Combination Antibacterial Therapy against β-lactam Drug Resistance

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Dedication

I would like to dedicate this thesis to my dad, Dae Hyun Shin, who would be happy to see me follow in his steps as a fellow scientist.

Also, I dedicate it to my mom, Young Boon Song, for your constant, unconditional and endless love, support, guidance and encouragement. You truly are the best parents anyone could have!

Acknowledgements

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Finally, I'm forever grateful for my wife, Young-Joo Cho; for constant love and support. You have been my partner, my best friend, and my personal cheerleader. I don't know if I could have done it without you. I look forward to many happy years together.

Abstract

The β-lactam antibiotics have been the primary therapeutic treatments to combat common bacterial infections. However, the emergence of β-lactamase producing multi-drug resistant bacterial pathogens has become a major problem for public health. To address this problem, antibiotics are administered in combination with β-lactamase inhibitors to treat drug resistance pathogens.

To date, there are only four β-lactamase inhibitors approved for combination therapy by the US Food and Drug Administration (FDA). With the continuing emergence of drug-resistant β-lactamase mutants worldwide, there is an urgent need to expand the repertoire of β-lactamase inhibitors for combination therapy.

The major objective of my research was to identify a new class of β-lactamase inhibitors that can restore β-lactam antibiotics activity and use them for combination antibacterial therapy.

I successfully established the use of sulfonyl oxadiazole and 1-hydroxypyridine-2-thiones-6-carboxylic acid as two novel classes of β-lactamase inhibitors against serine and metallo β-lactamases respectively that can effectively restore β-lactam antibiotic activity. Based on a cell-based and biochemical study, I further demonstrated the promising therapeutic potential of these compounds which were subsequently disclosed in two patent applications.

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List of Abbreviations

1, 2-HPT	1-hydroxypyridine-2(1H)-thiones
A. baumannii	Acinetobacter baumannii
Amox	Amoxicillin
AmpC	Ampicillin class C
ATCC	American type culture collection
CADD	Computer sided drug design
CC_{50}	Half maximal cytotoxicity concentration
CDC	Centers for Disease Control and Prevention
Clav	Clavulanic acid
CTX	active on Cefotaxime
DMSO	Dimethyl sulfoxide
E. coli	Escherichia coli
E. faecium	Enterococcus faecium
EC_{50}	Half maximal effective concentration
EDTA	Ethylenediaminetetraacetic acid
ESBL	Extended-spectrum β-lactamases
ESKAPE	Enterococcus faecium
	Staphylococcus aureus
	Klebsiella pneumoniae
	Acinetobacter baumannii
	Pseudomonas aeruginosa
	Enterobacter species
FDA	Food and Drug Administration
HEK293	Human embryonic kidney293
HTS	High Throughput Screening
IC_{50}	Half maximal inhibition concentration
K. pneumoniae	Klebsiella pneumoniae
MBL	Metallo β-lactamase

MD	Molecular dynamics
MDR	Multi-Drug Resistance
MIC	Minimum inhibitory concentrations
MRSA	Methicillin-resistant Staphylococcus aureus
MSA	Multi-sequence alignment
MSSA	Methicillin-resistant Staphylococcus aureus
MT	Mycobacterium tuberculosis
NA	Nutrient agar
NB	Nutrient broth
NDM	New Delhi metallo
OD	Optical density
P. aeruginosa	Pseudomonas aeruginosa
PBPs	Penicillin binding proteins
PDB	Protein Data Bank
qHTS	Quantitative high throughput screening
S. aureus	Staphylococcus aureus
SAR	Structure Activity Relationship
SAR	Structure activity relationship
SHV	Sulfhydryl variable
SO	Sulfonyl Oxadiazole
ST	Sulfonyl Thiadiazoles
Sul	Sulbactam
Tazo	Tazobactam
TB	Tuberculosis
TI	Therapeutic Index
TSB	Trypticase Soy Broth
VIM	Verona integrin-encoded metallo
VRE	Vancomycin-resistant *E. faecium*
VRSA	Vancomycin-resistant Staphylococcus aureus
XDR	extensively drug resistant

CHAPTER 1

INTRODUCTION

1.1. Project Description

β-lactam antibiotics are a broad class of antibiotics, consisting of all antibiotic agents that contain a β-lactam nucleus in its molecular structure. β-lactam antibiotics work by inhibiting cell wall synthesis by the bacterial organism and are the most widely used group of antibiotics [1-5]. The non-residue producing antibiotics agents are not believed to create resistant bacteria because of their rapid killing effect. However, resistance to these antibiotics has been found where these agents are used continuously. Specifically, bacterial enzymes, called β-lactamases, have evolved that can inactivate many clinically useful β-lactam antibiotics with extremely high efficiency, and constitute the major antibiotic-resistant mechanism in the bacterial world.

The increasing emergence of β-lactamases in the bacteria family is a worrying clinical problem [6, 7]. This clinical pressure has spurred extensive efforts to discover new and potent β-lactamases inhibitors, which are commonly encountered in many clinical cases of bacterial infection [8-15].

Bacteria often develop resistance to β-lactam antibiotics by synthesizing β-lactamase, an enzyme that attacks the β-lactam ring. To overcome this resistance, β-lactam antibiotics are often given with β-lactamase inhibitors. The goal of the project is the discovery of new β-lactamase inhibitors to restore β-lactam antibiotic activities for combination antibacterial treatment. This chapter includes the background of β-lactam antibiotics and its drug resistance with public health relevance and significance for the better understanding of our project aims.

1.2. β-lactam antibiotics

β-lactam antibiotics are a first line broad class of antibiotics, consisting of antibiotic agents that contain β-lactam nucleus in their molecular structure [1]. These include penicillin derivatives (penams), cephalosporins (cephems), monobactams, and carbapenems [16]. A β-lactam ring is present in certain antibiotics and β-lactam antibiotics work by inhibiting cell wall synthesis by the bacterial organism and are the most widely used group of antibiotics (Fig 1.1). This β-lactam ring is a lactam with a heteroatomic ring structure, consisting of three carbon atoms and one nitrogen atom.

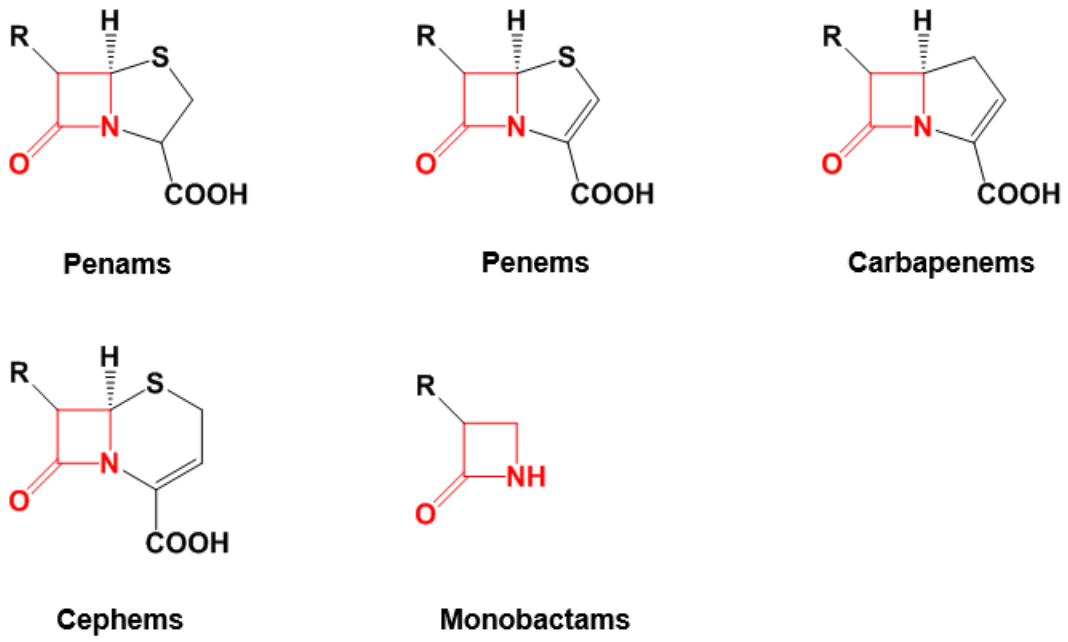


Figure 1.1. The classification of β-lactam antibiotics by its core structures. All class of β-lactam antibiotics includes the β-lactam ring in their chemical structure.

The penicillin-binding proteins (PBPs) and β-lactamases are key components of cell wall biochemistry [17]. PBPs are enzymes involved in the final stages of bacterial cell wall synthesis [18] (Fig 1.2). These enzymes have a wide range of molecular weights from 27 to120 kDa. PBPs are the targets in bacteria for β-lactam antibiotics [19]. β-lactam antibiotics inhibit bacterial PBP's which facilitates the synthesis of the peptidoglycan layer of the bacterial cell wall during growth. The peptidoglycan layer is important for cell wall structural integrity, especially in Gram-positive organisms. These drugs serve as potent antibacterial agents because they inhibit PBPs in the growing bacteria, preventing the crucial cross-linking of the cell wall. The final transpeptidation step in the synthesis of the peptidoglycan is facilitated by transpeptidases known as PBPs. In clinical use, β-lactam antibiotics are indicated for the prophylaxis and treatment of bacterial infections caused by susceptible organisms [20]. At first, β-lactam antibiotics were mainly active only against gram-positive bacteria, but the recent development of broad-spectrum β-lactam antibiotics that are active against various gram-negative organisms has increased their usefulness [21, 22].

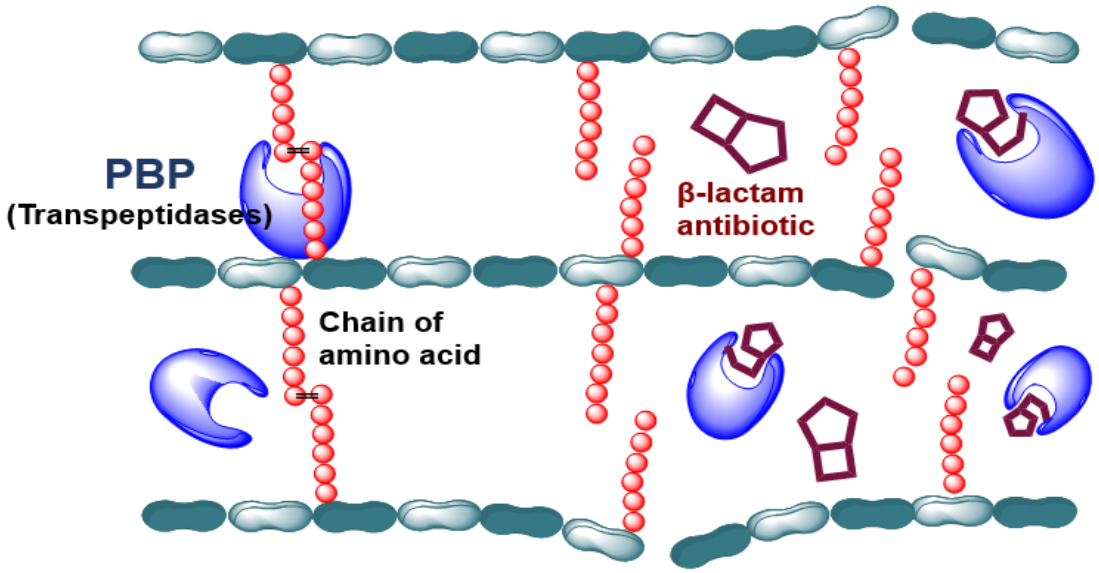


Figure 1.2. The simple scheme of β-lactam antibiotics mechanism. The improper formation of peptidoglycan layer induce cell lysis and death by changing of its osmotic pressure.

1.3. Drug Resistance and Current β-lactamase Inhibitors

The common occurrence of β-lactam antibiotic drug resistance is a global health concern to our antibiotic arsenals against bacterial infections [23-25]. Bacterial resistance to antibiotics is an important and growing concern in the treatment of infectious diseases. Bacteria often develop resistance to β-lactam antibiotics by synthesizing β-lactamase, an enzyme that attacks the β-lactam ring [1]. β-lactam drug resistance is mediated by the expression of a serine or metallo-β-lactamase, which enzymatically hydrolyses the cyclic β-lactam amide bond, rendering the β-lactam antibiotic inactive against their targeted

PBP's. Expression of β-lactamase from chromosomally encoded or acquired plasmid gene leads to the development of β-lactam drug resistance [26]. β-lactamase enzymatically cleaves open the β-lactam ring which renders β-lactam antibiotics inactive against their intended PBP target (Fig 1.3). Extended spectrum β-lactamases (ESBL) that hydrolyze extended-spectrum antibiotic cephalosporin include TEM, SHV, CTX and OXA types with hundreds of mutants within the TEM type alone [27-34]. These enzymes destroy the antibiotics before they exert their desired effect. Although β-lactam compounds still make up more than 50% of all prescribed antibiotics, the development of resistant strains represents a serious threat to the continued usefulness of these agents [35]. In particular, the emergence of mutant forms of the β-lactamase TEM, the single most prevalent β-lactamase found in gram-negative bacteria, provides a striking example of the evolution of antibiotic resistance. Expression of broad-spectrum β-lactamase or multiple classes of β-lactamases is one of the primary mechanism for the development of multi-drug resistance against multiple classes of β-lactam antibiotics [36-39]. There are several classes of β-lactam drug-resistant pathogens including *Klebsiella spp*., *Escherichia coli*, *Acinetobacter baumannii, Pseudomonas aeruginosa*, and Methicillin-resistant *Staphylococcus aureus* (MRSA) that have been identified as microorganisms with urgent or serious threat level in the United States [23]. Up to 90% of ampicillin resistance in drug-resistant, *E. coli* is due to the expression of TEM-1 β-lactamase. *Mycobacteria tuberculosis*, the causative agent of tuberculosis (TB), consists of chromosomally encoded β-lactamase (BlaC) that render them intrinsically resistant to β-lactam antibiotic drugs [23, 24].

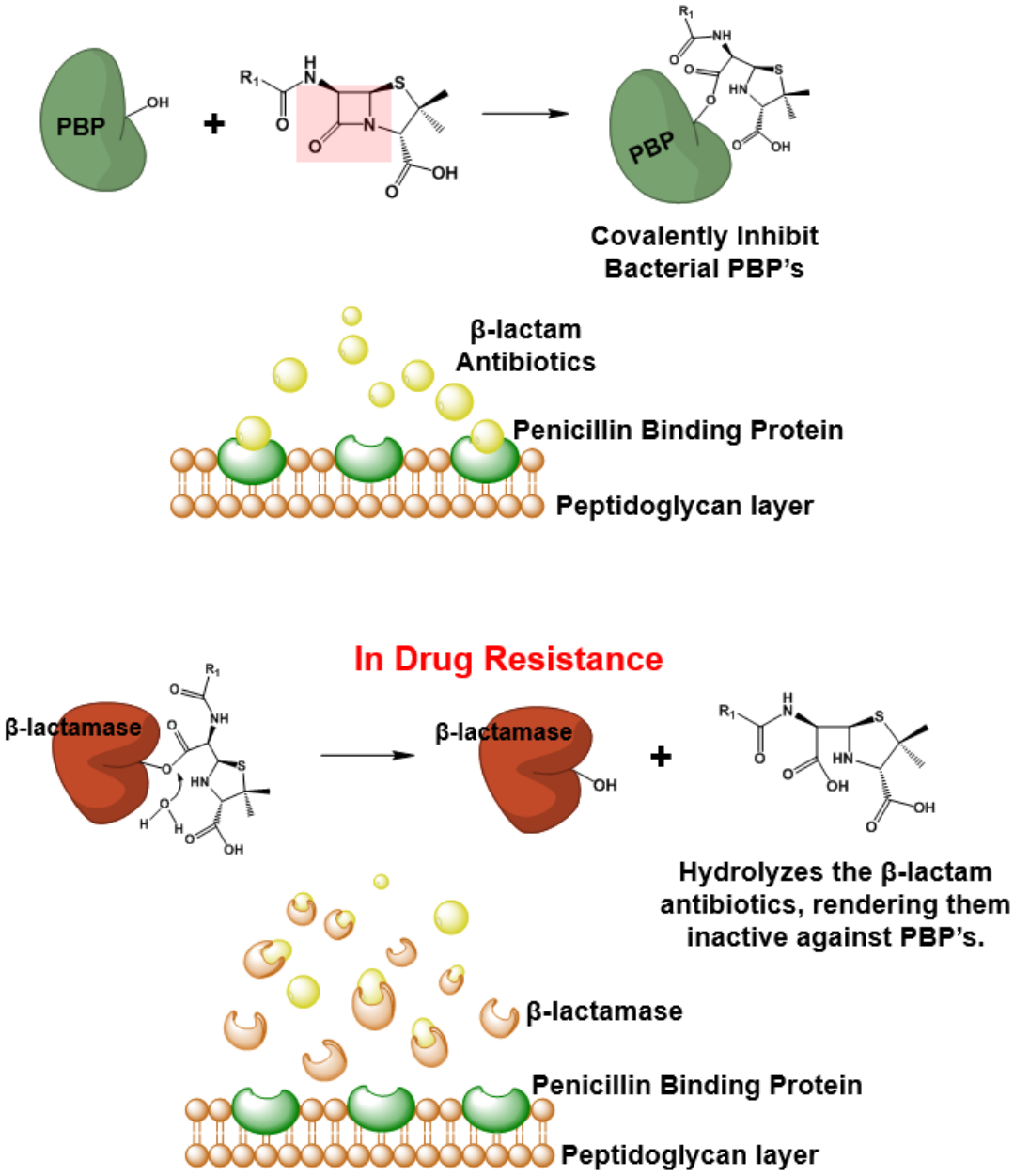


Figure 1.3. The simple mechanism of drug resistance action. Expression of plasmid-acquired β-lactamase induces β-lactam drug resistance

Inappropriate and unnecessary uses of antibiotics for maintaining human health and the health of food-producing livestock are the leading cause for the selection, retention, and spread of β-lactam antibiotic resistance bacterial pathogens [40-44]. Their continued emergence in community and health care settings has become a serious health threat as nosocomial infections and secondary infections against immune deficient, weakened or suppressed patients. To overcome this resistance, β-lactam antibiotics are often given with β-lactamase inhibitors such as clavulanic acid. Common treatments against β-lactam drug resistance involve combination therapy, which involves the use of a β-lactamase inhibitor acting as a re-sensitizing agent to re-establish β-lactam antibiotic activity [45].

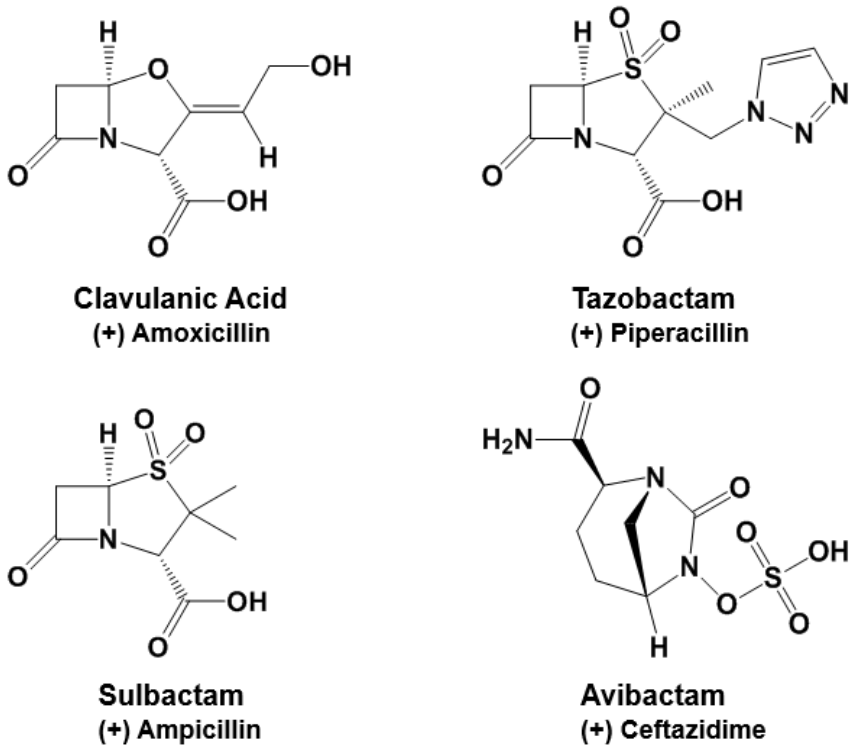


Figure 1.4. FDA approved combination therapy against β-lactam drug resistance

To date, there are only three β-lactam β-lactamase inhibitors (Clavulanic acid, Sulbactam, and Tazobactam) and one recently discovered non-β-lactam β-lactamase inhibitor (Avibactam) approved for the combination therapy against β-lactam drug-resistant infections (Fig 1.4, 1.5) [46]. A novel class of β-lactamase inhibitors that resuscitates and redeploys existing β-lactam antibiotic arsenals provides the maximum opportunity for developing novel combination therapies for combating β-lactam drug resistance.

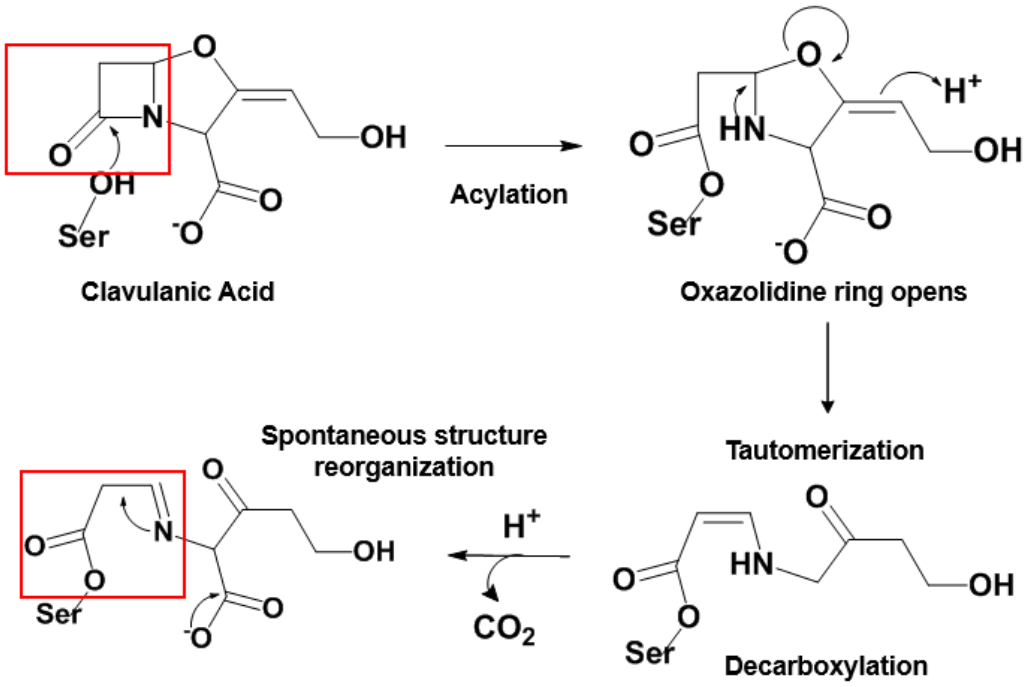


Figure 1.5. Mechanism of β-lactamase inhibitor as an Irreversible Suicide inhibitor. Clavulanic acid is a suicide inhibitor, covalently bonding to a serine residue in the active site of the β-Lactamase.

1.4. Enzyme Characteristic and Classification

The classification scheme for β-lactamases includes four molecular classes, A, B, C, and D, based on amino acid sequence information. Molecular classes A, C, and D comprise the serine β-lactamase, and class B the zinc-dependent metallo-enzymes (Fig 1.6, 1.7) [47-49]. Classes A and D include penicillinases and cephalosporinases, most of which are inhibited by clavulanic acid or tazobactam. Class C includes cephalosporinases that are not, or poorly, inhibited by clavulanic acid or tazobactam. Class B comprises the metallo-enzymes, which are inhibited by EDTA and have carbapenems as distinctive substrates.

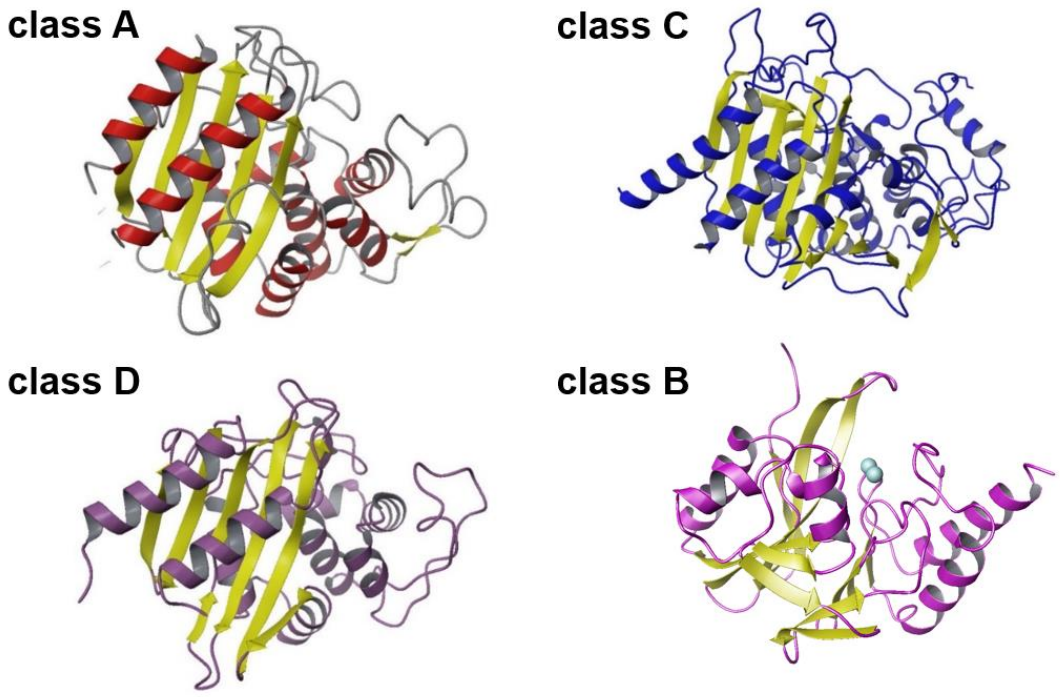


Figure 1.6. β-lactamase protein structure comparison. Class A TEM-1 (PBD ID: 1JTG), class C AmpC (PDB ID: 2PU4), class D OXA-23 (PDB ID: 4JF6), class B VIM-2 (PDB ID: 4BZ3)

Classes A, C and D β-lactamases and PBPs are structurally related with similar β-sheet subdomains (usually five strands associated with two or three helices); and share certain mechanistic features (Fig 1.6) [49, 50]. These enzymes probably have a common origin. They contain an active site serine residue to which the antibiotic is covalently bound, via an ester bond, as a catalytic intermediate. The ester bond is subsequently hydrolyzed, and the inactivated antibiotic is released from the enzyme. The β-lactamase is then ready for a new catalytic cycle. This mechanism is analogous to the binding of β-lactam antibiotics to PBPs. The main difference is that the covalent bond formed between the antibiotic and the active serine in the PBP is not, or very slowly, hydrolyzed. Therefore, the enzyme activity of the PBP is blocked.

The overall three-dimensional structure of the K15 PBP enzyme consists of a single polypeptide chain organized into two domains. One domain contains mainly α-helices, and the second one is of α/β-type. The K15 PBP enzyme bears the signature fold topology of the penicilloyl-serine transferase superfamily, but overall, it exhibits more similarity to the class A, C, and D serine β-lactamases [51]. Using the standard secondary structure of the serine β-lactamases, the first domain contains a central helix that is surrounded by four helices, a four-stranded antiparallel β-sheet and α-helix [50, 52-54]. The α/β -domain consists of a five-stranded antiparallel β-sheet that is covered on one face by the short α-helix and the long carboxyl-terminal α-helix and on the other face by α-helix and one turn of α-helix [48].

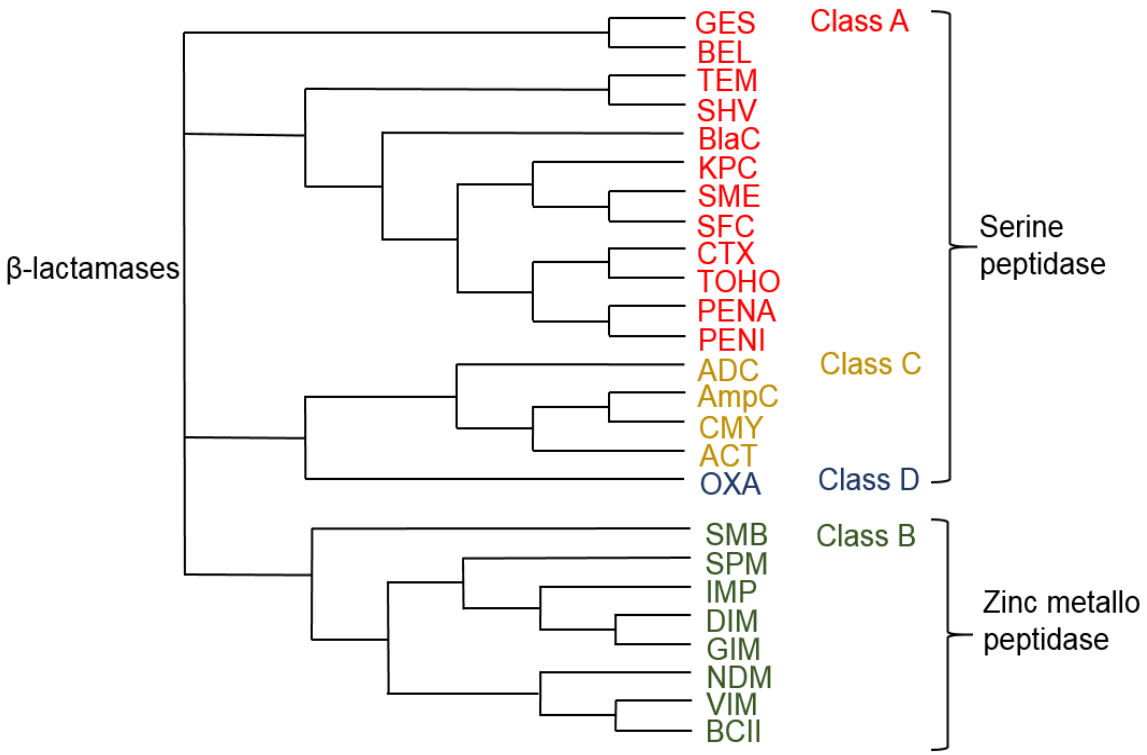


Figure 1.7. Classification of β-lactamase protein

1.5. Public Health Relevance and Significance of Multi-drug Resistance Pathogens

According to the Center for Disease Control and Prevention (CDC) report, each year in the United States, at least 2 million people acquire serious infections with bacteria that are resistant to one or more of the antibiotics designed to treat those infections[23, 24]. At least 23,000 people die each year as a direct result of these antibiotic-resistant infections. Also, worldwide, this continued emergence of β-lactamase mediated drug resistance is a major public health concern. Extended spectrum β-lactam (ESBL) resistance pathogens

such as methicillin-resistant *Staphylococcus aureus* (MRSA) can become life threatening as opportunistic co-infection to *Mycobacterium tuberculosis* (MT) infected patients and have also been linked to nosocomial infections, particularly in children and post-surgical patients in hospitals [23, 24]. The successful introduction of combination therapy such as clavulanate, a β-lactamase inhibitor, with β-lactam antibiotic amoxicillin has prompted us to investigate the similar combination therapeutic approaches of reinstating other β-lactam antibiotic activity. There are currently over fifty β-lactam antibiotics approved by the FDA, but only four β-lactamase inhibitors approved for combination therapy [55, 56]. A recent study has also demonstrated that such combination therapy approach using standard tuberculosis (TB) regimen with clavulanate, one of the three FDA-approved β-lactamase inhibitors, is also biologically active against extensively drug-resistant (XDR) *Mycobacterium tuberculosis* by inhibiting the chromosomally encoded serine β-lactamase C (BlaC) [23, 35, 57-59].

Because of the lack of incentive in antibacterial research in the pharmaceutical industry, there is an urgent need to develop alternative therapeutic treatments against drug resistance infections. Successful developments of a novel β-lactamase inhibitor will provide a safeguard against β-lactamase mediated drug resistance by reactivating existing β-lactam antibiotics and can ultimately have a profound impact on the treatment of common infections and the co-management of serious infections such as multi-drug resistant TB. It has been estimated that up to 90% of ampicillin resistance in *E. coli* is due to the production of TEM-1 β-lactamase [60]. Plasmids consisting of the β-lactamase gene are widely shared among pathogenic bacteria, and this is a major concern in the spread of

β-lactam drug resistance. With the continuing emergence of drug-resistant β-lactamase mutants worldwide, there is an urgent need to expand the therapeutic repertoire for combination therapy to maintain existing β-lactam antibiotics as our primary arsenal against common bacterial infections.

Estimating the relationship activity of the chemical structure of the compound is important in drug discovery. Activity is quantified by various explanatory variables, and used to further identify active compounds [61-63]. High Throughput Screening (HTS) was used in drug discovery to screen large numbers of compounds against a biological target [64]. Data on activity against the target are collected for a representative sample of compounds selected from a large library. The major goal of the project is to discover novel classes of potent β-lactamase inhibitors to rescue existing β-lactam antibiotic activities. The ability to preserve the efficacy of existing β-lactam antibiotics arsenal provides maximum opportunity for combination antimicrobial therapy development.We investigated a class of sulfonyl oxadiazoles and thiadiazoles compounds that were first reported in 2008 *J. Med. Chem.* by the Schoichet group as potent serine β-lactamase inhibitors with low micromolar activity. Also, we investigated 1-hydroxypyridine-2-thiones-6-carboxylic acid (1, 2-HPT) as potent metallo β-lactamase inhibitors with estimated therapeutic potential of the compound.

CHAPTER 2

IDENTIFICATION OF SERIEN

β–LACTAMASE INHIBITOR

2.1 Summary

Class A, C and D serine β-lactamase represent the major source of bacterial resistance [65-68]. These enzymes constitute a threat to human health since they can hydrolyze the β-lactamase antibiotics according to the acylation-decylation pathway. Therefore, they represent an important target for drug design. The expression of chromosomal and, more commonly observed, plasmid encoded β-lactamase protein is the primary cause for the development of β-lactam drug resistance [46, 69]. Its ability to hydrolyze the β-lactam amide bond renders many existing β-lactam antibiotics inactive against their targeted proteins. Common treatments against β-lactam drug resistance involve combination therapy, which is use of a β-lactamase inhibitor as a re-sensitizing agent to restore β-lactam antibiotic activity [70-77]. Recent high throughput screenings have identified a series of potent non-β-lactam β-lactamase inhibitors which contains a sulfonyl oxadiazole core with low micromolar inhibition activities against class C serine β-lactamase [78].

To identify a new class of serine β-lactamase inhibitor, we demonstrate the therapeutic potential of the most potent sulfonyl oxadiazole compound by cell and enzyme based study. This compound has broad spectrum activity and low cytotoxicity and can be used in combination antibacterial therapy to restore β-lactam antibiotic activity. It is a first-time study to establish the therapeutic potential of the sulfonyl oxadiazole compound as leading drug candidate.

2.2 Multi-drug Resistant Bacteria Pathogens

Bacterial infections caused by susceptible organisms to be a problem [79, 80]. In our research, we are focusing on resistance among gram-positive and gram-negative pathogens that cause infections in the hospital and the community. The "ESKAPE" pathogens (*Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* species) are a major threat of hospital nosocomial infections [81]. The data from the Centers for Disease Control and Prevention (CDC) show rapid increase of infection due to methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *E. faecium* (VRE), and *A. baumannii* pathogens [23, 24]. More people now die of multi-drug resistant bacteria infection in US hospitals than of HIV/AIDS and tuberculosis combined. Furthermore, several highly resistant gram-negative pathogens (*Acinetobacter baumannii*, multidrug-resistant (MDR) *Staphylococcus aureus*, and *Escherichia coli*) are emerging as significant pathogens in both the United States and other parts of the world.

2.2.1 *Acinetobacter baumannii*

Acinetobacter baumannii (*A. baumannii*) is typically a short, almost round, rod-shaped (coccobacillus) Gram-negative bacterium (Fig 2.1) [82]. It has also been isolated from environmental soil and water samples. *A. baumannii* is a pleomorphic aerobic gram-negative bacillus commonly isolated from the hospital environment and hospitalized

patients. The pathogen *A. baumannii* has been stealthily gaining ground as an agent of serious nosocomial and community-acquired infection. Historically, *Acinetobacter* spp. have been associated with opportunistic infections that were rare and of modest severity. The last two decades have seen an increase in both the incidence and seriousness of *A. baumannii* infection, with the main targets being patients in intensive-care units [83, 84]. Although this organism appears to have a predilection for the most vulnerable patients, community-acquired *A. baumannii* infection is an increasing cause for concern [85-88].

A. baumannii is also referred to as 'Iraqibacter' due to its seemingly sudden emergence in military treatment facilities during the Iraq War [89, 90]. The dry, sandy conditions associated with these desert campaigns provide an ideal environment for the physiologically robust *A. baumannii*, making it the main source of infection among injured soldiers. It has continued to be an issue for veterans and soldiers who served in Iraq and Afghanistan. Multidrug-resistant *A. baumannii* has spread to civilian hospitals in part due to the transport of infected soldiers through multiple medical facilities. Multidrug-resistant *Acinetobacter* deep wound infections, osteomyelitis, respiratory infections, and bacteremia have been reported among military personnel with traumatic injuries during the conflicts in Iraq and Afghanistan. Theories that previously colonized soldiers are auto-inoculated or that *Acinetobacter* species from local soil or water are introduced during traumatic injury have not been supported by cultures of specimens obtained from healthy soldiers, soil samples, water samples, or samples from fresh wounds [91-94].

A. baumannii has been identified as an ESKAPE pathogen, a group of pathogens with a high rate of antibiotic resistance that is responsible for the majority of nosocomial infections [95-97]. Multidrug-resistant *A. baumannii* is a rapidly emerging pathogen in the health care setting, where it causes infections that include bacteremia, pneumonia, meningitis, urinary tract infection, and wound infection [98-104]. The organism's ability to survive under a wide range of environmental conditions and to remain for extended periods of time on surfaces makes it a frequent cause of outbreaks of infection and a health care–associated pathogen. The increase in multidrug-resistant *A. baumannii* infections has paralleled the alarming development of resistance it has demonstrated. The persistence of *A. baumannii* in healthcare units and its inherent resistance to antibiotics result in it being a formidable emerging pathogen.

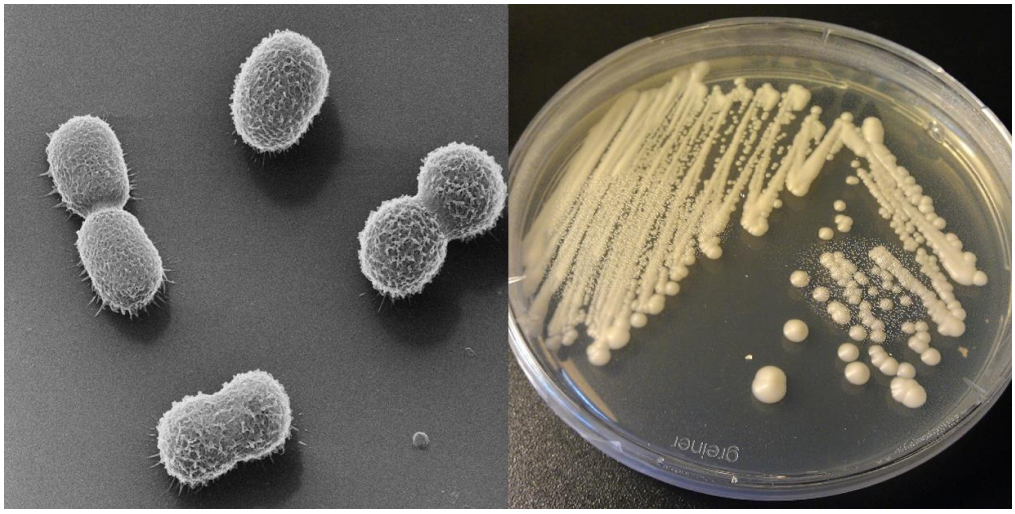


Figure 2.1. Acinetobacter baumannii bacterium shape and growth

2.2.2 Methicillin Resistant *Staphylococcus Aureus* (MRSA)

Staphylococcus aureus is a small round shaped gram-positive bacterium (Fig 2.2). It is frequently found in the human body such as the nose, respiratory tract, and on the skin [18]. *Staphylococcus aureus* is not always pathogenic, but it is a common cause of skin infections such as abscesses, respiratory infections such as sinusitis, and food poisoning [105]. MRSA is a strain of *Staphylococcus aureus* that has developed resistance to β-lactam antibiotics, which include the Penicillin (Methicillin, Ticarcillin, Piperacillin, Nafcillin, Temocillin, Benzylpenicillin, Oxacillin, etc.) and the Cephalosporins (Cephalothin, Cefixime, cefotaxime, etc.) (Fig 1.1) [106, 107]. Strains unable to resist these antibiotics are classified as Methicillin-sensitive *Staphylococcus aureus* (MSSA) [108]. The evolution of such resistance does not cause the organism to be more intrinsically virulent than strains of *Staphylococcus aureus* that have no antibiotic resistance, but resistance does make MRSA infection more difficult to treat with standard types of antibiotics and thus more dangerous.

Currently, MRSA is a worldwide major pathogen [109-112]. MRSA resistant to various classes of antibiotics is reported. Methicillin resistance in *Staphylococcus aureus* involves an altered target site due to an acquired penicillin-binding proteins (PBPs) with decreased affinity to β-lactams [113]. In the community, most MRSA infections are skin infections. In medical facilities, MRSA causes life-threatening bloodstream infections, pneumonia, and surgical site infections. MRSA is especially troublesome in hospitals, prisons, and nursing homes, where patients with open wounds, invasive devices, and weakened immune systems are at greater risk of nosocomial infection than the general

public. MRSA began as a hospital-acquired infection, but has developed limited endemic status and is now sometimes community-acquired and livestock-acquired.

The vancomycin has been treated as the first-line drug for treatment of MRSA. Unfortunately, there has been an increase in the use of vancomycin for other infections [114-117]. When vancomycin was introduced in 1800's, no resistance to this antibiotic as resistance was reported. Since 1997 from Japan, there has been an increase in the number of cases with vancomycin-resistant *S. aureus* (VRSA). Also, infection with vancomycin-resistant *S. aureus* (VRSA) strains have been described in the United States later, the clinical and epidemiological significance of this resistance phenotype is unclear at present [118-127].

MRSA and VRSA have been identified as ESKAPE pathogens with a high rate of antibiotic resistance [23]. With the continuing emergence of β-lactam drug-resistant *S. aureus* pathogens worldwide, there is an urgent need to discover a new class of β-lactamase inhibitors for further combination antibacterial therapy.

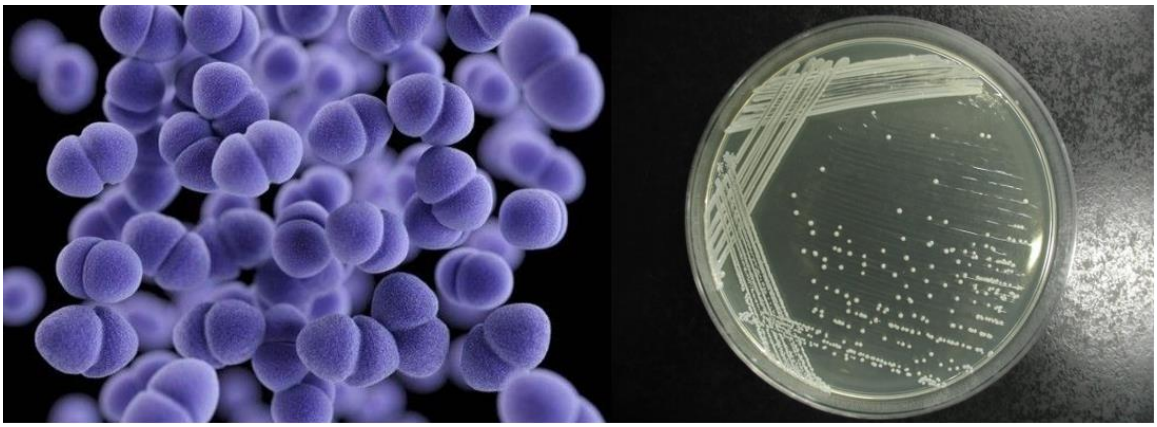


Figure 2.2. Gram stain of Staphylococcus aureus cells and laboratory agar plate growth scheme.

2.3. First Report of Sulfonyl Oxadiazole as β-lactamase Inhibitor

The new class of active molecules was identified by Schoichet groups in 2008. Quantitative HTS and structure-based docking of over 70,000 compounds were performed against the antibiotic resistance target β-lactamase [128]. The new class of non-β-lactam compound has a sulfonyl oxadiazole core in their chemical structure (Fig 2.3). All of the existing β-lactamase inhibitors are in conjunction with a β-lactam antibiotic. It means β-lactamase inhibitors have β-lactam rings in their molecular structure from the discovery of penicillin in 1928 [129-133]. The goal of the project is to determine the structural activity relationships (SAR) of a novel class of non-β-lactam β-lactamase inhibitors in both bacterial cell-based and biochemical assays to examine the molecular basis for bioactivity in combination therapy.

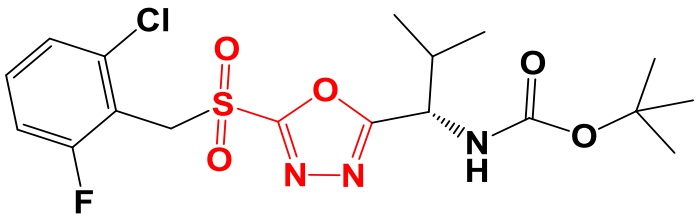


Figure 2.3. Sulfonyl oxadiazoles compound chemical structure by Schoichet group

In a cell-based assay, reported compounds (2008, *J. Med. Chem.*) (Fig 2.3) were tested with amoxicillin to six of ESKAPE pathogens for 18hours. The combinational

treatment of reported sulfonyl oxadiazole compound with amoxicillin shows most potent cell growth inhibition against *A. baumannii* strain up to 96%. However, none of the strains shows any growth inhibition activities. Based on first screening data, *A. baumannii* strains were selected for an initial target of sulfonyl oxadiazole screening study.

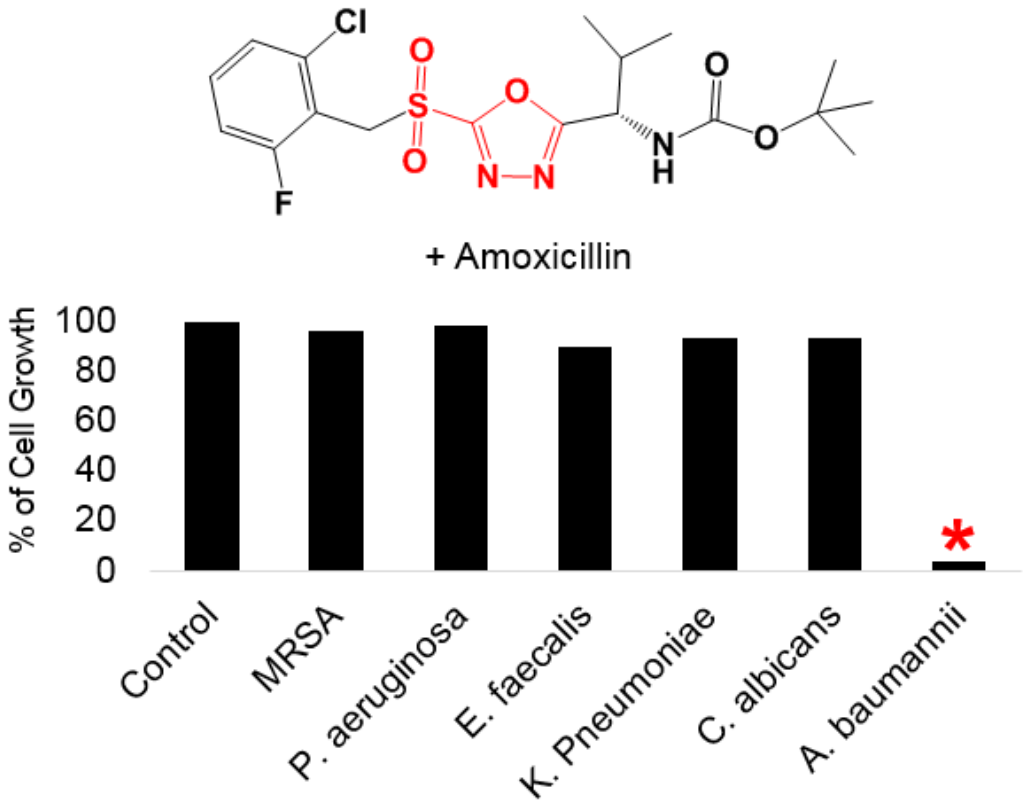


Figure 2.4 Combinational treatment of reported sulfonyl oxadiazole compound with amoxicillin against six of ESKAPE pathogens.

2.4 Bioactivities of Sulfonyl Oxadiazoles/Thiadiazoles against *A. baumannii*

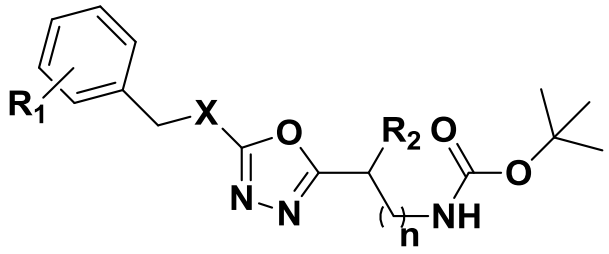
A total 99 series of sulfonyl oxadiazole and 12 of sulfonyl thiadiazole compounds were obtained from the commercial compound chemical library Interbioscreen, Inc., www.ibscreen.com.. The compounds were identified and screened to determine the minimum inhibitory concentration of the β-lactamase inhibitors of the invention in conjunction with the β-lactam antibiotic amoxicillin.

In a cell-based assay, our commercial compounds were tested with amoxicillin, clavulanic acid and Tetracycline (-) to *A. baumannii* (ATCC19606) for 24 hours. For identification of the optimal compounds for inhibiting the growth of amoxicillin-selected *A. baumannii* when treated in combination with amoxicillin, a screening study was carried out using a series of sulfonyl oxadiazoles and related compounds. Table, 2.1, 2.2, 2.3 indicate the results of the screening study for a compound of the invention of formula. Clavulanic acid was used as a positive control as a known effective β-lactamase inhibitor. The column indicating with – Amox indicates the % of the viability of the amoxicillin-selected *A. baumannii* strain in the presence of the sulfonyl oxadiazoles only. The studied inhibitors are a series of sulfonyl-oxadiazole/ thiadiazole, which is shown in Table 2.1. The Schoichet group compound was used for reference, and the series of compounds which have a similar scaffold are studied by cell based assay. All of them have an aromatic ring and short aliphatic substituents. Our SAR analysis showed that the replacement of the sulfone atom by sulfur (14-21) led to the decrease of the activity toward *A.baumannii*

pathogens. Moreover, the compounds with aliphatic substituents on the benzene ring with 2-chloro (3) and CH_3 (10-12) series displayed high activity with decreasing percentage of cell viability with Amoxicillin. These synergistic properties against *A.baumannii* pathogens are enough to compare with existing drug Augmentin™ (clavulanic acid with amoxicillin).

A low percentage means that our compound treatment inhibits the cell viability of *A. baumannii,* and high percentage means the compound treatment doesn't show any large effect against *A. baumannii* cell growth. This new class of compounds has shown promising therapeutic properties in our preliminary studies; including a synergistic property with amoxicillin against clinical drug resistant *A.baumannii* pathogens. Since *A. baumannii* has multi-drug resistance and expresses every class of β-lactamases, we can predict that our compound shows synergistic inhibition against most classes of β-lactamases. As can be seen, the sulfonyl oxadiazoles alone do not exhibit much, if any, growth inhibition against the bacterial strain tested. When the bacterial culture of the amoxicillin-selected *A. baumannii* was challenged with the sulfonyl oxadiazoles and amoxicillin (+ Amox), the indicated results obtained showed that the sulfonyl oxadiazoles used for the practice of a method of the invention are significantly more effective as inhibitors of the serine-type β-lactamase of *A. baumannii* than is clavulanic acid. These combinational treatment results were highly significant to inhibit the bacterial cell proliferation, as the combination of amoxicillin and clavulanic acid is a well-known pharmaceutical composition known as Augmentin®, used for the treatment of β-lactam resistant bacterial infections.

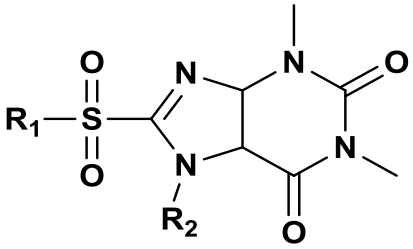
Table 2.1. Structure-Activity Relationship among the sulfonyl-Oxadiazole series with cell-based assay against Acinetobacter baumannii pathogen.



#	R_1	R_2	X	n	% of viability (+ Amox)	% of viability (only)
Tetracycline(-)					-	0
Amoxicillin					-	91
Clavulanic acid					9	24
85413	H	H	SO_2	4	9	94
85371	H	CH_3	SO_2	0	15	99
85380	H	isopropyl	SO_2	0	12	90
85388	H	isobutyl	SO_2	0	11	75
85363	H	*sec*-butyl	SO_2	0	25	85
85410	H	benzyl	SO_2	0	17	99
85395	2-CH_3	H	SO_2	4	11	91
85324	2-CH_3	CH_3	SO_2	0	17	91
85370	2-CH_3	isopropyl	SO_2	0	20	100
85368	2-CH_3	isobutyl	SO_2	0	22	104
85399	2-CH_3	*sec*-butyl	SO_2	0	41	97
85337	2-CH_3	benzyl	SO_2	0	57	77
85406	3-CH_3	isopropyl	SO_2	0	18	92
85387	3-CH_3	isobutyl	SO_2	0	9	84
85353	3-CH_3	*sec*-butyl	SO_2	0	17	67
85338	3-CH_3	benzyl	SO_2	0	27	90
85409	4-CH_3	H	SO_2	4	11	92
85361	4-CH_3	CH_3	SO_2	0	6	52
85376	4-CH_3	isobutyl	SO_2	0	71	101
85373	4-CH_3	*sec*-butyl	SO_2	0	56	101
85382	4-CH_3	benzyl	SO_2	0	30	99
85396	2,5-CH_3	H	SO_2	4	13	79
85366	2,5-CH_3	CH_3	SO_2	0	31	88
85402	2,5-CH_3	isopropyl	SO_2	0	16	83
85405	2,5-CH_3	isobutyl	SO_2	0	31	78
85360	2,5-CH_3	*sec*-butyl	SO_2	0	11	73
85362	2-fluoro	CH_3	SO_2	0	7	51

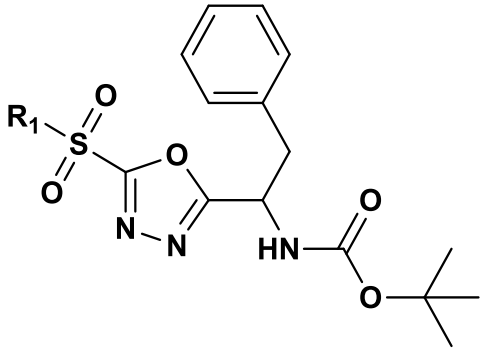
85369	2-fluoro	isobutyl	SO_2	0	28	96
85329	2-fluoro	*sec*-butyl	SO_2	0	14	87
85339	2-fluoro	benzyl	SO_2	0	22	84
85401	3-fluoro	H	SO_2	4	10	81
85411	3-fluoro	CH_3	SO_2	0	8	94
85344	3-fluoro	isopropyl	SO_2	0	18	75
85340	3-fluoro	isobutyl	SO_2	0	17	93
85330	3-fluoro	*sec*-butyl	SO_2	0	40	94
85352	3-fluoro	benzyl	SO_2	0	20	96
85355	4-fluoro	H	SO_2	4	65	97
85325	4-fluoro	CH_3	SO_2	0	11	92
85386	4-fluoro	isobutyl	SO_2	0	25	90
85385	2-chloro	H	SO_2	4	6	91
85327	2-chloro	CH_3	SO_2	0	13	90
85374	2-chloro	isopropyl	SO_2	0	43	99
85346	2-chloro	isopropyl	SO_2	0	15	60
85375	2-chloro	*sec*-butyl	SO_2	0	58	104
85355	2-chloro	benzyl	SO_2	0	34	100
85389	3-chloro	H	SO_2	4	5	77
85403	3-chloro	CH_3	SO_2	0	7	81
85390	3-chloro	isopropyl	SO_2	0	7	78
85400	3-chloro	isobutyl	SO_2	0	19	96
85404	3-chloro	*sec*-butyl	SO_2	0	23	83
85391	3-chloro	benzyl	SO_2	0	6	84
85326	4-chloro	CH_3	SO_2	0	15	88
85345	4-chloro	isopropyl	SO_2	0	16	68
85364	4-chloro	isobutyl	SO_2	0	24	57
85365	4-chloro	*sec*-butyl	SO_2	0	30	100
85383	4-chloro	benzyl	SO_2	0	12	100
85358	2,4-chloro	H	SO_2	4	10	69
85377	2,4-chloro	CH_3	SO_2	0	9	100
85350	2,4-chloro	isopropyl	SO_2	0	12	88
85381	2,4-chloro	isobutyl	SO_2	0	13	83
85354	2,4-chloro	benzyl	SO_2	0	22	89
85349	2,6-chloro	CH_3	SO_2	0	8	91
85347	2,6-chloro	isopropyl	SO_2	0	30	62
85342	2,6-chloro	isobutyl	SO_2	0	18	89
85367	2,6-chloro	*sec*-butyl	SO_2	0	59	96
85384	3,4-chloro	CH_3	SO_2	0	8	92
85407	3,4-chloro	isopropyl	SO_2	0	31	98
85356	3,4-chloro	*sec*-butyl	SO_2	0	15	56
85357	3,4-chloro	benzyl	SO_2	0	22	106
85336	4,5-chloro	H	SO_2	4	63	93
85328	2-fluoro-6-chloro	CH_3	SO_2	0	14	90
85412	2-fluoro-6-chloro	isopropyl	SO_2	0	13	104
85341	2-fluoro-6-chloro	isobutyl	SO_2	0	14	84
85348	2-fluoro-6-chloro	*sec*-butyl	SO_2	0	26	96

Table 2.2. Structure-Activity Relationship among additional compounds with cell-based assay against *Acinetobacter baumannii* pathogen.



#	R_1	R_2	% of viability (+ Amox)	% of viability (only)
Tetracycline(-)			-	0
Amoxicillin			-	91
Clavulanic acid			9	24
85314	CH_3	CH_3	13	77
85315	benzyl	CH_3	17	85
85414	CH_3	2-fluoro-benzyl	9	82

Table 2.3. Structure-Activity Relationship among additional compounds with cell-based assay against *Acinetobacter baumannii* pathogen.



#	R_1	% of viability (+ Amox)	% of viability (only)
Tetracycline(-)		-	0
Amoxicillin		-	91
Clavulanic acid		9	24
85372	CH_2CH_3	35	96
85398	CH_3	11	96

From the previous screening data, Figure 2.5 shows the chemical structures of four of the most effective sulfonyl oxadiazoles against *A. baumannii*. YYS-1 and YYS-2 showed most potent inhibition (< 5% survive) against *A. baumannii* within amoxicillin (Table 2.2) while YYS-3 and YYS-4 exhibit a balanced broad spectrum activity against *A. baumannii* (~80% inhibition), MRSA(~20% inhibition), *K. Pneumoniae*(~40% inhibition) and *P. aeruginosa* (~40% inhibition) (Figure 2.6).

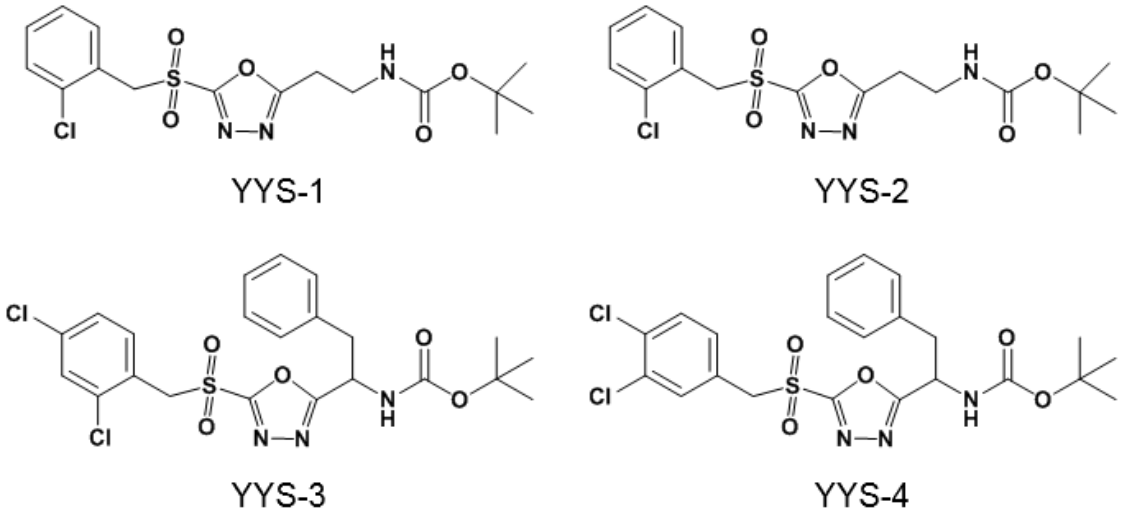


Figure 2.5. Four of most potent sulfonyl oxadiazoles compound structure scheme.

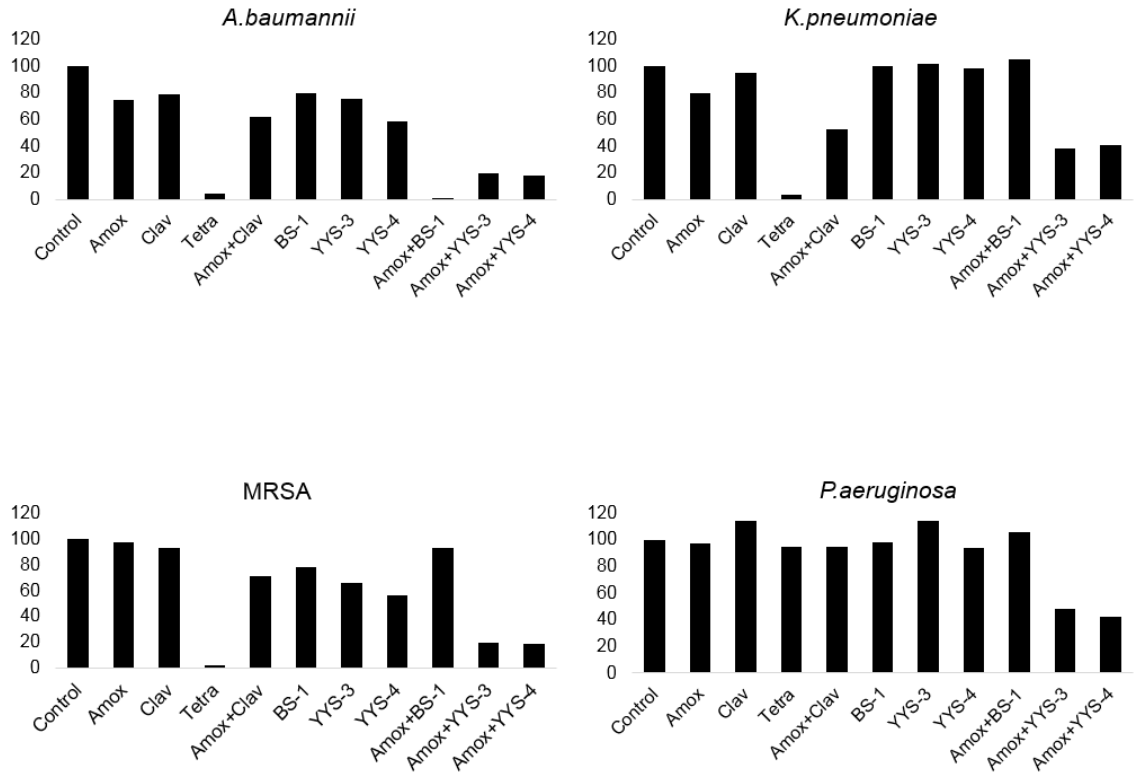


Figure 2.6. Combinational treatment of sulfonyl oxadiazole compound against various bacteria strains.

Figure 2.7 shows the results of a series of combinations of compounds YYS-1 and YYS-2 with various β-lactam antibiotics versus amoxicillin selected *A. baumannii.* In the relative effective antibacterial activity of a series of combinations of sulfonyl oxadiazole β-lactamase-inhibitory compounds and β-lactam antibiotics versus amoxicillin selected *A. baumannii* data, combinational treatment with amoxicillin shows dramatic cell growth inhibition up to 95% compare with other class of β-lactam antibiotics.

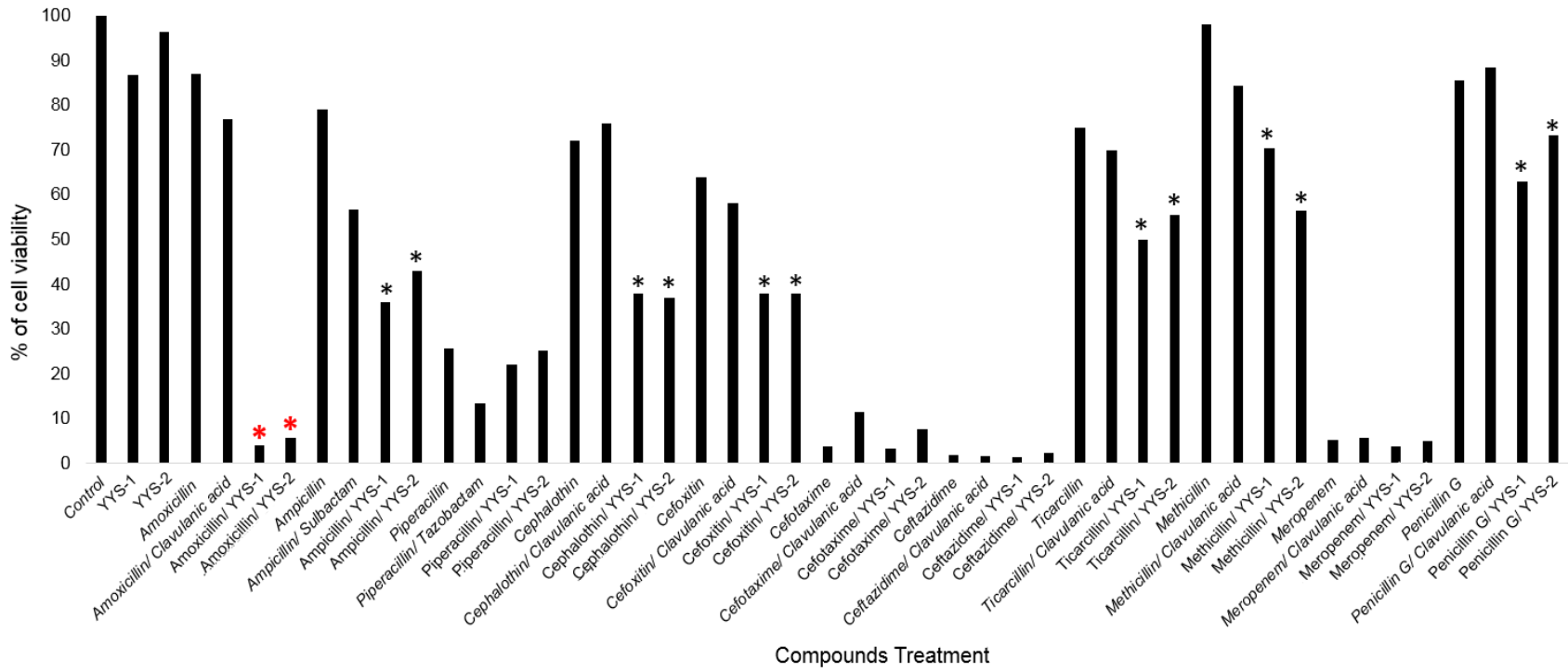


Figure 2.7. "Shotgun" evaluation of selected SO compounds with representative β-lactam antibiotics against MDR A. baumannii. A synergistic effect was observed for compounds represented with *.

Figure 2.8 provides a graphical representation of the dose-response curve for compounds YYS-85333 and YYS-85351 versus amoxicillin selected *A. baumannii* supernatant containing amoxicillin inducible expressed β-lactamase. Table 2.4 provides a summary of the inhibitory constants of these two compounds in comparison with the known β-lactamase inhibitor clavulanic acid.

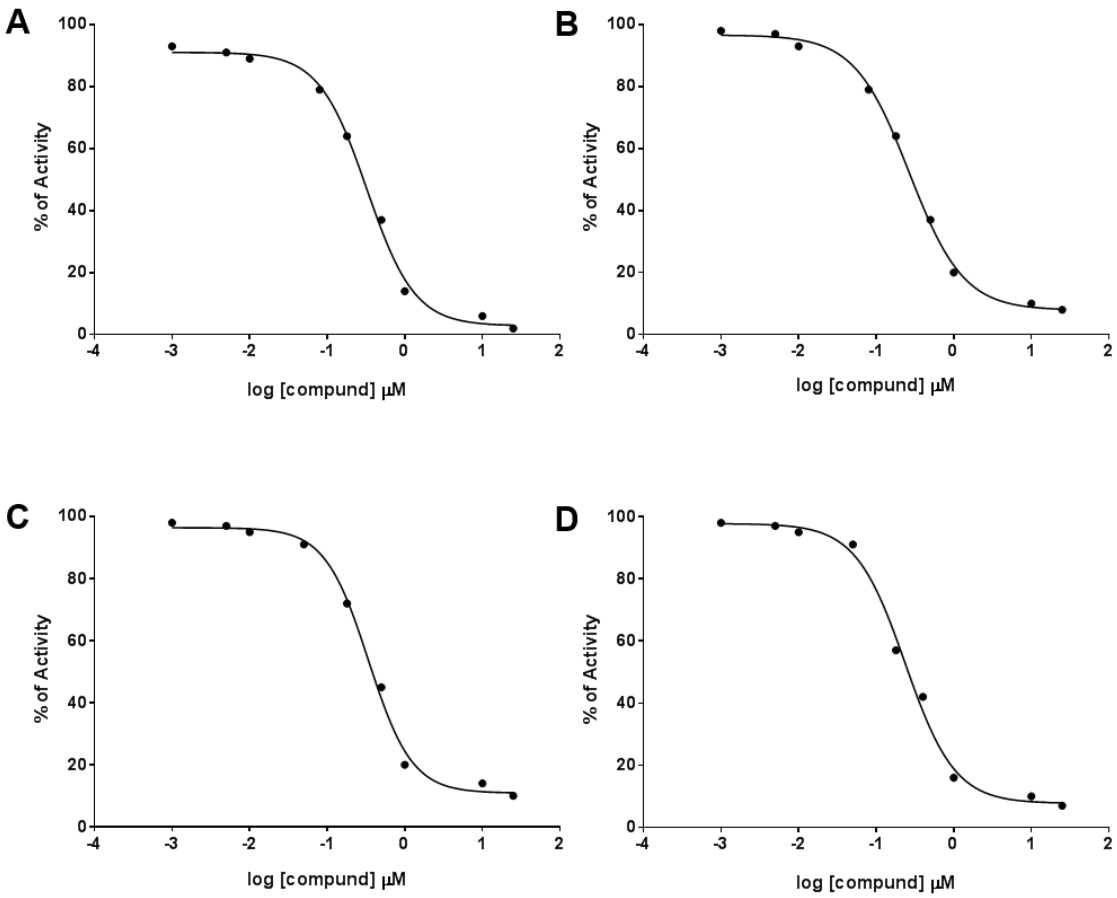


Figure 2.8. Effect of SO compounds on A. baumannii viability with amoxicillin in 18hrs treatment.

2.5 Sulfonyl Oxadiazole Compounds Cytotoxicity Assay

Cytotoxicity test assays are widely used by the pharmaceutical industry to screen for cytotoxicity in compound libraries [134-136]. Cytotoxicity can also be monitored using the 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) or MTS assay. To investigate compound cytotoxicity, the well-characterized human embryonic kidney (HEK293) cell line was chosen as a test system, given the widespread use of these cells to evaluate the cytotoxic effects of chemicals[137]. From the results obtained from MTT assay methods, by the comparison of the clavulanic acid and IC_{50} values and linearity of the activity, our compounds showed excellent cytotoxicity (28.6 µM <, 41.8 µM <) against the HEK293 human cell line (Fig 2.9). Clavulanic acid is an immunosuppressant drug, and it is commonly used for cytotoxicity study. The sulfonyl oxadiazole β-lactamase-inhibitory compounds were shown to exhibit a low degree of cytotoxicity, being of comparable or lower cytotoxicity than β-lactam antibiotics such as amoxicillin, sulbactam, and tazobactam, and also compared to the well-known β-lactamase inhibitor clavulanic acid (Table 2.4).

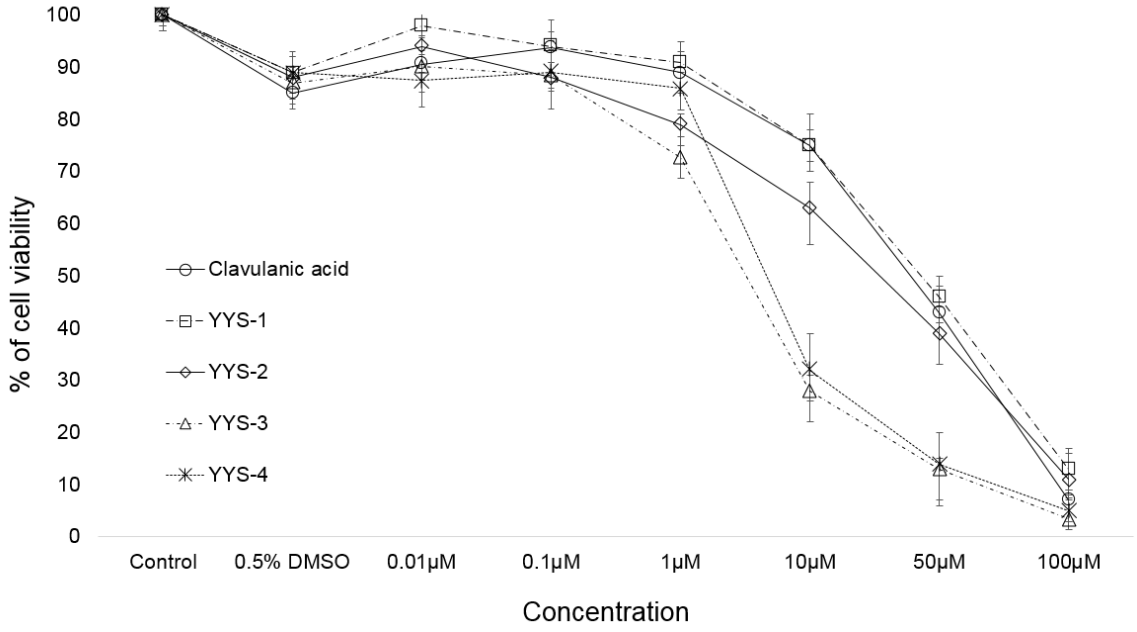


Figure 2.9. Effect of SO compounds on HEK293 viability. Cell viability was assessed by the MTT assays after exposure to different concentrations of SO compounds for 72hr.

Table 2.4. Therapeutic Index (TI) of selected SO compounds. TI is defined as the ratio of CC_{50} / EC_{50}. EC_{50} and CC_{50} are concentrations resulting in 50% cell viability against MDR *A. baumannii* and HEK293 cell, respectively.

Compounds	EC_{50} (μM)	CC_{50} (μM)	TI
Clavulanic acid	124 ± 16.8	22.4 ± 1.65	0.18
YYS-1	2.34 ± 0.28	41.8 ± 3.65	17.8
YYS-2	0.839 ± 0.13	28.6 ± 2.38	34.1
YYS-3	7.13 ± 1.4	3.8 ± 1.07	0.53
YYS-4	4.32 ± 0.81	5.37 ± 0.98	1.24

2.6 Bioactivities of Sulfonyl Oxadiazoles/Thiadiazoles against MRSA

In a cell-based assay, our total 111 series of sulfonyl oxadiazole and sulfonyl thiadiazoles commercial compounds were tested with amoxicillin, clavulanic acid and Tetracycline (-) to MRSA pathogen (ATCC 45300) for 24 hours. For identification of the optimal compounds for inhibiting the growth of amoxicillin-selected *Staphylococcus aureus* when treated in combination with amoxicillin, a screening study was carried out using a series of sulfonyl oxadiazoles and sulfonyl thiadiazoles compounds. Figure 2.10 indicates the results of the screening study for a compound of the invention of formula.

The chemical features of studied sulfonyl-oxadiazole/thiadiazoles inhibitors are represented at left four columns of Table 2.1. The amoxicillin, clavulanic acid and Augmentin (clavulanic acid with amoxicillin) were used for control. Our SAR analysis showed that 44 (> 40%) of sulfonyl-oxadiazole and 2 (24%) of sulfonyl- thiadiazoles compound shows more than 90% of MRSA cell growth inhibition with the treatment of amoxicillin. These synergistic properties against *Staphylococcus aureus* pathogens are enough to compare with existing drug Augmentin.

A low percentage of cell viability means that our sulfonyl oxadiazoles and thiadiazoles compounds inhibit MRSA cell growth, and a high percentage of cell viability means the bacterium is more difficult to kill by our combinational therapy. This new class of non-β-lactam compounds has shown promising therapeutic properties in our preliminary

studies; including a synergistic property with amoxicillin against clinical drug resistant MRSA pathogens. Since MRSA has multi-drug resistance and expresses the most class of β-lactamases, we can predict that our compound shows synergistic inhibition against various classes of MRSA expressed β-lactamases. Our other data shows that the sulfonyl oxadiazoles alone do not exhibit much if any, growth inhibition against the bacterial strain tested. When the bacterial culture of the amoxicillin-selected MRSA was challenged with the sulfonyl oxadiazoles and amoxicillin (+ Amox), the indicated results obtained showed that the sulfonyl oxadiazoles used for the practice of a method of the invention are significantly more effective as inhibitors of the serine-type β-lactamase of MRSA than clavulanic acid. These results were highly significant, as the combination of amoxicillin and clavulanic acid is a well-known pharmaceutical composition known as Augmentin®, used for the treatment of β-lactam resistant bacterial infections.

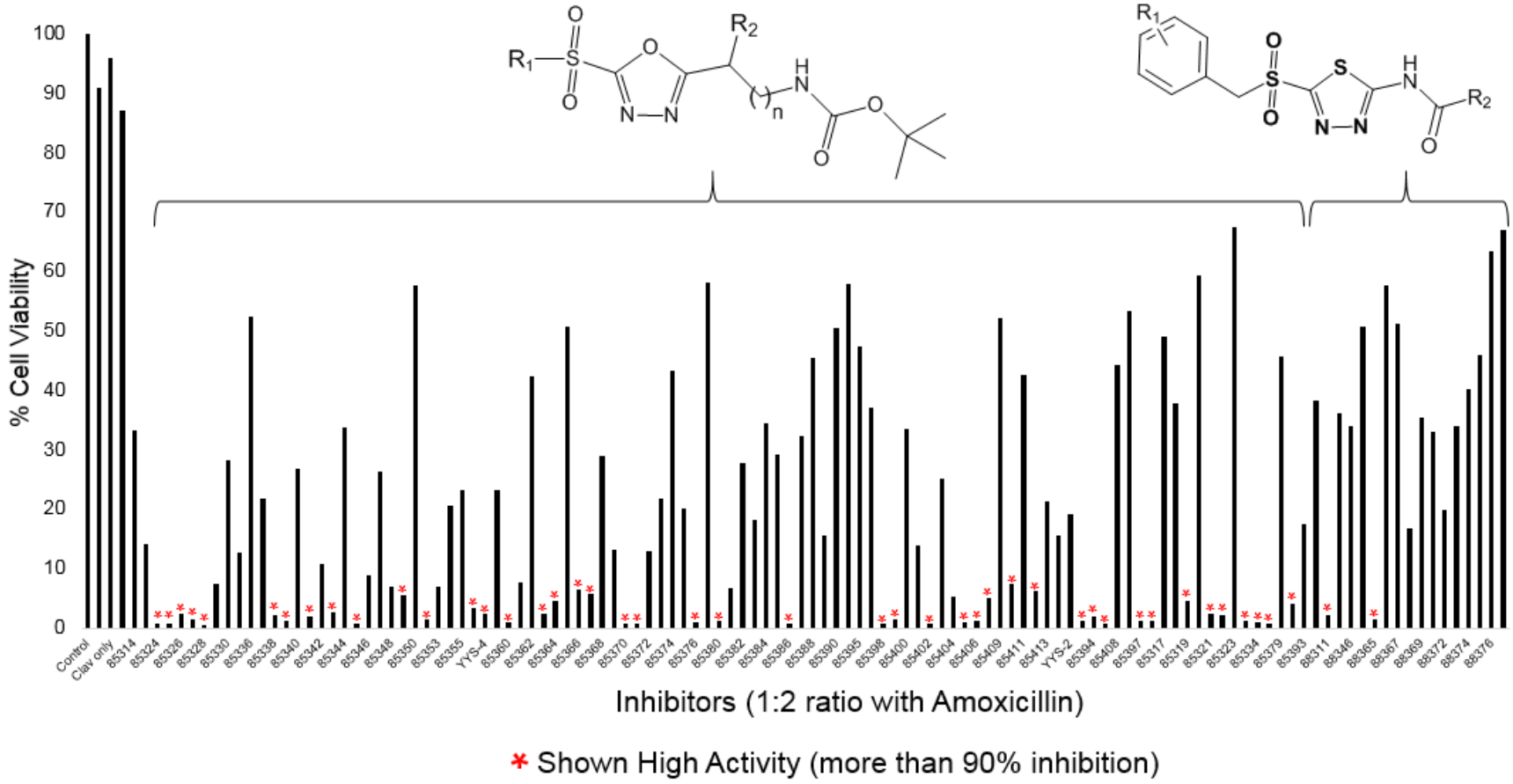


Figure 2.10. Activity Screening Assay against MRSA with Amoxicillin

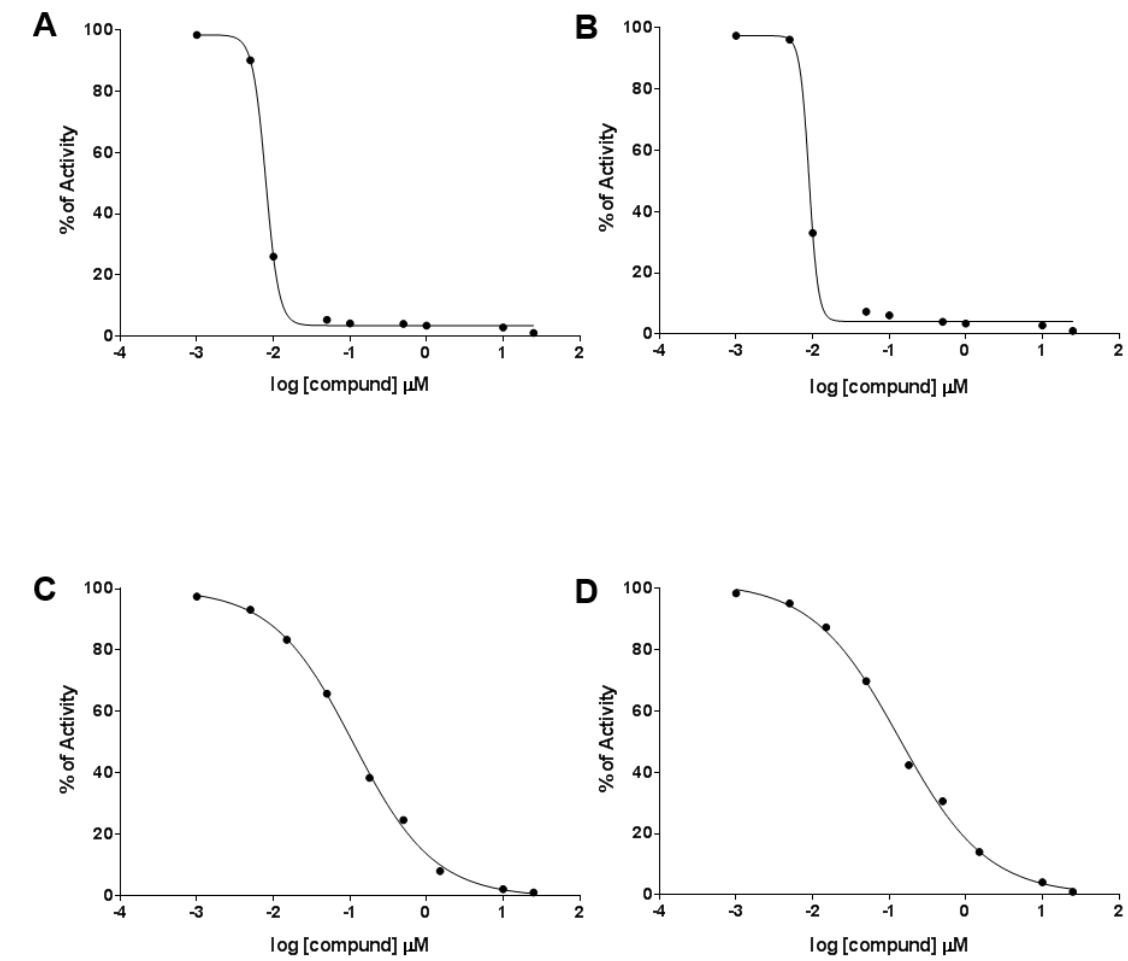


Figure 2.11. Half maximal effective concentration against MRSA with Amoxicillin

Figure 2.11 provides a graphical representation of the dose-response curve for four of the most potent YYS sulfonyl oxadiazoles compounds versus amoxicillin selected MRSA pathogens. Table 2.6 provides a summary of the inhibitory constants of these four compounds which are comparable with the known β-lactamase inhibitor clavulanic acid. Also, as we described in the previous study, our sulfonyl oxadiazole compounds were shown to exhibit a low degree of cytotoxicity, being of comparable or lower cytotoxicity than β-lactam antibiotics such as amoxicillin, sulbactam, and tazobactam, and also compared to the well-known β-lactamase inhibitor clavulanic acid (Table 2.5). For the therapeutic index (TI) which is a comparison of the amount of a therapeutic agent that causes the therapeutic effect to the amount that causes toxicity, our sulfonyl oxadiazole compounds show 522.5 and 59.6 which is extremely higher or comparable with existing β-lactamase inhibitor clavulanic acid (Table 2.4, 2.5).

Table 2.5. Half maximal effective concentration against MRSA with Amoxicillin

Compounds	EC_{50} (μM)	CC_{50} (μM)	TI
YYS-1	0.08 ± 0.007	41.8 ± 3.65	522.5
YYS-2	0.48 ± 0.06	28.6 ± 2.38	59.58
YYS-3	1.17 ± 0.14	7.6 ± 0.87	6.49
YYS-4	1.47 ± 0.12	9.37 ± 0.96	6.37

2.7 Sulfonyl Oxadiazole Compound Drug Resistant Test

A relative effectiveness of biostatic activity against amoxicillin selected *A. baumannii* with four of most potent sulfonyl oxadiazole compounds YYS-1, YYS-2, YYS-3 and YYS-4 verses clavulanic acid, which is exiting drug, over a period of 7 days. Figure 2.12 shows the relative effectiveness of biostatic activity versus *A. baumannii* of compounds YYS-1, YYS-2, YYS-3 and YYS-4 relative to clavulanic acid over a period of 7 days. As can be seen, sulfonyl oxadiazoles β-lactamase-inhibitory compounds disclosed and claimed herein for the practice of a method of the invention are more effective at comparable concentrations than clavulanic acid.

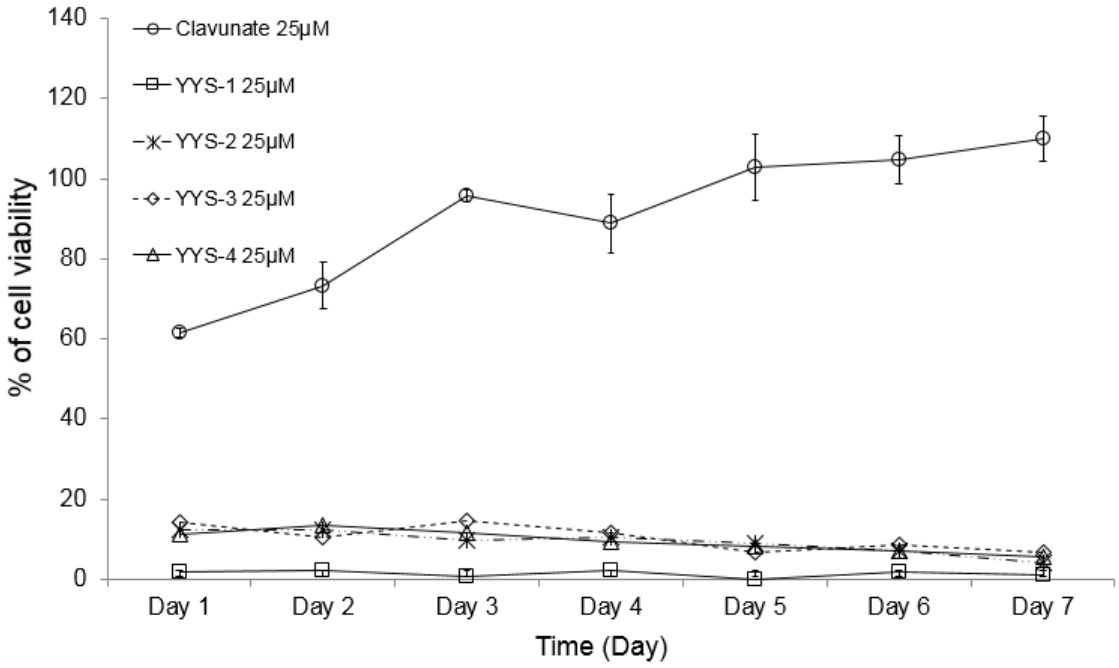


Figure 2.12. Biostatic activity Study: 7 day Growth Rate of Acinetobacter baumannii Pathogen.

2.8 Biochemical Assay against Serine β-lactamase

The purpose of the biochemical assays is to identify compounds that act as inhibitors of the bacteria expressed β-lactamase. This biochemical assay employs the cephalosporin Nitrocefin as the substrate and takes advantage of the fluorescent properties of white microtiter plates. Nitrocefin is a yellow chromogenic substrate (λ_{max}=395 nm) that is hydrolyzed by β-lactamases to yield a red product with increased absorbance properties (λ_{max}=495 nm) that quench plate fluorescence by absorbing the plate's emission light [138, 139] (Fig 2.13). In this assay, test compounds are incubated with purified β-lactamase enzyme and Nitrocefin in detergent-containing buffer at room temperature. The reaction is stopped by the addition of EDTA, followed by measurement of fluorescence. As designed, compounds that inhibit β-lactamase will inhibit Nitrocefin hydrolysis and the generation of red product. It will also inhibit the quenching of plate fluorescence, resulting in an increase in well fluorescence.

2.8.1 Sample Preparation and β-lactamase Detection

To determine the β-lactamase that is expressed by amox-selected *A. baumannii* strain, we used a Nitrocefin (Cayman, CAS 41906-86-9) as a colorimetric substrate of β-lactamase. This Nitrocefin is a chromogenic cephalosporin substrate routinely used to detect the presence of β-lactamase enzymes produced by various microbes. Nitrocefin contains a β-

lactam ring which is susceptible to β-lactamase mediated hydrolysis (Fig 2.13). Once hydrolyzed, the degraded Nitrocefin compound rapidly changes color from yellow to red. Figure 2.15 provides a graphical representation of amoxicillin selected *A. baumannii* (ATCC 19606), strain supernatant consisting of inducibly expressed β-lactamase.

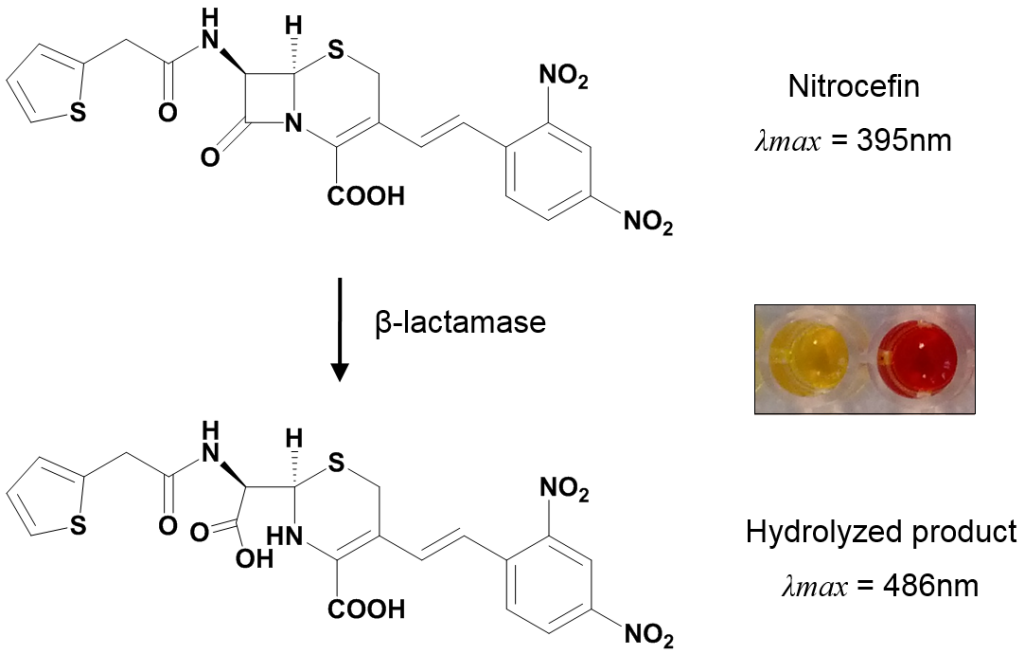


Figure 2.13. Nitrocefin use as Colorimetric β-lactamase Substrate [140].

The expressed enzymatic activity was detected in both cell-free supernatant and cell lysate in the variable concentration of Nitrocefin (0.01 to 100μM). The purified and expressed class A TEM-1 β-lactamase were used for positive control.

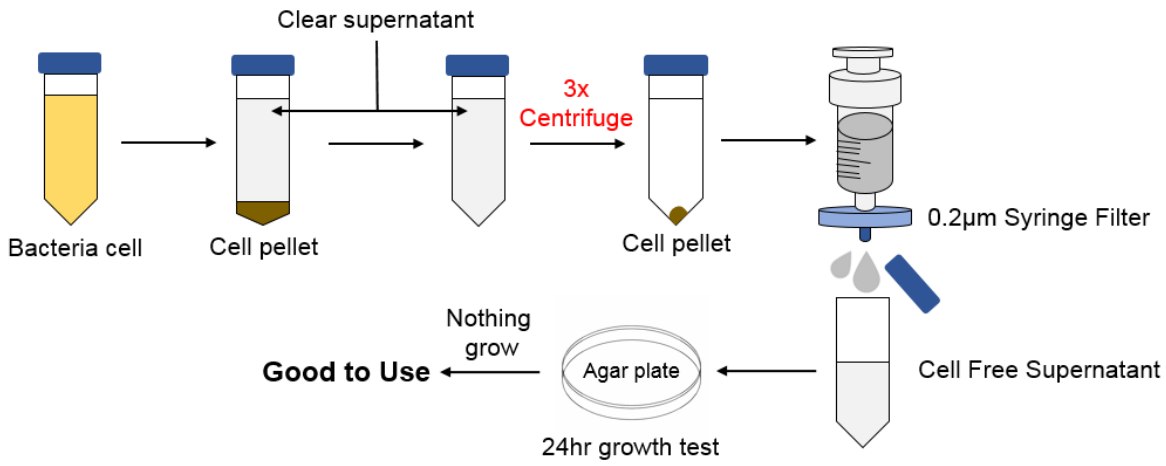


Figure 2.14. Sample preparation scheme for β-lactamase detection

For detection of β-lactamase from bacteria expression, the amoxicillin selected *A. baumannii* strains were grown for 72 h and the cultured bacterial were collected and centrifuged at 8000xg, 10 min.We transferred cell-free supernatants to clean 15ml Falcon tube without pellet and centrifuge at 8000xg, 10 min twice. We filtered the clarified supernatant through a 0.2µm syringe filter to remove any remaining bacteria pathogens (Fig 2.14).

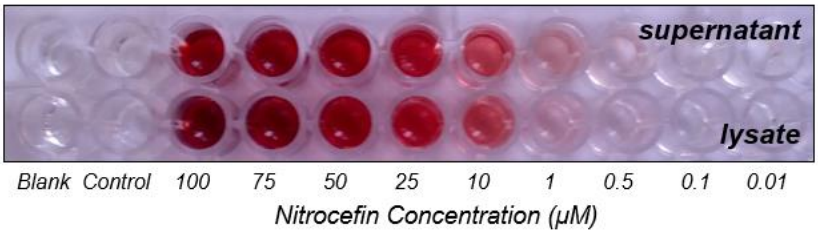


Figure 2.15. Colorimetric enzyme activity scheme of Acinetobacter baumannii cell lysate and supernatant against Nitrocefin.

2.8.2 Determine the Apparent Kinetic Parameters

Tables 2.6 and 2.7 show the apparent kinetic parameters of supernatant and lysate of amoxicillin-selected *A. baumannii* (ATCC 19606), *B. substiles* (ATCC 21332), *E. coli* (ATCC 35218), *K. pneumonia* (ATCC 13883), MRSA (ATCC 4330) and *P. aeruginosa* (ATCC 27853) strain with inducible expressed β-lactamase using Nitrocefin as a chromogenic substrate with the measured value and calculated apparent value of K_m and k_{cat}. The apparent kinetic parameters for the various bacterial strain expressed β-lactamase were determined by using its colorimetric substrate Nitrocefin. The apparent K_m and k_{cat} values were derived from at least four independent initial-velocity measurements by nonlinear regression using Michaelis-Menten enzyme kinetics Graphpad Prism 6. These results are comparable with TEM-1 control kinetic parameters with showing the reasonable range (k_{cat} /K_m). From these results, the β-lactamase in six of the different bacterial supernatant is showing the stabilized affinity with the substrate.

Table 2.6. Apparent kinetic parameters of supernatant and lysate of amoxicillin-selected bacterial pathogens with inducibly expressed β-lactamase using Nitrocefin as a chromogenic substrate.

Amoxicillin selected bacterial pathogens	K_m^{app} (μM)		k_{cat}^{app} (s^{-1})		k_{cat}^{app}/K_m^{app} ($\mu M^{-1}\ s^{-1}$)	
	supernatant	lysate	supernatant	lysate	supernatant	lysate
A. baumannii	15.1	35.4	270.3	703.3	17.88	19.8
B. subtilis	26.7	8.97	3.8	1.05	0.14	0.11
E. coli	1.29	4.32	7.12	8.00	5.49	1.85
K. pneumonia	274.3	78.5	172.4	110.5	0.63	1.40
MRSA	245.9	99.5	236.2	184.3	0.96	1.85
P. aeruginosa	181.0	116.7	146.7	126.3	0.81	1.08

Table 2.7. The apparent inhibition constant (Ki) against A.baumannii Supernatant

Enzyme	K_i^{app} (μM)				
	Clavulanic acid	YYS-1	YYS-2	YYS-3	YYS-4
A.baumannii supernatant	22.87 ± 4.47	0.013 ± 0.004	0.009 ± 0.004	0.002 ± 0.001	0.262 ± 0.036

For the apparent inhibitory constant, K_i^{app}, the four of most promising sulfonyl oxadiazoles compounds were tested (Table 2.8). Each compound was pre-incubated at various concentrations from 0.001 to 50μM with bacterially expressed β-lactamase at supernatant for 10mins at room temperature before the addition of 10μM Nitrocefin. Apparent K_i values were determined from initial maximum velocity fitted by nonlinear regression using competitive-inhibition enzyme kinetics from Graphpad Prism 6. The FDA approved β-lactamase inhibitor, Clavulanic acid, were used as a control. In our inhibition constant data with bacterially expressed β-lactamase, our sulfonyl oxadiazoles compound shows low nM to mid-nM range of inhibition.

Table 2.8. The apparent inhibition constant (*Ki*) against MRSA Supernatant.

Enzyme	Clavulanic acid		YYS-1		YYS-2		YYS-3		YYS-4	
	(μM)									
	IC_{50}^{app}	K_i^{app}	IC_{50}^{app}	K_i^{app}	IC_{50}^{app}	K_i^{app}	IC_{50}^{app}	K_i^{app}	IC_{50}^{app}	K_i^{app}
MRSA supernatant	>100	24.9 ± 1.116	< 0.001	< 0.001	< 0.001	< 0.001	0.5 ± 0.053	0.083 ± 0.016	16.81 ± 2.451	2.31 ± 0.184

The IC_{50} and inhibitory constant, K_i, for the four sulfonyl oxadiazoles compounds with the lowest single dose MRSA, expressed β-lactamase activity were determined and are shown in Table 2.9. Each Inhibitor was pre-incubated at various concentrations from

0.001 to 50μM with MRSA expressed β-lactamase for 10mins at room temperature before the addition of 10μM Nitrocefin. K_i values were determined from initial maximum velocity fitted by nonlinear regression using competitive-inhibition enzyme kinetics from Graphpad Prism 6. The determined IC_{50} for Clavulanic acid was over 100μM and was compared without sulfonyl oxadiazoles compounds data. Our sulfonyl oxadiazoles compound determined its K_i with low nM range and also it shows nM inhibition at IC_{50} determined. Our enzymatic data shows synergistic inhibition of sulfonyl oxadiazoles compounds against various classes of MRSA expressed β-lactamases. The colorimetric enzyme activity scheme of MRSA cell supernatant with Nitrocefin are represented in Figure 2.16.

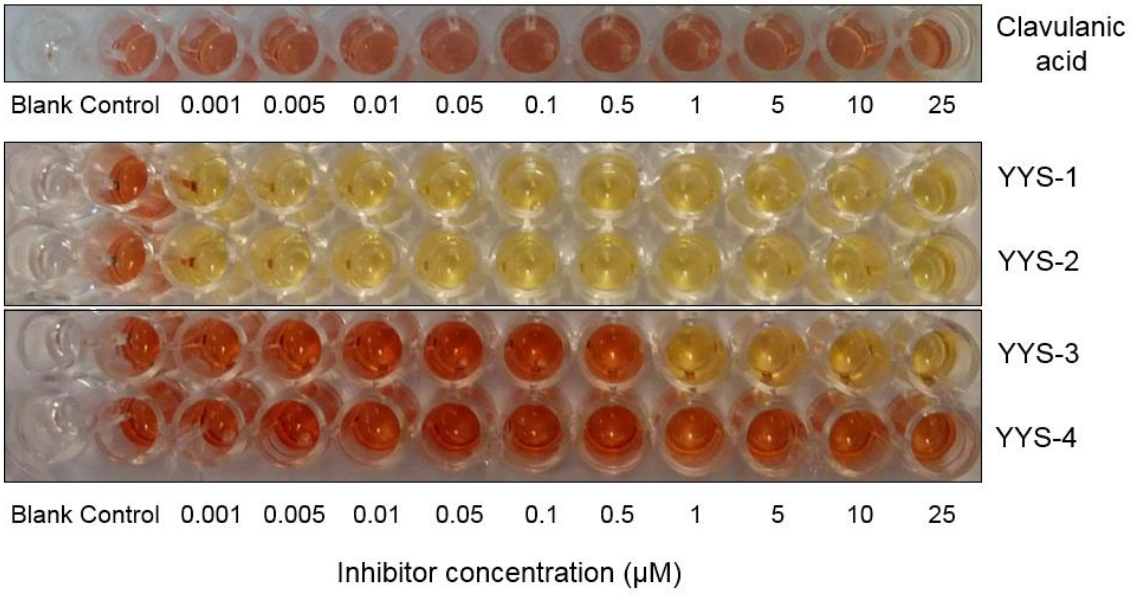


Figure 2.16. Colorimetric enzyme activity Inhibition scheme of MRSA cell supernatant against Nitrocefin.

2.8.3. Determine Kinetic Parameters against Purified β-lactamase

The β-lactamase activity of purified TEM-1, BlaC, and AmpC β-lactamase was determined spectrophotometrically (spectramax-M5-reader) using Nitrocefin (Cayman, CAS 41906-86-9) as the chromogenic substrate at room temperature in 50mM potassium phosphate buffer at pH 7.0. Its enzymatic activity was monitored based on the formation of the hydrolyzed product using a continuous measurement of λ_{max}=486nm absorbance in 0.1-s intervals for 30mins. The K_m and k_{cat} values for Nitrocefin were derived from 0.001 to 100μM concentration with at least four independent initial-velocity measurements by nonlinear regression using Michaelis-Menten Enzyme kinetics with Graphpad Prism 6. Figure 3.4 shows Steady-state kinetics for the hydrolysis of Nitrocefin by TEM-1, BlaC, and AmpC β-lactamase. The determined K_m and k_{cat} for TEM-1 were 66.49μM and 803.7(s^{-1}), BlaC 58.4μM and 160.2(s^{-1}) and AmpC 103μM and 1220(s^{-1}) respectively, comparable to literature.

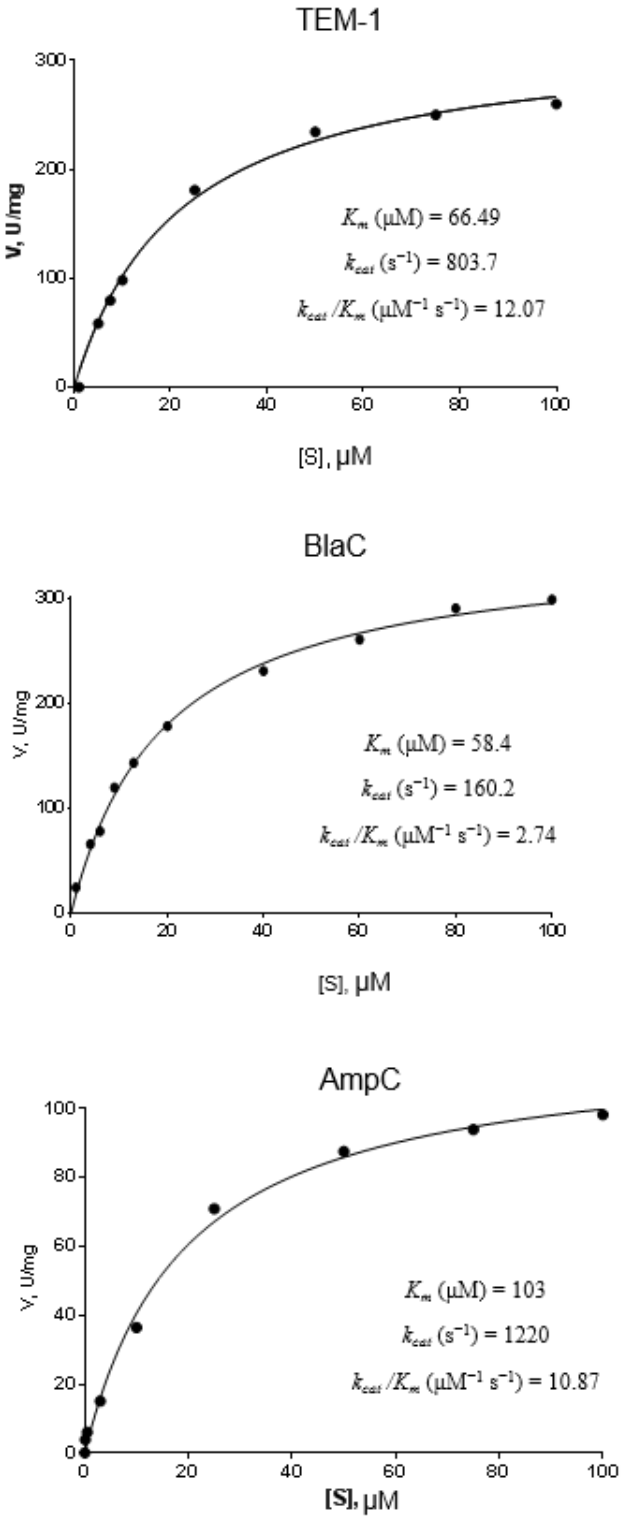


Figure 2.17. Kinetic parameters of Class A & C β-lactamase using Nitrocefin as Colorimetric Substrate.

The inhibitory constant, K_i, for the sulfonyl oxadiazoles, compounds with the lowest single dose serine β-lactamases activity was determined and are shown in Table 2.9. Each Inhibitor was pre-incubated at various concentrations from 0.001 to 50μM with 3~5nM serine β-lactamase for 10mins at room temperature before the addition of 10μM Nitrocefin. K_i values were determined from initial maximum velocity fitted by nonlinear regression using competitive-inhibition enzyme kinetics from Graphpad Prism 6. The determined K_i, for three FDA, approved β-lactamase inhibitor (Clavulanic acid, Sulbactam, and Tazobactam) were comparable to an earlier report [141-143]. In our inhibition constant data, we show the discovery of sulfonyl oxadiazoles and thiadiazoles compounds as a potent serine β-lactamase inhibitor with a K_i of 1nM to class C AmpC β-lactamase and mid-nM range of K_i against class A TEM-1 and BlaC β-lactamase (Table 2.9).

Table 2.9. The inhibition constant (Ki) of serine β-lactamase.

Compound	K_i (μM)		
	TEM-1	BlaC	AmpC
Clavulanic acid	0.093	0.110	NA
Sulbactam	0.240	0.019	
Tazobactam	0.008	0.018	
BKS-1	NA	32.39	0.056
YYS-1	NA	NA	0.004
YYS-2	2.712	0.541	0.001
YYS-3	0.210	0.158	0.934
YYS-4	0.370	1.330	0.118
YYS-S1	0.068	0.203	
YYS-S2	0.154	0.128	

2.9 Proteomics Analysis

Proteomic analysis is an important approach to characterizing the proteins and characterizing the function of specific proteins. It is also a powerful screening method for detecting expected fragments in protein expression in the biochemical study [144]. Expression of four class of serine and metallo β-lactamase confers the major source of drug resistance; therefore, they represent an important target for drug design [46] (Fig 2.18). In our previous cell-based study, the selection of amoxicillin induced drug resistance against existing combinatory therapy in *A.baumannii* ATCC 19606, MRSA ATCC 45300 and *K. pneumonia* ATCC BAA1705 strain. The apparent kinetic parameters for those three pathogens expressed β-lactamase were determined, and now it needs to identify and characterize the exact drug resistance causing β-lactamase expressed.

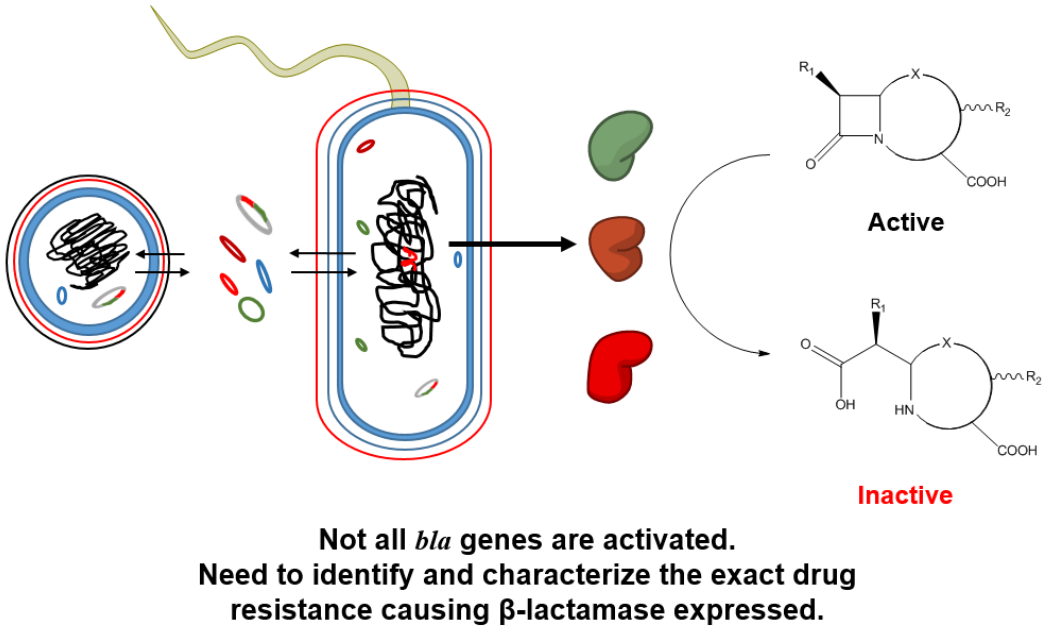


Figure 2.18. Expression of plasmid-acquired or chromosomal genes leads to β-lactam drug resistance

An important hurdle in the development of a precise combination therapy for overcoming β-lactam drug resistance is the identification of the innately or inducibly expressed β-lactamase that leads to β-lactam drug inactivation [145]. Bacteria can acquire drug resistance through the transfer of β-lactamase gene containing plasmids. Drug resistant bacteria carry various copies of β-lactamase genes. Not all genes are activated and expressed at the same time to provide cellular protection against β-lactam antibiotics due to the impractical utilization of available cellular resources for sustaining growth. To better understand the exact cause of β-lactam drug resistance, it is therefore important to characterize the β-lactamase being expressed, and whether direct inhibition of it can resuscitate β-lactam activity. We propose to analyze the expressed β-lactamase found within the supernatant of the growth media and whether they are directly responsible the observed β-lactam drug resistance.

In this study, we will characterize inducibly expressed β-lactamase with amoxicillin by proteomics study and determine whether β-lactam inhibitors that can potently inhibit the enzymatic activity of the supernatant can be directly used for combination therapy against amoxicillin-selected bacteria. The study should provide a novel strategy for developing a precise combination therapy for overcoming β-lactam drug resistance.

2.9.1 Sample Preparation and Gel Separation

To determine and characterize amoxicillin resistance of *A. baumannii* ATCC 19606 strain after treatment of amoxicillin, the strain ATCC 19606 were incubated with amoxicillin (10µg/ml) for 72h. The original ATCC 19606 cells were used for control. *A. baumannii* ATCC19606 was selected and cultured in nutrient media with amoxicillin. Cell-free supernatant was isolated from the media, concentrated and characterized biochemically for β-lactamase activity using colorimetric substrate Nitrocefin. The β-lactamase protein in amoxicillin selected bacterial pathogens supernatant detection were analyzed with SDS-PAGE and trypsin digested and identified by LC-MS (Figure 2.19).

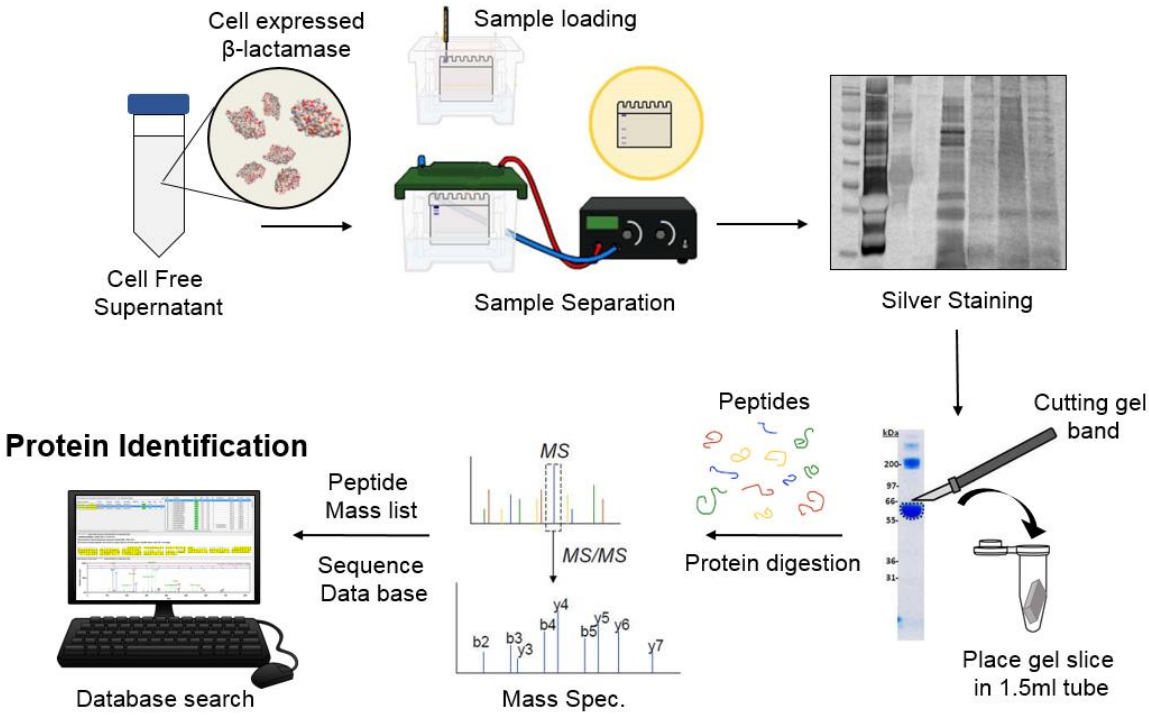


Figure 2.19. Proteomics Experimental Scheme

2.9.2. Detection of β-lactamase by Silver Staining

To further characterize these structural genes, an SDS-PAGE was performed. A major protein band and several minor protein bands were observed on the gel, with molecular weights ranging from approximately 10 to 170 kDa. After electrophoresis, the gel slab was fixed in 50% methanol, 5% acetic acid in water for 20 min. It was then washed for 10 min with 50% methanol in water and additionally for 10 min with water to remove the remaining acid. The gel was sensitized by a 1 min incubation in 0.02% sodium thiosulfate, and it was then rinsed with two changes of distilled water for 1 min each. After rinsing, the gel was submerged in chilled 0.1% silver nitrate solution and incubated for 20 min at 4 °C. After incubation, the silver nitrate was discarded, and the gel slab was rinsed twice with water for 1 min and then developed in 0.04% formalin [35% formaldehyde in water (Merck, Darmstadt)] in 2% sodium carbonate with intensive shaking. After the developer had turned yellow, it was discarded and replaced with a fresh portion.

Figure. 2.20 shows the SDS-Gel PAGE profile of a silver stained β-lactamase protein from amox-selected *A. baumannii* ATCC 19606, MRSA ATCC 45300, *A. baumannii* 5705 clinical isolated and *K. pneumonia* ATCC BAA1705 stain cell-free supernatant. This protein amount was readily visible after both Coomassie blue staining and silver staining. The class A TEM-1 (29kDa / line 1) and class C AmpC (38kDa / line 2) β-lactamase protein were used for positive control and the amox-selected supernatant (line 2, 3, 4, 5) shows the gray band at size 29~30kDa. However, the non-selected strain

sample (line 1) couldn't detect the band due to its low amount of β-lactamase protein expression.

A "control" piece of the gel was cut from a blank region of the gel and processed in parallel with the sample. After the gel pieces had been excised and shrunk by dehydration in acetonitrile, which was then removed, they were dried in a vacuum centrifuge. A volume of 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 sufficient to cover the gel pieces was added, and the proteins were reduced for one h at 56 °C. After cooling to room temperature, the DTT solution was replaced with roughly the same volume of 55mM iodoacetamide in 100 mM NH_4HCO_3. After 45 min incubation at an ambient temperature in the dark with occasional vortexing, the gel pieces were washed with 50-100 µL of 100 mM NH_4HCO_3 for 10 min, dehydrated by the addition of acetonitrile, swelled by rehydration in 100 mM NH_4HCO_3, and shrunk again by addition of the same volume of acetonitrile. The liquid phase was removed, and the gel pieces were completely dried in a vacuum centrifuge. The gel pieces were swollen in a digestion buffer containing 50mM NH_4HCO_3, five mM $CaCl_2$, and 12.5 ng/µL of trypsin (Boehringer Mannheim, sequencing grade) in an ice-cold bath. After 45 min, the supernatant was removed and replaced with 5-10 µL of the same buffer, but without trypsin, to keep the gel pieces wet during enzymic cleavage (37 °C, overnight). Peptides were extracted by one change of 20 mM NH_4HCO_3 and three changes of 5% formic acid in 50% acetonitrile (20 min for each change) at room temperature and dried down.

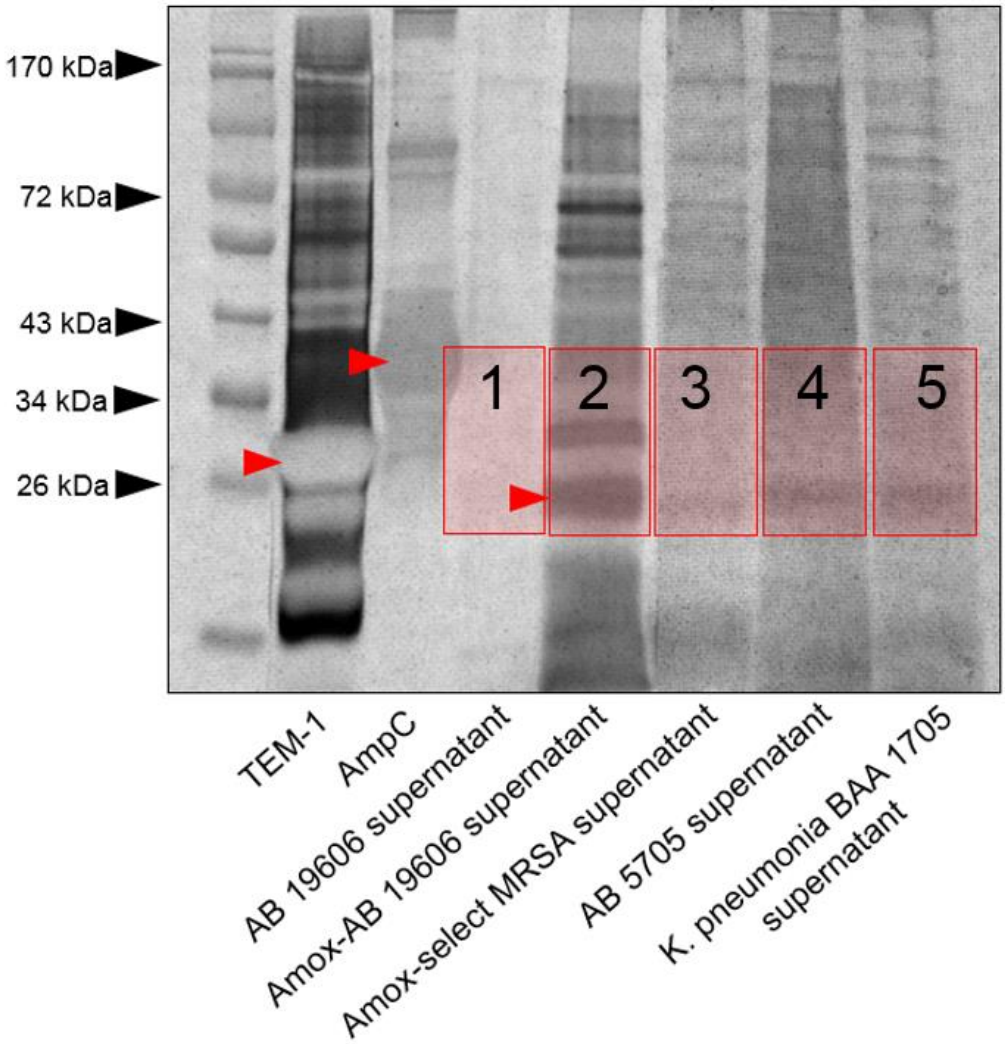


Figure 2.20. Silver staining of amoxicillin-selected A. baumannii cell-free supernatant with TEM-1 and AmpC β-lactamase as a control.

2.9.3. Protein Identification

Proteins from silver-stained gels were digested enzymatically and the resulting peptides analyzed and sequenced by mass spectrometry. In the case of 100% identity in sequence and length to an already assigned TEM type β-lactamase, the sequence was added to the respective protein entry and described by the TEM number and the corresponding mutation profile. The amox-induced β-lactamase were identified by LC-MS analysis from protein spots (29 ~ 41kDa) observed on silver stained gels. This protein was identified using MATCOT search of the obtained peptide mass fingerprint spectra as class A TEM type β-lactamase with 78% matching. Subsequent fragmentation of these ions from *m/z* 200 to 600 produced MS/MS spectra containing enough information for protein identification through database searching using peptide sequence tags (Fig 2.21). Details of identified protein peptides by MS analysis are given in Table 2.10.

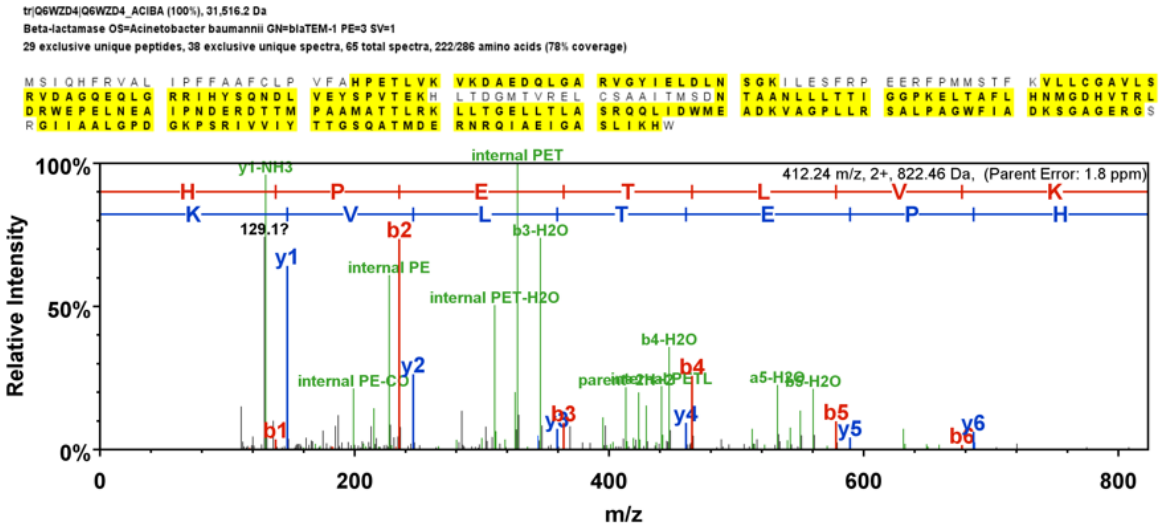


Figure 2.21. Exemplary spectra of the peptide from the tryptic digest. NCBI amino acids sequence and MALDI-TOF/TOF peptide spectra of class A β-lactamase. Highlighted areas are the matching peptides predicted by MALDI-TOF/TOF peptide spectra.

Table 2.10. Identified β-lactamase protein from detected fragment and database matching

Species	Coverage	# Peptide	Avg. Mass	Description
Acinetobacter baumannii ATCC19606 Amox-selected	75%	23	30350	TEM family class A β-lactamase
	73%	23	31515	Class A broad-spectrum TEM-1
	12%	7	41961	Class C extended-spectrum β-lactamase CMY-30
	3%	1	31169	Class A extended-spectrum β-lactamase GES-11
Methicillin-Resistant Staphylococcus Aureus ATCC 43300 Amox-selected	64%	16	31515	Class A broad-spectrum β-lactamase TEM-1
	45%	14	31399	BlaZ family class A β-lactamase
Acinetobacter baumannii 5705 Clinical Isolated	67%	21	31169	Class A extended-spectrum β-lactamase GES-11
	57%	19	28950	OXA-23 family carbapenem-hydrolyzing class D β-lactamase
	53%	19	30967	Class D β-lactamase OXA-23
	53%	19	30979	Carbapenem-hydrolyzing class D β-lactamase
	53%	19	30967	OXA-23 family carbapenem-hydrolyzing class D β-lactamase
	38%	12	43227	Class C β-lactamase
	38%	12	43238	Class C extended-spectrum β-lactamase ADC-11
	27%	6	30350	TEM family class A β-lactamase
	26%	6	31515	Class A broad-spectrum β-lactamase TEM-1
Klebsiella Pneumoniae ATCC BAA-1705 Amox-selected	68%	23	31469	SHV family class A β-lactamase
	48%	13	26199	TEM family class A β-lactamase

Amoxicillin-induced the overexpression of β-lactamase that led to multi-drug resistance. Proteomic analysis of cell-free supernatant confirmed for the first time the presence of class A TEM, SHV type β-lactamase at *Acinetobacter baumannii* (ATCC 19606), *Staphylococcus aureus* (ATCC 43300), *Klebsiella pneumonia* (ATCC 13883) strain. Also, clinically isolated stain *Acinetobacter baumannii* 5705 supernatant express various class of serine β-lactamase such as class A BlaZ, GES, TEM and class C and class D OXA type. Also, in our previous biochemical study, β-lactamase inhibitors identified using the isolated cell-free supernatant effectively resuscitate amoxicillin antibiotic activity.

2.9.4. Multiple Sequence Alignments

Multiple-sequence alignment of PBPs and β-lactamases were made via the program Maestro from the Schrodinger package. Based on the peptide fragment reported from proteomics study, we generated the sequence identity and similarity by multiple sequence alignment tools in Schrödinger package. The database of various class of β-lactamases was provided by Protein Data Bank. Fig 2.22 shows an alignment of the three amox-selected *A. baumannii* cell-free supernatant with the various representative class of β-lactamases from Protein Data Bank. It is seen that the sequences of tryptic peptide fragments derived from amox-selected *A. baumannii* show an almost exact match with TEM-type β-lactamases. This observation is highlighted with the red color box in sequence alignment table (Fig 2.22).

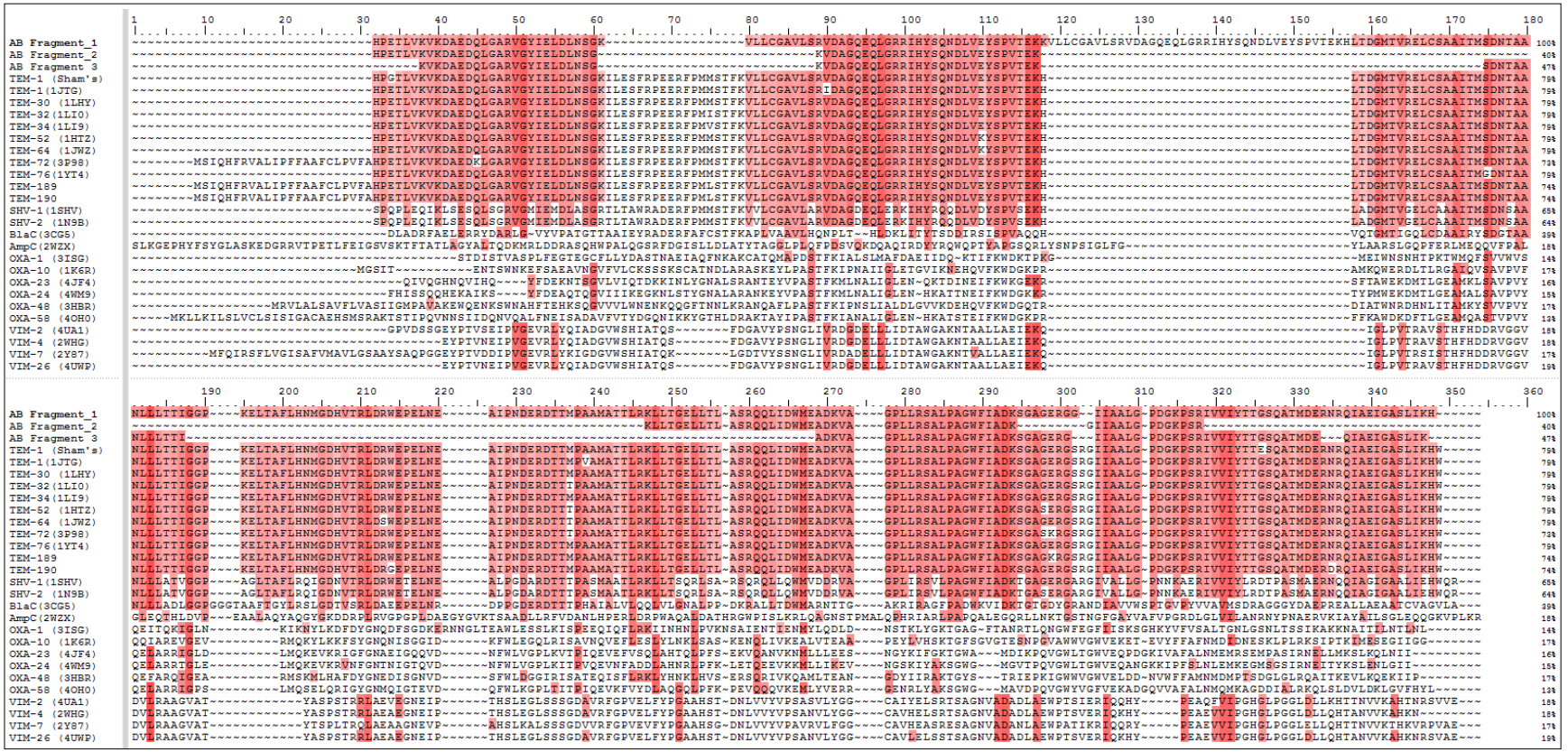
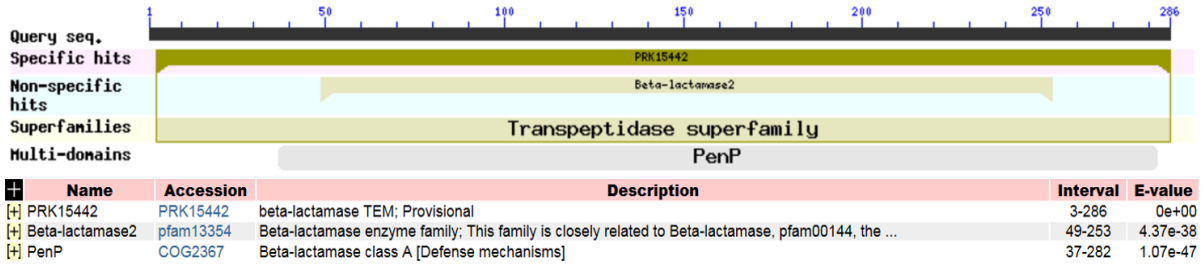


Figure 2.22. Multiple sequence alignment of peptide fragment from amox-selected A.baumannii cell-free supernatant with the various class of β-lactamases. The figure was generated using the program prime which is Schrodinger package.

2.9.5 Matching Experimental Sequences with Database Searches

The protein sequence database provides peptide sequences to be matched against the tandem mass spectra by the search engine. Our study shows the distinct tryptic peptides have yielded sufficient mass spectral data to permit assignment of 15 residue sequences. We applied the 15 peptides sequences from amox-selected *A.baumannii* supernatant into Blast search engine to compare the subsequences within each β-lactamase superfamilies. The Fig 2.23 shows our peptides sequences defined TEM-type of class A β-lactamase.



Name	Accession	Description	Interval	E-value
[+] PRK15442	PRK15442	beta-lactamase TEM; Provisional	3-286	0e+00
[+] Beta-lactamase2	pfam13354	Beta-lactamase enzyme family; This family is closely related to Beta-lactamase, pfam00144, the ...	49-253	4.37e-38
[+] PenP	COG2367	Beta-lactamase class A [Defense mechanisms]	37-282	1.07e-47

Figure 2.23. Database searches from Blast.

The presence of the TEM gene in *A. baumannii* ATCC 19606 strains was recently announced [146]. Our proteomics data show that, this is the first report that amoxicillin induced the overexpression of TEM β-lactamase resulting in multidrug resistance. Ability to characterize and identify inhibitors against the multi-drug resistance causing β-lactamase present a rational paradigm shift in developing a precise combination therapy for overcoming antibiotic-induced multidrug resistance.

2.10. Conclusion

Bacteria have developed resistance to various classes of antimicrobial agents currently in use. Due to the rapid evolution of bacterial resistances towards β-lactam-based drugs, it has become necessary to develop improved inhibitors to combat β-lactamase enzymes. In this study, I have demonstrated a new class of sulfonyl oxadiazole β-lactamase inhibitor with reasonable therapeutic properties for combination therapy against *A. baumannii*, MRSA infections or infections caused by other bacterial strains having resistance to β-lactam antibiotics (penicillins, cephalosporins, carbapenems, etc.), due to the presence of a resistance-conferring serine-type β-lactamase enzyme. This sulfonyl oxadiazole β-lactamase inhibitors, which do not possess a β-lactam structure, exhibit low cytotoxicity against human cells and are biologically active when administered in conjunction with β-lactam antibiotics in inhibition against ESKAPE pathogens. Moreover, it is a first-time study to establish the therapeutic potential of the sulfonyl oxadiazole compound as leading drug candidate.

The proteome analysis of culture supernatant proteins determines that the TEM type β-lactamase expressions are induced from Amoxicillin selected *A. baumannii* ATCC 19606 stains. It is the first report that amoxicillin selection of amoxicillin induced TEM type β- lactamase against *A.baumannii* ATCC 19606, MRSA ATCC 45300, and *K. pneumonia* ATCC BAA1705 stain. Also, our studies elegantly revealed the mechanisms of antibiotic resistance in A. baumannii ATCC 19606 strain.

2.11. Material and Methods

2.11.1. Bacterial Culture and Development of Amoxicillin Resistance

Acinetobacter baumannii (ATCC 19606), *Staphylococcus aureus* (ATCC 43300), *Klebsiella pneumonia* (ATCC 13883), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Bacillus subtilis* (ATCC 21332) were used for the assay. The bacteria (<10 mLs) was grown overnight in 50 mL capped culture tubes at a desirable temperature and in nutrient broth (NB) growth media. The original cells were selected with amoxicillin (10µg/ml) on nutrient agar (0.8g/100ml, pH 7.0) plate and incubated at 37^{o}C incubator. 72hr bacterial growth from nutrient agar plate was diluted into nutrient broth (NB) to a concentration of 1.5 OD_{600} and incubated by shaking at 37 ^{o}C.

2.11.2 Minimum Inhibitory Concentrations Assay

Minimum inhibitory concentrations (MICs) were determined by the standard broth dilution method. *Acinetobacter baumannii* strain ATCC 19606 was used for the assay. An overnight culture of the bacterial strain was subcultured to an optical density at 600 nm (OD_{600}) of 0.06 into the TSB (Trypticase Soy Broth, 3g/100ml, pH 7.3) medium at room temperature. Control experiments were conducted by growing bacteria with either (i) medium only (no antibiotic), (ii) medium and dimethyl sulfoxide (DMSO) (amount used to administer dissolved compound) or (iii) medium and antibiotics and β-lactamase

inhibitor (Tetracycline(10.0 µg/ml), amoxicillin(50µM), clavulanic acid(50µM)). Our commercial compounds were used with 50µM only and 25µM with amoxicillin (50µM). The sub-cultured bacterial suspension was seeded (max 200µl) into the wells of a 96 well microtiter plate by using the multichannel pipette. Medium alone was added to a subset of the wells to serve as a blank. Samples were then incubated at 37°C and shaken at 200 rpm for 18 h. The absorbance was measured on an ELIZA plate reader at 600 nm and analyzed with the Gen5^{TM} software suite (version 1.08).

2.11.3 Inhibitor Concentration to Inhibit 50% (IC_{50})

The inhibitor concentration required to inhibit 50% of enzyme activity (IC_{50}) of current β-lactamase inhibitors (clavulanic acid, sulbactam, and tazobactam) and our commercial compounds (YYS-85333, 85351, 85354, 85357, 85385, 85377) against *Acinetobacter baumannii* was determined. *Acinetobacter baumannii* were incubated at 37°C and shaken at 200 rpm for 24h. An overnight culture of the bacterial strain was subcultured to an optical density at 600 nm (OD_{600}) of 0.02 into the bacterial medium at room temperature. The sub-cultured bacterial suspension was seeded (max 200µl) into the wells of a 96 well microtiter plate by using the multichannel pipette. Medium alone was added to a subset of the wells to serve as a blank. Control experiments were conducted by growing bacteria with either (i) medium only (no antibiotic), (ii) medium and dimethyl sulfoxide (DMSO) (amount used to administer dissolved compound) or (iii) medium and antibiotics and β-lactamase inhibitor (Tetracycline (-) control (10.0 µg/ml), Amoxicillin (50µM)). β-lactamase inhibitors and our commercial compounds (0.001 to 25µM) were treated with Amoxicillin (50µM) per 18-20hr in 37 ^{o}C incubator. The absorbance was measured on an ELIZA plate

reader at 600 nm and analyzed with the Gen5^{TM} software suite (version 1.08). The IC_{50} values were obtained by fitting binding data to a sigmoidal dose-response equation using GraphPad Prism 6.

2.11.4 Inhibitor Resistance and Stability Test

Sub-cultured *Acinetobacter baumannii* suspension was seeded (max 200µl) into 96 well microtiter plate with optical density at 600 nm (OD_{600}) of 0.03 into Nutrient broth medium (0.8g/100ml, pH 7.0) at 37°C. Clavulanic acid and YYS-85333, 85351, 85385, 85377 (10 and 25µM) were treated with Amoxicillin (50µM) per 7-days in 37 ºC incubator. The absorbance was measured on an ELIZA plate reader at 600 nm at every 24 hr. Optical densities are analyzed with the Gen5^{TM} software suite with a non-treated control well after 24hrs.

2.11.5 Cytotoxicity Test against Human Embryonic Kidney Cells

Human embryonic kidney cell line (HEK 293) was grown in DMEM (Dulbecco's modifications of eagle's medium with L-glutamine & 4.5G/L glucose) supplemented with fetal bovine serum 100 units/ml of penicillin G and 0.1 mg/ml of streptomycin sulfate in a humidified atmosphere of a 5% CO_2 at 37°C.

Trypsin-treated monolayer HEK293 cell line is harvested, and cell counted using Vi-cell machine (Beckman Coulter Com.). The cells were seeded at a concentration of 8×10^4 cells/well in 200µl culture medium and incubated at 37ºC in 5 % CO_2 incubator for 24 hrs.

After 24 hours, when the monolayer formed, the supernatant was removed and added fresh media with different concentrations of compounds (0.001 to 100µM) and kept for incubation at 37ºC in 5 % CO_2 incubator for 72h.

After 72 hours, 15µl of MTT (5mg/ml) dye was added to each well and the plates were incubated for 4 hours at 37ºC in 5% CO_2 incubator. To prepare plates for reading spins plates in swinging bucket rotor down 1,500xg for 10 mins to remove the supernatant. Add 200µl of dimethyl sulfoxide (DMSO) and the plates were gently shaken to solubilize the formed formazan for 30 min. The absorbance was measured using a microplate reader at wavelength 590 nm. The IC_{50} values were obtained by fitting binding data to a sigmoidal dose-response equation using GraphPad Prism 6.

2.11.6. Protein Purification and Expression

The purity of the enzyme was ascertained by 10% SDS-polyacrylamide gel electrophoresis, and the protein concentration was determined by using the Bio-Rad protein assay kit. Enzyme activity was determined in 1× PBS buffer at pH 7.0 and room temperature using Nitrocefin as a substrate; the formation of the hydrolyzed Nitrocefin was monitored and quantitated using λ_{max}=485. The purified enzyme can be stored at -80 °C without losing any activity for a long period.

2.11.7. Sample Collection

The amoxicillin selected *A. baumannii* strains were grown for 72 h to optimal production of the target protein at 37°C shaking incubator (to an OD_{600} of 1.5). The cultured bacteria were collected and centrifuged at 8000 x g, 10 min. Transfer cell-free supernatants to clean 15ml Falcon tube without pellet and centrifuged at 8000 x g, 10 min twice. We filtered the clarified supernatant through a 0.2µm *syringe* filter to remove any remaining bacteria pathogens.

2.11.8. β-lactamase Activity and Kinetic Constants

TEM-1 was expressed in *Escherichia coli* BL21 (ʎDE3), extracted by osmotic shock (Tris pH 8.0, Sucrose, EDTA) and purified by Zn-chelating chromatography (Acetate, NaCl, pH 8.0). The Purified TEM-1 β-lactamase activity was determined spectrophotometrically (spectramax-M5-reader) at room temperature in 50mM potassium phosphate buffer (pH 7.0) a buffer that contributes to enzyme stability at these volumes in a total volume of 100µl under the conditions with Nitrocefin (ε486 nm = 20500 M-1.cm-1) as reporter substrate. Nitrocefin (0.001 to 100 µM) was freshly prepared in 50mM potassium buffer (pH 7.0). *Km* and *k*cat values were derived from at least four independent initial velocity measurements by nonlinear regression using Michaelis-Menten Enzyme kinetics Graphpad Prism 6.

Enzyme inhibition (K_i) value was determined also using Nitrocefin as substrate. Inhibitors, at various concentrations (Clavulanic acid (0.001 to 25 µM), commercial compounds (0.01

to 25 μM)), were pre-incubated with the purified TEM-1 β-lactamase enzyme (5nM) for 5 min at room temperature in the detergent buffer before addition of the substrate (10μM fixed). *Ki* values were derived from initial velocity measurements by nonlinear regression using competitive-inhibition Enzyme kinetics Graphpad Prism 6.

2.11.9. β-lactamase Detection

The production of β-lactamase by these bacteria was tested by the chromogenic cephalosporin method using Nitrocefin as directed by the manufacturer (P212121) using class A TEM-1 and class C AmpC β-lactamase as a positive control.

2.11.10. Supernatant Enzyme Activity and Kinetic Constants

The β-lactamase in Amoxicillin selected bacterial pathogens supernatant activities were determined spectrophotometrically (spectramax-M5-reader) at 37 ^{o}C using in the micro-tilter plate in a total volume of 100μl with Nitrocefin (ε486 nm = 20500 M-1.cm-1) as reporter substrate (0.001 to 100 μM). Apparent K_m and k_{cat} values were derived from at least four independent initial velocity measurements by nonlinear regression using Michaelis-Menten Enzyme kinetics Graphpad Prism 6.

Enzyme inhibition (*Apparent* K_i) value was determined also using Nitrocefin as substrate. Inhibitors, at various concentrations (Clavulanic acid (0.001 to 25 μM), commercial compounds (0.01 to 25 μM)), were pre-incubated with the Purified TEM-1 β-lactamase enzyme (5nM) for 5 min at room temperature in the detergent buffer before addition of the

substrate (10µM fixed). Apparent K_i values were derived from initial velocity measurements by nonlinear regression using competitive-inhibition Enzyme kinetics Graph pad Prism 6. The catalytic efficiency (k_{cat}/K_m) for TEM-1 β-lactamase was also determined.

2.11.11. SDS-page and Silver Staining

The protein in amoxicillin selected bacterial pathogens supernatant detection were analyzed with SDS-PAGE 10% gradient Novex Tris-glycine resolving gel (Invitrogen, Carlsbad, CA, USA). After separation at 130V for 1hr, the gel was fixed in 50% methanol, 5% acetic acid in water for 20 min. It was then washed for 10 min with 50% methanol in water and additionally for 10min with water to remove the remaining acid. The gel was sensitized by a 1 min incubation in 0.02% sodium thiosulfate, and it was then rinsed with two changes of distilled water for 1 min each. After rinsing, the gel was submerged in chilled 0.1% silver nitrate solution and incubated for 20 min at 4 C. After incubation, the silver nitrate was discarded, and the gel slab was rinsed twice with water for 1 min and then developed in 0.04% formalin in 2% sodium carbonate with intensive shaking. After the developer had turned yellow, it was discarded and replaced with a fresh portion. It is essential that the developing is carried out in a transparent solution. After the desired intensity of staining was achieved, the development was terminated by discarding the reagent, followed by washing of the gel slab with 5% acetic acid. Developed gels were completely transparent when the sensitization step with sodium thiosulfate was included. Silver-stained gels were stored in a solution of 1% acetic acid at 4 C until analyzed.

2.11.12. Gel Digestion

Excision of protein bands from SDS-PAGE 10% polyacrylamide gels. Cut into ~2 x 2 mm cubes and place in 1.5 ml snap-cap microfuge tube. Transfer 75µl 1:1 100mM ammonium bicarbonate: acetonitrile to gel pieces, and incubate 15 min at room temperature. Remove previous wash and add 75µl of 100% acetonitrile. Remove acetonitrile (~30 sec – 1 min) and Rehydrate gel pieces with 75µl 10mM DTT in 10mM ammonium bicarbonate. Incubate 1hr at 56 °C in a water bath and spin down tubes then remove DTT solution. Add 7 5ul of 55mM iodoacetamide in 100mM NH_4HCO_3 and incubate 30 min at room temperature in the dark. Wash gel plugs with 75µl 1:1 acetonitrile until pieces shrink and turn opaque white then remove acetonitrile (~30 sec-1 min). Rehydrate gel pieces in digestion buffer at 4 °C (50mM NH_4HCO_3, 5mM $CaCl_2$, 12.5 ng/µl trypsin). Add a sufficient volume of buffer to cover gel pieces, ~20 µl, inspect visually. Set on ice for 15 min. Remove supernatant and replace with 70µl 50mM NH_4HCO_3, 5mM $CaCl_2$. Incubate at 37°C overnight in a warm-air incubator.

2.11.13. Extraction of Peptides

Spin down tubes in a centrifuge at low speed and recover supernatant from overnight incubation. Place supernatant in new 1.5 ml tube labeled accordingly. Add sufficient volume of 50% acetonitrile, 0.3% formic acid to cover gel pieces (approximately 60µl), incubate 15 min. Recover supernatant and place in the corresponding tube. Add the 80% acetonitrile, 0.3% formic acid, incubate 15 min. Recover supernatant and place in the

corresponding tube. Freeze pooled extracts in -80 °C freezer 30 minutes, then dry in speed vac. Proceed to zip tip/stage tip protocol (for desalting) or store at -80 °C before submission for mass spec analysis.

2.11.14. Mass Spectrometric Identification

Protein samples were desalted and concentrated on C-18 ZipTips (Millipore) using the manufacturer's protocol. The data were interpreted by the software package PEAKS Studio ver. 7.5. Each slice was digested with trypsin and analyzed by nano-LC/MS/MS using a linear ion trap mass spectrometer. Submission of peak lists to the database using the UniProtKB/Swiss-Prot search engine to identify the proteins. The MS⁄MS spectra were calibrated internally to the precursor ion mass and used for sequence specific search at MACSOT/SwissProt database. Also, peptide mass fingerprint-based searches were carried out using only the set of peptide masses, in the same database without any constraints for isoelectric point (pI) and molecular mass. Identified proteins were classified into functional groups, including some hypothetical proteins without annotated functional identities, based on the presence of conserved domains and use of the Protein Cluster and BLASTp tools. The whole procedure was repeated twice times to ensure correct protein identification.

2.11.15. Multiple-Sequence Analysis

Our sequence alignment method was used for database search in a straightforward manner. The multiple sequence alignment tools in Schrodinger package ver.9.7 based on classic Smith-Waterman algorithm were used. The comparing sequence database were provided by NCBI Protein Data Bank.

CHAPTER 3

IDENTIFICATION OF METALLO β-LACTAMASE INHIBITOR

3.1 Summary

Class B metallo β–lactamase (MBL) were found in clinical isolates of ESKAPE pathogens. For drug development, currently, there is a lack of lead compounds with optimal therapeutic potential. Here we report the discovery of 1-hydroxypyridine-2(*1H*)-thiones-6-carboxylic acid as a potent VIM-2 and NDM-1 metallo β–lactamase inhibitor with potent activities and low cytotoxicity. We further show that this inhibitor can restore the antibiotic activity of amoxicillin against metallo β–lactamase producing *E. coli* in whole cell assays. Its potential mode of binding was examined by molecular modeling and its stability in mouse and human plasma studies is assessed.

We identify a new class of metallo β-lactamase inhibitor and demonstrate the therapeutic potential of the 1-hydroxypyridine-2(*1H*)-thiones (1, 2-HTP) compound. It has broad spectrum activity and low cytotoxicity, and can be used in combination antibacterial therapy to restore existing β-lactam antibiotic activity. It is a first-time study to establish the therapeutic potential of the 1, 2-HTP families as leading drug candidate.

3.2 Introduction

The ability to hydrolyze β-lactam antibiotics is necessary for the survival of antibiotic resistant bacteria. Two broad classes of mechanisms to hydrolyze lactam β-lactam rings have evolved, one using a serine residue and the other using zinc ion for the nucleophilic attack [147-150]. Enzymes using the second type of mechanism are classified as metallo-β-lactamases conferring resistance to a broad range of β-lactam antibiotics. This Metallo β-lactamase was first detected in a *Klebsiella pneumonia* isolate from a patient of India in 2008 [151]. It later spread to India, Pakistan, the United Kingdom, the United States, Canada, and Asia such as Japan and S. Korea. Since the discovery of the first metallo-β-lactamase, at least twelve new metallo-enzymes have been described [152-155].

Currently, several multi-drug resistance ESKAPE pathogens use metallo-β-lactamase enzymes to hydrolyze β-lactam rings found in many antibiotics, rendering them ineffective [96]. Metallo-β-lactamases have been the subject of growing interest in the past few years due to their increasing occurrence in pathogenic bacterial strains, their rapid dissemination by horizontal transfer, and the lack of efficient therapy to treat infected patients [156-160]. Among the class B metalloenzymes, NMD, IMP, and VIM Carbapenem-hydrolyzing β-lactamases have been genetically characterized in *Pseudomonas aeruginosa*. Metallo enzymes possess the broadest substrate of hydrolysis range among *Pseudomonas aeruginosa* β-lactamases, including penicillin, cephalosporins, and carbapenems, but not monobactams. Their activity is entirely dependent on zinc.

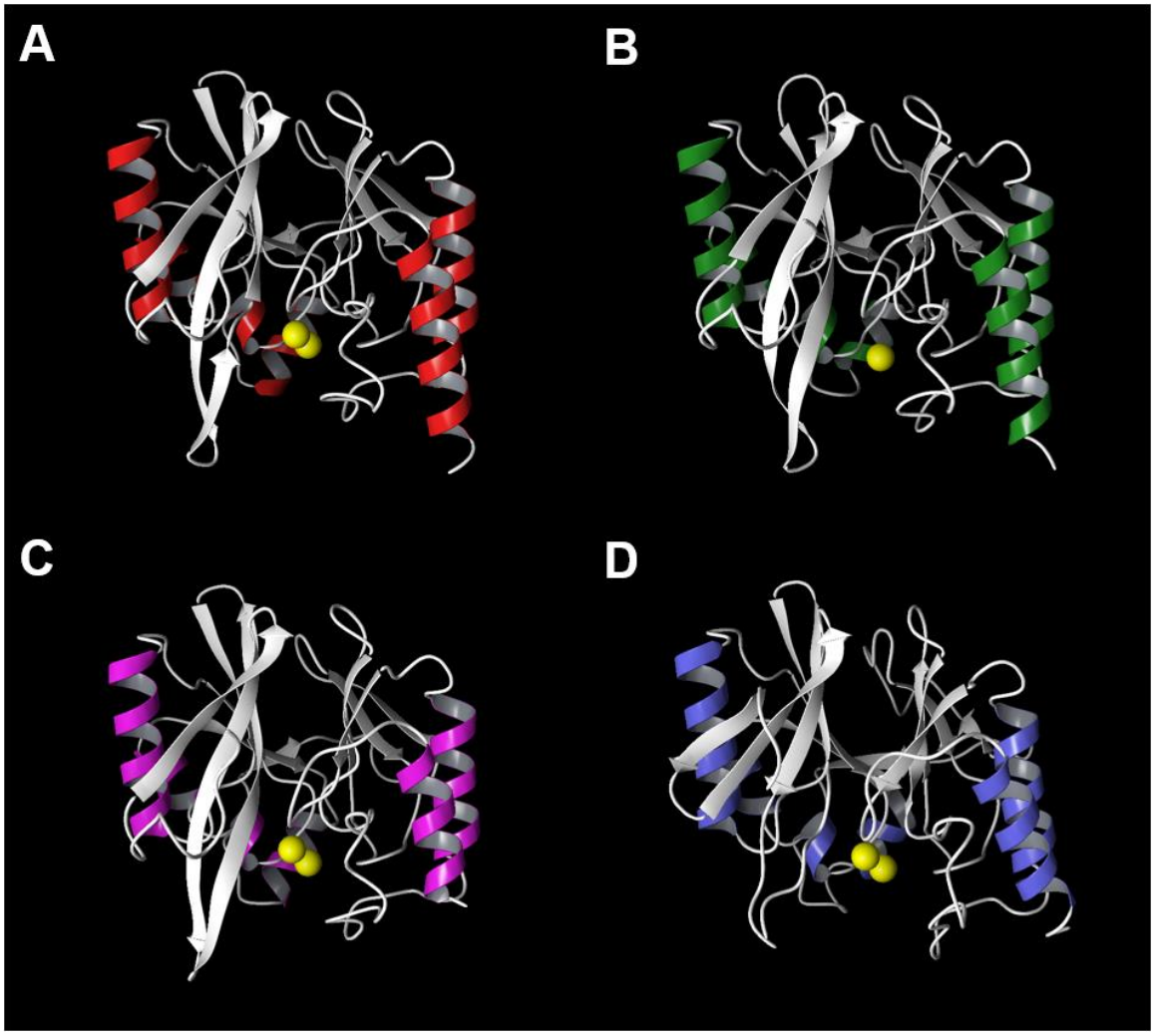


Figure 3.1. The X-ray protein crystal structure of metallo β-lactamase. The target enzyme, VIM-2 metallo β-lactamase are represented at A (PDB ID: 4BZ3). Other class of VIM-4, VIM-7 and NDM-1 metallo enzymes are represented at B (PDB ID: 2WRS), C (PDB ID: 2Y8A), and D (PDB ID: 4U4L)

A second growing family of carbapenemases, the VIM family, was reported from Italy in 2008 and now includes Europe, South America, and the United States [96]. VIM-1 was discovered in *P. aeruginosa* in Italy in 1996; since then, VIM-2 was repeatedly found in Europe and the Far East and also VIM-3 and -4 detected later [154, 155, 161, 162]. These VIM enzymes occur mostly in *P. aeruginosa*, *K. pneumonia* and, *Enterobacteriaceae*. The VIM-2, which is our target of study, is a carbapenem-hydrolyzing metallo β-lactamase (MBL) found in numerous clinically isolated ESKAPE pathogens.

The class B β-lactamases are classified into three subclasses B1, B2, and B3 by sequence alignments. IMP, VIM and NDM belong to the subclass B1 of the metallo β-lactamase, consisting of dinuclear zinc metal cofactors necessary for enzyme catalysis[163]. There is currently a lack of lead compounds with optimal therapeutic potential against MBLs available for development. To identify novel classes of metallo β-lactamase inhibitors (MBLi), we utilized VIM-2, a carbapenemase commonly found in clinically isolated ESKAPE pathogens, as the biochemical screening platform for MBLi discovery.

The general structure of metallo enzymes is similar and consists of a α-β-β-α structure, composed by two central β-sheets with five solvent-exposed α-helices [164]. The N-terminal and C-terminal parts of the molecule, each of them comprising a β-strand and two α helices, can be superposed by an 180° rotation around a central axis, and the active site is located at the external edge of the ββ sandwich (Fig 3.1). The N-terminal domain of metallo-β-lactamases incorporates a loop (residues 61–65) that can interact with substrate or inhibitor molecules which possess hydrophobic side-chains. This loop is very flexible in the native form of the enzyme. When the substrate or the inhibitor diffuses into the active site, the loop moves to block the molecule in the active site [165].

The active site of Metallo β-lactamase shows the presence of two zinc ions in the active site. One zinc is coordinated by three histidines and a water molecule, which supposedly acts as the nucleophile during β-lactam hydrolysis [166, 167]. The second zinc ion is bound to a three histidine (His116, His118, and His196), an aspartate, a cysteine (Cys198), and the nucleophilic water that forms a bridge between the two metals (Fig 3.2). An additional water molecule is usually bound to the second zinc and may serve as a proton donor during the catalytic process [168-171].

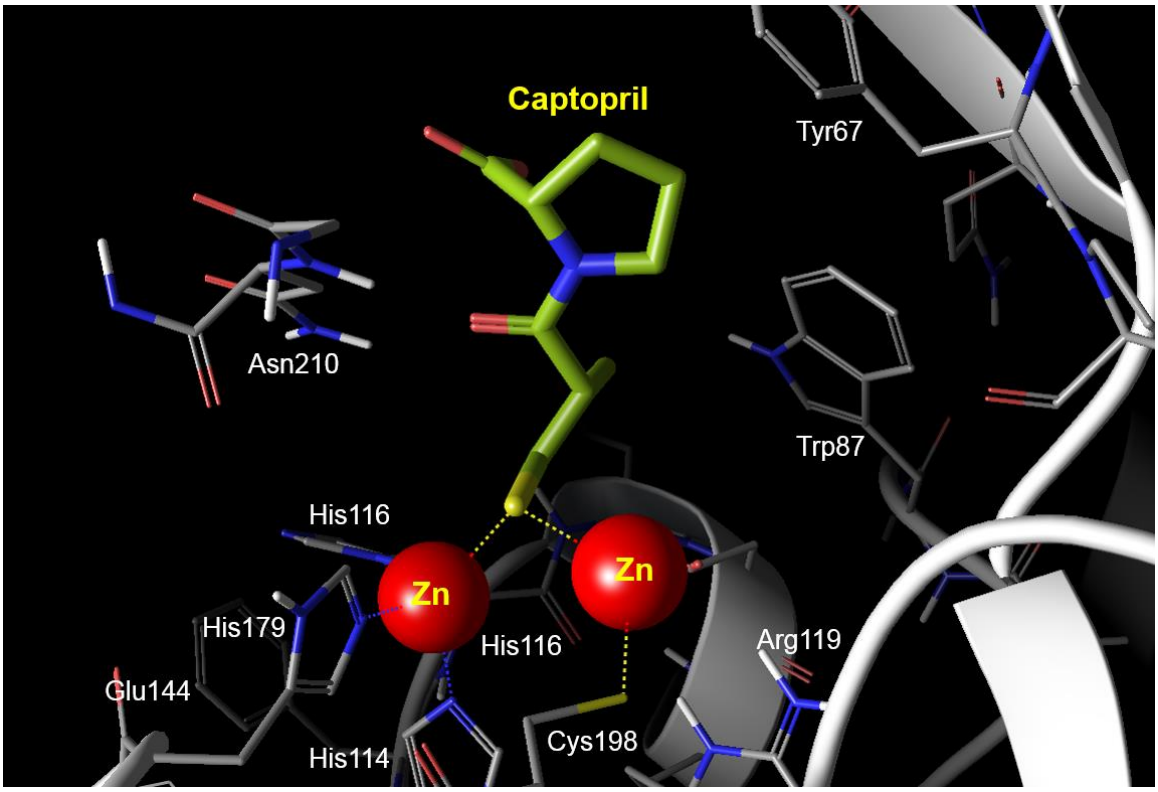


Figure 3.2. Graphical view of VIM-2 metallo β-lactamase active site with L-captopril (PDB ID: 4C1B)

3.3 Biochemical Assay of 1, 2-HPT compound against Metallo β-lactamase

3.3.1 1-hydroxypyridine-2(1H)-thiones-6-carboxylic acid

L-Captopril is an angiotensin converting enzyme inhibitor approved by the FDA for the treatment of hypertension and congestive heart failure [172, 173]. For over a decade, L-captopril and its stereoisomer have been shown to exhibit broad spectrum inhibitory activity against various MBLs [174, 175]. Its inhibitory potency stems from its thiol group which is viewed as a pharmacological liability for a non-specific zinc binding against other metallo enzymes and is prone to inactivation by metabolic oxidation[176] that can lead to reactive radical species. As such, captopril has never been further pursued clinically as a β-lactamase inhibitor for combination antibacterial therapy. To-date, its use has been limited to improve our understanding of MBL inhibition in antibacterial discovery [173, 177]. Recent structural characterization of Captopril stereoisomer against IMP-1, BcII, and VIM-2 have shown the common mode of binding involving a bridge chelation between the two zinc ions by its deprotonated thiolate ion (Fig 3.2).

1-Hydroxypyridine-2-thione (1,2-HPT), also referred to as pyrithione, is a heterocyclic thiohydroxamic acid [178] that forms a five-membered complex *via* their oxygen and sulfur atoms with zinc. Zinc pyrithione (ZPT) can be isolated from Chinese herbal roots *Polyalthia nemoralis* [179] and has been shown to possess a broad range of

antimicrobials activities[180-186]. Most recently, we have reported compounds with the 1,2-HPT moiety as zinc-specific chelating inhibitors of VanX for the re-sensitization of vancomycin against vancomycin-resistant *Enterococcus faecium* (VREF)[187] and as selective inhibitors of HDAC8 for their potential treatment of leukemia.[182] These earlier successes against zinc enzymes have prompted us to further explore the application 1,2-HPT as potential MBLi for overcoming β-lactam drug resistance in ESKAPE pathogens.

For comparison purposes, we included L-captopril and other representative compounds consisting the hydroxamic and cyclic hydroxamic acid moiety as alternative ZBGs (Fig 3.3). L-captopril, SAHA and zinc pyrithione (2) were commercially purchased from Sigma-Aldrich. Compounds **3-5** were taken from our earlier studies.[182, 187] Compounds **1** was from our in-house unpublished library and was included as a comparison compound to hydroxamic acid and 1,2-HPT.

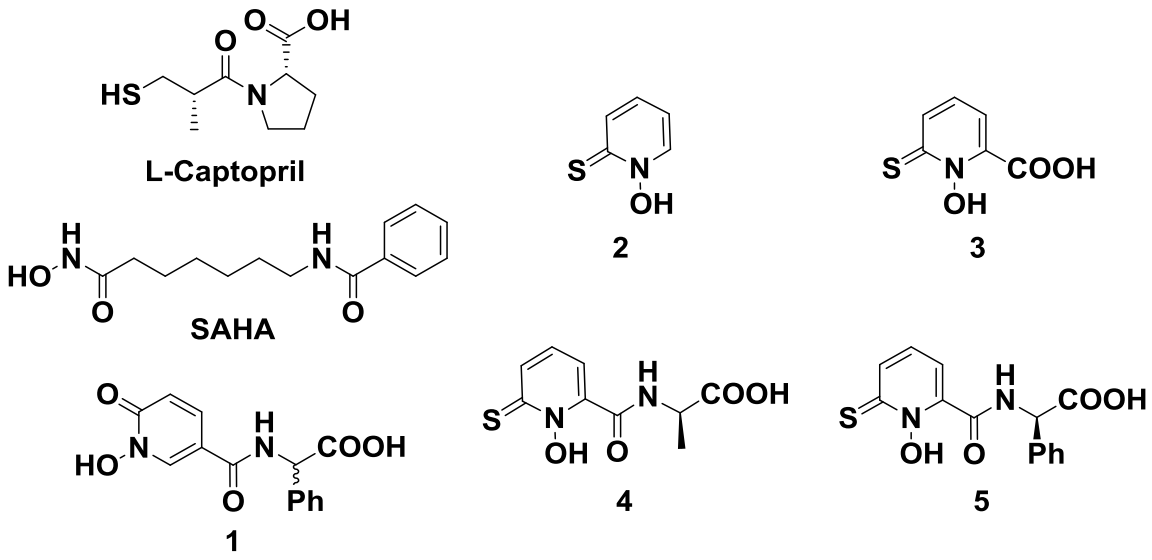


Figure 3.3. Structures of L-Captopril, SAHA, 1-hydroxypyridine-2(1H)-ones and 1-hydroxypyridine-2(1H)-thiones (1,2-HPT) analogues (2-5). L-captopril and SAHA were used for positive and negative control for the assay.

3.3.2. Determine Kinetic Parameters against Purified β-lactamase

For our biochemical assay, the bla_{VIM-2} gene, from a clinical strain of *P. aeruginosa*, was expressed using the pET24a (+) vector. The pET24a-VIM-2 plasmid was transformed into competent BL21 (DE3) *E. coli* cells. The β-lactamase activity of purified VIM-2 was determined spectrophotometrically (spectramax-M5-reader) using Nitrocefin (Cayman, CAS 41906-86-9) as the chromogenic substrate at room temperature in 50mM potassium phosphate buffer at pH 7.0. Its enzymatic activity was monitored based on the formation of the hydrolyzed product using a continuous measurement of λ_{max}=486nm absorbance in 0.1-s intervals for 30mins. The K_m and k_{cat} values for Nitrocefin were derived from 0.001 to 100μM concentration with at least four independent initial velocity measurements by nonlinear regression using Michaelis-Menten enzyme kinetics with Graphpad Prism 6. Figure 3.4 shows steady-state kinetics for the hydrolysis of Nitrocefin by VIM-2. The determined K_m and k_{cat} were 23.0μM and 212s^{-1}, respectively, comparable to literature values.[167, 188]

For comparison purposes, we included L-captopril and other representative compounds representing the hydroxamic and cyclic hydroxamic acid moiety as alternative ZBGs (Figure 3.3). L-captopril and zinc pyrithione (2) were commercially purchased from Sigma-Aldrich. Compounds 3-5 were taken from our earlier studies. Compound 1 was from our in-house unpublished library and was included as a comparison compound to hydroxamic acid and 1,2-HPT.

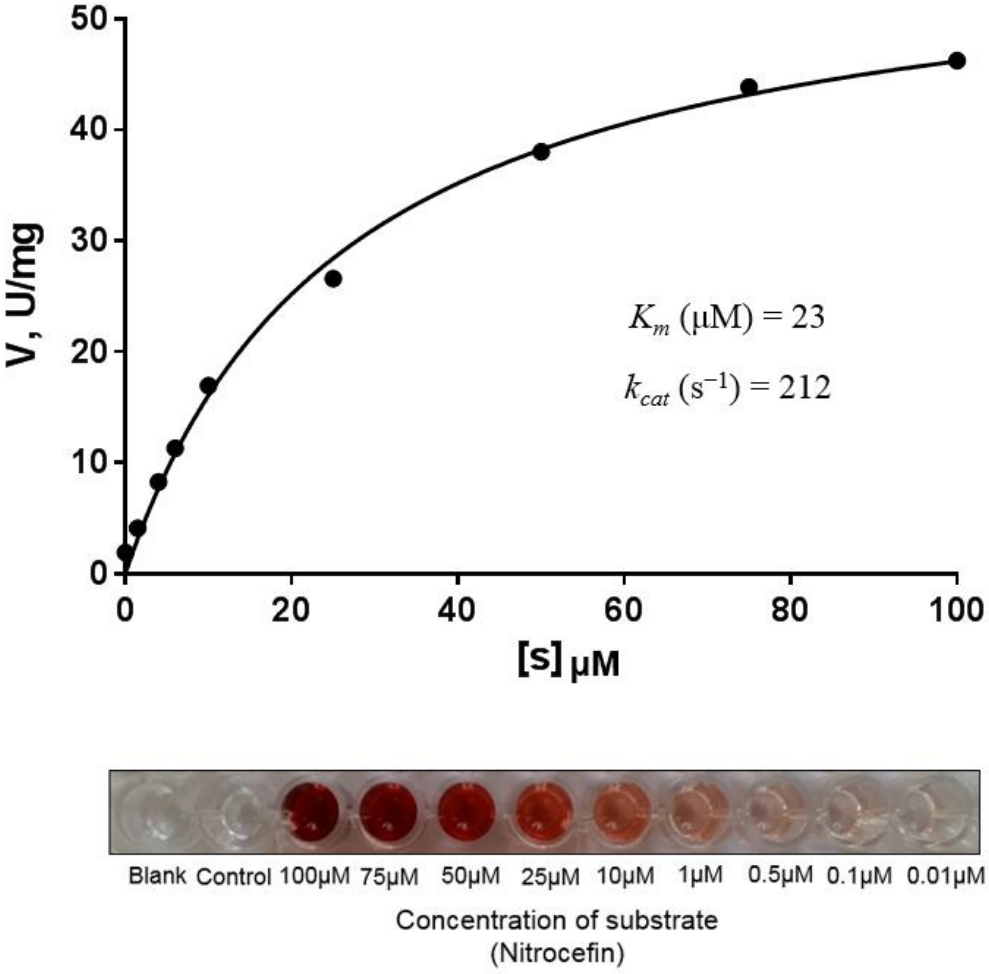


Figure 3.4. Steady-state kinetics for the hydrolysis of Nitrocefin by VIM-2

3.3.3. Bioactivity Screening of In-house Library against VIM-2 Metallo β-lactamase

To screen for potential MBL inhibition activity, 50μM of each selected compound was tested against VIM-2 in the presence of Nitrocefin. Each compound was incubated for 10mins with VIM-2, followed by the addition of Nitrocefin. The relative change in enzymatic activity was measured after 30mins at λ_{max}=486nm absorbance and is shown in Table 3.1. Both clavulanate and tazobactam, two of the FDA approved β-lactamase inhibitors, were also included as a control. Compound 3 and L-captopril showed remarkable inhibition with only 2% VIM-2 activity observed. Low inhibition was observed for SAHA and compound 1, suggesting both hydroxamic acid and cyclic hydroxamic acid as poor starting pharmacophore for MBLi design. Surprisingly, 1, 2-HPT from zinc pyrithione salt showed relatively weak inhibition activity as compared to compound 3. Varied inhibitory activities were also observed for compounds 4 and 5 between the methyl and phenyl substitution, indicating inhibitory affinity can be enhanced by the addition of an aromatic ring at the non-zinc-binding group.

Table 3.1. Single dose inhibition assay

Compound	% Activity at 50μM
L-Captopril	2.2
Clavulanate	85
Tazobactam	75
SAHA	71
1	88
2	85
3	2.1
4	82
5	66

3.3.4. Determine Kinetic Parameters against VIM-2 β-lactamase

The IC_{50} and inhibitory constant, K_i, for the three compounds with the lowest single dose VIM-2 activity, were determined and are shown in Table 3.2. Each inhibitor was pre-incubated at various concentrations from 0.001 to 50μM with 5nM VIM-2 β-lactamase for 10mins at room temperature before the addition of 10μM Nitrocefin. K_i values were determined from the maximum initial velocity fitted by nonlinear regression using

competitive inhibition enzyme kinetics from Graphpad Prism 6. The determined IC_{50} for L-captopril was 6.6µM and was comparable to an earlier report.[175] Its K_i was 630nM corresponding to a ligand efficiency of 0.51. For compound 3, the determined IC_{50} and K_i were 270nM and 13nM, respectively, resulting in a remarkable ligand efficiency of 0.99 and likely the highest ever determined for a reported MBLi. Incorporation of a single amino acid with phenyl side chain, 5, significantly diminishes its K_i by 576-fold from 0.013nM to 7.5µM. Given the observed data from both biochemical assays, the unexpected observed potency by the addition of the carboxylic acid group adjacent to the N1 position is likely due to both of its electronic effect on 1,2,-HPT zinc binding affinity and its interaction with nearby residues within the active site. Removal of the carboxylic acid from the adjacent N1 position or its displacement by three atomic spacers through amino acid addition could explain the significant of loss of inhibition potency in 2, 4 and 5.

Table 3.2. Inhibitory activity against VIM-2

Compound	K_i (µM)	IC_{50} (µM)	LE
L-Captopril	0.63	6.6 (4.4)	0.51
3	0.013	0.27	0.99
5	7.5	67.9	0.34

3.4 Computational Modeling Study

Numerous structural studies of VIM-2 have been carried out previously to examine the exact mechanism of ligand binding for various well-establish MBLi's.[175] To better understand the mechanism of MBL binding, we examined the previously solved X-ray structures complexes of VIM-2. As shown in Figure 3.5, in the absence of ligands, two water molecules (W1 and W2) with W1 acting as a bridge chelate between the two Zn1 and Zn2 ions. Zn1 is in tetra-coordinated to H114, H116, H179 and W1 while Zn2 is in pen-coordinated to D118, S198, H240, W1, and W2. The carboxylates of formic acid displace W2 from Zn2 while the thiolate ion of D-captopril replaces W1 as the bridging chelate. D-captopril also undergoes hydrogen bonding to the amide hydrogen of N210 sidechain and forms a direct salt bridge with R205. Cross-examination with other available MBLs (PDB: 1DD6, 3VQZ, 2QDT, 2FU9) with bound ligands consistently showed thiolate to be the preferred as the bridging chelate over carboxylate when both are present. Given the fact that pyrithione can undergo resonance state to form thiolate ion, the expert mode of binding involves thiolate ion as the bridging chelating atom between the two zinc ions.

To improve our understanding of the potential mode of binding for 3, molecular modeling was carried out using the Schrodinger modeling suites[189]. Docking with Glide into metallo enzymes did not reliably reproduce the structurally observed mode of binding with observed sulfur atoms acting as a bridge chelate between the two zinc ions. Due to

the presence of the charged carboxylate group, the dominant pose observed involved chelation of the carboxylate to the zinc ions. As such, comparative modeling based on the established structurally determined mode of binding was carried out to yield the most consistent model to corroborate with our biochemical data (Figure 3.5 D). The model of **3** binding to VIM-2 was developed through simple overlaying of the chelating S and O atoms from 1,2-HPT moiety and its carboxylate group to the two crystallographic water sites (W1 and W2) and D-captopril's carboxylate group.

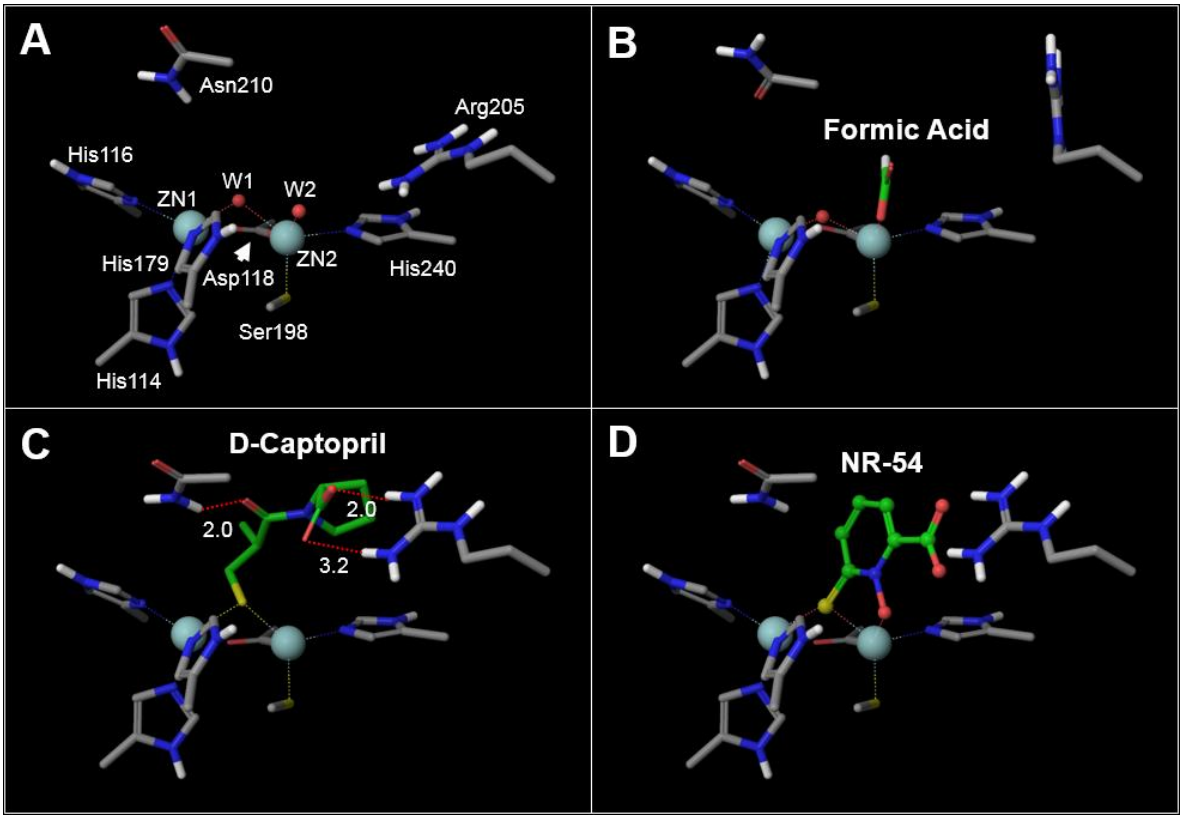


Figure 3.5. The vim-2 active site (A) without ligand (4BZ3), with (B) formic acid (C) D-captopril (4C1E) and (D) model compound 3. The chelated waters are labeled as W1 and W2.

3.5 Combination Therapy against VIM-2 Expressed Bacterial Cell

To demonstrate compound **3** clinical relevance, we further demonstrated its ability to re-sensitize amoxicillin efficacy against VIM-2 expressing *E. coli*. Previously reported E. coli DH10B and VIM-2/pBCSK cloned E. coli strains were cultured on a 0.8g/100ml nutrient agar plate at pH 7.0 and 37^{o}C. The bacterial growth medium was diluted in nutrient broth (NB) to a concentration-absorbance of 1.5 at OD_{600nm} and incubated overnight in 10ml capped culture tubes with shaking. An overnight culture of the bacterial strain was subcultured to an optical density of 0.06 at OD_{600nm} into the NB medium. Control experiments were conducted by growing each bacteria with either (i) medium only (no antibiotic), (ii) medium and dimethyl sulfoxide (DMSO) (amount used to administer dissolved compound) or (iii) medium and antibiotics and β-lactamase inhibitor, Amoxicillin (50μM), Clavulanic acid (25μM)). Each of the chosen compounds was tested at 25μM in the absence or the presence of 50μM Amoxicillin against both strains of *E. coli*.

The sub-cultured bacterial suspension was seeded at max 200μl into the wells of a 96 well microtiter plate. Nutrient medium was added to a subset of the wells to serve as a blank. Samples were then incubated at 37°C and shaken at 200rpm for 18h. The absorbance was measured on an ELIZA plate reader at 600nm and analyzed with the Gen5TM software suite (version 1.08). Both Clavulanate and SAHA were included as a control. As shown in Table 3.3, VIM-2 expression *E. coli* is resistant to the combination therapy with amoxicillin

and clavulanate. Both L-captopril (here L-captopril not D-captopril discussed) and three do not exhibit significant growth inhibition alone against either *E. coli* but are highly effective in inhibiting the growth of VIM-2 expression *E. coli* in combination antibacterial therapy with amoxicillin.

Table 3.3. Single Dose cell viability assay

Compound	*E. coli*		VIM-2 expressing *E. coli*	
	Amox (-)	Amox (+)	Amox (-)	Amox (+)
Amox	-	3.6	-	77
Clav	86	2.9	93	57
SAHA	83	3.6	79	60
L-Captopril	86	5.0	87	9.3
3	77	3.6	61	3.2

All compounds were tested at 25μM in the absence (-) or presence of 50μM amoxicillin (+).

To further explore the therapeutic potential of 1-hydroxypyridine-2-thiones-6-carboxylic acid, **3**, its stability in mouse and human plasma were also assessed. The compound was spiked into pooled mouse or human plasma at a final concentration of 10 μM (0.1% DMSO) and incubated at 37°C. The incubations were performed in triplicate. At different time points, 40μL of incubation mixture were removed and immediately quenched by 120 μL of acetonitrile containing appropriate internal standard and 0.5%

formic acid. The quenched samples were centrifuged at 14,000 rpm for 5 min at 4°C. The supernatants were injected into LC-MS/MS for analysis. The electrospray mass spectrum shows two prominent peaks that indicate these compounds exist predominantly as dimers but are at constant equilibrium to one another. Plasma stability was evaluated by monitoring the disappearance of both monomer and dimer of each compound over a certain period. The peak area ratios of the analysis versus internal standard were used to calculate the remaining percentage at each time point. The natural logarithm of remaining percentage is plotted against time, and the gradient of the line is used to determine the half-life $t^{1/2}$ in plasma. While the half-life for both compounds was determined to be quite short in mouse plasma, the half-life's of 1-hydroxypyridine-2-thiones-6-carboxylic acid in human plasma were determined to be 11.7 and 12.7 mins, respectively (Table 3.4), suggesting further PK optimization is necessary.

Table 3.4. Plasma Stability Assay

Compound	CC_{50} (μM)	EC_{50} (μM)*	TI	Human Plasma $t_{1/2}$ (mins)	Mouse Plasma $t_{1/2}$ (mins)
NR-54	97	0.110	880	11.7	12.7

* – For combination therapy, the EC_{50} of 3 was carried out in the presence of 50μM amoxicillin.
Therapeutic index (TI) = CC_{50}/IC_{50}.
$t_{1/2}$ – Half-life.

3.6 Conclusion

Many multi-drug resistance bacteria pathogens use metallo-β-lactamase enzymes to hydrolyze β-lactam rings found in many antibiotics, rendering them ineffective. In the metallo-β-lactamase study, we report the inhibition activity of three representative classes of zinc-specific chelators as potential MBL inhibitors, namely hydroxamic acid, cyclic hydroxamic acid and pyrithione.

The study demonstrated the therapeutic potential of the 1-hydroxypyridine-2-thiones-6-carboxylic acid, 3, as a potent nanomolar inhibitor of metallo-β-lactamase with broad spectrum activity and low cytotoxicity and can be used in combination antibacterial therapy to restore existing β-lactam antibiotic activity. In a further study, the molecular docking model supports a better understanding of its mechanism of action. It is the first study of 1,2-HPT analogs as a potent and a novel MBLi and should provide an alternative platform for further development against other MBLs.

3.7. Material and Method

3.7.1. Cell Culture and Single Dose Screening

E. coli DH10B and VIM-2/pBCSK cloned E. coli strain were used for the assay. The original cells were cultured on Nutrient Agar (0.8g/100ml, pH 7.0) plate and incubated at 37ºC incubator. The bacterial growth from nutrient agar plate was diluted into nutrient broth (NB) to a concentration of 1.5 OD_{600} and incubated overnight in 10 ml capped culture tubes by shaking at 37 ºC.

An overnight culture of the bacterial strain was subcultured to an optical density at 600 nm (OD_{600}) of 0.06 into the NB medium at 37 ºC. Control experiments were conducted by growing bacteria with either (i) medium only (no antibiotic), (ii) medium and dimethyl sulfoxide (DMSO) (amount used to administer dissolved compound) or (iii) medium and antibiotics and β-lactamase inhibitor, Amoxicillin (25µM), Clavulanic acid (25µM)). Our compounds were used on 25µM only and 25µM with Amoxicillin (25µM). The sub-cultured bacterial suspension was seeded (max 200µl) into the wells of a 96 well microtiter plate by using the multichannel pipette. Medium alone was added to a subset of the wells to serve as a blank. Samples were then incubated at 37°C and shaken at 200 rpm for 18 h. The absorbance was measured on an ELIZA plate reader at 600 nm and analyzed with the Gen5^{TM} software suite (version 1.08).

3.7.2. Half Maximal Effective Concentration (EC50)

The bacterial culture was prepared as described above. The diluted subculture bacteria in NB medium was then set up to a final volume of 200µl in clear flat-bottom 96-well plates containing various fold of each tested compounds (0.001µM ~50µM). The mixing of the bacterial culture plate was then incubated in a 37 °C stationary shaken incubator at 200rpm for 18h before measuring their OD_{600}. The EC_{50} were obtained by fitting binding data to a sigmoidal dose-response equation using GraphPad Prism 6.

3.7.3. Protein Expression and Purification

For our biochemical assay, the bla_{VIM-2} gene, from a clinical strain of *P. aeruginosa*, was expressed using the pET24a (+) vector. The pET24a-VIM-2 plasmid was transformed into competent BL21 (DE3) *E. coli* cells. The cells were plated onto an LB-agar plate with kanamycin (25µg/mL) and incubated overnight at 37°C. A single colony was used to inoculate 50mL of LB, containing 25µg/mL kanamycin, and the culture was shaken overnight at 37°C. From the overnight culture, 10mL were transferred to 4 X 1L LB medium containing 25µg/mL kanamycin. The cultures were grown at 37°C until the optical density (OD_{600nm}) reached 0.6-0.8, at which point protein production was induced with IPTG (0.5mM) and ZnCl2 (100µM). The temperature was reduced to 20°C, and the cells were shaken for an additional 18h. The cultures were harvested by centrifugation (8000g speed not weight) for 10min at 4°C. The resulting pellets were re-suspended with 25mL of 50mM HEPES, pH 7.5, containing 500mM NaCl (buffer B). The cells were lysed with three passes through a French Press. The lysate was centrifuged (15K x g) for 30min

at 4°C. The supernatant liquid was dialyzed against 2L of 50mM HEPES, pH 7.5 (buffer A), for 4h. Buffer A was used to equilibrate a 25mL Q-Sepharose column using an FPLC. The sample was loaded onto the column, and proteins were eluted with a linear gradient 0-500mM NaCl with buffer B. Fractions containing VIM-2, determined by SDS-PAGE, were pooled and concentrated to 2-3 mL in an Amicon ultraconcentrated equipped a YM-10 membrane. Further purification was conducted with a Sephacryl S-200 gel filtration column using 50mM HEPES, pH 7.5, containing 150mM NaCl. Fractions containing pure VIM-2 were pooled, and metal analysis was performed.

3.7.4 Cytotoxicity Test against Human Embryonic Kidney Cells

Human embryonic kidney cell line (HEK 293) was grown in DMEM (Dulbecco's modifications of eagle's medium with L-glutamine & 4.5G/L glucose) supplemented with fetal bovine serum 100 units/ml of penicillin G and 0.1 mg/ml of streptomycin sulfate in a humidified atmosphere of a 5% CO_2 at 37°C.

Trypsin-treated monolayer HEK293 cell line is harvested, and cell counted using Vi-cell machine (Beckman Coulter Com.). The cells were seeded at a concentration of 8×10^4 cells/well in 200µl culture medium and incubated at 37ºC in 5 % CO_2 incubator for 24 hrs. After 24 hours, when the monolayer formed, the supernatant was removed and added fresh media with different concentrations of compounds (0.001 to 100µM) and kept for incubation at 37ºC in 5 % CO_2 incubator for 72h.

After 72 hours, 15µl of MTT (5mg/ml) dye was added to each well and the plates were incubated for 4 hours at 37^{o}C in 5% CO_2 incubator. To prepare plates for reading spins plates in swinging bucket rotor down 1,500xg for 10 mins to remove the supernatant. Add 200µl of dimethyl sulfoxide (DMSO) and the plates were gently shaken to solubilize the formed formazan for 30 min. The absorbance was measured using a microplate reader at wavelength 590 nm. The IC_{50} values were obtained by fitting binding data to a sigmoidal dose-response equation using GraphPad Prism 6.

3.7.5. Synthesis of Compound 1

Unless otherwise stated all chemicals were purchased from commercial suppliers and used as received. Flash silica gel chromatography was performed using standard commercial source (40-60 µm mesh) Inert reactions were carried out under a nitrogen atmosphere (balloon), H1 NMR spectra were recorded at ambient temperature on a 300 MHz Varian FT-NMR instrument. Mass spectra were obtained in the department of chemistry, University of Minnesota. The synthesis of **1** started with the 6-chloropyridine 3-carboxylic acid **6**. The N-oxidation with H_2O_2 in trifluoroacetic acid anhydride (TFAA) did not lead to the completion of the reaction; a substantial amount of starting material was left even after the addition of the additional 2-3 equivalent of hydrogen peroxide. Further, separation of N-oxide from starting acid became cumbersome and led to unacceptable yields. Instead, we started the synthesis with the methyl ester of **7.** The N-oxidation was carried out using urea-hydrogen peroxide addition complex in trifluoroacetic acid

anhydride. The 6-chloro N-oxide **8** was converted into 6-oxo compound **9** using TFAA keeping the carboxylic acid ester intact. It was then benzylated with benzyl bromide in the presence of potassium carbonate. The methyl ester of N-benzyl compound **10** was hydrolyzed with NaOH in methanol. Initially, we tried coupling with (R) methyl 2-amino-2-phenylacetate hydrochloride **12** under a variety of peptide coupling condition. Racemization occurred in almost all cases and also gave poor yields after chromatography. We could not avoid the racemization but gave good yields with coupling reagent combination- EDC, HOBt, and DIPEA in DMF. Attempts to debenzylation with Pd/C/H2 was unsuccessful; all most all conditions the benzyloxy group was cleaved leaving 6-hydroxy pyridine derivative. However, first hydrolyzing the ester to a carboxylic acid with LiOH followed by hydrogenation, gave the desired compound **1** in acceptable yields with 80% enantiomer excess (chiral HPLC).

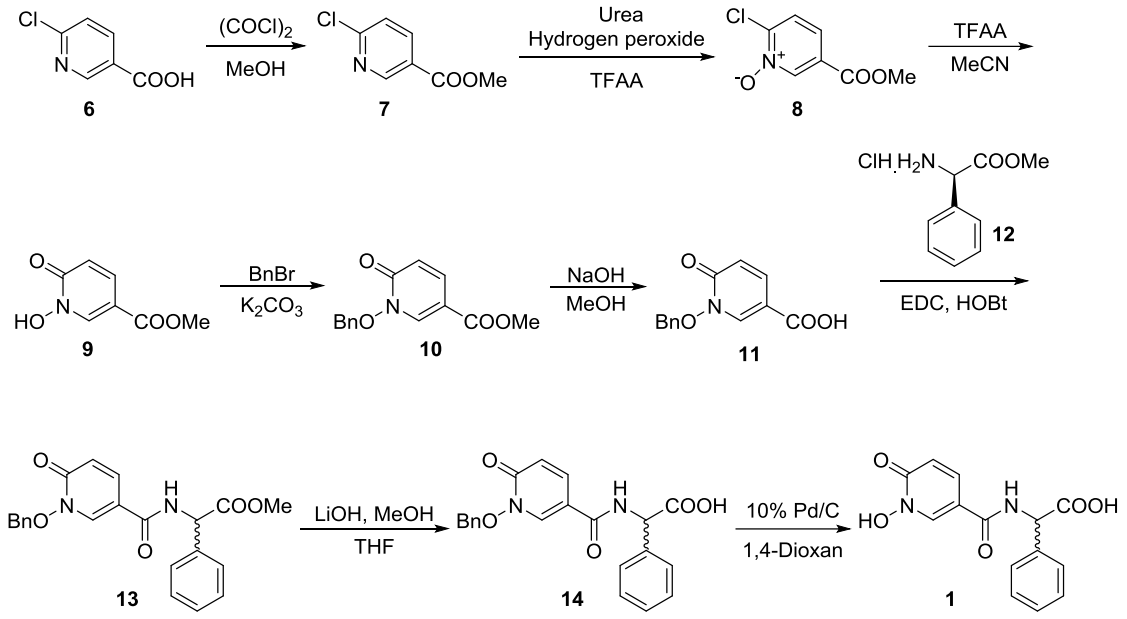


Figure 3.6. Synthesis of Compound 1

CHAPTER 4

CONTRIBUTION AND FUTURE DIRECTIONS

4.1 Contribution

The clinical overuse of β-lactam antibiotics has created major evolutionary pressures in bacteria to evolve towards drug resistance. Bacterial resistance to penicillin, cephalosporins, monobactams and carbapenems is most often mediated by expression of β-lactamases, which have emerged and evolved rapidly in both gram-positive and negative bacteria. A novel approach to countering bacterial β-lactamases is the treatment of a β-lactam antibiotic in combination with a β-lactamase inhibitor. Several combination therapies are currently FDA approved and available to use, containing inhibitors clavulanic acid, sulbactam, tazobactam, and avibactam. However, these combination therapies are not active against all β-lactamases and several ESKAPE pathogen resistant. Currently, limited efforts in β-lactamase inhibitor discovery rely exclusively on random screening, which scarcely contributes to the fundamental understanding of the mechanism of inhibition and which has not yielded inhibitors sufficiently potent for further development. Newer strategies need to be developed to counteract β-lactamase-mediated multi-drug resistance pathogens. One of the most promising approaches to the resistance problem is the novel design of β-lactamase inhibitor related by the tertiary structure of the enzyme and its mechanism of action.

By focusing on characterizing the inhibitor binding site and assembling pharmacophore models, our research aims at gaining a fundamental understanding of β-lactamase inhibition and constructing a platform for inhibitor design. Through our previous data, the sulfonyl oxadiazoles alone do not exhibit much, if any, growth inhibition against

the bacterial strain tested. However, when treated with amoxicillin combinational, the results showed that the sulfonyl oxadiazoles are significantly more effective as β-lactamase inhibitors of the serine-type β-lactamase than current drug clavulanic acid. These a novel class of non-β-lactam β-lactamase inhibitors with optimal therapeutic properties for drug development in combination therapy including 1) synergistic activity against *Acinetobacter baumannii* and MRSA drug resistance pathogens, 2) low cytotoxicity against human cells comparable existing drug and 3) improved chemical stability over traditional β-lactamase inhibitors drugs. This development of a novel class of non-β-lactam β-lactamase inhibitors study is a highly impacted model for the study of potent inhibition against β-lactamase. The insight gained will provide the molecular basis for drug development of a new class of β-lactamase inhibitors for combination therapy.

In our further finding, amoxicillin induced the overexpression of β-lactamase that led to multi-drug resistance. Proteomic analysis of cell-free supernatant confirmed for the first time the presence of TEM-type β-lactamase. β-lactamase inhibitors identified using the isolated cell-free supernatant effectively resuscitate amoxicillin antibiotic activity. The presence of the TEM gene in *A. baumannii* ATCC 19606 strains was recently announced. It is the first report that amoxicillin-induced the overexpression of TEM β-lactamase resulting in multidrug resistance. Ability to characterize and identify inhibitors against the multi-drug resistance causing β-lactamase present a rational paradigm shift in developing a precise combination therapy for overcoming antibiotic-induced multidrug resistance.

We also identified a new class of metallo β-lactamase inhibitor and demonstrated the therapeutic potential of the 1-hydroxypyridine-2(*1H*)-thiones-6-carboxylic acid compound. The data show this inhibitor can restore the antibiotic activity of amoxicillin against metallo β–lactamase producing bacteria pathogen. This development of 1, 2-HPT class of β-lactamase inhibitors study is first time report and highly impact model for the study of potent inhibition against metallo-β-lactamase.

Through on bacterial drug resistance, our research will be identifying and characterizing sensitizing agents against broad spectrum β-lactam drug-resistant bacterial pathogens. All the researches were based on SAR cell activity study, proteomics analysis, and enzymatic activity study against both serine and metallo β-lactamase laboratory assay studies and computational modeling work. This study will shed light on the new antibiotics mechanism of action for new compounds with great potential for new therapy.

4.2 Future Directions

β-lactam drug resistance remains a major public health threat. Developments of a single new β-lactamase inhibitor that resuscitates existing β-lactam antibiotic arsenals provides the maximum opportunity for novel combination antimicrobial therapy for combating drug resistance pathogens. Our sulfonyl oxadiazoles and thiadiazoles compounds is a novel and non-β-lactam β-lactamase inhibitor in combination with β-lactam antibiotic amoxicillin. *In vitro* studies show that sulfonyl oxadiazoles and thiadiazoles compounds may restore the broad-spectrum activity of amoxicillin against class A, and class C, serine β-lactamases. Also, we show the discovery of 1-hydroxypyridine-2(1H)-thiones-6-carboxylic acid as a potent metallo β-lactamases inhibitor with low nM K_i and low cytotoxicity against human cell lines.

Most modern drug design and discovery projects start with protein target identification and verification to obtain its target of the drug [190]. For protein structure-based drug design, the three-dimensional structure of the protein is essentially needed to be determined by high-resolution experimental methods such as X-ray protein crystallization study or NMR. Our protein modeling is based on X- ray protein crystal structures. Advances in crystallization methods and computational approaches have provided researchers with rapid and reliable access to three-dimensional structural information on a wide variety of protein drug targets. Structural base approach on protein–ligand complexes can eliminate much of the complexity involved in design and discovery of prospective drug leads [191]. Our research group developed 1.6 Å and 2.68 Å resolution

of TEM-1 and BlaC β-lactamases crystal structure by using hanging drop methods. We need to optimize our co-crystallization method to confirm the mechanism of our sulfonyl oxadiazoles and 1-hydroxypyridine-2-thiones-6-carboxylic acid compounds inhibition of β-lactamases. This application of X-ray crystallography protein–ligand β-lactamases complexes and further molecular modeling using this model can provide valuable insight into the optimization of the molecular interactions of a drug–protein complex to achieve potency and selectivity of a drug candidate. Additionally, these structural modifications help to improve potency and selectivity and other considerations that include solubility, bioavailability, metabolism, distribution, toxicology, and chemical stability in structure-based drug design.

Pharmacokinetics (pK) study with human plasma stability is important to obtain when planning for human clinical studies. Our sulfonyl oxadiazoles compound shows only a minutes of half-life time in human and mouse plasma pK studies. We need to improve delivery stability in human plasma. Therefore, compound water solubility is one of the important parameters to achieve desired concentration of drug in systemic circulation for desired pharmacological response. Any drug to be absorbed must be present in the form of a solution at the site of absorption. Our sulfonyl oxadiazoles compound has a low water solubility so; it can use with DMSO soluble. Currently, various techniques can be used to enhance the solubility of the drugs. We need to improve the water soluble problem with compound chemical stability over traditional β-lactamase inhibitors drugs.

Finally, our research examines a novel class of non-β-lactam β-lactamase inhibitors with optimal therapeutic properties for drug development in combination therapy in synergistic activity against *A. baumannii* and MRSA drug resistance ESKAPE pathogens, with low cytotoxicity against human cells and identified target β-lactamase from resistance strains. With our improved future work, our study will construct a platform for the design of a novel class of β-lactamase inhibitors for combination antibacterial therapy.

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