OPTIMIZATION OF β-LACTAM/β-LACTAMASE INHIBITOR DOSING

A Dissertation Presented to

The Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of

The Requirements for the Degree

Doctor of Philosophy

By

Henrietta Abodakpi

December 2018

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TABLE OF CONTENTS

ABSTRACT

Infections caused by extended-spectrum β-lactamase (ESBL)-producing Gram-negative bacteria are associated with increased mortality and cost of care. These pathogens are particularly challenging to treat due to their resistance to many antibiotics. Although β-lactam/β-lactamase inhibitors have proven *in vitro* activity against ESBL-producing pathogens, their use for ESBL infections remains controversial due to reports of treatment failure and a limited understanding of the pharmacokinetic/pharmacodynamics (PK/PD) governing these combinations. As a result, carbapenems have remained the preferred agents for treating infections due to ESBL-producing organisms. However, the heavy reliance on these carbapenems may have contributed to the rapid dissemination of carbapenemases that further limit treatment options. This has heightened the need to optimize the use of available antibiotics against these clinically-challenging pathogens.

Thus, the goal of this project was to improve the rational basis for the dosing and pairing of β-lactams and β-lactamase inhibitors that would extend their usefulness against resistant organisms. We used a commercially available and widely used combination (piperacillin/tazobactam) to illustrate our approach. In our third chapter, we explored the prevalence and mechanism of resistance for extended-spectrum β-lactamases produced by *Klebsiella pneumoniae* isolated at a local tertiary care hospital. In so doing, we were able to focus our efforts on the most clinically-relevant β-lactamases to our geographic region. Using a collection of β-lactamase-producing clinical isolates, in our fourth chapter, we demonstrated a novel approach to susceptibility profiling for β-lactam/β-lactamase inhibitor combinations that could better inform dosing. Using a theoretical concept called the

instantaneous MIC (MICi), we illustrated how susceptibility may be dependent on inhibitor concentrations and differed for each clinical isolate. In chapter 4, we also illustrated that the efficacy of combinations such as piperacillin/tazobactam was dependent on the concentration-response relationship between the β-lactamase producing isolate and the inhibitor (tazobactam). We showed that the current fixed dose ratio for piperacillin/tazobactam was inadequate against our test isolates, even when they tested susceptible by current susceptibility standards. Instead, escalated exposures of tazobactam were required to optimize dosing for certain clinical isolates. Finally, in chapter 5, we showed that for isolates that failed to respond to optimal piperacillin/tazobactam dosing, pairing piperacillin to an alternative inhibitor improved efficacy.

The results from our studies have contributed to a better understanding of the PK/PD governing the joint effect of β-lactam/β-lactamase inhibitor combinations like piperacillin/tazobactam. Moreover, our results also highlighted additional considerations for the optimal pairing of β-lactams and β-lactamase inhibitors. Our findings should provide an improved scheme for the design and evaluation of these combinations for clinical use.

LIST OF ABBREVIATIONS

AUC/MIC: Area under the 24 h concentration-time curve divided by MIC

CLSI: Clinical Laboratory Standards Institute

C_{max}/MIC: Maximum serum concentration divided by MIC

CMY β-lactamase: Cephamycin β-lactamase

CTX-M β-lactamase: Cefotaxime β-lactamase

ESBL: Extended-spectrum β-lactamase

***f*T>MIC**: Free-time above MIC

***f*T>MICi**: Free-time above instantaneous MIC

HFIM: Hollowfiber infection model

IMP β-lactamase: Imipenemase β-lactamase

KPC: *Klebsiella pneumoniae* carbapenemase

LC-MS/MS: Liquid chromatography-tandem mass spectrometry

MIC: Minimum inhibitory concentration

MICi: Instantaneous MIC

NDM β-lactamase: New Delhi metallo-β-lactamase

PK/PD: Pharmacokinetics/Pharmacodynamics

SHV β-lactamase: Sulfhydryl variable β-lactamase

TEM β-lactamase: Temoniera β-lactamase

OXA: Oxacillinase

VIM β-lactamase: Verona integron-encoded metallo-β-lactamase

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

The World Health Organization recognizes antimicrobial resistance as one of the greatest medical challenges to human health. According to the Centers for Disease Control (CDC), resistant bacteria account for more than 2 million illnesses and 23,000 deaths annually in the United States alone. In healthcare settings, infections caused by resistant Gram-negative bacteria may be especially challenging to treat because they often present with multiple mechanisms of resistance. These infections are commonly associated with inappropriate or suboptimal therapy. Several studies have shown that resistance in Gram-negative bacteria leads to additional costs and length of hospitalization, as well as high morbidity and mortality (Kollef, Sherman et al. 1999, Ibrahim, Sherman et al. 2000, Slama 2008, Mauldin, Salgado et al. 2010, Tam, Rogers et al. 2010, Cerceo, Deitelzweig et al. 2016). Although resistance to key antibiotics continues to rise, there is a shortage of new drug candidates in early development for the treatment of Gram-negative bacterial infections. Thus, there is a pressing need for a critical evaluation of how to optimally use existing agents and those under development.

1.1 Mechanisms of Resistance

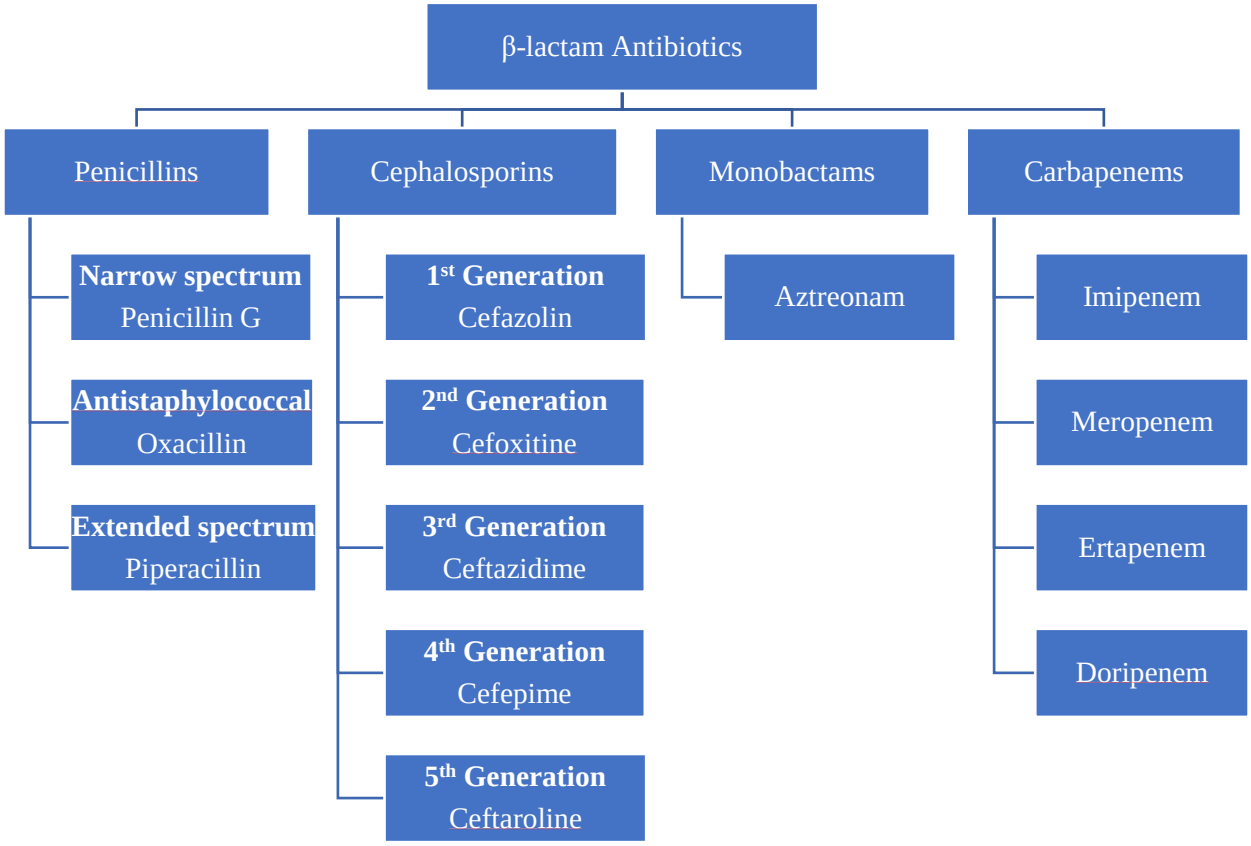
Gram-negative bacteria are highly adaptable pathogens capable of intrinsic and acquired resistance to multiple classes of antibiotics. Resistance to these agents is mediated through a variety of mechanisms that include: decreased permeability through loss of porins, extrusion of drug through overexpression of transmembrane efflux pumps, decreased binding of drugs through target mutations, and the production of inactivating enzymes such as β-lactamases and group transferases. Of these mechanisms, the production of β-lactamases is one of the most

commonly encountered resistance mechanisms in Gram-negative bacteria. The production of these β-lactamases has significant clinical implications because they target β-lactams, which are an important class of antibiotics in the treatment of Gram-negative bacterial infections.

1.2 β-lactam Antibiotics

β-lactams refer to a class of antibiotics that possess a characteristic β-lactam ring and a long history of clinical success. They cause bacterial cell death by binding irreversibly to penicillin-binding proteins (PBP) required for cell wall synthesis. The first β-lactam, penicillin, was first discovered by Alexander Fleming and revolutionized the treatment of bacterial infections. However, β-lactamase-mediated resistance (via penicillinases) to penicillin soon emerged, which led to the design of structural analogs capable of withstanding the effects of these early β-lactamases. As β-lactamases have continued to evolve, increasingly potent and/or broader spectrum β-lactams (such as cephalosporins and carbapenems) have been developed. Nonetheless, this approach alone has been insufficient to limit the activity of newer β-lactamases. Figure 1.1 highlights the different classes of β-lactams that are clinically available.

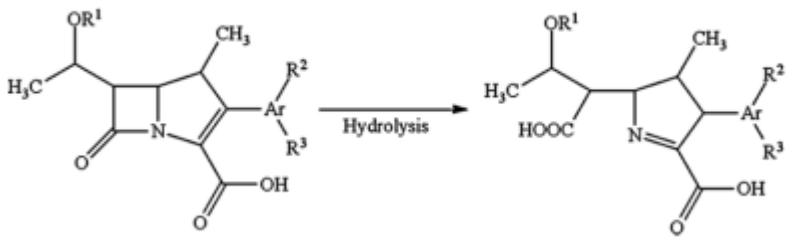
Figure 1.1. Different Families of β-lactam Antibiotics and Representative Examples



1.3 Clinically Relevant β-lactamases

β-lactamases represent a heterogenous group of enzymes capable of hydrolyzing and inactivating the core β-lactam ring of β-lactam antibiotics as shown below.

Figure 1.2. Hydrolysis of the β-lactam Ring by a β-lactamase



β-lactamases are classified either according to substrate and inhibitor profiles (Bush-Jacoby classification) or primary amino acid sequences (the Ambler classification). The Bush-Jacoby classification comprises 7 major numerical functional classes (1, 2b, 2be, 2d, 2df, 2f and 3) that correlate to the Ambler classes. The Ambler classification groups β-lactamases into classes A, B, C and D as shown in Table 1.1 (Bush and Jacoby 2010). Enzymes belonging to classes A, C and D possess a serine residue at their active site while class B enzymes have a characteristic Zn^{2+} that is required for activity (Bush and Jacoby 2010). According to the 2010 data from the SENTRY Antimicrobial Surveillance Program, 175 (89.7%) Enterobacteriaceae bloodstream isolates screened from 26 U.S. hospitals carried at least one β-lactamase gene (Castanheira, Farrell et al. 2013). In the United States, class A enzymes are the predominant β-lactamases reported in Gram-negative species. Nonetheless, the prevalence of other β-lactamases is also rising.

Table 1.1. Major Classes of β-lactamases of Clinical Importance

Ambler Class	Type of β-lactamase	Preferred Substrate(s)	Representative Enzymes
A	Narrow spectrum (penicillinase)	Penicillins, early cephalosporins	TEM-1/-2, SHV-1
A	Extended spectrum	Narrow and extended spectrum penicillins, cephalosporins	SHV-2, CTX-M-15
A	Serine carbapenemases	All β-lactams	KPC-2, KPC-3
B	Metallo-β-lactamases	β-lactams except aztreonam	IMP-1, VIM-1
C	Cephalosporinases	Cephalosporins	AmpC, CYM-2
D	Oxacillinases	Oxacillin/cloxacillin	OXA-1, OXA-2
D	Cephalosporinases	Oxacillin/cloxacillin, cephalosporins	OXA-11, OXA-15
D	Carbapenemases	Oxacillin, carbapenems	OXA-48

1.3.1 Class A β-lactamases

Genes encoding class A enzymes are generally located on plasmids which can be transferred between bacteria. These plasmids often co-harbor resistance mechanisms for other antibiotic classes, such as fluoroquinolones, thus further limiting treatment options. Class A β-lactamases

include penicillinases, extended-spectrum β-lactamases (ESBL) and carbapenemases. Penicillinases (such as SHV-1, TEM-1) primarily hydrolyze penicillins and early generation cephalosporins (Bush 2010, Bush and Jacoby 2010*)*. In addition to penicillins, ESBLs (of the CTX-M SHV, TEM subtypes) hydrolyze nearly all cephalosporins and monobactams (Bush 2010). Meanwhile, class A carbapenems (such as *Klebsiella pneumoniae* carbapenemases or KPCs) are especially worrisome because they can confer resistance to all currently available β-lactams. The 2010 and 2014 SENTRY data found $bla_{\text{CTX-M-15}}$ and $bla_{\text{KPC-2}}$ to be the most prevalent β-lactamase genes across all Gram-negative species, with the highest rates encountered in the Mid-Atlantic region of the country (Castanheira, Farrell et al. 2013, Castanheira, Farrell et al. 2014). In both surveillance reports, *Klebsiella pneumoniae* and *Escherichia coli* were key nosocomial pathogens that harbored these β-lactamases.

1.3.2 Class B β-lactamases

Class B β-lactamases (which are also plasmid-borne) are known as metallo-β-lactamases. Examples of such β-lactamases include the New Delhi metallo-β-lactamases (NDM), imipenemase (IMP) and Verona integron-encoded metallo-β-lactamases (VIM). These enzymes are capable of hydrolyzing all β-lactams except monobactams such as aztreonam (Bush 2010, Bush and Jacoby 2010). These β-lactamases are commonly found in carbapenem-resistant *Pseudomonas aeruginosa* and are growing increasingly prevalent in Enterobacteriaceae.

1.3.3 Class C β-lactamases

Class C enzymes are cephalosporinases (e.g. AmpC, CMY-2 enzymes) that are mostly chromosomally-encoded and can hydrolyze most cephalosporins (Bush and Jacoby 2010). These enzymes (especially the chromosomally-mediated ones) are known to be inducible enzymes that can cause resistance development to cephalosporins during treatment. These enzymes are inherent to species such as *Morganella, Serratia, Pseudomonas, Acinetobacter, Citrobacter and Enterobacter*. AmpC enzymes may also be encoded in plasmids and found in Enterobacteriaceae.

1.3.4 Class D β-lactamases

Finally, class D enzymes are oxacillinases or OXA enzymes named for their high hydrolytic activity against oxacillin. However, these enzymes are a diverse group comprised of penicillinases (OXA-1, OXA-2 and OXA-3) with narrow hydrolytic capability, extended-spectrum cephalosporinases (OXA-11, OXA-15) and carbapenemases such as the OXA-48 group (Bush and Jacoby 2010). The OXA enzymes may be chromosomally encoded or plasmid mediated among *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and some Enterobacteriaceae.

1.4 β-lactamase Inhibitors

The use of β-lactamase inhibitors is considered one of the most successful approaches for restoring β-lactam efficacy against β-lactamase producing organisms. Currently approved β-lactamase inhibitors mostly lack significant antibacterial effect at clinically relevant doses, but

inhibit β-lactamases to preserve the efficacy of β-lactams. They are unavailable as stand-alone agents: instead, they are co-formulated commercially with a partner β-lactam.

1.4.1 First Generation β-lactamase Inhibitors

The first generation of β-lactamase inhibitors include clavulanic acid, sulbactam and tazobactam, which are commercially available as combinations such as amoxicillin/clavulanic acid, ampicillin/sulbactam and piperacillin/tazobactam. All three inhibitors share a mechanism of action: they bind irreversibly to β-lactamases, forming an acyl-enzyme complex that shields partner β-lactam molecules from hydrolysis (Bush 1988, Wright 1999, Drawz and Bonomo 2010). Sulbactam, clavulanic acid and tazobactam have *in vitro* inhibitory activity against class A penicillinases, conventional and extended-spectrum β-lactamases (Bush 2015). However, they lack inhibitory activity against class A and B carbapenemases, as well as class D β-lactamases. In spite of their shared mechanism of action, sulbactam, clavulanic acid and tazobactam vary in potency and spectrum of activity. Based on IC_{50} and MIC assays, clavulanic acid and tazobactam have shown greater potency (than sulbactam) against ESBL enzymes (Kuck, Jacobus et al. 1989, Wright 1999). Although similar potencies have been reported for clavulanic acid and tazobactam, kinetic studies have illustrated differences in their inhibitory profiles against different β-lactamases (Payne, Cramp et al. 1994, Sader, Tosin et al. 2000).

1.4.2 Newer β-lactamase Inhibitors

Recently, avibactam and vaborbactam, which belong to a class of non-β-lactam inhibitors, have been added to the armamentarium of β-lactamase inhibitors. Avibactam was developed for use in combination with ceftazidime while vaborbactam is available in combination with

meropenem. Avibactam and vaborbactam possess inhibitory activity against Ambler class A and C β-lactamases, but lack activity against class B enzymes (Drawz, Papp-Wallace et al. 2014, Wong and van Duin 2017). Additionally, avibactam exhibits activity against select class D enzymes (Drawz, Papp-Wallace et al. 2014). These novel non-β-lactam inhibitors not only differ structurally from the traditional inhibitors; mechanistically, they bind reversibly to the enzyme active site without (in most cases) being hydrolyzed. This allows for recycling of the inhibitor and potential binding to additional β-lactamase molecules.

Several other non-β-lactam inhibitors, such as relebactam, zidebactam and nacubactam are currently under clinical development. Relebactam is under development for use with imipenem and has shown an inhibitory spectrum similar to avibactam (Wong and van Duin 2017). Pharmacologically, zidebactam and nacubactam perform a unique hybrid function: inactivation of important β-lactamases (i.e. metallo-β-lactamases, KPCs and class D carbapenemases) as well as selective inhibition of penicillin-binding proteins (Moya, Barcelo et al. 2017, Sader, Castanheira et al. 2017). As a result of their unique mechanisms of action, these newer agents have the potential to address lingering challenges in the inhibition of clinically relevant β-lactamases.

1.4.3 Clinical Utility of β-lactamase Inhibitors

While combinations like piperacillin/tazobactam have been widely used for decades, in recent years, there has been renewed interest in their utility against ESBL-producing Enterobacteriaceae. Piperacillin is a semi-synthetic ureidopenicillin with antibiotic activity against both Gram-positive and Gram-negative pathogens (Schoonover LL 1995). When administered alone, piperacillin is susceptible to inactivation by β-lactamases. To circumvent

this problem, it is co-administered with tazobactam to preserve its activity against narrow and extended-spectrum (TEM-, SHV- and CTX-M-type) β-lactamases (Schoonover LL 1995, Drawz and Bonomo 2010).

Traditionally, carbapenems have been considered the drugs of choice for ESBL infections. However, the rapid dissemination of carbapenemases has highlighted the need to evaluate alternative treatment options. β-lactam/β-lactamase inhibitor combinations show *in vitro* activity against ESBL-producing pathogens. In spite of this *in vitro* sensitivity, the role of β-lactam/β-lactamase inhibitor combinations in this setting has been a topic of considerable debate. One of the first studies to address the appropriateness of β-lactam/β-lactamase inhibitor combinations for ESBL bacteremia was a post-hoc analysis featuring data from 6 prospective bacteremia cohorts. In that study, 30 day mortality and length of hospitalization for patients with ESBL *E. coli* bacteremia was comparable for patients treated with β-lactam/β-lactamase inhibitor combinations (amoxicillin/clavulanic acid and piperacillin/tazobactam) or a carbapenem (Rodriguez-Bano, Navarro et al. 2012). Notably, the median piperacillin/tazobactam MIC in that study was quite low (2/4 μg/mL) given the current efficacy breakpoint of ≤ 16/4 μg/mL. Additionally, the bacteremia cases were mostly due to urinary and biliary infections, which are considered low to moderate inoculum infections. Following that landmark report, several observational clinical studies have further evaluated the efficacy of piperacillin/tazobactam for ESBL bacteremia. While some studies have since validated these findings, there is a lack of consensus regarding the efficacy of piperacillin/tazobactam for ESBL infections. The outcomes of key studies are summarized in Table 1.2. Variables such as the infecting pathogen, severity of infection (inoculum size) and primary infection site appear to impact the clinical efficacy of piperacillin/tazobactam (Rodriguez-Bano, Navarro et al. 2012,

Harris, Yin et al. 2015, Ofer-Friedman, Shefler et al. 2015, Tamma, Han et al. 2015, Gutierrez-Gutierrez, Perez-Galera et al. 2016, Ng, Khong et al. 2016). These studies have generally suggested that piperacillin/tazobactam might be associated with positive outcomes for isolates presenting with low MICs (< 8 μg/mL), and for bacteremia secondary to low or moderate inoculum infections (such as urinary and biliary infections). Nonetheless, in a recent randomized clinical trial focusing on bloodstream infections due to ESBL-producing *E. coli* and *K. pneumoniae*, treatment with piperacillin/tazobactam was associated with higher mortality than meropenem (Harris, Tambyah et al. 2018). Consistent with the observational studies, lower mortality rates (for the piperacillin/tazobactam treatment group) were observed in patients whose bacteremia was due to urinary infections. However, there did not appear to be a trend towards worsening outcomes at higher MICs as noted in the observational studies. Altogether, the data from these studies highlight discrepancies between *in vitro* susceptibility and observed clinical efficacy for piperacillin/tazobactam.

Given the broader inhibitory spectra of the new inhibitors, combinations such as ceftazidime/avibactam and meropenem/vaborbactam have been evaluated primarily against carbapenemase-producing organisms. Although both combinations have shown efficacy against KPCs, there have already been reports of resistance to ceftazidime/avibactam. Clinical resistance was first reported for a *K. pneumoniae* isolate harboring KPC-3 obtained from a patient who had no previous exposure to ceftazidime-avibactam (Humphries, Yang et al. 2015). Since that report, there have been clinical cases of resistance development following treatment with ceftazidime-avibactam in isolates harboring KPC-2 and KPC-3 (Giddins, Macesic et al. 2017, Shields, Chen et al. 2017). The rapid emergence of resistance soon after the commercial

availability of ceftazidime/avibactam may further illustrate shortcomings in our assessments and dosing of these combinations.

Table 1.2. Studies Comparing the Efficacy of Piperacillin/Tazobactam versus Carbapenems for ESBL Bacteremia

Author (Year)	Study Design	Primary Organism(s)	Primary Infection Source	30 Day Mortality			
				PTZ[a]	CBP[b]	*P*-value	Interpretation
Rodriguez-Baño (2004)	Prospective	*E. coli*	Urinary/ biliary	9%[c]	17%	> 0.05	Comparable
Harris (2012)	Retrospective	*E. coli* *K. pneumoniae*	Urinary/ biliary	8%	17%	> 0.05	Comparable
Tamma (2015)	Retrospective	*E. coli* *K. pneumoniae*	Catheter	26%	11%	< 0.05	Inferior
Ofer-Friedman (2015)	Retrospective	*E. coli* *K. pneumoniae*	Pneumonia	60%	34%	> 0.05	Comparable
Ng (2016)	Retrospective	*E. coli* *K. pneumoniae*	Urinary	31%	30%	> 0.05	Comparable
Gutiérrez-Gutiérrez (2016)	Retrospective	*E. coli* *K. pneumoniae*	Urinary	10%	14%	> 0.05	Comparable
Harris (2018)	Prospective	*E. coli* *K. pneumoniae*	Urinary	12.3%	3.7%	0.90[d]	Inferior

[a]PTZ = piperacillin/tazobactam; [b]CBP = carbapenem

[c]Composite mortality associated with amoxicillin/clavulanic acid and piperacillin/tazobactam

[d]*P*-value for non-inferiority

1.5 LIMITATIONS IN CURRENT PRACTICE WITH β-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS

1.5.1 Fixed Agent Pairings

The utility of β-lactams/β-lactamase inhibitors may be limited by current practices in the design of these combinations. β-lactams and β-lactamase inhibitors are paired most commonly based on shared pharmacokinetics (such as similar elimination half-lives, distribution and metabolic pathways). While matching the pharmacokinetics of the two agents is key to ensuring the presence of both agents at the site of infection and protecting the integrity of the β-lactam drug, these considerations alone may not infer optimal efficacy against all clinical isolates. Furthermore, when more than 1 β-lactamase is present, individual enzymes may display different affinities and susceptibilities (to the various inhibitors), thus a fixed agent combination may not always be optimal.

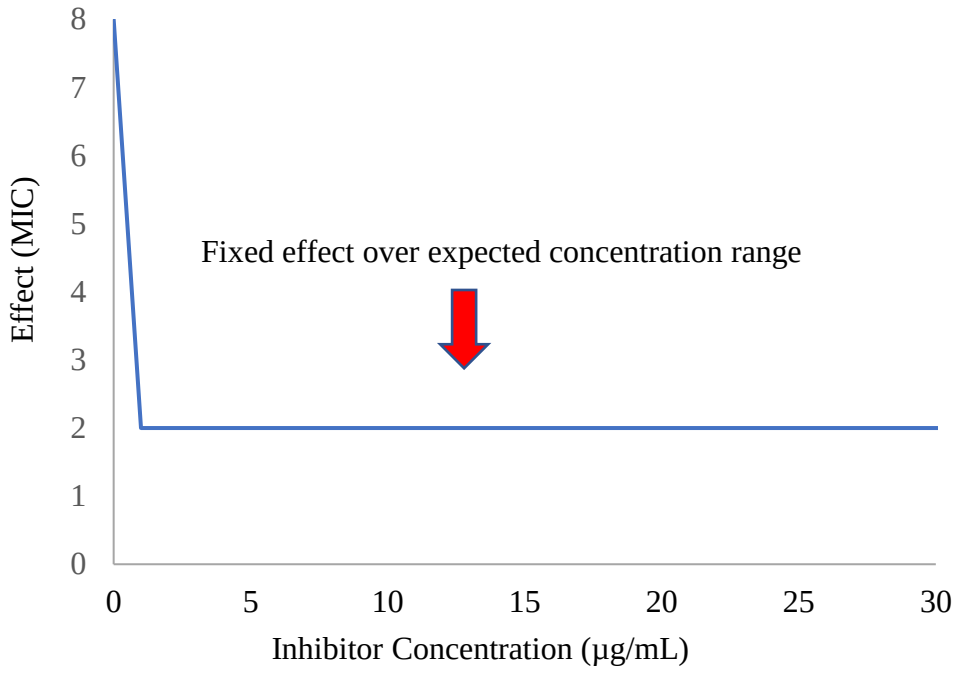
1.5.2 Fixed Dose Ratio Pairings

β-lactam/β-lactamase inhibitor pairs are generally available only as a fixed dose ratio combination. For instance, commercial piperacillin/tazobactam formulations all contain 8:1 (piperacillin to tazobactam). Yet, the rationale for this fixed ratio remains unclear. In some clinical scenarios, enzyme hyperproduction or severe (high) inoculum infections may require modification of this ratio to ensure adequate inhibitor exposures. The exception to this practice is with oral formulations of amoxicillin/clavulanic acid, where compositions of 2:1, 4:1, and 7:1 of β-lactam to inhibitor are available. This may allow some flexibility for clinicians to customize dosing to different clinical scenarios.

1.5.3 Susceptibility Testing

Since the advent of β-lactamase inhibitors, there has been much debate regarding the appropriate approach to assess *in vitro* susceptibility (MIC) for β-lactam/β-lactamase inhibitor combinations. For some combinations, susceptibility is evaluated using a fixed inhibitor concentration (such as 4 μg/mL of tazobactam) against a range of concentrations for the β-lactam (reflective of dynamic concentrations observed *in vivo*). The resulting MIC is designated as susceptible, intermediate or resistant based on established breakpoints for efficacy. This scheme is predicated on the assumption that the magnitude of enhanced susceptibility remains constant in the presence of an inhibitor, neglecting the contribution that varying concentrations may have on susceptibility, as shown in Figure 1.3. Hence, only a partial assessment of efficacy may be achieved with a single inhibitor concentration, and the resulting susceptibility data may not always predict clinical efficacy. For combinations like ampicillin/sulbactam, a fixed 2:1 ratio of β-lactam to inhibitor is used in susceptibility testing. For ampicillin/sulbactam, this latter approach reflects the 2:1 dose ratios used in commercial formulations. Hence, it has been argued that this could better reflect the *in vivo* concentration ratios achieved for the combination and provide improved predictions of *in vivo* efficacy (Pfaller, Barry et al. 1993, Thomson, Miles et al. 1995). However, reported ampicillin/sulbactam ratios in infected tissues and fluids are approximately 1:5 to 1:1 (Foulds 1986). Furthermore, major discrepancies have also been noted between *in vitro* activity and clinical outcomes for ampicillin/sulbactam when used for infections caused by *Acinetobacter* species (Oliveria, Costa et al. 2013). The limitations presented by both approaches underscore a need to identify and adopt a uniform susceptibility testing scheme that correlates more reliably to clinical outcomes.

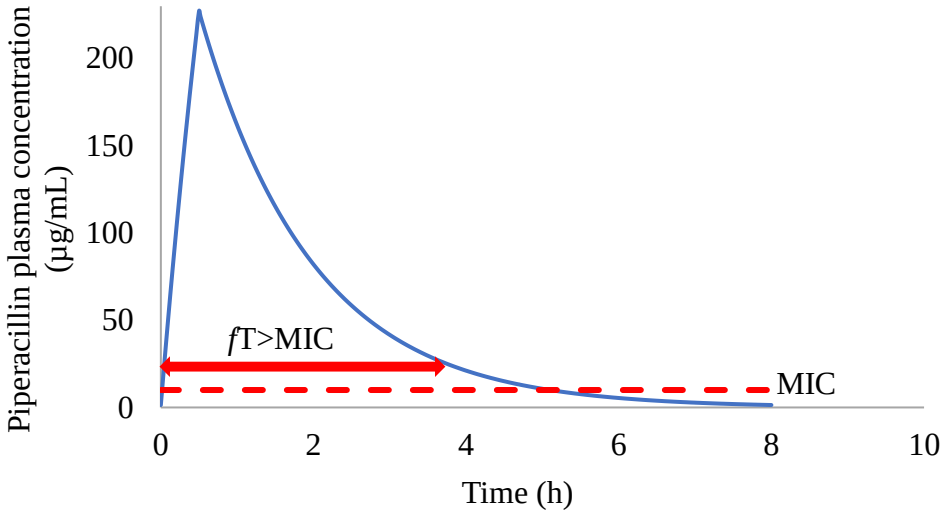
Figure 1.3. Hypothetical Pharmacodynamic Effect of Inhibitor. Inhibitor effect is assumed to remain constant over the range of observed concentrations *in vivo*.



1.5.4 Conventional PK/PD Characterization of Combinations

Pharmacokinetic/pharmacodynamic (PK/PD) indices such as the maximum concentration divided by the MIC (C_{max}/MIC), the area under the 24 h concentration-time curve divided by the MIC (AUC/MIC) and the percentage of free-time above MIC (%*f*T>MIC) are commonly used to characterize killing profiles for various antibiotics. For β-lactams like piperacillin and ceftazidime, the PK/PD index that best correlates with efficacy is the *f*T>MIC, illustrated in Figure 1.4, which represents the duration of the dosing interval that the β-lactam concentration exceeds the MIC.

Figure 1.4. Illustration of *f*T>MIC Index Used to Characterize Killing Activity of β-lactams. Shown is a typical concentration-time profile for piperacillin administered at 8 h intervals. The *f*T>MIC represents the fraction of the dosing interval that free (unbound) piperacillin concentrations exceed the MIC.



However, our understanding of the PK/PD of the β-lactam in combination with a β-lactamase inhibitor is more limited. When an inhibitor is co-administered, it is commonly assumed to have a fixed effect over the entire concentration range observed during a dosing interval. While traditional inhibitors lack appreciable killing ability, they are more likely to impact susceptibility in a concentration-dependent manner. Thus, the assumption of a fixed (all-or-nothing) inhibitory effect may not be appropriate and may hamper efforts to optimally dose these combinations.

1.6 Objectives of Research

The objective of our research was to provide a framework for rational dosing and pairing of β-lactam/β-lactamase inhibitor combinations, with the long-term goal of combatting β-lactamase-

mediated resistance. Historically, β-lactamase inhibitors have been critical to extending the viability of β-lactam antibiotics by circumventing β-lactamase-mediated resistance. However, there are gaps in our understanding of optimal dosing strategies for β-lactam/β-lactamase inhibitor combinations that may limit their clinical utility. Our central hypothesis was that the current approach to clinical susceptibility testing does not reflect the full benefit that β-lactamase inhibitors may confer to β-lactam efficacy. Furthermore, the current scheme does not allow the opportunity to vary the dose or choice of inhibitor with respect to the target pathogen.

Our lab previously used relebactam (previously MK7655) in combination with imipenem against KPC-producing *K. pneumoniae* to illustrate the combined effects of a β-lactam and β-lactamase inhibitor (Bhagunde, Chang et al. 2012). In that study, a full factorial design was used to explore susceptibility to the combination. MICs were determined using a range of inhibitor concentrations to better reflect the fluctuations in inhibitor concentration observed *in vivo*, and adapted to a modified inhibitory sigmoid E_{max} model. The model was used to characterize a theoretical concept called the instantaneous MIC (MICi), which reflected changing pathogen susceptibility as inhibitor concentrations varied over a typical dosing interval. Bhagunde et al. elaborated on this concept to define the percentage of free-time above instantaneous MIC (*f*T>MICi) as the PK/PD index that best correlated with the efficacy of imipenem/relebactam. Extending from this framework, our current research used piperacillin/tazobactam as a representative β-lactam/β-lactamase inhibitor combination to explore the dynamics between a β-lactam, β-lactamase inhibitor and β-lactamase-producing pathogens. We hypothesized that since the β-lactamase activity of a pathogen contributes to the elimination of the β-lactam antibiotic, the exposure and the choice of inhibitor may be instrumental to antimicrobial response and ultimately, the quality of patient care. We

accomplished the goals of this research by:

1) Determining the prevalence and mechanism of resistance for extended-spectrum β-lactamases produced by *K. pneumoniae* at a local tertiary care hospital.
2) Evaluating a novel approach to susceptibility profiling (to improve efficacy predictions) and determining the feasibility of nonconventional piperacillin/tazobactam dosing strategies.
3) Comparing the efficacy of alternative piperacillin/inhibitor pairings against ESBL-producing Enterobacteriaceae

It is anticipated that this research could provide a robust model for evaluating the joint action of β-lactams/β-lactamase inhibitors, which would better inform clinical dosing strategies and pairing of these combinations. Furthermore, it could help preserve the utility of β-lactam/β-lactamase inhibitor combinations in the face of evolving β-lactamase-mediated resistance.

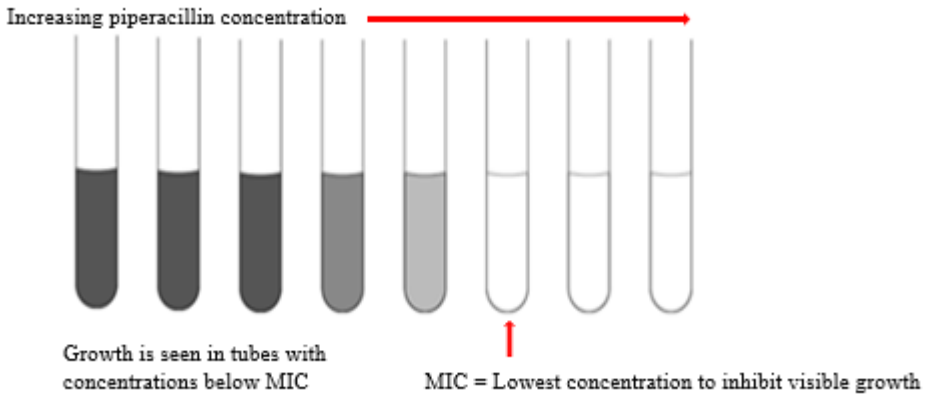
CHAPTER 2

GENERAL METHODOLOGY AND EXPERIMENTATION

2.1 Minimum Inhibitory Concentration (MIC)

Determination of the minimum inhibitory concentration (MIC) is the primary tool for evaluating bacterial susceptibility to an antibiotic in clinical microbiology laboratories. MIC testing by broth macrodilution was used in all our assessments. The antimicrobial agent (such as piperacillin) was diluted twofold serially (e.g. 0.25, 0.5, 1, 2, 4…µg/mL) in cation-adjusted Mueller-Hinton broth (Ca-MHB) and distributed across a collection of sterile test tubes. Each tube was inoculated with a bacterial suspension of approximately 5 x 10^5 cfu/mL. The mixture was then incubated at 37° C for 24 h. Following the incubation period, the lowest antibiotic concentration inhibiting visible growth in media was designated as the MIC, as shown in Figure 2.1 below.

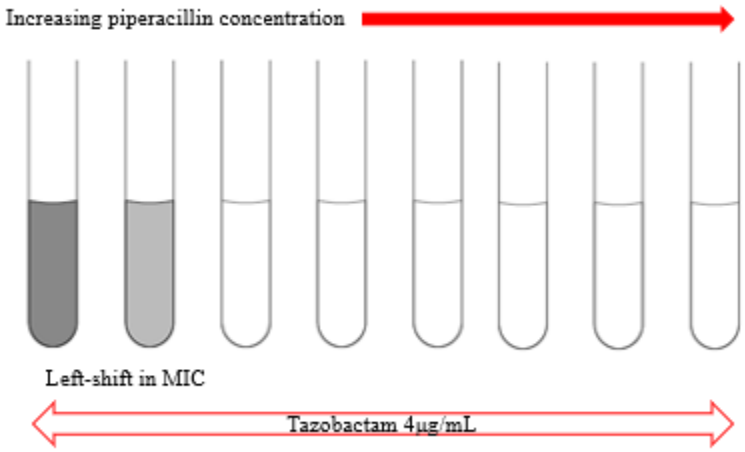
Figure 2.1. Illustration of Susceptibility Testing by Broth Dilution. Test tubes represent a range of piperacillin concentrations from lowest (left) to highest (right).



Traditionally, when assessing susceptibility to piperacillin in combination with tazobactam, the Clinical and Laboratory Standards Institute (CLSI) recommends using a fixed concentration of

the inhibitor against a range of concentrations for piperacillin. If the test microorganism expresses an enzyme that is susceptible to tazobactam, the resulting piperacillin MIC is lower than that observed in the absence of inhibitor, as shown in Figure 2.2.

Figure 2.2. MIC Determination Using a Fixed Inhibitor Concentration. 4µg/mL tazobactam is used across all tubes while piperacillin concentrations range from lowest (left) to highest (right).



For our studies, we expanded on this scheme to evaluate susceptibility as a function of changing inhibitor concentrations. Susceptibility was assessed with a range of inhibitor concentrations (0-256µg/mL) to capture the full range of the inhibitor effect as illustrated in Figure 2.3. The effect of inhibitor concentration on MIC was then profiled as shown in Figure 2.4

Figure 2.3. Characterization of Susceptibility Using a Range of Inhibitor Concentrations. Incremental reductions in piperacillin MIC are observed with increasing tazobactam concentrations.

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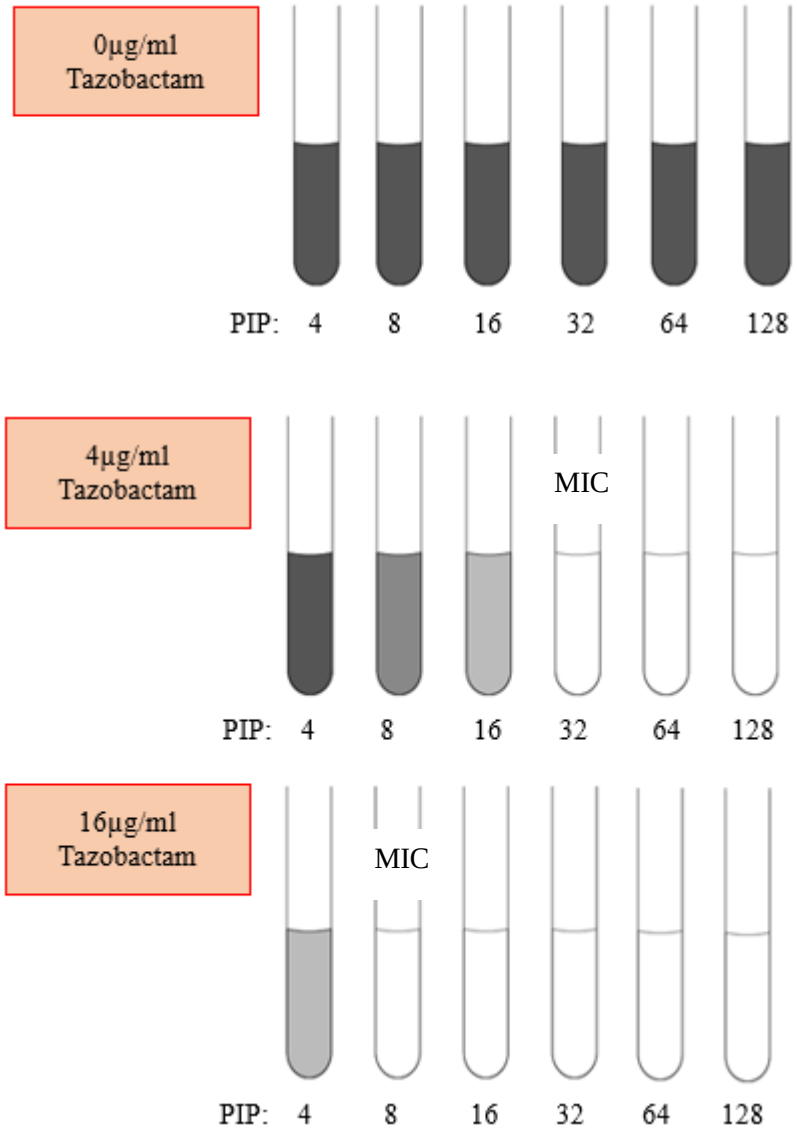
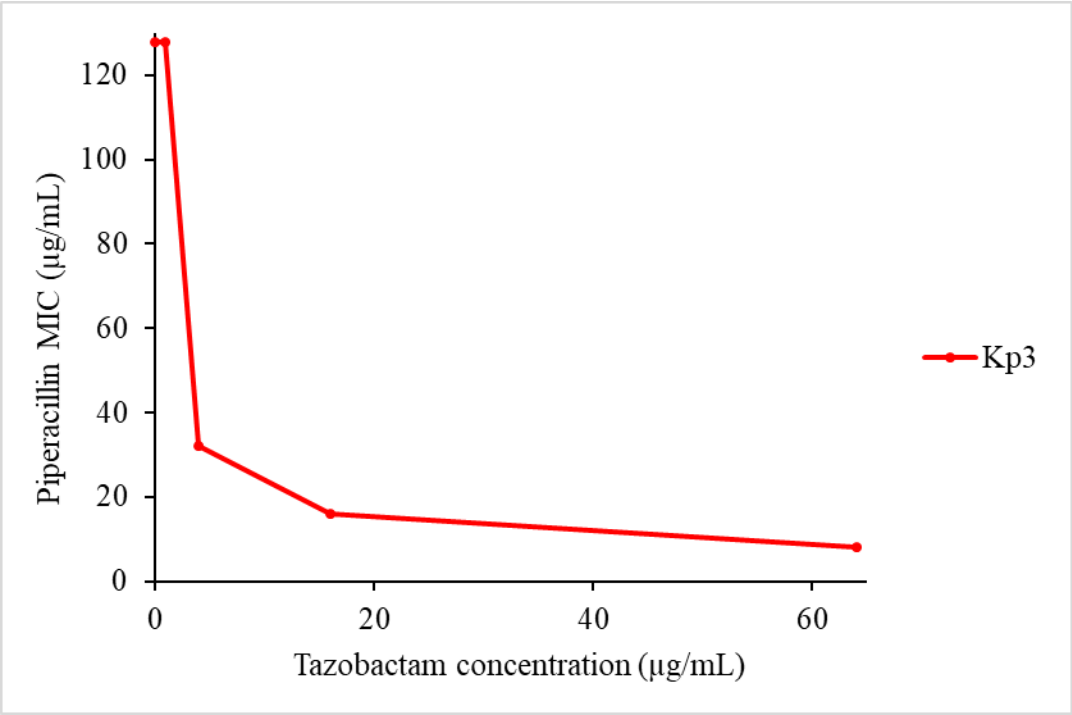


Figure 2.4. Profiling of MIC Dependence on Inhibitor Concentration. Reductions in piperacillin MIC plotted as a function of tazobactam concentration (0-64µg/mL)



2.2 Modeling of MIC Data

The resulting MIC data were fitted to a modified sigmoid inhibitory E_{max} model:

$$\log_2(MIC) = \log_2(MIC_0) - I_{max}\, I^H/(I^H + IC_{50}{}^H)$$

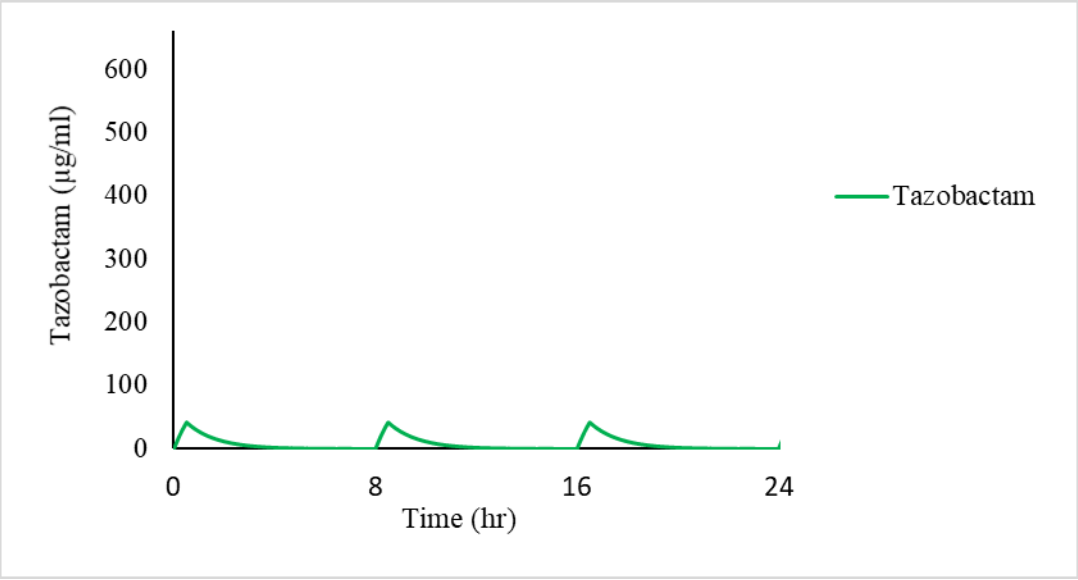
in which, *MIC* = MIC in the presence of inhibitor
MIC_0 = MIC in the absence of inhibitor
I_{max} = maximum inhibitor effect
H = sigmoidicity coefficient
I = inhibitor concentration
IC_{50} = *I* required for 50% maximal inhibition

Free (unbound) inhibitor pharmacokinetic profiles were simulated using ADAPT 5 as illustrated in Figure 2.5 (A). The model was conditioned with the inhibitor pharmacokinetic profiles to simulate theoretical instantaneous MIC (MICi) profiles reflective of changing

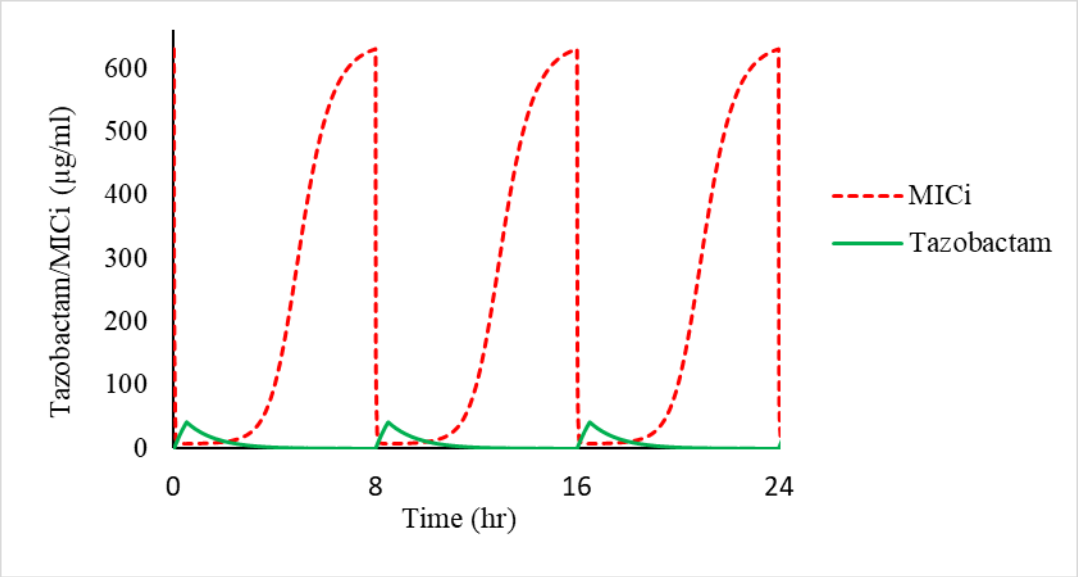
pathogen susceptibility as inhibitor concentrations fluctuate over a typical (8 h) dosing interval, as shown in Figure 2.5 (B). Unbound β-lactam pharmacokinetic profiles were then superimposed on the theoretical MICi profiles to estimate the duration of the dosing interval over which the piperacillin concentration exceeds the MICi, also known as the %*f*T>MICi as shown in Figure 2.5 (C). In the example provided, the %*f*T>MICi is 39.6%, indicating that free piperacillin concentrations exceed the instantaneous MIC for approximately 40% of the 8 h dosing interval.

Figure 2.5. Modeling of MIC Dependency on Inhibitor Concentration and Estimation of %*f*T>MICi. Pharmacokinetic profile corresponding to 0.5 g tazobactam administered every 8 h (green) and instantaneous MICi (red). Pharmacokinetic profile corresponding to 4 g piperacillin administered every 8 h (black).

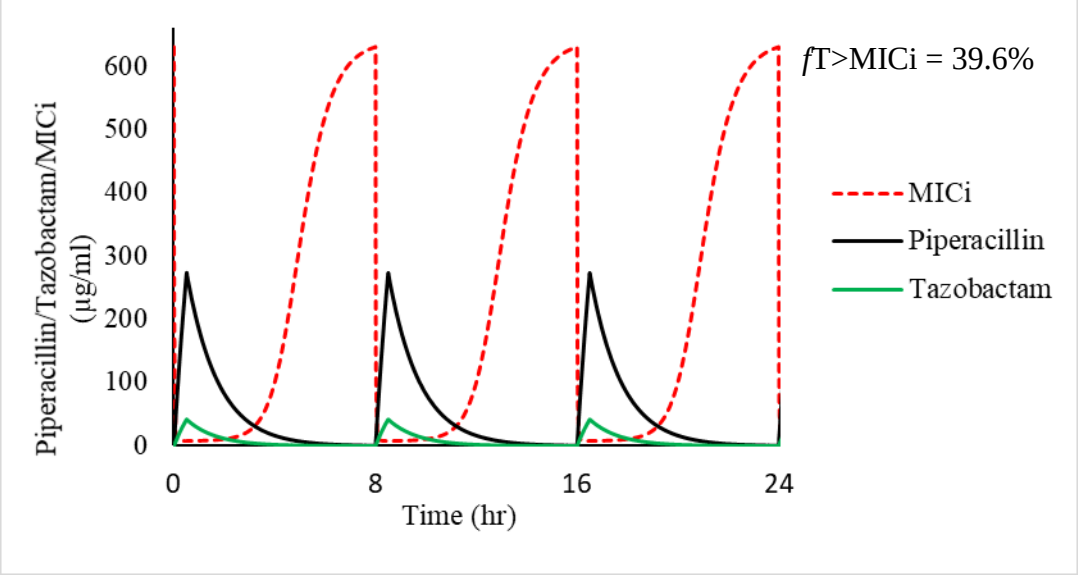
A



B


Tazobactam/MICi (µg/ml)
600
500
400
300
200
100
0
0
8
16
24
Time (hr)
MICi
Tazobactam

C

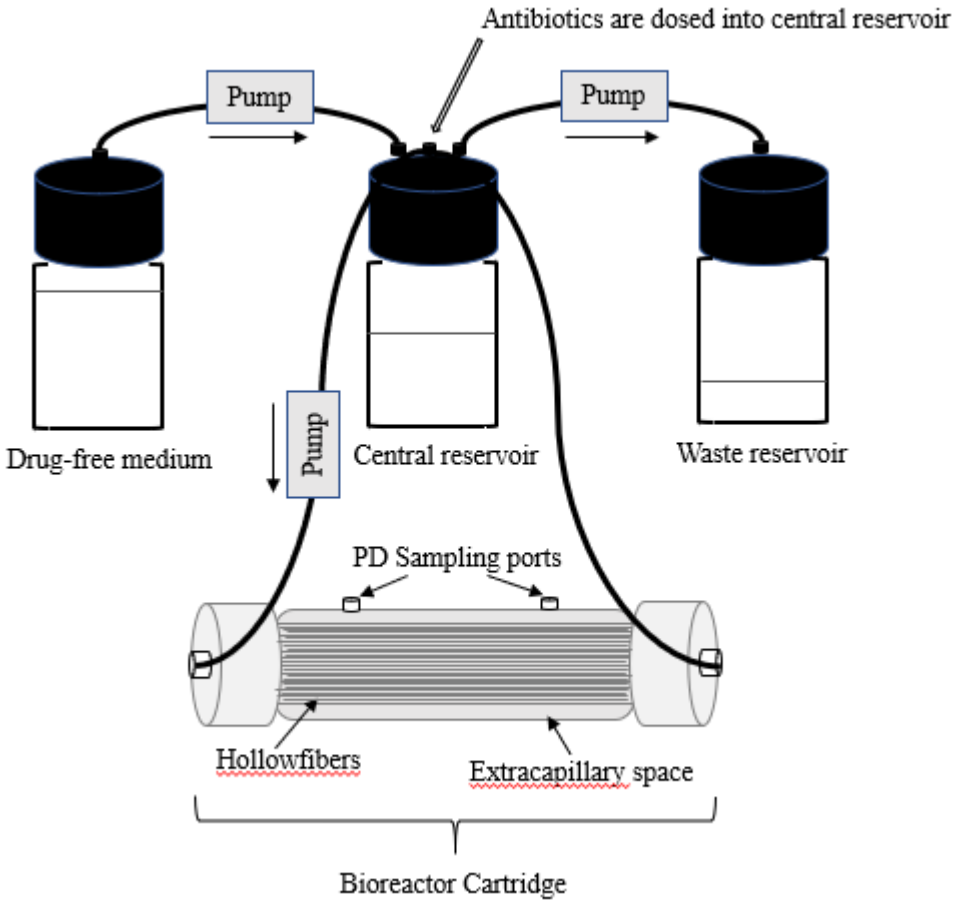

Piperacillin/Tazobactam/MICi (µg/ml)
600
500
400
300
200
100
0
0
8
16
24
Time (hr)
fT>MICi = 39.6%
MICi
Piperacillin
Tazobactam

2.3 Hollowfiber Infection Model Schematics

A hollowfiber infection model (HFIM) is a dynamic *in vitro* system that allows simulation of human concentration-time profiles of drugs. For our studies, a two-compartment model was used (as illustrated in Figure 2.6). The model consists of a hollowfiber cartridge, a central compartment, diluent and elimination reservoirs. Drugs are administered through a dosing port into the central compartment, which rapidly equilibrates with the peripheral (cartridge) compartment. The cartridge consists of tubular fibers that retain the bacteria (in the extracapillary space) while allowing free diffusion of nutrients and drug. Following equilibration of the two compartments, fresh media flows through the system to allow drug concentrations to decline over time with the same half-life as observed in humans. Medium from the central compartment continuously recirculates through the cartridge fibers providing nutrition and drug, while moving waste products and drug back to the central compartment.

For our studies, the pharmacokinetic simulations were considered acceptable if the best-fit peak concentrations and elimination half-lives were both within 20% of target values. Changes in bacterial density were monitored for up to 72 h by serially sampling (in duplicate) from the bioreactor cartridge. Samples were centrifuged, washed with saline to reduce drug carry-over, serially diluted and plated quantitatively on Mueller-Hinton agar plates. Viable colony counts were then determined following 24 h incubation at 35°C. Additionally, drug-supplemented agar plates (using 3× the baseline piperacillin/tazobactam MIC) were used selectively to detect regrowth associated with the development of resistance over time.

Figure 2.6. Representative Schematic of a Two-compartment HFIM



2.4 Analytical Assay of Antibiotics

To verify the concentration-time profiles of our antibiotics, drug concentrations were assayed with validated liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods using a Waters Acquity™ UPLC, and API5500 Qtrap triple quadrupole mass spectrometer (Applied Biosystem/MDS SCIEX, Foster City, CA) equipped with Turbo-Ion-Spray ™ source. Mobile phase A and B were 0.1% formic acid in water and acetonitrile respectively. All working solutions were prepared by 200-fold dilution of stock standard or sample solutions into LC-MS grade water containing 100µL of internal standard (320ng/mL ertapenem).

2.4.1 *Assay of Piperacillin and Tazobactam*

For analysis of piperacillin, an injection volume of 5µL was used whereas 10µL was used for tazobactam. The analytes were separated by a gradient elution at 45°C at a flow rate of 0.35mL/min using a Waters BEH C_{18} column (1.7µm 2.1x50mm). The gradient consisted of 0-0.5min: 95% A; 0.5-0.7min: 95-84% A; 0.7-1.2 min: 84-76% A; 1.2-1.7min: 76-70% A; 1.7-2.1min: 70-50% A; 2.1-2.5 min: 50-5% A; 2.5-3.2min: 5-95% A; 3.2-5min: 95% A. Multiple reaction monitoring (MRM) scan type in positive mode was used for identification of both piperacillin and tazobactam. The analyte specific conditions and MRM transitions are noted in Table 2.1.

The linearity of the calibration curves was determined by peak area ratios of the analyte and internal standard versus concentration, and fitted with a linear regression (with $1/x^2$ weighting) method. The linear range of quantification was 0.0625-128 µg/ml with linear regression coefficients of 0.97 for both piperacillin and tazobactam as shown in Figure 2.7. The assay was further validated based on the accuracy (within 85-115% of target concentrations), and precision (based on inter and intraday variabilities, ≤15%) of the calibration curve. The calculated intra-day variability was <9.4% while the inter-day variability was <13.4% for both analytes. Typical chromatograms are illustrated in Figure 2.8.

2.4.2 *Assay of Avibactam*

An injection volume of 10µL was used to analyze avibactam concentrations with a gradient elution at 45°C at a flow rate of 0.35mL/min using a Restek Ultra Biphenyl column (100mm x 2.1mm, 5µm). The gradient parameters were: 0-0.5min: 95% A; 0.5-0.7min: 95-84% A; 0.7-1.2min: 84-76% A; 1.2-1.7min: 76-70% A; 2.1-3.05 min: 65-0% A; 3.05-4.40min: 0-50% A;

4.40-4.50min: 50-95% A. Multiple reaction monitoring (MRM) scan type in negative mode was used for identification of avibactam.

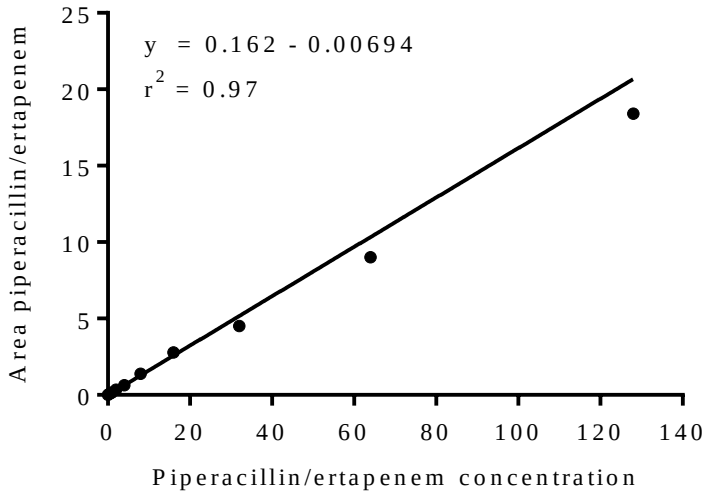
Assay validation was performed as described for piperacillin/tazobactam. The linear range of quantitation was 0.0625-128 µg/mL with linear regression coefficient of 0.99 as shown in Figure 2.7 (C). Intra and inter-day variabilities in the calibration curve were <10%. Representative chromatograms are shown in Figure 2.8.

Table 2.1. Analyte Specific MS parameters

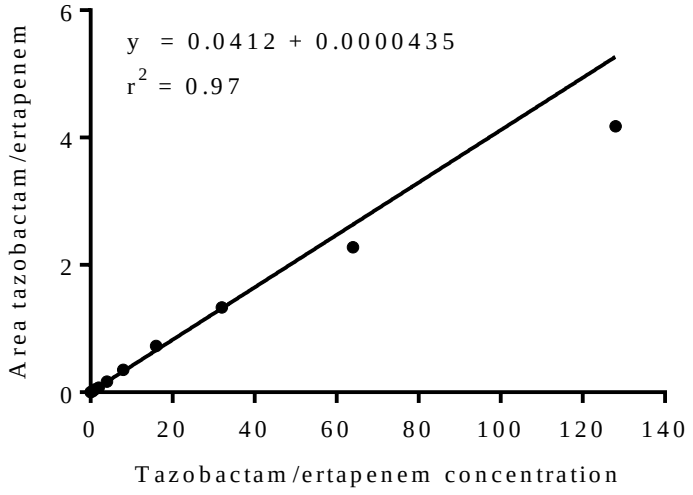
Compound	MRM Transitions [(Q1 → Q3) Da]	Declustering Potential (Volts)	Entrance Potential (Volts)	Collision Cell Exit Potential (Volts)
Piperacillin	m/z 518.1→143.2	45	25	5
Tazobactam	m/z 300.9→168.2	90	17	10
Avibactam	m/z 264→95	-80	-30	-15
Ertapenem (negative mode)	m/z 474→265	-104	-27	-14
Ertapenem (positive mode)	m/z 474→432	90	12	19

Figure 2.7. Piperacillin (A), Tazobactam (B) and Avibactam (C) Calibration Curves

A



B



C

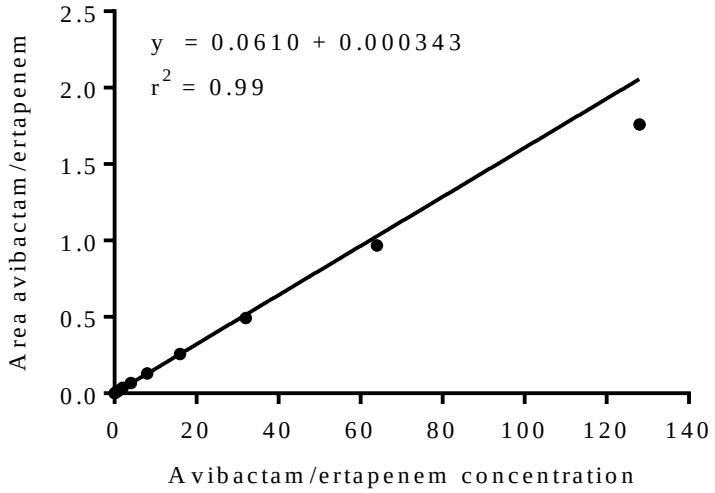
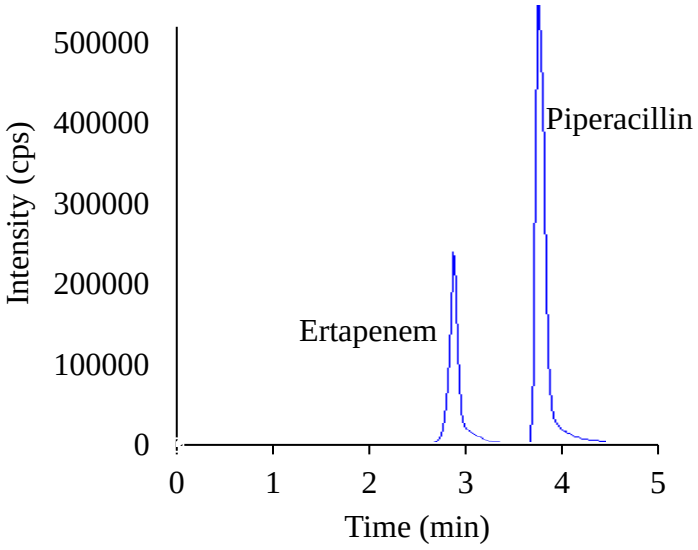
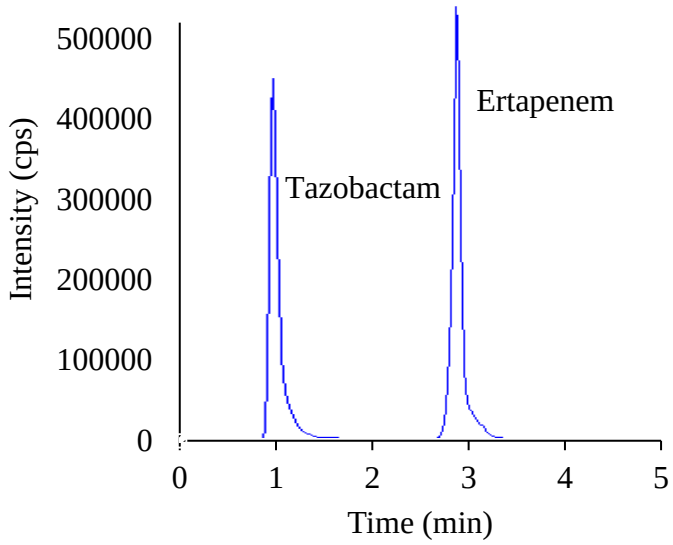


Figure 2.8. Representative Chromatograms for Piperacillin, Tazobactam and Avibactam

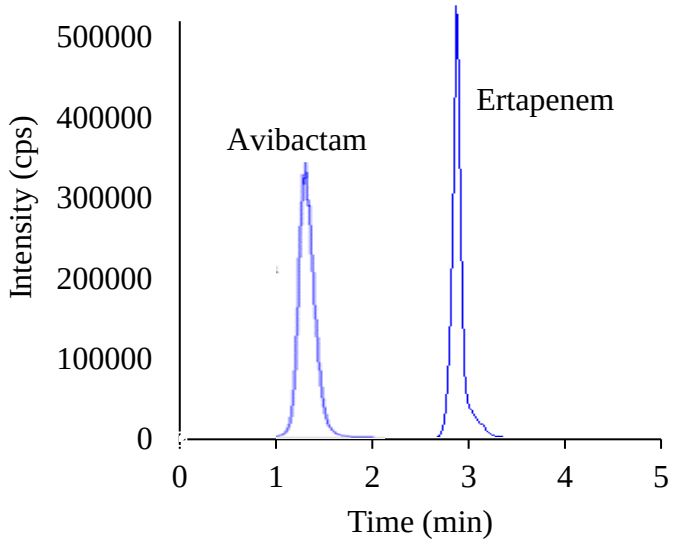
A



B


500000
400000
300000
200000
100000
0
Intensity (cps)
Tazobactam
Ertapenem
0
1
2
3
4
5
Time (min)

C


500000
400000
300000
200000
100000
0
Intensity (cps)
Avibactam
Ertapenem
0
1
2
3
4
5
Time (min)

CHAPTER 3

Prevalence of Extended-Spectrum β-Lactamases

3.1 Objective

In this study, we sought to determine the local prevalence and mechanism of resistance for extended-spectrum β-lactamases produced by *K. pneumoniae*

3.2 Rationale

Extended-spectrum β-lactamases represent a heterogenous family of enzymes comprised of CTX-M, SHV and TEM subtypes. In order to focus subsequent studies on the enzyme subtypes of clinical relevance, we identified the most prevalent genotypes in our geographic region.

3.3 Materials and Methods

3.3.1 Identification and Selection of Isolates

Bloodstream isolates of *K. pneumoniae* from December 2014 through December 2015 were identified from the Microbiology Laboratory of CHI Baylor St. Luke's Medical Center in Houston, Texas. The isolates were stored at -80 °C in Protect® (Key Scientific Products, Round Rock, TX) storage vials and sub-cultured twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA) for 24 h at 37° prior to use. Susceptibility data obtained by the VITEK 2 automated system (BioMérieux Inc., Durham, NC) was retrieved from electronic medical records. Each patient was included once unless he/she had an isolate obtained more than 2 weeks after the initial culture with different susceptibility to at least 3 classes of antibiotics. Isolates resistant to any third-generation cephalosporin (e.g., ceftazidime, ceftriaxone) based on

Clinical and Laboratory Standards Institute (CLSI) breakpoints were then screened for the presence of ESBLs (CLSI 2016). Ethical approval was obtained from the institutional review boards at the University of Houston and CHI Baylor St. Luke's Medical Center. Informed consent was not required as the isolates were obtained as part of standard patient care and the study was retrospective in nature.

3.3.2 *Phenotypic Screening for Enzyme Activity and Detection of ESBL-encoding Genes*

ESBL production was screened by the double-disc synergy test and confirmed by ceftazidime/ceftazidime + clavulanic acid Etest (BioMérieux, Marcy l'Etoile, France) (CLSI 2009). Characterization of genes encoding ESBLs (*bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$) was carried out by PCR and sequencing with primers/conditions previously described (Rasheed, Jay et al. 1997, Monstein, Ostholm-Balkhed et al. 2007)

3.3.3 *Evaluation of Hydrolytic Activity*

Enzymatic activity of crude cell lysate was quantified for isolates harboring ESBL genes using a spectrophotometric assay of nitrocefin degradation (Tam, Schilling et al. 2007). Nitrocefin is a chromogenic cephalosporin substrate that changes color from yellow to red as its β-lactam ring is hydrolyzed by β-lactamases. For each isolate, an inoculum of approximately 1 x 10^8 cfu/mL was prepared in cation-adjusted Mueller-Hinton broth from overnight cultures in a shaker water bath set at 37°C. Cells were lysed using 2 freeze-thaw cycles and centrifuged at 20,000G at 4°C. A working solution of 0.34 mM nitrocefin (Becton, Dickson and Co., Sparks, MD) diluted in 0.1 M phosphate buffer (pH 7) was used to evaluate changes in absorbance (486nm) in the presence of bacterial cell lysates. Enzymatic activity attributable to ESBL

production was further ascertained by LC-MS/MS quantification of ceftazidime hydrolysis by the cell lysates. *K. pneumoniae* ATCC 13883 was used as a negative control, while a well-characterized clinical strain harboring CTX-M-15 served as a positive control (Valverde, Coque et al. 2008). Enzymatic activity was normalized based on total protein content (Pierce BCA Protein Assay, Rockford, IL) and compared to the positive control. Finally, hydrolytic activity determined by the nitrocefin assay was correlated to the respective ceftazidime MICs of the isolates using linear regression.

3.3.4 *Clonality Assessment*

The clonal relatedness of isolates found to harbor ESBLs was evaluated by repetitive element-based PCR (Brolund, Haeggman et al. 2010). DNA fragments were separated and analyzed using the model 2100 bioanalyzer (Agilent Technologies Inc., Santa Clara, CA). Fingerprint patterns were compared using Diversilab software with a Pearson correlation coefficient (Bacterial Barcodes Inc., Athens, GA). Relatedness was defined by a similarity value of >95%.

3.4 Results

Of the 84 unique isolates identified from 81 patients, 13 isolates (15.5%) from different patients screened positive for β-lactamase activity based on our criteria. Of these, 5 isolates were found to express the ESBL phenotype and harbored $bla_{\text{CTX-M-15}}$. Three isolates co-harbored other β-lactamases, as detailed in Table 3.1. The susceptibilities of the isolates to ceftazidime, ceftriaxone, piperacillin/tazobactam, ceftazidime/avibactam and meropenem are also provided in Table 3.1. All isolates were also resistant to piperacillin/tazobactam, and except for isolate 2301, they were all susceptible to meropenem. Additionally, all of the isolates were susceptible

to ceftazidime/avibactam. β-lactamase genes were functionally expressed in all 5 isolates, and the linear portion of the nitrocefin reaction is shown in Figure 3.1. Comparison of overall hydrolytic activity to MIC yielded a correlation coefficient of 98% for these isolates (Figure 3.2). Additionally, all 5 isolates successfully hydrolyzed ceftazidime (Figure 3.3). Assessment of clonal relatedness revealed 4 unique clones expressing the ESBL phenotype, as shown in Figure 3.4.

Table 3.1. Phenotyping, Genotyping, and Susceptibility (MIC) Results of Isolates

Isolate	Phenotype	Gene(s) detected	CAZ	CTX	PIP/TAZ	CAZ/AVI	MEM
1255	ESBL	CTX-M-15, SHV-28	>**32**	≥**64**	>**128/4**	0.25/4	≤0.25
1416	ESBL	CTX-M-15, SHV, OXA-1	**8**	≥**64**	>**128/4**	0.25/4	≤0.25
1562	ESBL	CTX-M-15, OXA-1	**16**	≥**64**	**64/4**	0.25/4	≤0.25
2301	ESBL	CTX-M-15	>**32**	**64**	>**128/4**	1.5/4	**16**
2366	ESBL	CTX-M-15	>**32**	**64**	**128/4**	0.5/4	≤0.25

Note: MIC in μg/mL; bold fonts depict resistant phenotypes

ESBL: extended-spectrum β-lactamase

CAZ: ceftazidime; CTX: ceftriaxone; PIP/TAZ: piperacillin/tazobactam; CAZ/AVI: ceftazidime/avibactam; MEM: meropenem

Figure 3.1 Comparative Hydrolytic Activity of ESBL-positive Isolates. Activities based on equivalent mg of total protein. Shown are negative control Kp13883 (green), positive control Kp3 (blue) and the 5 ESBL-positive clinical isolates.

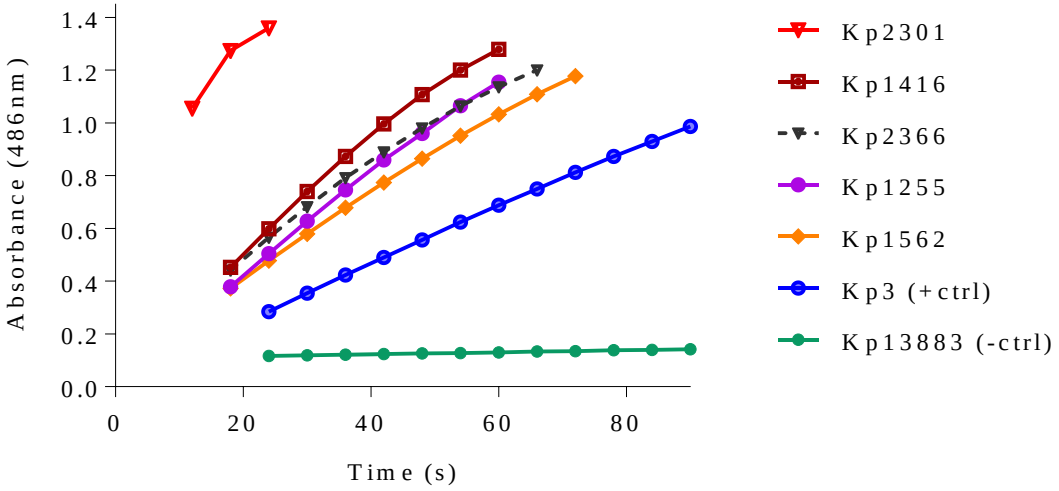


Figure 3.2. Correlation of MIC to Hydrolytic Activity for ESBL-positive Isolates. Activities and MICs expressed as ratios of each isolate's activity and MIC compared to estimates for positive control (Kp3)

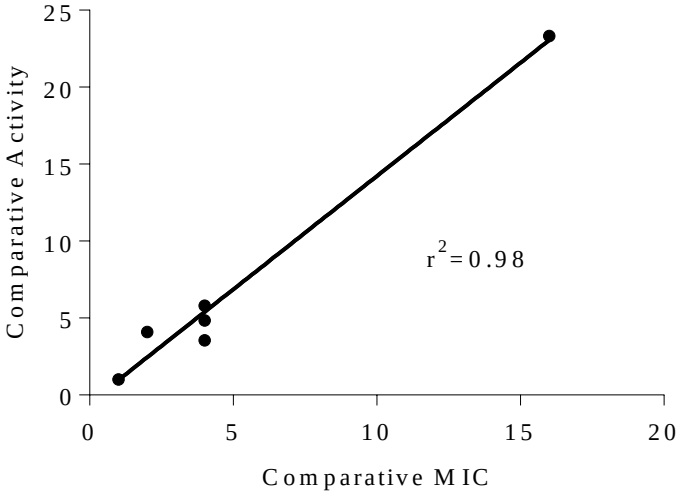


Figure 3.3. Representative Ceftazidime Hydrolysis for ESBL Isolates. Hydrolysis of ceftazidime by negative control (Kp13883), positive control (Kp3) and 2 clinical isolates (Kp1562 and Kp2301).

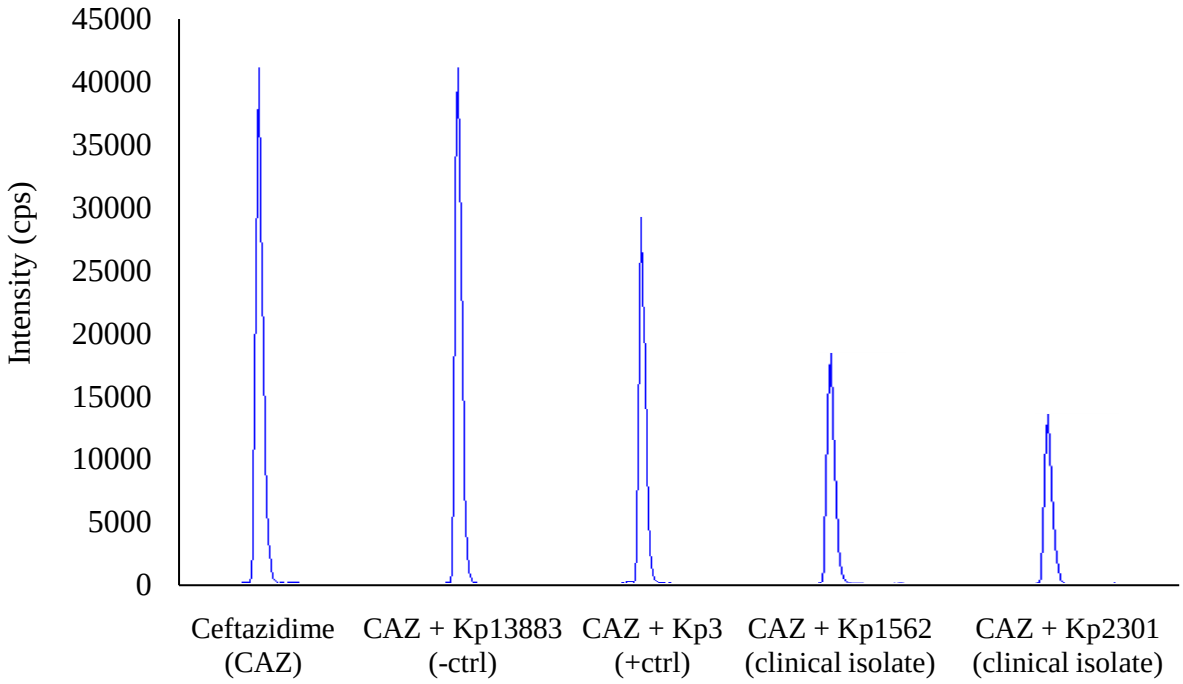
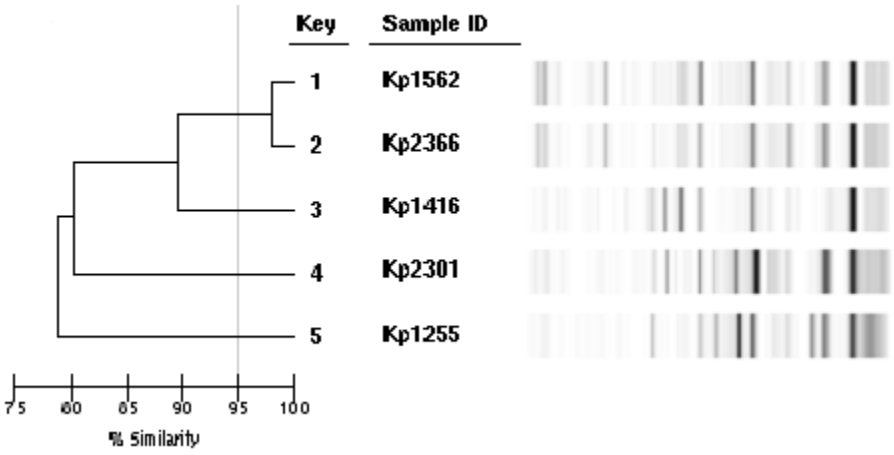


Figure 3.4. Clonal Uniqueness of Clinical Isolates Expressing ESBL Phenotype. Four unique clones identified based on >95% similarity cutoff.



CHAPTER 4

Modified Susceptibility Profiling and Alternative Piperacillin/Tazobactam Dosing Strategies

4.1 Objective

The objectives for this study were twofold: 1) to evaluate a novel approach to susceptibility testing to guide β-lactam/β-lactamase inhibitor dosing and 2) to determine the feasibility of alternative piperacillin/tazobactam dosing strategies using a hollowfiber infection model.

4.2 Rationale

While older inhibitors (such as tazobactam) lack appreciable intrinsic antimicrobial activity, they alter susceptibility (MIC) to the partner β-lactam in a concentration-dependent manner that is not captured in current susceptibility testing schemes. As a result, the conventional approach for establishing PK/PD indices may not directly apply to these β-lactam/β-lactamase inhibitor combinations. Additionally, the rationale for dosing piperacillin/tazobactam clinically in a fixed ratio of 8:1 (piperacillin to tazobactam) remains unclear. The rationale for this study was to improve efficacy predictions for piperacillin/tazobactam and better inform rational dosing strategies for piperacillin/tazobactam against ESBL-producing Enterobacteriaceae. Since current susceptibility testing for piperacillin in combination with tazobactam involves use of a fixed tazobactam concentration, we sought a more comprehensive characterization of susceptibility using a range of inhibitor concentrations. Moreover, piperacillin/tazobactam is dosed in a fixed 8:1 ratio that may not be optimal in all clinical scenarios. Extending from our previously proposed modeling framework (for capturing the dependence of susceptibility on

inhibitor concentration), we aimed to provide new insights into the efficacy of different piperacillin/tazobactam dose ratios.

4.3 Materials and Methods

4.3.1 *Antimicrobial Agents, Chemicals and Reagents*

Piperacillin was purchased from Sigma-Aldrich (St. Louis, MO). Tazobactam and ceftazidime were obtained from Chem-Impex International (Wood Dale, IL). LC-MS-grade water and acetonitrile were purchased from EMD Millipore Corporation (Billerica, MA). Stock solutions of piperacillin, tazobactam and ceftazidime were prepared in sterile water, aliquoted, stored at -80°C and thawed immediately before use.

4.3.2 *Bacteria*

Four representative clinical isolates commonly encountered in serious nosocomial infections were studied. Two CTX-M-15-producing *K. pneumoniae* (Kp3 and KpK91) and one SHV-12 producing *E. coli* (EcF65) isolates were obtained from a reference microbiology laboratory (Madrid, Spain). An additional CTX-M-15-producing *K. pneumoniae* (Kp2301) isolate was obtained from our local surveillance study (Abodakpi, Chang et al. 2018). These isolates were selected based on their MIC and susceptibility reversibility profiles in the presence of tazobactam. From a modeling perspective, these diverse isolates were expected to enhance the robustness (i.e., predictive capability) of our approach. The methods for the detection of resistance mechanisms in these isolates were detailed previously (Rasheed, Jay et al. 1997, Wang, Kelkar et al. 2003, Monstein, Ostholm-Balkhed et al. 2007, Endimiani, Carias et al. 2008). β-lactamase activity was assessed for all 4 isolates using a nitrocefin degradation assay

as described previously in chapter 3 (Abodakpi, Chang et al. 2018). The isolates were stored in Protect® storage vials (Key Scientific Products, Round Rock, TX) at -80 °C and subcultured twice on 5% blood agar plates (Hardy Diagnostics, Santa Maria, CA) for 24 h at 37 °C before use.

4.3.3 *Susceptibility and Effect of Inhibitor on MIC*

Initial ceftazidime and piperacillin/tazobactam susceptibilities (MICs) were determined by the broth dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI). Piperacillin MICs were further determined for each isolate using escalating concentrations of tazobactam (0-256 µg/ml) as described in the general methodology chapter. All MIC experiments were performed in triplicate and repeated at least once on a different day. The MIC reductions in the presence of tazobactam were modeled using a previously described sigmoid inhibitory E_{max} model (Bhagunde, Chang et al. 2012):

$$log_2\,(MIC) = log_2(MIC_o) - I_{max}\,I^H/(I^H + IC_{50}{}^H\,)$$

wherein the *MIC* represents the MIC with the addition of an inhibitor, MIC_0 (intrinsic MIC) is the MIC in the absence of inhibitor, I_{max} represents the maximum effect conferred by the inhibitor, *H* describes the sigmoidicity coefficient, *I* represents the concentration of inhibitor and IC_{50} represents the inhibitor concentration required for 50% of the maximal inhibitory effect. The E_{max} model was conditioned with fluctuating tazobactam concentrations associated with different dosing regimens. A theoretical instantaneous MIC (MICi) profile, which represents changing pathogen susceptibility over time (in the presence of changing inhibitor concentrations over a dosing interval), was then simulated using the best-fit parameter estimates for each isolate. Finally, each MICi profile was superimposed on a simulated free (unbound)

piperacillin pharmacokinetic profile to determine the percentage of *f*T>MICi (as illustrated in chapter 2).

4.3.4 *Hollowfiber Infection Model*

The schematics of the experimental setup have been described previously in the general methodology chapter. Fresh colonies of each isolate were inoculated in Ca-MHB and grown to late log phase. Based on the absorbance at 630 nm, the bacterial suspension was adjusted to ~1 x 10^6 cfu/mL. Simulated unbound exposures of piperacillin and tazobactam (dosed over 30 min) were given every 8 h for up to 72 h. For both piperacillin and tazobactam, 30% protein binding and 1 h elimination half-life were used to simulate the free-drug exposures.

4.3.5 *Drug Assay and Pharmacokinetic Modeling*

Analyte concentrations were assayed as described in the general methodology chapter. A 1-compartment model with zero-order infusion input was fit to the observed piperacillin and tazobactam concentration-time profiles using ADAPT 5 (D'Argenio 2009).

4.3.6 *Identification of Target Exposure*

Using Kp3 as the (arbitrary) reference isolate, a clinical dosing regimen of 4 g piperacillin every 8 h was evaluated alongside escalating exposures of tazobactam (0.5 g, 1 g, 1.5 g, 2 g) in our hollowfiber model. With a %*f*T>MICi identified for each exposure, the threshold for efficacy was defined as the lowest %*f*T>MICi associated with bacterial growth suppression (i.e., bacterial burden below the baseline inoculum) at the end of the experiment. This threshold

target exposure was subsequently validated in our 3 other clinical isolates (KpK91, Kp2301 and EcF65).

4.3.7 *Experimental Validations*

For the remaining isolates, predictions were made on the efficacy of different tazobactam exposures based on the corresponding %*f*T>MICi. Piperacillin/tazobactam regimens that exceeded the threshold value were expected to suppress growth. Conversely, exposures that failed to achieve the target %*f*T>MICi were predicted to result in bacterial regrowth over time. The %*f*T>MICi for 4 g piperacillin and 0.5 g tazobactam (every 8 h) was first determined for each isolate. If this initial exposure failed to achieve the threshold %*f*T>MICi, escalating tazobactam exposures up to 4 g were then simulated to meet the target %*f*T>MICi. The hollowfiber model was subsequently used to experimentally validate the predicted outcomes of the standard dosing regimen and either: 1) exposure(s) that exceeded the target %*f*T>MICi, or 2) the highest exposure evaluated if the target %*f*T>MICi was unattainable.

4.4 Results

4.4.1 *Bacteria*

The susceptibility profiles and known mechanisms of resistance for the four isolates examined are shown in Table 4.1 All isolates were resistant to ceftazidime, and all but EcF65 were also resistant to piperacillin/tazobactam. Functional expression of the ESBL genes was confirmed as shown in Figure 4.1. However, the rates of nitrocefin hydrolysis were dramatically different, suggesting different enzyme expression levels of the isolates.

4.4.2 *Effect of Inhibitor on MIC*

A tazobactam concentration-dependent reduction in piperacillin MIC was observed. The relationship between susceptibility and inhibitor concentrations was reasonably characterized by the sigmoid inhibitory E_{max} model for all 4 isolates ($r^2 \geq 0.94$). The model best-fit parameter estimates (Table 4.1) illustrate differences in isolate sensitivity to tazobactam, with Kp3, KpK91 and EcF65 displaying lower IC_{50} values than Kp2301. Additionally, I_{max} values indicate a more drastic reduction in MIC for Kp3, EcF65 and Kp2301 than for KpK91. Representative model fitting of the MIC data for Kp3, along with an associated MICi profile are shown in Figure 4.2.

Table 4.1. ESBL Genes Detected, Susceptibility (MIC in µg/mL), Inhibitory E_{max} Parameter Estimates and Model Fit for Clinical Isolates

Bacteria	ESBL gene	MIC			Model estimates and fit				
		CAZ	CTX	PIP/TAZ	$\log_2(MIC_0)$	I_{max}	IC_{50}	H	r^2
K. pneumoniae (Kp3)	CTX-M-15	**64**	**>32**	**32/4**	9.32	6.52	2.60	1.57	0.94
K. pneumoniae (KpK91)	CTX-M-15	**64**	**>32**	**32/4**	9.03	4.75	1.36	4.00	0.97
K. pneumoniae (Kp2301)	CTX-M-15	**>512**	**64**	**>512/4**	9.09	6.23	35.25	2.67	0.97
E. coli (EcF65)	SHV-12	**>512**	**>32**	4/4	8.67	6.99	2.71	3.41	0.98

CAZ: ceftazidime; CTX: ceftriaxone; PIP/TAZ: piperacillin/tazobactam

Note: Bold font denotes resistant phenotype according to CLSI breakpoints

Figure 4.1. Enzymatic Activity of the 4 Study Isolates

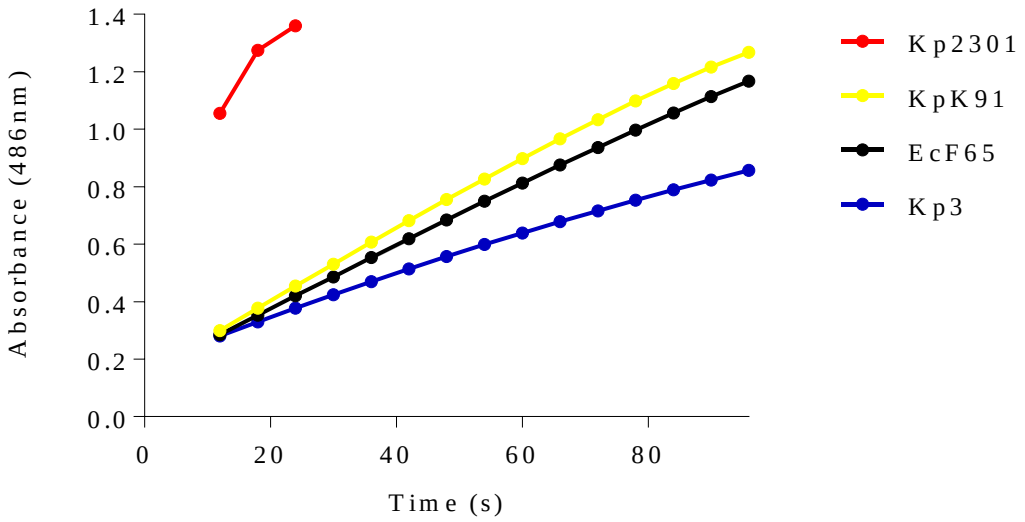
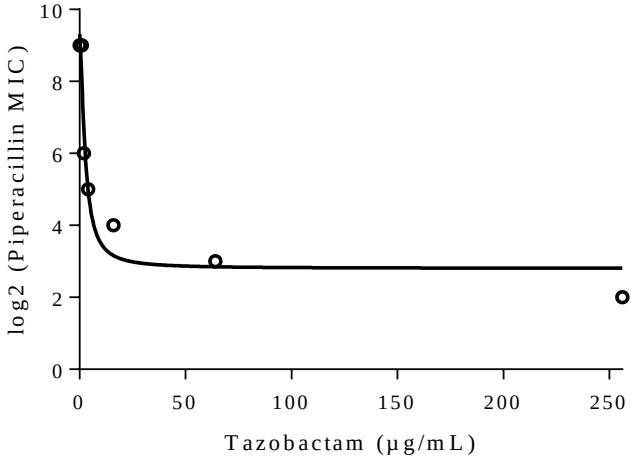


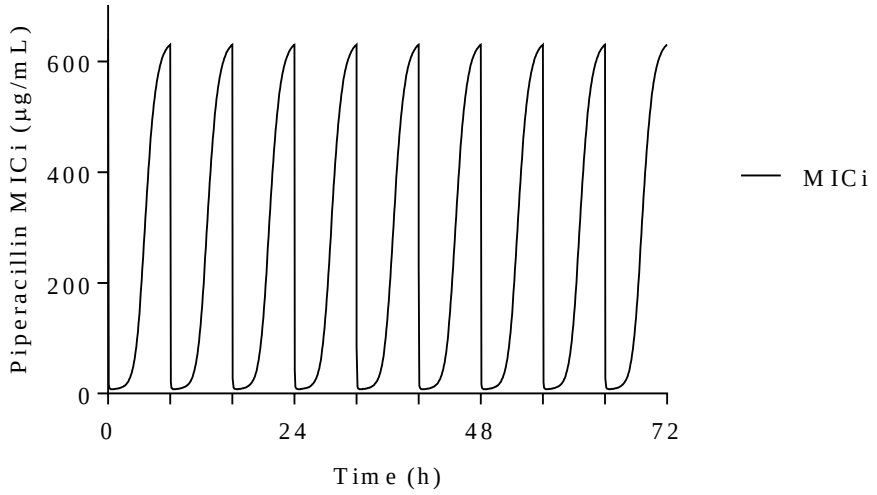
Figure 4.2. Representative Model Fit and Instantaneous MIC Profile. Model fit to piperaciilin MIC data for Kp3 in the presence of escalting tazobactam concentrations (A). Open circles indicate experimental data and the continuous line represents the best-fit model. MICi profile using 0.5 g tazobactam q8h is shown (B).

A



$r^2 = 0.94$

B

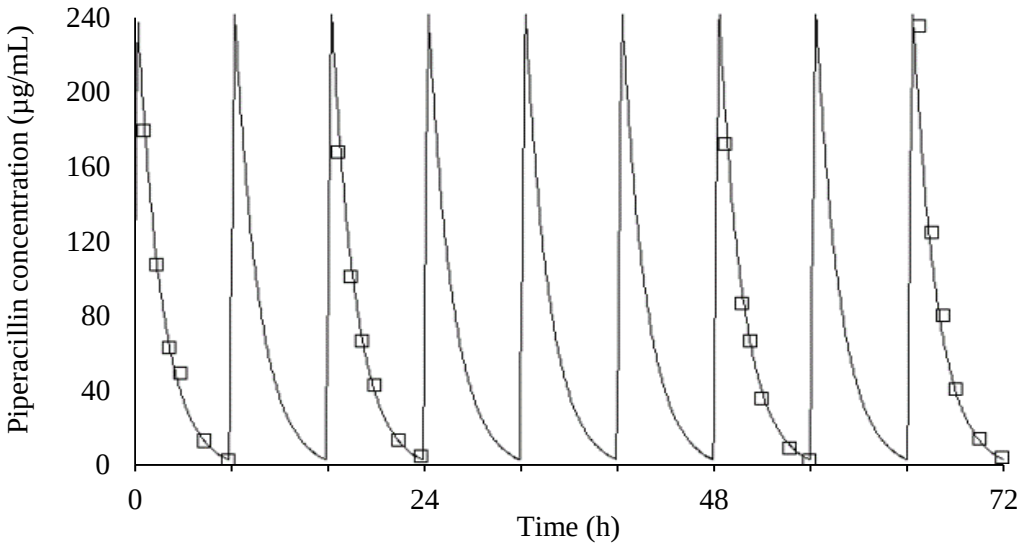


4.4.3 *Pharmacokinetics and Effect of Drug Exposures on Bacterial Burden*

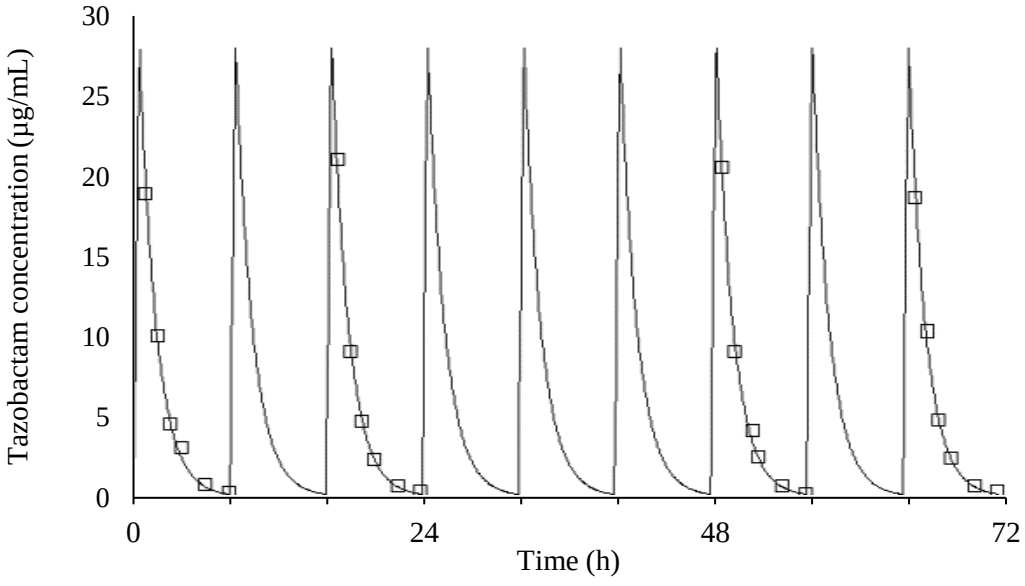
The targeted piperacillin and tazobactam pharmacokinetic profiles were reasonably well simulated in the hollowfiber infection model. Typical profiles for 4 g piperacillin and 0.5 g tazobactam are shown in Figure 4.3. For the reference isolate (Kp3), the bacterial burden declined initially for all treatment exposures. The clinical regimen of 4 g piperacillin and 0.5 g tazobactam was associated with %*f*T>MICi of 39.6% and resulted in bacterial regrowth after 8 h. An escalated tazobactam exposure of 1.5 g every 8 h resulted in a %*f*T>MICi of 55.1% and growth suppression. For the other isolates, the estimated %*f*T>MICi and predicted outcomes associated with different dosing exposures are summarized in Table 4.2.

Figure 4.3. Typical Simulated Pharmacokinetic Profile of 4 g Piperacillin (Target C_{max} = 240 µg/mL) (A) and 0.5 g Tazobactam (Target C_{max} = 30 µg/mL) (B) Administered Every 8 h. Elimination half-life of 1 h was simulated for both agents. Open squares represent observed concentrations and continuous lines represent the best-fit model.

A



B



4.4.4 *Experimental Validations*

Our model reliably predicted the outcomes of various piperacillin/tazobactam exposures for each validation isolate (EcF65, KpK91 and Kp2301), as shown in Table 4.2. The clinical dosing regimen of 4 g piperacillin and 0.5 g tazobactam was predicted to result in regrowth of all 3 isolates. For EcF65 (which was considered susceptible to piperacillin/tazobactam by the stardard susceptibilty testing method), the clinical regimen would achieve %*f*T>MICi = 43.8%; regrowth was noted by 8 h and the development of resistance was observed over time, as shown in Figure 4.4 (B). Instead, an elevated exposure equivalent to 4 g piperacillin and 1.0 g tazobactam (%*f*T>MICi = 60%) was necessary to suppress growth below the starting inoculum, as shown in Figure 4.4 (C). Further reduction of bacterial density (below the limit of detection) was observed with 4 g piperacillin and 1.5 g tazobactam (%*f*T>MICi = 65%) as shown in Figure 4.4 (D). For KpK91, the standard dosing regimen achieved a %*f*T>MICi of 44.5% and bacterial regrowth was observed. This isolate would be considered as equally susceptible to piperacillin/tazobactam compared to reference Kp3, but owing to the attenuated I_{max} for this isolate, higher exposures of tazobactam were predicted to be insufficient to suppress growth. At the highest tazobactam exposure evaluated (4 g), the %*f*T>MICi (50.9%) remained below the target exposure threshold and regrowth was seen. Finally for Kp2301, the efficacy threshold was predicted to be unattainable at the doses explored due to the high IC_{50} of the isolate. Simulated dosing regimens as high as 4 g piperacillin with 4 g tazobactam (%*f*T>MICi = 36.8%) resulted in regrowth (data not shown).

Table 4.2. Predicted and Observed Outcomes Associated with Different Piperacillin/Tazobactam Exposures

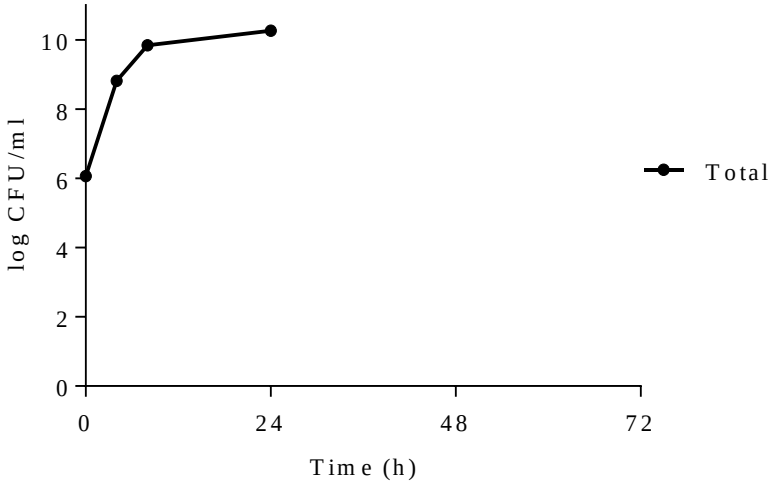
Isolate	Tazobactam dosing[a]	%*f*T>MIC$_i$	Predicted outcome	Observed outcome
Kp3	0.5 g	39.6	N/A	Regrowth
	1 g	51.6	N/A	Regrowth
	1.5 g	55.1	N/A	Suppression
	2 g	58.6	N/A	Suppression
EcF65	0.5 g	43.8	Regrowth	Regrowth
	1 g	60.0	Suppression	Suppression
	1.5 g	65.0	Suppression	Suppression
KpK91	0.5 g	44.5	Regrowth	Regrowth
	1 g	50.9	Regrowth	N/D
	1.5 g	50.9	Regrowth	N/D
	2 g	50.9	Regrowth	N/D
	4 g	50.9	Regrowth	Regrowth
Kp2301	0.5 g	13.5	Regrowth	Regrowth
	1 g	19.9	Regrowth	N/D
	1.5 g	25.5	Regrowth	N/D
	2 g	29.8	Regrowth	N/D
	4 g	36.8	Regrowth	Regrowth

[a] Co-administered with piperacillin 4 g every 8 h

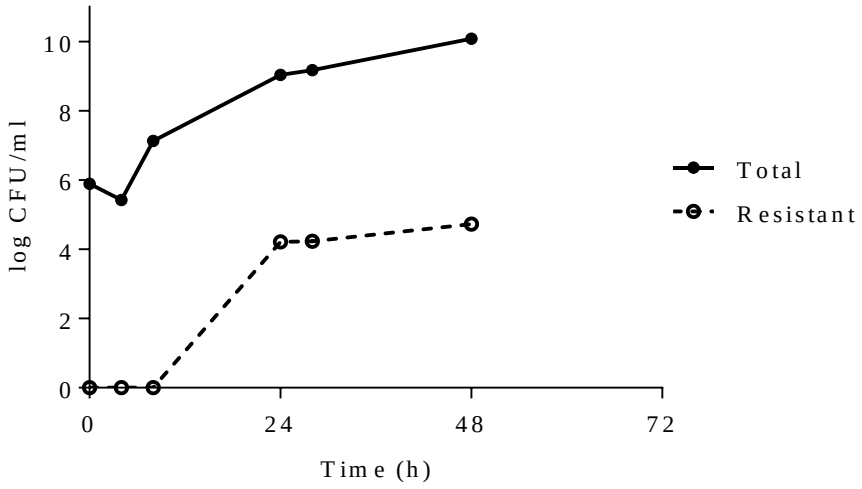
N/A – Not applicable; N/D – Not determined

Figure 4.4. Killing Profiles for EcF65. Shown are placebo control (a) along with killing profiles for 4 g piperacillin and 0.5 g tazobactam (%*f*T>MIC_i = 43.8%) (b); 4 g piperacillin and 1.0 g tazobactam (%*f*T>MIC_i = 60.0%) (c) and 4 g piperacillin and 1.5 g tazobactam (%*f*T>MIC_i = 65.0%) (d). Data displayed as mean ± SD.

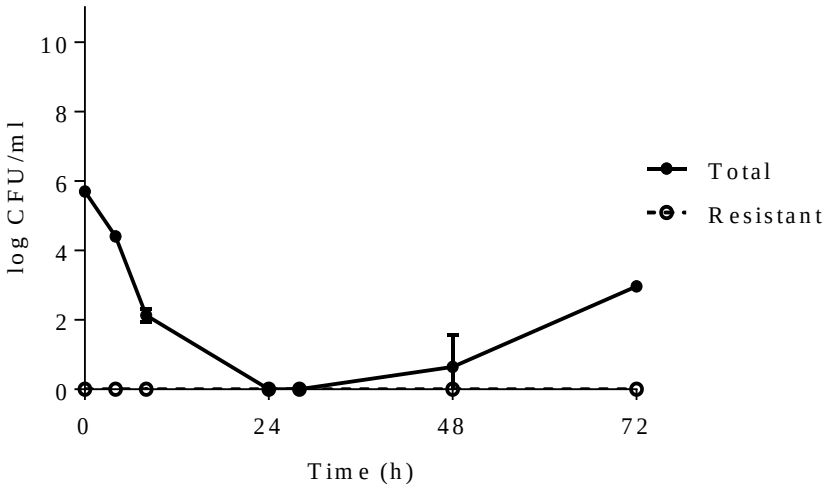
A



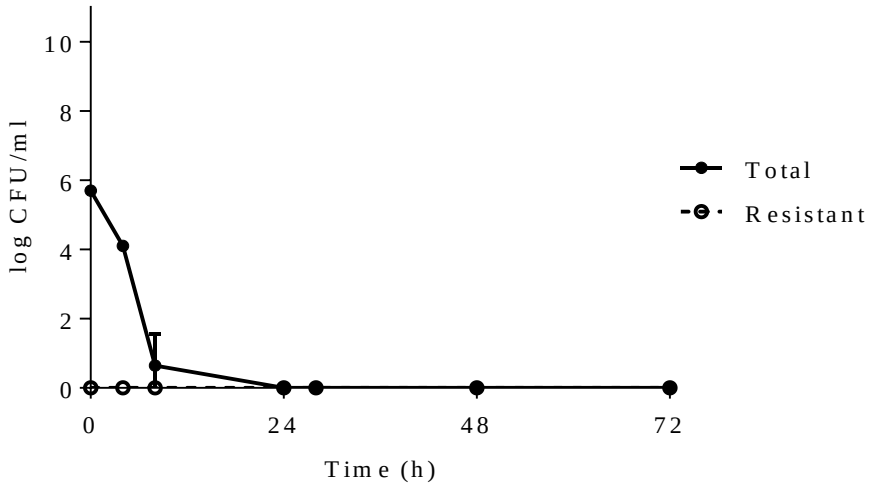
B



C


10
8
6
4
2
0
log CFU/ml
0
24
48
72
Time (h)
Total
Resistant

D


10
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log CFU/ml
0
24
48
72
Time (h)
Total
Resistant

CHAPTER 5

Comparing the Effectiveness of β-lactamase Inhibitors

5.1 Objective

The objective of this study was to provide a platform to compare the efficacy of different piperacillin/inhibitor pairings.

5.2 Rationale

Individual ESBLs may exhibit different affinities and susceptibilities to different inhibitors, thus a fixed β-lactam/β-lactamase inhibitor combination may not be optimal in all clinical situations. However, the unique concentration-response relationship between a β-lactamase inhibitor and an ESBL-producing pathogen could be harnessed to select inhibitors that could maximize the efficacy of piperacillin.

5.3 Materials and Methods

5.3.1 *Antimicrobial agents and Bacteria*

Piperacillin and tazobactam were purchased from Sigma-Aldrich and Chem-Impex International respectively, as noted in chapter 4. Relebactam and avibactam were obtained from Merck (Whitehouse Station, NJ) and Allergan (Bridgewater, NJ), respectively. Stock solutions were prepared for all drugs as described in chapter 4. All 4 ESBL-positive isolates used in this study (Kp3, KpK91, Kp2301 and EcF65) were previously characterized in chapter 4.

5.3.2 *Susceptibility Studies and Effect Modeling*

Piperacillin MICs were determined by broth dilution in triplicate for each isolate using escalating concentrations of each inhibitor (0-256 µg/mL) and repeated at least once on a different day as described in the chapter 4. Reductions in piperacillin MIC were characterized as a function of inhibitor concentration using our previously described sigmoid inhibitory E_{max} model:

$$log_2\,(MIC) = log_2(MIC_o) - I_{max}\,I^H/(I^H + IC_{50}{}^H\,)$$

5.3.3 *Model Predictions and Experimental Validations*

Using best-fit parameter values, the E_{max} model was conditioned with unbound concentrations of tazobactam, relebactam and avibactam associated with (proposed for relebactam) clinical dosing as summarized in Table 5.1.

T**able 5.1**. Simulated Piperacillin and β-lactamase Inhibitor Exposures

Agent	Dosing (g)	C_{max} (µg/mL)	$T_{1/2}$ (h)
Piperacillin	4.0	240	1.0
Tazobactam	0.5	30	1.0
Relebactam	0.25	13	1.2
Avibactam	0.5	15	2.5

C_{max} – Peak concentration; $T_{1/2}$ - elimination half-life

Theoretical instantaneous MIC (MIC_i) profiles were derived, which were reflective of changing isolate susceptibilities as inhibitor concentrations fluctuate within each dosing interval as

described in chapter 2. For each isolate, fluctuating free piperacillin exposures were then superimposed on the MIC_i profiles to estimate %*f*T>MIC_i for the inhibitors to be administered in combination with 4 g piperacillin every 8 h. An *in vitro* hollowfiber infection model was subsequently used to selectively validate predicted outcomes.

5.3.4 *Hollowfiber Infection Model*

Details regarding the schematics of the experimental setup have been previously outlined in the chapter 2. In order to simultaneously simulate first-order elimination kinetics for two agents with disparate half-lives, a supplemental drug compartment was included, as described by Blaser (Blaser 1985). Briefly, the longer half-life agent was continuously supplemented into the central compartment by a rate corresponding to the difference in clearance rates of the two agents. Fresh bacterial colonies grown to log phase in cation-adjusted Mueller-Hinton broth (Ca-MHB) were used in the hollowfiber model. A baseline inoculum of approximately 1.0 x 10^6 cfu/mL was used for all isolates. The targeted exposures for piperacillin and the inhibitors are summarized in Table 5.1.

5.3.5 *Drug Assays and Pharmacokinetic Modeling*

Analyte concentrations were assayed as described in the general methodology chapter. A 1-compartment model with zero-order infusion input was fit to the observed piperacillin and avibactam concentration-time profiles using ADAPT 5 (D'Argenio 2009).

5.4 Results

5.4.1 *Effect of β-lactamase Inhibitors on Piperacillin MIC*

For all 3 inhibitors, we observed a concentration-dependent decrease in piperacillin MIC. The relationship between susceptibility and inhibitor concentrations was reasonably characterized by the sigmoid inhibitory E_{max} model in all instances ($r^2 \geq 0.92$). The best-fit parameter estimates summarized in Table 5.2 indicated the differences in isolate susceptibilities to each of the inhibitors. For each isolate, IC_{50} values were the lowest with avibactam. Furthermore, I_{max} values also showed a more drastic reduction in MIC when paired with avibactam. These observations are reflective of the %*f*T>$MIC_{i,}$ values shown in Table 5.2, where the combination of piperacillin/avibactam is associated with the highest %*f*T>MIC_i values in all 4 isolates. Thus, piperacillin/avibactam was anticipated to have the highest likelihood of suppressing bacterial growth. For relebactam, IC_{50} values were either lower than observed with tazobactam (as with Kp2301), comparable (for Kp91 and EcF65) or higher (Kp3). There was a general trend toward higher I_{max} values for relebactam with Kp3, KpK91 and Kp2301. We previously showed that bacterial growth could be suppressed with more aggressive piperacillin/tazobactam dosing for Kp3 and EcF65. However, growth suppression was unattainable for KpK91 and Kp2301 using $\leq$ 4 g tazobactam (data not shown). Thus, we focused on validating efficacy predictions in this study for KpK91 and Kp2301 using piperacillin in combination with avibactam. Representative model fits for KpK91 and Kp2301 are shown in Figure 5.1.

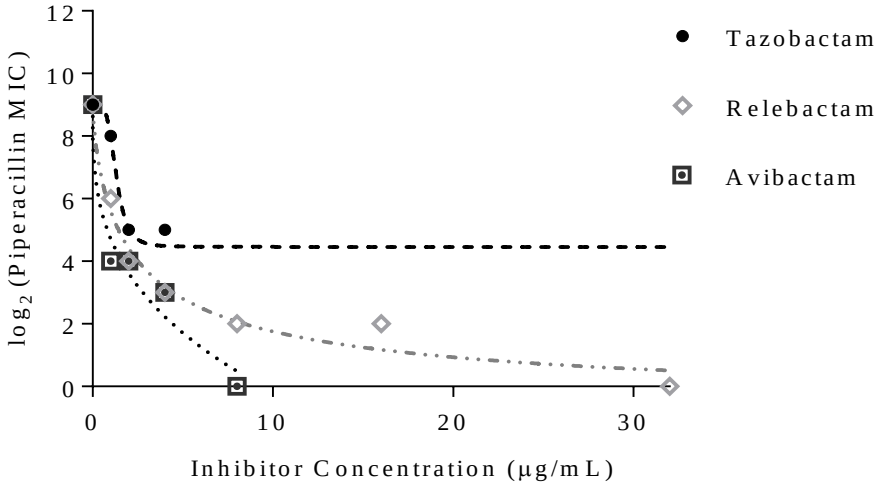
Table 5.2. Estimated E_{max} Model Parameters, %*f*T>MIC_i and Predicted Outcomes for Tazobactam, Relebactam and Avibactam

Inhibitor	Bacteria	$\log_2(MIC_0)$	I_{max}	IC_{50}	H	%*f*T>MIC_i [a]	r^2
Tazobactam	Kp3	9.32	6.52	2.60	1.57	39.6	0.94
	KpK91	9.03	4.75	1.36	4.00	44.5	0.97
	Kp2301	9.09	6.23	35.25	2.67	13.5 [b]	0.97
	EcF65	8.67	6.99	2.71	3.41	43.8 [b]	0.98
Relebactam	Kp3	9.04	8.28	3.72	1.27	37.0	0.99
	KpK91	9.02	7.86	1.39	1.18	48.5	0.99
	Kp2301	9.36	8.24	15.82	1.23	16.8	0.97
	EcF65	8.54	6.56	3.00	5.60	38.4	0.96
Avibactam	Kp3	9.00	11.22	0.62	0.21	73.6	0.98
	KpK91	9.19	8.02	1.05	1.39	73.6	0.92
	Kp2301	9.38	7.83	2.37	1.75	61.4 [b]	0.95
	EcF65	9.00	9.64	0.92	1.17	76.0 [b]	0.99

[a] Based on co-administration with piperacillin 4 g every 8 h; [b] Selected for prospective validations

Figure 5.1. Comparative Model Fit for KpK91 (A) and Kp2301 (B) for Tazobactam, Relebactam and Avibactam. Symbols represent experimental observations and continuous lines represent the best-fit model.

A



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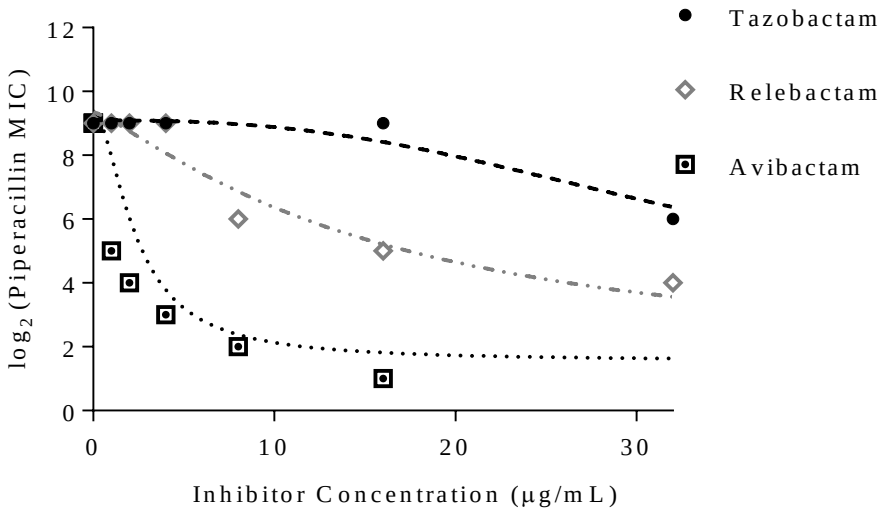
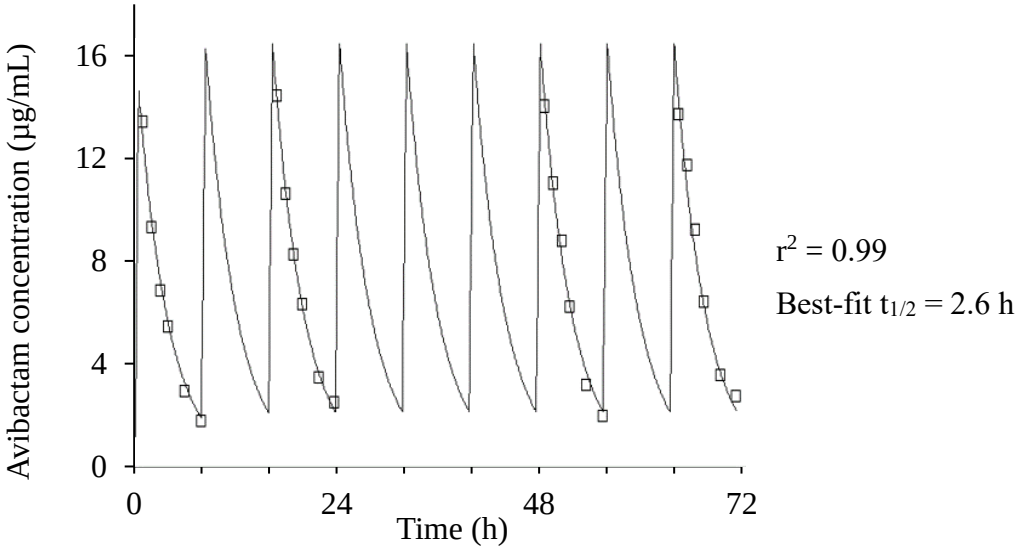


5.4.2 *Pharmacokinetics and Pharmacodynamics of Combinations*

The target peak concentrations and elimination half-lives were reasonably well simulated in the hollowfiber models. Representative pharmacokinetic profiles for avibactam and piperacillin are showed in Figure 5.2. Changes in bacterial density are shown for KpK91 and Kp2301 in Figure 5.3. For both isolates, regrowth was observed with a clinical regimen of 4 g piperacillin co-administered with 0.5 g tazobactam. However, co-administration of piperacillin with 0.5 g avibactam suppressed bacterial growth over time. These observations are generally consistent with our expectations that higher magnitudes of %*f*T>MIC_i correspond to improved efficacy.

Figure 5.2. Typical Simulated Pharmacokinetic Profile for 0.5 g Avibactam (Target C_{max} = 15 µg/mL) (A) and 4 g Piperacillin (Target C_{max} = 240 µg/mL) (B) Administered Every 8 h. Target half-lives were 2.5 h and 1.0 h for avibactam and piperacillin respectively. Open squares represent observed concentrations and continuous lines represent the best-fit model.

A



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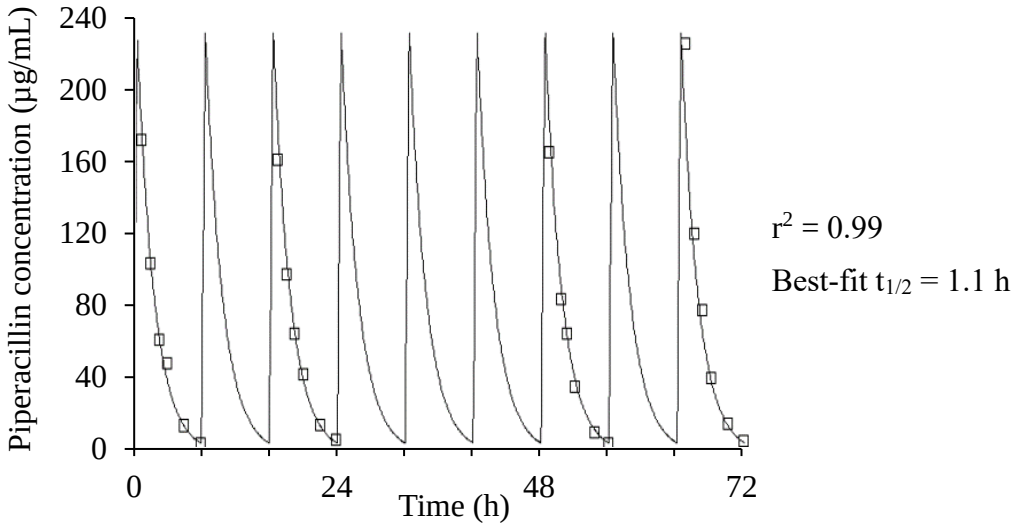
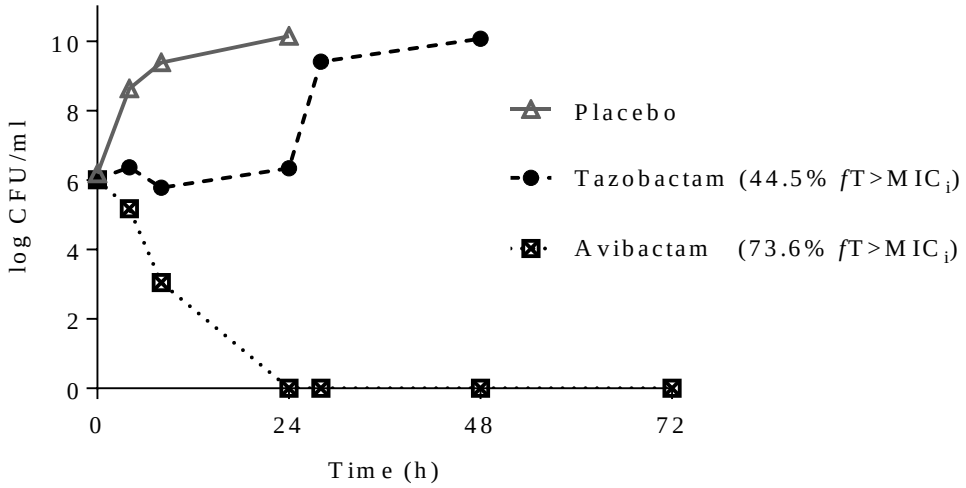


Figure 5.3. Killing Profiles for Piperacillin in Combination with Tazobactam and Avibactam against KpK91(A) and Kp2301 (B). Open triangles represent placebo (no drug treatment), closed circles represent killing profiles for 4 g piperacillin in combination with 0.5 g tazobactam and squares represent killing profiles for 4 g piperacillin in combination with 0.5 g avibactam. Data displayed as mean ± SD.

A



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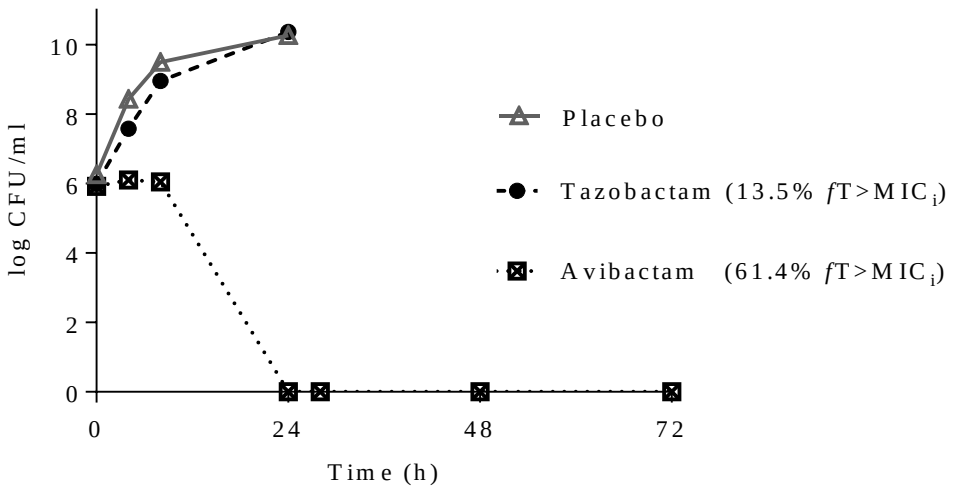
log CFU/ml
10
8
6
4
2
0
0
24
48
72
Time (h)
Placebo
Tazobactam (13.5% fT>MIC$_i$)
Avibactam (61.4% fT>MIC$_i$)

CHAPTER 6

DISCUSSION

In light of the global spread of carbapenemases, there is growing effort to reduce the heavy reliance on carbapenems for ESBL infections. β-lactam/β-lactamase inhibitor combinations such as piperacillin/tazobactam, while exhibiting promising *in vitro* profiles against ESBL-producing Enterobacteriaceae, remain a controversial option for these infections. The conflicting clinical data regarding the efficacy of piperacillin/tazobactam suggest that *in vitro* susceptibility may not always correlate to clinical efficacy. This is due to (at least in part) technical limitations in susceptibility testing which may impact efficacy predictions. Additionally, characterization of inhibitor activity and subsequent dose optimization remain a challenge because traditional PK/PD indices are not directly applicable.

Reservations regarding the use of combinations like piperacillin/tazobactam also stem from *in vitro* observations of reduced bactericidal activity in the presence of high inocula (>1x 10^7 cfu/mL) (López-Cerero, Picón et al. 2010). Currently, tazobactam is the only inhibitor approved by the U.S. Food and Drug Administration (FDA) for use in combination with piperacillin. Given the similar half-lives of piperacillin and tazobactam, it is believed that tazobactam can preserve the integrity of piperacillin for the entire duration of a dosing interval. However, this may not be applicable for all ESBL infections, as the presence of multiple enzymes and/or over-production of a single enzyme may render tazobactam less effective at conventional doses. For these reasons, there is a dire need for a more robust platform to optimize the dosing of existing β-lactam/β-lactamase inhibitor combinations.

There are more than 100 different types of resistance genes encoding ESBLs. In order to maintain the clinical relevance of our research, we assessed the scope of ESBL-mediated

resistance in our local region. We focused on clinically pervasive enzymes by exploring the prevalence of various ESBL genotypes in a local collection of *K. pneumoniae* bloodstream isolates. Overall, the prevalence of ESBLs was lower than that reported in national surveillance studies (Castanheira, Farrell et al. 2014). Despite the single center nature of our study, our isolates were clonally diverse and depicted a predominance of $bla_{\text{CTX-M-15}}$ in agreement with the national profile. Generally, the susceptibility profiles of these isolates were consistent with the ESBL phenotype. Interestingly, isolate 2301 also displayed resistance to meropenem, pointing to the possible involvement of altered outer membrane porin channels. Nonetheless, for all these isolates, we demonstrated a trend towards elevated ceftazidime MICs with increased enzymatic activity (r^2 = 98%). This indicated that β-lactamase activity had a notable impact on MIC, suggesting that optimal enzyme inhibition could be instrumental to susceptibility and ultimately, antimicrobial efficacy.

Based on the findings from our epidemiological study, we explored the efficacy of different dosing strategies for piperacillin/tazobactam to understand how maximizing enzyme inhibition could be leveraged to improve efficacy. Piperacillin/tazobactam was selected because it is a widely used β-lactam/β-lactamase inhibitor combination for nosocomial infections involving Gram-negative bacteria. To resolve some of the inconsistences between observed *in vitro* effects and clinical outcomes for piperacillin/tazobactam, we based our approach on a modified susceptibility profiling scheme that would improve efficacy predications.

Previous work by Nicasio et al. delineated the PK/PD index that best predicted the efficacy of tazobactam within the context of a fixed β-lactam (ceftolozane) (Nicasio, VanScoy et al. 2016). Using data from MIC studies, the percentage of time above a threshold inhibitor concentration (%Time > threshold) was identified as the index that best correlated to

tazobactam efficacy. This threshold value signified a critical concentration (dependent on enzyme transcription levels) at which enzyme inhibition was maximal. These findings suggested that tazobactam exposures may need to be customized for individual isolates (based on differences in enzyme expression) to meet efficacy targets. From a dosing perspective, this approach was more informative than the current scheme for characterizing β-lactam/β-lactamase inhibitor combinations. However, by overlooking inhibitor effects below and above the proposed critical threshold value, this approach was subject to similar inherent limitations as the current paradigm.

Other investigators have used a semi-mechanistic model to describe the combined activities of aztreonam/avibactam, another β-lactam/β-lactamase inhibitor combination (Sy, Zhuang et al. 2017). In their approach, Sy et al. incorporated data from time-kill studies to develop a sophisticated mathematical model that characterized bacterial killing as a function of varying β-lactamase inhibitor and β-lactam concentrations. The model was used to simulate humanized dosing of both agents to provide a comprehensive evaluation of dose-dependent changes in bacterial response. Additionally, the model accounted for β-lactam degradation (in the presence of varied inhibitor concentrations) against different resistant bacterial populations. While this framework is likely useful for predicting the efficacy of a β-lactam/β-lactamase inhibitor dosing regimen, this approach is somewhat limited in its clinical application since its implementation is dependent on time-kill data that are not routionely generated in clinical settings. Additionally, the model validations were limited to only 24 h, thus the effect of β-lactam/β-lactamase inhibitor exposures during an extended timeframe (beyond the initial experimentation) was not explored.

In our study, we captured the fluctuations in pathogen susceptibility associated with intermittent dosing of tazobactam. Modeling of the susceptibility reversibility profiles revealed unique characteristics related to inhibitor affinity and the maximum inhibition achievable for each tazobactam-pathogen combination. We also showed that the inhibitory sigmoid E_{max} model parameter estimates could be used to derive the $fT>MIC_i$ associated with various piperacillin/tazobactam exposures and provide a target efficacy threshold. Based on conventional susceptibility breakpoints, Kp3, KpK91 and Kp2301 were all resistant to piperacillin/tazobactam, thus standard dosing regimens would be expected to yield inadequate exposures. Yet we demonstrated that each isolate responded distinctly to escalating tazobactam exposures and a tailored tazobactam dosing approach could facilitate meeting the efficacy target. For instance, Kp3 and KpK91 shared an identical piperacillin MIC (using 4 µg/mL of tazobactam), hence would be expected to respond similarly to piperacillin/tazobactam. However, a more nuanced effect was observed and could be attributed to differences in I_{max} values. Consequently, growth suppression was achieved with a more aggressive dosing approach for Kp3, but was unattainable for KpK91 (using ≤ 4 g tazobactam). Consistent with our expectations, the efficacy threshold was also unattainable for Kp2301 due to high level of enzymatic activitiy, as reflected by the comparatively high IC_{50} for this isolate. With EcF65, our model further illustrated the shortcomings of predicting efficacy with a fixed tazobactam concentration. Although regarded as susceptible by current interpretation criteria, our findings indicated that dosing 4 g piperacillin and 0.5 g tazobactam every 8 h would be inadequate against this isolate. Instead, a higher tazobactam exposure (equivalent to 1.0 g) was needed to suppress bacterial growth below the starting inoculum.

For our clinical isolates that did not respond to optimal piperacillin/tazobactam dosing, we also evaluated the efficacy of different inhibitors in extending the viability of piperacillin. Switching a partner agent in an established β-lactam/β-lactamase inhibitor combination to combat specific mechanisms of resistance is not entirely novel, as illustrated with the pairing of ceftolozane with tazobactam. However, there is a lack of a standardized platform to evaluate nonconventional β-lactam/β-lactamase inhibitor combinations clinically. Several new β-lactamase inhibitors with activity against ESBLs have been introduced, but they are formulated commercially in fixed combinations with a partner β-lactam.

Using avibactam (co-formulated with ceftazidime) and relebactam (currently under development for use with imipenem), we derived and compared the $fT>MIC_i$ associated with alternative piperacillin/inhibitor pairings to the traditional combination of piperacillin/tazobactam. In our preceding study, we had established a threshold $fT>MIC_i$ of 55.1% for bacterial growth suppression. Piperacillin/avibactam achieved $fT>MIC_i$ ($\geq$ 61.4%) greater than estimated for piperacillin/tazobactam ($\leq$ 44.5%) and piperacillin/relebactam ($\leq$ 48.5%), and suppressed bacterial growth. Thus, these outcomes were consistent with our observed threshold $fT>MIC_i$ for suppressing bacterial growth.

Our approach was novel for four reasons: 1) in addition to the genetic detection of resistance elements, we used generalized phenotypic and biochemical assays to ascertain that the genes detected in our isolates were functionally expressed, 2) we attempted to address the drawbacks in conventional susceptibility testing with limited efficacy predictions, 3) we explored the adequacy of the standard 8:1 dosing ratio of piperacillin to tazobactam for different scenarios, and 4) we demonstrated an alternative scheme in which a different partner inhibitor may be used to enhance the efficacy of a β-lactam. Instead of characterizing the activity of

piperacillin/tazobactam based on a single tazobactam concentration, we described this relationship more comprehensibly using a range of concentrations. This approach was more informative, as it better reflected the changing β-lactamase inhibitor concentrations observed *in vivo*. It resulted in a more robust model framework in assessing the efficacy of various piperacillin/tazobactam dosing regimens against commonly encountered clinical isolates of *K. pneumoniae* and *E. coli* producing ESBL enzymes. Morever, extending this approach to other piperacillin/inhibitor pairings allowed us to profile the concentration-dependent changes in MIC unique to different piperacillin/inhibitor combinations. This provided better guidance for partnering β-lactam/β-lactamase inhibitors not just on the basis of matching pharmacokinetic properties, but also for optimal pharmacodynamic effect.

There were notable limitations to our studies. Firstly, our model only focused on ESBL-mediated resistance, thus the effect of additional mechanisms of resistance (such as efflux pump hyperexpression, porin deficiency) in our isolates was not explored. The hollowfiber studies were limited to 72 h, hence the predictive value of the model for longer durations of exposure is unknown. Additionally, since all our studies involved a moderate inoculum (~1 x 10^6 cfu/mL), the impact of a high inoculum (~1 x 10^8 cfu/mL) on efficacy remains unclear. Given our limited sample size, our proposed framework for optimizing inhibitor dosing and comparing the efficacy of different inhibitors warrants further validation against a larger collection of clinical isolates. Lastly, since the study was confined to a fixed piperacillin backbone regimen, further investigations are required to determine generalizability to other dosing options and different β-lactam/β-lactamase inhibitor combinations.

CONCLUSION

In summary, our research has provided four key insights:

1) Susceptibility assessments using a range of both inhibitor and β-lactam concentrations is more informative to efficacy predictions than using a fixed inhibitor concentration
2) Efficacy of a β-lactam/β-lactamase inhibitor combination can be correlated to the concentration-response relationship between the ESBL-producing bacteria using a modified standard PK/PD metric (*f*T>MICi)
3) The fixed ratio approach to dosing β-lactam/β-lactamase inhibitor combinations may not be appropriate for all ESBL-producing Enterobacteriaceae since individual isolates differ in their degree of enzyme expression
4) Fixed agent combinations may not be optimal for all ESBL-producing Enterobacteriaceae, thus pairing the β-lactam to a different partner inhibitor may be warranted for challenging clinical isolates

The platform we have proposed for susceptibility profiling is relatively easily implemented to evaluate the utility of an inhibitor for a specific ESBL-producing isolate. While it may not be immediately feasible in a clinical laboratory setting, it may be adapated to automated diagnostic device. Our proposed framework may be instrumental to optimal dosing of old and newer β-lactam/β-lactamase inhibitor combinations, and expedite the identification of optimal pairings of drugs that are already available clinically.

CHAPTER 7

FUTURE DIRECTIONS

7.1 *Validation of Proposed Model against other ESBL Genotypes*

Future studies will determine the generalizability of our proposed framework in a larger collection of ESBL-producing Enterobacteriaceae that harbor other ESBL enzymes (e.g. TEM-type and additional SHV-types). We will explore the benefit of additional dosing schemes, such as continuous infusion of β-lactam/β-lactamase inhibitors, which has been shown to improve the efficacy of the combination in clinical settings. We will also evaluate the utility of asymmetric dosing frequency (i.e. dosing the inhibitor and β-lactam independently at different intervals), and evaluate the impact of staggered dosing (i.e., dosing the β-lactamase inhibitor first and the β-lactam later after a lag time) in maximizing the effect of β-lactam/β-lactamase inhibitor combinations.

7.2 *Impact of Higher Inoculum and Suppression of Resistance Development*

A number of studies have illustrated that for some β-lactams, the observed MIC of a bacterial isolate may be dependent on the initial inoculum. This is termed the inoculum effect, and is described as ≥ 8-fold change in MIC when an inoculum 100-fold higher than the standard 5 x 10^5 cfu/mL is used (Udekwu, Parrish et al. 2009, Smith and Kirby 2018). For β-lactamase producing strains, a higher bacterial burden is associated with increased involvement of β-lactamase enzymes which can further contribute to the degradation of the antibiotic. Our laboratory previously demonstrated that for isolates presenting with β-lactamase and/or non-β-lactamase mediated resistance, C_{min}/MIC ratios correlated well with observed trends in

regrowth and resistance suppression (Tam, Chang et al. 2017). In our future studies, we will determine if a threshold ratio may be proposed for suppressing resistance to β-lactam/β-lactamase inhibitor combinations. Preliminary experimental data indicate that for EcF65, co-administration of 4 g piperacillin with 4 g tazobactam (C_{min}/MIC = 2.3) against a 1.0 x 10^8 cfu/mL starting inoculum could not prevent bacterial regrowth or resistance development beyond 72 h. However, when piperacillin was used in combination with avibactam (C_{min}/MIC = 4.5), we did observe sustained growth and resistance suppression over 120 h.

7.3 *Optimization of β-lactam/β-lactamase Inhibitor Dosing against Carbapenemase-producing Enterobacteriaceae*

The next phase of our research will focus on optimizing the dosing of β-lactam/β-lactamase inhibitor combinations against carbapenemases. As part of our epidemiological study of the most prevalent β-lactamase enzymes in our geographic area, we also determined the prevalence of key carbapenemases (KPCs, OXA-type and metallo-β-lactamases). Eight of the isolates in our collection expressed non-metallo carbapenemase activity. As with our study of ESBL-positive isolates, the overall prevalence of carbapenemases was lower than described nationally. Nonetheless, our isolates predominantly harbored *bla*$_{KPC-2}$, a finding that was in agreement with national surveillance data. The correlation of hydrolytic activity to enzyme activity was lower in our carbapenemase-harboring isolates (r^2 = 56%) than observed with isolates harboring ESBLs; this indicates that additional non-enzymatic mechanisms, such as porin deletion or efflux pump over-expression may be crucial to resistance in these isolates. It is noteworthy that even in isolates that presented with concomitant polymyxin B resistance (i.e. multidrug resistance), the addition of avibactam restored susceptibility to ceftazidime. Thus, in spite of

the presence of other resistance modalities, the addition of an active β-lactamase inhibitor did restore the activity of ceftazidime. Nonetheless, optimal dosing of ceftazidime/avibactam is warranted, as resistance development has already been reported in clinical isolates harboring *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ (Humphries, Yang et al. 2015, Giddins, Macesic et al. 2017).

7.4 *Automated Longitudinal Data Capture for Dynamic Modeling*

Exploring a range of inhibitor concentrations as proposed in our research would be labor-intensive and time-consuming in a traditional clinical laboratory setting, where rapid and accurate antimicrobial susceptibility testing is critical to clinical decision-making. However, adapting our proposed scheme to a rapid diagnostic platform could expedite the assessment of the concentration-response relationships between β-lactamase producing isolates and inhibitors. One such technology, the BacterioScan, is a Forward Laser Light Scattering (FLLS) device that measures the density of microorganisms in a liquid medium and has shown promise in susceptibility testing for other β-lactams (such as cefepime) against *E. coli* (Hayden, Clinton et al. 2016). Our future studies will determine the feasibility of adapting our susceptibility testing scheme to this platform. In addition to lessening the technical work required to test numerous inhibitor concentrations, our initial studies indicate that the Bacterioscan follows the time-course for bactericidal effect, generating a profile similar to that observed in time-kill studies. As such, the data generated on this platform could be more informative (than a traditional MIC test) in characterizing the dynamic killing profiles for different β-lactam/inhibitor combinations and may further the predictive value and utility of our model.

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