are cephalosporinases coded for in chromosomal DNA; and Class D enzymes are oxacillinases.[16] The classes most relevant to the CDC report are Class A and Class B β-lactamases.

Among the "urgent threat" strains of bacteria exist the carbapenem-resistant *Enterobacteriaceae* (CRE).[2] Carbapenems are very important and effective broad-spectrum antibiotics that historically have had no problems with resistance; however, the recent development of carbapenem-hydrolyzing β-lactamases has increased the occurrence of resistance.[14,16] The most common carbapenemase now is the *Klebsiella pneumoniae* carbapenemase (KPC), a Class A enzyme that is very capable of inactivating β-lactam antibiotics. KPC-producing bacteria were first seen in North Carolina in 2001 and in the following years were seen throughout the Northeastern United States. As of 2010, KPC-producing microbes have been reported or received from 36 states, Washington DC, and Puerto Rico. Beginning in 2006, reports from foreign countries also showed the presence of KPC-producing bacteria. Studies and sample analyses have shown that in the United States, approximately 70% of KPC-producing bacteria belong to the same dominant strain.[14]

As mentioned previously, Class B enzymes are called metallo-β-lactamases (MBL's). These enzymes are different from Class A enzymes because of their use of zinc to hydrolyze the β-lactam ring.[14] A unique MBL, the New Delhi Metallo-β-Lactamase (NDM-1), has arisen from this class and is proving to be a problematic mechanism because of its ability to inactivate all β-lactam antibiotics except for aztreonam.[14,17] The

first known case of NDM-1-producing bacteria was found in 2007 in a Swedish patient that had been treated in a hospital in New Delhi, India; soon thereafter, NDM-1 producers were seen in the United Kingdom, India, and Pakistan.[17] Many of the UK patients infected with NDM-1-producing bacteria were previously treated in India or Pakistan, demonstrating the speed with which resistant microbes can be transferred from one country to another.[14,17] One of the factors that distinguishes NDM-1 from other resistance mechanisms is its tendency to spread to numerous unrelated bacterial strains. It has been seen in *E. coli* and *K. pneumoniae*, as well as other species of the same family. Another clinical problem presented by NDM-1 is its tendency to occur in bacterial strains that possess resistance to many other antibiotics, making these strains resistant to many, and possibly all, clinically used antibiotic therapies. Many strains carrying the NDM-1 gene are vulnerable only to colistin and tigecycline. There have been only small numbers of cases in multiple countries around the world, but the potential to spread is very high.[17] This potential for international travel mixed with the extent of resistance provided by NDM-1 makes bacterial strains containing this mechanism of resistance extremely dangerous and an urgent threat.

A third distinct clinical threat is the spread and emergence of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* strains. These strains produce β-lactamases that are able to inactivate a variety of newer β-lactam antibiotics, including third-generation cephalosporins (such as cefotaxime) and monobactams (such as aztreonam).[18] Many of these strains are also able to obtain resistance genes that confer resistance to other classes of antibiotics while still keeping the resistance to older antibiotics. Because of their ability to become more resistant and their frequent presence

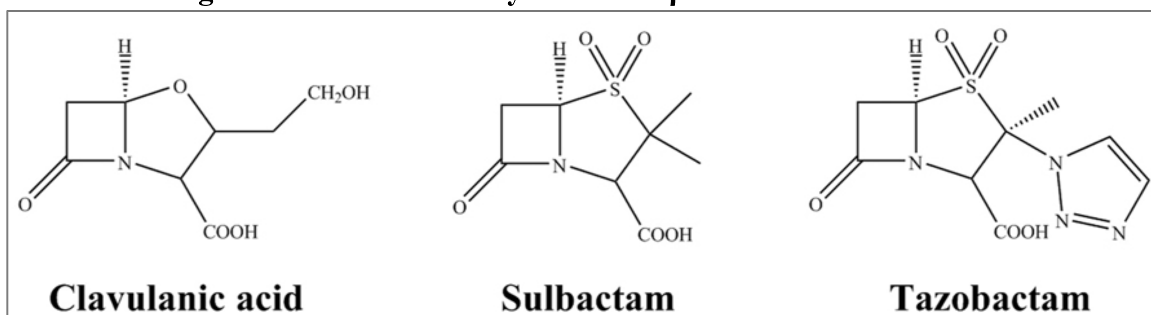
in human infections, ESBL-producing *K. pneumoniae* and *E. coli* were listed by the Infectious Diseases Society of America as microbes that need new treatment options as soon as possible.[19] Most ESBL's are separated into three groups (TEM, SHV, and CTX-M) based on the gene in which there is a mutation.[18,20] Bacteria displaying CTX-M-mediated resistance are not limited to hospital infections, and the epidemiology of such bacteria is very different than that of bacteria with TEM or SHV resistance.[18] TEM and SHV derived ESBL's both arise from one or more amino acid substitution(s) on their respective genes. TEM ESBL's are very common in *E. coli* and are one of the most common mechanisms of resistance against β-lactam antibiotics in gram-negative bacilli worldwide. The SHV family of β-lactamases is widespread in *K. pneumonia*, and actually originated in the chromosomes of species of the *Klebsiella* genus before being incorporated into a plasmid and spread to other *Enterobacteriaceae*.[20] The three genes cannot be differentiated by phenotypic measures and therefore must be genotyped for identification.[18,20] These three different types of ESBL-producing genes vary in their degree of resistance and susceptibility to certain antibiotics, but all convey resistance and are proving to be clinically-relevant threats.[20]

Reversing Resistance and Current Research Methodologies

Although antibiotic resistance is a growing problem, there is a sense of hope in the form of resistance-reversing compounds. Currently, these compounds are relatively rare, but research is being conducted to discover new agents that could be marketed for use in infections caused by resistant strains of bacteria.[16] Some of the most prominent types of resistance-reversing agents in ESBL-producing bacteria are the β-lactamase

inhibitors. As their names imply, these compounds work by preventing the activity of β-lactamases, thereby restoring the activity of β-lactam antibiotics.[21] Currently, there are three commercially available β-lactamase inhibitors: clavulanic acid (clavulanate), sulbactam, and tazobactam.[16] As seen in Figure 2-1, these compounds all contain a β-lactam ring similar to that present in β-lactam antibiotics, but have little inherent antibacterial activity.[16,21]

Figure 2-1: Commercially Available β-Lactamase Inhibitors



Source: Watkins R, Papp-Wallace K, Drawz S, Bonomo R. Novel β-lactamase inhibitors: A therapeutic hope against the scourge of multidrug resistance. Front Microbiol. 2013 24 December 2013;4

When in the presence of β-lactamases, these compounds irreversibly bind to the enzymes (using their inherent β-lactam structure), thereby permanently disabling the β-lactamases and "protecting" the antibiotics. This mechanism is responsible for the restored activity of antibiotics against resistant bacteria when in the presence of these compounds. There are different combinations of antibiotics and β-lactamase inhibitors, the most popular of which is clavulanate/amoxicillin, available orally under the name Augmentin as well as under various other trade names. These compounds are effective against Class A ESBL's except for the carbapenemases, but are ineffective against Class C and most Class B and Class D β-lactamases.[21] Because of its resistance-reversing activity, a β-lactamase inhibitor such as clavulanate can be used in combination with a β-

lactam antibiotic such as cefotaxime to phenotypically identify ESBL-producing bacteria or to inhibit bacterial colony growth.[22] This application could be useful as a positive control in screens detecting resistance-reversing compounds.

While the previous compounds work well with many Class A ESBL's, they lack effectiveness against carbapenemase-resistant *Enterobacteriaceae* (CRE); however, boronic acids are a group of compounds known to reverse carbapenem resistance.[16] Boronic acid (BA) compounds have been known to reversibly bind to and inactivate β-lactamases; however, recent studies have shown that this mechanism can lead to the inactivation of carbapenemases such as KPC.[23] A specific boronic compound, 3-aminophenylboronic acid (APB), has been found to be very successful in phenotypically determining KPC-producing bacteria through the use of disk-based assays by adding an antibiotic to a small disc and placing it on a petri dish with bacteria and comparing the zone of inhibition to that of a disk with the same antibiotic plus APB. According to one study, this works very well when the carbapenem used is meropenem; zones of inhibition were increased by more than 5mm in all KPC-producing strains when APB was combined with meropenem. The increase in zone of inhibition in the combination of meropenem and APB versus meropenem alone shows that the APB is able to work synergistically to restore or increase the activity of meropenem.[24] The CDC has also seen similar results in combinations of APB with carbapenems in their research efforts. In one study, the MIC of meropenem in a KPC-producing strain of *K. pneumoniae* was reduced 16.67-fold upon addition of APB (meropenem alone = 2 μg/mL; meropenem+APB = 0.12 μg/mL).[25] The ability of boronic acid compounds, especially APB, to synergistically work to restore activity to carbapenems, especially meropenem, provides a desirable

positive control for screens testing for resistance-reversing effects. However, as yet, BA is only effective in the laboratory and development of clinically used BA compounds is in its early stages, with only one product approaching clinical trials.[16]

One of the more difficult mechanisms of resistance to overcome is NDM-1 mediated resistance. The NDM-1 lactamase is a metallo-β-lactamase, meaning that it requires zinc to hydrolyze β-lactam antibiotics. This makes MBL's such as NDM-1 very problematic for chemists, which is shown by the few numbers of inhibitors present today. Few *in vivo* clinical inhibitors have been described over the years, but there are some promising *in vitro* possibilities, one of which is ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA). EDTA has well-known chelating properties, meaning that it can bind and remove metal ions, such as the zinc required for NDM-1 cleavage of β-lactams. Although this chelating effect makes EDTA useful in inhibiting NDM-1 and other MBL's, it also causes major concerns about toxicity when used clinically. Because of the large number of human metalloproteins, non-specific chelation by an agent such as EDTA could cause serious biological harm.[26] One *in vitro* use is the phenotypic detection of MBL-producing bacteria via disk assays and observation of zones of inhibition. Studies have shown that EDTA can work synergistically with imipenem, a type of carbapenem drug, to inhibit growth of MBL-producing bacteria.[27] The synergistic effect of EDTA with carbapenem drugs could prove to be very useful as a positive control in future resistance-reversing assays.

Chapter III

Methods

Preparation of Samples

Plant samples were delivered to the National Center for Natural Products Research (NCNPR) from various sources, including the Missouri Botanical Garden in St. Louis, MO, freeze-dried, ground, and stored in the sample repository. Ground material was extracted using the Accelerated Solvent Extraction System (ASE, Dionex) with 95% ethanol three times under 1500 psi at 40°C for 10 minutes. The ethanol extracts were evaporated using a Rocket evaporation system (SP Scientific) with final drying in the HT-12 evaporator (SP Scientific). Extracts were stored in dimethyl sulfoxide (DMSO) at 20mg/mL at -80°C in 96-well plates for testing. A portion of this extract (~150-200mg) was sent to St. Jude Children's Research Hospital in Memphis, Tennessee, while the rest was kept in the repository for future use.

At St. Jude, the delivered plant extracts were fractionated as described by Tu et al. Briefly, samples had polyphenols removed using a 700 mg polyamide-filled cartridge (Sigma-Aldrich, St. Louis, MO) and a 48-place positive pressure SPE manifold (SPEware Corporation, Baldwin Park, CA). Approximately 100 mg of extract were dissolved and added to the cartridge. The column was washed with five column values of methanol, and the effluent was dried using a stream of nitrogen from a Zymark TurboVap LV Concentration Workstation. After this prefractionation step, fractions were dissolved in 2

mL of DMSO. Samples were separated into 24 fractions using High-performance liquid chromatography (HPLC) and collected in preweighed 16 X 100 mm glass tubes. HPLC was performed on a Gemini 5 μm C_{18} 110A column, and a Shimadzu LC-8A binary preparative pump with a Shimadzu SCL-10A VP Controller was connected to the Gilson 215 auto sampler and Gilson 215 fraction connector. A Shimadzu SPD-M20A diode-array detector and a Shimadzu ELSD-LT II evaporative light scattering detector were used to perform detections. The fractions were sent to the NCNPR in deep 96-well plates at 2 mg/mL in DMSO. These samples are referred to as COMBI's and were later tested as such.[28]

In addition to NCNPR plant extracts and COMBIs, other samples tested in this project include microbial extracts from the National Cancer Institute (NCI), a collection of pure compounds isolated at NCNPR, and the FDA Approved Drug collection (SelleckChem).

Selection and Preparation of Bacterial Strains

Before a bioassay was designed, a preliminary literature review was conducted to determine the best possible strains to include in the study. After reviewing the available literature, it was determined that strains of *Klebsiella pneumoniae* and *Escherichia coli* were high priority research targets because of their clinical relevance in infections. It was also determined that the most important mechanisms of resistance were attributed to the β-lactamases (ESBL, KPC, and NDM-1). Bacterial strains were acquired from the American Type Culture Collection (ATCC) and include *Klebsiella pneumoniae* 700603, *Klebsiella pneumoniae* BAA-1705, *Klebsiella pneumoniae* BAA-2146, *Escherichia coli*

BAA-201, *Escherichia coli* BAA-2340, and *Escherichia coli* BAA-2452. These strains, their mechanisms of resistance, and the positive controls used in the screens can be found in **Table 3-1**. The strains were suspended in broth, spread on a Eugon agar plate, and allowed to incubate for 24 hours. The plates were then stored at 4°C until needed for assays. Fresh agar plates were prepared each week from frozen bacterial stocks. These bacteria were tested using Clinical and Laboratory Standards Institute (CLSI) methods. Briefly, bacterial strains were diluted and their optical densities (OD) were recorded using a Biotek Powerhouse XS Plate Reader. These diluted solutions were then added to agar plates in such a way that individual colonies could be counted. These recordings were done in triplicate to create a calibration curve to which all OD measurements could be compared to yield a colony-forming unit (CFU)/mL value. This procedure was done to ensure that in theory, every well in every assay contained a similar number of CFU's (5.0 X 10^5/mL), which provides consistency in testing.

Table 3-1: Tested Strains, Mechanisms of Resistance, and Positive Controls

Bacterial Strain	Mechanism of Resistance	Test Antibiotic	Positive Control
Klebsiella pneumoniae 700603	SHV-18 ESBL	Cefotaxime	Clavulanate
Klebsiella pneumoniae BAA-1705	KPC-1	Meropenem	3-Aminophenyl boronic acid
Klebsiella pneumoniae BAA-2146	NDM-1	Meropenem	EDTA
Escherichia coli BAA-201	TEM-3 ESBL	Cefotaxime	Clavulanate
Escherichia coli BAA-2340	KPC	Meropenem	3-aminophenyl boronic acid
Escherichia coli BAA-2452	NDM-1	Meropenem	EDTA

Developing and Conducting the Bioassay

To determine the ideal sample and antibiotic concentration combinations for the primary screen, preliminary checkerboard assays were performed. These tests were run in all six strains of bacteria with twofold serial dilutions of the appropriate test antibiotic and positive control compound. Different concentrations of antibiotic and positive control were used in different strains based on information obtained during a literature review. Graphs of the results of these optimizing checkerboards can be found in the Appendices.

The assay was designed to test samples against bacteria in the presence and absence of a sub-inhibitory concentration of the test antibiotic. By doing this, inherent antibacterial activity of the test sample could be separated from synergistic resistance-reversing effects of the sample in combination with the antibiotic.

Test samples from stored plates were diluted in saline at the same concentration and 4 µL of the samples and controls were transferred in duplicate to a 384-well plate by the Tecan Evo liquid automated handler to afford final test concentrations of 20-40 µg/mL for fractions and pure compounds and 80-200 µg/mL for extracts. These 384-well plates were stored in a 4°C cold room overnight in preparation for the assay. The next morning, inoculum was prepared in cation-adjusted Mueller-Hinton to afford 5 X 10^5 CFU/mL and supplemented with either the target concentration of antibiotic or an equivalent volume of DMSO. These antibiotic concentrations were chosen based on optimized results from checkerboard studies (shown in appendices) and can be found in **Table 3-2**.

Table 3-2: Antibiotic Concentrations in Bacterial Inocula

Bacterial Strain	Test Antibiotic	Antibiotic Concentration (µg/mL)
Klebsiella pneumoniae 700603	Cefotaxime	1.0
Klebsiella pneumoniae BAA-1705	Meropenem	2.5
Klebsiella pneumoniae BAA-2146	Meropenem	5.0
Escherichia coli BAA-201	Cefotaxime	5.0
Escherichia coli BAA-2340	Meropenem	1.0
Escherichia coli BAA-2452	Meropenem	1.5

Once the inocula (with antibiotic and without antibiotic) were prepared, 50 µL were added to their designated 384-well plates using a Thermo Scientific Multidrop Combi. The plates were read at 530 nm prior to and after incubation at 35°C for 24 hours. Using Microsoft Excel, the percent inhibitions of the samples (compared to blank and negative controls) were calculated. Samples showing synergizing activity were considered to have little to no inherent activity alone, but pronounced activity in the presence of the test antibiotic. These hits were further analyzed in checkerboard assays, where samples were diluted vertically (down columns), while antibiotic was diluted horizontally (across rows). Fractional inhibitory concentrations (FIC), which show the extent of compound synergy, were calculated as described by Li and Rinaldi. FIC's were calculated from the IC_{50}s (using XLFit, model 201, Alameda, CA) of the two compounds alone and in combination using the following formula: FIC = (IC_{50} of compound A in combination/IC_{50} of compound A alone) + (IC_{50} of compound B in combination/ IC_{50} of compound B alone).[29] A drug was considered synergistic if its FIC was ≤0.5.

Chapter IV

Results

Over the course of this research, over 5,000 samples were tested in this bioassay. A breakdown of the sample types tested can be found in **Table 4-1**. From these samples, 35 showed activity in the primary assay, giving a hit rate of 0.7%. **Table 4-2** shows the most promising hits from the screens, as well as the strain in which they were active and the extent of their activity.

Table 4-1: Types of Samples Tested in Primary Assays

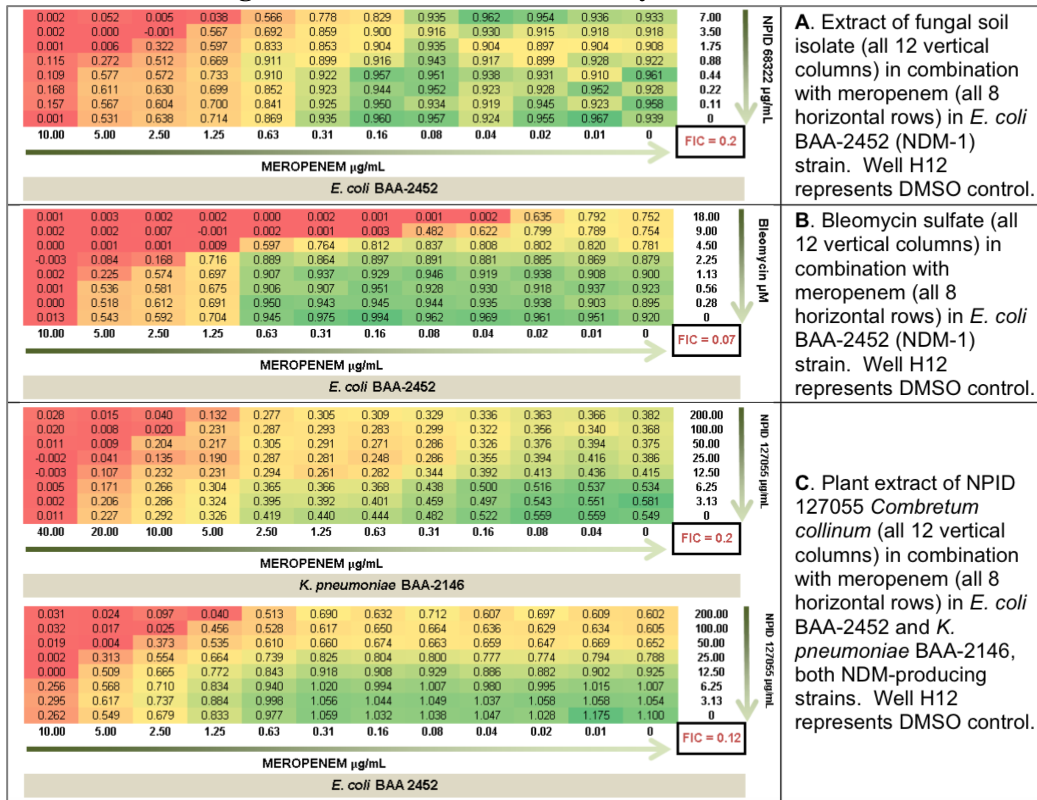
Sample Type	Number of Samples Tested in Primary Assay
St. Jude COMBI's	2451
NCNPR Plant Extracts	1048
NCNPR Pure Compounds	264
FDA Collection	88
NCI Microbial Extracts	1232

Table 4-2: Select Primary Hits with Promising Results

Sample ID	Strain	Extent of Activity
NPID 68322 (fungal soil isolate)	*Escherichia coli* BAA-2452	100% growth reduction in combination with meropenem
NPID 143149 Bleomycin sulfate	*Escherichia coli* BAA-2452	73% growth reduction in combination with meropenem
NPID 127055 *Combretum collinum*	*Escherichia coli* BAA-2452	48% growth reduction in combination with meropenem
NPID 57605 *Oenothera drummondii*	*Klebsiella pneumoniae* BAA-2146	47% growth reduction in combination with meropenem
NPID 57599 *Tamarix chinensis*	*Klebsiella pneumoniae* BAA-2146	40% growth reduction in combination with meropenem
NPID 81943 *Sanguisorba officinalis*	*Klebsiella pneumoniae* BAA-2146	35% growth reduction in combination with meropenem

Three samples displayed potent synergizing activity in checkerboard assays. As seen in **Figure 4-1**, all samples tested showed varying degrees of synergistic growth inhibition, verifying their activities in the primary assay. In this figure, each cell represents the differences in OD values of "after" readings and "before" readings. Smaller values (red) represent small increases in OD values and little bacterial growth, while larger values (green) represent greater differences in OD values and increased bacterial growth. Well H12 on each plate contained a DMSO control to represent uninhibited bacterial growth. All compounds tested in checkerboard assays had FIC's <0.5, confirming synergism. Since bleomycin sulfate was the only pure compound tested in the checkerboard assay, it is the only sample with a known structure, seen in **Figure 4-2**.

Figure 4-1: Checkerboard Assay of Select Hits



10.00	5.00	2.50	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01	0	NPID 68322 µg/mL
0.002	0.052	0.005	0.038	0.566	0.778	0.829	0.935	0.962	0.954	0.936	0.933	7.00
0.002	0.000	-0.001	0.567	0.692	0.859	0.900	0.916	0.930	0.915	0.918	0.918	3.50
0.001	0.006	0.322	0.597	0.833	0.853	0.904	0.935	0.904	0.897	0.904	0.908	1.75
0.115	0.272	0.512	0.669	0.911	0.899	0.916	0.943	0.917	0.899	0.928	0.922	0.88
0.109	0.577	0.572	0.733	0.910	0.922	0.957	0.951	0.938	0.931	0.910	0.961	0.44
0.168	0.611	0.630	0.699	0.852	0.923	0.944	0.952	0.923	0.928	0.952	0.928	0.22
0.157	0.567	0.604	0.700	0.841	0.925	0.950	0.934	0.919	0.945	0.923	0.958	0.11
0.001	0.531	0.638	0.714	0.869	0.935	0.960	0.957	0.924	0.955	0.967	0.939	0

FIC = 0.2

MEROPENEM µg/mL

E. coli BAA-2452

A. Extract of fungal soil isolate (all 12 vertical columns) in combination with meropenem (all 8 horizontal rows) in *E. coli* BAA-2452 (NDM-1) strain. Well H12 represents DMSO control.

10.00	5.00	2.50	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01	0	Bleomycin µM
0.001	0.003	0.002	0.002	0.000	0.002	0.001	0.001	0.002	0.635	0.792	0.752	18.00
0.002	0.002	0.007	-0.001	0.002	0.001	0.003	0.482	0.622	0.799	0.789	0.754	9.00
0.000	0.001	0.001	0.009	0.597	0.764	0.812	0.837	0.808	0.802	0.820	0.781	4.50
-0.003	0.084	0.168	0.716	0.889	0.864	0.897	0.891	0.881	0.885	0.869	0.879	2.25
0.002	0.225	0.574	0.697	0.907	0.937	0.929	0.946	0.919	0.938	0.908	0.900	1.13
0.001	0.536	0.581	0.675	0.906	0.907	0.951	0.928	0.930	0.918	0.937	0.923	0.56
0.000	0.518	0.612	0.691	0.950	0.943	0.945	0.944	0.935	0.938	0.903	0.895	0.28
0.013	0.543	0.592	0.704	0.945	0.975	0.994	0.962	0.969	0.961	0.951	0.920	0

FIC = 0.07

MEROPENEM µg/mL

E. coli BAA-2452

B. Bleomycin sulfate (all 12 vertical columns) in combination with meropenem (all 8 horizontal rows) in *E. coli* BAA-2452 (NDM-1) strain. Well H12 represents DMSO control.

40.00	20.00	10.00	5.00	2.50	1.25	0.63	0.31	0.16	0.08	0.04	0	NPID 127055 µg/mL
0.028	0.015	0.040	0.132	0.277	0.305	0.309	0.329	0.336	0.363	0.366	0.382	200.00
0.020	0.008	0.020	0.231	0.287	0.293	0.283	0.299	0.322	0.356	0.340	0.368	100.00
0.011	0.009	0.204	0.217	0.305	0.291	0.271	0.286	0.326	0.376	0.394	0.375	50.00
-0.002	0.041	0.135	0.190	0.287	0.281	0.248	0.286	0.355	0.394	0.416	0.386	25.00
-0.003	0.107	0.232	0.231	0.294	0.261	0.282	0.344	0.392	0.413	0.436	0.415	12.50
0.005	0.171	0.266	0.304	0.365	0.366	0.368	0.438	0.500	0.516	0.537	0.534	6.25
0.002	0.206	0.286	0.324	0.395	0.392	0.401	0.459	0.497	0.543	0.551	0.581	3.13
0.011	0.227	0.292	0.326	0.419	0.440	0.444	0.482	0.522	0.559	0.559	0.549	0

FIC = 0.2

MEROPENEM µg/mL

K. pneumoniae BAA-2146

10.00	5.00	2.50	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01	0	NPID 127055 µg/mL
0.031	0.024	0.097	0.040	0.513	0.690	0.632	0.712	0.607	0.697	0.609	0.602	200.00
0.032	0.017	0.025	0.456	0.528	0.617	0.650	0.664	0.636	0.629	0.634	0.605	100.00
0.019	0.004	0.373	0.535	0.610	0.660	0.674	0.663	0.659	0.647	0.669	0.652	50.00
0.002	0.313	0.554	0.664	0.739	0.825	0.804	0.800	0.777	0.774	0.794	0.788	25.00
0.000	0.509	0.665	0.772	0.843	0.918	0.908	0.929	0.886	0.882	0.902	0.925	12.50
0.256	0.568	0.710	0.834	0.940	1.020	0.994	1.007	0.980	0.995	1.015	1.007	6.25
0.295	0.617	0.737	0.884	0.998	1.056	1.044	1.049	1.037	1.058	1.058	1.054	3.13
0.262	0.549	0.679	0.833	0.977	1.059	1.032	1.038	1.047	1.028	1.175	1.100	0

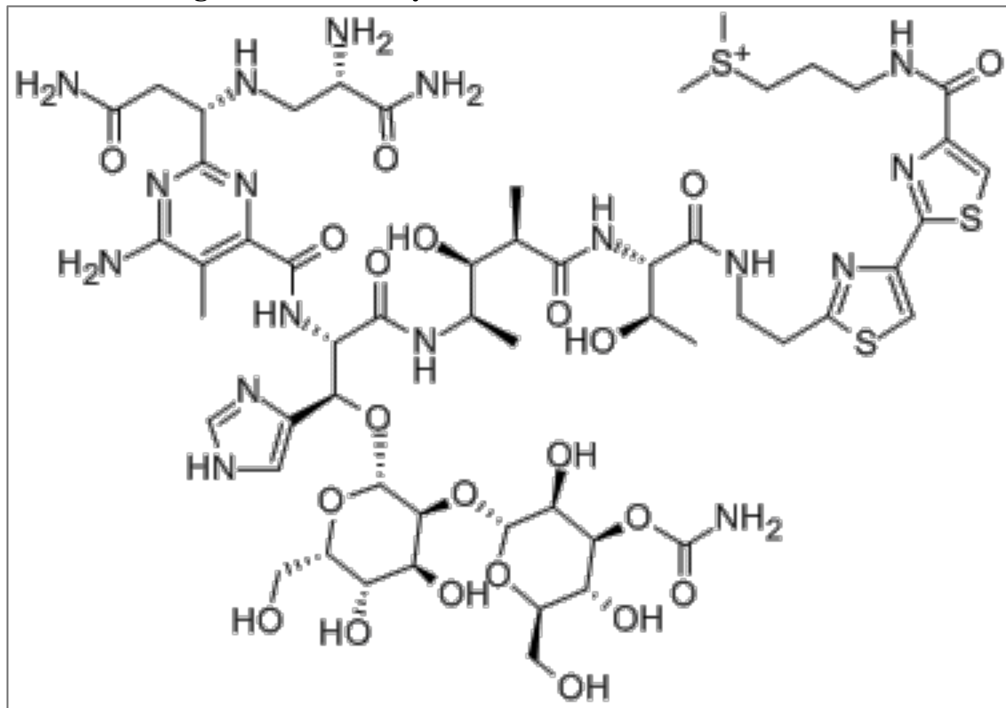
FIC = 0.12

MEROPENEM µg/mL

E. coli BAA 2452

C. Plant extract of NPID 127055 *Combretum collinum* (all 12 vertical columns) in combination with meropenem (all 8 horizontal rows) in *E. coli* BAA-2452 and *K. pneumoniae* BAA-2146, both NDM-producing strains. Well H12 represents DMSO control.

Figure 4-2: Bleomycin Sulfate Molecular Structure



Source: http://www.chemicalbook.com/ProductChemicalPropertiesCB8391148_EN.htm

Chapter V

Discussion

Data Discussion

The primary purpose of this exploratory research was to discover new compounds capable of reversing bacterial mechanisms of resistance (β-lactamases in particular) and therefore restoring the activity of clinically used antibiotics. This was accomplished through the implementation of a new primary screen and subsequent checkerboard assays of promising hits. While the hit rate for the primary assay was only 0.7%, it seems to be effective in finding potent hits. This low hit rate is understandable because of the rarity of resistance-reversing compounds, and may be favorable moving forward with a high-volume screening approach, especially because the NCNPR has over 50,000 samples that can be tested. The lower percentage of hits will allow reasonable and sustainable chemical purification and fractionation that could potentially result in therapeutically effective compounds to be tested *in vivo*.

One of the most interesting hits was that of a fungal soil isolate obtained from the National Cancer Institute (NCI). The isolate was unidentified, and contact has been made to obtain a culture for future assays and identification. Based on these results, the NCI has elevated this sample for identification studies. Once the culture is received, it will be grown, extracted, and tested to confirm activity. If activity is confirmed, isolation studies

will be performed on this sample. Its potent activity and natural source make this sample a high priority for future research.

Another interesting hit was that of bleomycin sulfate. Bleomycin is a glycopeptide antibiotic produced by *Streptomyces verticillus* that is used as an anticancer agent to cause DNA strand breaks in cancerous cells. Bleomycin resistance genes are usually found in bleomycin-producing strains, but through genetic exchange they have been transferred to other bacterial strains, including *Enterobacteriaceae.* Interestingly, analysis of NDM-1-containing strains of *E. coli* has shown a bleomycin resistance gene downstream of the NDM-1 gene, and it is likely that these genes are coexpressed under a common promoter.[30] The implications of this gene and the bleomycin hits in this bioassay are unknown, and further research should be conducted to determine the possible mechanism of action.

The final sample that was tested in a checkerboard assay was a plant extract from the fruits of *Combretum collinum*, commonly known as the bushwillow plant. This plant sample was collected in Kenya, Africa. Studies have shown some antibacterial activity in this genus, but no studies were found linking this genus or species to NDM-1.[31] This lack of information demands further research on this plant species and its role in antibiotic resistance reversal.

Limitations

There are over 50,000 samples in the NCNPR repository, but only ~5,000 could be tested due to time constraints. A majority of the time for this project was dedicated to multiple troubleshooting experiments towards the development of a robust assay to detect

resistance-reversing samples. Once conditions (bacterial strain selections, antibiotic test concentrations, positive control selections) were determined, little time was available for testing NCNPR samples. Therefore, little comprehensive follow-up studies, fractionations, and identifications could be performed on the confirmed hits. Because of the number of hits that were extracts, it would have been very beneficial to fractionate and retest to find a single compound or a small group of compounds that confer the resistance-reversing effects of the sample. This will be done in the next stages of the project. Also, once the NCI fungal isolate was identified as a hit, the NCI was notified to provide a culture, for which they have to prepare for shipping, and the sample has not yet been received for confirmation studies. This isolate could be very promising, and its purification and identification could have greatly added to this study. The lack of time also limited the number of St. Jude COMBI's that could be tested. While a large number of COMBIs were tested, there still remain 35,000. These COMBI's are very interesting samples that could result in promising hits. Moreover, the COMBIs have already been chemically analyzed via UV, ELSD and mass spectrometry, and therefore this data can serve to facilitate compound identity. Future studies will focus heavily on testing these COMBI's for activity.

Another limitation could be the utilization and interpretation of the bioassay used. This bioassay was designed and the cutoff values and concentrations were recently developed by the research group. While the initial sub-inhibitory concentrations of the test antibiotics have been initially determined, it may be necessary to alter these concentrations if the hit rate is too low (not enough antibiotic is present to achieve synergy). The antibiotic test concentrations were chosen based on the positive control's

ability to reverse resistance. Since in many cases there are complex mixtures of compounds in the form of crude extracts, and synergizing compounds may be diluted by inherently active antibacterial compounds, changes to the test antibiotic concentration may be necessary. As well, the differences in the inhibition of the sample in the presence and absence of antibiotic (ideally a delta of 100) will be continually evaluated as more primary data becomes available. Smaller deltas, especially when the sample is unique or little is known about the extract, may be considered as a hit, and subsequent confirmation assays will be used to justify if this smaller difference was valid.

A final limitation to this study could be the *in vitro* approach the bioassay used. Although very helpful in preliminary drug discovery studies, *in vitro* techniques provide no insight into how compounds will work in the body or how safe they may be when used in humans. Caution must be taken when analyzing these results, as they do not reflect accurate *in vivo* results.

Implications

As previously discussed, antibiotic resistance is a growing problem worldwide that is only getting worse. With the decrease in new antibiotic development, it is crucial that researchers discover new methods of reversing resistance to ensure positive patient outcomes and increased patient survival. By implementing high volume screens such as the one developed for this study, scientists can test large numbers of samples, both synthetic and natural, with different antibiotics and different strains of bacteria containing different mechanisms of resistance. While ESBL, KPC, and NDM-1 mechanisms of resistance are very dangerous and clinically relevant in today's society, there are also

numerous other mechanisms that could be tested with similar screens. There are also many pathogenic strains of bacteria other than *K. pneumoniae* and *E. coli* that could be tested in a similar fashion. This screening format is convenient because of its ease and efficiency, as well as the large number of samples it can test per assay. Tests such as these run in multiple laboratories worldwide will result in a large breadth of samples to be tested, identified, and purified.

Questions still remaining after the completion of this study are the identities of active compounds within samples. Compounds need to be isolated using a bioassay-guided fractionation method, a common bottleneck in natural product research, and these isolated compounds should be tested to determine their mechanisms of action. These issues will be addressed in future studies, as this is an ongoing research project.

Future research in the field may hope to expand this study to include more realistic health models and to optimize the screen. Future tests should also include *in vivo* testing to discover toxicity issues and possible therapeutic uses for active compounds. By focusing efforts on testing active compounds *in vivo*, researchers can move closer to discovering clinically significant therapeutic compounds. Future research should also focus on different strains of bacteria and different mechanisms of resistance to ensure diverse and comprehensive discoveries. The arms race against bacterial resistance mechanisms is ever changing, and only through innovative thinking and research strategies can researchers and clinicians hope to overcome the clinical challenges presented by antibiotic resistance.

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Appendices

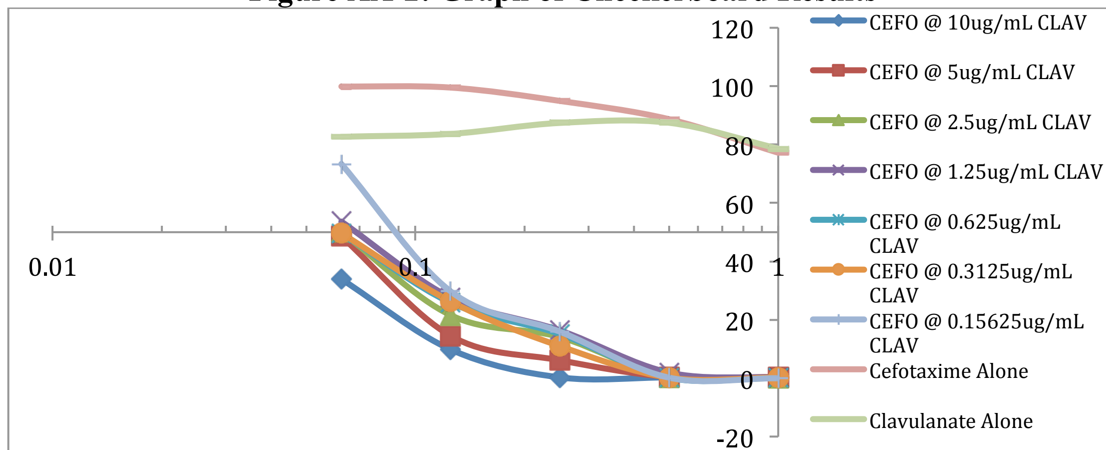
Appendix A: Checkerboard Assay of *Klebsiella pneumoniae* 700603 (ESBL) using Cefotaxime (Cefo) and Clavulanate (Clav) [FIC = 0.06]

Figure AA-1: Relative Optical Densities at 530 nm

	Cefo 1	Cefo 0.5	Cefo 0.25	Cefo 0.125	Cefo 0.0625	Clav Alone
Clav 10	0.003	0.004	0.004	0.111	0.382	0.884
Clav 5	0.005	0.005	0.070	0.164	0.547	0.984
Clav 2.5	0.001	0.003	0.159	0.243	0.564	0.983
Clav 1.25	0.002	0.022	0.185	0.311	0.603	0.941
Clav 0.625	0.003	0.002	0.171	0.291	0.558	0.930
Clav 0.3125	0.003	0.002	0.124	0.296	0.559	0.932
Clav 0.15625	0.001	0.003	0.180	0.335	0.825	1.010
Cefo Alone	0.869	0.998	1.068	1.119	1.124	1.125

Axes values represent concentration in µg/mL

Figure AA-2: Graph of Checkerboard Results



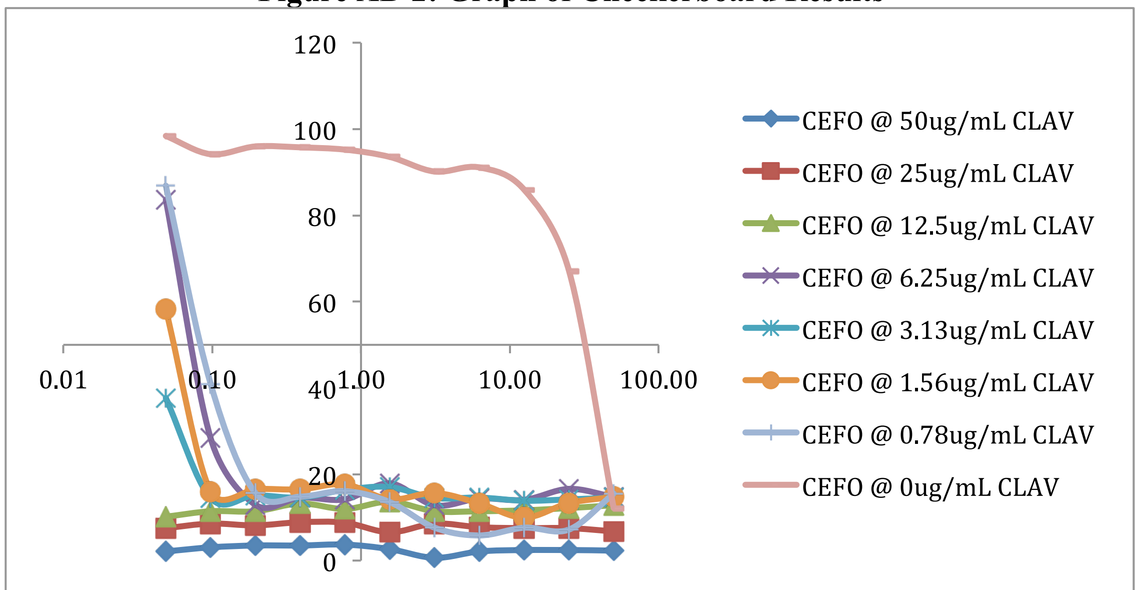
Appendix B: Checkerboard Assay of *Escherichia coli* BAA-201 (ESBL) using Cefotaxime (Cefo) and Clavulanate (Clav) [FIC = 0.05]

Figure AB-1: Relative Optical Densities at 530 nm

	Cefo 50	Cefo 25	Cefo 12.5	Cefo 6.25	Cefo 3.13	Cefo 1.56	Cefo 0.78	Cefo 0.39	Cefo 0.2	Cefo 0.1	Cefo 0.05	Clav Alone
Clav 50	0.011	0.012	0.012	0.010	0.003	0.013	0.018	0.017	0.017	0.015	0.010	0.001
Clav 25	0.032	0.036	0.036	0.037	0.041	0.031	0.042	0.042	0.039	0.041	0.036	0.093
Clav 12.5	0.061	0.058	0.055	0.054	0.054	0.065	0.057	0.063	0.054	0.054	0.048	0.410
Clav 6.25	0.070	0.079	0.067	0.068	0.061	0.085	0.067	0.069	0.061	0.134	0.395	0.406
Clav 3.13	0.069	0.067	0.066	0.069	0.069	0.081	0.078	0.069	0.072	0.069	0.178	0.435
Clav 1.56	0.070	0.063	0.047	0.063	0.074	0.067	0.084	0.078	0.079	0.076	0.275	0.481
Clav 0.78	0.074	0.034	0.036	0.028	0.036	0.064	0.076	0.070	0.074	0.194	0.411	0.459
Cefo Alone	0.058	0.317	0.406	0.431	0.426	0.442	0.450	0.453	0.454	0.445	0.465	0.473

Axes values represent concentration in µg/mL

Figure AB-2: Graph of Checkerboard Results



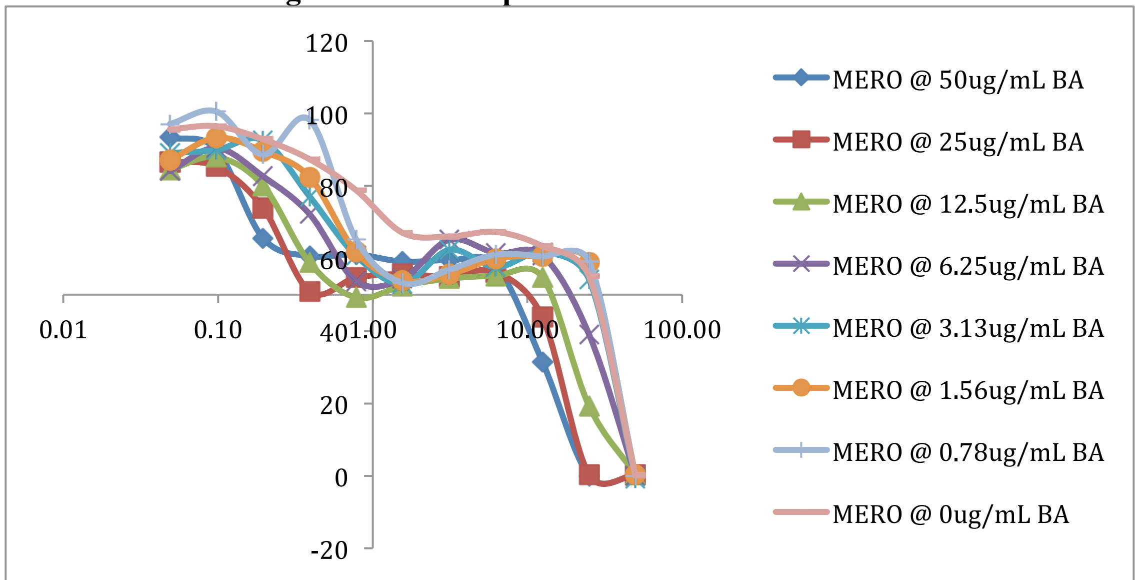
Appendix C: Checkerboard Assay of *Klebsiella pneumoniae* BAA-1705 (KPC) using Meropenem (Mero) and 3-Aminophenyl boronic acid (BA) [FIC = 0.35]

Figure AC-1: Relative Optical densities at 530 nm

	Mero 50	Mero 25	Mero 12.5	Mero 6.25	Mero 3.13	Mero 1.56	Mero 0.78	Mero 0.39	Mero 0.2	Mero 0.1	Mero 0.05	BA Alone
BA 50	0.001	0.000	0.283	0.522	0.536	0.532	0.550	0.546	0.591	0.811	0.841	0.845
BA 25	0.002	0.002	0.394	0.506	0.495	0.501	0.493	0.458	0.666	0.768	0.780	0.769
BA 12.5	0.003	0.174	0.493	0.497	0.490	0.472	0.443	0.530	0.721	0.792	0.761	0.775
BA 6.25	-0.001	0.351	0.547	0.553	0.587	0.490	0.484	0.650	0.744	0.815	0.757	0.776
BA 3.13	-0.006	0.488	0.555	0.519	0.562	0.478	0.547	0.694	0.834	0.810	0.802	0.812
BA 1.56	0.003	0.530	0.546	0.539	0.501	0.485	0.556	0.742	0.806	0.840	0.784	0.876
BA 0.78	0.000	0.533	0.545	0.550	0.512	0.479	0.587	0.885	0.798	0.905	0.874	0.827
Mero Alone	0.001	0.496	0.571	0.606	0.595	0.604	0.710	0.787	0.836	0.869	0.861	0.900

Axes values represent concentration in µg/mL

Figure AC-2: Graph of Checkerboard Results



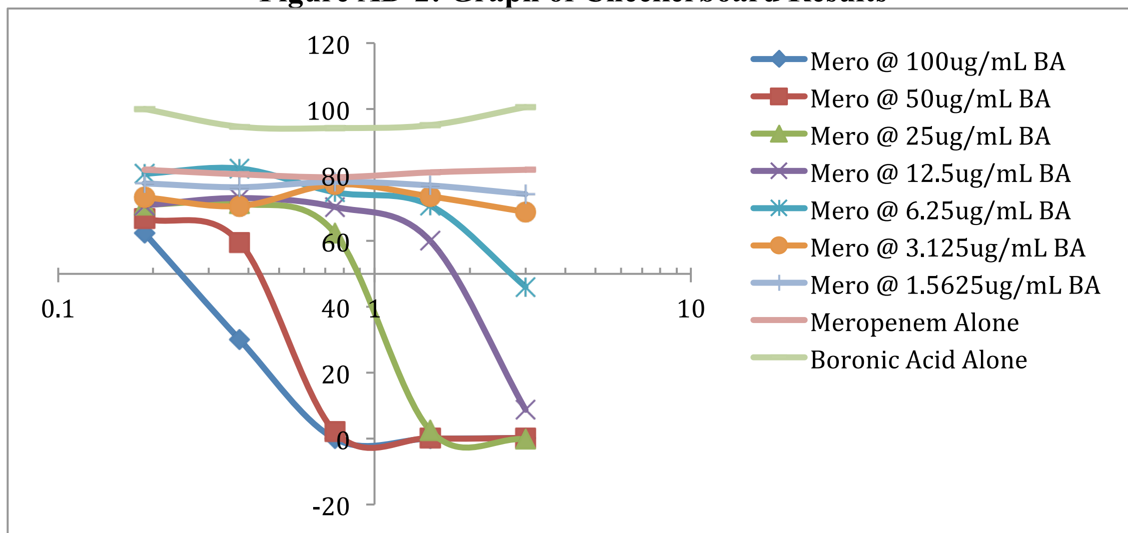
Appendix D: Checkerboard Assay of *Escherichia coli* BAA-2340 (KPC) using Meropenem (Mero) and 3-Aminophenyl boronic acid (BA) [FIC = 0.2]

Figure AD-1: Optical Densities at 530 nm

	Mero 3	Mero 1.5	Mero 0.75	Mero 0.375	Mero 0.19	BA Alone
BA 100	0.001	0.000	0.000	0.213	0.443	0.714
BA 50	0.001	0.001	0.015	0.422	0.474	0.675
BA 25	-0.001	0.017	0.442	0.506	0.503	0.668
BA 12.5	0.062	0.426	0.499	0.518	0.502	0.671
BA 6.25	0.327	0.502	0.530	0.581	0.570	0.710
BA 3.125	0.488	0.521	0.547	0.501	0.520	0.684
BA 1.5625	0.526	0.545	0.553	0.541	0.549	0.676
Mero Alone	0.579	0.573	0.563	0.569	0.579	0.709

Axes values represent concentration in μg/mL

Figure AD-2: Graph of Checkerboard Results



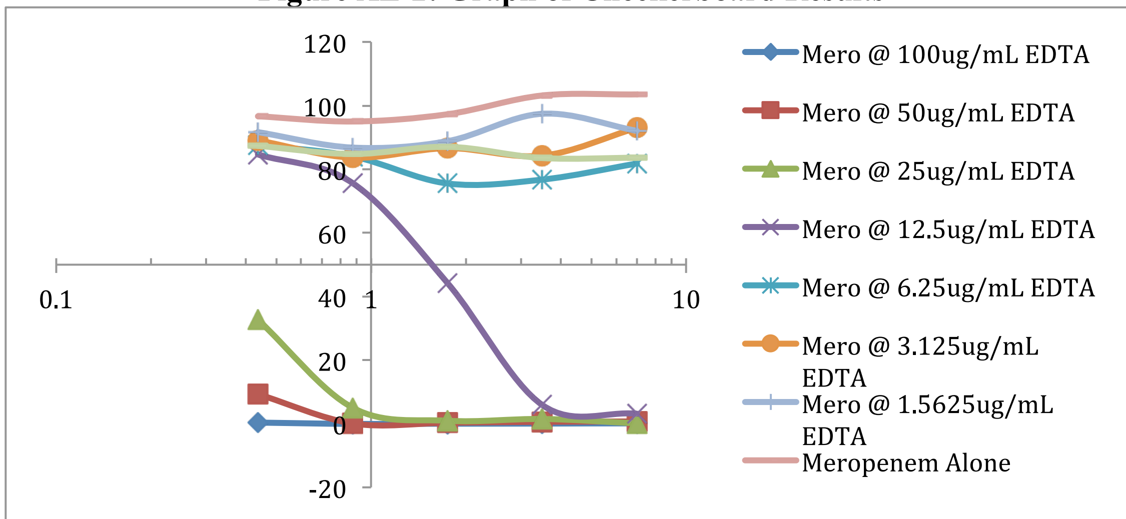
Appendix E: Checkerboard Assay of *Klebsiella pneumoniae* BAA-2146 (NDM) using Meropenem (Mero) and EDTA [FIC = 0.07]

Figure AE-1: Optical Densities at 530 nm

	Mero 7	Mero 3.5	Mero 1.75	Mero 0.875	Mero 0.438	EDTA Alone
EDTA 100	0.001	0.000	0.000	0.000	0.003	0.611
EDTA 50	0.005	0.005	0.002	0.001	0.068	0.610
EDTA 25	0.001	0.011	0.007	0.037	0.239	0.636
EDTA 12.5	0.024	0.043	0.323	0.552	0.618	0.619
EDTA 6.25	0.597	0.560	0.551	0.614	0.640	0.638
EDTA 3.125	0.679	0.615	0.632	0.611	0.650	0.642
EDTA 1.5625	0.672	0.711	0.648	0.634	0.669	0.648
Mero Alone	0.755	0.753	0.710	0.694	0.706	0.730

Axes values represent concentration in µg/mL

Figure AE-2: Graph of Checkerboard Results



Appendix F: Checkerboard Assay of *Escherichia coli* BAA-2452 (NDM) using Meropenem (Mero) and EDTA [FIC = 0.02]

Figure AF-1: Relative Optical Densities at 530 nm

	Mero 50	Mero 25	Mero 12.5	Mero 6.25	Mero 3.13	Mero 1.56	Mero 0.78	Mero 0.39	Mero 0.2	Mero 0.1	Mero 0.05	EDTA Alone
EDTA 50	0.000	-0.001	-0.002	-0.003	-0.003	-0.003	-0.004	-0.003	-0.005	-0.003	-0.002	1.445
EDTA 25	0.000	0.000	0.003	-0.002	0.002	-0.001	-0.003	-0.002	-0.003	-0.002	0.000	1.453
EDTA 12.5	0.001	-0.001	0.000	-0.002	0.002	-0.003	-0.002	-0.002	-0.001	-0.003	-0.001	1.463
EDTA 6.25	0.000	0.005	0.000	0.001	0.000	-0.001	-0.001	-0.001	0.000	-0.002	0.000	1.469
EDTA 3.13	0.001	0.001	-0.001	-0.001	0.002	-0.002	0.001	-0.002	0.002	-0.001	0.000	1.461
EDTA 1.56	0.003	0.001	0.001	0.000	0.000	0.000	0.001	0.000	0.001	0.000	1.329	1.453
EDTA 0.78	0.002	0.001	0.001	0.001	0.004	0.001	0.385	1.412	1.420	1.404	1.413	1.405
Mero Alone	0.003	0.002	0.002	1.015	1.231	1.371	1.363	1.429	1.405	1.436	1.430	1.427

Axes values represent concentration in µg/mL

Figure AF-2: Graph of Checkerboard Results