UNIVERSITY OF
KWAZULU-NATAL

INYUVESI
YAKWAZULU-NATALI

EVALUATION OF TEIXOBACTIN DERIVATIVES AS POTENTIAL ANTIMICROBIAL AGENTS

Estelle J. Ramchuran

209534898

Submitted in fulfillment of the requirements for the degree of Masters in Medical Sciences in the School of Health Science, University of KwaZulu-Natal

December 2017

Evaluation of teixobactin derivatives as potential antimicrobial agents

Estelle J. Ramchuran

209534898

A dissertation submitted to the School of Health Sciences, College of Health Science, University of KwaZulu-Natal, Westville, for the degree of Master of Medical Science.

This is a dissertation by manuscript with an overall introduction and final summary.

This is to certify that the content of this dissertation is the original research work of Miss Estelle J. Ramchuran, approved by:

_____	_____
Name:	*Date*
_____	_____
Dr Linda A. Bester	*Date*

DECLARATION

I, Miss Estelle J. Ramchuran, declare as follows:

1. That the work described in this dissertation has not been submitted to UKZN or any other tertiary institution for purposes of obtaining an academic qualification, whether by myself or any other party.

2. That my contribution to the project was as follows:

    a. The research reported in this dissertation, except where otherwise indicated, is my original work

3. This dissertation does not contain other person's writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:

    a. Their words have been re-written but the general information attributed to them has been referenced.

    b. Where their exact words have been used, then their writing has been placed in italics, inside quotation marks and duly referenced.

_____ _____

Signature *Date*

E.J. Ramchuran

DEDICATION

I dedicate this work to my Papa, the late Rev. SG Jackson.

His legacy will live on forever.

ACKNOWLEDGEMENT

All praise and glory to Jesus Christ, this is only possible through Him.

My sincere thanks and appreciation to Dr. Linda Bester, thank you for the continuous support and encouragement. You truly are the epitome of a role model.

This study would not have been successful without my family, especially my dad (Anesh Ramchuran), mum (Sue- Rita Ramchuran) and my brother (Travis Ramchuran). Thank you for motivating me and helping me overcome the many challenges I faced this year.

To my fiancé Jayd Singh, thank you for being my pillar of strength. I could not have done this without you. I hope you're ready to walk the next road with me… PhD.

My sincere thanks to my Biomedical Resource Unit research team, especially Anou dit Moise Somboro, Daniel Gyamfi Amoako and Christiana Shobo.

I would also like to express my deepest gratitude and respect to Prof Fernando Albericio, Beatriz de la Torre and Shimaa Abdel Monaim, it was a pleasure working on this Peptide project with you.

My greatest appreciation goes to Prof Essack, thank you for your encouragement and advice during this study.

I would also like to thank my friends and colleagues Samke Gumede, Chantal Molechan, Nondu Ntuli, Melissa Ramtahal, Stephanie Pillay and others.

I would like to acknowledge the financial support from the National Research Foundation and the College of Health Sciences.

TABLE OF CONTENTS

DECLARATION.....i
DEDICATION.....ii
ACKNOWLEDGEMENT.....iii
LIST OF PUBLICATIONS.....iii
LIST OF FIGURES.....v
LIST OF TABLES.....xi
LIST OF ABBREVIATIONS AND ACRONYMS.....xii
ABSTRACT.....xii
OUTLINE OF THE DISSERTATION.....xiii
CHAPTER ONE: Introduction.....1
1.1 BRIEF INTRODUCTION.....1
1.2. BACKGROUND AND STATEMENT OF THE PROBLEM.....1
1.2.1 Penicillin: mode of action and mechanism of resistance.....2
1.2.2 Vancomycin: mode of action and mechanism of resistance.....5
1.3. TEIXOBACTIN.....7
1.4. *IN VITRO* ASSAYS.....10
1.4.1 Antimicrobial susceptibility techniques.....10
1.4.1.1 Agar dilution
1.4.1.2 Broth dilution
1.4.1.3 Disk diffusion test
1.4.2 Synergy test.....12
1.4.3 Serum effect.....13
1.5. JUSTIFICATION.....14
1.6. AIMS.....14
1.6.1 Specific objectives.....14
REFERENCES.....15
CHAPTER TWO:
***In vitro* evaluation of teixobactin derivatives against clinically relevant bacterial isolates**.....22
Abstract:.....23
2.1. Introduction.....24
2.2 - Materials and methods.....25
2.2.1- Antibiotics and reagents.....25

2.2.2- Bacterial strains 26
2.2.3 Synthesis, purification, and characterization of teixobactin derivatives 26
2.2.4- Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) determination 26
2.2.5- Human serum effect in the MICs 27
2.2.6- Time-kill kinetic assays 27
2.2.7- Cell culture 27
2.2.8- Haemolysis Assay on Red Blood Cells (RBCs) 28
2.2.9- Cytotoxicity on peripheral blood mononuclear cells (PBMCs) 29
2.3- Results 30
2.3.1- Teixobactin derivatives 30
2.3.2- Antimicrobial activity of teixobactin derivatives and the effects of human serum on the MICs 32
2.3.3- Time-kill kinetics 36
2.3.4- Haemolysis and Cytotoxicity 37
2.4- Discussion 37
REFERENCES 40
CHAPTER THREE: Conclusion and Recommendations
3.1 CONCLUSION 45
3.2 RECOMMENDATIONS 45
APPENDIX I 47
Submission confirmation from the Journal of Frontiers Microbiology ID: 340217] 47
APPENDIX II 48
Ethics Approval from Biomedical Research Ethics Committee UKZN, Ref BE394/15 48
APPENDIX III 49
Publications not included in this dissertation 49

LIST OF PUBLICATIONS

Publications included in this dissertation:

Estelle J. Ramchuran, Anou M Somboro, Daniel G Amoako, Shimaa A. H. Abdel Monaim, Raveen Parboosing, Beatriz G. de la Torre, Fernando Albericio and Linda Bester; *In vitro* evaluation of teixobactin derivatives against clinical relevant bacterial isolates.

Contribution: Estelle conceived and designed the experiment, conducted the antimicrobial assays, and wrote the manuscript.

Publications not included in this dissertation

Shimaa A. H. Abdel Monaim, Yahya E. Jad, **Estelle J. Ramchuran**, Ayman El-Faham, Thavendran Govender, Hendrik G. Kruger, Beatriz G. de la Torre, and Fernando Albericio; Lysine Scanning of Arg10−teixobactin: Deciphering the Role of Hydrophobic and Hydrophilic Residues (Abdel Monaim et al., 2016)

Contribution: Carried out the *in vitro* antimicrobial assays, and the biological analysis of the compounds and wrote the microbiology part of the article.

Shimaa A.H. Abdel Monaim, Yahya E. Jad, Gerardo Acosta, **Estelle J. Ramchuran**, Ayman El-Faham, Thavendran Govender, Hendrik G. Kruger, Beatriz G. de la Torre and Fernando Albericio; Re-Evaluating the N-Terminal Substitution and the D-Residues on teixobactin (Abdel Monaim et al., 2016)

Contribution: Carried out the *in vitro* antimicrobial assays, and the biological analysis of the compounds and wrote the microbiology part of the article.

Shimaa A.H. Abdel Monaim, **Estelle J. Ramchuran**, Ayman El-Faham, Fernando Albericio and Beatriz de la Torre; Converting teixobactin into a Cationic Antimicrobial Peptide (AMP***)*** (Abdel Monaim et al., 2017b)

Contribution: Carried out the *in vitro* antimicrobial assays, and the biological analysis of the compounds and wrote the microbiology part of the article.

Shimaa A.H. Abdel Monaim, Noki Sikabwe, **Estelle J. Ramchuran**, Ayman El-Faham, Fernando Albericio and Beatriz de la Torre; Investigation of the N-Terminus Amino Function of Arg10-teixobactin (Abdel Monaim et al., 2017a)

Contribution: Carried out the *in vitro* antimicrobial assays, and the biological analysis of the compounds and wrote the microbiology part of the article.

LIST OF FIGURES

CHAPTER ONE

Figure 1: Structural representation of teixobactin

CHAPTER TWO

Figure 1: Chemical structure of teixobactin

Figure 2: Chemical structure of teixobactin derivatives (2, 3, 4 and 5)

Figure 3: Time killing kinetics at different concentrations of teixobactin derivative 4. Gram-positive bacteria (*S. aureus* and *B. subtilis*) were challenged with compound 4 at 1x, 2x and 4x MIC levels.

Figure 4: Haemolysis and cytotoxicity effects of teixobactin derivatives at various concentrations A) Viability (%) of RBCs and PBMCs treated with DMSO; B) Viability (%) of RBCs and PBMCs treated with compound 3; C) Viability (%) of RBCs and PBMCs treated with compound 4; D Viability (%) of RBCs and PBMCs treated with compound 5.

LIST OF TABLES

CHAPTER TWO

Table 1: Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and MIC in presence of 50% human serum of teixobactin derivatives against susceptible reference strains of bacteria.

Table 2: Minimum inhibitory concentration (MIC) of teixobactin derivatives against methicillin resistant *Staphylococcus aureus (*MRSA).

Table 3: Minimum inhibitory concentration (MIC) of teixobactin derivatives against Vancomycin-Resistant Enterococci (VRE).

LIST OF ABBREVIATIONS AND ACRONYMS

CLSI	Clinical Laboratory Standards Institute
iChip	Isolation Chip
ICU	Intensive Care Unit
PBP	Penicillin Binding Proteins
PBMC's	Peripheral Blood Mononuclear cells
RBC's	Red blood cells
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin resistant *Staphylococcus aureus*
MSSA	Methicillin-susceptible *Staphylococcus aureus*
NDM1	New Delhi metallo-beta-lactamase 1
VISA	Vancomycin intermediate-resistant *Staphylococcus aureus*
VRE	Vancomycin resistant *Enterococcus* or enterococci
WHO	World Health Organization

ABSTRACT

Methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) are on the World Health Organization (WHO) high priority list of pathogens that require serious attention. Therefore, the need for novel class of compounds is vital in overcoming this problem. Teixobactin is a new class of antibiotic that has exhibited antimicrobial activity against resistant bacteria. In this study we are expanding the investigation of teixobactin derivatives against clinically relevant bacterial isolates from South African patients. The minimum inhibitory concentration (MIC), the minimal bactericidal concentration (MBC), the serum effect on the MIC's and the time kill kinetics studies of three of our synthesized teixobactin derivatives 3, 4 and 5 were ascertained by broth microdilution according to the CLSI 2017 guidelines. Haemolysis on red blood cells (RBCs) and cytotoxicity on peripheral blood mononuclear cells (PBMCs) were performed to investigate the safety of these derivatives. MIC's of the teixobactin derivatives against ATCC reference strains were between 4-64 µg/ml (3), 2-64 µg/ml (4) and 0.5-64 µg/ml (5). The MIC's for MRSA were 32 µg/ml for (3), 2-4 µg/ml for (4) and 2-4 µg/ml for (5) whilst the MIC's obtained for VRE's were 8-16 µg/ml for (3), 4 µg/ml for (4) and 2-16 µg/ml for (5). 50% human serum had no effect on the MIC's. All these derivatives did not show any effect on cell viability at their effective concentration. Teixobactin derivatives (3, 4 and 5) were capable of inhibiting bacterial growth in drug resistant bacteria and thus serve as potential antimicrobial agents.

OUTLINE OF THE DISSERTATION

This study evaluated teixobactin derivatives as potential antimicrobial agents, through screening both susceptible and resistant bacteria.

Chapter 1 is the literature review and briefly outlines the need for this study.

Chapter 2 focuses on the evaluation of teixobactin derivatives and a number of antimicrobial assays conducted. This section was submitted to the accredited journal Frontiers in Microbiology and is formatted accordingly.

Chapter 3 presents the concluding remarks of this study as well as the recommendations and limitations.

The dissertation is concluded with the appendixes that includes the proof of journal submission and ethical approval documents.

CHAPTER ONE

Introduction

1.1 BRIEF INTRODUCTION

Antimicrobial resistance and the lack of novel antibiotics are of worldwide concern. The production of antibiotics dates back to the 1900's however because of misuse, resistance has become a huge obstacle in the treatment of infections. Methicillin-resistant *Staphylococcus aureus* (MRSA) and Vancomycin-resistant *Enterococcus* (VRE) are pathogens that are of alarming concern. Thus the need for novel antibiotics to treat such infections is essential in curbing antimicrobial resistance. Potential antimicrobial agents are developed using current antibiotics that are no longer effective as a result of resistance. These antibiotics are the backbone in the design of novel antimicrobial agents, with the hope to broaden the activity of the antibiotics.

Teixobactin is a new class of antibiotic that has proven to be very effective against Gram-positive bacteria. In this study novel teixobactin derivatives were tested as potential antimicrobial agents. A number of *in vitro* assays were conducted using Gram-positive, Gram-negative bacteria as well as drug-resistant bacteria, especially MRSA and VRE.

1.2. BACKGROUND AND STATEMENT OF THE PROBLEM

Bacterial resistance has become a public health problem as a result of the negligent usage of antibiotics. MRSA and VRE are progressively emerging as common pathogens. As the existing antibiotics have failed to circumvent the genes known to convey resistance, they are unable to thoroughly treat infections caused by these bacteria. The development of novel antibiotics is fundamental in the search for methods to curb the problem of resistance.

1.2.1 Penicillin: mode of action and mechanism of resistance

The discovery of Penicillin by Alexander Fleming in 1928 has paved the path in the treatment of bacterial infections. Penicillin is still the most widely used antibiotic despite it being the oldest known antibiotic and a number of bacteria have developed resistance as a result of its extensive use. Penicillin was derived from a fungus, *Penicillin chrysogenum*. The presence of a bicyclic 'penam' nucleus illustrates the biological activity of this group of antibiotics. The 'penam' nucleus forms a 4- membered Kalley Heiligenthal ring that is fused to a 5- membered thiazolidine ring and an acyl side chain to the β-lactam (Dumancas et al., 2014). Penicillin antibiotics were among the first antibiotics that were effective against bacterial infections that resulted from streptococci and staphylococci. Penicillin is the termed used broadly to refer to benzylpenicillin (penicillin G originally found in 1928), benzathine benzylpenicillin (benzathine penicillin), benzylpenicillin (procaine penicillin) and phenoxymethylpenicillin (penicillin V). Penicillin G and V are natural penicillins that were active against susceptible Gram-positive cocci (Dumancas et al., 2014; Goodman, 2011).

Most β-lactam antibiotics function by inhibiting cell wall biosynthesis in bacteria; this is achieved by covalently binding to penicillin binding proteins (PBPs). PBPs are responsible for linking together the peptidoglycan molecules. This linkage is an essential step in cell wall synthesis (Rang et al., 2015). Penicillin derivatives (penams), carbapenems, cephalosporins (cephems) and monobactams all belong to this class and have the same mechanism of action but vary in their antimicrobial activity (Papp-Wallace et al., 2011). Bacteria are able to overcome the effects of β-lactam antibiotics by developing an enzyme known as β-lactamase that attacks the β-lactam ring resulting in β-lactam resistance. In order to curb this resistance, β-lactam antibiotics are prescribed in conjunction with β-lactamase inhibitors.

Ampicillin was the first major development from penicillin and presented a broader spectrum of activity than the original penicillins. It exhibited activity against *Streptococcus pyogenes* and

Streptococcus pneumonia as well as some isolates of *Staphylococcus aureus* (Rang et al., 2014). Dicloxacillin, methicillin and flucloxacillin were β-lactamase-resistant penicillins that were further developed but these were only significant for their activity against β-lactamase-producing species. It is however ineffective against MRSA.

MRSA has developed into the leading nosocomial Gram-positive pathogen contributing to high rates of mortality and morbidity (Sista et al., 2004; Drago et al., 2007; Khan et al., 2015; Ventola, 2015). More than 50% of hospital *Staphylococcus aureus* infections in intensive care unit (ICU) are a result of MRSA. There have also been increased reports of multi-drug resistant MRSA and increasingly virulent MRSA. Along with its broad based β-lactam resistance, MRSA is also known to have a multi-drug resistance genotype, which includes aminoglycosides, cephalosporins, fluoroquinolones and macrolide resistances (Pantosti et al., 2007; Hope et al., 2008). Strategies to control and eliminate MRSA can include a combination of vaccine development, development of novel anti-MRSA drugs, infection control and other non- traditional methods (Chopra, 2003; Drago et al., 2007).

MRSA like all *Staphylococcus aureus* is a Gram-positive, spherical (cocci), non-spore forming, non-motile bacterium and forms grape-like clusters or chains (Murray et al., 2015). Any strain of *S. aureus* that mutated through horizontal gene transfer, natural selection and multiple drug resistance to β-lactam antibiotics is known to be MRSA. β-lactam antibiotics are a group of broad-spectrum antibiotic, which includes some penams – penicillin derivatives such as methicillin and oxacillin, and cephems such as the cephalosporins (Gurusamy et al., 2013). Strains that are susceptible to or unable to resist these antibiotics are classified as methicillin-susceptible *Staphylococcus aureus*, or MSSA. MRSA can be present as mixed colonies in MSSA and also shows slower growth on media as compared to MSSA. (Murray et al., 2015). The prevalence of MRSA is high in hospitals, nursing homes, where patients that have catheters, open wounds and weakened immune systems are at risk of hospital- acquired

infections. MRSA can be hospital-acquired (HA-MRSA), community-acquired (CA-MRSA) or livestock-acquired (LA-MRSA). Methods used to identify MRSA include polymerase chain reaction (PCR) and selective culture media supplemented with relevant antibiotics (Murray et al., 2015). MRSA can also be identified through the presence of the *mecA* gene, which is generally absent in MSSA. However, some MSSA possess the *mecA* gene but it is not expressed. The *mecA* gene is known to confer resistance to a large number of antibiotics, which include methicillin, penicillin, and other penicillin-like antibiotics. This *mecA* gene, which encodes for a novel penicillin- binding protein (PBP), facilitates methicillin resistance in *S. aureus*. Methicillin exposure inactivates the four-high binding affinity PBPs that are usually present. This results in PBP-2a, which has a low affinity to methicillin to take over the functions of these PBPs, allowing the cell to grow. Other genes influence the production of PBP-2a and the regulation of the methicillin resistance phenotype. *MecR1* and *mec*l, two genes that are located upstream from *mecA*, control the expression of PBP-2a. Although β-lactam antibiotics with a high affinity for PBP-2a have shown efficacy against MRSA *in vivo*, none of these antibiotics were able to make it further than the exploration phase (Chambers, 2001; Thabit et al., 2015).

Vancomycin remains the preferred drug for the treatment of infections caused by MRSA, despite it being less active than penicillins. Combination therapy using vancomycin with ss-lactam may be synergistic *in vivo* against MRSA. However because of the increasing prevalence of MRSA in hospitals and communities, alternative approaches (antimicrobial stewardships, hand washing, proper use od antibiotics) are required to treat MRSA (Chambers, 2001; Thabit et al., 2015). In addition to the concerns surrounding MRSA, several reports have shown that the emergence of VRE is increasing drastically and therefore requires some intervention.

1.2.2 Vancomycin: mode of action and mechanism of resistance

Vancomycin is produced by *Amycolatopsis orientalis*, a soil bacterium (Levine, 2006). Vancomycin is administrated intravenously as a first line treatment option for a number of serious bacterial infections caused by Gram-positive bacteria (Levine, 2006; Liu et al., 2011). It is also used to treat meningitis that is caused by MRSA. Vancomycin, when taken orally, is absorbed very poorly therefore intravenous administration is preferred however, it is recommended orally to treat *Clostridium difficile* colitis (Edlund et al., 1997; Van Bambeke, 2006). The mechanism of action of vancomycin includes the inhibition of cell wall synthesis in Gram-positive bacteria. Vancomycin is not active against Gram-negative bacteria because of the different mechanism used by Gram-negatives in cell wall synthesis as well as factors linked to the permeability of outer membrane (Wright, 2015).

Enterococci are present as common bacteria on human skin, and are part of the normal microbiota make-up in human intestines (Miller et al., 2014). However, resistance to antibiotics occurs as a result of selective pressure subsequently causing symptomatic infections, particularly in patients that are immune-compromised. Bacteria are able to mutate therefore antibiotics that are first-line treatment options are no longer effective. Vancomycin much like teixobactin, also binds to lipid cell wall precursors, however resistance to this antibiotic only emerged almost 40 years after its discovery. This was as a result of the mobilization of self-resistance mechanisms that is employed by vancomycin-producing bacteria, through horizontal gene transfer (Marshal et al., 1998; Wright, 2015). Vancomycin resistant enterococci (VRE) are bacterial strains that belong to the genus *Enterococcus* and are resistant to vancomycin (CDC, 2011). Six vancomycin resistant genes are currently known, viz.*VanA*, *VanB*, *VanC*, *VanD*, *VanE* and *VanG* (Fong and Drlica, 2008). The mechanism of resistance to vancomycin that has been observed involved the alteration of the peptidoglycan synthesis pathways (Arias and Murray, 2012; Thabit et al., 2015). The D-alanyl-D-lactase variation results in the loss of one

hydrogen bond interaction (4 as opposed to 5 for D-alanyl-D-alanine) between vancomycin and the peptide. A six-fold loss of affinity between vancomycin and the peptide is caused by the D-alanyl-D-serine variation, possibly due to steric hindrance (Coates, 2012; Meziane-Cherif et al., 2012).

Reports from U.S. hospitals indicate a rapid increase in colonization and infection with VRE. These increases bring about several complications within the health care sector, which include, i) potential gene transfer of the vancomycin resistant gene to other Gram-positive organisms namely *S. aureus*, and ii), the lack of antimicrobials for the treatment of VRE infections (CDC, 2011; Orsi and Ciorba, 2013). Most VRE are multi-drug resistant strains; e.g., ampicillin and aminoglycosides that were previously used to treat infections brought about by these organisms. A number of elements contribute to the increased risk of VRE infection and colonization. These include severe underlying disease, previous therapy with vancomycin and multi antimicrobial drugs, immunosuppression and intra-abdominal surgery. The presence of enterococci in the normal gastrointestinal or female genital tract is the source of most enterococcal infections because of its presence in the patient. Outbreaks and endemic infections due to enterococci, including VRE, have indicated that patient-to-patient transmission can occur through direct or indirect contact via hands of individuals or contaminated patient- care equipment or surfaces (Tacconelli and Cataldo, 2008).

The prevalence of both MRSA and VRE in South Africa has increased in recent years and is the leading cause of morbidity and mortality (Mahabeer. et al., 2016). There has not been much success in reducing the spread of resistant bacteria in communities and healthcare settings, despite the implementation of precautionary measures. These precautionary measures include (hand washing with an antiseptic, routine screening for VRE, reduced use of antibiotics unnecessarily and antimicrobial stewardship). (Orsi and Ciorba, 2013). Failure to follow through with these precautionary measures, more so in undeveloped countries, leads to

increased prevalence and therefore, new antibiotics that have shown great promise such as teixobactin, plays an important role in overcoming drug resistant bacteria.

1.3. TEIXOBACTIN

Teixobactin (Figure 1) is a new class of antibiotic that has been discovered using the iChip (isolation Chip), a culturing method that enables uncultured bacteria to be isolated from soil (Nichols et al., 2010; Ling et al., 2015; Stone and Judy, 2015; Wright, 2015). This method allowed researchers to grow *Eleftheria terrae* (*E. terrae*), a previously uncultured bacterium that produces the antibiotic teixobactin (Nichols et al., 2010). The iChip culture cells in a block of plastic are inoculated with soil which is diluted to insert one bacterium in each cell, this is then sealed with semi-permeable membranes and is planted in a box containing the origin soil (Nichols et al., 2010; Stone and Judy, 2015). Nutrients and growth factors diffuse from the soil into each cell through the membrane, this promotes bacterial growth into a self-sustaining colony *in vitro*, allowing for growth of one species in the cells (Nichols et al., 2010). Teixobactin is the first novel antibiotic with drug potential, isolated from bacteria in decades.

Teixobactin is an 11- residue, macrocyclic depsipeptide synthesized by *E. terrae* through the non-ribosomal peptide synthetases Txo1 and Txo2 (Ling et al., 2015). These peptides have unusual features which includes a methylated phenylalam=nine, four D-amino acids and the non-proteinogenic amino acid enduracidine. The amino acid sequence of teixobactin is MeHN—d-Phe—Ile—Ser——d-Gln—d-Ile—Ile—Ser—d-Thr*—Ala—enduracididine—Ile—CO—*. The carboxy terminus forms a lactone with the l-threonine residue (*), this is common in microbial nonribosomal peptides. The catalysation of the lactone-forming ring closure reaction by two C-terminal thioesterase domains of Txo2, forms a lactone (Ling et al., 2015). Txo1 and Txo2 are composed of 11 modules; these modules are said to sequentially add one

amino acid to a growing peptide chain. The first module has a methyltransferase domain that methylates the N-terminal phenylalanine (Ling et al., 2015).

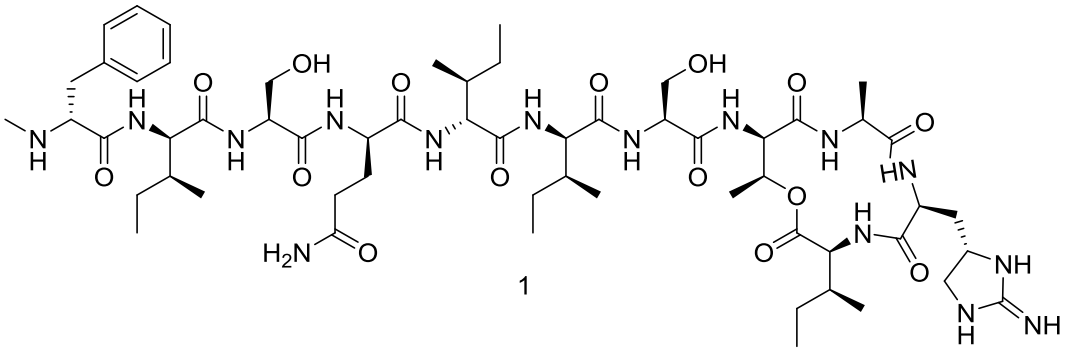


Figure 1: Structural representation of teixobactin

This small molecule antibiotic exerts its effect by binding to two precursors of bacterial cell-wall polymers lipid II (peptidoglycan) and lipid III (teichoic acid), preventing cell wall synthesis that results in lysis of susceptible bacteria (Ling et al., 2015; Piddock, 2015; Wright, 2015). This mechanism of action explains the antibiotics efficacy against Gram-positive bacteria rather than Gram-negative bacteria which have an outer membrane that prevents access to lipid II and lacks teichoic acid (Ling et al., 2015; Wright, 2015). Teixobactin is reported to be potent *in vitro* against all Gram-positive bacteria and has shown great activity against *Staphylococcus aureus*, resistant enterococci and *Mycobacterium tuberculosis* as well as *Bacillus anthracis* and *Clostridium difficile* (Ling et al., 2015; Piddock, 2015; Wright, 2015). *In vivo*, teixobactin was effective when used to treat MRSA and *Streptococcus pneumoniae* infected mice (Ling et al., 2015). Teixobactin is however not active against Gram-negative bacteria because of the presence of an outer membrane, more specially carbapenem resistant *Enterobacteriaceae* or pathogens that contain the New Delhi metallo-beta-lactamase 1(NDM1).

A detailed model for teixobactin activity and potential mechanisms of resistance is still in the foundation phases (Fiers et al., 2017).

Resistance to teixobactin has not been demonstrated in *S. aureus* and *M. tuberculosis* when these pathogens were exposed to high doses of teixobactin (Lewis, 2017; Ling et al., 2015; Piddock, 2015). It has been observed that resistance is less likely to develop against antibiotics that target the lipid molecules that are essential for cell-wall synthesis than antibiotics that target proteins (Anna and January, 2015; Wright, 2015). Thus implies that resistance through horizontal gene transfer from the producing organism is unlikely (Fiers et al., 2017; Ling et al., 2015). However numerous scientists caution that is it still too early to conclude that teixobactin resistance cannot develop (Anna and January, 2015). Vancomycin once showed promise and similar claims were made when it was discovered, however resistance developed very soon after large-scale use around the 1980s. With the continuation of antibiotic misuse and prolonged use in patients, mutations can occur thus resistance is inevitable (Arias and Murray, 2015). It is highly probable that genes encoding resistance to teixobactin may already be present in soil bacteria and it is only a matter of time until they emerge. The need for teixobactin derivatives is required to broaden the spectrum of activity of this antibiotic and to include derivatives that are also active against Gram-negative bacteria. Teixobactin is active against *M. tuberculosis*, thus offering an opportunity for a new treatment regime for TB (Piddock, 2015).

For novel compounds to be suitable as treatment options for infections in patients, preliminary *in vitro* screening is required to determine the cytotoxicity. This is followed by *in vivo* studies to understand the pharmacokinetics and pharmacodynamics of these compounds (Piddock, 2015).

1.4. *IN VITRO* ASSAYS

In vitro assays are used to examine the antibacterial activity of potential drugs/compounds; these include the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) determination assays. These assays are essential in determining the level of activity of novel compounds and establishes their dosing decisions.

1.4.1 Antimicrobial susceptibility techniques

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) are standard quantitative techniques used to determine antimicrobial susceptibility to novel agents (Brown and Brown, 1991). MIC refers to the minimum concentration of an antibiotic that inhibits the visible growth of a microorganism after incubation. MBC refers to the minimum concentration of the antibiotic that completely kills the microorganism (Andrews, 2001).

Agar dilution, broth dilution and disk diffusion are the standardized assays which are used for MIC testing (Andrews, 2001; Wiegand et al., 2008). To quantitatively obtain the *in vitro* activity of an antimicrobial agent, the agar dilution and broth dilution assays are employed. These assays are executed by introducing a standardized suspension of the test microorganism to a series of two-fold dilutions of the antimicrobial agent that is prepared in a solid or liquid medium. The MIC is verified as the lowest concentration of the antimicrobial agent that inhibited growth following an incubation period, specific to the microorganism (Wiegand et al., 2008).

1.4.1.1 Agar dilution

The agar dilution method is an established reference method used to determine MIC. This technique involves the combination of antimicrobial agents into agar with varying

concentrations on each agar plate. The microorganisms being tested are concurrently inoculated onto the agar surface. This is achieved using an inoculum-replicating apparatus. 32-36 different microorganisms can be inoculated onto each plate (CLSI, 2017). These plates are then incubated overnight and the MIC is read as the minimum concentration at which the microorganism's growth is inhibited.

1.4.1.2. Broth dilution

The broth dilution assay is the commonly used assay in antimicrobial susceptibility testing. Tube dilution method or Macrobroth is one of the first techniques used and is the gold standard for MIC determination. Antimicrobial agents are diluted (two-fold) in a test tube that contains a liquid (broth) growth medium. The limitation of this assay is that it requires a large amount of reagents and there is a very high possibility of human technical error when preparing the solutions of the antibiotics. By making use of 96 well microtitre plates to conduct this test, increased its practicality and popularity. This is known as the microdilution assay (Reller et al., 2009). The microtitre plates are able to accommodate up to 8 antibiotics in 12 two-fold dilutions or 12 antibiotics in 8 two-fold dilutions. According to CLSI guidelines, this assay has an accuracy of approximately 1 two-fold concentration (CLSI, 2017). Following incubation, MICs can either be determined by using an automated or manual device to inspect the growth of the microorganism (Reller et al., 2009). Resazurin is a cell viability dye that is also used to confirm if viable cells present in the solution (Collins and Franzblau, 1997; Moshayedi et al., 2012; Rampersad, 2012).

1.4.1.3. Disk diffusion test

Disk diffusion is a standardized susceptibility assay most commonly utilsed in diagnostic routine bacteriology laboratories. The antibiotic diffuses from a disk (containing a specific antibiotic concentration) into the agar medium containing the microorganism being tested

(CLSI., 2017). A zone of inhibition will appear if the antibiotic is able to inhibit the growth of the microorganism. The zone diameters are then compared to standardized references within the CLSI guidelines to determine the susceptibility profile of the microorganisms, making this a qualitative assay rather than quantitative. Various factors can affect the results of this assay; these factors include the thickness of the medium, the type of media used, the incubation times, and the concentration of the inoculum (Griffin et al., 2000).

1.4.2 Synergy test

Synergism is employed to determine if the effects of combination therapy results in better antimicrobial activity as compared to when the antimicrobial agents are tested individually (Doern, 2014). The checkerboard method and time kill kinetic assay is commonly used to determine this (White et al., 1996, 2001). The checkerboard assay is a technique employed decades ago where varying concentrations of an antimicrobial agent is combined with varying concentrations of a second antimicrobial agent, through agar dilutions or broth dilutions. The microdilution is the most commonly used method for checkerboard assay however both methods can be used to mix two antimicrobial agents in the plates. This assay can be achieved by either diluting the two antimicrobial agents before pipetting into the microdilution plates for serial dilutions or one antimicrobial agent is serial diluted in the microtitre plate and then the second antimicrobial agents, which has already been diluted, is added to the wells containing the first antimicrobial agent (Dougherty et al., 1977). The plates incubated and the results determined as per instructions for micro-broth dilution assay.

1.4.3 Serum effect

The serum effect assay, when conducted on novel compounds, aids in estimating the *in vivo* activity. Therefore, if the MIC of the novel compound significantly changes upon the introduction of serum, this indicates possible cellular interaction of the compounds being tested.

Based on whether the MIC increases or decreases when in the presence of serum, will determine if the interaction is extracellular or intracellular (Levison, 2004).

Antibodies are present in normal human serum; these antibodies destroy the cell wall/ membrane of bacteria through the classical and alternate complement pathways, thus resulting the dissolution of most bacterial cells (Melching and Vas, 1971; Osawa and Muschel, 1964; Pillemer et al., 1954). By heating the serum to 56^0C, the bactericidal and bacteriolytic proteins are damaged (Taylor, 1983). This assay also aids in determining the level of protein binding of the potential antimicrobial agents. Heating at 56^0C for 30 minutes inactivates the serum, after which it is then filtered and added to the Mueller-Hinton broth (Keepers et al., 2014; Osawa and Muschel, 1964). This assay determines the serum effect on the MIC of potential antimicrobial agents without the intervention of immunological effects. This assay along with those discussed earlier are huge contributors in the design and development of *in vivo* protocols for evaluating novel compounds and also influences the compounds dosage for preclinical trials.

1.5. JUSTIFICATION

The increase in bacterial resistance and the lack of new antimicrobial agents is a serious concern. The search for novel antimicrobial agents and their derivatives is a growing focus globally. Possible future lack of treatment options of resistant bacterial infections necessitates for detailed *in vitro* studies of potential antimicrobial agents for subsequent *in vivo* analysis of these agents.

1.6. AIMS

To evaluate the potential of novel teixobactin derivatives as antimicrobial agents using *in-vitro* pharmacodynamic parameters.

1.6.1 Specific objectives

The objectives of this study were:

- To determine the minimum inhibitory concentrations (MICs) of teixobactin derivatives according to the Clinical Laboratory Standards Institute guidelines using the broth microdilution assay (CLSI, 2017).
- To ascertain the minimum bactericidal concentrations (MBCs) and the serum effect on teixobactin derivatives according to Keepers et al., 2014.
- To determine the synergistic effect of teixobactin derivatives and vancomycin using the checkerboard method according to the CLSI guidelines (CLSI, 2017).
- To understand the time-kill kinetics of the derivatives according to Wang et al., 2015.
- To investigate the safety of these compounds using their haemolysis on red blood cells (RBCs) and cytotoxicity on peripheral blood mononuclear cells (PBMCs).

REFERENCES

Andrews, J. M. (2001). Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.* 48, Suppl. S I .5-16. doi:10.1093/jac/48.suppl_1.5.

Anna, B., and January, A. (2015). New Antibiotic from Soil Bacteria. *Nature* 517, 455–9. doi:10.1038/nature14098.

Arias, C. A., and Murray, B. E. (2012). The rise of the *Enterococcus*: beyond vancomycin resistance. *Nat. Rev. Microbiol.* 10, 266–278. doi:10.1038/nrmicro2761.

Arias, C. A., and Murray, B. E. (2015). A New Antibiotic and the Evolution of Resistance. *N. Engl. J. Med.* 372, 1168–1170. doi:10.1056/NEJMcibr1500292.

Brown, D. F. J., and Brown, L. (1991). Evaluation of the E test, a novel method of quantifying antimicrobial activity. *J. Antimicrob. Chemother.* 27, 185–190. doi.org/10.1093/jac/27.2.185

Centers for Disease Control and Prevention (CDC) (2011). Vancomycin-resistant *enterococci* (VRE) in healthcare settings. Available at: https://www.cdc.gov/hai/organisms/vre/vre.html. [Accessed: July 2017].

Chambers, H. F. (2001). Methicillin-resistant *Staphylococcus aureus*. Mechanisms of resistance and implications for treatment. *Postgrad. Med.* 109, 43–50. doi:10.3810/pgm.02.2001.suppl12.65.

Chopra, I. (2003). Antibiotic resistance in *Staphylococcus aureus*: concerns, causes and cures. *Expert Rev. Anti. Infect. Ther.* 1, 45–55. doi:10.1586/14787210.1.1.45.

CLSI (2017). *Clinical and Laboratory Standards Institute.* doi:10.4103/0976-237X.91790.

Coates, A. R. M. (2012). *Antibiotic resistance*. M.U. F. B. Hofmann, (Ed.), Handbook of

Experimental Pharmacology, Springer, London. 194

Collins, L. A., and Franzblau, S. G. (1997). Microplate Alamar blue assay versus BACTEC 460 system for high- throughput screening of compounds against Mycobacterium tuberculosis and Mycobacterium avium. *Antimicrob. Agents Chemother.* 41, 1004–1009.

Doern, C. D. (2014). When does 2 plus 2 equal 5? A review of antimicrobial synergy testing. *J. Clin. Microbiol.* 52, 4124–4128. doi:10.1128/JCM.01121-14.

Dougherty, P. F., Yotter, D. W., and Matthews, T. R. (1977). Microdilution transfer plate technique for determining in vitro synergy of antimicrobial agents. *Antimicrob. Agents Chemother.* 11, 225–228. doi: 10.1128/AAC.11.2.225

Drago, L., De Vecchi, E., Nicola, L., & Gismondo, M. R. (2007). *In vitro* evaluation of antibiotics' combinations for empirical therapy of suspected methicillin resistant *Staphylococcus aureus* severe respiratory infections. *BMC Infect Dis, 7*, 111. http://doi.org/10.1186/1471-2334-7-111

Dumancas, G. G., Hikkaduwa Koralege, R. S., Mojica, E. R. E., Murdianti, B. S., and Pham, P. J. (2014). "Penicillins," in *Encyclopedia of Toxicology*. Elsevier. 3, 768–772. doi:http://dx.doi.org/10.1016/B978-0-12-386454-3.00764-8.

Edlund, C., Barkholt, L., Olsson-Liljequist, B., and Nord, C. E. (1997). Effect of vancomycin on intestinal flora of patients who previously received antimicrobial therapy. *Clin. Infect. Dis.* 25, 729–732. doi.org/10.1086/513755

Fiers, W. D., Craighead, M., and Singh, I. (2017). Teixobactin and Its Analogues: A New Hope in Antibiotic Discovery. *ACS Infect. Dis.* 3, 688–690. doi:10.1021/acsinfecdis.7b00108.

Fong, I. W., and Drlica, K. (2008). Antimicrobial resistance and implications for the 21st

century. *Emerg. Infect. Dis.*, 411.

Goodman, G. L. (2011). Gilman's the pharmacological basis of therapeutics McGraw-Hill. *NY, USA*.

Griffin, S. G., Markham, J. L., and Leach, D. N. (2000). An agar dilution method for the determination of the minimum inhibitory concentration of essential oils. *J. Essent. Oil Res.* 12, 249–255. doi:10.1080/10412905.2000.9699509.

Gurusamy, K. S., Koti, R., Toon, C. D., Wilson, P., and Davidson, B. R. (2013). Antibiotic therapy for the treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) in non surgical wounds. *Cochrane Database Syst Rev* 11. doi 10.1002/14651858.CD010427

Hope, R., Livermore, D.M., Brick, G., Lillie, M. and Reynolds, R. (2008). Non-susceptibility trends among staphylococci from bacteraemias in the UK and Ireland, 2001–06. *J. of antimicrob. chemother.*, *62*(suppl_2), pp.ii65-ii74. doi.org/10.1093/jac/dkn353

Keepers, T. R., Gomez, M., Celeri, C., Nichols, W. W., and Krause, K. M. (2014). Bactericidal activity, absence of serum effect, and time-kill kinetics of ceftazidime-avibactam against β-lactamase-producing Enterobacteriaceae and *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* 58, 5297–5305. doi:10.1128/AAC.02894-14.

Khan, H. A., Ahmad, A., & Mehboob, R. (2015). Nosocomial infections and their control strategies. *Asian Pacific J. of Trop. Biomed*, *5*(7), 509-514.

Levine, D. P. (2006). Vancomycin: a history. *Clin. Infect. Dis.* 42, S5–S12. doi.org/10.1086/491709

Levison, M. E. (2004). Pharmacodynamics of antimicrobial drugs. *Infect. Dis. Clin. North Am.* 18, 451–465. doi:10.1016/j.idc.2004.04.012.

Lewis, K. (2017). New approaches to antimicrobial discovery. *Biochem. Pharmacol.* 134, 87–98. doi:10.1016/J.BCP.2016.11.002.

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature* 517, 455–459. doi:10.1038/nature14098

Liu, C., Bayer, A., Cosgrove, S. E., Daum, R. S., Fridkin, S. K., Gorwitz, R. J. (2011). Clinical practice guidelines by the Infectious Diseases Society of America for the treatment of methicillin-resistant Staphylococcus aureus infections in adults and children. *Clin. Infect. Dis.* 52. doi:10.1093/cid/ciq146.

Mahabeer, Y., Lowman, W., Govind, C.N., Swe-Swe-Han, K., and Mlisana, K. P. (2016). First outbreak of vancomycin-resistant *Enterococcus* in a haematology unit in Durban, South africai. *South. African J. Epidemiol. Infect.* 31, 26–30. doi:10.1080/23120053.2015.1118819.

Marshall, C. G., Lessard, I. A. D., Park, I. S., and Wright, G. D. (1998). Glycopeptide antibiotic resistance genes in glycopeptide-producing organisms. *Antimicrob. Agents Chemother.* 42, 2215–2220.

Melching, L., and Vas, S. I. (1971). Effects of serum components on Gram-negative bacteria during bactericidal reactions. *Infect. Immun.* 3, 107–115.

Meziane-Cherif, D., Saul, F. A., Haouz, A., and Courvalin, P. (2012). Structural and functional characterization of VanG D-Ala:D-Ser ligase associated with vancomycin resistance in *Enterococcus* faecalis. *J. Biol. Chem.* 287, 37583–37592. doi:10.1074/jbc.M112.405522.

Miller, W. R., Munita, J. M., & Arias, C. A. (2014). Mechanisms of antibiotic resistance in enterococci. *Expert review of anti-infective therapy*, *12*(10), 1221-1236.

Moshayedi, M., Barneh, F., Mirmohammadsadeghi, H., Sabzghabaee, A., and Javanmard, S. H. (2012). Set-up an alamarblue based viability assay as an alternative method to routine MTT tests. *Res. Pharm. Sci.* 7, 491.

Murray, P. R., Rosenthal, K. S., and Pfaller, M. A. (2015). *Medical microbiology 8th Edition*. Elsevier Health Sciences. [Accessed: June 2017].

Nichols, D., Cahoon, N., Trakhtenberg, E. M., Pham, L., Mehta, A., Belanger, A. (2010). Use of ichip for high-throughput in situ cultivation of "uncultivable" microbial species. *Appl. Environ. Microbiol.* 76, 2445–50. doi:10.1128/AEM.01754-09.

Orsi, G.B. and Ciorba, V., (2013). Vancomycin resistant enterococci healthcare associated infections. *Ann Ig*. *25*(6), pp.485-92. doi:10.7416/ai.2013.194885

Osawa, E., and Muschel, L. H. (1964). Studies relating to the serum resistance of certain Gram-negative bacteria. *J. Exp. Med.* 119, 41–51. doi 10.1084/jem.119.1.41

Pantosti, A., Sanchini, A. and Monaco, M., 2007. Mechanisms of antibiotic resistance in *Staphylococcus aureus*. *Future microbiol*. *2*(3), pp.323-334. doi.org/10.2217/17460913.2.3.323

Papp-Wallace, K. M., Endimiani, A., Taracila, M. A., and Bonomo, R. A. (2011). Carbapenems: Past, present, and future. *Antimicrob. Agents Chemother.* 55, 4943–4960. doi:10.1128/AAC.00296-11.

Piddock, L. J. V (2015). Teixobactin, the first of a new class of antibiotics discovered by ichip technology? *J. Antimicrob. Chemother.* 70, 2679–2680. doi:10.1093/jac/dkv175.

Pillemer, L., Blum, L., Lepow, I. H., Ross, O. A., Todd, E. W., and Wardlaw, A. C. (1954). The properdin system and immunity: I. Demonstration and isolation of a new serum protein,

properdin, and its role in immune phenomena. *Science (80).* 120, 279–285.

Rampersad, S. N. (2012). Multiple applications of alamar blue as an indicator of metabolic function and cellular health in cell viability bioassays. *Sensors (Switzerland)* 12, 12347–12360. doi:10.3390/s120912347

Rang, H. P., Ritter, J. M., Flower, R. J., and Henderson, G. (2014). *Rang & Dale's Pharmacology, 7th Edition.* Elsevier Churchill Livingstone. London, UK.

Rang, H.P., Ritter, J.M., Flower, R.J., Henderson, G. (2015). *Rang & Dale's Pharmacology, 8th Edition*. Elsevier Churchill Livingstone. London, UK.

Reller, L. B., Weinstein, M., Jorgensen, J. H., and Ferraro, M. J. (2009). Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clin. Infect. Dis.* 49, 1749–1755. doi:10.1086/647952.

Sista, R. R., Oda, G., & Barr, J. (2004). Methicillin-resistant *Staphylococcus aureus* infections in ICU patients. *Anesthesiology Clinics of North America*, *22*(3), 405-435.

Stone, J., and Judy, S. (2015). Teixobactin. "iChip Promise Hope against Antibiotic Resistance." Forbes.

Thabit, A.K., Crandon, J.L. and Nicolau, D.P., 2015. Antimicrobial resistance: impact on clinical and economic outcomes and the need for new antimicrobials. *Expert opinion on pharmacotherapy*, *16*(2), pp.159-177. doi.org/10.1517/14656566.2015.993381

Tacconelli, E. and Cataldo, M.A., 2008. Vancomycin-resistant enterococci (VRE): transmission and control. *Int. J. of antimicrob. agents*, *31*(2), pp.99-106. doi.org/10.1016/j.ijantimicag.2007.08.026

Taylor, P. W. (1983). Bactericidal and bacteriolytic activity of serum against Gram-negative

bacteria. *Microbiol. Rev.* 47, 46.

Van Bambeke, F. (2006). Glycopeptides and glycodepsipeptides in clinical development: a comparative review of their antibacterial spectrum, pharmacokinetics and clinical efficacy. *Curr. Opin. Investig. Drugs* 7, 740–749.

Ventola, C.L., 2015. The antibiotic resistance crisis: part 1: causes and threats. *Pharm. and Therap*, *40*(4), p.277.

Wang, D., Zhang, W., Wang, T., Li, N., Mu, H., Zhang, J. & Duan, J. (2015). Unveiling the mode of action of two antibacterial tanshinone derivatives. *Int. J. Mol. Sci.* 16**,** 17668-17681.

White, R. L., Burgess, D. S., Manduru, M., and Bosso, J. A. (1996). Comparison of three different in vitro methods of detecting synergy: Time-kill, checkerboard, and E test. *Antimicrob. Agents Chemother.* 40, 1914–1918.

White, R. L., Friedrich, L. V, Manduru, M., Mihm, L. B., and Bosso, J. A. (2001). Comparative in vitro pharmacodynamics of imipenem and meropenem against ATCC strains of Escherichia coli, Staphylococcus aureus and Bacteroides fragilis. *Diagn. Microbiol. Infect. Dis.* 39, 39–47.

Wiegand, I., Hilpert, K., and Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc.* 3, 163–175. doi:10.1038/nprot.2007.521.

Wright, G. (2015). Antibiotics: An irresistible newcomer. *Nature* 517, 442–444. doi:10.1038/nature14193.

CHAPTER TWO

In vitro evaluation of teixobactin derivatives against clinically relevant bacterial isolates

Estelle J. Ramchuran[1], Anou M. Somboro[1], Shimaa A. H. Abdel Monaim[2], Daniel Gyamfi Amoako[1], Raveen Parboosing[3], Beatriz G. de La Torre[4], Fernando Albericio[2,5,6], and Linda A. Bester[1*]

[1] Biomedical Resource Unit, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa

[2] Catalysis and Peptide Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa

[3] Department of Virology, National Health Laboratory Service, University of KwaZulu-Natal, Durban 4000, South Africa

[4] KRISP, College of Health Sciences, University of KwaZulu-Natal, Westville, Durban 4001, South Africa

[5] School of Chemistry and Physics, University of KwaZulu-Natal, University Road, Westville, Durban 4001, South Africa

[6] CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, and Department of Organic Chemistry, University of Barcelona, 08028-Barcelona, Spain

*__Corresponding author:__ Linda A. Bester, Biomedical Resource Unit, School of Laboratory Medicine and Medical Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban 4000, South Africa. **Email:** besterl@ukzn.ac.za, Tel: +27(0) 312607671.

Running title: Evaluation of teixobactin derivatives

Abstract:

Background: Methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) are part of the WHO high priority list of pathogens that needs urgent invention hence emphasis needs to be placed on developing novel class of molecules to overcome this scourge. Teixobactin is a new class of antibiotic, which has demonstrated antimicrobial activity ATCC strains and Gram-positive resistant bacteria. Herein, we are broadening the investigation of antimicrobial properties of teixobactin derivatives against clinically relevant bacterial isolates from South African patients.

Methods: The minimum inhibitory concentration (MIC), the minimal bactericidal concentration (MBC), the effect of serum on the MIC's and the time kill kinetics studies of three of our synthesized teixobactin derivatives **3**, **4** and **5** were ascertained according the CLSI 2017 guideline by the broth microdilution method. Haemolysis on red blood cells (RBCs) and cytotoxicity on peripheral blood mononuclear cells (PBMCs) were performed to investigate the safety of these compounds.

Results: MICs of the teixobactin derivatives against reference strains were between 4-64µg/ml **(3)**, 2-64µg/ml **(4)** and 0.5-64µg/ml **(5)**. The MIC's observed for MRSA were 32µg/ml for **(3)**, 2-4µg/ml for **(4)** and 2-4µg/ml for **(5)** whilst MIC's for VRE's were 8-16µg/ml for **(3)**, 4µg/ml for **(4)** and 2-16µg/ml for **(5)**. In the presence of 50% human serum, there was no significant effect on the MIC's. All the compounds did not have any effect on cell viability at their effective concentration.

Conclusion: Teixobactin derivatives (**3**, **4** and **5**) were able to inhibit bacterial growth in drug resistant bacteria and hence serve as potential antimicrobial agents.

Keywords: Teixobactin derivatives, biological activity, antimicrobial agents, resistant bacteria, antimicrobial peptides.

2.1. Introduction

The rate of antibiotic resistance is increasing faster than the development of new compounds for clinical practice; this is causing a public health crisis. Unfortunately, in an extremely short period, resistance to antibiotics has become a significant cause of disease and death globally (Brown and Wright, 2016; Penesyan et al., 2015; Hamilton and Wenlock, 2016). This worldwide collapse in collective research action to synthesize novel and efficient compounds, has contributed to the existing drug resistance disaster and the lack of new and efficient treatment options.

The first antibiotics were produced through screening soil microorganisms, but this limited resource of cultivable bacteria was over mined by the 1960s (Lewis, 2012). Synthetic approaches to produce antibiotics have been unable to replace this platform. An available source of new antibiotics is the uncultured bacteria which makes up 99% of all species in external environments (Kaeberlein et al., 2002; Fang et al., 2012; Nichols et al., 2010).

Teixobactin (**1,** Figure 1) is a new class of antibiotic that was discovered through screening of uncultured bacteria using the i-Chip (isolation chip), which is a revolutionary method for culturing bacteria (Ling et al., 2015; Piddock, 2015; von Nussbaum and Süssmuth, 2015). It was identified as an effective agent against Gram-positive bacteria, which inhibits cell wall synthesis through binding to two lipid cell wall precursors, lipid II (peptidoglycan precursor) and lipid III (teichoic acid precursor) (Homma et al., 2016; Jad et al., 2015; Ling et al., 2015). Teixobactin binds to the undecaprenyl-PP-sugar region of these precursors, which is known to be unmodified. Thus, making teixobactin the first example of a target-specific compound essentially free of resistance. Vancomycin also targets lipid II however, teixobactin binds to a different region, this is confirmed by its activity against VRE that posses modified lipid II (Ling et al., 2015; Homma et al., 2016; Jad et al., 2015; Parmar et al., 2016).

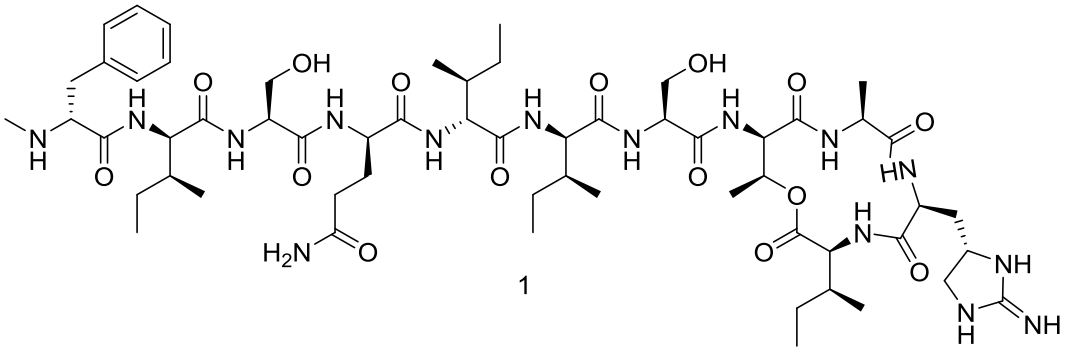


Figure 1: Chemical structure of teixobactin

Although much attention has shifted towards combating Gram-negative bacteria there is still a need for new compounds with novel mechanisms and low resistance profiles against Gram-positive bacteria. Hence Texiobactin can satisfy this need and aid in the treatment of resistant Gram-positive bacteria such as VRE and MRSA's. The main aim of this study is to evaluate novel derivatives of teixobactin (**3,4** and **5**) and investigate their biological properties toward clinically relevant Gram-positive resistant bacteria, including sensitive ATCC Gram-negative species.

2.2 - Materials and methods

2.2.1- Antibiotics and reagents

All the derivatives were dissolved in 5% DMSO. GIBCO® RPMI-1640 cell culture media (with HEPES, L-glutamine and sodium pyruvate) was obtained from Life Technolgies™ (Carlsbad, California, United States). Hyclone™ fetal bovine serum was purchased from GE Healthcare Life Sciences (Chicago, Illinois, United States). Phosphate Buffered Saline (PBS) was obtained from Lonza (Basel, Switzerland). Nunclon™ Delta Surface sterile microtiter plates (including the Edge 2.0 plate) were bought from Thermo Fisher Scientific™ (Waltham, Massachusetts, United States). Human serum from male AB plasma, sterile and filtered, antibiotics, antimycotic solution and all other reagents were obtained from Sigma (St. Louis, Missouri, United States).

2.2.2- Bacterial strains

Clinical isolates of MRSA and VRE were obtained from Lancet Laboratories, Durban, South Africa, ethical approval BE394/15 Biomedical Research Ethical Committee of the University of KwaZulu-Natal. Four reference strains of bacteria: *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Bacillus subtilis* ATCC 6051 and *Staphylococcus aureus* ATCC 29213 were obtained from American type of collection culture.

2.2.3 Synthesis, purification, and characterization of teixobactin derivatives

Teixobactin derivatives (**3, 4,** and **5**) (Figure 2) were synthesized, purified, and characterized as previously described (Abdel Monaim et al., 2017).

2.2.4- Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) determination

The MICs of the synthesized teixobactin derivatives were determined using the broth microdilution method according to the Clinical Laboratory Standard Institute CLSI (CLSI., 2017) Two-fold dilutions of each compound solution were prepared using cation adjusted Mueller–Hinton Broth (CAMHB) in a microtiter plate. A 0.5 McFarland-standardized bacterial inoculum (Biosan SIA DEN-1B densitometer) was used to prepare a total volume of 200µl in each microtiter well. The plates were incubated at 37°C for 18 - 20h. The MIC was determined as the lowest concentration at which there was no visible growth. Control wells for bacteria and media were also included. Meropenem, vancomycin and ampicillin were employed as standard control drugs. The plates containing VRE's were incubated using the Scientific series 2000 incubator at 35 °C under aerobic conditions. The MBC was determined as the lowest concentration of the test compound that was able to produce a 99.9% decrease in bacterial viable count on the agar plates. Control wells included the amount of solvent used in dissolving drug candidates, medium and bacteria.

2.2.5- Human serum effect in the MICs

The effect of serum on the MIC was performed similar to the MIC method described above however, in this case a 50 % human serum: Mueller-Hinton broth (Sigma Aldrich) was prepared.

2.2.6- Time-kill kinetic assays

Time-kill assays were performed using the broth micro-dilution method in accordance with the CLSI guidelines (CLSI., 2017). In this study, inoculum suspensions with approximately 10^6 (colony-forming units) CFU/ml of exponentially growing bacterial cells were used. 1:10. 1:100 and 1:1000, serial dilutions were performed. The test compound was added to 10mL of inoculum suspensions with final concentrations corresponding to 1x MIC, 2x MIC and/or 4x MIC.

Each test included a growth control that comprised of the bacterial strain without the test compound. The inoculum cultures were incubated at 37 °C. Aliquots were removed from the inoculum cultures after timed intervals of incubation (i.e. 0, 1, 2, 4, 6, 8 and 24 h), and serial ten-fold dilutions were prepared in CAMHB. The numbers of viable cells were determined by the plate count technique, which involved plating 100 µL of each dilution on a MHA plate (Wang et al., 2015).

2.2.7- Cell culture

Buffy coat was obtained from a healthy anonymous blood donor via the South African National Blood Service following approval by their Ethics Committee (National Health Laboratory Service Clearance Certificate number 2013/18). Aseptic technique and appropriate biosafety precautions were observed.

2.2.8- Haemolysis Assay on Red Blood Cells (RBCs)

The haemolysis assay was performed as previously described (Tramer et al., 2012), with modifications to allow for a 96-well microtiter plate format. Approximately 10 mL of blood was decanted from the buffy coat pack and spun down at 600g for 10 min in a Jouan BR4 centrifuge. The supernatant was removed by gentle aspiration and the pellet washed several times by centrifugation and re-suspension in buffer (PBS with EDTA and then PBS alone). After the final wash, the red cell pellet was re-suspended in 4 volumes of PBS (to obtain a hematocrit of approximately 20%). 10µL of the cells aliquoted into the wells of a clear, round bottom 96-well microtiter plate containing 170µL PBS and then lysed by addition of 20µL 1% Triton™ X-100 solution. A blank, containing 180µL PBS and 20µL Triton™ X-100 (1%) was also prepared. After 30 min, the samples were spun down at 3000 g for 5 min in an Orto Alresa Digicen 21R plate centrifuge.

The absorbance was read at 405nm in a Tecan Sunrise™ plate reader. The absorbance of the blank was subtracted from that of the lysed RBCs, and the concentration of the re-suspended RBCs was adjusted so that they would have an absorbance of approximately 1.5 upon lysis. This was confirmed by repeating the procedure on the adjusted red blood cell preparation.

Seven, serial 5-fold dilutions of the compounds were then prepared in triplicate by adding 25µL of the compound to 100µL PBS. Untreated controls (i.e. 0% hemolysis) and 100% hemolysis samples (with 20µL Triton™ X-100 and 80µL PBS) were included. Then appropriately diluted RBCs (10µL RBC and 90µL PBS per well) was added to the plate using a multichannel multistepper pipette.

The plate was incubated at 37°C for 30 min, spun down at 3000 g for 5 minutes in a plate centrifuge, the supernatants transferred to a new microtiter plate, and the absorbance read at 405nm. The viability of the red cells at each concentration of the compound was calculated as

follows: % viability = 100 x [1 – [A_t / (A_{100} - A_0)]] where A_t = mean absorbance of the test compound at a given concentration, A_0 = mean absorbance of the untreated control and A_{100} = mean absorbance of the sample lysed with Triton™ X-100. The results were represented graphically.

2.2.9- Cytotoxicity on peripheral blood mononuclear cells (PBMCs)

The cytotoxicity assay was performed as previously described (Araújo et al., 2013; Azumah et al., 2016; Pannecouque et al., 2008; Pinto et al., 2011). Briefly, PBMCs were isolated from the buffy coat pack using the Ficoll-Paque density gradient protocol (GE Healthcare, Munich, Germany) which entails layering of the buffy coat on the Ficoll-Paque reagent, centrifugation for 30 min and gentle aspiration of the lymphocyte layer, as described by the manufacturer. The lymphocytes were washed as recommended and the viability and cell count were determined on the Countess™ Automated Cell Counter (Thermo Fisher Scientific™, Waltham, Massachusetts, United States). The cells (100 000 viable cells/well) were then dispensed into wells of a Nunclon™ Delta Surface Edge 2.0 plate microtiter plate containing 100µl complete media (RPMI-1640 with 10% fetal bovine serum, 1% Antibiotic Antimycotic solution and 3% phytohemagglutinin). The cells were incubated in a Scientific Series 2000 incubator for 24 hours at 37°C and 5% CO_2. Then seven, serial 5-fold dilutions of the compounds were prepared in triplicate by adding 25µL of the compound to 100µL complete media in the wells of a separate microtiter plate. The dilutions were transferred to the appropriate wells of the plate containing the cells, which was then incubated for 72 hours at 37°C and 5% CO_2. Thereafter, 20ul of MTT salt (7.5mg/ml) was added to each well, and the plate was incubated for a further 4 hours. Then 100µl of the media was carefully removed from each well (avoiding agitation of the crystals) and replaced with 100µl of solubilisation solution (containing acidified isopropanol and Triton™ X-100). The plate was shaken on a plate shaker for 30 minutes to facilitate

complete dissolution of the crystals. The absorbance was read at 550nm (background: 690nm). The results were shown graphically.

2.3- Results

2.3.1- Teixobactin derivatives

Teixobactin is an 11-amino acid "head to side-chain" cyclodepsipeptide (**1,** Figure 1) with a D-Thr as a bridge head forming the ester with the carboxylic group of a L-Ile. L-Ala and the post-translational modified L-*allo*-enduracidine (End), which contains a cyclic guanidine, are also part of the cycle (Giltrap et al., 2016; Dhara et al., 2016; Jin et al., 2016; Yang et al., 2016; Abdel Monaim et al., 2016a; Yang et al., 2017; Wu et al., 2017; Jin et al., 2017; Parmar et al., 2017a; Parmar et al., 2017b; Abdel Monaim et al., 2017; Schumacher et al., 2017). The tail is formed by 2 moieties of L-Ser, 2 moieties of L-Ile, D-*allo*-Ile, D-Gln, ending with a *N*-Me-D-Phe. As L-*allo*-End was not commercially available, our group and others have concentrated their efforts in the synthesis of Arg_{10}-teixobactin (**2,** Figure 2), which L-*allo*-End has been substituted by Arg (Jad et al., 2015; Parmar et al., 2016)**.**

Arg_{10}-teixobactin (**2**), which has been converted as the parent teixobactin analogue, has slightly lower activity than teixobactin. Our group has prepared a short library of teixobactin analogues containing more than one Lys residue using a Lys-scanning strategy, which was absent in the natural structure (Abdel Monaim et al., 2016b; Abdel Monaim et al., 2017). From this collection of peptides, three (**3, 4 and 5,** Figure 2**)** identified as having good MIC's against ATCC bacteria and were selected for further *in vitro* evaluation in this study.

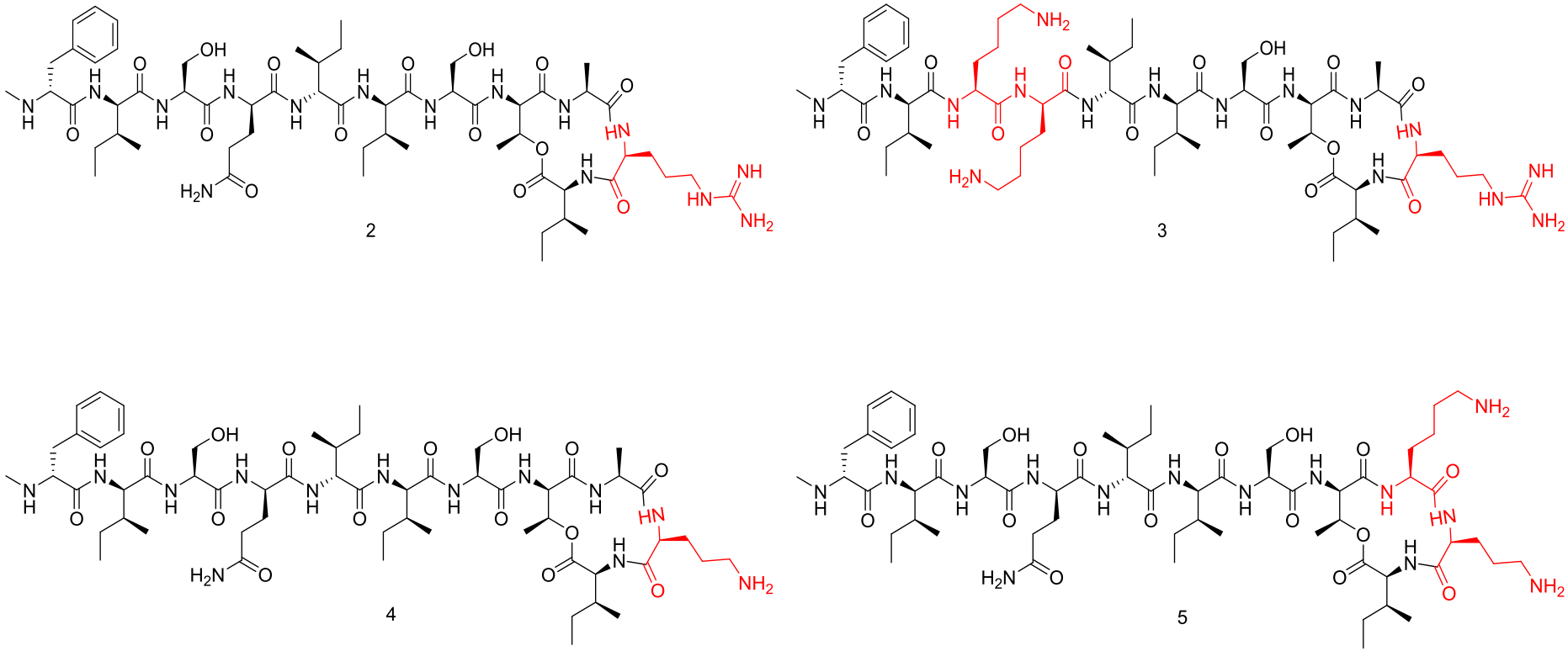


Figure 2: Chemical structure of teixobactin derivatives (**2**, **3**, **4** and **5**)

2.3.2- Antimicrobial activity of teixobactin derivatives and the effects of human serum on the MICs

The antimicrobial activity of the three derivatives (**3, 4** and **5**) was investigated by *in vitro* screening against drug-resistant and sensitive bacteria using the broth micro-dilution method in accordance with the CSLI guideline. As shown in Tables 1, 2 and 3, the derivatives were able to induce inhibition in both sensitive and resistant strains of the tested bacterial isolates. Teixobactin derivatives demonstrated potent antimicrobial activity against Gram-positive bacteria as opposed to Gram-negative bacteria. Three conventional antibiotics (meropenem, vancomycin and ampicillin), were used as control antimicrobial agents, these drugs only exhibited activity against the drug-sensitive strains of bacteria, but showed no activity against the resistant isolates with the exception of vancomycin which is known to be efficient against MRSA. The MIC_{50} were 32 μg/ml (**3**), 2 μg/ml (**5**/**4**) for MRSA, as well as 16 μg/ml (**3**) and 4 μg/ml (**5/4**) for VRE. In this study teixobactin derivatives yielded MIC's as low as 2 μg/ml and 0.5μg/ml for Gram-positive reference strains, *S. aureus* and *B. subtilis* respectively. The MIC's of the experimental compounds against susceptible Gram-negative bacteria were 32 μg/ml for *E. coli* and 64 μg/ml for *P. aeruginosa*. These compounds also inhibited drug-resistant clinical isolates of MRSA at concentrations of 32μg/ml, 4 μg/ml and 2 μg/ml for 3, 5 and 4 respectively. Vancomycin, being the current drug of choice for the treatment of MRSA, had an MIC of 1 μg/ml; while the MIC of ampicillin against MRSA was ≥512 μg/ml. Against VRE, the MIC's for 3, 5 and 4 were 16μg/ml, 4μg/ml and 8μg/ml respectively.

No significant effect of serum on the MIC were observed when the reference bacterial strains where tested with varying concentrations of the derivatives in presence of 50% human serum; the values only varied by ±1 in fold dilutions. Teixobactin derivatives (**3, 4** and **5**) demonstrated bactericidal activities against Gram- positive ATCC strains, which yielded a

99.9% decrease in viable cells on the agar plates at concentrations ≤ 4x the MIC values. All the experiments were conducted in triplicate to confirm the outcomes.

Table 1: Minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and MIC in presence of 50% human serum of teixobactin derivatives against susceptible reference strains of bacteria.

Antimicrobial agents	**Organism**											
	Gram-positive						**Gram-negative**					
	S. aureus ATCC 29213			***B. subtilis ATCC 6051***			***E. coli ATCC 25822***			***P. aeruginosa ATCC 27853***		
	MIC (µg/ml)	MBC (µg/ml)	50% serum MIC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	50% serum MIC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	50% serum MIC (µg/ml)	MIC (µg/ml)	MBC (µg/ml)	50% serum MIC (µg/ml)
3	32	**64**	64	4	**8**	2	64	**64**	>64	64	**128**	>64
4	4	**16**	4	2	**8**	1	64	**64**	>64	>64	**128**	>64
5	2	**8**	**4**	0.5	**1**	**0.5**	32	**64**	**>64**	64	**128**	**>64**
Meropenem	**0.25**	**ND**	**ND**	**0.125**	**ND**	**ND**	**0.125**	**ND**	**ND**	**1**	**ND**	**ND**

Table 2: Minimum inhibitory concentration (MIC) of teixobactin derivatives against methicillin resistant *Staphylococcus aureus (*MRSA).

Isolates	**Origin**	**Species**	**3**	**4**	**5**	**Vancomycin**	**Ampicillin**
			MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)
B11970	Blood	*S. aureus*	32	2	2	1	>512
P10781	Nasal	*S. aureus*	32	2	2	1	>512
P10747	CVP	*S. aureus*	32	2	2	1	>512
S37938	-	*S. aureus*	32	2	2	1	>512
S18155	ETT	*S. aureus*	32	2	2	0.5	>512
B13178	Blood	*S. aureus*	32	2	2	1	>512
440260	-	*S. aureus*	32	4	4	1	>512
S18970	-	*S. aureus*	32	2	2	1	>512
P11520	Pus	*S. aureus*	32	4	4	1	512
T5683	Nasal	*S. aureus*	32	2	2	1	>512
MIC50			32	2	2	1	>512

[a.] ETT, Endotracheal tube; CVP, Central venous catheter; ICU, - Missing data.

Table 3: Minimum inhibitory concentration (MIC) of teixobactin derivatives against Vancomycin-Resistant Enterococci (VRE).

Isolates	**Species**	**3**	**4**	**5**	**Vancomycin**
		MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)	MIC (µg/ml)
951245262 (A)	*Enterococcus faecium*	8	4	4	>128
951234856 (B)	*Enterococcus faecium*	16	4	4	>128
951208931 (C)	*Enterococcus faecium*	16	4	4	>128
938636470 (D)	*Enterococcus faecium*	16	8	4	>128
938666613 (E)	*Enterococcus faecium*	16	16	4	>128
938600912 (F)	*Enterococcus faecium*	16	2	8	>128
938072607 (G)	*Enterococcus faecium*	16	8	4	>128
944414000 (H)	*Enterococcus faecium*	16	8	4	>128
945530665 (I)	*Enterococcus faecium*	16	4	4	>128
U43821 (J)	*Enterococcus faecium*	16	8	4	>128
MIC50		16	4	4	>128

2.3.3- Time-kill kinetics

Time-kill kinetic assays were performed to determine whether teixobactin derivatives exhibited time-dependent or concentration-dependent properties as well as if their effects were bacteriostatic or bactericidal. The time-kill curves of the teixobactin derivative **4** against Gram-positive bacteria; *S. aureus* and *B. subtilis both* ATCC strains are represented in (Figure 3). The killing kinetics of the derivative indicated that the bacterial killing is time and concentration dependent, and the bactericidal effect was observed at concentration of 2 and 4x MIC levels at 6 hours, as well as at 1x MIC at 24 hours against *S. aureus* and *B. subtilis*. Exposure of *S. aureus and B. subtilis* to the test compound at 2x and 4x MIC resulted in decreasing bacterial cell count greater than 3 $\log_{10}$ relative to the initial density from 6 and 4 hours respectively, which was also indicative of a bactericidal effect. At a concentration of 1x the MIC, significant reduction in $\log_{10}$ CFU occurred after 6 hours, upon the addition of the compound 4 (Figure 3).

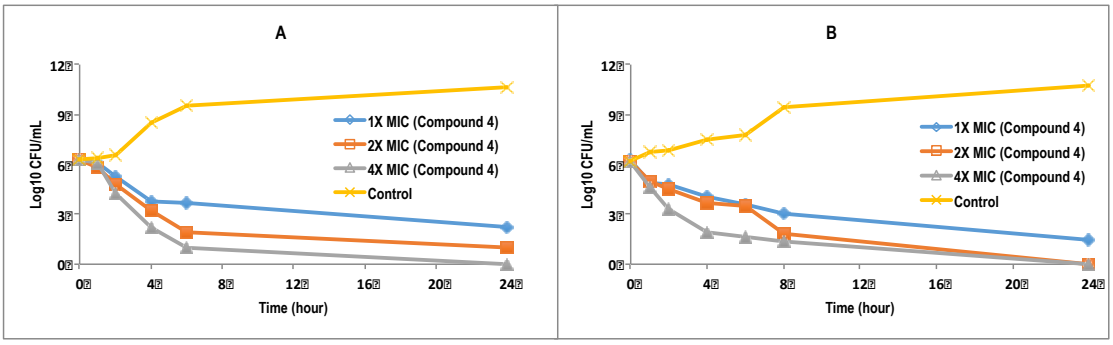


Figure 3*:* Time killing kinetics at different concentrations of teixobactin derivative 4. Gram-positive bacteria (*S. aureus and B. subtilis*) were challenged with compound 4 at 1x, 2x and 4x MIC levels.

2.3.4- Haemolysis and Cytotoxicity

The haemolysis and cytotoxicity effects were evaluated by exposing RBCs and PBMCs to teixobactin derivatives at varying concentrations. The tested concentrations of the compounds had neither cytotoxic effect on PBMCs nor any hemolytic effect on erythrocytes. The percent viability of RBCs and PBMCs were above 90% at the highest concentration used in this study (64ug/mL) (Figure 4).

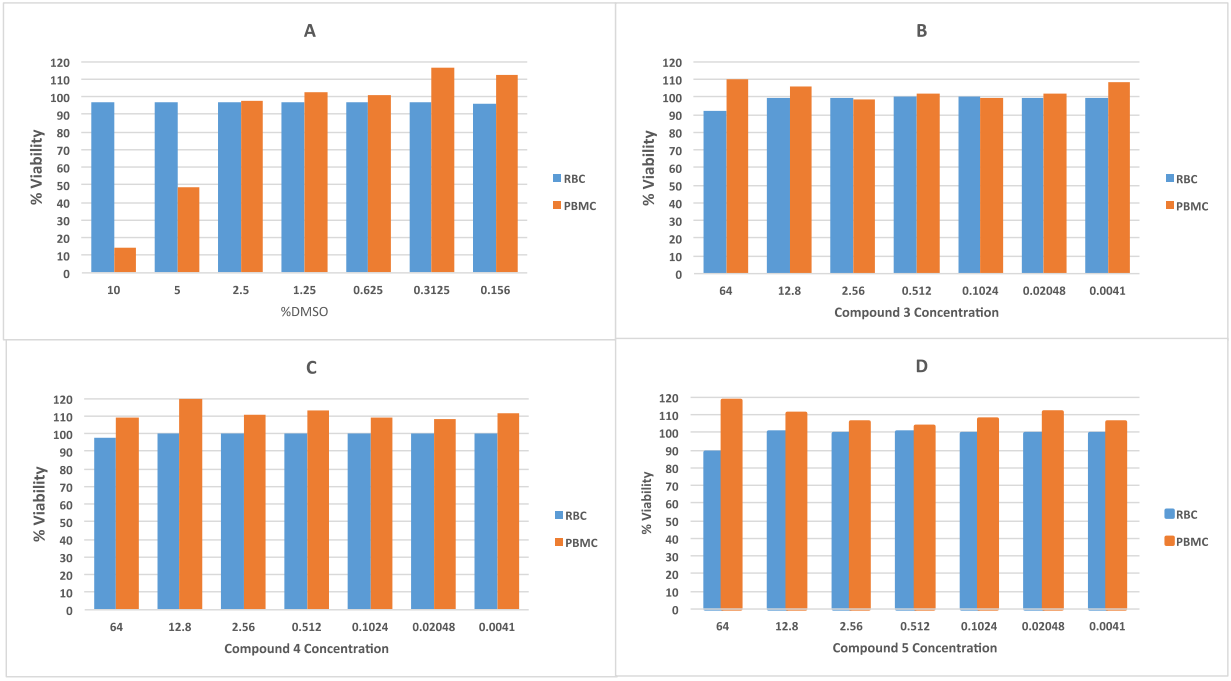


Figure 4: Haemolysis and cytotoxicity effects of teixobactin derivatives at various concentrations A) Viability (%) of RBCs and PBMCs treated with DMSO; B) Viability (%) of RBCs and PBMCs treated with compound 3; C) Viability (%) of RBCs and PBMCs treated with compound 4; D Viability (%) of RBCs and PBMCs treated with compound 5.

2.4- Discussion

Antibiotic resistance in Gram-positive bacteria is on the rise globally as indicated by the WHO list of high priority pathogens (i.e. VRE and MRSA) though much attention has shifted to combating Gram-negative bacteria. Teixobactin has been demonstrated as effective against

Gram-positive bacteria and no detectable resistance has been reported yet. This is because teixobactin is structurally distinct from glycopeptides and is the first member of a new class of lipid II binding antibiotics.

Compounds **4** and **5** MIC's for reference strains *S. aureus* and *B. subtilis* were between the ranges of 0.5-4µg/ml (Table 1), and demonstrated a range of 2-4µg/ml against the clinical MRSA isolates. **3** had an MIC of 32µg/ml, this was much higher than those reported for the control antibiotic, vancomycin, 0.5-1µg/ml. Other groups reported an MIC of 4µg/ml against MRSA using compound (Parmar et al., 2017a; Jin et al., 2016; Schumacher et al., 2017). These results were echoed in this study, as the MIC's observed against MRSA using compound 3 was between 2-4µg/ml. The MIC's observed for the three derivatives make them good candidates as potential antimicrobial agents

The MBC reported by Ling et al, 2015 was 2x the MIC of teixobactin. The bactericidal activity of teixobactin and its derivatives against Gram-positive bacteria is superior to that of vancomycin, and it retains excellent bactericidal activity against VRE (Ling et al., 2015). This excellent bactericidal activity of teixobactin and its derivatives is reported to be due to not only inhibition of peptidoglycan synthesis but also the synergistic inhibition of wall teichoic acid synthesis. These derivatives echo the same bactericidal activity that is observed for teixobactin. The MIC/MBC ratios were ≤4 for all the three derivatives (**3**, **4** and **5**).

Time kill kinetic assays were carried out with compound **4** as it exhibited the best MIC's against *S. aureus* and *B. subtilis*. Complete bactericidal activity was observed at concentrations of 16 and 8µg/ml at 4h. Much like that observed in studies on other teixobactin derivatives, these derivatives have no cytotoxic or hemolytic effect *in vitro*. In the presence of 50% serum there was no drastic change in the MIC's. The MIC's obtained with serum were 1x higher or equivalent to the MIC (Table 1).

Thus we can conclude that human serum has no effect on the antibacterial activity of the derivatives (**3, 4** and **5**). These results are similar to those observed by Parmar et al, 2017 (Parmar et al., 2017a). Although not sufficient to conclude, the serum effect is essential as it aids in speculating the probable *in vivo* activity of the drug. These derivatives will possibly have low protein binding properties because they bind to multiple target sites on the bacterial cell, none of which are proteins. The present study confirms that Teixobactin derivatives **3, 4** and **5** are safe and can thus be considered potential treatment options against sensitive and resistant bacteria. These results suggest that urgent *in vitro* pharmacodynamics and *in vivo* studies are required.

Interestingly in this study we observed that the derivatives were also active against Gram-negative bacteria but at higher concentrations (Table 1), which can be an important result taking into consideration the low toxicity of these compounds. These derivatives may exert their activity against Gram-negative bacteria by disrupting the outer membrane.

In conclusion, we have demonstrated the highly potent antimicrobial activity of three teixobactin derivatives against clinically significant isolates of bacteria. Unlike vancomycin, these derivatives have produced early stage killing kinetics. This study will further aid in the development of other teixobactin derivatives that can also lead to high potent antimicrobial activity against resistant bacterial strains and contribute to the development of novel peptide based antimicrobial agents to assist with the global threat of drug resistance.

Author Contributions

Conceived and designed the experiments: EJR, AMS, SAHAM, BGLT and FA. Performed the experiments: EJR, AMS, SAHAM and DGA. Analyzed the data: EJR, AMS, DGA and RP. Contributed to reagents/materials/analysis tools: RP, BGLT, FA and LAB. Wrote paper: EJR. Critical revision for Manuscript: AMS, DGA, SAHAM, BGLT, FA and LAB.

Acknowledgements: This study was supported by College of Health Sciences, University of KwaZulu- Natal, Durban, South Africa and the South African National Research Foundation (NRF).

Conflict of Interest: None

Abbreviation

EJR, Estelle J. Ramchuran; AMS, Anou M. Somboro; SAHAM, Shimaa A. H. Abdel Monaim; DGA, Daniel Gyamfi Amoako; RP, Raveen Parboosing; BGLT, Beatriz G. de La Torre; FA, Fernando Albericio; LAB, Linda A. Bester.

REFERENCES

Abdel Monaim, S. A., Jad, Y. E., Ramchuran, E. J., El-Faham, A., Acosta, G. A., Naicker, T., Govender, T., Kruger, H. G., De La Torre, B. G. & Albericio, F. (2016a). Re-evaluation of the N-terminal substitution and the D-residues of teixobactin. *RSC Advances*. 6**,** 73827-73829.

Abdel Monaim, S. A., Jad, Y. E., Ramchuran, E. J., El-Faham, A., Govender, T., Kruger, H. G., De La Torre, B. G. & Albericio, F. (2016b). Lysine scanning of Arg10–teixobactin: deciphering the role of hydrophobic and hydrophilic residues. *ACS Omega*. 1**,** 1262-1265.

Abdel Monaim, S. A., Ramchuran, E. J., El-Faham, A., Albericio, F. & De La Torre, B. G. (2017a). Converting teixobactin into a cationic antimicrobial peptide (AMP). *J. Med.Chem*. 60**,** 7476-7482.

Abdel Monaim, S. A., Noki, S., Ramchuran, E. J., El-Faham, A., Albericio, F. & Torre, B. G. (2017b). Investigation of the N-Terminus Amino Function of Arg10-teixobactin. *Molecules*. 22**,** 1632.

Araújo, L. C. C., Aguiar, J. S., Napoleão, T. H., Mota, F. V. B., Barros, A. L. S., Moura, M. C., Coriolano, M. C., Coelho, L. C. B. B., Silva, T. G. & Paiva, P. M. G. (2013). Evaluation of cytotoxic and anti-inflammatory activities of extracts and lectins from Moringa oleifera seeds. *PLoS One*. 8**,** e81973.

Azumah, R., Dutta, J., Somboro, A., Ramtahal, M., Chonco, L., Parboosing, R., Bester, L., Kruger, H., Naicker, T. & Essack, S. (2016). In vitro evaluation of metal chelators as potential metallo-β-lactamase inhibitors. *J. Appl. Microbiol*. 120**,** 860-867.

Brown, E. D. & Wright, G. D. (2016). Antibacterial drug discovery in the resistance era. *Nature*. 529**,** 336-343.

Clinical and Laboratory Standards Institute. *CLSI*. (2017).

Dhara, S., Gunjal, V. B., Handore, K. L. & Srinivasa Reddy, D. (2016). Solution-phase synthesis of the macrocyclic core of teixobactin. *Eur. J. Org. Chem.* 4289-4293.

Fang, X., Chen, H., Xu, L., Jiang, X., Wu, W. & Kong, J. (2012). A portable and integrated nucleic acid amplification microfluidic chip for identifying bacteria. *Lab on a Chip*. 12**,** 1495-1499.

Giltrap, A. M., Dowman, L. J., Nagalingam, G., Ochoa, J. L., Linington, R. G., Britton, W. J. & Payne, R. J. (2016). Total Synthesis of teixobactin. *Org. Lett*. 18**,** 2788-2791.

Hamilton, W. L. & Wenlock, R. (2016). Antimicrobial resistance: A major threat to public health. *Cambridge Med. J*. Doi:10.7244/cmj.2016.01.001.

Homma, T., Nuxoll, A., Gandt, A. B., Ebner, P., Engels, I., Schneider, T., Götz, F., Lewis, K. & Conlon, B. P. (2016). Dual targeting of cell wall precursors by teixobactin leads to cell lysis. *Antimicrob. Agents and Chemoth*. 60**,** 6510-6517.

Jad, Y. E., Acosta, G. A., Naicker, T., Ramtahal, M., El-Faham, A., Govender, T., Kruger, H. G., Torre, B. G. D. L. & Albericio, F. (2015). Synthesis and biological evaluation of a teixobactin analogue. *Org. lett*. 17**,** 6182-6185.

Jin, K., Sam, I. H., Po, K. H. L., Lin, D. A., Ghazvini Zadeh, E. H., Chen, S., Yuan, Y. & Li, X. (2016). Total synthesis of teixobactin. *Nat. Commun.* 7**,** 12394. DOI: 10.1038/ncomms12394.

Jin, K., Po, K. H. L., Wang, S., Reuven, J. A., Wai, C. N., Lau, H. T., Chan, T. H., Chen, S. & Li, X. (2017). Synthesis and structure-activity relationship of teixobactin analogues via convergent Ser ligation. *Bioorg. Med. Chem*. 25: 4990-4995.

Kaeberlein, T., Lewis, K. & Epstein, S. S. (2002). Isolating" uncultivable" microorganisms in pure culture in a simulated natural environment. *Science*. 296**,** 1127-1129.

Lewis, K. (2012). Antibiotics: recover the lost art of drug discovery. *Nature*. 485**,** 439-440.

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., Mueller, A., Schäberle, T. F., Hughes, D. E. & Epstein, S. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature*. 517**,** 455-459.

Nichols, D., Cahoon, N., Trakhtenberg, E., Pham, L., Mehta, A., Belanger, A., Kanigan, T., Lewis, K. & Epstein, S. (2010). Use of ichip for high-throughput in situ cultivation of "uncultivable" microbial species. *Appl. and Env. Microbio.* 76**,** 2445-2450.

Pannecouque, C., Daelemans, D. & De Clercq, E. (2008). Tetrazolium-based colorimetric assay for the detection of HIV replication inhibitors: revisited 20 years later. *Nature protocols*. 3**,** 427-434.

Parmar, A., Iyer, A., Lloyd, D. G., Vincent, C. S., Prior, S. H., Madder, A., Taylor, E. J. & Singh, I. (2017a). Syntheses of potent teixobactin analogues against methicillin-resistant Staphylococcus aureus (MRSA) through the replacement of l-allo-enduracididine with its isosteres. *Chem. Comm*. 53**,** 7788-7791.

Parmar, A., Iyer, A., Vincent, C. S., Van Lysebetten, D., Prior, S. H., Madder, A., Taylor, E. J. & Singh, I. (2016). Efficient total syntheses and biological activities of two teixobactin analogues. *Chem. Comm*. 52**,** 6060-6063.

Parmar, A., Prior, S. H., Iyer, A., Vincent, C. S., Van Lysebetten, D., Breukink, E., Madder, A., Taylor, E. J. & Singh, I. (2017b). Defining the molecular structure of teixobactin analogues and understanding their role in antibacterial activities. *Chem. Commu*. 53**,** 2016-2019.

Penesyan, A., Gillings, M. & Paulsen, I. T. (2015). Antibiotic discovery: combatting bacterial resistance in cells and in biofilm communities. *Molecules*. 20**,** 5286-5298.

Piddock, L. J. (2015). Teixobactin, the first of a new class of antibiotics discovered by iChip technology? *J. Antimicrob. Chemoth*. 70**,** 2679-2680.

Pinto, M. C., Dias, D. F., Del Puerto, H. L., Martins, A. S., Teixeira-Carvalho, A., Martins-Filho, O. A., Badet, B., Durand, P., Alves, R. J. & Souza-Fagundes, E. M. (2011). Discovery of cytotoxic and pro-apoptotic compounds against leukemia cells: Tert-butyl-4-[(3-nitrophenoxy) methyl]-2, 2-dimethyloxazolidine-3-carboxylate. *Life Sci*. 89**,** 786-794.

Schumacher, C. E., Harris, P. W., Ding, X.-B., Krause, B., Wright, T. H., Cook, G. M., Furkert, D. P. & Brimble, M. A. (2017). Synthesis and biological evaluation of novel teixobactin analogues. *Org. Biomol. Chem.* 15**,** 8755-8760.

Tramer, F., Da Ros, T. & Passamonti, S. (2012). Screening of fullerene toxicity by hemolysis assay. *Nanotoxicity: Methods and Protocols*. 203-217.

Von Nussbaum, F. & Süssmuth, R. D. (2015). Multiple attack on bacteria by the new antibiotic teixobactin. *Ang. Chemie Inter. Edition*. 54**,** 6684-6686.

Wang, D., Zhang, W., Wang, T., Li, N., Mu, H., Zhang, J. & Duan, J. (2015). Unveiling the mode of action of two antibacterial tanshinone derivatives. *Int. J. Mol. Sci.* 16**,** 17668-17681.

Wu, C., Pan, Z., Yao, G., Wang, W., Fang, L. & Su, W. (2017). Synthesis and structure–activity relationship studies of teixobactin analogues. *RSC Advances*. 7**,** 1923-1926.

Yang, H., Chen, K. H. & Nowick, J. S. (2016). Elucidation of the teixobactin pharmacophore. *ACS Chem. Biol*. 11**,** 1823-1826.

Yang, H., Du Bois, D., Ziller, J. & Nowick, J. (2017). X-ray crystallographic structure of a teixobactin analogue reveals key interactions of the teixobactin pharmacophore. *Chem. Commun*. 53**,** 2772-2775.

CHAPTER THREE

Conclusion and Recommendations

3.1 CONCLUSION

This study evaluated novel teixobactin derivatives as potential antibiotics. Teixobactin derivatives have shown good activity against sensitive Gram- positive and Gram- negative bacteria as well as clinically significant bacterial isolates. The MIC's observed for MRSA was between 2-4μg/ml. The MIC's obtained for VRE's were between 4-16μg/ml. The MIC/MBC ratio was ≤4, this result was consistent with all three derivatives and retains excellent bactericidal activity against VRE. The derivatives were able to inhibit bacterial growth in drug resistant bacteria and therefore serve as potential antimicrobial agents. Complete bactericidal activity was observed at 4 h at concentrations of 16 and 8μg/ml. These derivatives have no cytotoxic and hemolytic effect *in vitro*. In the presence of 50% human serum, no drastic change (values were the same as the MIC or one concentration higher than the MIC) in the MIC's was observed indicating that human serum has no effect on the antimicrobial activity of the derivatives. Interestingly in this study we observed that at higher concentrations (32- 64μg/ml), the derivatives were also active against Gram-negative bacteria. This observation is vital, considering the low toxicity of these compounds. This study confirms that these derivatives are safe and can serve as potential antimicrobial agents for the treatment of both sensitive and resistant bacteria.

3.2 RECOMMENDATIONS

- During this study, *in vitro* assays were largely examined. Based on our findings these molecules are potential antimicrobial drug candidates that need further investigations.

Therefore, we recommend *in vivo* studies to ascertain their potential therapeutic efficacy and to provide in-depth understanding of their pharmacokinetic and pharmacodynamic properties.

- Designing and developing analogues/derivatives of these potent teixobactin derivatives investigated in this project might improve their pharmacological properties and extend their inhibitory spectrum to cover more Gram positive and negative resistant pathogenic bacteria.

APPENDIX I

Submission confirmation to the Journal of Frontiers Microbiology, manuscript ID 340217

In vitro evaluation of Teixobactin derivatives against clinically relevant bacterial isolates

Estelle J. Ramchuran[1], Anou M. Somboro[1], Shimaa A. Abdel Monaim[2], Daniel G. Amoako[1], Raveen Parboosing[3], Beatriz G. De la Torre[4], Fernando Albericio[2, 5, 6], Linda A. Bester[1*]

[1]Biomedical Resource Unit, University of KwaZulu-Natal, South Africa, [2]School of Health Sciences, University of KwaZulu-Natal, South Africa, [3]Department of Virology, University of KwaZulu-Natal, South Africa, [4]KRISP, University of KwaZulu-Natal, South Africa, [5]School of Chemistry and Physics, University of KwaZulu-Natal, South Africa, [6]CIBER-BBN, University of Barcelona, Spain

Submitted to Journal:
Frontiers in Microbiology

Specialty Section:
Antimicrobials, Resistance and Chemotherapy

Article type:
Original Research Article

Manuscript ID:
340217

Received on:
13 Dec 2017

Frontiers website link:
www.frontiersin.org

APPENDIX II

Ethics Approval from Biomedical Research Ethics Committee UKZN, Ref BE394/15

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

11 November 2015

Mr DG Amoako (214583994)
Department of Pharmaceutical Sciences
School of Health Sciences
castlicky2010@gmail.com

Dear Mr Amoako

Protocol: Molecular characterization of genetic determinants of resistance and virulence in methicillin resistant staphylococcus aureus (MRSA) from the private sector in KwaZulu-Natal, South Africa.
Degree: MMedSc
BREC reference number: BE394/15

EXPEDITED APPLICATION

A sub-committee of the Biomedical Research Ethics Committee has considered and noted your application received on 02 September 2015.

The conditions have been met and the study is given full ethics approval.

This approval is valid for one year from **11 November 2015.** To ensure uninterrupted approval of this study beyond the approval expiry date, an application for recertification must be submitted to BREC on the appropriate BREC form 2-3 months before the expiry date.

Any amendments to this study, unless urgently required to ensure safety of participants, must be approved by BREC prior to implementation.

Your acceptance of this approval denotes your compliance with South African National Research Ethics Guidelines (2015), South African National Good Clinical Practice Guidelines (2006) (if applicable) and with UKZN BREC ethics requirements as contained in the UKZN BREC Terms of Reference and Standard Operating Procedures, all available at http://research.ukzn.ac.za/Research-Ethics/Biomedical-Research-Ethics.aspx.

BREC is registered with the South African National Health Research Ethics Council (REC-290408-009). BREC has US Office for Human Research Protections (OHRP) Federal-wide Assurance (FWA 678).

The sub-committee's decision will be RATIFIED by a full Committee at its meeting taking place on 08 December 2015.

We wish you well with this study. We would appreciate receiving copies of all publications arising out of this study.

Yours sincer[redacted]

Pr[redacted] Tsoka-Gwegweni
Chair: Biomedical Research Ethics Committee

cc supervisor: [redacted]
cc postgrad: [redacted]

Biomedical Research Ethics Committee
Professor J Tsoka-Gwegweni (Chair)
Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
Telephone: +27 (0) 31 260 2486 Facsimile: +27 (0) 31 260 4609 Email: [redacted]
Website: [redacted]

1910 - 2010
100 YEARS OF ACADEMIC EXCELLENCE

Edgewood Howard College Medical School Pietermaritzburg Westville

APPENDIX III

Publications not include in this thesis

molecules

MDPI

Communication

Investigation of the *N*-Terminus Amino Function of Arg_{10}-Teixobactin

Shimaa A. H. Abdel Monaim [1], Sikabwe Noki [1], Estelle J. Ramchuran [1], Ayman El-Faham [2,3], Fernando Albericio [1,2,4,5,6,*] and Beatriz G. de la Torre [1,7,*]

1 Catalysis and Peptide Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban 4001, South Africa; shimaa.amin10@yahoo.com (S.A.H.A.M.); sikabwenoki7@gmail.com (S.N.); ramchurane@ukzn.ac.za (E.J.R.)
2 Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia; aelfaham@ksu.edu.sa
3 Chemistry Department, Faculty of Science, Alexandria University, P.O. Box 426, Ibrahimia, Alexandria 12321, Egypt
4 School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001, South Africa
5 Department of Organic Chemistry, University of Barcelona, Barcelona 08028, Spain
6 CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, Barcelona 08028, Spain
7 KRISP, College of Health Sciences, University of KwaZulu-Natal, Durban 4001, South Africa
* Correspondence: albericio@ukzn.ac.za (F.A.); garciadelatorreb@ukzn.ac.za (B.G.d.l.T.); Tel.: +27-614-047-528 (F.A.); +27-614-009-144 (B.G.d.l.T.)

Received: 28 August 2017; Accepted: 25 September 2017; Published: 28 September 2017

Abstract: Teixobactin is a recently described antimicrobial peptide that shows high activity against gram-positive bacteria as well as *mycobacterium tuberculosis*. Due to both its structure as a head-to-side chain cyclodepsipeptide and its activity, it has attracted the attention of several research groups. In this regard, a large number of analogs with substitutions in both the cycle and the tail has been described. Here, we report the contribution of the *N*-terminus residue, *N*-Me-D-Phe, to the activity of Arg_{10}-teixobactin. On the basis of our findings, we conclude that the *N*-terminus accepts minimum changes but not the presence of long alkyl chains. The presence of a positive charge is a requirement for the activity of the peptide. Furthermore, acylation of the *N*-terminus leads to total loss of activity.

Keywords: antimicrobial peptides; teixobactin; lipophilicity; solid-phase peptide synthesis; cyclic depsipeptides

1. Introduction

The isolation of a new anti-microbial peptide (AMP) called teixobactin in 2015 by Ling et al. raised great expectations because it is one of the few new antibiotics to have been reported in recent years [1,2]. This antibiotic shows high activity against gram-positive bacteria as well as *Mycobacterium tuberculosis*. Teixobactin (Figure 1) is a head-to-side chain cyclodepsipeptide that contains hydrophilic and hydrophobic residues [3,4]. Thus, the tail of the natural peptide contains four polar amino acids, L-*allo*-enduracididine, two Ser, and D-Gln, and four non-polar Ile residues, one Ala and one *N*-Me-D-Phe residue at the *N*-terminus. Finally, a D-Thr is the branching unit to form the cyclic moiety.

Journal of Medicinal Chemistry

Article

pubs.acs.org/jmc

Converting Teixobactin into a Cationic Antimicrobial Peptide (AMP)

Shimaa A. H. Abdel Monaim,[†] Estelle J. Ramchuran,[†] Ayman El-Faham,[‡,§] Fernando Albericio,[*,†,‡,∥,⊥,#] and Beatriz G. de la Torre[*,†,¶]

[†]Catalysis and Peptide Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban 4001, South Africa

[‡]Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

[§]Chemistry Department, Faculty of Science, Alexandria University, P.O. Box 426, Ibrahimia, Alexandria 12321, Egypt

[∥]School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001, South Africa

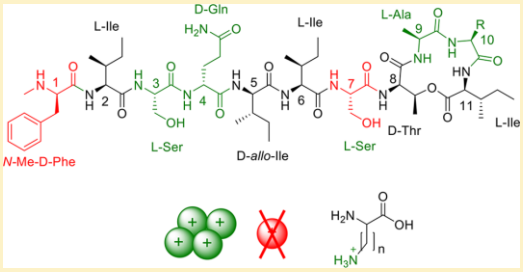
[⊥]Department of Organic Chemistry, University of Barcelona, 08028-Barcelona, Spain

[#]CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, 08028-Barcelona, Spain

[¶]KRISP, College of Health Sciences, University of KwaZulu-Natal, Durban 4001, South Africa

Supporting Information

ABSTRACT: Teixobactin is a head to side chain cyclodepsipeptide that contains two positive charges. One is found in the cycle, as a result of the presence of the guanidino-unusual amino acid L-*allo*-End, while the other is at the *N*-terminal. Here we introduce 26 new Teixobactin analogues with an increasing number of positive charges. In an attempt to fine-tune the biological activity of Teixobactin, we examined the effect of cationicity on the SAR of these analogues. The maximum number of positive charges to maintain the activity are three to four. Analogues with five positive charges show the lowest activity.



INTRODUCTION

Cationic antimicrobial peptides (AMPs) are a crucial component of the innate immune defense of almost all living organisms, since they are natural antibiotics with the capacity to kill a wide range of pathogens. Moreover, cationic AMPs also display high therapeutic potential. In this regard, they have been reported to exert diverse immunomodulatory activity,[1,2] and activity against skin cancer and also infectious skin diseases.[3,4]

Cationic AMPs are short−medium size amphipathic peptides. As such, they have a hydrophilic character as a result of the presence of a relatively high percentage of Arg and Lys residues. These cationic amino acids confer aqueous solubility to the peptide and also allow for electrostatic interaction with the negatively charged bacterial cell wall,[5,6] while the hydrophobic character of the peptide facilitates interaction with the lipid bilayer membrane of bacteria.[7,8]

One of the proposed mechanisms of action for cationic AMPs is inhibition of the cell wall biosynthesis via binding to lipid II, the precursor of cell wall synthesis in Gram-positive microorganisms. For example, plectasin binds to lipid II and prevents its incorporation into the peptidoglycan chain.[9] Human β-defensin 3 (hBD3), which is also a highly charged (+11) cationic host defense peptide, inhibits cell wall synthesis by interacting with the protein involved in lipid II synthesis.[10]

A new AMP, Teixobactin, was isolated from *Eleftheria terraea*, a nonculturable Gram-negative bacterium that belongs to the class of beta-proteobacteria.[11] Teixobactin is one of the first new antibiotics to be discovered in several decades and is attracting attention not only because of its strong activity against Gram-positive bacteria and *Mycobacterium tuberculosis*[12−14] but also because of its lack of known bacterial resistance. It is believed that the mode of action of Teixobactin is the inhibition of cell wall synthesis via the interaction with lipid II and teichoic acid, the precursor of lipid III. This mode of action explains the nonresistance of bacteria against Teixobactin.[11]

From a chemical point of view, Teixobactin is an 11-mer cyclodepsipeptide (head to side chain) (Figure 1) consisting of a seven-residue tail connected to the D-Thr residue at position 8, which is the bridge head unit and forms a four-residue cyclic moiety upon lactonization of the D-Thr hydroxyl function with the Ile carboxy terminus. It contains five nonproteinogenic amino acids: four D-amino acids, namely, *N*-methyl-D-Phe at the *N*-terminus, D-Gln at position 4, D-allo-Ile, at position 5, D-Thr, at position 8 (involved in the cyclic part), and L-*allo*-enduracididine (End) (Figure 1, A) as part of the cycle, which is the source of a positive charge in the molecule.[11]

Received: June 9, 2017
Published: August 10, 2017

DOI: 10.1021/acs.jmedchem.7b00834
J. Med. Chem. 2017, 60, 7476−7482

DOI: 10.1039/C6RA17720D

JournalName

COMMUNICATION

Re-Evaluation of the *N*-Terminal Substitution and the D-Residues of Teixobactin

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

Shimaa A. H. Abdel Monaim,[a] Yahya E. Jad,[a] Gerardo A. Acosta,[b] Tricia Naicker,[a] Estelle J. Ramchuran,[a] Ayman El-Faham[c,d] ThavendranGovender,[a] Hendrik G. Kruger,[a] Beatriz G. de la Torre,[a] and Fernando Albericio[a,b,d,e,f]

Teixobactin is a head to side-chain cyclic depsipeptide with a guanidino based residue within the cycle, three D-amino acids in the tail, and a *N*-methylated terminal residue. The synthesis of the first analogue, containing Arg, was recently described by our group. Herein, we demonstrated that analogues of Arg.Teixobactin bearing either (a) three L-amino acids in the tail and keeping the *N*-methyl at the *N*-terminal or (b) with three D-amino acids, but with acetylation of the *N*-terminal, are inactive against gram (+) and gram (-) bacteria. These results complement those published by the groups of Madder, Taylor, and Singh that have shown that both modifications: L-amino acids and *N*-acetylation also led to loss of biological activity.

The development of new antimicrobial drugs have became essential and crucial due the dramatic increase of bacterial resistance to the traditional antibiotics.[1-3] Amongst these new antimicrobial compounds, antimicrobial peptides (AMP), especially cyclic peptides, have shown to be very appealing due to the potent activities and stability.[4-6] Very recently (2015), a new head to side chain antimicrobial cyclodepsipetides called Teixobactin (**1**) was discovered by Ling *et al.* using an iChip multichannel device as a new discovery technique to simultaneously isolate and grow the uncultured bacteria.[7] This new antimicrobial peptide exhibited higher activity against gram positive than gram negative bacteria. Furthermore, Teixobactin has been reported to kill bacteria in absence of any detectable resistance. The reason for that was ascribed to its mechanism of action; where it blocks one of the membrane-associated steps of peptidoglycan biosynthesis and then causes inhibition of lipid II ,as a predominant target, and lipid III which plays a critical role in the synthesis of teichoic acid.[7, 8]

Since the publication of isolation, characterization, and antimicrobial activity of Teixobactin,[7] several groups started the development of synthetic strategies for a convenient synthesis that is suitable for running a medicinal chemistry program. Our group published in 2015, the first synthesis of an analogue of Teixobactin **(2a)** in 2015 where the non-proteinogenic guanidine-based amino acid enduracididine was substituted with the proteinogenic Arginine.[9] This analogue showed similar biological trends, being active against the gram-positive bacteria and inactive against gram-negative bacteria. However, Teixobcatin was one order of magnitude more active than the Arg analogue.[9, 10]

At the beginning of 2016, the groups of Madder, Taylor, and Singh published the synthesis of the same analogue, Arg-teixobactin (**2a**),[11] using a identical strategy and reporting the same biological activity. In the same manuscript, the authors stated that "(they) established that the D-amino acids are critical for the antimicrobial activity". They supported this statement with the synthesis of a second analogue **(3b)** where the three D-amino acids of the tail were substituted by the L-amino acid residues (Phe, Gln, Ile). However, the *N*-Me of the terminal Phe for this new analogue (**3b**) was substituted with N-Ac. Substitution of *N*-Me-Phe residue with Ac-Phe should have a significant impact in the biological activity. The impact of this Ac-Phe terminal could potentially be more critical than the change to L-amino acids, since the peptide (**3b**) has lost its cationic character (a normal requirement for antimicrobial peptides)[12] due to introduction of the acetyl group. In addition to that, the hydrogen bond map of the peptide is also dramatically altered.

Herein, we report the synthesis and biological evaluation of two unpublished analogues: (**2b)** that has three L-amino acids while keeping the *N*-Me group at the Phe terminal; and **(3a)** with D-amino acids, but where the D-Phe terminal is acetylated.

a)Catalysis and Peptide Research Unit, School of Health Sciences, University of KwaZulu-Natal, Durban 4001, South Africa; b) CIBER-BBN, Networking Centre on Bioengineering, Biomaterials and Nanomedicine, Barcelona Science Park, 08028-Barcelona, Spain; c) Department of Chemistry, Faculty of Science, Alexandria University P.O. Box 426, Ibrahimia, Alexandria 21321, Egypt; d) Department of Chemistry, College of Science, King Saud University P.O. Box 2455,Riyadh11451, Saudi Arabia; e) School of Chemistry and Physics, University of KwaZulu-Natal, Durban 4001, South Africa; f) Department of Organic Chemistry, University of Barcelona, 08028-Barcelona, Spain.

Electronic Supplementary Information (ESI) available: [Synthetic method, HPLC, HRMS and MALDI]. See DOI: 10.1039/x0xx00000x

ACS OMEGA

Article

http://pubs.acs.org/journal/acsodf

Lysine Scanning of Arg_{10}–Teixobactin: Deciphering the Role of Hydrophobic and Hydrophilic Residues

Shimaa A. H. Abdel Monaim,[†] Yahya E. Jad,[†] Estelle J. Ramchuran,[†] Ayman El-Faham,[‡,§] Thavendran Govender,[†] Hendrik G. Kruger,[†] Beatriz G. de la Torre,*[,†] and Fernando Albericio*[,†,‡,⊥,#,∥]

[†]Catalysis and Peptide Research Unit, School of Health Sciences and [∥]School of Chemistry and Physics, University of KwaZulu-Natal, University Road, Durban 4001, South Africa

[‡]Department of Chemistry, College of Science, King Saud University, P.O. Box 2455, Riyadh 11451, Saudi Arabia

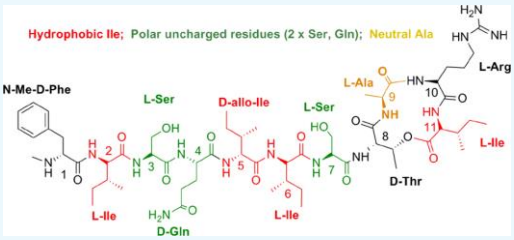
[§]Department of Chemistry, Faculty of Science, Alexandria University, P.O. Box 426, Ibrahimia, Alexandria 21321, Egypt

[⊥]Department of Organic Chemistry, University of Barcelona, Martí i Franqués 1-11, Barcelona 08028, Spain

[#]Networking Centre on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, Barcelona Science Park, Baldiri Reixac 10, Barcelona 08028, Spain

Supporting Information

ABSTRACT: Teixobactin is a recently discovered antimicrobial cyclodepsipeptide with good activity against Gram positive bacteria. Taking Arg_{10}–teixobactin as a reference, where the nonproteinogenic residue L-allo-enduracididine was substituted by arginine, a lysine scan was performed to identify the importance of keeping the balance between hydrophilic and hydrophobic amino acids for the antimicrobial activities of this peptide family. Thus, the substitution of four isoleucine residues present in the natural sequence by lysine led to a total loss of activity. On the other hand, the substitution of the polar noncharged residues and alanine by lysine allowed us to keep and in some cases to improve the antimicrobial activity.



1. INTRODUCTION

Infectious diseases, including tuberculosis (TB), cause major global health problems.[1−5] This awareness accompanied by the lobbying of some nongovernmental organizations has fueled the renaissance of the search for new antibiotics.[5] In this scenario, the publication on teixobactin (**1**) (Figure 1) at the beginning of 2015 by Ling et al. raised a pronounced expectation.[6] Teixobactin is a head to side-chain cyclodepsipeptide that exhibits good activity against Gram-positive bacteria and TB.[6−11] At the end of 2015, our group published the first synthesis of an analogue of teixobactin, Arg_{10}–teixobactin (**2**) (Figure 1), where the nonproteinogenic amino acid L-allo-enduracididine was substituted by arginine.[7,12]

Arg_{10}–teixobactin (**2**) exhibits similar biological trends as teixobactin (**1**) by showing better activity against Gram-positive bacteria than against Gram-negative bacteria. However, Arg_{10}–teixobactin (**2**) was shown to be 1 order of magnitude less active than the parent compound. In 2016, two groups, Payne and co-workers and Li and co-workers,[13,14] published the total synthesis of the natural product (**1**).

At a glance, the structure of teixobactin, an 11 amino acid peptide, can be defined as a scaffold formed by a linear segment constituted with seven amino acid residues, which we named tail, followed by a cyclic structure with four residues. In the cycle, a D-Thr is at the bridgehead linked to an L-Ile, which contains a guanidine side-chain amino acid, and the neutral alanine completes the sequence. The tail end is an *N*-Me-D-Phe and contains two more D-amino acids. Another characteristic feature of this peptide is the presence in total of four isoleucine and three polar uncharged residues (two serine and one glutamine).

Although the structure of this peptide was known only relatively recently, there are already a few reports on its structure activity relationship. In this regard, our group and Singh and co-workers independently found that the activity was lost when the three D-amino acids in the tail were substituted by the corresponding L-amino acids (phenylalanine, glutamine, and isoleucine). This was also observed when the *N*-Me-D-Phe at the *N*-terminal was substituted with *N*-Ac-D-Phe, indicating the importance of a positive charge at the *N*-terminal.[8,10]

More recently and during the completion of this study, Nowick and co-workers have reported another bioactivity study, employing Arg_{10}–teixobactin (**2**) as a reference.[15] The main conclusions of their work are that the Lys_{10}–teixobactin (substitution of the arginine by lysine) is more active than (**2**). Furthermore, the absolute stereochemistry is insignificant.

Received: October 31, 2016
Accepted: December 5, 2016
Published: December 19, 2016

DOI: 10.1021/acsomega.6b00354
ACS Omega 2016, 1, 1262−1265