University of Tennessee Health Science Center
UTHSC Digital Commons

Theses and Dissertations (ETD)

College of Graduate Health Sciences

12-2015

Optimization of Lead Spectinamide Compounds as Novel Anti-tuberculosis Agents with a Pharmacometric Approach

Ashit Rasendu Trivedi

University of Tennessee Health Science Center

Follow this and additional works at: <https://dc.uthsc.edu/dissertations>

Part of the [Medicinal and Pharmaceutical Chemistry Commons](#), and the [Pharmaceutics and Drug Design Commons](#)

Recommended Citation

Trivedi, Ashit Rasendu , "Optimization of Lead Spectinamide Compounds as Novel Anti-tuberculosis Agents with a Pharmacometric Approach" (2015). *Theses and Dissertations (ETD)*. Paper 399.
<http://dx.doi.org/10.21007/etd.cghs.2015.0323>.

This Dissertation is brought to you for free and open access by the College of Graduate Health Sciences at UTHSC Digital Commons. It has been accepted for inclusion in Theses and Dissertations (ETD) by an authorized administrator of UTHSC Digital Commons. For more information, please contact jwelch30@uthsc.edu.

Optimization of Lead Spectinamide Compounds as Novel Anti-tuberculosis Agents with a Pharmacometric Approach

Abstract

In an effort to combat the global Tuberculosis pandemic, Dr. Richard E. Lee and his group at St. Jude Children's Research Hospital designed a novel series of anti-tuberculosis agents, spectinamides – semi-synthetic analogs of spectinomycin. Spectinamides are a potent inhibitor of mycobacterial ribosomes and overcome efflux mediated drug resistance in *M. tb*. Spectinamides have shown an excellent in vitro activity, which makes them well suited for further lead optimization and preclinical development. We hypothesized that through pharmacokinetic (PK) and pharmacodynamics (PD) model-based dosing optimization studies, we could strategically guide the selection and refinement of more potent and effective anti-TB spectinamides. Biopharmaceutical in vitro screening demonstrated that spectinamides in general have low plasma protein binding and are stable against hepatic microsomal metabolism. In vivo pharmacokinetic studies in rats revealed that the kidneys are the major route of elimination for spectinamides in their unchanged form. Radiolabeled biodistribution studies showed 84.7% of radioactivity accumulated 70% in urine, 12.6% in feces, and the remainder in the blood and other major organs. The unaccounted for residual 15.3% likely distributed into the epidermis and other surface tissue. In multiple-dose accumulation studies, the C_{max} of radiolabeled compound after the 1st dose and the 8th dose of twice-daily dosing regimen was similar: 3.39 μ Ci/mL and 3.55 μ Ci/mL, suggesting no relevant accumulation of parent drug and metabolites. The concentration of radiolabeled compound was three times more in lungs and spleen as compared to whole blood, suggesting good tissue penetration. Macrophage uptake studies showed that Lee 1329, Lee 1445 and Lee 1599 had significantly higher macrophage uptake than spectinomycin and streptomycin. Lee 1329 showed 6-fold and 2.2-fold higher uptake than streptomycin and spectinomycin, respectively. Based on the results of the in vitro experiments and preliminary PK/PD studies in rats, Lee 1599 was selected as the lead candidate compound. To predict PK/PD indices of antimicrobial efficacy, we performed model-based dosing optimization studies with Lee 1599. We used an in vitro PK/PD model system to simulate the rat PK conditions while evaluating antibacterial activities to predict effective dosing regimens for further in vivo efficacy studies. Our results have shown that Lee 1599 exhibits dose-dependent bactericidal effect. Lee 1599 showed up to 4-log reductions in bacterial counts at 100mg QD dosing. The PK/PD indices demonstrated that Lee 1599 elicits a concentration- and time-dependent killing with AUC/MIC as the optimal index. The model was put through numerical simulations to predict the effect of Lee 1599 in mice at various dosing regimens. The in vitro PK/PD simulated profile has suggested that high doses with frequent dosing intervals may demonstrate optimum in vivo efficacy. Consequently, we aimed to determine the pharmacodynamic interaction between Lee 1599 and existing anti-tuberculosis agent. We selected rifampicin as a model compound and applied a parametric approach to quantitatively assess the pharmacodynamic drug interaction between Lee 1599 and rifampicin. The three dimensional surface response assay demonstrated that there is an additive effect between both the agents as opposed to the conventional checkerboard assay, which suggested synergism between these agents. The results of surface response assay were validated using an in vitro PK/PD model for combination agents and in vivo efficacy trials, which showed an additive effect between Lee 1599 and rifampicin. Thus, quantitative assays such as the surface response assay seem to provide more reliable information on pharmacodynamic interactions as opposed to qualitative methods such as checkerboard assay. In conclusion, we have successfully supported the further development of spectinamides using a pharmacometric approach. We have identified a lead candidate compound Lee 1599 using an iterative PK/PD approach for its pre-clinical drug development. The application of PK/PD knowledge is essential for translating the in vitro screening assay findings to the in vivo stage, thus accelerating the drug development process. The results of the above studies can be used as a roadmap for the optimization of anti-infective agents in the early drug discovery and pre-clinical developmental phase.

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Pharmaceutical Sciences

Research Advisor

Bernd Meibohm, Ph.D., FCP

Keywords

Anti-infectives, Modeling, Pharmacodynamics, Pharmacokinetics, Pharmacometrics, Simulations

Subject Categories

Medicinal and Pharmaceutical Chemistry | Medicine and Health Sciences | Pharmaceutics and Drug Design | Pharmacy and Pharmaceutical Sciences

OPTIMIZATION OF LEAD SPECTINAMIDE COMPOUNDS AS NOVEL ANTI-TUBERCULOSIS AGENTS WITH A PHARMACOMETRIC APPROACH

A Dissertation
Presented for
The Graduate Studies Council
The University of Tennessee
Health Science Center

In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy
From The University of Tennessee

By
Ashit Rasendu Trivedi
December 2015

Chapter 1 © 2013 by Informa Healthcare.
All other material © 2015 by Ashit Rasendu Trivedi.
All rights reserved.

DEDICATION

It is not the destination; it is about the journey...

ACKNOWLEDGEMENTS

Although the journey for the doctor of philosophy degree was certainly lengthy, adventurous and eventful, there are number of people that I want to acknowledge and express my gratitude for making it beautiful and memorable. Firstly, I would like to thank and appreciate my mentor Dr. Bernd Meibohm who believed me and provided me with a wonderful opportunity of working with him under his direct supervision. It would not have been easier for me to learn and accomplish multiple things without your support and guidance. My committee members: Dr. Richard Lee, Dr. Ryan Yates, Dr. David Rogers and Dr. Julian Hurdle have played an instrumental role in my academic and scientific progress towards my PhD degree. I want to thank Dr. Richard Lee especially for allowing me to work on spectinamide project without which my research work would not have been possible. Special thanks to Dr. Richard Lee's lab members: Robin Lee, Dr. David Bruhn, Marcus Maddox and Dr. Jiuyu Liu for teaching me microbiology skills, giving me the compounds for performing all research work and MIC data; our collaborators Dr. Anne Leanarts and her team for providing *in vivo* data to support and navigate my research findings. I am also grateful to Dr. Neeraj Gupta, Director in Clinical Pharmacology at Takeda (Millennium) Pharmaceuticals, Cambridge who offered me with an internship position and contributed to my scientific and career development.

Secondly, I wish to extend the appreciation to my previous and current lab members (also my friends) for being my support system and helping me troubleshoot the research problems during the course of my research work. These amazing people are Dr. Nageshwar Budha, Dr. Pavan Kumar Vaddady, Dr. Satyendra Suryawanshi, Dr. Yi Zhang, Wararat Limothai, Dr. Margaret Thomson, Dr. Lei Diao, Dr. Dorababu Madhura, Chetan Rath, Dr. Sumit Rawal, Dr. Josiah Ryman, Karoline Kromke and Sophie Krauel. My sincere thanks to the staff members Jennifer Stabenow, Dr. Jan Peters, Samuel Woods, Robert Benson and John Shelton of Regional Biocontainment Lab for making my life easier by providing us a space to carry out the microbiology experiments and for extending their technical support at any given point of time. I also wish to acknowledge Brenda Thornton, Corliss Finlay and Benita Williams in the Department of Pharmaceutical Science for providing administrative aid. A special note of appreciation to my friends and colleagues Jordan Toutounchian, Jeffrey Pressly, Dileep Janagam, Diwa Koirala, Shajila Siricilla, Dr. Nivesh Mittal, Dr. Sarah Whaley, Dr. Andrew Nishimoto and Beth Ladner Berkow.

It was not an easy task for me to leave behind old good friends and settle in Memphis without any known person. It was possible to move forward only because of my dear friends Varun, Ramya, Nikunj, Dipti, Pallavi, Satyajeet, Spurthi and Vishwanath who encouraged and supported me at every step during my graduate studies. I also met new people here in Memphis and they definitely became good friends over the period of five years making me feel home. Thanks a lot Vaibhav, Viraj, Sumana, Rajashree, Kavisha, Kiraat, Satya and Saini for some great memories that we will cherish together in years to come.

None of the above things would have been possible without my dedicated parents who taught me everything and supported me with every decision and correcting me whenever I was wrong. Thanks mummy and papa for this awesome upbringing and my sister, Sneha for entertaining me every single day over the phone and making me feel I am home with them in India.

ABSTRACT

In an effort to combat the global Tuberculosis pandemic, Dr. Richard E. Lee and his group at St. Jude Children's Research Hospital designed a novel series of anti-tuberculosis agents, spectinamides – semi-synthetic analogs of spectinomycin. Spectinamides are a potent inhibitor of mycobacterial ribosomes and overcomes efflux mediated drug resistance in *M. tb*.

Spectinamides have shown an excellent *in vitro* activity, which makes them well suited for further lead optimization and preclinical development. We hypothesized that through pharmacokinetic (PK) and pharmacodynamics (PD) model-based dosing optimization studies, we could strategically guide the selection and refinement of more potent and effective anti-TB spectinamides.

Biopharmaceutical *in vitro* screening demonstrated that spectinamides in general have low plasma protein binding and are stable against hepatic microsomal metabolism. *In vivo* pharmacokinetic studies in rats revealed that the kidneys are the major route of elimination for spectinamides in their unchanged form. Radiolabeled biodistribution studies showed 84.7% of radioactivity accumulated 70% in urine, 12.6% in feces, and the remainder in the blood and other major organs. The unaccounted for residual 15.3% likely distributed into the epidermis and other surface tissue. In multiple-dose accumulation studies, the C_{max} of radiolabeled compound after the 1st dose and the 8th dose of twice-daily dosing regimen was similar: 3.39 μ Ci/mL and 3.55 μ Ci/mL, suggesting no relevant accumulation of parent drug and metabolites. The concentration of radiolabeled compound was three times more in lungs and spleen as compared to whole blood, suggesting good tissue penetration. Macrophage uptake studies showed that Lee 1329, Lee1445 and Lee 1599 had significantly higher macrophage uptake than spectinomycin and streptomycin. Lee 1329 showed 6-fold and 2.2-fold higher uptake than streptomycin and spectinomycin, respectively.

Based on the results of the *in vitro* experiments and preliminary PK/PD studies in rats, Lee 1599 was selected as the lead candidate compound. To predict PK/PD indices of anti-microbial efficacy, we performed model-based dosing optimization studies with Lee 1599. We used an *in vitro* PK/PD model system to simulate the rat PK conditions while evaluating antibacterial activities to predict effective dosing regimens for further *in vivo* efficacy studies. Our results have shown that Lee 1599 exhibits dose-dependent bactericidal effect. Lee 1599 showed up to 4-log reductions in bacterial counts at 100mg QD dosing. The PK/PD indices demonstrated that Lee 1599 elicits a concentration- and time-dependent killing with AUC/MIC as the optimal index. The model was put through numerical simulations to predict the effect of Lee 1599 in mice at various dosing regimens. The *in vitro* PK/PD simulated profile has suggested that high doses with frequent dosing intervals may demonstrate optimum *in vivo* efficacy.

Consequently, we aimed to determine the pharmacodynamic interaction between Lee 1599 and existing anti-tuberculosis agent. We selected rifampicin as a model

compound and applied a parametric approach to quantitatively assess the pharmacodynamic drug interaction between Lee 1599 and rifampicin. The three dimensional surface response assay demonstrated that there is an additive effect between both the agents as opposed to the conventional checkerboard assay, which suggested synergism between these agents. The results of surface response assay were validated using an *in vitro* PK/PD model for combination agents and *in vivo* efficacy trials, which showed an additive effect between Lee 1599 and rifampicin. Thus, quantitative assays such as the surface response assay seem to provide more reliable information on pharmacodynamic interactions as opposed to qualitative methods such as checkerboard assay.

In conclusion, we have successfully supported the further development of spectinamides using a pharmacometric approach. We have identified a lead candidate compound Lee 1599 using an iterative PK/PD approach for its pre-clinical drug development. The application of PK/PD knowledge is essential for translating the *in vitro* screening assay findings to the *in vivo* stage, thus accelerating the drug development process. The results of the above studies can be used as a roadmap for the optimization of anti-infective agents in the early drug discovery and pre-clinical developmental phase.

TABLE OF CONTENTS

CHAPTER 1. APPLICATION OF PHARMACOMETRICS IN THE CLINICAL DRUG DEVELOPMENT OF ANTI-INFECTIVES	1
Introduction.....	1
Pharmacometrics in Anti-infective Drug Development	1
Prerequisites for the Application of Pharmacometrics	3
PK/PD Indices and Susceptibility Breakpoints	9
Development of Optimal Dosing Regimens	13
Optimization of Clinical Studies and Clinical Applications.....	15
Future Prospects.....	16
CHAPTER 2. CENTRAL HYPOTHESIS	19
Specific Aim 1	19
Specific Aim 2	19
Specific Aim 3	20
CHAPTER 3. PHARMACOKINETICS AND BIODISTRIBUTION OF A NOVEL SERIES OF ANTI-TUBERCULOSIS AGENTS: SPECTINAMIDES.....	21
Introduction.....	21
Materials and Methods.....	21
Chemicals and reagents.....	21
Protein binding.....	22
Formulation preparation and administration.....	22
Pharmacokinetic studies.....	22
Pharmacokinetic data analysis	24
Macrophage uptake assay	25
Sample preparation and LC-MS/MS analysis.....	25
Biodistribution	26
Sample preparation of biological samples for ³ H-1329	26
Blood.....	26
Serum and urine	27
Organs and muscle tissue.....	27
Feces	27
Sample analysis of biological samples for ³ H-1329	27
Results and Discussion	28
Biopharmaceutical properties of spectinamides	28
Macrophage uptake assay	37
Biodistribution	37
Mass balance.....	41
Tissue distribution.....	41
Pharmacokinetic profile of radioactivity in serum.....	41
Conclusion	48

CHAPTER 4. A PHARMACOMETRICS-BASED APPROACH USING <i>IN VITRO</i> PK/PD MODELING TO DEVELOP DOSING REGIMENS FOR <i>IN VIVO</i> STUDIES FOR A NOVEL SPECTINAMIDE ANTI-TUBERCULOSIS AGENT	49
Introduction.....	49
Materials and Methods.....	50
Culture, media and antibiotics	50
<i>In vitro</i> PK/PD model	50
Time kill curves for Lee 1599	52
PK/PD model building.....	52
Numerical simulations	54
Results.....	55
Time kill curves for Lee 1599.....	55
Numerical simulations	63
Discussion.....	63
Conclusions.....	71
 CHAPTER 5. APPLICATION OF PHARMACOMETRIC TOOLS FOR IDENTIFYING PHARMACODYNAMIC INTERACTIONS IN ANTIBIOTIC COMBINATIONS	72
Introduction.....	72
Materials and Methods.....	73
Antimicrobial agents	73
Microorganisms	73
Checkerboard assay	73
Response surface assay	74
<i>In vitro</i> PK/PD model	75
Time kill curves for Lee 1599.....	77
<i>In vivo</i> efficacy studies.....	77
Results.....	77
Checkerboard assay	77
Response surface assay	78
<i>In vitro</i> PK/PD model for combination agents.....	78
<i>In vivo</i> studies	85
Discussion.....	85
Conclusions.....	93
 CHAPTER 6. SUMMARY.....	94
 LIST OF REFERENCES.....	96
 VITA.....	104

LIST OF TABLES

Table 1-1.	Examples of the application of pharmacometrics analyses for anti-infectives.	4
Table 3-1.	Physico-chemical properties and pharmacokinetic parameters for spectinamides after intravenous administration at 10mg/kg dose.	29
Table 3-2.	Mean concentration of drug uptake into J774A.1 murine macrophage cell line at 24h for two different doses.	38
Table 3-3.	Tissue distribution and recovery of radioactivity (mean(%CV)) following IV administration of ³ H-1329 at 0.9mg/kg BID for 4.5days in rats.	44
Table 3-4.	Serum pharmacokinetic parameters after the 1st and 8th intravenous dose of 0.9mg/kg ³ H-1329 (values are expressed as mean (%CV)).	47
Table 4-1.	<i>In vitro</i> PK/PD parameter estimates for Lee 1599.	58
Table 4-2.	Empirical PK/PD indices based on day 4 data.	62
Table 5-1.	Checkerboard assay results to determine pharmacodynamic interaction between Lee 1599 and RIF.	79
Table 5-2.	Results of three dimensional surface response assay.	83
Table 5-3.	Summary of results of pharmacodynamics interactions from different methods.	89
Table 5-4.	Comparison of %AUC ratios (the %AUC were normalized with AUC of control group).	91

LIST OF FIGURES

Figure 1-1.	Illustration of interplay of pharmacology, microbiology, physiology, disease and their association with pharmacometric evaluations in the clinical development and application of anti-infectives.....	2
Figure 1-2.	Illustration of the PK/PD indices at steady state during multiple dosing: (i) T>MIC: Time above MIC (ii) C _{max} /MIC (iii) AUC/MIC.	10
Figure 3-1.	Chemical structure of lead spectinamides with MIC values.....	23
Figure 3-2.	Measured plasma concentration-time profiles (mean ± SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1544 in rats.	30
Figure 3-3.	Measured plasma concentration-time profiles (mean ± SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1599 in rats.	31
Figure 3-4.	Measured plasma concentration-time profiles (mean ± SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1601 in rats.	32
Figure 3-5.	Measured plasma concentration-time profiles (mean ± SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1603 in rats.	33
Figure 3-6.	Measured plasma concentration-time profiles (mean ± SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1661 in rats.	34
Figure 3-7.	Measured plasma concentration-time profiles (mean ± SEM) after intravenous (10mg/Kg) administration of Lee 1666 in rats.	35
Figure 3-8.	Measured plasma concentration-time profiles (mean ± SEM) after intravenous (10mg/Kg) administration of spectinomycin in rats.	36
Figure 3-9.	Mean average concentrations (± SEM) of drug uptake into J774A.1 murine macrophage cell line at 24h.	39
Figure 3-10.	Fold differences in drug uptake in J774A.1 murine macrophage cell line.	40
Figure 3-11.	Mass balance and percentage distribution of Lee 1329-T at 1h post 9 th dose of 0.9mg/kg.	42
Figure 3-12.	Amount of radioactivity (mean ± SD) excreted in urine after each dosing interval (0.9mg/kg ³ H-1329, BID for 4.5days).	43

Figure 3-13. Fold difference (mean $\pm$ SD) in tissue distribution of radioactivity compared to amount in whole blood (after normalizing the distributions to $\mu\text{Ci/mL}$ or $\mu\text{Ci/mg}$ of blood or tissue).	45
Figure 3-14. Comparison of single and multiple dose pharmacokinetics showing average ($\pm$ SD) serum concentration-time profiles of radioactivity after the 1st dose and 8th dose during multiple dosing with 0.9mg/kg ^3H -1329 given intravenously every 12h.	46
Figure 4-1. A representation of working unit of an <i>in vitro</i> PK/PD model.	51
Figure 4-2. Schematic representation of PK/PD model to describe the rate of change in the number of bacteria in the <i>in vitro</i> PK/PD model system.	53
Figure 4-3. Timekill effect of Lee 1599 against <i>M. bovis</i> BCG (N_0 : 6-7 log CFU/mL) with observed log CFU/mL plotted against time for different dosing regimens.	56
Figure 4-4. Timekill effect of Lee 1599 against <i>M. bovis</i> BCG (N_0 : 7-8 log CFU/mL) with observed log CFU/mL plotted against time for different dosing regimens.	57
Figure 4-5. Panels showing goodness of fit plots for the Lee 1599 PK/PD model fit (A.) DV vs PRED (B.) DV vs IPRED (C.) PRED vs CWRES (D.) TIME vs CWRES.	59
Figure 4-6. Plots showing measured (DV is observed data), individual predictions IPRED (black lines) and population predictions (red lines) for <i>M. bovis</i> BCG CFU/mL over time for the Lee 1599 PK/PD model.	60
Figure 4-7. Relationship between PK/PD indices and microbial kill on Day 4 after treatment.	61
Figure 4-8. Schematic representation of PK/PD model used for simulations.	64
Figure 4-9. Time kill curves calculated from 1000 numerical simulations for once daily dosing regimen of Lee 1599 in mice.	65
Figure 4-10. Time kill curves calculated from 1000 numerical simulations for twice daily dosing regimen of Lee 1599 in mice.	66
Figure 4-11. Time kill curves calculated from 1000 numerical simulations for thrice daily dosing regimen of Lee 1599 in mice.	67
Figure 4-12. Time kill curves calculated from 1000 numerical simulations for once daily (5 days a week) dosing regimen of Lee 1599 in mice.	69

Figure 4-13. Time kill curves calculated from 1000 numerical simulations for twice daily (5 days a week) dosing regimen of Lee 1599 in mice.....	70
Figure 5-1. A representation of <i>in vitro</i> PK/PD model set up for dynamic time-kill studies.....	76
Figure 5-2. Model fits of bacterial burden at 24h in static time kill studies for Lee 1599 (A) and RIF (B) to obtain the parameter estimates such as E_0 , E_{max} and EC_{50}	80
Figure 5-3. Observed effect of combination agent Lee 1599 and RIF.	81
Figure 5-4. Model predicted effect of the combination compound Lee 1599 and RIF. ...	82
Figure 5-5. <i>In vitro</i> PK/PD dynamic time kill study for Lee 1599, RIF and its combination with different doses.	84
Figure 5-6. The bacterial log reduction with different doses using an <i>in vitro</i> PK/PD dynamic time kill experiment.	86
Figure 5-7. <i>In vivo</i> efficacy trial data showing bacterial burden reduction (log CFU/mL) in the lungs of <i>M. tb</i> infected mice, N=6 for Lee 1599 and Lee 1599+RIF, N=5 for RIF.	87
Figure 5-8. Comparison of %AUC ratios after the %AUC were normalized with AUC of control group indicating higher the %AUC ratio, greater the bacterial killing.	92

LIST OF ABBREVIATIONS

ABC	ATP binding cassette
AIC	Akaike information criterion
ATCC	American type culture collection
AUC	Area under the curve
AUC _{ss,0-24}	Area under the curve at steady state plasma concentration
BAL	Bronchoalveolar lavage fluid
BCG	Bacillus calmette–guérin
BIC	Bayesian information criterion
BID	Twice daily
CAP	Community acquired pneumonia
CFU	Colony forming unit
CKD	Chronic kidney disease
CL	Clearance
CL _{Cr}	Creatinine clearance
CL _r	Renal clearance
CLSI	Clinical laboratory standards institute
C _{max}	Maximum plasma concentration
CSF	Cerebrospinal fluid
CVVHDF	Continuous venovenous hemodiafiltration
DI	Deionized water
DMEM	Dulbecco's modified eagle's medium
DPBS	Dulbecco's phosphate-buffered saline
DPM	Disintegrations per minute
EBA	Early bactericidal activity
ELF	Epithelial lining fluid
EMA	European medicines agency
F	Oral bioavailability
f _e	Fraction excreted unchanged in the urine
FICI	Fractional inhibitory concentration index
f _{up}	fraction unbound in plasma
GFR	Glomerular filtration rate
HAART	Highly active antiretroviral therapy
HDA	High dose aerosol
HFIM	Hollow fiber infection model
HIV	Human immunodeficiency virus
IC ₅₀	Concentration required to produce half-maximal effect
II	Interaction index
I _{max}	Maximum bacterial kill rate
IV	Intravenous
LC-MS/MS	Liquid chromatography-mass spectrometry
LDA	Low dose aerosol
M&S	Modeling and simulations
MDR	Multi drug resistant

MIC	Minimum inhibitory concentration
MPC	Mutant prevention concentration
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MRT	Mean residence time
<i>M. tb</i>	<i>Mycobacterium Tuberculosis</i>
NAT2	N-acetyl transferase-2
NCA	Non compartmental analysis
NDA	New drug applications
OD	Optical density
PB	Protein binding
PBPK	Physiologically based PK and PK/PD modeling
PCP	<i>Pneumocystis carinii</i> pneumonia
PD	Pharmacodynamic
PK	Pharmacokinetic
PK/PD	Pharmacokinetics/Pharmacodynamics
PO	Per oral
PTA	Pharmacodynamic target attainment
Q6h	Once every 6 hours
Q8h	Once every 8 hours
QD	Once daily
RIF	Rifampicin
RSE	Residual standard error
SEM	Standard error of mean
SNP	Single nucleotide polymorphisms
SP	Spectinomycin
ST	Streptomycin
TB	Tuberculosis
TID	Thrice daily
TR-TB	Totally drug resistant tuberculosis
US FDA	United states food and drug administration
USP	United states pharmacopoeia
VPC	Visual predictive check
V_{ss}	Volume of distribution at steady state
VUP	Volume under the plane
WHO	World health organization
XDR	Extremely drug resistant
%SCLSI	CLSI susceptibility breakpoints
%SPD	Between-subject variability to define PD derived breakpoints
-2LL	-2 log likelihood

CHAPTER 1. APPLICATION OF PHARMACOMETRICS IN THE CLINICAL DRUG DEVELOPMENT OF ANTI-INFECTIVES*

Introduction

With the increased emergence of anti-infective resistance in recent years, much focus has recently been drawn to the development of new anti-infectives and the optimization of treatment regimens and combination therapies for established antimicrobials. In this context, the field of pharmacometrics using quantitative numerical modeling and simulation techniques has in recent years emerged as an invaluable tool in the pharmaceutical industry, academia and regulatory agencies to facilitate the integration of preclinical and clinical development data and to provide a scientifically based framework for rationale dosage regimen design and treatment optimization. The following chapter highlights the usefulness of pharmacometric analyses in anti-infective drug development and applied pharmacotherapy with select examples.

Pharmacometrics in Anti-infective Drug Development

The increased emergence of multidrug resistant (MDR) microbial strains in recent years and the resulting complications in the clinical care for infected patients is a strong reminder for the urgent need to develop new anti-infective agents. As the battle against drug-resistant microorganisms rages on in clinical centers around the globe, the development of novel single agent and combination therapies against microbial infections has challenged research scientists in the pharmaceutical industry and academia as well as clinicians and other healthcare providers to seek a multi-disciplinary approach; one that encompasses microbiology, clinical expertise, and drug development sciences. Pharmacometrics has in this context arisen as a formidable tool to bridge and link between key concepts in these disciplines and to provide a rationale, scientific framework throughout the drug development continuum as well as in clinical applications [1, 2]. There are case studies which suggest that basing optimization of treatment on pharmacometric assessments improves the risk-management of MDR pathogens in the clinical realm by minimizing the failures and mistakes associated with inappropriate or careless use of antibiotic therapies [3, 4].

Pharmacometrics is the scientific discipline that uses mathematical models based on biology, pharmacology, physiology, and disease for quantifying the interactions between drugs and patients (**Figure 1-1**). Data and information from various sources are bridged together and quantitatively related to each other. Computer-based modeling and simulation of pharmacokinetic (PK) and pharmacodynamic (PD) data (denoted as PK/PD M&S) usually forms the basis for a pharmacometric analysis, but is frequently

* Adapted with permission. Ashit Trivedi, Richard E. Lee, and Bernd Meibohm. (2013). Applications of pharmacometrics in the clinical development and pharmacotherapy of anti-infectives. Expert Review of Clinical Pharmacology, 6(2), 159-170.

Figure 1-1. Illustration of interplay of pharmacology, microbiology, physiology, disease and their association with pharmacometric evaluations in the clinical development and application of anti-infectives.

supplemented by models characterizing important other aspects of drug efficacy and/or safety in a given situation, such as disease progression, adherence to therapy or bacterial growth and infection [5]. PD measures used in pharmacometrics are frequently biomarkers, but can also be surrogate endpoints or clinical endpoints.

Pharmacometrics is aimed at establishing models that provide guidance and decision support in drug development such as trials design, efficacy comparisons, dosage regimen optimization and endpoint analysis, but also in supporting regulatory decisions and improving clinical care in specific patient populations [6, 7]. Its purpose is thereby to reduce cost and shorten development time by optimizing the clinical assessment of efficacy and safety.

An increased application of pharmacometric methodologies in drug development and applied pharmacotherapy has in the last decade been strongly promoted by industry, academia and especially regulatory agencies, with specific emphasis from the U.S. Food and Drug Administration [8, 9]. Pharmacometrics has been extensively used in the development and application of novel antibiotics [10-12]. In addition, increasing emphasis is more recently being placed on dose selection of approved antibiotics already in clinical use, with heavy reliance on quantitative benefit-risk evaluations [13].

This chapter aims to provide an overview on the usefulness and utility of pharmacometrics and in specific modeling and simulation approaches (M&S) in anti-infective drug development and clinical application (**Table 1-1**), and will highlight these in examples on the application of M&S approaches in three specific areas:

1. The determination of PK/PD indices and susceptibility breakpoints indicative of anti-microbial activity in clinical populations.
2. The development of optimal dosing regimens for effective and safe anti-microbial therapy in both general and specialized population groups, such as pediatric patients.
3. The optimization of key attributes of study design in clinical development and postmarketing evaluations.

Prerequisites for the Application of Pharmacometrics

One of the major prerequisites for the application of M&S in clinical drug development and applied pharmacotherapy is the availability of a mathematical framework that captures the dose-exposure relationship for the antibiotic in relevant tissues of interest (i.e. the plasma and tissue pharmacokinetics), the growth characteristics of the pathogen and its relevant subpopulations in the *in vivo* environment (microbiology), and the dynamic interplay between pathogen and antibiotic exposure with regard to pathogen survival, death, latency or resistance (pharmacodynamics).

Table 1-1. Examples of the application of pharmacometrics analyses for anti-infectives.

Drug	Organism	Disease	Target site	Objective for performing pharmacometrics analysis	Outcome	Population	Ref.
Biapenem	Gram positive and gram negative anaerobes	Continuous venovenous hemodiafiltration (CVVHDF)	Plasma and filtrate-dialysate	Determine appropriate dosage recommendation for patients on CVVHDF	300mg BID, intravenous (2 h infusion)	Adult Japanese (mean 65.1 yr), N=7	[14]
Cefditoren	<i>Streptococcus pneumoniae</i>	Lower respiratory tract infections	Plasma and BAL	PD profiling & determining PTA (Dose: 400mg QD, oral)	PTA with 33% T>MIC is <80% for MIC = 0.06mg/L	Adult Caucasian (35-78 yr) N=24	[15]
Cefepime	<i>Streptococcus pneumoniae</i>	Extracerebral infections	Serum and CSF	PD profiling & determining PTA (2g BID, 0.5h IV infusion)	PTA in CSF with 50% T>MIC is 91.8%, 100% T>MIC is 82%	Adult females (mean 58.9 yr) N=7	[16]
Ceftobiprole	<i>Staphylococcus aureus</i>	Staphylococcal pneumonia	ELF, serum, BAL	PD profiling & determining PTA (500mg, TID, 2h IV infusion)	PTA with T>MIC is 15% for 1-log ₁₀ CFU/g reduction; 25% for 2-log ₁₀ CFU/g reduction	Adults (>18 yr) N=25	[17]
	<i>Staphylococcus aureus</i> , <i>Streptococcus pneumoniae</i>	Nosocomial pneumonia	Skin, Plasma	PD profiling & determining PTA & renal dose adjustments (500 mg BID, 1h infusion; 500mg TID, 2h infusion)	With 500mg, BID: PTA of 30% and 50% T>MIC exceeded 90%. With 500 \mg, TID: PTA of 40% and 60% T>MIC exceeded 90%. 500mg, BID is optimal dose for CL _{Cr} ≤ 50mL/min	Adults, N=150	[18]

Table 1-1. (Continued).

Drug	Organism	Disease	Target site	Objective for performing pharmacometrics analysis	Outcome	Population	Ref.
Ceftriaxone	<i>Streptococcus pneumoniae</i>	Extracerebral infections	CSF	PD profiling & determining PTA (2g BID, 0.5h infusion)	PTA is 76% for 50% T>MIC in CSF; PTA is 65% for 100% T>MIC in CSF	Adult females (mean age 58.9 yr) N=7	[16]
Garenoxacin	<i>Streptococcus pneumoniae</i>	Community-acquired pneumonia (CAP)	Serum, ELF	Evaluate exposure-response relationship by population PK/PD	$fAUC_{0-24}/MIC_{90} > 200$ 400mg QD, Oral dosing is safe and adequate for efficacy	Adults (≥ 18 yr) N=580	[19, 20]
	<i>Streptococcus pneumoniae</i> , <i>Staphylococcus aureus</i> , <i>Klebsiella pneumoniae</i> , <i>Moraxella catarrhalis</i> , <i>Haemophilus influenzae</i>	Pneumonia, secondary infection of chronic respiratory diseases, bronchitis, sinusitis, otitis media, laryngopharyngitis, tonsillitis		Determine PTA in serum and ELF	PTA $> 95\%$ for $fAUC_{0-24}/MIC_{90}$ is 100 (serum) and 120 (ELF)	Adult Japanese (≥ 18 yr) N=136	[21]
Gatifloxacin	<i>Streptococcus pneumoniae</i>	Community-acquired pneumonia (CAP)	N/A	PD profiling & determining PTA at 400mg QD, oral (young) and 200 mg QD, oral (elderly)	PTA for $AUC_{0-24}/MIC_{all} \geq 30$ is 92.3% in young; 91.4% in elderly	Adults Young (< 65 yr) Elderly (> 65 yr) N=183	[22]
Gemifloxacin	<i>Streptococcus pneumoniae</i>	Community-acquired pneumonia (CAP)	Serum, ELF	PD profiling & PTA in serum and ELF (320mg QD, oral)	PTA ($> 95\%$, or $> 99\%$ [23]) for $fAUC_{0-24}/MIC_{90}$ is 100 (ELF) and 78.3-88 (Serum)	N/A	[20, 23]

Table 1-1. (Continued).

Drug	Organism	Disease	Target site	Objective for performing pharmacometrics analysis	Outcome	Population	Ref.
Levofloxacin	<i>Escherichia coli</i> , <i>Chlamydia</i>	Prostatitis	Prostatic tissue	Determine penetration at site of action (500mg QD)	$AUC_{prostate}/AUC_{plasma}$ is 2.96	Adult (47-94 yr) N=22	[24]
	Organisms causing Pneumonia	Nosocomial pneumonia	Plasma, ELF	Determine penetration ratio in ELF (500mg QD; 750mg QD)	AUC_{ELF}/AUC_{plasma} is 1.16	Adult (> 18 yr) N=24	[25]
Metronidazole	<i>Bacteroides fragilis</i>	N/A	Plasma and urine	PD profiling & determining PTA at 500mg, TID; 1000mg QD; 1500mg QD	PTA for AUC/MIC ≥ 70 is 99%	Adults males, N=18 (10 healthy, 8 patients)	[26]
Moxifloxacin	<i>Streptococcus pneumoniae</i>	Community-acquired pneumonia (CAP)	Serum, ELF	PD profiling & determining PTA (400mg QD, 1h infusion)	PTA (>95%) for $fAUC_{0-24}/MIC_{90}$ is 120 (ELF) and 78.3-88 (Serum); $C_{max}/MIC_{90} > 10$, $AUC/MIC_{90} \sim 100$	Adults (18-80 yr) N=16	[20, 27]
Norvancomycin	<i>Staphylococcus aureus</i>	Gram-positive bacterial infections	Serum	PD profiling & prediction of CL estimates in population (400mg QD, intravenous)	95% cured clinical outcome with AUC_{0-24}/MIC of 579.9 $CL=2.54(CL_{Cr}/50)$ in patients with renal dysfunction $CL=6.0(Body\ Weight/60)^{0.52}$ in healthy subjects	Adult, N=166	[28]

Table 1-1. (Continued).

Drug	Organism	Disease	Target site	Objective for performing pharmacometrics analysis	Outcome	Population	Ref.
Oseltamivir and Oseltamivir carboxylate (OC)	Influenza A (H1N1 virus)	Influenza A and B	Serum	To determine dosing in neonates and infants	3mg/kg, BID, Oral in infants 1.7mg/kg, BID, Oral in neonates	Adult males N=6; Infants (<2 yr) N=43; Neonates (1.5-17.5 weeks) N=20	[29, 30]
Piperacillin/Tazobactam (combination)	<i>Escherichia coli</i> , <i>S. aureus</i> , <i>Klebsiella pneumonia</i> , <i>Pseudomonas aeruginosa</i> , <i>Bacteroides fragilis</i> , <i>Acinetobacter baumannii</i>	Gram-negative bacterial infections	Serum	To determine PK/PD parameters & <i>in vivo</i> effectiveness with doses 3.375g, Q4h, Q6h; 4.5g, Q6h, Q8h (intravenous, 0.5h infusion)	T>MIC is >60% for all doses	Adult males N=12	[31, 32]
Rifampin	<i>Mycobacterium tuberculosis</i>	Tuberculosis	Plasma, ELF, BAL, Alveolar Cells (ACs)	Determine pulmonary PK/PD in lungs & evaluate recommended dose (600mg, QD, oral)	$C_{max}/MIC \geq 175$ are 95% (ACs), 48.8% (plasma), 35.9% (ELF); $AUC_{0-24}/MIC \geq 271$ is 100% (plasma); Higher doses (1200 mg) required as pulmonary concentration is too low with recommended dosing	Adults, N=40	[33]

Table 1-1. (Continued).

Drug	Organism	Disease	Target site	Objective for performing pharmacometrics analysis	Outcome	Population	Ref.
Telavancin	<i>Staphylococcus aureus</i>	Health care associated pneumonia	Plasma and ELF	To determine penetration in ELF with 10mg/kg QD (intravenous, 1h infusion)	AUC_{ELF}/AUC_{plasma} is 0.73	Adult Caucasians, N=20	[34]
Vancomycin	<i>Staphylococcus aureus</i>	Ventilator-associated pneumonia	Plasma, Bronchoalveolar lavage fluid, ELF	To determine penetration in ELF with 1000mg BID (intravenous)	AUC_{ELF}/AUC_{plasma} is 0.675 $AUC/MIC \geq 400$	Adults (>18 yr) N=10	[35]
Voriconazole	<i>Candida</i> strains	N/A	Plasma	To investigate the effect of concomitant fluconazole on Voriconazole PK	Coadministration is not recommended, Monitor for adverse events if voriconazole is sequentially administered after fluconazole	Adult males (21-55 yr) N=10	[36]

Relevant PK and PD parameters may be derived from a variety of sources, including preclinical and clinical pharmacokinetic studies, studies assessing tissue concentration-time courses with techniques such as imaging or microdialysis, *in vitro* MIC data, *in vitro* time-kill curves, disease pathology, and microbiologic characteristics of the pathogen/infective agent itself [37, 38]. These data can be combined with knowledge about the between- and within-subject variability in relevant PK parameters as well as microbiologic differences among different pathogen strains. Since between-subject variability is oftentimes a major complicating factor in drug development and pharmacotherapy, the potential impact of M&S approaches is usually higher the larger is the between-subject variability. The resulting models help identify optimal drug concentrations necessary to achieve at the target site to be efficacious in overcoming an infection (i.e. bacterial eradication) and minimizing bacterial resistance, and the corresponding dosing regimens that provide a high likelihood of therapeutic success in a specific target population [10, 39]. Once this basic framework has been established, additional factors including human physiological, biochemical, and genetic variability and differences to preclinical species that complicate the translation of preclinical results to the clinic and of data from controlled clinical studies to clinical practice may be considered and implemented in the modeling framework.

PK/PD Indices and Susceptibility Breakpoints

Susceptibility breakpoints, or critical antimicrobial concentrations, are valuable parameters in assessing the degree of drug susceptibility and/or resistance to an antimicrobial pharmacotherapy. Breakpoints refer to antibiotic concentrations or exposures that separate bacterial strains where there is a high likelihood of treatment success from those bacteria where treatment is more likely to fail. Historically, pharmacokinetic exposure parameters such as the area-under-the-plasma-concentration-time-curve (AUC) or the maximum or peak concentration (C_{max}) have been the gold standard for monitoring drug action under the assumption that plasma concentration are indicative of target site concentrations and that a defined and fixed target site exposure is necessary for drug action. For antimicrobial pharmacotherapy, however, target site exposure is not sufficient to predict efficacy since the susceptibility of the microorganism to the target site concentration is variable among different microorganisms and strains and may change over time in the event of for example resistance development. Susceptibility of a microorganism to an antibiotic is frequently described by its minimum inhibitory concentration (MIC), an *in vitro* parameter that quantifies growth inhibition to a static antibiotic concentration. Combination of the pharmacodynamic parameter MIC with pharmacokinetic exposure parameters such as AUC or C_{max} allows defining PK/PD indices that are hybrid parameters that integrate PK and PD information related to target exposure and microbial sensitivity, and can be used to derive PK/PD breakpoints. Dependent on the predominant mechanism of action of the antibiotic, the following three PK/PD indices (as shown in **Figure 1-2**) have been found useful in characterizing *in vivo*.

Figure 1-2. Illustration of the PK/PD indices at steady state during multiple dosing: (i) $T > MIC$: Time above MIC (ii) C_{max}/MIC (iii) AUC/MIC .

1. $T > MIC$: Time T of exposure of microorganisms to plasma concentrations exceeding its MIC.
2. C_{max}/MIC : Ratio of peak plasma concentration to MIC.
3. AUC/MIC : Ratio of area under plasma concentration curve to MIC.

These indices are frequently used to classify the pattern of killing activity for antimicrobials, concentration-dependent vs. time-dependent. Antibiotics for which $T > MIC$ best describes their antimicrobial activity follow time-dependent killing with minimal persistent effects that requires a maximum duration of exposure above MIC for maximum activity. Antibiotics for which C_{max}/MIC best describes their antimicrobial activity follow concentration-dependent killing with prolonged persistent effects that require a maximum peak antibiotic concentration for maximum activity. Antibiotics for which AUC/MIC best describes their antimicrobial activity exhibit both time-dependent and concentration-dependent killing with moderately persistent effects that require a combination of high concentration and long duration above MIC for maximum activity. The AUC in these indices is calculated at pharmacokinetic steady state over 24h ($AUC_{ss,0-24}$) and is usually derived from Bayesian parameter estimates from population PK analysis approaches.

As indicated in these relationships, the MIC is consistently used as a denominator or ‘normalization factor’. The integration of drug exposure (PK) and the actual drug activity based on microbial sensitivity (PD) allows to develop generalized breakpoints with which multiple drugs can be evaluated for efficacy related to both their PK and PD parameters [40-42]. The efficacy is the desired clinical effect that a particular drug is producing and is the combined effect of PK and PD and other, for example patient-related, factors. The term ‘efficacy’ cannot be interchanged for PD. Thus, breakpoints are often used as a valuable tool in drug development and applied pharmacotherapy to optimize dosing regimens of an antibiotic for the treatment of a specific pathogen and to minimize the increase of resistant strains with optimal dosing.

It should be stressed, however, that many antibiotics are bound to variable degree to proteins in plasma and tissue, and that only the free, unbound concentration at the target site is pharmacologically active and exerts the antimicrobial activity. Since MIC is frequently determined with no or limited binding proteins present, it usually represents an unbound concentration [43]. Thus, PK/PD indices also need to be based in free drug exposures rather than total concentrations to avoid any bias introduced by considering protein binding only in the PD parameter MIC, but not the PK parameters AUC and C_{max} . Consequently, $fT > MIC$, fC_{max}/MIC , and $fAUC/MIC$ based on free drug concentrations are now usually applied as appropriate PK/PD indices [40-42, 44].

PK/PD indices assessed in M&S studies can be used to assess and compare the likelihood of achieving effective target exposure for different antibiotics and thus prioritize compounds for the treatment of specific infections. Noreddin *et al.* used this approach by applying simulation studies to determine the probability of target attainment

in serum and epithelial lining fluid (ELF) defined as a $fAUC_{0-24}/MIC_{90}$ ratio of 30, with bacterial eradication as efficacy endpoint, for standard clinical regimens of azithromycin, clarithromycin, and telithromycin against *S. pneumonia* strains with different susceptibility [45].

Another example for establishing PK/PD indices by M&S is the case of isoniazid which displays a multimodal elimination pattern in the population based on single nucleotide polymorphisms (SNPs) in the gene encoding for N-acetyl transferase-2 (NAT2). Since the allelic frequency of these genetic variants differs among different ethnic groups, these groups differ in the number of “slow” and “fast” acetylators based on NAT2 phenotype. Thus, standard recommended doses would obviously result in different bactericidal activities among different populations. *In vitro* models showed that AUC_{0-24}/MIC is associated with both microbial death and emergence of resistance for isoniazid. Based on this approach, M&S was used to predict the bactericidal effects of different doses of drug in various ethnic populations, accounting for the multimodal elimination of isoniazid. The model allowed to predict the dose with highest early bactericidal activity (EBA) and this dose was used in subsequent clinical studies [46].

Based on susceptibility breakpoints microorganisms are classified as susceptible, intermediate and resistant based on predefined. The identification of susceptibility breakpoints is being performed using standard tests implemented by the Clinical Laboratory Standards Institute (CLSI) by examining MIC distributions across different strains of a bacterial species. MIC values are measured using Mueller-Hinton broth, as recommended by CLSI guidelines [42, 47]. While these test methodologies achieve a precise estimate of breakpoints *in vitro*, the major challenge lies in the ability for clinical translation. Since target site exposure and microbial susceptibility will vary among different individuals with different infection sites and different pathogen populations, susceptibility breakpoints are inherently variable in a clinical population [40]. Thus, *in vitro* derived breakpoints have to be interpreted as point estimates in a breakpoint distribution with a confidence interval that presents itself in the clinic [48]. M&S approaches using clinical data may contribute to derive and increase the exactness or confidence in the derived breakpoints.

Multiple studies have indicated inconsistencies between the probability of PD target attainment (PTA) and susceptibility as per CLSI defined breakpoints. DeRyke et al. demonstrated that CLSI susceptibility breakpoints (%SCLSI) overestimated probability for achieving bactericidal exposure for antibiotics such as ciprofloxacin, levofloxacin and piperacillin-tazobactam against gram negative bacteria such as *P.aeruginosa*, *A.baumannii*, *E. coli* and *Klebsiella spp.* [48]. To overcome this limitation, the authors used Monte-Carlo simulations based on published PK data and their between-subject variability to define PD derived breakpoints (%SPD). This %SPD accounts for between-subject variability in human pharmacokinetics as well as exposure differences resulting from different dosing regimens. In this approach, %SPD was defined as the highest MIC at which at least 90% of the population achieve the targeted bactericidal exposure, and bactericidal exposure was defined as $fT > MIC$ (time of free concentration above MIC) of 40% for carbapenems, 50% for cephalosporins and the piperacillin component of the

piperacillin-tazobactam combination, and 125 for the AUC/MIC ratio of fluoroquinolones. The analysis indicated that although there was a general lack of agreement between PTA and %SPD or %SCLSI, only %SCLSI overestimated the ability of the investigated dosing regimens to achieve bactericidal exposures. In contrast, %SPD often underestimated PTA, a deviation clinically far less concerning than overestimation. The underestimation of PTA using %SPD leads to an overestimation of dose in a particular subject (which is preferable), but the general overestimation of doses for a population can lead to an increase in bacterial resistance. Based on the performed simulations, the authors suggest pathogen and dosing regimen specific breakpoints as a more appropriate way to classify bacteria as susceptible [48].

Development of Optimal Dosing Regimens

One of the top priorities in antibiotic pharmacotherapy is to optimize the duration of therapy with proper dosing regimens that are effective and safe, and minimize the occurrence of bacterial resistance. However, these goals are often requiring opposing measures and can often not be achieved simultaneously. The concept of clinical utility can help in optimizing these goals. Clinical utility allows to quantify and optimize “trade-offs” between multiple factors such as efficacy, safety, microbiology, and usage of drugs [49]. Pharmacometrics allows the integration of clinical utility with M&S, where “trade-offs” can be derived and explored mathematically.

The clinical efficacy (i.e. bacterial eradication) of drugs such as garenoxacin, telithromycin and doripenem is more than 90%. With such high efficacy it is challenging to design dosing regimens on the basis of efficacy and safety at varying doses. Population based pharmacokinetic M&S using Monte Carlo simulations helped in deciding on a dosage regimen by taking into account the between-subject variability in PK parameters and varying MIC distributions by various clinical isolates. This approach helped in determining the optimal dose for garenoxacin (400mg) on the basis of:

1. Selecting AUC₀₋₂₄/MIC as an appropriate PK/PD index derived from *in vivo* efficacy data
2. Performing Monte Carlo Simulations using population PK parameters, MIC distributions and mutant prevention concentration (MPC)
3. Determining a recommended dose based on probability of target attainment
4. Validation of the recommended dose based on PK/PD data acquired from phase III studies for respiratory tract infection [21].

Design of dosing regimens is especially challenging for patients who are critically ill with severe bacterial infections, and who have renal failure and are on continuous venovenous hemodiafiltration (CVVHDF). In the event of renal failure, dosing regimen needs of individual patients can be adjusted by assessing the impact of kidney

dysfunction on the disposition of drugs [50]. For example the PK and PD properties of biapenem change when a patient is on CVVHDF. Ikawa et al. performed PK M&S to determine PD exposure of $T > MIC_{4\mu g/mL}$ and suggested that for $T > MIC_{4\mu g/mL}$ for 30% of the dosing interval biapenem dosage should be 300mg BID, 600mg BID, or 600mg BID for dialysate flow rates of 1.4L/h, 2.8L/h, or 5.6L/h, respectively [14]. As the dialysate flow rate increased, $T > MIC_{4\mu g/mL}$ decreased. The 600mg BID dose was the optimum to reach $T > MIC_{4\mu g/mL}$ of 30% for both dialysate flow rates of 2.8L/h and 5.6L/h. These simulations illustrate that low doses or increased dosing intervals should not be recommended for patients who are on CVVHDF or renal replacement technique.

Since critically ill patients often have altered drug distribution and elimination, they may experience inadequate dosing with antibiotics and may thus be at a much higher risk of infections with antibiotic resistant organisms [51]. For doripenem, a carbapenem, $T > MIC$ for 35% of the dosing interval has been the recommendation in patients with normal kidney function. Since doripenem is primarily excreted in unchanged form into the urine, PK-based Monte Carlo simulations were performed to develop dosing recommendations for patients with renal impairment. For mild-stage 2 chronic kidney disease (CKD) patients with a creatinine clearance (CL_{Cr}) $> 60 mL/min$, 500mg should be infused over 1h every 8h for bacteria with $MIC \leq 1 \mu g/mL$; however, in the more severe stage 3 CKD patients with $CL_{Cr} \sim 30-50 mL/min$, an infusion of 250mg of doripenem over 1h every 8h for bacteria with $MIC \leq 2 \mu g/mL$ is recommended. For patients with severe renal impairment with a CL_{Cr} of 10-29mL/min, 250mg was recommended to be infused over longer durations, 4h instead of 1h, every 12h for $MIC \leq 2 \mu g/mL$. This is consistent with the time-dependent ($T > MIC$) killing pattern of carbapenems. Dosing strategies like this can only be achieved from PK/PD M&S where the creatinine clearance builds the parameter framework for achieving set exposure levels, as PK studies in critically ill patients would be extremely difficult to perform [52].

In order to be efficacious, an antibiotic needs to reach the site of infection in therapeutically effective concentrations. In patients with severe lung infections, the concentration of antibiotics in ELF is an essential measure to decide on an optimal dosage regimen [25]. It has been reported recently that methicillin-resistant *S. aureus* (MRSA) is the likely culprit in causing more than 25% of all ventilator-associated pneumonias in intensive care unit patients [53]. A population PK model with Monte Carlo simulations was employed to determine the range of ELF exposure relative to that in plasma. It was determined that vancomycin penetrates ELF at approximately 50% of plasma concentrations [35]. Under the assumption that the PK/PD in ELF is similar to the AUC/MIC of ≥ 400 used in plasma, the M&S exercise showed that target attainment is expected to be suboptimal for MRSA with MICs in excess of 1 mg/L under current dosing regimens. A similar approach was used for PD profiling of cefditoren and telavancin against penicillin-susceptible and penicillin-intermediate *S. pneumonia* [15] and methicillin-resistant *S. aureus*, respectively [34].

The MIC value for an antibiotic varies depending on which organism it acts upon. Carbapenems require higher MIC to be effective against *P. aeruginosa* as compared to other bacteria. The antibacterial efficacy of four commonly used dosage regimen for

doripenem was determined using an integrated PK/PD M&S approach based on *in vitro* time-kill data and human PK data. The simulations indicated time-dependent antimicrobial activity of doripenem, in which the 250mg TID regimen had a higher bactericidal effect than did 500mg BID [54].

While dosage regimens for most antibiotics can be derived by PK/PD-based M&S as outlined in the presented examples, the therapeutic concentration obtained from those recommendations may be insufficient to safeguard optimum clinical efficacy and bacterial clearance from the site of infection, especially if they are based on studies in healthy individuals rather than patients. Hence, it is of utmost importance to take into account the disposition (PK) and target site accessibility of antibiotics in diseased individuals having abnormal physiological conditions such as renal dysfunction. Along these lines, it is often preferred to use in these PK/PD M&S evaluations drug resistant bacteria that constitute a worst case scenario. An example for such an approach is the dose optimization of norvancomycin against methicillin-resistant rather than non-resistant *Staphylococcus* isolates for patients with different physiological and pathological conditions in the Chinese population. The optimized norvancomycin dosing recommendations were 800-1000mg BID for patients with normal renal function, 800mg QD or BID for patients with mild renal dysfunction ($CL_{Cr} > 50$ to ≤ 85 mL/min), 800mg QD to 2.5days for patients with modest renal dysfunction ($CL_{Cr} > 10$ to ≤ 50 mL/min), and 600mg over 5-11days or 800mg over 6-13days for patients with severe renal dysfunction ($CL_{Cr} > 5$ to ≤ 10 mL/min) [28].

Optimization of Clinical Studies and Clinical Applications

Over the last decade, it has become obvious in many examples that fine tuning of phase II/III clinical trials as well as post-marketing studies a priori is essential to reduce cost and optimize study performance and outcomes. PK/PD based M&S has played a major role in this process by integrating observations and data obtained from preclinical studies in animals and phase I studies in healthy volunteers as well as phase IIa studies in patients. The factors taken into consideration for mathematical modeling are often:

1. MIC distribution patterns in clinical isolates
2. The range of individual PK parameters in the target patient population
3. Identified PD targets from efficacy studies in animals
4. The degree of protein binding.

The combination of microbiological, preclinical and clinical PK and PD data in mathematical models is essential in designing target antibiotic exposures. M&S with such data is not only beneficial for single drug therapy but also for the evaluation of multiple concurrently used antibiotics and their potential additive or synergistic effects [41].

There is a sustained need for monitoring the PK/PD of antibiotics even after their approval and marketing. Rifampin is widely used for treatment of tuberculosis since several decades, but there is only limited data on rifampin concentrations in the lungs. Using an M&S based simulation study, Goutelle *et al.* found that the existing dosage regimen (600mg/day) for rifampin is suboptimal in most patients. This could be a potential contributing factor for the increasing number of tuberculosis infections with multi drug-resistant and extremely drug-resistant strains. The authors suggest considering higher doses (1200mg/day) of rifampin for an increase in pulmonary concentrations, thereby keeping in mind the toxicity and tolerability of such high doses in patients [33].

M&S may also be useful in developing strategies to minimize the effect of drug-drug interactions. Voriconazole and fluconazole are two widely used antifungal drugs with similar mechanism of action, but a strong drug-drug interaction when immediately switching between the two agents. Fluconazole is the most commonly used antifungal agent but unlike voriconazole it does not have anti-aspergillosis coverage sometimes necessitating a clinical switch. Fluconazole is a CYP2C19 inhibitor and its presence inhibits voriconazole metabolism causing substantial voriconazole-related adverse effects. Physiologically based PK modeling was used to determine the time lag required between the two therapies to avoid potential toxicity, but also minimize the time period without effective antifungal exposure which is essential in the event of serious fungal infections. The PK simulations demonstrated that a time lag of at least 24h is required to switch from fluconazole to voriconazole in order to avoid relevant interactions [36].

The use of M&S is not only restricted to optimizing dosing regimen and efficacy of treatment, but can also be used for building up clinical treatment guidelines. Goldie *et al.* constructed a population model of HIV infection to answer lifetime cost, life expectancy, quality-adjusted life expectancy, and quality-adjusted life years in patients receiving highly active antiretroviral therapy (HAART) and *Pneumocystis carinii* pneumonia (PCP) prophylaxis. The simulation exercises were performed to compare different criteria of CD4 cell counts for discontinuing primary PCP prophylaxis in patients with CD4 cell count increases receiving HAART and second-line PCP prophylaxis (such as dapsone, atovaquone, and aerosolized pentamidine) due to intolerance to trimethoprim-sulfamethoxazole. The outcome of the exercise showed that treatment is cost-effective when PCP prophylaxis is discontinued with CD4 count >300/ μ L, and that in patients intolerant to trimethoprim-sulfamethoxazole dapsone therapy is more cost-effective than atovaquone [55].

Future Prospects

The application of pharmacometric analyses using PK/PD based M&S approaches has in the recent decade developed into one of the most essential tools for interpreting and integrating large and diverse pools of preclinical and clinical data and translating them into an informative knowledgebase [5]. It is expedient not only for industry professionals to promote internal decision making at critical drug development steps, but is also increasingly utilized by regulatory authorities for compiling and analyzing data for

approval and labeling. A recent survey shows that during the last decade, the drug approval and labeling decisions of more than 60% of submissions for New Drug Applications (NDA) to the US FDA were influenced by pharmacometric analyses. For 30% of the submissions during this period, FDA reviewers performed independent pharmacometric analysis [13]. For example, among anti-infectives, an appropriate dosing regimen of micafungin for the treatment of esophageal candidiasis was selected based on an exposure-response analysis by FDA personnel. The FDA reviewers utilized the phase II and III study data to model the relationship between dose and effectiveness, which led them to recommend approval of the 150mg over the 100mg micafungin dose due to similar maximum efficacy, but a 15% lower rate of relapse [9].

Another prime example for use of M&S in regulatory decision making is the pharmacometrics-based dose selection of levofloxacin for post exposure treatment of inhalational anthrax in children. The available data from two PK studies on 47 adults at different doses (500mg/kg and 750mg/kg) and 90 pediatric patients who received a dose of 7mg/kg were used to develop an integrated population PK model. The model was then utilized to derive dosing recommendations for pediatric patients that match adult exposure after a 500mg/kg dose with regard to AUC, and maximum and minimum concentration during multiple dosing at steady state. Based solely on this M&S approach, a dose regimen of 8mg/kg BID for children <50kg and ≥ 6 months of age and 500mg QD for children weighing ≥ 50 kg was recommended and included in the labeling information [56].

The US FDA and the European Medicines Agency (EMA) both acknowledge the impact of M&S on regulatory approvals, but assess the investigational plans and scientific information differently. FDA seems to prefer to conduct pharmacometric reviews including independent data analyses for all the submitted applications irrespective of whether the sponsor submitted an analysis or not. In contrast, EMA seems to consider a pharmacometric assessment only if it is included in the submission by the sponsor. EMA might ask for additional pharmacometric analyses, but these should be conducted by the sponsor. European regulatory bodies are especially encouraging and emphasizing the use of M&S for drugs in pediatrics and for anti-infectives [57-59]. Irrespective of these different philosophies in the review process, the emphasis and demand of M&S based pharmacometric analyses in drug approval will likely further increase the application of M&S throughout the drug development process [8, 30, 60].

More recently, physiologically based PK and PK/PD modeling (PBPK) is gaining more widespread application, predominantly because of improvements in the associated M&S methodologies and the availability of more robust and user-friendly software tools [61-63]. It is predicted that the use of PBPK will increase over the next years for identifying the effects of intrinsic and extrinsic factors modifying drug efficacy and safety in patients. PBPK will play a significant role in predicting and deciphering drug-drug interactions and will be the basis for M&S in special population such as pediatric patients or in critically-ill patients [63].

Evidently, pharmacometric analyses have proven their merits in myriad ways during the entire process of drug development. However, there is always room for improvements for more accurate and precise estimates resulting in more confident predictions. This fine-tuning can likely be accomplished by a more prospective integration of M&S approaches in each individual development phase, including goal-oriented and intelligent sample and data collection that specifically addresses and supports pharmacometric analyses. Some of the questions that need to be addressed relate to the transfer of knowledge from controlled studies to clinical practice: PK parameter estimates are generally derived from healthy subjects. How can we assure that PK/PD predictions are accurate in patients with variable physiologic function and variable sites and types of infection? How can we account for patients with multiple disease conditions? How will their confounding illnesses or polypharmacy affect drug exposure for the considered anti-infective? How will variability in the strains of microorganisms and their response to the survival stress elicited by the anti-infective such as latency and resistance development affect treatment outcome? A more systematic and intensive assessment of antibiotic concentrations at the site of action such as ELF over the time course of therapy might help to provide some of the needed answers. Techniques such as microdialysis have been proven of substantial value in assessing active drug concentrations in target tissues not as easily accessible as ELF [64, 65].

In summary, more complex M&S models will likely be established in the future that integrate all therapeutic considerations when treating microbial infections. Using precise estimates of PK and PD parameters as well as their distributions in populations of patients and microorganisms, including their potential interdependencies, will help in facilitating more realistic and thus also even more clinically meaningful predictions for the *in silico* evaluation and development of optimal antibiotic dosing regimens, including combination therapies. To combat the growing bacterial resistance, we need to rapidly increase the available number of effective antibiotics in our armamentarium by relatively fast, safe, smart and inexpensive clinical development programs. We believe that M&S based pharmacometric approaches hold great promise in facilitating this process and thus may serve as a key technology for the development of future generations of anti-infectives.

CHAPTER 2. CENTRAL HYPOTHESIS

Among the most deadly infectious microorganisms, tuberculosis is a second greatest killer worldwide [66]. According to WHO [67], tuberculosis kills approximately 1.5 million people annually and it was estimated that 480,000 patients developed multi-drug resistant TB (MDR-TB) in 2013 alone. The existing drug therapies requires treatment spans of 4-24 months and efficacy is hindered severely by poor adherence to dosing regimens and drug adverse effects. This alarming situation undoubtedly calls for the urgent need for the development of novel and more effective treatment options to defeat TB [68, 69]. The primary goal for current anti-tuberculosis drug development efforts is to shorten the treatment regimen and circumvent drug-resistance mechanisms.

Our group has designed a novel semi-synthetic series of spectinomycin analogs, or spectinamides. All compounds in this series were initially screened using *in vitro* assays. The results from these preliminary studies aided in refining the chemical structure of lead compounds and improved their drug-likeness properties for clinical readiness. The lead optimization process comprised *in vitro* and *in vivo* pharmacological, biopharmaceutical, pharmacokinetic (PK) and pharmacodynamic (PD) profiling of candidate compounds [70, 71]. Using a pharmacometric approach, we integrated the PK and PD properties of spectinamides to better characterize the interplay and interdependencies between drug concentration and drug efficacy in models of disease. We hypothesized that a pharmacometrics-directed quantitative approach for integrated PK/PD characterization is useful for antibiotic lead characterization and highly predictive of *in vivo* antibacterial efficacy.

Specific Aim 1

In this Aim 1 (Chapter 3), we investigated the biopharmaceutics and pharmacokinetic properties of select spectinamide agents to determine lead candidate compounds and provide the basis for pharmacometrics-directed PK/PD assessments. An important aspect in the efficacy and safety of novel drug candidates is the extent of distribution and potential accumulation in tissues. In this context, intracellular uptake into macrophages is a prerequisite for antibiotic activity against *M. tb* residing inside infected macrophages. To address these questions, we also evaluated the biodistribution profile of a radio-labeled spectinamide and cellular uptake of spectinamides into murine macrophages.

Specific Aim 2

In Aim 2, we further evaluated the PK/PD relationships of lead compound, Lee 1599 using an *in vitro* PK/PD model system which characterizes a dose-response relationship in order to facilitate a rational dose-selection for *in vivo* efficacy trials (Chapter 4). While PD assessments under static conditions such as MIC determinations

provided only a point estimate of anti-bacterial activity. A more effective way of determining the appropriate dosing regimen with optimum efficacy and with high correlation between *in vitro* and *in vivo* efficacy is through an integrated PK/PD model approach. An *in vitro* PK/PD model is a convenient tool to predict the efficacy, dosing regimen of single or combination drug candidates and PK/PD indices (concentration and/or time dependent killing) based on dynamic time-kill curves for supporting *in vivo* studies. To test this hypothesis we determined the time-kill effect of Lee 1599 at various dosing regimens against *M. bovis* BCG as model organism. With this data, we developed a PD model to describe Lee 1599 mediated bacterial killing. Based on these *in silico* simulations, we were able to predict the optimal dosing regimen for *in vivo* efficacy studies.

Specific Aim 3

In Aim 3 (Chapter 5), we expanded the pharmacometrics-directed quantitative PK/PD assessment of Lee 1599 to combination therapies with rifampicin as model compound. Traditionally, qualitative methods such as the checkerboard assay have been widely used to evaluate possible pharmacodynamic interaction between two agents. We applied a three dimensional surface response assay as quantitative pharmacometric tool for characterizing the interaction between Lee 1599 and rifampicin and compared them to the checkerboard assay. We validated the results of both assays by expanding our previously applied with an *in vitro* PK/PD model (Chapter 3) to drug combination therapy and by a comparison to results of *in vivo* studies in a mouse model of *M. tb* infection.

CHAPTER 3. PHARMACOKINETICS AND BIODISTRIBUTION OF A NOVEL SERIES OF ANTI-TUBERCULOSIS AGENTS: SPECTINAMIDES

Introduction

Tuberculosis (TB) is a global pandemic that has a mortality rate of approximately 1.5 million people per year. The existing drug therapy requires treatment for a span of 4-24 months. The flaws of this treatment regimen become very apparent once it is administered to patient populations, as it is lengthy and complicated resulting in poor patient adherence. As a result, many patients discontinue therapy before complete eradication of the disease, which subsequently may lead to the development of drug resistant strains such as multi drug resistant (MDR), extremely drug resistant (XDR) and totally drug resistant tuberculosis (TR-TB) [67, 68, 72-76].

With the help of a structure based design process, novel semisynthetic analogs of spectinomycin called spectinamides were recently discovered. Spectinamides inhibit protein synthesis by selectively binding to the head domain of the 30S ribosomal subunit and blocking its translocation [77]. They have also exhibited overcoming an intrinsic resistance mechanism by avoiding the tap efflux pump Rv1258c in *M. tb*. Rv1258c is an efflux pump belonging to the ABC (ATP-binding cassette) transporters and is known to play an important role in mediating the efflux of different chemical classes and antibiotics [78-80]. While spectinomycin is a substrate for Rv1258c resulting in no relevant activity against *M. tb*, the structural modification in spectinamides renders them unsuitable as Rv1258c substrate. Thus, the potency of the spectinamide series is a product of high affinity for the mycobacterial ribosome and the avoidance of Rv1258c efflux pumps [81].

The primary goal of this chapter is to gain knowledge of the preclinical pharmacokinetics of the select compounds in the spectinamide series. We also investigated the biodistribution properties of a spectinamide lead, Lee 1329, using a radioisotope technique, as well as uptake into macrophages. Knowledge about the extent and pattern of biodistribution are crucial PK characteristics that may determine the efficacy and safety profile of drug candidates.

Materials and Methods

Chemicals and reagents

All tested spectinamides were synthesized in the laboratory of Dr. Richard.E. Lee at St.Jude Children's Research Hospital, Memphis, TN, including Lee 1329 (3'-dihydro-3'-deoxy-4(R)-(pyridin-2-yl) acetylamino spectinomycin), Lee 1544 (3'-Dihydro-3'-deoxy-4(R)-(4-chloropyridin-2-yl) acetylamino spectinomycin), Lee 1599 (3'-Dihydro-3'-deoxy-3'(R)- (5-chloropyridin-2-yl)acetylamino spectinomycin Trihydrobromide), 3'-

Dihydro-3'-deoxy-3'(R)- (4-(4-chlorophenyl)pyridin-2-yl)acetylamino spectinomycin Trihydrobromide (Lee 1601), 3'-Dihydro-3'-deoxy-3'(R)- (2-(pyridin-2-ylamino)thiazol-4-yl)acetylamino spectinomycin Tetrahydrobromide (Lee 1603), 3'-Dihydro-3'-deoxy-3'(R)- (4-chloro-5-fluoropyridin-2-yl)acetylamino spectinomycin Trihydrobromide (Lee 1661), 3'-Dihydro-3'-deoxy-3'(R)- (1-methyl-1H-benzo[d]imidazol-2-yl)acetylamino spectinomycin Trihydrobromide (Lee 1663) as shown in **Figure 3-1**. Acetonitrile, methanol, HPLC grade water, formic acid and ammonium formate were purchased from Fisher Scientific (Pittsburgh, PA). Drug free rat plasma was purchased from Innovative Research (Novi, MI).

Protein binding

The plasma protein binding is determined using equilibrium dialysis with ready to use RED device inserts from Thermo Scientific (Rockford, IL) in base plates containing plasma and buffer chamber for analysis. High and low concentrations (500ng/mL and 5µg/mL) of test compounds are prepared in rat plasma. 200µL of the plasma sample was added to the central compartment and 350µL of blank isotonic phosphate buffer, pH 7.4 to the peripheral compartment of dialysis base plate. The base plate is covered with sealing tape and incubated at 37°C at approximately 100 rpm on an orbital shaker for 4h to achieve equilibrium. The drug concentration is determined using an LC-MS/MS assay. The free fraction of the drug is calculated as ratio of the concentrations in the buffer and in plasma. The results are expressed in terms of %-bound to plasma proteins.

Formulation preparation and administration

Spectinamides (10mg/kg and 100mg/kg) were formulated in plasmalyte solution and sterilized by filtration (0.2 micron syringe filter). The IV doses were administered via femoral vein catheter followed by flushing the catheter with locking solution. A group of 5 rats were dosed for each compound and dose level.

Pharmacokinetic studies

The PK studies are performed on catheterized (jugular vein alone for oral study (PO) and jugular vein and femoral vein for intravenous (IV) study) male Sprague-Dawley rats weighing approximately 225g (8-10 weeks old). The animals were housed in the animal facility with 12h of light/dark cycle with food and water available *ad libitum* at University of Tennessee Health Science Center following protocol number 13-4263A. the protocol was approved by the Institutional Animal Care and Use Committee at University of Tennessee Health Science Center. Test compounds were administered for IV and PO studies at a dose of 10mg/kg and 100mg/kg, respectively. Serial blood samples were collected pre-dose and at predetermined intervals until 72h. Plasma was separated immediately by centrifugation at 10,000 rpm, 4°C for 10min and stored at -80°C until analysis. Plasma and urine samples were analyzed for drug concentrations by

Figure 3-1. Chemical structure of lead spectinamides with MIC values.

LC-MS/MS analysis. Blood samples (250µL) were collected at 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 12.0, 24.0, 36.0 and 48.0h after drug administration. Urine samples were collected at intervals of 0-6, 6-12, 12-24, 24-36 and 36-48h post-dose and stored at -20°C until analysis.

Pharmacokinetic data analysis

The concentration-time profiles for both PO and IV administration were analyzed by non-compartmental analysis. The IV concentration-time profiles were also analyzed by nonlinear regression analysis using a 2-compartment open model with bolus input and first order output. The area under curve (AUC_{inf}) from time zero to infinity was calculated using the trapezoidal rule by extrapolating to time infinity. Mean residence time (MRT), defined as the average amount of time a drug particle remaining in a compartment of a system upon IV administration was calculated using

$$MRT = \frac{AUMC_{inf}}{AUC_{inf}}$$

where $AUMC_{inf}$ is the area under the moment curve when the concentration-time curve is extrapolated to infinity. The systemic clearance (CL) was calculated using the equation

$$CL = \frac{Dose_{iv}}{AUC_{inf,iv}}$$

where $Dose_{iv}$ and $AUC_{inf,iv}$ are the IV dose and area under the plasma concentration-time curve from time 0 to infinity, respectively. An estimate of volume of distribution at steady state (V_{ss}) was obtained from IV data using

$$V_{ss} = MRT * CL$$

Oral bioavailability (F) was calculated using

$$F = \frac{AUC_{inf,oral} * Dose_{iv}}{AUC_{inf,iv} * Dose_{oral}}$$

where $Dose_{oral}$, $Dose_{iv}$, $AUC_{inf,iv}$, and $AUC_{inf,oral}$ are the oral and IV doses and the corresponding areas under the plasma concentration-time curves from time zero to infinity, respectively. Urinary excretion parameters were calculated from the cumulative dose excreted unchanged in urine and the fraction unbound in plasma (fup). Glomerular filtration rate (GFR) was taken from the physiologic parameters published by Davies *et al* [82]. The fraction (fe) of the test compound excreted in urine was calculated as the cumulative amount of dose excreted unchanged in urine divided by the dose of the test compound administered. Renal clearance (CL_r) values were determined from the total plasma CL and the fraction excreted in urine using $fe * CL_{total}$. The net processes such as

filtration, reabsorption and secretion a drug undergoes in the kidney can be evaluated using excretion ratio (Eratio). This was calculated using $CL_r/(f_{up} \times GFR)$.

Macrophage uptake assay

Uptake of spectinamide compounds (Lee 1329, 1443, 1445, 1544, 1599), SP and ST into murine macrophages was studied in the J774A.1 cell line (ATCC, Manassas, VA). J774A.1 cells were cultured in T25 flasks in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, USA) with 10% Fetal bovine serum (Sigma-Aldrich, USA) and maintained at 37°C, 5% CO₂. Cells were scraped using teflon cell scraper from the T25 flasks and the final cell density of 2 to 4 × 10⁵ cells/mL was adjusted using fresh DMEM. The cells were allowed to grow for 48h at 37°C until 60-80% confluent in a 6-well plate (each plate representing individual time point and concentration). The media was aspirated from the wells and fresh DMEM containing a final test compound concentration of 25 µg/mL or 100 µg/mL was added. The plates were incubated at 37°C, 5% CO₂ for 0.5, 1, 4, 8 and 24h. Additional plates were also incubated for 48, 72 and 96h. The media containing test compound was aspirated at every time point and the cells were washed with ice-cold Dulbecco's Phosphate-Buffered Saline (DPBS) for three times. The cells were suspended in 1mL of water and sonicated for 2min. The cell suspension was centrifuged for 5min at 10,000 rpm and the supernatant collected was used to determine the drug uptake using LC-MS/MS assay. The drug uptake was expressed in terms of µL/mg of protein. The protein content in cells was determined using the Pierce BCA assay kit (Thermo Scientific, Pierce Biotechnology, IL, USA).

Sample preparation and LC-MS/MS analysis

The plasma and urine samples were prepared by the protein precipitation method by adding 50 µL aliquots to 200 µL methanol (spiked with the internal standard 3'-dihydro-3'-deoxy-3'(R)-isopropylacetyl amino spectinomycin) followed by vortexing for 1min, centrifugation at 10,000g for 10min at 4°C. The supernatant was separated via chromatography on a Luna 3 µM hydrophobic interaction liquid chromatography (HILIC) 100 × 4.6mm column (Phenomenex, Torrance, CA) using a gradient mobile phase of methanol and 10mM ammonium formate, pH 2.75, at a flow rate of 0.4mL/min. Detection was performed with an API 3000 triple-quadrupole mass spectrometer (ABI-Sciex, Foster City, CA) with electrospray ionization in multiple reaction monitoring mode using the compound-specific mass transfers of m/z 453.3/247.3 for Lee 1329, m/z 459.2/207.2 for Lee 1443, m/z 471.3/207.1 for Lee 1445, m/z 487.2/128.3 for Lee 1544, m/z 487.2/207.1 for Lee 1599 and m/z 418.3/207.1 for Lee 1369.

A calibration curve (range: 1.95 to 5,000 µg/L) was constructed for each test compound and validated with spiked samples of rat plasma or urine. The peak area ratios of analyte to internal standard were linear over the concentration range tested for all compounds, with all correlation coefficients (weighted least-square linear regression analyses) >0.997. Accuracy (deviation of the analyzed quality-control samples from

nominal values) was within $\pm 3\%$ over the entire range of the calibration curve, and the precision (coefficient of variation of repeated measurements of the quality-control samples) was $< 2\%$ for all compounds.

Biodistribution

Radiolabeling of Lee 1329 (3'-Dihydro-3'-deoxy-3'(R)-(pyridin-2yl) acetylamino spectinomycin Trihydrobromide) compound was performed by Dr. Richard E. Lee's group at St. Jude Children's Research Hospital. The biodistribution study was conducted in Sprague-Dawley rats (N=7, one control and 6 in the treatment group). The rats were administered ^3H -1329 intravenously (IV) via a femoral vein catheter at a dose of 0.9mg/kg body weight (0.87mCi/kg) every 12h for 4.5days. The control rat received normal saline only. Serial blood samples were collected at 0 (pre-dose), 0.25, 2, 8 and 12h after the 1st dose and at 0 (before 8th dose), 0.25, 0.5, 1, 2, 4, 6, and 12h after the 8th dose to evaluate the pharmacokinetics after single and multiple dosing. Plasma was separated immediately after sample collection by centrifugation (10,000g for 10min at 4°C) and stored at -80°C until analysis of radioactivity by scintillation counting. Urine and feces specimens were collected at 0h (pre-dose) and every 12h after dosing up to the end of the study and stored at -80°C until analysis. The volume of urine and weight of feces were noted at every sampling point for the calculation of mass balance. One hour after the administration of the 9th dose, the animals were euthanized and all major organs such as liver, kidneys, spleen, lung, heart, brain, thigh muscle and blood were collected to determine tissue distribution and accumulation. Total blood was collected by cardiac puncture. Major organs and tissues were collected and stored at -80°C until analysis. Total volume of blood, urine, total weight of the organs, muscle and feces was noted for the calculation of mass balance.

Sample preparation of biological samples for ^3H -1329

Blood

0.2mL of blood was added to 1mL of Solvable™ (aqueous based solubilizer, PerkinElmer, Waltham, MA) in a glass scintillation vial and incubated at 60°C for 1h. 0.1mL of 0.1 M EDTA-di-sodium solution was added to reduce foaming due to H_2O_2 . 0.5mL of 30% H_2O_2 was added in 0.1mL increments and gently agitated after each addition to allow reaction and foaming to subside. H_2O_2 treatment helps to reduce the amount of color present, and thus reduces color quench in the final mixture. The mixture was allowed to stand for 30min at room temperature to complete the reaction and was incubated in a water bath at 60°C for 1h, venting any slight pressure occasionally. The sample was then cooled to room temperature and 15mL of liquid scintillation cocktail Ultima Gold™ (Perkin Elmer, Waltham, MA) was added. The samples were light adapted for one hour before counting the radioactivity using a scintillation counter.

Serum and urine

The direct addition method was acquired to determine the amount of radioactivity in serum and urine. 1.5mL of ethanol and 15mL of liquid scintillation cocktail Ultima Gold™ was added in a glass scintillation vial to 0.1mL of serum or 0.5mL of urine and shake well. The samples were light adapted for at least one hour before scintillation counting.

Organs and muscle tissue

Organs (liver, kidney, spleen, lung, heart and brain) and muscle samples were prepared by the solubilization method for the determination of accumulated radioactivity. 50mg of organs or muscle tissue was weighed into a scintillation vial and 2mL of Solvable™ was added and heated on a water bath at 60°C for 1 to 3.5h with occasional swirling. After cooling to room temperature, 0.2mL of 30% H₂O₂ was added slowly with swirling between additions, and the mixture was allowed to subside any reaction between additions of the H₂O₂. The mixture was heated again at 60°C for 30min for complete decolorization. 10mL of liquid scintillation cocktail Ultima Gold™ was added, and the samples were light adapted for at least one hour before scintillation counting.

Feces

The amount of radioactivity excreted in feces was determined by sample combustion with a sample oxidizer (PerkinElmer Model 307 Sample Oxidizer, Waltham, MA) and the recovered ³H was analyzed by scintillation counting. Briefly, feces specimens of each animal collected at different time intervals were combined together and homogenized with 4 volumes of water. Triplicate samples from each animal of approximately 300mg of homogenate were placed in the sample oxidizer and combusted in an oxygen rich atmosphere to oxidize the tritium present in the samples to water (³H₂O). The water was separated into a scintillation vial and mixed with 10mL of scintillation cocktail Monophase-S (PerkinElmer, Waltham, MA). The samples were light adapted for at least one hour before scintillation counting.

Sample analysis of biological samples for ³H-1329

The blood, serum, urine, organs, muscle and feces samples mixed with liquid scintillation cocktail as described above were placed in a Tri-carb 2800 TR liquid scintillation analyzer (PerkinElmer, Waltham, MA) to determine the tritium activity (³H). The radioactivity was measured in terms of disintegrations per minute (DPM) setting the energy window from channel 0 to channel 20. The luminescence from the sample was reduced by setting a 10min pre-count delay. Samples were counted in the luminescence correction mode. The average counting efficiency for ³H is 65.45%. The counting time for each sample was 1min and each sample was analyzed three times. The quench indicator tSIE (transformed spectral index of the external standard) was used as an indicator of a good spectrum.

Results and Discussion

Biopharmaceutical properties of spectinamides

As shown in **Table 3-1**, the molecular weight of spectinamides ranged from between 450 to 600Da and is larger as compared to the spectinomycin with weighs around 332.3Da. All of the spectinamides are highly hydrophilic in nature with clogP between -3 and 0.

Plasma protein binding is an essential parameter in determining the drug distribution, metabolism and elimination. A drug molecule that is circulating in the system exists in two variants, protein bound and unbound (free), assuming that the protein binding is reversible. The unbound and the bound form exist in equilibrium. The unbound (free) drug is the only pharmacologically active drug as it can permeate the cell membranes and exert the pharmacological effect on binding to target structure. Thus, it is important to estimate the extent of plasma protein binding [43, 83, 84].

In general, spectinamides showed low to intermediate plasma protein binding in rats. The only exception was Lee 1601 with 75% protein binding which can be attributed to its increased molecular weight (563Da) as compared to the other lead compounds in this series. The low plasma protein binding suggests that a large fraction of molecules is freely available to enter the intracellular space for eliciting their therapeutic effect.

Lee 1544, Lee 1599, Lee 1601, Lee 1603, Lee 1661 and Lee 1666 are 2-heteroaryl acetic acid amide analogs of spectinomycin that showed potent *in vitro* activity against *M. tb*. The pharmacokinetic parameters for all these compounds are shown in **Table 3-1**. Following intravenous administration of 10mg/kg in rats, all molecules exhibited bi-exponential concentration time profiles with distinct distribution and elimination phase (**Figures 3-2 to 3-8**) except Lee 1601.

All the lead candidate compounds in the spectinamide series showed comparable alpha half-life of 0.58 to 1.06h, except for Lee 1601 with a very low alpha half-life of 0.28h. The compounds showed prolonged beta half-life, except Lee 1601. This prolonged beta phase did not significantly contribute to the overall systemic exposure (AUC) because of 100 to 1000 fold lower plasma concentrations than C_{max}. This trend is commonly seen in aminoglycosides and other amines and is associated with slow release of the trapped molecules from deep tissue compartments. This phenomenon may be attributed to lysosomal trapping and/or complexation with membrane phospholipids [85-89].

The compounds showed a steady state volume of distribution in the range of 0.8 to 1.48L/kg except for Lee 1601 and Lee 1661 with V_d of 0.17 and 0.4L/kg, respectively. The AUC ranged from 8.3 to 19.3h.mg/L. The AUC for Lee 1599 was slightly lower than other spectinamide compounds. The MRT ranged from 0.2 to 1.84h and the mean systemic clearance ranged from 0.5 to 1.22L/h/kg. The percent dose excreted unchanged

Table 3-1. Physico-chemical properties and pharmacokinetic parameters for spectinamides after intravenous administration at 10mg/kg dose.

Name of the compound	MW (Da)	cLogP	PB (%)	Cmax mg/L	t _{1/2} h	AUC h*mg/L	CL L/h/kg	Vd L/kg	MRT h	Fe	Eratio	% F
Lee 1544	486.9	-2.51	15.0	12.1	1.06	10.7	0.935	0.868	0.931	0.520	1.70	4.00
Lee 1599	486.9	-2.51	13.0	9.94	0.58	8.37	1.22	0.820	0.680	0.885	3.02	3.47
Lee 1601	563.0	-0.697	75.0	15.9	0.297	12.7	0.828	0.167	0.197	0.129	1.08	0.377
Lee 1603	550.6	-2.30	55.0	16.2	1.05	13.7	0.748	1.41	1.84	0.283	1.82	2.03
Lee 1661	504.9	-2.34	40.0	23.4	0.732	19.3	0.521	0.358	0.687	0.860	1.91	1.75
Lee 1666	500.9	-2.01	42.0	15.2	1.04	13.6	0.740	1.48	1.98	0.74	2.33	-
Spectinomycin	332.3	-2.87	13.0	20.6	1.25	16.8	0.602	0.756	0.757	0.553	1.00	-

Notes: MW: Molecular Weight

cLogP: Calculate logarithm of partition coefficient

PB(%): Percentage protein binding

Cmax: Peak concentration in plasma

t_{1/2}: Alpha half-life

Fe: Fraction excreted unchanged in the urine

Eratio: Excretion ratio

%F: Percent bioavailability is calculated using the AUC values obtained after oral administration at 100mg/kg

Figure 3-2. Measured plasma concentration-time profiles (mean $\pm$ SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1544 in rats.

Figure 3-3. Measured plasma concentration-time profiles (mean $\pm$ SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1599 in rats.

Figure 3-4. Measured plasma concentration-time profiles (mean $\pm$ SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1601 in rats.

Figure 3-5. Measured plasma concentration-time profiles (mean $\pm$ SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1603 in rats.

Figure 3-6. Measured plasma concentration-time profiles (mean $\pm$ SEM) after intravenous (10mg/Kg) and oral (100mg/Kg) administration of Lee 1661 in rats.

Figure 3-7. Measured plasma concentration-time profiles (mean $\pm$ SEM) after intravenous (10mg/Kg) administration of Lee 1666 in rats.

Figure 3-8. Measured plasma concentration-time profiles (mean $\pm$ SEM) after intravenous (10mg/Kg) administration of spectinomycin in rats.

in the urine (fe) ranged from 13% to 89%. Except for Lee 1601 and Lee 1603, all the compounds had a high fe value 0.5, suggesting that the urinary excretion is the major route of elimination for these compounds. The Eratio >1 for all the compounds suggests active secretion as the net urinary elimination process.

Following 100mg/kg oral administration in rats, all compounds showed similar bi-exponential concentration-time profiles (**Figures 3-2 to 3-6**) except for Lee 1601. However, all tested spectinamides had poor oral bioavailability (F) ranging from 0.4 to 4%. Due to higher hydrophilicity, these molecules have limited membrane permeability that likely leads to the poor oral bioavailability. One of the strategies to improve oral bioavailability is by increasing the lipophilicity and reducing the polar surface area without compensating the solubility of these molecules. Alternative route of administration such as intrapulmonary route can also be assessed to determine an improvement in the bioavailability [90-93].

Macrophage uptake assay

It has been commonly observed that macrophage ingest the *M. tb* as a defense mechanism. After being ingested, *M. tb* continue to survive and multiply within macrophages. Hence, it is important that the antimycobacterial agents reach the intracellular region where bacteria thrive within macrophage [94-97]. The macrophage uptake of spectinamides was evaluated by incubating the spectinamide agents with macrophages and quantifying the intracellular uptake of the agents by macrophages. Spectinomycin and streptomycin were used as controls for macrophage uptake. Spectinamides are chemically derived from spectinomycin and streptomycin is an aminoglycoside and also a second line agent to treat tuberculosis. The concentration of drug uptake by macrophages after 24h incubation are shown in **Table 3-2** and **Figure 3-9**. Lee 1329 and Lee 1599 showed significant penetration into macrophages as compared to spectinomycin and streptomycin. As shown in **Figure 3-10**, Lee 1329 has a 6-fold and Lee 1599 has 5-fold higher uptake into macrophages as compared to streptomycin. 1329 also has 2.2 fold higher uptake than spectinomycin. The results indicate that spectinamides have substantial intracellular penetration into macrophages.

Biodistribution

Biodistribution studies with radiolabeled compounds provide a foundation in the assessment of drug disposition as they provide understandings into mass balance, tissue distribution, drug accumulation, circulatory and excretory metabolites, clearance mechanisms and pharmacokinetics. This information is crucial to identify potential toxicity and safety concerns of novel drug candidates, but also provides basic information on systemic exposure [6].

Table 3-2. Mean concentration of drug uptake into J774A.1 murine macrophage cell line at 24h for two different doses.

Compounds	25µg/mL		100µg/mL	
	ng/µg protein	SEM	ng/µg protein	SEM
Lee 1329	1.17	0.287	4.04	0.753
Lee 1443	0.44	0.205	0.624	0.187
Lee 1445	0.762	0.125	2.71	0.411
Lee 1544	0.413	0.026	1.37	0.036
Lee 1599	1.04	0.032	3.31	0.055
SP	0.599	0.099	1.86	0.503
ST	0.184	0.017	0.68	0.052

Figure 3-9. Mean average concentrations ($\pm$ SEM) of drug uptake into J774A.1 murine macrophage cell line at 24h.

Figure 3-10. Fold differences in drug uptake in J774A.1 murine macrophage cell line.

Mass balance

The scintillation assay results revealed that 86.6% of administered radioactivity was recovered in urine, feces, blood, muscle and organs throughout the length of the study that included 9 doses of 0.9mg/kg ^3H -Lee 1329 administered intravenously every 12h. It is challenging to achieve 100% recovery in mass balance studies due to several practical limitations influenced by methodological, analytical or biological factors and the quantitative investigation were not performed on all organs and tissues at the termination of the study. These results, as shown in **Figure 3-11**, are in accordance with other mass balance studies [98-100]. The samples were collected post 1h after the 9th dose for mass balance determination. Approximately 70% of administered ^3H -Lee 1329 was recovered in urine, 12.6% in feces and only 4% from all major organs, muscle tissue and whole blood. The residual amount of 13.4% can likely be found in the animal carcasses. Urine samples collected every 12h showed similar amount of radioactivity in the range of 119.3 to 138.9 μCi as shown in **Figure 3-12**. These results indicate that there is no considerable accumulation of drug in the body during multiple dose administration.

Tissue distribution

The tissue distribution, penetration and accumulation of ^3H -Lee 1329 and its labeled metabolite was determined by analyzing the distribution of radioactivity to major organs and muscles post 1h after administering the 9th dose. The observed data for relative and absolute radioactivity for each major organ and tissue are presented in **Table 3-3**. The degree of tissue penetration was identified by measuring the fold differences in amount of radioactivity per mg of tissue as compared to whole blood **Figure 3-13**. The amount of radioactivity in lungs and spleen was 2.79 and 3.06 fold higher as compared to whole blood. This infers that ^3H -Lee 1329 possesses good tissue penetration into major organs which are the primary site of infections by *M. tb*. Moreover, the amount of radioactivity was less than one fold in other organs such as heart and brain.

Pharmacokinetic profile of radioactivity in serum

The concentration-time profiles for ^3H -Lee 1329 after administration of 1st dose and 8th dose are compared in **Figure 3-14**. The profiles are comparable for both the doses for the first two hours with identical C_{max} values. However, the concentrations of radioactivity was 50% greater after the 8th dose as compared to the 1st dose. This differences in concentrations at later time points indicates the possibility of metabolite formation circulating with longer half-life than the parent drug.

Conversely, the systemic exposure ($\text{AUC}_{0-\tau}$) of radioactivity after the 1st dose and 8th dose were found to be very similar because of two reasons (i) The concentrations of ^3H -Lee 1329 2h post dose was very low compared to the C_{max} and (ii) these concentrations added only 21% of the total exposure ($\text{AUC}_{0-\tau}$) (**Table 3-4**). A comparison of the systemic exposure characterized by C_{max} and $\text{AUC}_{0-\tau}$ following the 1st dose and 8th dose indicated an accumulation ratio R of 1.1, providing further evidence for the absence of any substantial drug accumulation. R is a parameter used to quantify the

Figure 3-11. Mass balance and percentage distribution of Lee 1329-T at 1h post 9th dose of 0.9mg/kg.

Figure 3-12. Amount of radioactivity (mean $\pm$ SD) excreted in urine after each dosing interval (0.9mg/kg ^3H -1329, BID for 4.5days).

Table 3-3. Tissue distribution and recovery of radioactivity (mean(%CV)) following IV administration of ³H-1329 at 0.9mg/kg BID for 4.5days in rats.

Sample/Organ	Relative amount of radioactivity [μCi/g tissue]	Total amount of radioactivity [μCi]	% of radioactivity recovered
ADMINISTRATION			
Total amount administered		1432 (3.44)	
RECOVERY			
Blood	0.24 (12.2)	3.47	0.242 (26.8)
Liver	1.19 (13.7)	11.4	0.796 (5.99)
Kidney	7.92 (8.98)	16.4	1.15 (10.9)
Lung	0.64 (9.35)	0.805	0.056 (12.1)
Spleen	0.69 (11.5)	0.658	0.046 (13.8)
Heart	0.22 (9.32)	0.219	0.015 (24.9)
Brain	0.10 (18.1)	0.160	0.011 (39.6)
Muscle	0.23 (21.8)	24.7	1.72 (34.9)
Urine	17.90 (21.2)	1003	70.0 (2.99)
Feces	5.91 (39.6)	181	12.6 (29.8)
Total amount recovered		1242	86.6 (5.09)

Figure 3-13. Fold difference (mean $\pm$ SD) in tissue distribution of radioactivity compared to amount in whole blood (after normalizing the distributions to $\mu\text{Ci}/\text{mL}$ or $\mu\text{Ci}/\text{mg}$ of blood or tissue).

Figure 3-14. Comparison of single and multiple dose pharmacokinetics showing average ($\pm$ SD) serum concentration-time profiles of radioactivity after the 1st dose and 8th dose during multiple dosing with 0.9mg/kg ^3H -1329 given intravenously every 12h.

Table 3-4. Serum pharmacokinetic parameters after the 1st and 8th intravenous dose of 0.9mg/kg ³H-1329 (values are expressed as mean (%CV)).

Parameter	Dosing interval	
	1 st	8 th
t _{max} (h)	0.25	0.25
C _{max} [mCi/L]	3.39 (7.4)	3.55 (28.9)
AUC _{0-τ} [mCi*h/L]	5.08 (6.8)	5.17 (20.5)

extent to which a compound will accumulate during multiple dosing relative to the first dose and is calculated as ratio between AUC or C_{max} during one dosing interval during multiple dosing compared to the first dosing interval. An R value of 1 indicates absence of any drug accumulation [101]

These results confirm that there is no substantial accumulation of ³H- Lee 1329 or its metabolites. The recovery of 70% radioactivity in urine and 35-fold higher concentration in kidneys compared to blood suggests that renal excretion is the major route of elimination (or kidney is the major eliminating organ) for Lee 1329 and its metabolites.

Conclusion

Select molecules from the spectinamide series were identified and characterized via screening prototype involving various *in vitro* and *in vivo* assays. Spectinamides demonstrated low to intermediate protein binding, shorter *in vivo* half-life at clinically relevant concentrations and very low oral bioavailability. The compounds in the series also expressed higher cellular uptake into macrophages as compared to streptomycin and spectinomycin.

The biodistribution and mass balance study emphasized the favorable pharmacokinetic properties of spectinamides as anti-TB agents; augmenting that spectinamide Lee 1329 has excellent penetration into target tissues, did not accumulate in tissue during multiple dosing, and forms low levels of circulating metabolites that are excreted into urine and feces.

The pharmacokinetic parameters and tissue distribution data obtained in this chapter provide the basis for the pharmacometrics-directed integrated PK/PD characterizations in Chapters 4 and 5.

CHAPTER 4. A PHARMACOMETRICS-BASED APPROACH USING *IN VITRO* PK/PD MODELING TO DEVELOP DOSING REGIMENS FOR *IN VIVO* STUDIES FOR A NOVEL SPECTINAMIDE ANTI-TUBERCULOSIS AGENT

Introduction

The increasing emergence of resistant mycobacterial strains in recent years and the complications arising from treatment regimens are a strong reminder of the urgent need for the development of new and more effective treatment options for tuberculosis patients worldwide [67, 68, 72, 73]. Spectinamides, a novel class of spectinomycin analogs, were designed to combat the increasing TB pandemic [81]. Spectinamides have shown potent activity against mycobacteria *in vitro* and *in vivo*. Based on *in vitro* screening assays and pharmacokinetic studies in rodents, Lee 1599 emerged as one of the lead candidate molecules from the class of synthesized compounds. Lee 1599 has a minimum inhibitory concentration (MIC) of 1.6 µg/mL, and a favorable pharmacokinetic profile with low (12.5%) protein binding, high microsomal metabolic stability, and renal excretion as the primary elimination pathway. Although the half-life at effective concentrations in rats was with 0.58h is relatively low, this property is counterbalanced by a long post-antibiotic effect [81, 102, 103] that last approximately 133h when mycobacteria were exposed to Lee 1599 at 10X the MIC.

The preliminary screening assays for new anti-infectives routinely include MIC testing as one of the major pharmacodynamic measures of potency. Although the MIC parameter is very informative at the early discovery phase, the limitations of this testing method become evident as the *in vitro* knowledge about MIC can often not correlate very well with *in vivo* efficacy in clinical settings. MIC is just a point estimate of pharmacodynamic effect and does not describe the nature of bacterial killing over time [39, 104, 105]. Thus, it is essential to have a more dynamic approach that has the ability to translate the *in vitro* bacterial killing effect to *in vivo* conditions. Various *in vitro* PK/PD modeling approaches have been utilized in recent years to capture the interaction of fluctuating drug concentrations with bacterial killing over time. The benefits and pitfalls of each method have been described elsewhere [10]. To obtain initial assessments on PK/PD drivers of efficacy for new leads in drug discovery programs, a spinner-flask based *in vitro* PK/PD model is a simple tool to generate time-kill curves for mycobacteria under exposure to antibiotics. The fluctuating concentration-time profiles of the free, pharmacologically active antibiotic as experienced during multiple dosing *in vivo* are simulated in this approach using previously determined *in vivo* pharmacokinetic parameters of the compound

In this article, we describe the dynamics of bacterial killing by Lee 1599 using an *in vitro* PK/PD model. The Lee 1599 facilitated bacterial killing was characterized at multiple dosing regimens by means of *M. bovis* BCG as model organism. An integrated PK/PD model was generated based on the generated bacterial time-kill curves and the simulated pharmacokinetic profiles of Lee 1599. Numerical simulations were subsequently performed to predict the anti-mycobacterial activity of Lee 1599 for

untested dosing regimens. This methodology has potential to guide researchers for optimizing the dose selection process and study designs for further *in vivo* efficacy testing.

Materials and Methods

Culture, media and antibiotics

M. bovis BCG was grown in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD). The cultures were stored at -80°C in Middlebrook 7H9 broth. For each experiment, cultures were thawed and incubated at 37°C in Middlebrook 7H9 broth until the bacteria reaches logarithmic phase (OD₆₀₀ 0.4-0.7). Lee 1599 (3'-Dihydro-3'-deoxy-3'(R)- (5-chloropyridin-2-yl)acetylamino spectinomycin Trihydrobromide) was synthesized and provided by our collaborators at St.Jude Children's Research Hospital. A solution of Lee 1599 was freshly prepared in sterile water before the start of each experiment.

In vitro PK/PD model

As previously reported [39], the *in vitro* PK/PD model system consisted of a two-armed, water jacketed flask (1965 series spinner flask, Bellco Glass, Vineland, NJ) as depicted in **Figure 4-1** [71]. A filter unit attached to a hollow steel tube is introduced into the system. The filter unit contains a prefilter (5µm, Millipore, Billerica, MA) and filter membrane (0.22µm, Millipore, Billerica, MA) to prevent leakage of bacteria during the dilution process. The other end of the tube is connected to plastic tubing (PharMed tubing, Cole-Parmer, Vernon Hills, IL) from which the media is withdrawn continuously at a defined flow rate using a peristaltic pump (Masterflex L/S, Cole-Parmer, Vernon Hills, IL). The flask is placed on a magnetic stirrer for homogeneity of the culture and preventing membrane blockage. The temperature of the system is maintained at 37°C with the help of thermostatic water bath attached to the water-jacketed flask. One arm of the flask is connected by plastic tubing to the fresh sterile media reservoir and the other arm is used for dosing and repeated sampling. The solution of Lee 1599 is added through the sidearm. The concentration of Lee 1599 in flask decreases exponentially as per the equation:

$$C = C_0 * e^{-k*t}$$

C₀ is the initial concentration of Lee 1599 in the flask following administration, C is the concentration of Lee 1599 at any given time t, k is the elimination rate constant.

$$k = \frac{CL}{Vd} = \frac{F}{Vm} = \frac{0.693}{t_{1/2}}$$

Figure 4-1. A representation of working unit of an *in vitro* PK/PD model.

where CL is the clearance of 1599, Vd is the Volume of distribution of 1599, F is the flow rate of the media depicting Clearance, Vm is the volume of media in the flask, and $t_{1/2}$ is the half-life of 1599. All the above mentioned PK parameters were from obtained by performing PK study in rats. The *in vitro* set up is assembled in a biosafety cabinet in a sterile environment. The flask and the tubes are cleaned and sterilized by autoclaving before starting every experiment.

Time kill curves for Lee 1599

M. bovis BCG was used as a test organism for evaluating the effect of Lee 1599 *in vitro* model. The organism was grown to early logarithmic phase (O.D₆₀₀. 0.4-0.7) in Middlebrook 7H9 medium. This actively growing bacteria was used as an inoculum for the *in vitro* model making the final density in the culture as 10⁶ CFU/mL.

In separate experiments, different dose levels and dosing intervals of Lee 1599 were tested. The dosing regimens tested were 1mg QD, 5mg QD and BID, 10mg QD and BID, 20mg QD, 25mg QD and BID, 50mg QD and BID and 100mg QD. Samples with an approximate volume of 200µL were collected from the flask every 12h. The samples were centrifuged at 10,000 rpm for 10min at 37°C. The pellets were collected and re-suspended in an antibiotic-free medium. This step ensured that there was no drug carry over effect. The samples were serially diluted and the dilutions were plated on 7H11 agar plates in duplicates. The agar plates were incubated for 21days at 37°C. The effect of Lee 1599 was characterized by measuring the reduction in the number of viable bacteria, in terms of CFU/mL. There was a control arm included in the study to determine the bacterial growth in absence of drug.

PK/PD model building

The chemostat flask in the *in vitro* system resembled the central compartment and total amount of drug administered in the central compartment. The concentration-time profiles of the drug in the *in vitro* PK/PD model system were described with a simple one-compartment model with first-order elimination:

$$C = C_0 * e^{-k*t}$$

where C is the concentration of Lee 1599 at any given time t, C₀ is the concentration of Lee 1599 at time 0h, k is the elimination rate constant. The rate of change in the number of bacteria in the presence of drug was modeled using the full PK/PD model as shown in **Figure 4-2**. The bacterial submodel and the PK model were combined and the parameters are combined to characterize the effect of antibiotic Lee 1599 on *M. bovis* BCG [105, 106] as shown in the equation below.

$$\frac{dN}{dt} = \left[k_0 * \left(1 - \frac{N}{N_{max}} \right) - (1 - e^{-at}) * \left(\frac{I_{max} * C}{IC_{50} + C} \right) \right] * N$$

Figure 4-2. Schematic representation of PK/PD model to describe the rate of change in the number of bacteria in the *in vitro* PK/PD model system.

Notes: C: Concentration of drug in central compartment
 F: Bioavailability
 k_e : Elimination rate constant
 k_0 : Growth rate constant
 N_s : Total number of susceptible bacteria in the system
 α : Delay rate constant

The parameters in the equation are as follows: N is the number of viable bacteria in the system, k_0 is the bacterial growth rate constant, N_{max} is the maximum number of bacteria grown in the system in the absence of antibiotic, α is the delay rate constant for growth, I_{max} is the maximum kill rate of the bacteria, C is the concentration of the drug in the system at a given time point, IC_{50} is the inhibitory concentration at half maximal kill rate. The logistic growth function added to the equation implies that the entry into the stationary phase is attributed to self-limiting growth of bacteria. The delay rate constant is incorporated to take into account the time taken by the drug to reach the site of action in bacteria.

The time kill curves were analyzed using non-linear mixed effects modeling (NONMEM v.7.2, ICON plc). The pharmacokinetic parameters were fixed in the described PK/PD model to the following values: $V=55\text{mL}$, volume of media in the system; half-life $t_{1/2} = 0.58\text{h}$. The first order conditional estimate method within NONMEM was used to analyze the experimental time-kill curves. The between experimental variability was modeled as proportional error. The model was evaluated using various goodness of fit plots.

The appropriate PK/PD index associated with the bacterial kill by Lee 1599 was determined by calculating the individual indices and subsequently fitting an inhibitory E_{max} model to the colony count at a specific time point versus PKPD index data using nonlinear regression:

$$E = E_0 - \left(\frac{E_{max} * PKPD_{index}}{EC_{50} + PKPD_{index}} \right)$$

where, E is the observed *M. bovis* BCG counts in Log CFU/mL, E_0 is the bacterial count in the control experiment, E_{max} is the maximum anti-bacterial effect in Log CFU/mL and EC_{50} is the value of PK/PD index that produces half-maximal antibacterial effect. $PKPD_{index}$ is one of three empirical PK/PD indices frequently used for antibiotics pharmacotherapy such as AUC_{0-24}/MIC , C_{max}/MIC or $T>MIC$. The most appropriate PK/PD index that best described the bacterial killing was selected based on visual inspection and goodness of fit criteria.

Numerical simulations

Numerical simulations were performed using the developed PK/PD model and the parameter estimates derived from the time kill experiments to predict the effect of different untested doses and dosing regimens in a murine *in vivo* infection model of mycobacteria. The PK parameters of Lee 1599 in mouse were obtained from a mouse bridging studies as published elsewhere[81]. Dosing regimens ranging from 1mg/kg to 200mg/kg with QD, BID and TID dosing were simulated using the *in vivo* PK parameters and PK/PD estimates obtained from experimental time kill curves. 1000 datasets were simulated for each dose/dosing regimen using NONMEM 7.2

Results

Time kill curves for Lee 1599

The bacillary load in tuberculosis patients varies from 6-9 log CFU/mL in cavity walls [107]. Thus, the experiment was initiated with a bacterial burden of approximately 6 log CFU/mL and 7 log CFU/mL. The time kill experiments were carried out for Lee 1599 against *M. bovis* BCG with various dose and dosing regimens as shown in **Figure 4-3** (N_0 : 6-7 log CFU/mL) and **Figure 4-4** (N_0 : 7-8 log CFU/mL). The samples were collected every 12h up to 96h (Day 4) for all the studies. All the studies were performed in duplicate ($N=2$). LC-MS analysis has demonstrated that Lee 1599 is stable in 7H9 media for up to 144h. The untreated control group showed a 2-log increase in bacterial growth through the length of the study. The lowest treatment dose of 1mg/kg QD dosing did not show any change in bacterial counts, indicating similar doubling time and death rate. At 5mg/kg QD and 10mg/kg QD, Lee 1599 showed a bactericidal effect and a decrease in bacterial count was observed. However, Lee 1599 did not show a significant difference ($p>0.05$) in bacterial counts at a very high dose of 100mg/kg QD compared to 50mg/kg QD ($p=0.0729$ at 48h and $p=0.278$ at 72h). The antibiotic effect improved substantially with BID and TID dosing as compared to QD dosing using the same daily dose. An increase in bacterial killing was observed with 5mg/kg BID (10mg/kg/day) and 3.33mg/kg TID (10mg/kg/day) as compared to 10mg/kg QD dosing. Similarly, 50mg/kg BID (100mg/kg/day) dosing was more effective than 100mg/kg QD.

The time kill curves for *M. bovis* BCG were described using a semi-mechanistic PK/PD model. The effect of Lee 1599 on bacteria was characterized by incorporating an inhibitory sigmoidal Emax model with delay rate constants that reduced the growth rate in a concentration-dependent fashion. The growth rate constant was estimated to be 0.0368h^{-1} that equals to a doubling time of 18.83h. The doubling time is in accordance with the values reported in the literature [108, 109] indicating that the growth rate was not affected by the *in vitro* PK/PD model system. A maximum bacterial kill rate ($I_{\max}$) was calculated as 0.0355h^{-1} , suggesting that it takes nearly 19.5h for the bacterial counts to be halved. The concentration of the drug required to produce half-maximum inhibition (IC_{50}) was calculated as 0.0437mg/mL. A delay rate constant was introduced in the equation to explain the delay in the initiation of the actual killing effect. The delay rate constant was estimated to be around 0.0209h^{-1} . The results of this analysis are shown in **Table 4-1** and the model diagnostics and predictions for various dosage regimens are shown in **Figures 4-5** and **4-6**.

The PK/PD index for Lee 1599 was determined using a sigmoidal inhibitory Emax model. The bacterial kill on Day 2, 3 and 4 (only Day 4 data shown) suggested that Lee 1599 follows both AUC/MIC and $C_{\max}$ /MIC as PK/PD index (**Figure 4-7**). Besides visual inspections, various model diagnostics such as AIC, BIC and -2LL are used to estimate the relative quality of statistical models and as a means of model selection. These goodness-of-criteria also demonstrated that AUC/MIC and $C_{\max}$ /MIC are appropriate PK/PD indices as compared to $T>\text{MIC}$ (**Table 4-2**). AUC/MIC index

Figure 4-3. Timekill effect of Lee 1599 against *M. bovis* BCG (N_0 : 6-7 log CFU/mL) with observed log CFU/mL plotted against time for different dosing regimens.

Figure 4-4. Timekill effect of Lee 1599 against *M. bovis* BCG (No: 7-8 log CFU/mL) with observed log CFU/mL plotted against time for different dosing regimens.

Table 4-1. *In vitro* PK/PD parameter estimates for Lee 1599.

Parameter	Estimate (%RSE)	Between experimental variability (%RSE)	Residual error (%RSE)
α (h ⁻¹)	0.0209 (49%)	0.754 (38%)	0.00591 (32%)
K_o (h ⁻¹)	0.0368 (40%)	0.124 (106%)	
N (CFU/mL)	6.82 (2%)	0.00304 (85%)	
N_{max} (CFU/mL)	8.27 (4%)	0.00483 (57%)	
I_{max} (h ⁻¹)	0.0355 (27%)		
IC ₅₀ (mg/mL)	0.0437 (61%)		

Notes: RSE: Residual Standard Error

α : Delay rate constant

K_o : Growth rate constant

N: Initial number of bacteria

N_{max} : Maximum number of bacteria

I_{max} : Maximum bacterial kill rate

IC₅₀: Concentration required to produce half-maximal effect

Figure 4-5. Panels showing goodness of fit plots for the Lee 1599 PK/PD model fit (A.) DV vs PRED (B.) DV vs IPRED (C.) PRED vs CWRES (D.) TIME vs CWRES.

Notes: (A.) Observed effect (DV) vs population prediction (PRED)

(B.) Observed effect (DV) vs individual prediction (IPRED)

(C.) Population prediction (PRED) vs conditional weighted residuals (CWRES)

(D.) Time vs conditional weighted residuals (CWRES)

CWRES is a model diagnostic for evaluating model misspecification. It is calculated based on first order conditional estimate (FOCE).

Figure 4-6. Plots showing measured (DV is observed data), individual predictions IPRED (black lines) and population predictions (red lines) for *M. bovis* BCG CFU/mL over time for the Lee 1599 PK/PD model.

Figure 4-7. Relationship between PK/PD indices and microbial kill on Day 4 after treatment.

Notes: Panels A, B and C shows AUC/MIC, Cmax/MIC and T>MIC, respectively. The red dots are observed data points and blue lines are model predicted lines.

Table 4-2. Empirical PK/PD indices based on day 4 data.

Parameter	AUC/MIC (%CV)	C _{max} /MIC (%CV)	T>MIC (%CV)
EC ₅₀	14.7 (71.03%)	3.63 (79.06%)	1.42 (211.8%)
E _{control}	10.2 (20.7%)	11.2 (28.4%)	12.2 (65.2%)
E _{max}	8.09 (24.09%)	8.97 (32.5%)	9.70 (68.6%)
AIC	48.3	47.9	56.4
BIC	50.4	50.09	58.5

Notes: EC₅₀: Effective concentration to achieve 50% of maximal effect

E_{control}: Initial *M. bovis* BCG counts in absence of drug (at time 0, pre-treatment)

E_{max}: Maximum effect achieved on Day 3

AIC: Akaike information criterion

BIC: Bayesian information criterion

%CV: % Coefficient of Variation

suggests that Lee 1599 follow both concentration and time dependent killing as opposed to time dependent killing pattern observed commonly with aminoglycosides. The potential explanation for the difference in PK/PD index of Lee 1599 and other aminoglycosides can be related to the lengthy post-antibiotic effect (PAE) of 133h for Lee 1599 at 10X MIC. This can be achieved clinically by administering higher doses more frequently so that higher concentration of drug stays above MIC for longer periods in spite of clinically relevant shorter half-life for Lee 1599.

Numerical simulations

The pharmacokinetic parameters from a mice bridging study and pharmacodynamic parameters from the *in vitro* time kill experiments were combined in a PK/PD model as shown in **Figure 4-8**. Numerical simulations for time-kill curves were performed utilizing parameters from this PK/PD model to predict the effect ascertained by Lee 1599 at various untested dosing regimens in mice. The simulated profiles for QD, BID and TID dosing regimens are shown in **Figure 4-9, 4-10** and **4-11** respectively. The simulations also suggest that there is a dose dependent killing indicating an evident dose response relationship. The efficacy was higher with increasing doses for all the three regimes, however, for TID dosing regimen, there was a ceiling effect observed at very high doses of 50mg, 100mg and 200mg TID. Although the total daily doses were the same, BID and TID dosing showed better efficacy as compared to QD dosing regimens.

Discussion

The urge to combat tuberculosis is constantly on the rise and numerous research groups have discovered new drugs and tested globally. The preliminary screening of these drugs during the early discovery program are often based on target inhibition studies and MIC testing. The monitoring of the dynamic interplay between the drug and bacteria becomes more relevant as the concentration of drug changes over time *in vivo* as opposed to the static time kill studies of MIC testing where the concentration of drug remains constant throughout the course of the study. Several research groups have performed static time kill studies using bacteria that have very fast doubling time [110]. Since, *M. tb* have a lengthy doubling time, it might be even more misleading to perform a time kill study under static conditions. Hence, the need for dynamic *in vitro* PK/PD models are evident. The utility of dynamic models such as the applied *in vitro* PK/PD model is primarily to identify the nature and time course of the anti-bacterial effect obtained from the antibiotic in question and to apply these findings to extrapolate the PD effect to the *in vivo* situation.

The time kill curves for Lee 1599 showed dose-dependent killing with the tested dosing regimens. The estimates obtained from semi-mechanistic PK/PD model were used for numerical simulations of the kill dynamics of varying dosing regimen in a mouse model of tuberculosis infection under the assumption of an absence of the animal's immune response. For this purpose, PK parameter estimates were used from a mouse

Figure 4-8. Schematic representation of PK/PD model used for simulations.

Notes: * indicates that the bioavailability of 1 was assumed for modeling and simulation purposes. SC is subcutaneous route of administration, C is the central compartment and P is the peripheral compartment. The pharmacokinetic parameter estimates were obtained from PK studies performed in mice.

Figure 4-9. Time kill curves calculated from 1000 numerical simulations for once daily dosing regimen of Lee 1599 in mice.

Figure 4-10. Time kill curves calculated from 1000 numerical simulations for twice daily dosing regimen of Lee 1599 in mice.

Figure 4-11. Time kill curves calculated from 1000 numerical simulations for thrice daily dosing regimen of Lee 1599 in mice.

bridging studies previously performed by our group. The simulations using mouse PK parameters suggested that Lee 1599 elicits exposure (concentration and time) dependent killing as opposed to only dose dependent killing. For QD dosing regimens, the simulations indicate regrowth in the bacterial counts once the drug is cleared from the system. This can be possible due to short half-life of the Lee 1599 at clinically relevant concentrations. The regrowth could not be captured *in vitro* model system because of the limited number of sampling time points. The compound still causes a significant bacterial reduction with QD regimen at higher doses. With BID and TID regimen, a similar effect in efficacy is observed for higher doses 50mg, 100mg and 200mg. At such high doses, there is a dramatic reduction in bacterial counts reaching sterilization. However, prolonged administration at such high doses is not necessary as similar efficacy can be achieved at a dose lower than 50mg BID.

We also performed simulation exercises with the dosing regimens used for the *in vivo* efficacy trials by our collaborator Dr. Lenaert's and her group at Colorado (5 days a week with 2 consecutive drug holidays) in order to visualize the effect of dosing holidays with regards to efficacy of Lee 1599 in mice. The simulations are shown for QD and BID dosing in **Figure 4-12** and **4-13**. These simulations are stochastic simulations taking into account the drug holidays and prediction intervals. The simulated effect was the bacterial log reduction in the central compartment. There is a regrowth of bacteria observed during the holiday period. With QD dosing, only 200mg/kg showed a significant effect as compared to other dose groups. There was no efficacy observed at doses up to 40mg/kg. In BID dose groups, there was a substantial decrease in bacterial counts at doses above 20mg/kg BID (i.e. 40mg/kg/day). This suggests that higher doses with shorter dosing intervals are desirable for accomplishing an optimal antibacterial effect for Lee 1599. The simulations of the *in vivo* efficacy trials also helped us in understanding the relevant PK/PD index. It clearly suggests that, both, higher exposure of drug and shorter dosing intervals are required to achieve optimal effect. This finding correlates well with the identified relevant PK/PD index of AUC/MIC.

However, the simulations based on the *in vitro* PK/PD model may not be extrapolated precisely and quantitatively to predict the *in vivo* conditions due to several factors. The PK/PD model established from the *in vitro* PK/PD model provides information about direct interaction of drug with bacteria in the absence of the immune system. Also, 100% of drug is available at the site of action i.e. the bacteria in the central compartment of the *in vitro* PK/PD model without any barrier for drug distribution such as biomembranes. In contrast, in the *in vivo* situation, the bacteria reside in the lungs and localized within the alveolar cellular compartment (and spleen) as well as within macrophages and the drug has to permeate into the lung tissues to cause the desired effect. The results might be more accurate if we obtain further information such as biodistribution in lung tissues and subcellular compartment with respect to plasma.

In addition, the drug is administered subcutaneously in the *in vivo* efficacy trials in mice and the bioavailability of Lee 1599 is unknown for this route of administration in mouse. The model prediction assumes bioavailability of 100% reaching the lung tissues for numerical simulations, which may not be the case. Also, different bacterial strains

Figure 4-12. Time kill curves calculated from 1000 numerical simulations for once daily (5 days a week) dosing regimen of Lee 1599 in mice.

Notes: The shaded region (grey zone) is the 90% confidence interval and the dark lines are the mean bacterial counts in log CFU/mL.

Figure 4-13. Time kill curves calculated from 1000 numerical simulations for twice daily (5 days a week) dosing regimen of Lee 1599 in mice.

Notes: The shaded region (grey zone) is the 90% confidence interval and the dark lines are the mean bacterial counts in log CFU/mL.

were used for the *in vivo* studies (*M. tb.* Erdman) and the *in vitro* PK/PD model (*M. bovis* BCG) which may affect the model based predictions of *in vivo* efficacy trials. The doubling times and the logarithmic growth phase of bacteria can be effected under *in vitro* and *in vivo* conditions. Despite these shortcomings, it is beneficial to perform studies using an *in vitro* PK/PD model and execute pharmacometric analyses to gain knowledge about the dose fractionation studies and identifying PK/PD indices. These *in vitro* studies helped us in facilitating the decision making process during the transition to *in vivo* studies. It aided us in selecting an appropriate dosing regimen for *in vivo* trials in order to characterize an exposure-response relationship.

Conclusions

We successfully determined the time kill profiles for dosing regimens of Lee 1599 against *M. bovis* BCG. The semi-mechanistic PK/PD model was competent to describe the effect of Lee 1599 on the growth and survival of bacteria. The simulations suggested that higher doses are more effective than very low doses irrespective of the frequency of dosing. The optimal effect in *in vivo* mouse models can likely be achieved with twice-daily dosing regimens. These findings support the rational of utilizing a PK/PD based *in vitro* model system to characterize the dose response relationship of spectinamides and obtain an understanding of the best PK/PD index as driver of efficacy and the effect of dose fractionation studies to maximize efficacy for *in vivo* trials.

CHAPTER 5. APPLICATION OF PHARMACOMETRIC TOOLS FOR IDENTIFYING PHARMACODYNAMIC INTERACTIONS IN ANTIBIOTIC COMBINATIONS

Introduction

The mechanism of action for spectinomides is similar to that of spectinomycin-inhibiting protein synthesis by selectively binding to the head domain of the 30S ribosomal subunit and blocking its translocation [77]. Although there can be multiple redundancies or alternate pathways in response to such inhibition by a single antibiotic, most often this results in drug resistance or clinical relapse. For these reasons, combination therapies are needed for more durable and effective treatment [111]. Combination therapy of new and existing anti-TB agents are constantly being evaluated to determine the pharmacodynamic interaction between two agents. The selection of combination compounds will aid in treating patients with MDR-TB and also shorten the duration of treatment [112].

The primary objective of this research study was to find a combination partner agent for lead spectinamide molecule Lee 1599. We selected rifampicin (RIF) as a model compound for combination agent as rifampicin has been in use for TB therapy for several decades. The elementary justification for combination therapy is expecting an enhanced pharmacodynamic effect with two or more agents. The net effect of any pharmacodynamic interaction can be synergistic, antagonistic or additive. The screening for an efficacious partner agent for a new molecule is a critical step in the early discovery phase before performing any extensive *in vivo* trials.

To determine the interaction between the two agents, we applied the widely accepted checkerboard assay method as a primary screening tool. The checkerboard assay can be used for qualitative interpretation only as it does not provide any information on the degree of interaction between Lee 1599 and RIF. An alternative approach is to quantitatively assess the interaction by utilizing a parametric method known as three dimensional surface response assay. The basis for both these methodologies is set on different principles [113-115]. Thus, the expected outcome for an interaction between two drugs may or may not be similar from these methodologies. It is essential to identify an appropriate screening tool for selection of candidate compounds as it helps in accelerating the preclinical drug development process.

Herein, we illustrate the differences in outcomes of the checkerboard assay and the response surface assay, which is instrumental in screening the potential combination compounds that are urgently required to curb evolving resistant strains. The results of the surface response assay were validated using an *in vitro* PK/PD model for combination therapy by generating dynamic time kill curves at different dose levels and dosing regimens. Further, we compared these results of the pharmacodynamic interaction between Lee 1599 and RIF with *in vivo* studies in a mouse model of infection to further validate our outcomes.

Materials and Methods

Antimicrobial agents

The spectinamide Lee 1599 (3'-Dihydro-3'-deoxy-3'(R)- (5-chloropyridin-2-yl) acetylamino spectinomycin Trihydrobromide) was synthesized in the laboratory of Dr. Richard.E. Lee at St.Jude Children's Research hospital. Rifampicin (RIF) was purchased from Sigma (St. Louis, MO). A stock solution of each agent was prepared and stored at 4°C. Prior to each susceptibility testing, an aliquot of each agent was diluted to the desired concentration with 7H9 media.

Microorganisms

M. bovis BCG was grown in Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD). The cultures were stored at -80°C in Middlebrook 7H9 broth. For each experiment, cultures were thawed and incubated at 37°C in Middlebrook 7H9 broth until the bacteria reaches logarithmic phase (OD₆₀₀ 0.4-0.7). *M. tb* Erdman (TMC 107, ATCC 35801) was used as testing organism for animal studies.

Checkerboard assay

The pharmacodynamic interaction between Lee 1599 and RIF against *M. bovis* BCG was evaluated using the checkerboard assay. The assay was carried out in 96-well plate format with varying concentrations of Lee 1599 and RIF serially diluted in 7H9 media across rows and columns, respectively. The concentrations for Lee 1599 and RIF ranged from 0.003 to 6.4µg/mL and 0.00049 to 2µg/mL, respectively, with a final volume of 100µL in each well. Additionally, 100µL of *M. bovis* BCG culture with OD₆₀₀ of 0.01 was also added to each well. The plate was incubated for 7 days at 37°C and the endpoint assessment was based on visual growth inspection. The fractional inhibitory concentration index (FICI) calculated for each concentration in each combination is represented by the following equations:

$$FICI = FIC_{Lee\ 1599} + FIC_{Rif}$$

$$FIC_{Lee1599} = \frac{MIC_{Lee1599\ in\ combination}}{MIC_{Lee1599\ alone}}$$

$$FIC_{Rif} = \frac{MIC_{Rif\ in\ combination}}{MIC_{Rif\ alone}}$$

FICI represents the extent of interaction between two drugs in question and the interaction can be defined as synergistic (FICI < 0.5), antagonistic (FICI > 4.0) or additive (0.5 < FICI < 4.0).

Response surface assay

Static time-kill studies were performed in a 5x5 array with different concentration combinations for Lee 1599 and RIF. Five concentrations of each agent, 0 (Control), 0.05, 0.5, 1 and 2X MIC, were used. *M. bovis* BCG was grown to the logarithmic phase (OD₆₀₀ 0.4-0.7) in Middlebrook 7H9 medium. The logarithmically growing culture was then used to initiate the time kill studies (starting OD₆₀₀ 0.15). The final concentration of bacterial culture in each tube was approximately 10⁸ CFU/mL. The culture was treated with different antibiotic concentrations for 24h at 37°C on a shaker (200 rpm). Bacterial samples were collected from each tube after 24h for quantification. Prior to quantification, the samples were centrifuged at 37°C at 10,000 rpm for 10min and the supernatant was discarded. This step is required to ensure that there is no antibiotic carry over effect. The bacterial pellet was diluted with the same amount of fresh 7H9 media and serially diluted with 7H9 media. The dilutions were plated on 7H11 agar plates for 21 days at 37°C and the colonies were counted manually on each plate post-incubation. The anti-bacterial effect was determined as the decrease in number of viable bacteria in terms of CFU/mL.

The antibacterial effect after 24h of treatment with drugs was described using an inhibitory Emax model. The parameter estimates describing the killing effect were estimated by nonlinear regression analysis using Phoenix WinNonlin 6.3 (Certara, NJ). The total bacterial density for the treatment groups was then modeled with the obtained parameters using three dimensional surfaces. Null interaction (or additivity) was used to model the effect summation for combination treatment as follows:

$$Effect_{total} = Effect_{Lee1599} + Effect_{Rif}$$

$$Predicted \log CFU/mL = E_0 - \left(\frac{E_{max, Lee1599} * C_{Lee1599}}{EC_{50, Lee1599} + C_{Lee1599}} \right) - \left(\frac{E_{max, Rif} * C_{Rif}}{EC_{50, Rif} + C_{Rif}} \right)$$

where E_0 is the growth at 24h in the absence of antibiotics, $E_{max, Lee 1599} / E_{max, Rif}$ is the maximal effect of Lee 1599/ RIF, $EC_{50, Lee 1599} / EC_{50, RIF}$ is the effective concentration of Lee 1599/RIF to achieve 50% of maximal effect, $C_{Lee 1599} / C_{RIF}$ is the concentration of Lee 1599/RIF.

Volume under the plane (VUP) for both observed and predicted effect was calculated using MATLAB 8.2. The interaction index (II) is the ratio of VUP_{observed}/VUP_{predicted} and it defines the pharmacodynamic interaction between two agents as synergism (II<1) and antagonism (II>1). An II around 1 suggests no interaction, i.e. an additive effect.

***In vitro* PK/PD model**

The dynamic time kill curves for different doses of Lee 1599 and RIF were established using a modified version of our *in vitro* PK/PD model. As described previously, [39] the *in vitro* PK/PD model system consists of a two-armed, water jacketed flask (1965 series spinner flask, Bellco Glass, Vineland, NJ) as depicted in **Figure 4-1**. A filter unit attached to a hollow steel tube was introduced into the system. The filter unit contained a prefilter (5µm, Millipore, Billerica, MA) and filter membrane (0.22µm, Millipore, Billerica, MA) to prevent leakage of bacteria during the dilution process. The other end of the tube was connected to plastic tubing (PharMed tubing, Cole-Parmer, Vernon Hills, IL) from which the media was withdrawn continuously at a defined flow rate using a peristaltic pump (Masterflex L/S, Cole-Parmer, Vernon Hills, IL). The flask was placed on a magnetic stirrer for homogeneity of the culture and preventing membrane blockage. The temperature of the system was set at 37°C with the help of a thermostatic water bath attached to the water-jacketed flask. One arm of the flask was connected by plastic tubing to the fresh sterile media reservoir and the other arm was used for dosing and repeated sampling.

For simultaneously generating the concentration-time profiles for two antibiotics, additional components were added to the model utilized for a single drug treatment. The additional component is a supplementing compartment that contains the second administered drug. As shown in **Figure 5-1**, one of the sidearm of the flask is connected to the reservoir containing sterile medium and the supplementing compartment. The drug having the higher clearance was administered as bolus dose from the side arm. The flow rates (F) of the inlet and outlet in the central compartment and supplementing compartment were computed based on equations described by Jürg Blaser [116]. The individual values of each flow rate in and out of supplementing and central compartment are shown in **Figure 5-1**. A supplementing reservoir contains media with the drug with the slower clearance. The change in concentration per unit time will be same in both the compartments. Thus, the volume of supplementing compartment and flow rate is calculated as:

$$V_s = \left(\frac{CL_A - CL_B}{CL_B} \right) * V_c$$

$$F = \frac{V * 0.693}{t_{1/2}}$$

Where, V_s is volume of media in the supplementing compartment; CL_A and CL_B are the clearance of Drug A and B; V_c is volume of media in the supplementing compartment (55mL); F is the flow rate of media and $t_{1/2}$ of drug was obtained from *in vivo* studies in rats. The $t_{1/2}$ of Lee 1599 was experimentally determined and RIF was obtained from literature [117].

The combination tested in this experiment was Lee 1599 and rifampicin. Lee 1599 (Drug A) has a higher clearance than rifampicin (Drug B). The *in vitro* set up is

Figure 5-1. A representation of *in vitro* PK/PD model set up for dynamic time-kill studies.

Notes: Drug A is Lee 1599 and Drug B is RIF.

assembled in a biosafety cabinet in a sterile environment. The flask and the tubes are cleaned and sterilized by autoclaving before starting every experiment.

Time kill curves for Lee 1599

M. bovis BCG was used as a test organism for evaluating the effect of drug *in vitro* model. The organism was grown to early logarithmic phase (O.D.₆₀₀ 0.4-0.7) in Middlebrook 7H9 medium. This actively growing bacteria was used as an inoculum for the *in vitro* model making the final density of the culture as 10⁶ CFU/mL.

Samples of 200µL were collected every 12h from the central compartment flask with volume replacement containing fresh media. The samples were centrifuged at 10,000 rpm for 10min at 37°C. The formed pellets were collected and re-suspended in antibiotic-free media. This step was to ensure that there is no drug carry-over effect. The samples were serially diluted and the dilutions were plated on 7H11 agar plates in duplicate. To check if there are any resistant colonies forming bacteria, the samples were also plated on agar plates with 1X, 2X and 4X MIC of each drug. The agar plates were incubated for 21days at 37°C. The efficacy of Lee 1599 was characterized by measuring the reduction in number of viable bacteria, in terms of CFU/mL. There was a control arm included in the study to determine the bacterial growth in absence of drug.

***In vivo* efficacy studies**

The results of the checkerboard assay, the response surface assay and the *in vitro* PKPD model were compared to results of *in vivo* efficacy studies for Lee 1599 and RIF. The methodology for the animal studies and the endpoint analysis are described as Model 2 and 3 in a previous study [81]. The formulation for Lee 1599 was freshly prepared using 5 parts of USP Multiple Electrolytes Solution (PlasmaLyte, Baxter Healthcare Corp., Deerfield, IL) and 1 part sterile deionized water. RIF was ground to a fine powder using mortar and pestle, and sterile deionized water was added incrementally to the total volume. The formulation was stored at 4°C until required. Lee 1599 was administered subcutaneously at 200mg/kg QD and RIF was administered orally at 10mg/kg QD. For the combination studies, both drugs were administered simultaneously at the same doses and route of administration as given in monotherapy.

Results

Checkerboard assay

The drug aliquots were stable at the beginning of experiments and this was confirmed using LC-MS analysis. In the checkerboard assay, the positive control wells that did not contain any drug showed bacterial growth at the end of the incubation period

as expected. The MIC of Lee 1599 was found to be 0.8µg/mL, which is consistent with the MIC determination that had been performed during the initial screening of the spectinamide series. The MIC of Lee 1599 was found to be similar for *M. bovis* BCG and *M. tb*. The FICI score for Lee 1599 was 0.375 as shown in **Table 5-1**. The MIC of both, RIF and Lee 1599 was significantly reduced in the presence of each other. As the FICI score for this tested combinations is below 0.5 it suggests that RIF has a synergistic interaction with Lee 1599.

Response surface assay

Lee 1599 and RIF exhibited similar concentration-killing profiles in pilot studies as shown in **Figure 5-2**. The optimal concentration was found to be approximately 6.4µg/mL for 1599 and 1.6µg/mL for RIF. There was no ceiling effect observed for both of the drugs. A reasonable model fit was obtained for Lee 1599 ($r^2=0.992$) and RIF ($r^2=0.996$) for the applied inhibitory sigmoid Emax-model based on model diagnostics. The concentrations for capturing the most precise parameter estimates describing the killing effect for Lee 1599 were found at 0.04, 0.4, 0.8, 1.6, 3.2 and 6.4µg/mL, whereas the concentrations to obtain parameter estimates for RIF were 0.02, 0.2, 0.4, 0.8 and 1.6µg/mL.

To analyze the pharmacodynamics interaction between Lee 1599 and RIF, we plotted the combined effect of both the agents using a three-dimensional response surface approach (**Figure 5-3**). A three dimensional plot of bacterial count vs. concentration was plotted with Lee 1599 concentration on the x-axis, RIF concentration on the y-axis and bacterial count in Log CFU on the z-axis. The “null interaction” surface was constructed in a separate plot using a set of points representing model predicted additive effect of combinations of Lee 1599 and RIF and is shown in **Figure 5-4**. In all different sets of concentrations of the compounds in combination, an increased killing effect was seen as compared to either drug alone at the same concentration. The killing effect by one agent was supplemented by the presence of the other agent. The volumes under the plane (VUP) for the observed effect and model predicted effect as shown in **Table 5-2** are 274.84 and 271.86 log CFU µg²/mL³. The interaction index calculated based on the obtained VUPs was found to be 1.01 indicating an additive effect exerted by the combination of Lee 1599 and RIF.

In vitro PK/PD model for combination agents

For the *in vitro* PK/PD model, three different dosing regimens for Lee 1599 and two for RIF were selected to be investigated in combination experiments: 100mg/kg QD, 10mg/kg QD and 1mg/kg QD for Lee 1599 and 10mg/kg QD and 0.5mg/kg QD for RIF. As shown in **Figure 5-5**, there was a marginal increase in the average bacterial killing when both the agents were used in combination as compared to when administered individually. The bacterial counts were below limit of quantification for the timepoints without any data points beyond 48h or 72h in the time-kill curves. The bacterial counts at

Table 5-1. Checkerboard assay results to determine pharmacodynamic interaction between Lee 1599 and RIF.

Agent	MIC of agent alone (µg/mL)	MIC in presence of Lee 1599 (µg/mL)	MIC of Lee 1599 in presence of agent (µg/mL)	FICI score	Indication
Rifampicin	0.002	0.0005	0.1	0.375	Synergy

Note: The MIC of Lee 1599 alone is 0.8µg/mL.

$$\text{Log}_{10}\text{CFU/mL} = 9.311 - \left(\frac{3.53 * C_{\text{Lee1599}}}{1.26 + C_{\text{Lee1599}}} \right)$$

$$\text{Log}_{10}\text{CFU/mL} = 8.11 - \left(\frac{6.15 * C_{\text{RIF}}}{0.377 + C_{\text{RIF}}} \right)$$

Figure 5-2. Model fits of bacterial burden at 24h in static time kill studies for Lee 1599 (A) and RIF (B) to obtain the parameter estimates such as E_0 , E_{max} and EC_{50} .

Figure 5-3. Observed effect of combination agent Lee 1599 and RIF.

Figure 5-4. Model predicted effect of the combination compound Lee 1599 and RIF.

Table 5-2. Results of three dimensional surface response assay.

Model	VUP value	Ratio (VUPobs/VUPpred)	Indication
Observed effect	274.84	1.01	Additive effect
Model predicted effect	271.86

Figure 5-5. *In vitro* PK/PD dynamic time kill study for Lee 1599, RIF and its combination with different doses.

48h for all the treatment groups were arbitrarily selected for quantitative comparison as shown in **Figure 5-6**. At higher doses of 100mg/kg Lee 1599 and 10mg/kg RIF, there is an average of 4 log CFU/mL reduction in bacterial counts when the agents are administered in combination as compared to 2.44 log CFU/mL by Lee 1599 alone or 3 log CFU/mL by RIF alone. Similarly, an approximate 4 log CFU/mL bacterial killing was observed when 10mg/kg Lee 1599 was co-administered with 10mg/kg RIF as compared to 1.73 log CFU/mL killing with 10mg/kg Lee 1599 alone. The efficacy observed with both dose group including the higher RIF dose (100mg/kg Lee 1599 + 10mg/kg RIF and 10mg/kg Lee 1599 + 10mg/kg RIF) were almost identical. This can be possibly due to a ceiling effect in the killing pattern by Lee 1599 when given with high doses of RIF.

At much lower doses, an average of 2.5 log CFU/mL bacterial reduction was observed with 1mg/kg 1599 + 0.5mg/kg RIF. A dose of 1mg/kg Lee 1599 did not show any evidence for sterilizing activity. However, the same dose when co-administered with 0.5mg/kg RIF could improve bacterial killing in contrast to individual Lee 1599 dose at 48h and 72h ($p < 0.05$). All three sets of combination doses showed an increased bacterial killing when both the agents were administered in combination. This is an indication of an additive effect of both the anti-bacterial agents in combination.

***In vivo* studies**

The results of our pharmacodynamic interaction assays of the Lee 1599-RIF combination were subsequently compared to *in vivo* efficacy results after 4 weeks of treatment in an acute *M. tb* infection model in mice performed by our collaborators, Dr. Anne Lenaerts and her group at Colorado State University. The studies were carried out in two different groups: A high dose aerosol (HDA) and low dose aerosol (LDA) infection with 200mg/kg QD Lee 1599 (subcutaneously) and 10mg/kg QD RIF (oral) administered as shown in **Figure 5-7**. The HDA group was inoculated with 3.5 log CFU per mouse and LDA group was inoculated with 2 log CFU per mouse. For the HDA group, the observed bacterial reduction was 2.14, 1.08 and 2.97 log CFU with Lee 1599, RIF and combination of both drugs, respectively. The expected combined effect of Lee 1599 and RIF assuming there is no interaction between the two drugs can be calculated as 3.22 log CFU. Similarly, for the LDA group, the observed effect with combination was 2.79 log CFU and theoretical summation was 2.84 log CFU. The close proximity of the observed and the expected value for efficacy of the combination regimens suggests that Lee 1599 and RIF have an additive effect when given in combination.

Discussion

Spectinamide Lee 1599 has excellent anti-TB activity both *in vitro* and *in vivo* and has demonstrated potency against MDR- and XDR-TB strains obtained from several clinical isolates. It is a well-known fact that TB therapeutic regimen require a combination of multiple anti-TB drugs. It is evident that eventually every drug against

Figure 5-6. The bacterial log reduction with different doses using an *in vitro* PK/PD dynamic time kill experiment.

Figure 5-7. *In vivo* efficacy trial data showing bacterial burden reduction (log CFU/mL) in the lungs of *M. tb* infected mice, N=6 for Lee 1599 and Lee 1599+RIF, N=5 for RIF.

Notes: Lee 1599 was administered subcutaneously at 200mg/kg and RIF was dosed orally at 10mg/kg. The results are significant for all dose groups at $p < 0.05$ by pairwise multiple comparison procedures (Tukey test).

TB will be co-administered with other anti-TB drugs [21]. In our ongoing research studies, we hypothesized an increase in pharmacodynamic effect when spectinamide Lee 1599 is co-administered with another established anti-TB drug and that this increase in *in vivo* effect can be predicted by adequate *in vitro* assays. The model drug selected for the purpose of our study was RIF, a first-line antibiotic used for TB therapy. The change in pharmacodynamic effect upon co-administration of two drugs is frequently determined using techniques such as the checkerboard assay, the E-test and time-kill curves both *in vitro* and *in vivo* [118, 119]. We applied two of these methods for primary screening: the checkerboard assay and the three dimensional surface response assay. As shown by our results, there is a clear disconnect between the outcomes of these two methods as shown in **Table 5-3**. The checkerboard assay suggested a synergistic interaction between Lee 1599 and RIF. However, the three dimensional surface response assays indicated an additive effect between the two compounds.

The primary reason for the difference between these two methods is likely rooted in the underlying principles of both methods. The basis of null interaction for the FICI score derived from the checkerboard assay is the Loewe additivity method. However, there are several pitfalls associated with the checkerboard assay: (1) The initial bacterial load for testing interactions is much lower (OD_{600} 0.01 or 10^5 CFU/mL) than what is observed in *in vivo* studies or in patients [120, 121]. (2) The endpoint is often based on visual inspection and very subjective, increasing the possibility of erroneous results. Since, it follows a 2-fold dilution technique; there is a high possibility of one-well errors. Though this error may be undervalued assuming a miniscule marginal error, the error is magnified and the extent of allowance increases at higher dilutions [122]. (3) Moreover, there are various methods to calculate the FICI index using the checkerboard assay and there has been variability reported with different methods of interpretation of checkerboard synergy testing [123].

To overcome these limitations, we advanced with a quantitative, parametric three dimensional surface response approach proposed by Tam *et al.* The surface response approach has several advantages over the checkerboard assay: (1) The endpoint is the quantification of bacterial counts on agar plates as opposed to visual inspection. (2) The initial number of bacteria pre-dose, approximately 10^7 - 10^8 CFU/mL, is much higher relative to the checkerboard assay and comparable to *in vivo* mouse infection models. (3) The bacterial inoculum is in higher volumes of media providing a more conducive environment with fresh nutrients for bacterial growth. (4) The difference in bacterial counts in dosing and control groups after 24h of incubation normalizes the bacterial reduction thus providing us with a measure for extent of bacterial killing. Based on these facts, the indication of combination effects from the response surface assay seems to be more reliable compared to the checkerboard assay based on the above mentioned rationales.

It is obligatory to validate the results obtained from two different screening assays, particularly if the two applied methodologies provide discordant results as in our study. We therefore performed an *in vitro* PK/PD model based dynamic time-kill study as opposed to static time kill studies to validate the preliminary findings and confirm the

Table 5-3. Summary of results of pharmacodynamics interactions from different methods.

Method	Indication
Checkerboard assay	Synergy
3D surface response assay	Additive effect
Dynamic time kill curves	Additive effect
<i>In vivo</i> mouse studies	Additive effect

authenticity of the above mentioned assays. The *in vitro* PK/PD model based time-kill curves were performed under dynamic conditions and take into account the fluctuating concentration of drug over time mimicking the pharmacokinetic conditions observed *in vivo*. Although the hollow fiber infection model (HFIM) is now used by an increasing number of research groups for this purpose, our previously used *in vitro* PK/PD model has several advantages over the use of HFIM if used for the compound screening. Some of the major limitations of the HFIM is non-specific drug binding (especially highly lipophilic compounds) to the hollow fiber capillaries producing misleading results, relatively high cost and complex operation. Our *in vitro* model uses a substantially easier setup, and can be sterilized and reused between two cycles of different studies. This makes the *in vitro* model very cost-effective and ideal for use in exploratory trials to test multiple dose and dosing regimens [39].

The results from the time-kill curves experiments indicate a moderate increase in bacterial reduction with combination treatment as compared to monotherapy. The net bacterial reduction with combination is approximately the algebraic sum of bacterial reduction caused by the two agents during monotherapy. Thus, the time-kill results indicated an additive effect between Lee 1599 and RIF. To compare the efficacy among the treatment regimens, we selected the 48h timepoint as the bacterial counts were below the quantification limit at later timepoints in the Lee 1599 100mg/kg QD and RIF 10mg/kg QD dose groups. The bacterial killing increases with increasing doses of Lee 1599 or RIF alone, which is expected based on their previously observed non-linear dose response relationships. The combined effect at low doses of Lee 1599 1mg/kg and RIF 0.5mg/kg was marginally higher than RIF 0.5mg/kg. However, when higher doses for both Lee 1599 and RIF are co-administered there is a substantial increase in the reduction of bacterial counts. Theoretically, the bacterial reduction for the Lee 1599 100mg/kg + RIF 10mg/kg group should be higher than Lee 1599 10mg/kg + RIF 10mg/kg group. The observed effect for both groups, Lee 1599 100mg/kg+RIF 10mg/kg and Lee 1599 10mg/kg + RIF 10mg/kg group, however, showed similar efficacy, that was significantly higher than for the groups treated with Lee 1599 alone ($p < 0.05$). This clearly suggests that doses of 10mg/kg and 100mg/kg of Lee 1599 when given in combination with 10mg/kg RIF have the same efficacy possibly due to a ceiling effect.

There is always experimental variability associated with individual data points. The method of using a specific time point to compare the efficacy of different dose group as we applied can be erroneous, as it does not capture the cumulative effect of the treatment. To overcome this limitation, we calculated the area-under the curve (AUC_{0-t}) for the individual time-kill curves to establish a cumulative measure of the overall effect. As the time-kill experiments were terminated at different time points, we determined AUC_{0-48} for all time-kill curves and normalized them with the control group. The percentages of AUC_{0-48} ratios were calculated to compare different groups as shown in **Table 5-4**. The higher the percentages, the higher the killing. The %ratios were larger for combination therapy as compared to monotherapy. The %ratios for Lee 1599 100mg/kg + RIF 10mg/kg was larger than Lee 1599 10mg/kg + RIF 10mg/kg indicating more bacterial kill for the former group. Also, at lower doses (**Figure 5-8**), the %ratio for Lee 1599 1mg/kg + RIF 0.5mg/kg group was larger than the individual Lee 1599 and RIF

Table 5-4. Comparison of %AUC ratios (the %AUC were normalized with AUC of control group).

Group	%AUC ratio
Lee 1599 100mg/kg + RIF 10mg/kg	94.1
Lee 1599 10mg/kg + RIF 10mg/kg	91.7
Lee 1599 100mg/kg	93.9
Lee 1599 10mg/kg	86.1
RIF 10mg/kg	84.5
Lee 1599 1mg/kg + RIF 0.5mg/kg	85.9
RIF 0.5mg/kg	74.9
Lee 1599 1mg/kg	68.8
Control	0.0

Figure 5-8. Comparison of %AUC ratios after the %AUC were normalized with AUC of control group indicating higher the %AUC ratio, greater the bacterial killing.

treatment arms. The enhanced killing in the combination group suggests an additive effect of both the agents. Thus, the AUC_{0-t} ratios may be a better estimate for comparison of kill curves than a point estimate as it captures the dynamic range of the bacterial time-kill effect.

The above *in vitro* results are corroborated by *in vivo* findings suggesting a additive effect between Lee 1599 and RIF. The *in vivo* studies were performed using different bacterial loads (HDA and LDA bacterial burden) for treatment. There is no “gold standard” definition of synergy observed under *in vitro* or *in vivo* conditions. Of the many definitions reported in the literature, synergy is commonly defined as ≥ 2 log CFU/mL reduction by combination as compared to single therapy [124, 125]. Based on this assessment, we interpreted the *in vivo* results to be an additive effect. However, these definitions of synergy are not necessarily translatable to clinical scenarios where the endpoint of treatment is an improvement in the patient’s condition. It is challenging to identify an additive effect or synergistic effect under clinical conditions as one can usually not quantify bacterial counts in patients. The primary application of these drug interaction characteristics determined under preclinical conditions is to adjust the dose and dosing regimen for the combination therapy and balancing the efficacy profile of the drugs without imparting any toxicity to the patient. In view of these considerations, drawing a line between additive effect and synergy at the preclinical stage may not give any edge for dose optimization in the clinic. Hence, the purpose of the drug interaction assays should focus on whether the combination is more efficacious or less toxic as compared to single dose treatment.

Conclusions

In summary, it is crucial to carry out *in vitro* experiments on pharmacodynamic interactions for antibiotics early during the preclinical drug development phase to test potential interactions between two or more molecules. The results from the checkerboard assay or any other qualitative analysis assay should be used with caution. It is advised to confirm the results with quantitative methods such as three dimensional surface response assay, dynamic time kill curve experiments or *in vivo* studies to get precise information about the drug interaction in question. Our studies show that the preclinical drug development process can be hindered or misguided if decisions are based on only one qualitative assay. In addition, the purpose of the drug interaction assays should focus on whether the combination is more efficacious or less toxic as compared to single dose treatment rather than trying to decipher between additivity and synergism.

CHAPTER 6. SUMMARY

The number of resistant strains of *M. tb* that have increased at an alarming rate has convinced researchers to focus on the discovery of new anti-tuberculosis drugs to combat this pandemic. To contribute to solutions for this problem, our research group discovered a novel series of anti-tuberculosis agents called spectinamides, semi-synthetic analogs of spectinomycin. Spectinamides are potent inhibitors of mycobacterial ribosomes and overcomes efflux mediated drug resistance in *M. tb*. Multiple of the compounds in the spectinamide series have shown an excellent *in vitro* activity and acceptable drug-like properties, thus making them excellent preclinical drug candidates. We hypothesized that a pharmacometrics-directed quantitative approach for integrated PK/PD characterization can guide the selection of lead candidate compounds and is more predictive of *in vivo* efficacy than some qualitative or semi-quantitative standard approaches.

The screening for potential lead drug candidates included multiple experiments to determine the pharmacokinetic parameters. *In vitro* screening demonstrated that spectinamides have low plasma protein binding and are stable against hepatic microsomal activity. The pharmacokinetics studies in rats revealed that renal excretion is the major route of elimination for most spectinamides. This route of elimination suggests that there is a low potential for drug-drug interactions during combination pharmacotherapy as seen with agents that undergo extensive hepatic metabolism. Because spectinamides are highly hydrophilic, distribute well in aqueous distribution spaces, and show low plasma protein binding, they are advantageous as therapeutic candidates over other new anti-TB agents where most tend to be lipophilic and have high plasma protein binding. This results in spectinamides being able to target bacterial subpopulations that are unreachable by more lipophilic compounds.

An important aspect in the safety of novel drug candidates is their biodistribution and potential accumulation in tissues. On the other hand, intracellular uptake into macrophages is a prerequisite for antibiotic activity against *M. tb* residing inside infected macrophages. Our quantitative macrophage uptake studies showed that Lee 1329, Lee1445 and Lee 1599 had significantly higher macrophage uptake than spectinomycin and streptomycin.

The biodistribution of spectinamides was determined using a tritium labelled Lee 1329. About 70% of administered radioactivity was recovered in urine and 12.6% in feces, with a total mass balance of 84.7%. Total radioactivity showed similar activity-time profiles for the first 2h after I.V. administration. Deviation at later time points suggests formation of circulating metabolites with longer half-life than the parent drug. C_{max} after the 1st dose and the 8th dose is similar: 3.39 μ Ci/mL and 3.55 μ Ci/mL, suggesting no relevant accumulation of parent drug and metabolites. The activity of radiolabel was 3 times higher in lungs and spleen as compared to whole blood, suggesting good tissue penetration of Lee 1329. The results support the earlier findings that the major elimination pathway for Lee 1329 is renal excretion and that the compound

accumulates neither in blood nor in tissues during multiple dosing with a BID regimen in rats, which is an important aspect in the safety profile of spectinamides.

The minimum inhibitory concentration (MIC) is one of the key pharmacodynamic parameters used to compare and differentiate drug candidates. However, point estimates of pharmacodynamic activity such as MIC do not take into account the dynamic interaction between bacteria and fluctuating exposure to unbound drug during dosing *in vivo*. We thus applied an *in vitro* PK/PD model system to establish the quantitative interaction between bacterial growth and time-varying drug concentration for the emerging lead Lee 1599. The obtained results were used to identify the optimal PK/PD index driving Lee 1599's efficacy, and to perform model-based numerical simulations to determine appropriate dosing regimens for further *in vivo* efficacy trials. The results indicate that Lee 1599 exhibits a dose-dependent bactericidal effect. Lee 1599 showed up to 4-log reductions in bacterial counts at 100mg QD dosing. The PK/PD indices demonstrate that Lee 1599 elicits a concentration- and time-dependent killing with AUC/MIC as the optimal index. The *in vitro* PK/PD simulated profile suggests that higher doses with shorter dosing intervals may demonstrate optimal efficacy *in vivo*.

With an increasing frequency of mycobacterial resistant strains, there is an emerging need for robust and effective combination therapy. The conventional screening method for determining pharmacodynamic drug interactions using the Checkerboard assay depends on a subjective endpoint of growth inhibition and fails to provide any information on extent of bacterial growth or kill over time. We applied parametric approach to quantitatively assess the pharmacodynamics drug interaction between the lead candidate compound Lee 1599 and rifampicin. The three dimensional surface response assay demonstrated that there is an additive effect between both agents as opposed to the checkerboard assay, which showed synergism between these agents. The results of the surface response assay were validated using an *in vitro* PK/PD model for combination agents and *in vivo* efficacy trials. These results suggest that the quantitative response surface assay may be a screening tool for drug interactions that is more predictable of *in vivo* interactions than the traditional checkerboard assay. In addition, the additive effect between Lee 1599 and rifampicin suggest that a combination therapy may warrant optimal efficacy results at lower doses than single drug therapy of either drug.

In conclusion, we have successfully supported the further development of spectinamides using a pharmacometric approach. We have contributed to the selection of lead candidate compounds such as Lee 1599 using quantitative PK/PD approaches in its pre-clinical development. The application of PK/PD knowledge is essential for translating the *in vitro* screening assay findings to the *in vivo* stage. The results of the above studies may serve to illustrate the optimization of anti-infective agents in the early drug discovery and development.

LIST OF REFERENCES

1. Zhang, L., M. Pfister, and B. Meibohm, *Concepts and challenges in quantitative pharmacology and model-based drug development*. AAPS J, 2008. **10**(4): p. 552-9.
2. Lalonde, R.L., et al., *Model-based drug development*. Clin Pharmacol Ther, 2007. **82**(1): p. 21-32.
3. Sotto, A. and J.P. Lavigne, *A mathematical model to guide antibiotic treatment strategies*. BMC Med, 2012. **10**: p. 90.
4. Joyner, M.L.P., C.C. Manning, and B.N. Canter, *Modeling the effects of introducing a new antibiotic in a hospital setting: A case study*. Math Biosci Eng, 2012. **9**(3): p. 601-625.
5. Suryawanshi, S., et al., *The current role of model-based drug development*. Expert Opin Drug Discov, 2010. **5**(4): p. 311-21.
6. Meibohm, B. and H. Derendorf, *Pharmacokinetic/pharmacodynamic studies in drug product development*. J Pharm Sci, 2002. **91**(1): p. 18-31.
7. Romero, K., et al., *Pharmacometrics as a discipline is entering the "industrialization" phase: standards, automation, knowledge sharing, and training are critical for future success*. J Clin Pharmacol, 2010. **50**(9 Suppl): p. 9S-19S.
8. Manolis, E. and R. Herold, *Pharmacometrics for regulatory decision making: status and perspective*. Clin Pharmacokinet, 2011. **50**(10): p. 625-6.
9. Bhattaram, V.A., et al., *Impact of pharmacometric reviews on new drug approval and labeling decisions--a survey of 31 new drug applications submitted between 2005 and 2006*. Clin Pharmacol Ther, 2007. **81**(2): p. 213-21.
10. Vaddady, P.K., R.E. Lee, and B. Meibohm, *In vitro pharmacokinetic/pharmacodynamic models in anti-infective drug development: focus on TB*. Future Med Chem, 2010. **2**(8): p. 1355-69.
11. Pasipanodya, J. and T. Gumbo, *An oracle: antituberculosis pharmacokinetics-pharmacodynamics, clinical correlation, and clinical trial simulations to predict the future*. Antimicrob Agents Chemother, 2011. **55**(1): p. 24-34.
12. Preston, S.L., et al., *Levofloxacin population pharmacokinetics and creation of a demographic model for prediction of individual drug clearance in patients with serious community-acquired infection*. Antimicrob Agents Chemother, 1998. **42**(5): p. 1098-104.
13. Lee, J.Y., et al., *Impact of pharmacometric analyses on new drug approval and labelling decisions: a review of 198 submissions between 2000 and 2008*. Clin Pharmacokinet, 2011. **50**(10): p. 627-35.
14. Ikawa, K., et al., *Pharmacokinetic modeling and dosage adaptation of biapenem in Japanese patients during continuous venovenous hemodiafiltration*. J Infect Chemother, 2008. **14**(1): p. 35-9.
15. Lodise, T.P., et al., *Use of population pharmacokinetic modeling and Monte Carlo simulation to describe the pharmacodynamic profile of cefditoren in plasma and epithelial lining fluid*. Antimicrob Agents Chemother, 2008. **52**(6): p. 1945-51.

16. Lodise, T.P., Jr., et al., *Comparison of the probability of target attainment between ceftriaxone and cefepime in the cerebrospinal fluid and serum against Streptococcus pneumoniae*. Diagn Microbiol Infect Dis, 2007. **58**(4): p. 445-52.
17. Rodvold, K.A., et al., *Identifying exposure targets for treatment of staphylococcal pneumonia with ceftobiprole*. Antimicrob Agents Chemother, 2009. **53**(8): p. 3294-301.
18. Lodise, T.P., Jr., et al., *Probability of target attainment for ceftobiprole as derived from a population pharmacokinetic analysis of 150 subjects*. Antimicrob Agents Chemother, 2007. **51**(7): p. 2378-87.
19. Van Wart, S., et al., *Population pharmacokinetics and pharmacodynamics of garenoxacin in patients with community-acquired respiratory tract infections*. Antimicrob Agents Chemother, 2004. **48**(12): p. 4766-77.
20. Noreddin, A.M., et al., *Comparative pharmacodynamics of garenoxacin, gemifloxacin, and moxifloxacin in community-acquired pneumonia caused by Streptococcus pneumoniae: a Monte Carlo simulation analysis*. Clin Ther, 2007. **29**(12): p. 2685-9.
21. Tanigawara, Y., K. Nozawa, and H. Tsuda, *Optimal dose finding of garenoxacin based on population pharmacokinetics/pharmacodynamics and Monte Carlo simulation*. Eur J Clin Pharmacol, 2012. **68**(1): p. 39-53.
22. Noreddin, A.M., D.J. Hoban, and G.G. Zhanel, *Comparison of gatifloxacin and levofloxacin administered at various dosing regimens to hospitalised patients with community-acquired pneumonia: pharmacodynamic target attainment study using North American surveillance data for Streptococcus pneumoniae*. Int J Antimicrob Agents, 2005. **26**(2): p. 120-5.
23. Owens, R.C., Jr., S.M. Bhavnani, and P.G. Ambrose, *Assessment of pharmacokinetic-pharmacodynamic target attainment of gemifloxacin against Streptococcus pneumoniae*. Diagn Microbiol Infect Dis, 2005. **51**(1): p. 45-9.
24. Drusano, G.L., et al., *A population pharmacokinetic analysis of the penetration of the prostate by levofloxacin*. Antimicrob Agents Chemother, 2000. **44**(8): p. 2046-51.
25. Drusano, G.L., et al., *Levofloxacin penetration into epithelial lining fluid as determined by population pharmacokinetic modeling and monte carlo simulation*. Antimicrob Agents Chemother, 2002. **46**(2): p. 586-9.
26. Sprandel, K.A., et al., *Population pharmacokinetic modeling and Monte Carlo simulation of varying doses of intravenous metronidazole*. Diagn Microbiol Infect Dis, 2006. **55**(4): p. 303-9.
27. Simon, N., et al., *Population pharmacokinetics of moxifloxacin in plasma and bronchial secretions in patients with severe bronchopneumonia*. Clin Pharmacol Ther, 2003. **74**(4): p. 353-63.
28. Zhang, J., et al., *Population pharmacokinetic and pharmacodynamic modeling of norvancomycin*. Eur J Clin Microbiol Infect Dis, 2008. **27**(4): p. 275-84.
29. Acosta, E.P., et al., *Oseltamivir dosing for influenza infection in premature neonates*. J Infect Dis, 2010. **202**(4): p. 563-6.
30. Parrott, N., et al., *Development of a physiologically based model for oseltamivir and simulation of pharmacokinetics in neonates and infants*. Clin Pharmacokinet, 2011. **50**(9): p. 613-23.

31. Occhipinti, D.J., et al., *Pharmacokinetics and pharmacodynamics of two multiple-dose piperacillin-tazobactam regimens*. Antimicrob Agents Chemother, 1997. **41**(11): p. 2511-7.
32. Frei, C.R. and D.S. Burgess, *Pharmacokinetic/pharmacodynamic modeling to predict in vivo effectiveness of various dosing regimens of piperacillin/tazobactam and piperacillin monotherapy against gram-negative pulmonary isolates from patients managed in intensive care units in 2002*. Clin Ther, 2008. **30**(12): p. 2335-41.
33. Goutelle, S., et al., *Population modeling and Monte Carlo simulation study of the pharmacokinetics and antituberculosis pharmacodynamics of rifampin in lungs*. Antimicrob Agents Chemother, 2009. **53**(7): p. 2974-81.
34. Lodise, T.P., Jr., et al., *Telavancin penetration into human epithelial lining fluid determined by population pharmacokinetic modeling and Monte Carlo simulation*. Antimicrob Agents Chemother, 2008. **52**(7): p. 2300-4.
35. Lodise, T.P., et al., *Penetration of vancomycin into epithelial lining fluid in healthy volunteers*. Antimicrob Agents Chemother, 2011. **55**(12): p. 5507-11.
36. Damle, B., M.V. Varma, and N. Wood, *Pharmacokinetics of voriconazole administered concomitantly with fluconazole and population-based simulation for sequential use*. Antimicrob Agents Chemother, 2011. **55**(11): p. 5172-7.
37. Czock, D. and F. Keller, *Mechanism-based pharmacokinetic-pharmacodynamic modeling of antimicrobial drug effects*. J Pharmacokinet Pharmacodyn, 2007. **34**(6): p. 727-51.
38. Schmidt, S., et al., *PK/PD: new insights for antibacterial and antiviral applications*. Curr Opin Pharmacol, 2008. **8**(5): p. 549-56.
39. Budha, N.R., et al., *A simple in vitro PK/PD model system to determine time-kill curves of drugs against Mycobacteria*. Tuberculosis (Edinb), 2009. **89**(5): p. 378-85.
40. Stass, H. and A. Dalhoff, *The integrated use of pharmacokinetic and pharmacodynamic models for the definition of breakpoints*. Infection, 2005. **33 Suppl 2**: p. 29-35.
41. Drusano, G.L., et al., *Use of preclinical data for selection of a phase II/III dose for evernimicin and identification of a preclinical MIC breakpoint*. Antimicrob Agents Chemother, 2001. **45**(1): p. 13-22.
42. Turnidge, J. and D.L. Paterson, *Setting and revising antibacterial susceptibility breakpoints*. Clin Microbiol Rev, 2007. **20**(3): p. 391-408, table of contents.
43. Zeitlinger, M.A., et al., *Protein binding: do we ever learn?* Antimicrob Agents Chemother, 2011. **55**(7): p. 3067-74.
44. Barbour, A., F. Scaglione, and H. Derendorf, *Class-dependent relevance of tissue distribution in the interpretation of anti-infective pharmacokinetic/pharmacodynamic indices*. Int J Antimicrob Agents, 2010. **35**(5): p. 431-8.
45. Noreddin, A.M., et al., *Pharmacodynamic target attainment potential of azithromycin, clarithromycin, and telithromycin in serum and epithelial lining fluid of community-acquired pneumonia patients with penicillin-susceptible, intermediate, and resistant Streptococcus pneumoniae*. Int J Infect Dis, 2009. **13**(4): p. 483-7.

46. Gumbo, T., et al., *Isoniazid bactericidal activity and resistance emergence: integrating pharmacodynamics and pharmacogenomics to predict efficacy in different ethnic populations*. Antimicrob Agents Chemother, 2007. **51**(7): p. 2329-36.
47. Wiegand, I., K. Hilpert, and R.E. Hancock, *Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances*. Nat Protoc, 2008. **3**(2): p. 163-75.
48. DeRyke, C.A., J.L. Kuti, and D.P. Nicolau, *Reevaluation of current susceptibility breakpoints for Gram-negative rods based on pharmacodynamic assessment*. Diagn Microbiol Infect Dis, 2007. **58**(3): p. 337-44.
49. Korsan, B., *Transparent Trade-offs: A clinical utility index (CUI) openly evaluates a product's attributes—and chance of success*. Pharmaceutical Executive, 2005.
50. Matzke, G.R., et al., *Drug dosing consideration in patients with acute and chronic kidney disease-a clinical update from Kidney Disease: Improving Global Outcomes (KDIGO)*. Kidney Int, 2011. **80**(11): p. 1122-37.
51. Drusano, G.L., *Antimicrobial pharmacodynamics: critical interactions of 'bug and drug'*. Nat Rev Microbiol, 2004. **2**(4): p. 289-300.
52. Samtani, M.N., et al., *Pharmacokinetic-pharmacodynamic-model-guided doripenem dosing in critically ill patients*. Antimicrob Agents Chemother, 2010. **54**(6): p. 2360-4.
53. American Thoracic, S. and A. Infectious Diseases Society of, *Guidelines for the management of adults with hospital-acquired, ventilator-associated, and healthcare-associated pneumonia*. Am J Respir Crit Care Med, 2005. **171**(4): p. 388-416.
54. Katsube, T., et al., *Pharmacokinetic/pharmacodynamic modeling and simulation to determine effective dosage regimens for doripenem*. J Pharm Sci, 2010. **99**(5): p. 2483-91.
55. Goldie, S.J., et al., *Prophylaxis for human immunodeficiency virus-related Pneumocystis carinii pneumonia: using simulation modeling to inform clinical guidelines*. Arch Intern Med, 2002. **162**(8): p. 921-8.
56. Li, F., et al., *Pharmacometrics-based dose selection of levofloxacin as a treatment for postexposure inhalational anthrax in children*. Antimicrob Agents Chemother, 2010. **54**(1): p. 375-9.
57. European Medicines Agency Committee for Proprietary Medicinal Products (CPMP) *Points to consider on pharmacokinetics and pharmacodynamics in the development of antibacterial medicinal products*. 2009.
58. European Medicines Agency Committee for Proprietary Medicinal Products (CPMP) *Guideline on the clinical evaluation of medicinal products intended for treatment of hepatitis B* 2009.
59. European Medicines Agency Committee for Proprietary Medicinal Products (CPMP) *Guideline on clinical investigation of medicinal products for the treatment of sepsis*. 2009.
60. Jonsson, S., et al., *Role of modelling and simulation: a European regulatory perspective*. Clin Pharmacokinet, 2012. **51**(2): p. 69-76.

61. Rowland, M., C. Peck, and G. Tucker, *Physiologically-based pharmacokinetics in drug development and regulatory science*. Annu Rev Pharmacol Toxicol, 2011. **51**: p. 45-73.
62. Huang, S.M. and M. Rowland, *The role of physiologically based pharmacokinetic modeling in regulatory review*. Clin Pharmacol Ther, 2012. **91**(3): p. 542-9.
63. Barrett, J.S., et al., *Physiologically based pharmacokinetic (PBPK) modeling in children*. Clin Pharmacol Ther, 2012. **92**(1): p. 40-9.
64. Brunner, M., H. Derendorf, and M. Muller, *Microdialysis for in vivo pharmacokinetic/pharmacodynamic characterization of anti-infective drugs*. Curr Opin Pharmacol, 2005. **5**(5): p. 495-9.
65. Chaurasia, C.S., et al., *AAPS-FDA Workshop White Paper: microdialysis principles, application, and regulatory perspectives*. J Clin Pharmacol, 2007. **47**(5): p. 589-603.
66. Heron, M., *Deaths: leading causes for 2010*. Natl Vital Stat Rep, 2013. **62**(6): p. 1-96.
67. <http://www.who.int/topics/tuberculosis/en/>, World Health Organization. 2015.
68. Gillespie, S.H. and K. Singh, *XDR-TB, what is it; how is it treated; and why is therapeutic failure so high?* Recent Pat Antiinfect Drug Discov, 2011. **6**(2): p. 77-83.
69. <http://www.tballiance.org/>.
70. Budha, N.R., *A PHARMACOKINETICS AND PHARMACODYNAMICS (PK/PD) GUIDED APPROACH TO LEAD OPTIMIZATION OF NITROFURANYLAMIDE ANTI-TUBERCULOSIS AGENTS*, in *Graduate Health Sciences*. May 2009, The University of Tennessee.
71. Vaddady, V.N.R.P.K., *PHARMACOKINETIC AND PHARMACODYNAMIC STUDIES OF A NOVEL SPECTINAMIDE SERIES OF ANTITUBERCULOSIS AGENTS*, in *Graduate Health Sciences*. May 2011, The University of Tennessee.
72. van den Boogaard, J., et al., *New drugs against tuberculosis: problems, progress, and evaluation of agents in clinical development*. Antimicrob Agents Chemother, 2009. **53**(3): p. 849-62.
73. Caminero, J.A., et al., *Best drug treatment for multidrug-resistant and extensively drug-resistant tuberculosis*. Lancet Infect Dis, 2010. **10**(9): p. 621-9.
74. Mitnick, C.D., S.C. Appleton, and S.S. Shin, *Epidemiology and treatment of multidrug resistant tuberculosis*. Semin Respir Crit Care Med, 2008. **29**(5): p. 499-524.
75. Casali, N., et al., *Evolution and transmission of drug-resistant tuberculosis in a Russian population*. Nat Genet, 2014. **46**(3): p. 279-86.
76. Mdluli, K., T. Kaneko, and A. Upton, *The tuberculosis drug discovery and development pipeline and emerging drug targets*. Cold Spring Harb Perspect Med, 2015. **5**(6).
77. Borovinskaya, M.A., et al., *A steric block in translation caused by the antibiotic spectinomycin*. ACS Chem Biol, 2007. **2**(8): p. 545-52.
78. Louw, G.E., et al., *A balancing act: efflux/influx in mycobacterial drug resistance*. Antimicrob Agents Chemother, 2009. **53**(8): p. 3181-9.

79. Balganesch, M., et al., *Efflux pumps of Mycobacterium tuberculosis play a significant role in antituberculosis activity of potential drug candidates*. Antimicrob Agents Chemother, 2012. **56**(5): p. 2643-51.
80. Ramon-Garcia, S., et al., *Functional and genetic characterization of the tap efflux pump in Mycobacterium bovis BCG*. Antimicrob Agents Chemother, 2012. **56**(4): p. 2074-83.
81. Lee, R.E., et al., *Spectinamides: a new class of semisynthetic antituberculosis agents that overcome native drug efflux*. Nat Med, 2014. **20**(2): p. 152-8.
82. Morris, B.D.a.T., *Physiological parameters in laboratory animals and humans*. Pharmaceutical Research, 1993. **10**: p. 1093-1095.
83. Scheife, R.T., *Protein binding: what does it mean?* DICP, 1989. **23**(7-8 Suppl): p. S27-31.
84. PE, R., *Plasma protein binding displacement interactions--why are they still regarded as clinically important?* British Journal of Clinical Pharmacology, 1994. **37**(2): p. 125-128.
85. Daniel, W.A. and J. Wojcikowski, *Lysosomal trapping as an important mechanism involved in the cellular distribution of perazine and in pharmacokinetic interaction with antidepressants*. Eur Neuropsychopharmacol, 1999. **9**(6): p. 483-91.
86. Kazmi, F., et al., *Lysosomal sequestration (trapping) of lipophilic amine (cationic amphiphilic) drugs in immortalized human hepatocytes (Fa2N-4 cells)*. Drug Metab Dispos, 2013. **41**(4): p. 897-905.
87. Daniel, W.A. and J. Wojcikowski, *Contribution of lysosomal trapping to the total tissue uptake of psychotropic drugs*. Pharmacol Toxicol, 1997. **80**(2): p. 62-8.
88. Goldman, S.D., et al., *Mechanisms of amine accumulation in, and egress from, lysosomes*. Bioanalysis, 2009. **1**(8): p. 1445-59.
89. Anderson, N. and J. Borlak, *Drug-induced phospholipidosis*. FEBS Lett, 2006. **580**(23): p. 5533-40.
90. Gonzalez-Juarrero, M., et al., *Mouse model for efficacy testing of antituberculosis agents via intrapulmonary delivery*. Antimicrob Agents Chemother, 2012. **56**(7): p. 3957-9.
91. De Luca, D., et al., *Intrapulmonary drug administration in neonatal and paediatric critical care: a comprehensive review*. Eur Respir J, 2011. **37**(3): p. 678-89.
92. Chono, S., et al., *Pharmacokinetic and pharmacodynamic efficacy of intrapulmonary administration of ciprofloxacin for the treatment of respiratory infections*. Drug Metab Pharmacokinet, 2007. **22**(2): p. 88-95.
93. Vavilin, G.I., et al., *[Intrapulmonary administration of antitubercular agents in experimental tuberculosis of the lungs]*. Probl Tuberk, 1979(7): p. 52-6.
94. Li, Y.J., M. Petrofsky, and L.E. Bermudez, *Mycobacterium tuberculosis uptake by recipient host macrophages is influenced by environmental conditions in the granuloma of the infectious individual and is associated with impaired production of interleukin-12 and tumor necrosis factor alpha*. Infect Immun, 2002. **70**(11): p. 6223-30.
95. Ernst, J.D., *Macrophage receptors for Mycobacterium tuberculosis*. Infect Immun, 1998. **66**(4): p. 1277-81.

96. Fenton, M.J., *Macrophages and tuberculosis*. Curr Opin Hematol, 1998. **5**(1): p. 72-8.
97. Podinovskaia, M., et al., *Infection of macrophages with Mycobacterium tuberculosis induces global modifications to phagosomal function*. Cell Microbiol, 2013. **15**(6): p. 843-59.
98. Shaffer, C.L., et al., *Using a tritiated compound to elucidate its preclinical metabolic and excretory pathways in vivo: exploring tritium exchange risk*. Drug Metab Dispos, 2006. **34**(9): p. 1615-23.
99. Yang, S., et al., *Pharmacokinetics and disposition study of calf thymus DNA in rats by applying 3H-labeling method*. J Pharm Biomed Anal, 2012. **64-65**: p. 35-9.
100. Roffey, S.J., et al., *What is the objective of the mass balance study? A retrospective analysis of data in animal and human excretion studies employing radiolabeled drugs*. Drug Metab Rev, 2007. **39**(1): p. 17-43.
101. Muir, K.T. and R.O. Gomeni, *Non-compartmental analysis: Pharmacokinetics in Drug Development, In Pharmacokinetics in Drug Development: Clinical Study Design and Analysis*, P.L. Bonate and D.R. Howard, Editors. 2004 AAPS Press: USA.
102. Vogelman, B., et al., *In vivo postantibiotic effect in a thigh infection in neutropenic mice*. J Infect Dis, 1988. **157**(2): p. 287-98.
103. MacKenzie, F.M. and I.M. Gould, *The post-antibiotic effect*. J Antimicrob Chemother, 1993. **32**(4): p. 519-37.
104. Trivedi, A., R.E. Lee, and B. Meibohm, *Applications of pharmacometrics in the clinical development and pharmacotherapy of anti-infectives*. Expert Rev Clin Pharmacol, 2013. **6**(2): p. 159-70.
105. Nielsen, E.I. and L.E. Friberg, *Pharmacokinetic-pharmacodynamic modeling of antibacterial drugs*. Pharmacol Rev, 2013. **65**(3): p. 1053-90.
106. Treyaprasert, W., et al., *Pharmacokinetic/pharmacodynamic modeling of in vitro activity of azithromycin against four different bacterial strains*. Int J Antimicrob Agents, 2007. **29**(3): p. 263-70.
107. Palaci, M., et al., *Cavitary disease and quantitative sputum bacillary load in cases of pulmonary tuberculosis*. J Clin Microbiol, 2007. **45**(12): p. 4064-6.
108. Mostowy, S., et al., *The in vitro evolution of BCG vaccines*. Vaccine, 2003. **21**(27-30): p. 4270-4274.
109. Beste, D.J., et al., *Compiling a molecular inventory for Mycobacterium bovis BCG at two growth rates: evidence for growth rate-mediated regulation of ribosome biosynthesis and lipid metabolism*. J Bacteriol, 2005. **187**(5): p. 1677-84.
110. Nielsen, E.I., O. Cars, and L.E. Friberg, *Pharmacokinetic/pharmacodynamic (PK/PD) indices of antibiotics predicted by a semimechanistic PKPD model: a step toward model-based dose optimization*. Antimicrob Agents Chemother, 2011. **55**(10): p. 4619-30.
111. Woodcock, J., J.P. Griffin, and R.E. Behrman, *Development of Novel Combination Therapies*. New England Journal of Medicine, 2011. **364**(11): p. 985-987.

112. Zumla, A.I., et al., *New antituberculosis drugs, regimens, and adjunct therapies: needs, advances, and future prospects*. Lancet Infect Dis, 2014. **14**(4): p. 327-40.
113. Lim, T.P., et al., *Quantitative assessment of combination antimicrobial therapy against multidrug-resistant Acinetobacter baumannii*. Antimicrob Agents Chemother, 2008. **52**(8): p. 2898-904.
114. Tam, V.H., et al., *Novel approach to characterization of combined pharmacodynamic effects of antimicrobial agents*. Antimicrob Agents Chemother, 2004. **48**(11): p. 4315-21.
115. Yuan, Z., et al., *Quantitative assessment of combination antimicrobial therapy against multidrug-resistant bacteria in a murine pneumonia model*. J Infect Dis, 2010. **201**(6): p. 889-97.
116. Blaser, J., *In-vitro model for simultaneous simulation of the serum kinetics of two drugs with different half-lives*. J Antimicrob Chemother, 1985. **15 Suppl A**: p. 125-30.
117. Shim, C.-K. and J.-U. Lee, *Comparative study of rifampicin pharmacokinetics administered orally and intravenously in the fasted and non-fasted rats*. Archives of Pharmacal Research, 1985. **8**(3): p. 177-186.
118. Sopirala, M.M., et al., *Synergy testing by Etest, microdilution checkerboard, and time-kill methods for pan-drug-resistant Acinetobacter baumannii*. Antimicrob Agents Chemother, 2010. **54**(11): p. 4678-83.
119. Bonapace, C.R., et al., *Evaluation of antibiotic synergy against Acinetobacter baumannii: a comparison with Etest, time-kill, and checkerboard methods*. Diagn Microbiol Infect Dis, 2000. **38**(1): p. 43-50.
120. Hurdle, J.G., et al., *A microbiological assessment of novel nitrofuranylamides as anti-tuberculosis agents*. J Antimicrob Chemother, 2008. **62**(5): p. 1037-45.
121. Institute, C.a.L.S., *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically*, in *Approved Standard-Ninth Edition*. 2012.
122. Hsieh, M.H., et al., *Synergy assessed by checkerboard. A critical analysis*. Diagn Microbiol Infect Dis, 1993. **16**(4): p. 343-9.
123. Bonapace, C.R., et al., *Comparison of methods of interpretation of checkerboard synergy testing*. Diagn Microbiol Infect Dis, 2002. **44**(4): p. 363-6.
124. Cappelletty, D.M. and M.J. Rybak, *Comparison of methodologies for synergism testing of drug combinations against resistant strains of Pseudomonas aeruginosa*. Antimicrob Agents Chemother, 1996. **40**(3): p. 677-83.
125. Chadwick, E.G., S.T. Shulman, and R. Yogev, *Correlation of antibiotic synergy in vitro and in vivo: use of an animal model of neutropenic gram-negative sepsis*. J Infect Dis, 1986. **154**(4): p. 670-5.

VITA

Ashit Trivedi was born in 1985 in Ahmedabad, India. He was brought up and finished his schooling in 2002 in Mumbai, India. He obtained his Bachelors of Science in Biochemistry in 2005 and Masters of Science in Bioanalytical Sciences in 2007 from Ramnarain Ruia College, University of Mumbai. During his Bachelors and Master's degree, he did multiple internships at Tata Memorial Hospital, Haffkines Research Institute, Wockhardt Hospitals, Animal research center and Lupin Pharmaceuticals. He worked as Research Associate at Macleods Pharmaceuticals, Mumbai and Research Associate in Drug Metabolism and Pharmacokinetics at Lupin Pharmaceuticals, Pune, India. Later, he received another Master's degree in Pharmaceuticals Sciences from Northeastern University, Boston, MA in 2010. During his Masters, he did an internship at Abbott Bioresearch Center in Molecular Pharmacology, Worcester, MA and in Formulations at Vertex Pharmaceuticals, Cambridge, MA in 2009 and 2010, respectively.

Consequently, he joined the PhD Program in Pharmaceutical Science at University of Tennessee Health Science Center in 2010 with Dr. Bernd Meibohm as his major advisor. During his PhD, he was involved with various leadership positions such as Chair-elect of the American Association of Pharmaceutical Scientist (AAPS) Student Chapter at the University of Tennessee in 2012, Chair of the AAPS Student Chapter in 2013, Trainee Chair for the American Conference on Pharmacometrics in 2014 and Student Representative for the Clinical Pharmacology and Translational Research section in AAPS from 2013-2015. He received multiple travel awards and was a recipient of the Graduate Research Award in Pharmacokinetics, Pharmacodynamics and Drug Metabolism and Clinical Pharmacology and Translational Research at the AAPS Annual Meeting in 2014. During this period, he was also a summer intern at Takeda (Millennium) Pharmaceuticals, Cambridge MA in summer of 2014. After completion of his PhD, he will be joining the Clinical Pharmacology group at Amgen, Thousand Oaks, CA as a Scientist.