November 2017

Design, Synthesis, Applications of Polymers and Dendrimers

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Design, Synthesis, Applications of Polymers and Dendrimers

by

Alekhya Nimmagadda

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
Department of Chemistry
College of Arts and Sciences
University of South Florida

Major Professor: Dr. Jianfeng Cai, Ph.D.
Dr. James Leahy, Ph.D.
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November 15th, 2017

Keywords: Antimicrobial polymers, Dendrimers, amphiphilic, γ-AA peptide, host defense peptide

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DEDICATION

To Himaja Potluri
ACKNOWLEDGEMENTS

I would like to thank my research advisor, Dr. Jianfeng Cai, for the support and guidance he has given during my academic study at USF. I would like to thank him for his guidance, endless support and patience. It is very rare to find an advisor who always have time for their students to listen to their little problems, I feel I am fortunate and I thank my advisor for always finding time and encouraging to overcome the barriers in the course of my research work.

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<th>Description</th>
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<tbody>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>HDPs</td>
<td>Host-defense peptides (HDPs)</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-Resistant Staphylococcus Aureus</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>ACS</td>
<td>American Chemical Society</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharides</td>
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<td>Triethylamine</td>
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<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
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<tr>
<td>GPC</td>
<td>Gel permeation chromatography</td>
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<tr>
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<td>Trifluoracetic acid</td>
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<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimum inhibitory concentration</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>HRBCs</td>
<td>Human red blood cells</td>
</tr>
<tr>
<td>MRSE</td>
<td>Methicillin-resistant S. epidermidis</td>
</tr>
<tr>
<td>VREF</td>
<td>Vancomycin-resistant E. faecalis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Name</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’, 6-Diamidino-2-phenylindole dihydrochloride</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PAMAM</td>
<td>Polyamidoamine</td>
</tr>
<tr>
<td>HOBT</td>
<td>1-hydroxybenzotriazole monohydrate</td>
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<tr>
<td>DIC</td>
<td>Diisopropylcarbodiimide</td>
</tr>
<tr>
<td>Boc</td>
<td>tert-Butyloxycarbonyl</td>
</tr>
<tr>
<td>Fmoc</td>
<td>Fluorenlymethyloxycarbonyl chloride</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionization time-of-flight</td>
</tr>
<tr>
<td>MTT</td>
<td>Methyltrityl</td>
</tr>
<tr>
<td>TIS</td>
<td>Triisopropylsilane</td>
</tr>
<tr>
<td>NMP</td>
<td>N-Methyl-2-pyrrolidone</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>FM</td>
<td>Florencescence Microscopy</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
</tr>
<tr>
<td>DIPEA</td>
<td>Diisopropylethylamine</td>
</tr>
<tr>
<td>TFE</td>
<td>Tetrafluoroethylene</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>CTC</td>
<td>Chlorotrityl chloride</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide</td>
</tr>
<tr>
<td>PEEP</td>
<td>Positive and expiratory pressure</td>
</tr>
<tr>
<td>CD</td>
<td>Crystal violet</td>
</tr>
<tr>
<td>H &amp; E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>BAL</td>
<td>Bronchoalveolar lavage</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>KP</td>
<td>Klebsiella pneumoniae</td>
</tr>
<tr>
<td>PA</td>
<td>Pseudomonas aeruginosa</td>
</tr>
<tr>
<td>pH</td>
<td>Potential of hydrogen</td>
</tr>
<tr>
<td>NaHCO$_3$</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>NaBH$_3$CN</td>
<td>Sodium cyanoborohydride</td>
</tr>
<tr>
<td>LiAlH$_4$</td>
<td>Lithium aluminium hydride</td>
</tr>
<tr>
<td>EtOAc</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>Sodium sulfate</td>
</tr>
<tr>
<td>CNBr</td>
<td>Cyanogen Bromide</td>
</tr>
<tr>
<td>NaOH</td>
<td>Sodium hydroxide</td>
</tr>
<tr>
<td>DCC</td>
<td>Dicyclohexylcarbodiimide</td>
</tr>
<tr>
<td>TSA</td>
<td>Tryptic soy agar</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>Enterococcus faecalis</td>
</tr>
<tr>
<td>Rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>M</td>
<td>Molarity</td>
</tr>
<tr>
<td>Cu</td>
<td>Copper</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>Dde</td>
<td>Dichlorodiphenyldichloroethylene</td>
</tr>
<tr>
<td>MDC</td>
<td>5-methyl-2-oxo-1,3-dioxane-5-carbonyl chloride</td>
</tr>
<tr>
<td>TU</td>
<td>1-(3,5-bis(trifluoromethyl)-phenyl)-3-cyclohexyl-2-thiourea catalyst</td>
</tr>
<tr>
<td>DBU</td>
<td>1,8-diazabicyclo [5.4.0] undec-7-ene</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity Index</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut-Off's</td>
</tr>
<tr>
<td>CH₃CN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>DIC</td>
<td>Diisopropylcarbodiimide</td>
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ABSTRACT

WHO has reported that antibiotic resistance is the third major cause of human death all over the globe. Recent study, has focused on the development of new antibacterial resistance drugs. Herein, we tried to synthesis a series of polymers that can mimic the HDPs. HDPs can target the bacterial cell membrane and they have less chances to develop bacterial resistance. We synthesized the amphiphilic polycarbonates that are highly selective to Gram-positive bacteria, including multidrug resistant pathogens. The membrane disruption activity of these polymers was proved by fluorescence and TEM studies and the drug resistance study showed that the polymers don’t develop bacterial resistance.

In order to further design the molecules that can target a broad spectrum of bacteria, we have designed a series of lipidated dendrimers that can target the Gram-positive and Gram-negative bacteria. These dendrimers mimic the HDPs and target the bacterial cell membrane. Dendrimers are reported to inhibit the formation of bacterial biofilm which makes them promising for their future development of antibiotic agents.

Apart from the synthesis of polymers and dendrimers as antibacterial agents, we have designed a series of small molecular antibacterial agents that are based on the acylated reduced amide scaffold and small dimeric cyclic guanidine derivatives. These molecules display good potency against a panel of multidrug-resistant Gram-positive and Gram-negative bacterial strains. Meanwhile, they also effectively inhibit the biofilm formation. Mechanistic studies suggest that these compounds kill bacteria by compromising bacterial membranes, a mechanism analogous to that of host-defense peptides (HDPs). Lastly, we also demonstrate that these molecules have excellent in vivo activity against MRSA in a rat model. This class of compounds could lead to an appealing class of antibiotic agents combating drug-resistant bacterial strains.
CHAPTER 1: INTRODUCTION

1.1 Necessity of new Antimicrobial agents

Bacterial infections pose a great threat to health of common people.\(^1\) The World Health Organization (WHO) has recognized that antimicrobial pathogens have developed resistance against most of the commercially available antibiotics that are currently present in the market.\(^2\) WHO have reported that there has been a significant increase in the infections caused by the drug resistant bacterial strains over a decade, especially from methicillin-resistant staphylococcus aureus (MRSA).\(^3\) About 50-60% of the infections develop due to the lack in the sterility of the instruments used during surgeries.\(^4\) Infections caused due to sterility issues will lead to another post-surgical operation and in some extreme cases results in the removal of the infected part from the patient body.\(^5\) Therefore, there is a serious demand in the development of new antibacterial agents to combat the resistance developed by the bacteria against commercially available antibiotics.\(^2\)

1.2 General Characteristics of broad spectrum Antimicrobial drugs

Most of the commercially available antimicrobial drugs are amphiphilic in nature. Although the mechanism of the antimicrobial peptides is in debate, most scientists believe that the peptides fold into their secondary alpha helix or beta sheet then interact with the bacterial cell membrane. Upon binding to the membrane peptides separate into distinct hydrophobic and hydrophilic chains.\(^6\) The hydrophilic groups of the peptide bind to the negatively charged bacterial cell surface by electrostatic attraction. The peptides show selectivity in binding to bacterial cells over human cells, as the bacterial surface is more abundantly negatively charged over the human cells which is almost considered to be neutral charged.\(^7\) The hydrophobicity of the drug helps in binding to the phospholipid bilayer of the
bacteria and helps in easy penetration of the drug. This amphiphilic nature of the drug helps in disrupting the bacterial cell membrane by altering the trans membrane potential which causes leakage of the cytoplasmic contents and ultimately leading to death.\textsuperscript{8} Although most of the currently synthesized antimicrobial peptides are believed to show their mechanism of action by membrane disruption, there are still peptides available that show their action by altering the cellular pathways of the bacteria and inhibiting the DNA/RNA synthesis, folic acid synthesis etc.\textsuperscript{6}

1.3 Differentiation between mammalian and bacterial cells

Bacterial cells lack cholesterol in their membrane, whereas the mammalian cells have 25\% cholesterol in their membranes which acts as a stabilizing factor for the cell.\textsuperscript{9} So due to lack of stability, disruption of the membrane becomes quite easier. Eukaryotic cells mainly contain phospholipids such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin. These cells carry a net neutral charge, whereas the negative charge is located in the inner leaflet of the membrane. Bacterial cells, both gram positive and gram negative have a net negative charge on the membrane due to the presence of phospholipids such as phosphatidyl glycine, phosphatidylserine and cardiolipin on the outer leaflet membrane making it more susceptible to the drugs over the mammalian cells.\textsuperscript{5} In addition to this, gram positive bacteria has a thick peptidoglycan layer with lipoteichoic acids,\textsuperscript{10} which further contributes to the negative charge on the membrane for electrostatic interaction. The gram-negative bacteria have a thin peptidoglycan layer and an additional outer membrane layer that is composed of lipopolysaccharides that are anionic.\textsuperscript{11} It becomes a little difficult for the drugs to act on gram negative than gram positive due to more cell membrane layers.

1.4 Polymer Overview\textsuperscript{12}

Polymers are macromolecular substances composed of groups of monomers which are linked to each other through controlled chemical process. This process is known as polymerization. The basic
requirement of the polymerization reaction is that one monomer must have the capability of being linked with the other monomer, that is, monomers must have a functionality of one or two. The physical and chemical properties of polymers are different from the monomers from which the polymer is formed. The difference in the molecular size is responsible for the variation in the physical properties between the polymer and the monomer.

Homopolymers are a class of polymer which is made up from only one kind of monomer. A copolymer is made up of two or more different types of repeating units of monomers. Based on the particular arrangement of the repeating units along the polymer chain, there are several categories of copolymers such as statistical copolymers, alternating copolymers, graft copolymers, block copolymers, and random copolymers.

1.5 Dendrimer Overview

Dendrimers are highly branched globular star like structures that exhibit symmetry in their structure. Dendrimers consist of a central core from which identical fragments are built up to make a macromolecular structure. Based on the number of identical fragments grown from the center of the core, the generation of the dendrimer is defined. Dendrimers are in the nanosize scale and have narrow polydispersity and tunable surface entities. The end groups of the dendrimer will determine the solubility and reactivity of the dendrimer. These have gained importance as it is easier for the further functionalization of the end groups and can be used for various biomedical applications. Currently, dendrimers have been used in target based drug delivery systems for the treatment of cancer, as antimicrobial agents, enzyme catalysis and surface engineering techniques.

1.6 Host-defense peptides (HDPs)

More exposure of the bacteria to conventional antibiotics has driven a way for the bacteria to develop resistance against most of the antibiotics available in the market. In recent times, bacteria have
developed resistance to the third-generation antibiotics available in the market. Researchers have focused on developing new antibacterial drugs that can combat bacterial resistance. Host-defense peptides (HDPs) may be an alternative strategy to the conventional antibiotics. HDPs are found in the innate system of human body and can act against pathogens. These HDPs show their mode of action by bacterial membrane disruption. It is known that HDPs are diverse in structure and can form helices, β-sheets, and extended structures. However, they frequently attain globally amphipathic nature when approaching the bacterial surface. The hydrophilic cationic groups direct the initial association of HDPs with the negatively charged bacterial membranes, whereas the hydrophobic groups assist in the penetration of HDPs into the bacteria, leading to rupture of the bacterial cell membranes. This kind of mechanism makes it difficult for bacteria to develop resistance. These characteristics have inspired researchers to design a wide range of HDP mimic that can act as antibacterial agents. However, HDPs are prone to proteolytic degradation, moderately active and are not cost effective. Thus, there has been considerable interest in developing peptidomimetics that mimic HDPs to overcome the disadvantages. Dr. Cai’s lab has focused on developing γ-AApeptides that mimic the mechanism of action of HDPs. γ-AApeptides are proved for their stability against enzymatic degradation and capability of forming secondary structures.

1.7 γ-AApeptides

The peptidomimetic oligomers have started to find important applications in chemical biology and biomedical sciences in the past two decades. Consisting of unnatural backbones, peptidomimetics could overcome obstacles associated with canonical peptides, including low bioavailability, susceptibility to proteolytic degradation, and limitation in chemodiversity. Thus, they could lead to the development of promising lead compounds and drug candidates. Indeed, peptidomimetics, such as β-peptides, α/β-peptides, peptoids, oligoureas, show capability to
mimic hierarchical structures of proteins, and are extensively studied for therapeutic applications. To expand the family of peptidomimetics and to facilitate their application in biomedical sciences, a new class of peptidomimetics - "γ-AApeptides" was developed. They are termed “γ-AApeptides”, as they are oligomers of γ-substituted-N-acylated-N-aminoethyl amino acids (Figure 1.1), similar to γ-PNAs.

![Chemical structures](image)

Figure 1.1. The chemical structure of α-peptide and γ-AApeptide.

1.8 Outline of the Dissertation

In this dissertation, we discuss the design, synthesis, and antibacterial efficacy of polymers, dendrimers and HDPs.

In chapter 2, we describe the synthetic design of the positively charged polycarbonates that shows activity against Gram-positive bacteria.

In chapter 3, we discuss the design, synthesis of dendrimers and the appropriate amphiphilicity required to show broad spectrum antibacterial activity was evaluated.

In chapter 4, we describe the design and development of a class of small antimicrobial molecules containing acylated reduced amide that has potent and broad spectrum antimicrobial activity.
In chapter 5, we discuss that bis-cyclic guanidine compounds could rationally adopt cationic amphipathic structures, and thus capable of mimicking HDPs and revive as a promising approach to combat bacterial resistance.

In chapter 6, we summarize the research findings and conclude the future directions of our research study.

1.9 References
(1) Palermo, E. F.; Kuroda, K., Chemical structure of cationic groups in amphiphilic polymethacrylates modulates the antimicrobial and hemolytic activities. *Biomacromolecules* 2009, 10 (6), 1416-28.


CHAPTER 2: POLYCARBONATES WITH POTENT AND SELECTIVE ANTIMICROBIAL ACTIVITY

Note to Reader

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2.1 Introduction

Bacterial infections pose a great threat to the public health. The World Health Organization (WHO) recently acknowledged that certain bacterial pathogens have acquired significant resistance to most of the commercially available antibiotics. In hospitals about 50-60% of the infections are caused by the lack of the sterility of medical devices used during surgeries, especially due to the contamination of notorious methicillin-resistant staphylococcus aureus (MRSA) strains. The Center for Disease Control (CDC) has reported that, 2 million people develop antibiotic resistance after antibiotic treatment and 23,000 people die due to the lack of proper treatment annually. Therefore, there is an escalating demand for the development of new antibacterial agents to combat the emerging resistance.

One promising approach to circumvent bacterial resistance is to develop derivatives of host-defense peptides (HDPs). Although antimicrobial mechanisms of HDPs are still in debate, it is generally believed that these cationic peptides fold into discrete secondary structures such as α-helices or β-sheets upon binding to bacterial membranes, on which distinct hydrophobic and cationic patches are formed on the peptides. The cationic groups of HDPs bind to the negatively charged bacterial cell surface by electrostatic attraction, while the hydrophobic patch interacts with hydrophobic lipid bilayer of the bacteria, leading to penetration of the peptides. It is known that eukaryotic cells mainly contain
zwitterionic phospholipids such as phosphatidylcholine, phosphatidylethanolamine and sphingomyelin on their outer leaflet of membranes, whereas the negative charged lipids are largely sequestered in the inner leaflet of the membranes. As a result, these cells generally carry a net neutral charge on their surface. In contrast, both Gram-positive and Gram-negative bacteria bear a negative charge on their membrane surface due to the presence of phospholipids including phosphatidylglycine, phosphatidylserine and cardiolipin, making them more selective to cationic HDPs over mammalian cells due to charge attraction. In addition, Gram-positive bacteria have a thick peptidoglycan layer embedded with techoic and lipotechoic acids, whereas lipopolysaccharides (LPS) are an important component on the outer membranes of Gram-negative bacteria. These molecules all further contribute to the overall negative charge on the bacterial membranes. Another factor aiding to the selectivity of HDPs towards bacterial cells is that bacteria lack cholesterol in their membranes, whereas mammalian cells have 25% cholesterol or more in their membranes, which stabilizes their membrane integrity. As such, disruption of bacterial membranes is relatively less challenging. The disruption of the bacterial cell membranes causes the leakage of the cytoplasmic contents and ultimately cell death. As this biophysical interaction lacks defined membrane proteins and other targets, development of resistance is believed to be more challenging. Therefore, HDPs and their derivatives have been extensively explored for antimicrobial development. However, HDPs have intrinsic drawbacks such as tedious and costly synthesis and purification. The pharmacokinetic properties and chemical instability of the peptides are other obstacles that hamper therapeutic applications of HDPs. Moreover, HDPs exhibit moderate selectivity and are reported to be toxic toward mammalian cells. One of the successful examples of HDPs is magainin II, which exhibits broad-spectrum but weak antimicrobial activity against bacteria. The synthetic analog of magainin, Pexiganan (also known as MSI-78) has shown much improved activity and entered Phase III clinical trials for the treatment of
diabetic foot ulcers. However, it failed eventually due to its moderate in vivo efficacy and high cost.\textsuperscript{12} Due to drawbacks of HDPs, considerable effort has been extended to develop cationic antimicrobial polymers that mimic the function of HDPs, in the hope to supplement the potential application of HDPs. Compared to HDPs, polymers have some apparent advantages. The procedure for the preparation of polymers is generally very straightforward. Most of the polymer synthesis involves one pot polymerization reaction, which makes it easy to scale up to obtain products in large quantity.\textsuperscript{13} In addition, polymers are cost-effective. Examples of antimicrobial polymers include poly(\(\alpha\)-amino acid)s,\textsuperscript{13} metallopolymers,\textsuperscript{14} nylon-3 polymers,\textsuperscript{15} polyacrylates,\textsuperscript{16} polyvinyl pyridines,\textsuperscript{17} polystyrenes,\textsuperscript{18} polycarbonates,\textsuperscript{19} etc. Similar to HDPs, these polymers generally exert their activity by acting on bacterial membranes.

Yang and Hedrick et al recently reported cationic antimicrobial polycarbonates containing quaternary ammonium salts.\textsuperscript{20,21} These quaternary ammonium moieties were introduced through post-modification.\textsuperscript{22} Our previously reported antimicrobial peptidomimetics bear primary amino groups and show potent antimicrobial activity.\textsuperscript{10,10a} It is thus intriguing to study the antibacterial activity of polycarbonates bearing same groups. Herein, we report our investigation on the design and study of antimicrobial polycarbonates containing primary amino groups. Such a design eliminates the post-modification step, and surprisingly, these polycarbonates are highly selective to bacteria, and show virtually no toxicity to blood cells under the tested condition.

2.2 Materials and Methods

Synthesis of the monomer 1 (Figure 2.1)

The 5-methyl-2-oxo-1,3-dioxane-5-carbonyl chloride (MDC) was prepared according to the previous reported work by Yang and Hedrick.\textsuperscript{22} To synthesize the monomer 1, MDC (6.8 g, 38 mmol) was dissolved in 30 mL DCM in a 100 mL round bottom flask, to which a solution of Boc-protected
ethanolamine (6.15 g, 38 mmol) and TEA (7.85 mL, 57 mmol) in 10 mL DCM was added drop wise. The reaction was allowed to continue for 4 h in an ice bath. The solution was washed with 1 N HCl (100 mL×3), water (75 mL×3), brine (50 mL×1), and then dried over sodium sulfate. The solvent was removed in vacuo to give a yellow colored oil, which was further purified by flash chromatography (ethyl acetate/hexane 2:1) to give the final product monomer 1 (6.9 g, 22 mol, 60 %) as a white solid.

![Monomer 1](image)

**Figure 2.1** Synthesis of the monomer 1.

**Synthesis of the monomer 2 (Figure 2.2)** The monomer 2 was prepared according to the previous reported protocol by Yang and Hedrick.\(^{22}\)

![Monomer 2](image)

**Figure 2.2** Synthesis of the monomer 2.

**Synthesis of polycarbonate polymers.** All the polymers were prepared in a similar fashion using ring opening polymerization\(^{21}\) (Figure 2.3 and Table 2.1). In order to synthesize Boc-protected random copolymer polycarbonate P6\(^{'\,}\) which was formed by the mixed monomers of 1 and 2, the initiator benzyl alcohol (0.05 g, 0.46 mmol) was dissolved in 10 mL of DCM in a \(N_2\) purged round bottom
flask. Then 20 eq of hydrophobic monomer 2 (2.31 g, 9.0 mmol) and 20 eq of hydrophilic monomer 1 (2.8 g, 9.0 mmol) were added to the flask together (Scheme 2.3A), followed by the addition of 1-(3,5-bis(trifluoromethyl)-phenyl)-3-cyclohexyl-2-thiourea catalyst (TU) (2 eq, 0.34 g, 0.9 mmol) and (1,8-diazabicyclo [5.4.0] undec-7-ene (DBU) (2 eq, 0.14 g, 0.9 mmol) base. The reaction was allowed to stir for 4 h under nitrogen, and then quenched by addition of benzoic acid (1.1 eq, 0.06 g, 0.5 mmol). The other Boc-protected random copolymers were prepared in the similar way.

For the synthesis of Boc protected di-block polymers P4', the two monomers 1 and 2 were added in two batches separately in order to form two segments in the polymers (Scheme 2.3B). In brief, the Boc protected monomer 1 was first added to the reaction vessel in the presence of TU and DBU in 20 mL DCM and the reaction was allowed to run for 4 h under nitrogen. Subsequently, the hydrophobic monomer 2 was added and the reaction was allowed to continue for another 4 h. The reaction was quenched by benzoic acid at the end.

Table 2.1 Structures of the synthesized polymers. *based on NMR integration; †based on GPC.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of co-polymer</th>
<th>Hydrophobic units</th>
<th>Hydrophilic units</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Theoretical</td>
</tr>
<tr>
<td>P1</td>
<td>Single</td>
<td>0</td>
<td>20</td>
<td>4168</td>
</tr>
<tr>
<td>P2</td>
<td>Di-block</td>
<td>10</td>
<td>15</td>
<td>5653</td>
</tr>
<tr>
<td>P3</td>
<td>Random</td>
<td>10</td>
<td>10</td>
<td>4638</td>
</tr>
<tr>
<td>P4</td>
<td>Di-block</td>
<td>10</td>
<td>10</td>
<td>4638</td>
</tr>
<tr>
<td>P5</td>
<td>Random</td>
<td>15</td>
<td>10</td>
<td>5888</td>
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<tr>
<td>P6</td>
<td>Random</td>
<td>20</td>
<td>20</td>
<td>9168</td>
</tr>
<tr>
<td>P7</td>
<td>Random</td>
<td>15</td>
<td>15</td>
<td>6903</td>
</tr>
</tbody>
</table>
Figure 2.3 Synthesis of the amphiphilic polycarbonates.

A, synthesis of random polymer P6’ and P6, in which the monomer 1 and 2 were added in one batch; B, synthesis of di-block polymer P4’ and P4, in which the monomer 1 and 2 were added in two batches.

Dialysis. The polymers were dissolved in 2 mL methanol and dialyzed against methanol (dialysis tubing MWCO=3500) for three days, with methanol being replaced twice a day.\(^\text{13}\)

De-protection of Boc in the polymers. The solvent was evaporated and the products were treated with 50% TFA in 10 mL DCM for 2 h. The solvent was removed in vacuo and the residues were dissolved in DMSO and dialyzed against methanol for three days. After methanol was removed, the samples were dissolved in 5 mL of water and lyophilized to give the final polymers P1-P8 as colorless sticky oils, which were characterized by \(^1\)H NMR and GPC subsequently (Table 2.1).

MIC (Minimum Inhibitory Concentration)/Antimicrobial Activity. Minimum inhibitory concentration (MIC) was used to measure the efficacy of the synthesized polycarbonates against bacteria.\(^\text{23}\) MIC is defined as the lowest concentration of the compound by which it can completely inhibit the growth of the bacteria for a period of 20 h. Three clinically relevant Gram-positive strains, *Methicillin-resistant S. epidermidis* (MRSE, RP62A), *Vancomycin-resistant E. faecalis* (VREF, ATCC 700802), and *Methicillin-resistant S. aureus* (MRSA, ATCC 33591) were used in the assay.
Briefly, a single colony was isolated from the agar plate and allowed to grow in 4 mL TSB solution overnight in a digital shaking incubator at 37 °C. The following day the culture was diluted by 100-fold and the diluted culture was shaken for 6 h in order for the bacteria to reach the mid-logarithm phase. In a 96-well plate 50 µL of the polymer solution in 2-fold serial dilutions (50 µg/mL to 1.6 µg/mL) were added to the wells. Next, aliquots of the bacterial solution (50 µL, 1 × 10⁶ CFU/mL) were added to those polymer solutions, respectively, and the plate was incubated at 37 °C. After 20 h, the plate was read at 600 nm wavelength on a Biotek Synergy HT microtiter plate reader and the MIC values were determined. Results were repeated at least three times in duplicates each time.²⁴

**Hemolytic Activity Study.** To determine the selectivity of the polymers, the compounds were incubated with the human red blood cells and the HC₅₀ for the compounds was calculated. HC₅₀ is defined as the concentration that causes 50% hemolysis of the human red blood cells (hRBCs).²⁵,²⁶,²⁷ In this assay, freshly drawn blood was centrifuged, and erythrocytes were separated and washed a couple of times with PBS buffer, and the supernatant was removed. The polymer samples (50 µL) of various concentrations were made in a 96 well plate using 2-fold serial dilution technique. The erythrocytes were diluted to a final concentration of 5% (v/v) in PBS buffer. 50 µL of the erythrocytes diluted samples were added to the serial-diluted polymer solutions and incubated for 1 h at 37 °C. The 96 well plate was centrifuged at 3500 rpm for 10 min, and 30 µL supernatant liquid was transferred to a new 96 well plate containing 100 µL of PBS in each vial. The absorbance of these solutions was taken at a wavelength of 540 nm. For this assay PBS was used as negative control and 0.1% Triton x-100 was used as positive control. Hemolysis is calculated using the formula below:

\[
\%\text{hemolysis} = \frac{(\text{Abs of Sample} - \text{Abs of PBS negative control})}{(\text{Abs of positive Control} - \text{Abs of PBS negative control})} \times 100
\]
**Bacterial Resistance Assay.** MICs of the samples were determined by the method described above. After determination of the MIC, the bacterial solution from the well that contained the polymer at one-half concentration of the MIC value was used to dilute to $1 \times 10^6$ CFU/mL. Next, 50 µL of this bacterial solution was added to the 50 µL of 2-fold serial-diluted polymer samples, and the new MIC was measured. This assay was repeated for 14 passages. If the polymer had virtually the same MIC after every passage it indicated that the polymer did not develop resistance in the bacteria.\(^{28}\)

**Fluorescence Microscopy.** DAPI (4', 6-Diamidino-2-phenylindole dihydrochloride) and PI (Propidium iodide) were used in the assay.\(^{29,29b}\) DAPI is the dye that stains all the dead and living bacteria, whereas the PI dye stains only the dead bacteria with damaged membranes as itself is not cell permeable and it has to interact with the nucleic acids of the bacteria and fluorescing in bright red color. Briefly, the bacteria were allowed to grow to the mid logarithmic phase and then incubated with the polymer **P6** (6 µg/mL) at 37 °C for 3 h. The solution was centrifuged at 10,000 g for 10 min in an Eppendorf tube. The supernatant was removed and the bacterial pellets were washed with PBS three to four times. PI (5 µg/mL) was added and incubated for 15 min in dark at 0°C. The excess of the dye was removed by PBS washes (×3). Next, the cells were incubated with DAPI (10 µg/mL in water) for 15 min in dark at 0 °C and excess of the dye was removed, followed by PBS washes (×3). The bacteria were then examined under oil-immersion objective (100×) by using the Zeiss Axio Imager Z1 optical microscope.\(^{30}\)

**Time Kill Assay.** This assay determines bacterial killing kinetics for the polymer. Different concentrations of the polymer were incubated with bacterial suspension, at time intervals of 0 min, 10 min, 30 min, 1 h, and 2 h. At these time points, 100 µL of the solution were taken and diluted $10^2$ to $10^4$ times and then spread on respective agar plates for incubation at 37 °C. After 20 h, the bacterial colonies were counted. The assay was repeated at least three times.\(^{31}\)
**Dead Bacteria TEM.** The control and the polymer treated bacterial samples were made in the similar way as in fluorescence microscopy assay. The samples were spread on 200-mesh copper grids and were left for 1 h for adsorption onto the grid, and stained by 1% uranyl acetate for 30 sec. The grids were analyzed at 60 kV with FEI Morgagni 268D TEM instrument.\(^{29b}\)

2.3 Results and Discussion

**Synthesis of cationic polycarbonate polymers containing primary amino groups.**

Yan and Hedrick et al developed antimicrobial polycarbonates containing quaternary ammonium salts\(^{20}\) that show good activity towards a range of Gram-positive bacteria. However, the formation of quaternary ammonium salts involves post-modification. Based on our previous findings, we envisioned that polycarbonates with primary amino groups should also be active and maybe even more selective toward bacteria. As such, we designed and synthesized a series of such type of polymers (P1-P8) and investigated their antibacterial activity. The synthesis is straightforward as described earlier. The polymers are well characterized by NMR and GPC (Table 2.1, Figure 2.4, and supporting information).
Antimicrobial activity of the polymers.

In order to understand the effect of sequence composition on the antimicrobial activity of the polymers, we synthesized both random (in which hydrophilic and hydrophobic monomers were randomly arranged) and diblock (in which there are defined hydrophobic and hydrophilic segments) polycarbonate copolymers. These amphiphilic polycarbonate polymers (P1 - P8) were tested for their antimicrobial activity against three different Gram-positive bacterial strains, Methicillin-resistant *S. epidermidis* (MRSE, RP62A), Vancomycin-resistant *E. faecalis* (VREF, ATCC 700802), and Methicillin-resistant *S. aureus* (MRSA, ATCC 33591), all of which are clinically relevant threatening strains (Table 2.2). The polymer P1, containing only hydrophilic amino groups but no hydrophobic groups, failed to show any activity against the bacterial strains under tested concentrations. In contrast,
the other polymers containing both hydrophilic and hydrophobic groups all show good antibacterial activity. This data clearly demonstrates that although positive charges are necessary for initial recognition and selective association with bacteria, cationic groups alone are not sufficient for bacterial killing. Hydrophobic groups are critical for membrane interaction and disruption, and ultimate death of the bacteria.32 The diblock copolymers P2 and P4, differing in the number of hydrophobic groups, show similar activity against three different Gram-positive bacterial strains. We hypothesized that diblock copolymers may form stable nanomicelles structures in solution, in which their hydrophobic groups are sequestered, leading to comparable antimicrobial activity. To test our hypothesis, we synthesized the random polymer P3 that contains same number of hydrophobic and hydrophilic groups as P4. Consistent to our hypothesis, the antibacterial activity of P3 was better than P4. Although the activity against VREF remained the same for both P3 and P4, P3 exhibited good activity of 2.2 µM and 2.6 µM against MRSA and MRSE, indicating it is 2-fold more potent than P4 toward these two strains. We thus focused on the study of activity of random polymers, and further investigated how the activity of this type of polymers was related to the ratio of hydrophobic and hydrophilic groups. With the same number of hydrophilic groups, increasing the number of hydrophobic groups enhanced antimicrobial activity, as seen for polymers P3, P5 and P8. Containing 20 hydrophobic groups, P8 exhibited an excellent activity of 0.7 µM, 1.4 µM and 2.8 µM against MRSA, MRSE and VREF respectively. Similar trend was also revealed with the change of positively charged groups. Containing 20 hydrophilic and 20 hydrophobic groups, the random copolymer P6 showed potent activity against all three bacterial strains, with the activity of 0.17 µM, 0.55 µM and 0.55µM against MRSA, MRSE and VREF respectively. It is reasonable because the sequences containing more hydrophobic and hydrophilic groups have stronger interaction with bacterial membranes. The findings again suggested that both cationic and hydrophobic groups are essential for
the development of antimicrobial polymers, and their arrangement in the sequences plays a very important role.

We next conducted hemolytic assays to evaluate the selectivity of the polymers (Table 2.2). Surprisingly, none of the polymers was toxic at the tested condition even up to 1 mg/mL. The most potent polymer P6 displayed a selectivity of at least 600fold for MRSA. The results indicate that these polymers are highly selective toward bacteria and thus hold great promise for the development of potent antibacterial agents.

**Table 2.2** Antibacterial activity of polycarbonates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of co-polymer</th>
<th>Hydrophobic units</th>
<th>Hydrophilic units</th>
<th>Gram Positive, µg/mL (µM)</th>
<th>Hemolysis (HC50) (µg/mL)</th>
<th>Selectivity Index (HC50/MIC MRSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P1</strong></td>
<td>Single</td>
<td>0</td>
<td>20</td>
<td>&gt;50</td>
<td>&gt;50</td>
<td>&gt;1000</td>
</tr>
<tr>
<td><strong>P2</strong></td>
<td>Di-block</td>
<td>10</td>
<td>15</td>
<td>25 (4.47)</td>
<td>25 (4.47)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td><strong>P3</strong></td>
<td>Random</td>
<td>10</td>
<td>10</td>
<td>10 (2.2)</td>
<td>12 (2.6)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td><strong>P4</strong></td>
<td>Di-block</td>
<td>10</td>
<td>10</td>
<td>25 (5.44)</td>
<td>20 (4.35)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td><strong>P5</strong></td>
<td>Random</td>
<td>15</td>
<td>10</td>
<td>10 (1.7)</td>
<td>20 (3.4)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td><strong>P6</strong></td>
<td>Random</td>
<td>20</td>
<td>20</td>
<td>1.6 (0.17)</td>
<td>5.0 (0.55)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td><strong>P7</strong></td>
<td>Random</td>
<td>15</td>
<td>15</td>
<td>10 (1.45)</td>
<td>20 (2.89)</td>
<td>&gt;1000</td>
</tr>
<tr>
<td><strong>P8</strong></td>
<td>Random</td>
<td>20</td>
<td>10</td>
<td>5 (0.7)</td>
<td>10 (1.4)</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>
Morphology of polymers.

As we predicted that the antimicrobial activity of the polymers is related to their nanomorphology, we next conducted Transmission Electron Microscopy (TEM) experiment to test our hypothesis. As expected, most of the polymers formed into micelles in water with the size ranging from 80 to 200 nm (Figure 2.5). We particularly compared the morphology of P3, P4 and P6. These micelles show hydrophobic cores and hydrophilic coronas. The size of the particle increases as the hydrophobic and hydrophilic entities increase, as observed for P3 and P6 (Figure 2.5A and 2.5B). The diblock copolymer P4 (Figure 2.5C) was found to be larger than the random copolymer P3 (Figure 2.5A) which comprised of same number of hydrophobic and hydrophilic entities. This may be because P4 forms more stable core-shell micelle structure due to its diblock structure, and therefore more sequences can self-assemble together to form large-sized micelles. In the contrast, although P3 also contains both hydrophobic and hydrophilic groups, their random arrangement renders less ability of the sequences to assembly into defined nanostructure. This is also demonstrated by their zeta potentials (Table 2.3). P4 has a zeta potential of 58.98 mv, which is much larger than P3 and P6. This is consistent to our hypothesis, that random polymers are more active to bacteria than diblock polymers as they can quickly dissociate into smaller aggregates or single molecules, and as a result, they effectively interact and disrupt bacterial membranes.

![TEM micrographs showing the size of the polycarbonates.](image)

A: P3  B: P6  C: P4

Figure 2.5 TEM micrographs showing the size of the polycarbonates.
**Table 2.3** Size and Zeta potential of polycarbonates.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Type of co-polymer</th>
<th>Hydrophobic units</th>
<th>Hydrophilic units</th>
<th>Z-Average (d. nm)</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>Single</td>
<td>0</td>
<td>20</td>
<td>255.7</td>
<td>44.2</td>
</tr>
<tr>
<td>P2</td>
<td>Di-block</td>
<td>10</td>
<td>15</td>
<td>154.0</td>
<td>54.7</td>
</tr>
<tr>
<td>P3</td>
<td>Random</td>
<td>10</td>
<td>10</td>
<td>81.20</td>
<td>26.1</td>
</tr>
<tr>
<td>P4</td>
<td>Di-block</td>
<td>10</td>
<td>10</td>
<td>212.0</td>
<td>58.9</td>
</tr>
<tr>
<td>P5</td>
<td>Random</td>
<td>15</td>
<td>10</td>
<td>209.0</td>
<td>31.7</td>
</tr>
<tr>
<td>P6</td>
<td>Random</td>
<td>20</td>
<td>20</td>
<td>288.4</td>
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<td>Random</td>
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<td>15</td>
<td>328.7</td>
<td>9.70</td>
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<tr>
<td>P8</td>
<td>Random</td>
<td>20</td>
<td>10</td>
<td>435.1</td>
<td>7.73</td>
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</table>

**Antimicrobial mechanism**

The mode of action of the polymers against bacteria was initially evaluated by fluorescence microscopy using double staining method ([Figure 2.6](#)). The most potent sequence P6 was selected for the study. The control, which is just MRSA bacteria themselves, showed blue fluorescence under DAPI channel (a1). They were not observed under PI channel (a2) because their membranes were intact, and therefore they could not be stained by DAPI. In contrast, after MRSA were treated with P6, they were stained by both PI and DAPI (b1, b2), and observable under both channels, indicating MRSA membranes were disrupted.
Figure 2.6 Fluorescence micrographs of MRSA treated with 10 µg/mL of P6.

The mechanism of action was further supported by TEM (Figure 2.7), under which the morphology of untreated MRSA and the polymer P6 treated MRSA was revealed. The control (bacteria without treatment) show spherical shape with intact cell membranes (Figure 2.7A), a typical morphology of MRSA. However, after the treatment of bacteria with P6, most bacterial lost their spherical morphology. Instead, the leakage of the contents from the bacterial cells was observed (Figure 2.7B). These results suggest that bacterial membranes were disrupted by the polymer P6.

Figure 2.7 TEM micrographs of Control MRSA(4A) and MRSA treated with P6 - 10 µg/mL (4B).
Based on antimicrobial activity and nanomorphology of the polymers, we propose their mechanism of action (Figure 2.8). The polymers are initially believed to exist in micelle conformation in solution. As polymers approach the bacterial surface, due to change in the electrostatic interactions, the polymers lose their stability and start to dissociate into small entities or free polymer chains, which could bind to the bacterial membranes more effectively. The free polymer chain penetrates the surface of the bacteria due to its amphipathic nature and ultimately disrupts the bacterial membrane, leading to bacterial cell death.

**Figure 2.8** Proposed mechanism of action of the polymers on Gram-positive bacteria.

**Bacterial Kinetics Assay.**

To determine the time of action and efficacy of the polymers, the most potent polymer P6 was chosen for time kill study (Figure 2.9). Cell viability was determined by the colony county method on agar plate at regular intervals of 30 min, 1 h, 2 h, 3 h, 6 h, 9 h, 12 h and 24 h. At the concentration of 50 µg/mL and 25 µg/mL, the bacteria were completely eradicated after 6 h, suggesting that the polymer has the bactericidal mechanism and the action is rapid.
Drug Resistance Assay of polymers.

The TEM and florescence microscopy suggest the polymers act by membrane disruption mechanism on the bacteria. Under this mechanism, it is accepted that the bacteria are less prone to develop resistance. To evaluate the probability of the polymer to induce resistance in bacteria, drug resistance study was conducted for the most potent compound P6 against MRSA. As shown in the Figure 2.10, the MIC values were virtually constant even after 14 passages, indicating that the bacteria do not develop resistance readily towards the polymer.

Figure 2.9 Time kill study of P6 for MRSA.
2.4 Conclusion

We reported the development of potent and highly selective antimicrobial polycarbonate polymers containing primary amino groups. Our results suggest that amphiphilic nature is necessary for bacterial killing, and random block polymers are more potent than diblock polymers, possibly due to stable nanostructures of diblock polymers which prevent them from interacting with bacterial membranes more effectively. The fluorescence microscopy and the TEM data suggest that these polymers have the mechanism of action of bacterial membrane disruption. Remarkably, these polymers are highly selective towards bacterial cells and show no discernable hemolytic activity. In vivo study of these polymers on the mouse model to evaluate their efficacy is currently underway.
2.5 References


CHAPTER 3: LIPIDATED DENDRIMERS AS POTENT AND SELECTIVE BROAD SPECTRUM ANTIBACTERIAL AGENTS

3.1 Introduction

The resistance developed by bacteria against antibiotics have contributed to the sharp rise in illness and deaths that were once curable.¹ Conventional antibiotics are generally small molecules that exert their activity by targeting specific cellular nucleic acids and proteins, or cell wall enzymes of bacteria. Bacteria will likely develop mutations rapidly on the targets upon prolonged antibiotic treatment, leading to the development of drug resistant bacterial strains.¹ In order to combat the emerging resistance, research efforts have been focused on developing host-defense peptides (HDPs) as bacteria are believed to have less probability to develop resistance to HDPs due to their distinct antimicrobial mechanisms.² It is known that HDPs are naturally occurring peptides that are rich in cationic and hydrophobic residues. Despite the diversity in three-dimensional structure, upon association with the bacterial membranes, HDPs generally obtain a globally amphipathic structure which is critical for the membrane action on bacteria.³ The interaction occurs considerably selectively for bacteria as the outer leaflet of membranes of the bacteria is predominantly rich in negatively charged phospholipids.⁴ In addition, in Gram-positive bacteria, techoic acids or lipo-techoic acids are frequently identified on the peptidoglycan layer, whereas lipopolysaccharides are common components on the outer membranes of Gram-negative bacteria.⁵ These negative charges greatly contribute to the initial attraction of the catatonically charged HDPs onto the bacterial membranes. Subsequently, the hydrophobic patches of the HDPs help to further penetrate into the bacterial membranes through hydrophobic interactions with phospholipids. In contrast, the outer surface of
mammalian cell membranes is largely zwitterionic as they are dominated by neutral phospholipids such as cholesterol, phosphatidylcholine, and sphingomyelin, while their negatively charged phospholipids are essentially found in the inner leaflet of membranes. As such, HDPs have less probability to interact with mammalian cells compared with bacteria, which is believed to account for at least a significant part of the selectivity of HDPs. As such membrane action lacks specific molecular targets, it is generally believed that HDPs are less prone to development of antibiotic resistance.

Owing to the abovementioned advantages, HDPs have received notable attention as a new generation of antimicrobial agents combating antibiotic resistance. However, there are still limitations of HDPs, which include low-to-moderate activity, potential high cost of manufacturing, proteolytic degradation etc. It is conceivable that the antimicrobial agents which can mimic the mechanism of HDPs but with enhanced selectivity and antimicrobial activity will be one viable strategy for antibiotic development. To date, unnatural peptidomimetics such as β-peptides, peptoids, oligoureas, AApeptides, have been developed to mimic HDPs and target a wide range of bacterial strains. These peptidomimetics are generally more active than natural HDPs, however, they still suffer from potential high manufacturing cost and difficulty in scale-up. Another alternative approach is to develop cationic antimicrobial polymers such as poly (α-amino acid)s, nylon-3 polymers, polyacrylates, polycarbonates, and dendrimers such as PAMAM, poly(propylene imine), etc. Dendrimers are highly branched globular structures that exhibit symmetry in their structure. They consist of a central core from which identical fragments are built up to make star-like macromolecular structures. Compared with linear polymers, dendrimers are normally in the nanosize scale and have narrow polydispersity, uniform nanomorphology, and tunable surface entities. The terminal groups of the arms of the dendrimer determine its solubility and reactivity, and are also available for further
modification. Thus, dendrimers have attracted interest in exploring their potential application in the biomedical and materials sciences. For instance, dendrimers have been used in targeted drug delivery systems for the treatment of cancer, as antimicrobial agents, enzyme catalysis and surface engineering techniques.

Different attempts have been done to synthesize dendrimer compounds by introducing functional groups pertaining to antimicrobial activity, e. g., poly(amidoamine) dendrimers with quaternary ammonium salts. Similarly, lysine dendrimers were synthesized by coupling with other peptides and confer activity against bacteria. Herein, we are presenting dendrimers that encompass lysine amino acid to present a multicharged cationic surface and bear a hydrophobic domain which is composed of different lengths of C tails. We evaluated the desired amphiphilicity of the dendrimers to show antibacterial activity and evaluated their activity and mechanism of action. The synthesis of these dendrimers is less tedious and can be easily scaled up by solid phase peptide synthesis technique and are cost effective, augmenting their potential in vitro and in vivo applications.

3.2 Results & Discussion

Solid phase Synthesis of lipidated dendrimers

Lipidated dendrimers were synthesized on Rink amide resin by the standard Fmoc chemistry protocol used for the synthesis of peptides (Solid phase peptide synthesis). 20% piperidine in DMF was used to remove the Fmoc protecting group in every coupling cycle which was followed by the coupling of 2 equiv of the desired monomer that is to be attached on the beads with 4 equiv of HOBT (1-hydroxybenzotriazole monohydrate) and DIC (diisopropylcarbodiimide) in DMF for 4 h. The coupling reaction time was increased to 6-8 h upon increasing the number of generations of the dendrimer. In order to synthesize lipidated dendrimer, initially MTT-Lys(Fmoc)-OH was attached
onto the rink amide resin. After having the monomer attached to the solid support, the MTT (4-Methyltrityl) protecting group was selectively removed under 2% TFA.

To synthesize D-A type of dendrimer, the carbon tail is attached to the first monomer and later followed by the standard Fmoc coupling chemistry with the Fmoc-Lys(Fmoc)-OH monomer. In order to synthesize D-B type of dendrimer, after removing the MTT protecting group, Dde-Lys(Dde)-OH monomer was coupled on to the first monomer on the solid support, followed by the deprotection of the Dde protecting group with hydroxyl amine (1.8 mmol) and imidazole (1.35 mmol) which was suspended in 5 mL NMP solution and was sonicated until dissolved. A ratio of 5:1 NMP and DMF solution was added on to the solid support and ran for 2h for deprotection of Dde. To each of the two unprotected amines, one C_{16} carbon tails was attached. Later the standard Fmoc coupling chemistry with the Fmoc-Lys(Fmoc)-OH monomer was done until the desired dendrimer was obtained.

The lipidated dendrimers were cleaved from the solid support in 50:48:2 TFA/CH_{2}Cl_{2}/TIS (triisopropylsilane) for 2 h. The solvent was evaporated, and the dendrimers were purified by Waters HPLC system monitored at 215 nm and the desired product peaks were collected and lyophilized. By Bruker AutoFlex MALDI-TOF mass spectrometer, the product peaks were analyzed and the dendrimer that has the desired molecular weight were separated and further used for antimicrobial activity assays.
Figure 3.1 Solid phase Synthesis of D-A and D-B dendrimers.

Figure 3.2 Solid phase Synthesis of a series of D-A dendrimers.
Figure 3.3 Solid phase Synthesis of a series of D-A-2 dendrimers.

<table>
<thead>
<tr>
<th>Dendrimer</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-A-2a</td>
<td>12</td>
</tr>
<tr>
<td>D-A-2b</td>
<td>10</td>
</tr>
<tr>
<td>D-A-2c</td>
<td>8</td>
</tr>
<tr>
<td>D-A-2d</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 3.4 Solid phase Synthesis of a series of D-B dendrimers.
Antimicrobial and Hemolytic activity

Table 3.1 The Antibacterial and Hemolytic Activity of Lipidated Dendrimers.

<table>
<thead>
<tr>
<th>Dendrimer</th>
<th>MW</th>
<th>Terminal amine groups</th>
<th>Carbon tail length</th>
<th>Gram positive bacteria (µM)</th>
<th>Gram negative bacteria (µM)</th>
<th>Hemolysis (HC₅₀, µg/mL)</th>
<th>Selectivity index of MRSA (HC₅₀/ MIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-A-1</td>
<td>512</td>
<td>2</td>
<td>16</td>
<td>5.8</td>
<td>2.9</td>
<td>5.8</td>
<td>125</td>
</tr>
<tr>
<td>D-A-2</td>
<td>768</td>
<td>4</td>
<td>16</td>
<td>0.9</td>
<td>0.9</td>
<td>3.9</td>
<td>&gt;250</td>
</tr>
<tr>
<td>D-A-3</td>
<td>1280</td>
<td>8</td>
<td>16</td>
<td>1.17</td>
<td>1.17</td>
<td>4.6</td>
<td>&gt;250</td>
</tr>
<tr>
<td>D-A-4</td>
<td>2306</td>
<td>16</td>
<td>16</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-A-2a</td>
<td>740</td>
<td>4</td>
<td>14</td>
<td>2.02</td>
<td>2.02</td>
<td>-</td>
<td>125</td>
</tr>
<tr>
<td>D-A-2b</td>
<td>712</td>
<td>4</td>
<td>12</td>
<td>4.2</td>
<td>2.1</td>
<td>-</td>
<td>125</td>
</tr>
<tr>
<td>D-A-2c</td>
<td>684</td>
<td>4</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-A-2d</td>
<td>656</td>
<td>4</td>
<td>8</td>
<td>5.8</td>
<td>2.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D-A-1</td>
<td>512</td>
<td>2</td>
<td>16</td>
<td>0.9</td>
<td>0.9</td>
<td>3.9</td>
<td>125</td>
</tr>
</tbody>
</table>

In order to determine the amphiphilicity of the dendrimers that was required to exhibit broad spectrum antimicrobial activity a series of dendrimers were synthesized. These dendrimers were grouped as two types D-A and D-B, which differ mainly in the number of C₁₆ carbon tails present. The synthesized amphiphilic dendrimers (D-A-1 to D-A-4 and D-B-1 to D-B-4) were tested for their antimicrobial activity against three clinically threatening Gram-positive bacterial strains, methicillin-resistant S. epidermidis (MRSE, RP62A), vancomycin-resistant E. faecalis (VREF, ATCC 700802), and methicillin-resistant S. aureus (MRSA, ATCC 33591), and two Gram-negative bacterial strains, E. coli (ATCC 25922), P. aeruginosa (ATCC 27853). Primarily, comparing the two major types of the dendrimers synthesized, D-B failed to show activity (D-B-1 to D-B-4) against any bacterial strains. The highest concentration that was used for testing the antimicrobial activity was 25 µg/mL. Even upon doubling the hydrophilic pattern from D-B-1 to D-B-4, the dendrimers did not exhibit activity.
This might be because of the excess hydrophobic nature obtained from the two carbon tails in the dendrimers D-B. Our hypothesis is that the two carbon tails are sterically hindering the amine groups by folding over and blocking their interaction on to the membrane of the bacteria. In contrast, the dendrimer D-A-1 which had only two terminal amine groups and one C_{16} carbon tail exhibited good antibacterial activity against Gram-positive bacterial strains. The activity was reported to be 6-3 µg/mL against MRSA and E. faecalis and 3-1.5 µg/mL against MRSE bacterial strain. Excited by the positive results, D-A-2 was synthesized which had twice the hydrophilic nature and same hydrophobic nature as D-A-1. To our delight, we found that the dendrimer was active against both Gram-positive and Gram-negative bacterial strains with an activity of 1.5-0.75 µg/mL against Gram-positive strains and 6-3 µg/mL against Gram-negative strains. Further, D-A-3 and D-A-4 dendrimers were synthesized by increasing the terminal cationic amine groups to 8 and 16 and having one C_{16} hydrophobic carbon tail respectively. Unexpectedly, the D-A-3 activity reduced against both bacterial strains when compared to D-A-2. The activity was reported to be 3-1.5 µg/mL against MRSA, MRSE, and VREF Gram-positive strains and 12-6 µg/mL against E. coli and P.A Gram-negative strain. D-A-4 was found to be inactive against both bacterial strains. From the above results, we were able to identify the optimum hydrophilic and hydrophobic nature that was required to show potent and broad-spectrum activity.

To evaluate further on the required hydrophobicity of the dendrimers to display the activity, a series of dendrimers (D-A-2a to D-A-2d) were synthesized which had the structure similar to the most active dendrimer D-A-2 but varying in the length of the carbon tail; D-A-2a, D-A-2b, D-A-2c, D-A-2d had 14, 12, 10, 8 carbon tail length respectively. The dendrimers D-A-2a, D-A-2b were reported to be active against only Gram-positive bacteria and the activity ceased as the length of the carbon tail
decreased from D-A-2c and D-A-2d dendrimer (Table 1). From the results, it is evident that a minimum length of C\textsubscript{16} hydrophobic tail is necessary for the broad-spectrum activity of dendrimers.

In order to know the selectivity and further application of the dendrimers, a comparison of the antimicrobial and hemolytic data study was found to be crucial. Therefore, hemolytic study was conducted and the results were analyzed for the active dendrimers. The hemolysis for the most active dendrimer D-A-2 was reported to be >250 \(\mu\)g/mL which proves that the dendrimer was more selective to the bacterial cell membrane than the human cellular membrane. D-A-2 was further reported to be 165 times more selective towards MRSA than the human cells.

**Mechanism of Action**

These above findings suggest that cationic and anionic groups are required for the dendrimer to exhibit antibacterial activity. An appropriate ratio of both the groups are required to exhibit potent and broad-spectrum activity which has been evaluated and reported. We hypothesize that our dendrimers exhibit antibacterial activity by disrupting the cellular membrane similar to the antibacterial action of HDPs. The cationic amine groups on the dendrimer were hypothesized to bind to the negative teichoic acid groups on the bacterial surface and having made an interaction, the hydrophobic carbon tail helps in the penetration of the dendrimer into the bacterial membrane resulting in the leakage of the cellular contents of the bacteria and eventually leads to bacterial cell death.

In order to confirm our proposed hypothesis for the mechanism of action, florescence microscopy (FM) and transmission electron microscopy (TEM) experiments were done for the controlled bacteria and the drug treated bacteria. As the dendrimer displayed broad spectrum activity, a Gram-positive strain (MRSA) and a Gram-negative strain (E. coli) were used for the FM and TEM study.

In FM assay, two types of dyes were used to stain the bacteria. 4,’6-diamidino-2-phenylindole (DAPI) is a membrane permeable dye that can stain all the cells as it binds to the DNA double strand;
whereas propidium-iodide (PI) is the non-membrane permeable dye that can stain only the dead cells, as it interacts with the nucleic acids of the bacteria and gives a bright red fluorescent complex. So, PI can interact only when the cell membrane of the bacteria is disrupted. Hence by this method we can determine if the dendrimers display their action by membrane disruption. The most active dendrimer D-A-2 was used for the FM study to evaluate its mechanism of action. From Figure 3.5 & 3.6, it is clearly evident that D-A-2 dendrimer was able to disrupt the membrane of MRSA and E. Coli respectively as PI stain was seen with the bacteria treated with D-A-2 but not with the control bacteria. This indicates that dendrimer D-A-2 showed its bactericidal action against Gram-positive and Gram-negative bacteria by membrane disruption technique.

![Figure 3.5 Fluorescence micrographs of MRSA treated with 10 µg/mL of D-A-2 for 2 h.](image)

(C1) Control MRSA, DAPI stained, no treatment with D-A-2;

(C2) Control MRSA, PI stained, no treatment with D-A-2;

(D1) MRSA, DAPI stained, treated with the D-A-2;

(D2) MRSA, PI stained, treated with the D-A-2.
**Figure 3.6** Fluorescence micrographs of *E. Coli* treated with 10 µg/mL of **D-A-2** for 2 h.

(C1) Control *E. Coli*, DAPI stained, no treatment with D-A-2;

(C2) Control *E. Coli*, PI stained, no treatment with D-A-2;

(D1) *E. Coli*, DAPI stained, treated with the D-A-2;

(D2) *E. Coli*, PI stained, treated with the D-A-2.

In order to further prove our hypothesis of the dendrimer mimicking the mechanism of action of HDPs, TEM microscopy images were used to analyze the morphology for the drug treated and non-drug treated bacteria. **Figure 3.7 & 3.8**, clearly shows the membrane disruption when treated with the dendrimer D-A-2 for Gram-positive and Gram-negative bacteria respectively.

**Figure 3.7** TEM micrographs of *MRSA* treated with 10 µg/mL of **D-A-2** for 2 h.

(a) Control MRSA, no treatment with the drug;
(b) MRSA treated with D-A-2, image representing the initial rupture of the membrane;
(c) MRSA treated with D-A-2, image representing the damage of the cell membrane, cell rupture.

**Figure 3.8** TEM micrographs of *E. Coli* treated with 10 µg/mL of D-A-2 for 2 h.

(a) Control *E. coli*, no treatment with the drug;
(b) *E. coli* treated with D-A-2, image representing the initial rupture of the membrane;
(c) *E. coli* treated with D-A-2, image representing the damage of the cell membrane, cell rupture.

**Bacterial Kinetic Study**

To future investigate the time of bactericidal action of the dendrimers, bacterial kinetics assay was conducted. The most active dendrimer **D-A-2** was taken and the time required to show its bactericidal action was analyzed for Gram-positive and Gram-negative bacteria. Colony forming units of the Gram-positive and Gram-negative bacteria on the agar plate were counted for three different concentrations (50 µg/mL, 25 µg/mL, 12.5 µg/mL) of the D-A-2 treated bacteria and the control bacteria at regular intervals of 10min, 30 min, 1 h, 2 h. From **Figure 3.9 & 3.10**, it is clearly evident that at the concentration of 50 µg/mL, 25 µg/mL 12.5 µg/mL, the Gram-positive bacteria MRSA were eradicated completely after 1h, whereas the Gram-negative bacteria, *E. Coli* was eradicated after 2h at the concentration of 50 µg/mL, 25 µg/mL 12.5 µg/mL. This delay in bactericidal action on the Gram-negative bacteria might be due to the presence of the extra outer membrane layer, which is not present in the Gram-positive bacterial strains.
Bacterial Biofilm Assay

The infections caused by biofilms are a great threat to human life. Recent reports have shown that 80% of the bacterial infections are caused due to biofilms. Bacterial biofilm has become a severe problem specially in the cases of patients who suffer from infections that occur from the antibiotic resistant bacteria. Biofilms formed by MRSA and E. Coli have become major concern in hospitals as they contaminate the surgical tools and organ catheters. Therefore, developing new drugs that act against biofilm formation has become a major concern to treat bacterial infections. Thus, we analyzed the biofilm inhibiting efficiency of the most active dendrimer D-A-2 against MRSA and E. Coli. As shown in Figure 3.11, at 0.17 μg/mL, D-A-2 was able to inhibit 85% of biofilm formation of E. Coli and was able to completely eradicate the biofilm formation of MRSA. At the concentration of 0.8
μg/mL, D-A-2 was able to eradicate biofilm formation of E. Coli completely. The above stated results confirm that the dendrimers can act as biofilm inhibitors.

Figure 3.11 Biofilm study of D-A-2 against MRSA and E. Coli.

3.3 Materials and Methods

Antimicrobial Assay. Twelve synthesized lipidated dendrimers were tested against different strains of bacteria to determine their Minimum inhibitory concentration (MIC). Three clinically threatening Gram-positive bacterial strains including methicillin-resistant S. epidermidis (MRSE, RP62A), vancomycin-resistant E. faecalis (VREF, ATCC 700802), and methicillin-resistant S. aureus (MRSA, ATCC 33591) and two Gram-negative bacterial strains, E. coli (ATCC 25922), P. aeruginosa (ATCC 27853) were tabbed for testing. A serial dilution method was performed for determining the antimicrobial activities. In a 4ml of TSB solution a single colony of bacteria was grown and incubated at 37°C overnight after which the cultured bacteria were diluted by 100-fold and were shaken until their mid logarithmic phase was obtained. 50μL of medium containing different concentrations of the lipidated dendrimers was made in each vial of the 96 well plate. 50μL of aliquots of bacterial suspension is added to the medium with different drug concentrations. The 96 well plate was incubated at 37 °C overnight and the cell growth was monitored using Biotek synergy HT microtiter plate reader.
under the 600nm wavelength absorbance. The assay was repeated two times and showed comparable results.33,33b,34

**Hemolysis Assay.** The selectivity of the lipidated dendrimers was determined through hemolysis assay. To perform this assay fresh K2EDTA treated human red blood cells (hRBCs) were centrifuged at 1000g for 10 minutes. The step was done three times until the supernatant was clear, then the desired suspended erythrocytes were collected and washed with PBS buffer a couple of times. The collected RBC were then diluted into 5% v/v suspension. From the diluted solution 50 μL was taken and added to the already prepared serially- diluted lipidated dendrimer solutions and were left for incubation at 37 °C for 1 hr. The incubated solutions were centrifuged at 3500 rpm for 10 minutes. To measure the absorbance, 30 μL of supernatant was diluted with 100 μL of PBS and absorbance at a wavelength of 540 nm and 410 nm was recorded. Selectivity of the lipidated dendrimers (% Hemolysis) was then calculated by applying the formula  

\[
\text{% hemolysis} = \frac{(\text{Abs sample} - \text{Abs PBS})}{(\text{Abs Triton} - \text{Abs PBS})} \times 100\%
\]

where PBS was used as a negative control and 0.1% Triton x-100 was used as a positive control. Results were repeated two times with duplicates each time.35

**Fluorescence Microscopy.** To assess whether the lipidated dendrimers acts on the bacterial membrane or not, a double staining florescence microscopy assay was used. Both, 6-diamidino-2-phenylindole dihydrochloride (DAPI) and propidium iodide (PI) dyes were selected as fluorophores to differentiate between the dead bacterial cells and viable cells, DAPI is capable of staining both live and dead cells but PI can only stain dead cells with damaged membrane cell due to its impermeable nature through the membrane. The bacteria were grown to mid logarithmic phase and were incubated with lipidated dendrimers at 37 °C for 2 h, the mixture was centrifuged at 1000g for 10 min. Once the bacterial pellets were collected and washed with PBS three to four times. They were subsequently incubated with PI (5 μg/mL) in the dark for 15 min at 0 °C, the excessive PI was rinsed using PBS several times. Lastly,
the cells were incubated with DAPI (10 μg/mL) for 15 min on ice and the excess DAPI was removed by washing with PBS buffer. After the samples were ready,10 μL of the bacteria were used for testing under Zeiss Axio Image Zloptical microscope using 100x oil-immersion objective.35

**Time kill study.** The kinetic assay of the lipidated dendrimers was tested against MRSA and E. COLI. The bacteria were grown to midlogarithmic phase in TSB medium and then diluted into 10⁶ CFU mL⁻¹ suspensions. Different concentrations of the lipidated dendrimers were then added to the diluted suspensions and incubated for 10 min, 30 min, 1 h, and 2 h, respectively. After incubation, the mixture was diluted into 10² to 10⁴ times, then dispersed on TSB agar plates and incubated overnight at 37 °C. The colonies on the plates were counted and graphed against colony forming unit of bacteria and incubation time.36

**TEM.** Similar procedure was used to grow the bacteria to mid logarithmic phase as incase of MIC study. Two samples were made, one batch has only bacteria and the other batch was the bacteria treated with D-A-2. Samples were incubated at 37 °C for 2 h and the solution was centrifuged at 1000g for 10 min. Bacterial pellets were collected at the bottom of the tube. Pellets were washed with PBS three to four times before dissolving in DMSO to a 10⁻⁶ M.37 A drop of the solution was added on the carbon-coated Cu grid and the excess sample was wiped off with the filter paper to avoid any aggregation. The grid was then left for 30 min on the bench top so that the sample can be absorbed onto the grid. Later the grid was washed with acetonitrile and left in oven for few minutes to remove any further traces of the solvent. The samples on the grids were analyzed and images were taken with FEI Morgagni 268D TEM instrument.38,39

**Biofilm assay.** The bacteria were grown and the treated with the dendrimers in 96 well plate in a similar fashion as incase of MIC study. The 96 well plates were left for incubation at 37°C for 48
hours. The plate was shaken over an empty tray to remove all the planktonic bacteria. The 96-well plate was then rinsed in a large tray of water and shaken. Rinsing of the plate in water was done a couple of times. The plate was hit against the paper towel to get rid of the water from the wells and was laid on another paper towel and left to dry overnight. The wells were stained with 125 μL 0.1% crystal violet solution for 10 minutes. The plate was shaken over the empty tray to remove the solution and the plate was rinsed with water a couple of times until the wells are free of any liquid crystal violet and left to dry overnight. 200 μL of 30% acetic acid were added to the wells to solubilize the stained crystal violet and left for 10 min. 125 μL of the solution was transferred from each well into a flat-bottom 96-well plate. The plate was read at OD 595nm with a plate reader.\(^{34}\) The average of the blank wells was subtracted from the OD of each sample that contained sample and the average of the wells with samples were calculated. The averages of the sample wells were normalized to the average of the control wells.\(^{4}\)

### 3.4 Conclusion

We have developed a new class of antimicrobial lipidated dendrimer that can mimic the HDPs. The amphiphilicity that was required for the dendrimer to display potent and broad-spectrum activity against a range of multidrug-resistant Gram-negative and Gram-positive bacteria was identified. These lipidated dendrimers were also proven to act as good biofilm inhibitors providing an exciting route to develop cost efficient and less tedious drugs against biofilm and drug resistant bacterial species. Furthermore, these dendrimers can be used for other biological applications to treat various fungal, viral infections and their activity is yet to be explored.
3.5 References


(16)  

(17)  


CHAPTER 4: SMALL ANTIMICROBIAL AGENTS BASED ON ACYLATED REDUCED AMIDE SCAFFOLD

Note to Reader

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4.1 Introduction

Antibiotic resistance in bacteria is one of the most critical concerns faced in global public health.¹ Notorious multidrug-resistant strains, including Gram-positive bacteria methicillin-resistant Staphylococcus epidermidis (MRSE), methicillin-resistant Staphylococcus aureus (MRSA), Vancomycin-Resistant Enterococci faecalis (VRE), and Gram-negative bacteria Escherichia coli (E. Coli), Klebsiella pneumoniae, Pseudomonas aeruginosa, have emerged to be the major cause of hospital and community-acquired infections.² ³ As conventional antibiotics are observed to elicit escalating resistance, significant efforts have been extended to the investigation of host-defense peptides (HDPs) and their derivatives as alternative therapeutic strategies.⁴⁻⁶ Natural antimicrobial peptides, also known as HDPs, have attracted substantial interest in the past decade, and a few of them, such as PMX30063 and LTX109, are currently in clinical trials.⁴⁻⁷ HDPs are produced by living organisms as the first line of defense against a wide variety of infections.⁵ Unlike conventional antibiotics which target specific membrane or intracellular components of bacteria, HDPs preferentially interact with bacterial membranes and lead to the destruction of membranes eventually. The specificity of HDPs toward bacteria cells over mammalian cells lies on the intrinsic difference between bacterial and mammalian cell membranes. It is known that the outer leaflet of mammalian
cell membranes are comprised of zwitterionic lipids such as cholesterol, phosphatidylcholine, and sphingomyelin. Their negatively charged phospholipids are generally sequestered in the inner leaflet of membranes. In contrast, negatively charged phospholipids including cardiolipin and phosphatidylglycerol are frequently identified in the outer leaflet of bacterial membranes. In addition, negatively charged molecules such as teichoic acids and lipoteichoic acids are membrane components of most Gram-positive bacteria, while lipopolysaccharides are characteristic molecules on the outer membranes of Gram-negative bacteria. As a result, bacterial membrane surfaces are generally more negatively charged than those of mammalian cells, leading to interaction with cationic HDPs preferentially. Although detailed antimicrobial mechanism of HDPs are still under debate, a few models including barrel stave, carpet, toroidal pores have been proposed, all of which more or less possess surfactants-like behavior. For instance, lipopeptides have been considered as the most potent natural biosurfactants. As the interaction is based on physical charge-charge interaction and lacks defined membrane targets, it is believed that HDPs may decrease the risk of resistance development. This is also why HDPs possess broad-spectrum antibacterial activity and they are often bactericidal rather than bacteriostatic observed for most conventional antibiotics. It should be noted that some HDPs do have defined intracellular targets including proteins, nucleic acids and ribosomes, however, their cationic amphipathic structure is still crucial for their entry into bacterial cells, and membrane disruption may be the additional mechanism of action which further synergizes their antimicrobial activity.

Nevertheless, HDPs face considerable challenges for further antibiotic development, among which low stability, moderate activity and cost for production are major obstacles. Oligomeric peptidomimetics are resistant to proteolytic degradation, and through optimization, peptidomimetics such as peptoids, β-peptides, oligoureas, and AApeptides can mimic the structure and
function of HDPs and show potent and broad-spectrum activity against multidrug-resistant bacterial pathogens. Thus, they hold promise for future development of new class of antimicrobial agents. However, their structural complexity and large molecular weights (>1500 Da) make the synthetic process tedious and time consuming, significantly hindering the translation of HDPs into clinical applications. To overcome the drawbacks, considerable endeavors have been devoted to the development of small molecular mimetics of HDPs of simplicity. Examples include arylamide foldamers,25 small peptoid mimics,26,27 β-amino acid derivatives,28 dipeptide derivatives,29 and aryl compounds.30 In particular, it is exciting to witness that arylamide foldamers PMX series and dipeptide derivatives LTX compounds are currently either in Phase II or III clinical trials (Figure 4.1), demonstrating the therapeutic potential of small HDP-mimicking antibiotic agents.

**Figure 4.1** Structure of PMX-30063 and LTX-109, and design of small antimicrobial agents based on the acylated reduced amide. R₁ represents cationic groups, R₂, R₃, and R₄ represent hydrophobic groups.

Reduced amide derivatives are peptides containing reduced amide bond, and have been widely used as isosteric replacements for peptides (Figure 4.2).31 Even with one amide bond reduced to give secondary amine, the peptide sequences display significantly enhanced resistance to enzymatic degradation in contrast to canonical peptides.31 Recently there is growing interest in the exploration of biological application of reduced-amide-containing compounds, such as the use as enzyme inhibitors,32 analogues of endogenous peptides,33 synthetic vaccines,34 and DNA-complexing agents
for gene delivery. Several reduced-amide-containing sequences were also reported to mimic HDPs as they are more resistant to proteolytic degradation. Indeed, AApeptides as oligomeric acylated reduced-amides were shown to display broad-spectrum antibacterial activity. However, these reduced-amide-containing oligomers are fairly long and large (MW >1800) sequences, and therefore their further optimization and potential for antibiotic therapeutics is still limited.

Figure 4.2 The general chemical structures of α-peptide and reduced amide pseudopeptide.

4.2 Results and Discussion

To identify antibiotic agents with potentially more practical applications, herein we report the design and development of a class of small antimicrobial molecules containing acylated reduced amide (Figure 4.1). They are essentially built on a reduced dipeptide scaffold however with variable side chains. We hypothesized that if \( R_1 \) is a cationic group, while \( R_2, R_3 \) and \( R_4 \) are hydrophobic, the global structure of the molecules should be amphipathic, and thus they are expected to mimic HDPs and kill bacterial via membrane disruption. We anticipated that there are advantages associated with the molecular design. Reduced amide has been demonstrated for their biological potential and stability. The design is also very straightforward and universal, as functional groups can be introduced very easily. Moreover, the activity of the molecules could be tuned by varying the hydrophobicity of \( R_2, R_3 \) and \( R_4 \) groups. As such, it is intriguing to investigate the antimicrobial activity of this class of small molecules, and to evaluate their potential as antibiotic therapeutics.
Figure 4.3 The general scheme for the preparation of small reduced dipeptide derivatives.

The synthesis of these molecules was straightforward (Figure 4.3). Please see supporting information for details. Briefly, the building block\textsuperscript{39} bearing the \( R_2 \) side chain was attached to chlorotrityl resin. After Fmoc group was removed, the \( R_3 \) moiety was attached to the secondary amine. Next, the alloc protecting group was removed, followed by capping with the \( R_1 \) group. The molecule was then cleaved from the solid support, to which the \( R_4 \) functionality was appended to the c-terminus to give the final product. We synthesized a series of such small molecules, and the structures of some lead compounds are shown in Table 4.1. Their antibacterial activity was also tested against a few clinically relevant bacteria, including multi-drug resistant Gram-positive and Gram-negative bacterial pathogens (Table 4.1)\textsuperscript{23,40}

Table 4.1 The structure of small antimicrobial molecules and their antibacterial activity.

<table>
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<tr>
<th>#</th>
<th>Structure</th>
<th>MIC (µg/mL)</th>
<th>Hemolysis (HC\textsubscript{50}, µg/mL)</th>
<th>IC\textsubscript{50} (µg/mL)</th>
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<td></td>
<td></td>
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<td>Gram +</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>E. coli</em></td>
<td><em>P. aeruginos</em></td>
<td><em>K. pneumoniae</em></td>
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*a* NT, Not tested because compounds are not active.

*b* NC, No cytotoxicity toward HK-2 and K562 cells at 25 μg/mL.
The microbial organisms used in the study were *K. pneumoniae* (ATCC 13383), *P. aeruginosa* (ATCC 27853), *E. coli* (ATCC 25922), Methicillin-resistant *S. epidermidis* (MRSE) (RP62A), Methicillin-resistant *S. aureus* (MRSA) (ATCC 33591), vancomycin-resistant *E. faecalis* (ATCC 700802). The minimum inhibitory concentration (MIC) is the lowest concentration that completely inhibits microbial growth after 20 h.\textsuperscript{24,37} HC\textsubscript{50} is the concentration causing 50% hemolysis. Hemolysis activity was not measured for peptidomimetics that did not exhibit antimicrobial activity. Daptomycin\textsuperscript{41} and colistin,\textsuperscript{42} which are last-resort antibiotics and active against either Gram-positive or Gram-negative bacteria, were included for comparison.

As shown in Table 4.1, with MWs ranging from 600 to 800, many molecules have shown good antibacterial activity against a panel of multi-drug-resistant Gram-positive and Gram-negative bacteria. The structure-activity relationship is also obvious in this class of molecules allowing for their further optimization and development in the future. In compound 1, R\textsubscript{1} and R\textsubscript{4} are hydrophobic naphthyl groups, while R\textsubscript{2} and R\textsubscript{3} are cationic groups. This compound did not show any antibacterial activity at the tested condition, possibly because the structure doesn’t have segregated hydrophobic and cationic domains. We next kept R\textsubscript{1} as a cationic group, and started to change the hydrophobicity of R\textsubscript{2}, R\textsubscript{3} and R\textsubscript{4}. As seen in compounds 2–4, when R\textsubscript{2} is hydrophilic, the compounds were still not effective, albeit compound 2 exhibited some activity against all strains. This is likely due to the insufficient hydrophobicity of the molecules which do not endow the molecules with membrane activity. However, when R\textsubscript{2}, R\textsubscript{3} and R\textsubscript{4} groups are all hydrophobic, the compound 5 started to show activity against both Gram-positive and Gram-negative bacteria. It suggested that all R\textsubscript{2}, R\textsubscript{3} and R\textsubscript{4} groups have to be hydrophobic so as to possess sufficient interactions with bacterial membranes. We also noticed that the guanidino cationic group is slightly weaker than the amino group in eliciting antimicrobial activity, thus we focused on the development of derivatives containing cationic amino
groups. It was found that the antibacterial activity is highly related to the overall hydrophobicity of \( \textbf{R}_2, \textbf{R}_3 \) and \( \textbf{R}_4 \). Change of \( \textbf{R}_3 \) to adamantyl group seemed significantly improved the antimicrobial activity of \textbf{7}, as its MIC against MRSE was in the range of 3.12–6.25 µg/mL already. When \( \textbf{R}_4 \) became more hydrophobic, for instance, being two phenyl groups, or biphenyl group, the activity of compound \textbf{8} and \textbf{9} was further improved to be 3.12–6.25 µg/mL for most of strains. Substitution on the aromatic group on \( \textbf{R}_4 \) was also likely to boost activity, as seen for \textbf{10}, \textbf{11} and \textbf{12}, which contain the CF\(_3\) or \textit{t}-butyl groups on the aromatic ring, displayed further enhanced activity. For instance, both \textbf{11} and \textbf{12} had the activity of less than 6.25 µg/mL for all tested strains. It should be noted that these compounds are quite selective as their hemolytic activity are all more than 100 µg/mL. We hypothesized that the antibacterial activity could be further enhanced by increasing the hydrophobicity of \( \textbf{R}_4 \), and thus the compound \textbf{13} was designed. This compound contained adamantyl groups on both \( \textbf{R}_3 \) and \( \textbf{R}_4 \) position, and as anticipated, it displayed more potent activity against MRSA with MIC of 1.56 µg/mL. It is known that hemolytic activity could be mitigated through the introduction of cationic charges.\(^{43}\) We next designed the compound \textbf{14}, which contained one more lysine residue on the \( \textbf{R}_1 \) position compared with \textbf{13}. As expected, with one more cationic charge, compound \textbf{14} exhibited less hemolytic activity. However, the antibacterial activity of compound \textbf{14} was also less than \textbf{13}. Similar results were observed for \textbf{15} and \textbf{16} as well, suggesting that more lysine residues may not be preferred for bacterial killing. It is also interesting that with the replacement of lysine residue in \textbf{14} by an ornithine residue, compound \textbf{15} shows slightly increased hemolytic activity, even though the overall cationic charges remain the same. It maybe because the side chain of lysine is slightly longer than that of ornithine, which decreases the potential non-specific interactions. In addition, replacement of the lysine residue with arginine residue didn’t impact significantly either on antibacterial activity or on hemolytic activity, as seen for \textbf{14} and \textbf{16}, both of which show comparable selectivity towards bacteria. Next, we
also tested the cytotoxicity of these compounds against HK-2 and K562 cells. Interestingly, although amphipathic agents are often cytotoxic, our compounds did not show noticeable cytotoxicity at the concentration of 25 μg/mL against both HK-2 and K562 cells. The lead compound 13 has EC\textsubscript{50} of 86.3 and 83.2 μg/mL against HK-2 and K562 cells, respectively, >50 fold of selectivity for MRSA.

As these acylated reduced-amide molecules were designed to possess global amphipathic structure of HDPs, we hypothesized they could mimic the mechanism of action of HDPs and exert their bactericidal activity by disrupting bacterial membranes. To evaluate the ability of these compounds to compromise bacterial membranes, fluorescence microscopy was used to visualize the impact of compounds on membranes of \textit{S. aureus}.\textsuperscript{19} Although 11–13 have similar activity against the tested panel of bacteria, 13 and 14 were chosen in the study as they show most potent activity in killing MRSA. Two dyes, 4',6-diamidino-2-phenylindole (DAPI), and propidium iodide (PI) (Figure 4.4), were employed in the study. It is known that DAPI could stain membranes of bacterial cells with blue fluorescence irrespective of cell viability, however, the red fluorescence of PI due to the DNA intercalation could only be observed in the presence of impaired membranes that are permeable to PI. As shown in Figure 4.4, in the DAPI channel (460 nm), \textit{S. aureus} in the control groups are visible. However, they are not visible under the PI channel (620 nm). After bacteria were incubated with either 13 or 14, they fluoresce under both PI and DAPI channels, suggesting the membranes of \textit{S. aureus} were damaged.
<table>
<thead>
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<tr>
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<td><img src="#" alt="b2" /></td>
<td><img src="#" alt="c2" /></td>
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</table>

**Figure 4.4** Fluorescence micrographs of *S. aureus* that are treated or not treated with 25 µg/mL of 13 or 14 for 2 h. a1, control, no treatment, DAPI stained; a2, control, no treatment, PI stained. b1, treatment with 13, DAPI stained; b2, treatment with 13, PI stained. c1, treatment with 14, DAPI stained; c2, treatment with 14, PI stained. Scale bar = 10 µm.

It is known that membrane-active antibiotic agents could exert their bactericidal activity very rapidly, a phenomena that has been widely observed in HDPs.44 We thus next conducted time-kill study to investigate if 13 and 14 could rapidly kill MRSA in a dose-dependent fashion. Cell colonies were counted in agar plates at different time points at 4-, 8-, 16-fold of the MIC. As shown in Figure 4.5, the compounds 13 and 14 at the concentration of either 25 or 50 µg/mL could remove all bacteria completely in just two hours. This observation indicates that 13 and 14 act in a similar manner to that of HDPs.
Figure 4.5 Time-kill curves of 13 (a) and 14 (b) for MRSA. The killing activity was monitored for the first 2 h. The concentrations were 4 ×MIC, 8 ×MIC, and 16 ×MIC, respectively.

It is known that HDPs do not induce resistance in bacteria readily because they disintegrate bacterial membranes, a mechanism lacking of defined molecular targets. To evaluate the probability of these reduced-amide based small molecules to elicit resistance in bacteria, we carried out the drug resistance studies of 13 and 14 against MRSA. Their MICs towards MRSA were first measured, and then these compounds were incubated with bacteria in the well of the half-MIC, and tested for their activity again. As shown in Figure 4.6, after 14 passages, MICs of both 13 and 14 are virtually very constant, indicating that these compounds do not induce drug-resistance in MRSA, which suggests their mode of action is analogues to that of HDPs.

Figure 4.6 Drug-resistance study of 13 and 14 toward MRSA.
The biofilm is a notorious problem because bacteria in biofilm are generally more difficult to eradicate than planktonic cells. Thus, it could lead to severe chronic infectious diseases which complicate the treatment. In addition, the biofilm is also known to contaminate medical devices contributing to hospital acquired infections.\textsuperscript{46} The biofilm formed by Gram-negative bacteria are even more challenging due to their stronger resistance. It is known than biofilms of \textit{E. coli} and \textit{A. baumannii} have posted great threat to community, and thus compounds which prevent their biofilm formation could lead to novel therapeutics.\textsuperscript{47,48} To evaluate the potential of the reduced-amide derived molecules in the biofilm prevention, the compound \textbf{13} was evaluated for its ability to inhibit the biofilm formation of \textit{E. coli} and \textit{A. baumannii}. As shown in Figure 4.7, at just a concentration of 0.6 µg/mL, \textbf{13} could inhibit more than 50\% of biofilm formation of both \textit{E. coli} and \textit{A. baumannii}. At the concentration of 2 µg/mL, it almost completely eradicated their biofilms.

![Image](image-url)

**Figure 4.7** Biological activity of compound \textbf{13} on inhibition of biofilm by \textit{A. baumannii} and \textit{E. coli}.

Pneumonia is one of the most severe diseases caused by bacterial infection.\textsuperscript{49} Community-acquired MRSA induced pneumonia has led to significant medicare cost and modality. Encouraged by the \textit{in vitro} anti-bacterial profiles of our compounds, we tested their efficacy on a rat model bearing MRSA-induced ventilator-associated pneumonia,\textsuperscript{50} in order to evaluate their therapeutic potential. In this model anti-inflammatory activity is used as a surrogate for clearing the infection. The pathological
analysis (Figure 4.8a) show that local nasal administration of MRSA infection stimulated acute inflammation response in lungs, as indicated by the prevalence of neutrophils (blue spots in H&E staining). In the control group which was treated with saline, typical inflammation caused by pneumonia persisted over three days. In contrast, significant reduction of lung infiltrated pro-inflammatory cells could be observed post i.v. administration of compound 13 (10 mg/kg), strongly support the effectiveness of our antibacterial agents. To further support the observation, the level of the inflammatory cytokine TNF-α was also quantified by the ELISA assay. As shown in Figure 4.8b, the level of TNF-α cytokine increased over time in bronchoalveolar lavage fluid from the control rat group received saline only, suggesting the persistence of lung inflammation. However, with the treatment of 13, the cytokine level was lowered down even after 1 day. After 3 days post treatment, the level of TNF-α level decreased, which was almost half of the level in control. The in vivo studies suggest that the compound 13 is capably of effectively suppressing the acute lung inflammation caused by pneumonia.

**Figure 4.8** In vivo efficacy of the compound 13 on a MRSA-induced ventilator-associated pneumonia bearing rat model. **a**, pathological assay via H&E staining; **b**, TNF-α cytokine level in the rats by ELISA assay.
4.3 Conclusion

To summarize, we have developed a series of small antimicrobial agents based on the acylated reduced amide scaffold. These molecules display good potency against a panel of multidrug-resistant Gram-positive and Gram-negative bacteria. Although other antimicrobial mechanisms cannot be excluded, our mechanistic studies suggest that these compounds could kill bacteria rapidly by disrupting bacterial membranes, a mechanism analogous to that of host-defense peptides (HDPs). The hypothesis is further supported by the fact that the susceptibility of MRSA bacteria to the lead compounds remains nearly unchanged even after 14 passages. Meanwhile, they also exhibit high potency to inhibit the formation of biofilms. Furthermore, anti-inflammatory potential of these molecules was confirmed in the MRSA-induced pneumonia-bearing rat model. Although these types of agents have moderate to good selectivity so far, our work shed light on the development of more potent compounds. Compared to HDPs, facile synthesis and high cost-effectiveness of these compounds make them appealing classes of antibiotic agents combating drug-resistant bacterial strains. Further studies on optimization of activity and selectivity, as well pharmacokinetic analysis is underway in our lab.

4.4 Experimental section

General information. All Fmoc protected α-amino acids and Rink-amide resin (0.7 mmol/g, 200-400 mesh) were purchased from Chem-Impex International, Inc. Solvents and other reagents were purchased from either Sigma-Aldrich or Fisher Scientific and were used without further purification. Solid-phase synthesis of the compounds was carried out in the peptide synthesis vessel on a Burrell Wrist-Action shaker. The products were purified on a Waters Breeze 2 HPLC system, and lyophilized on a Labconco lyophilizer. The purity of the compounds was determined to be >95% by analytical
HPLC (1 mL/min flow, 5% to 100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 40 min was used). The NMR spectra were obtained on a Varian Inova 400 instrument.

**Synthesis of desired compounds.** Synthesis of 7 is shown below.

![Synthetic route for compound 7](image)

The other compounds were synthesized following the similar procedure of 7. 2-Chlorotrityl chloride (CTC) resin (200 mg, 0.2 mmol) was swelled in 3 mL DCM for 15 min. The attachment of the first building block to the resin was achieved by adding γ-BB-1 (154 mg, 0.3 mmol) and N,N-diisopropylethylamine (DIPEA) (173 μL, 1 mmol) to the beads in the reaction vessel, which was allowed to shake at room temperature for 2 h. After that, the reaction solution was drained, followed by washing with DMF (2 mL×3) and DCM (2 mL×3). The unreacted 2-chlorotrityl chloride moieties were capped with 3 mL methanol for 30 min. The beads were washed with DCM (3 mL×3) and DMF (3 mL×3). The Fmoc group was removed by treating beads with 20% piperidine/DMF (v/v) solution for 10 min (×2) at room temperature. The solution was drained and washed with DMF (3 mL×3) and DCM (3 mL×3). The beads were reacted with 1-admantaneacetyl chloride (72 μL, 0.4 mmol) and DIPEA (87 μL, 0.5 mmol) for 30 min (×2) at room temperature, and the solution was removed. After
DMF (2 mL×3) and DCM (2 mL×3) wash, the beads were treated with Pd(PPh3)4 (24 mg, 0.02 mmol) and Me2NH·BH3 (70 mg, 1.2 mmol) in 2 mL DCM for 10 min (×2) to remove the alloc protecting group. After the reaction, the solution was drained, and the beads were washed with DCM (3 mL×3) and DMF (3 mL×3). Next, Fmoc-Lys(Boc)-OH (187 mg, 0.4 mmol), N,N-diisopropylcarbodiimide (DIC) (84 μL, 0.8 mmol), and hydroxybenzotriazole (HOBt) (122 mg, 0.8 mmol) were pre-mixed in 2 mL DMF for 5 min before getting transferred to the reaction vessel. The reaction was shaken at room temperature for 3 hours, and the solution was drained. Then the intermediate was cleaved from resin with 5 mL of the cleavage cocktail (acetic acid:2,2,2-trifluoroethanol: DCM 1:1:8, v/v/v) for 2 h, the solution was collected and the remaining beads were washed with 3 mL of the cleavage cocktail solution for three times. All the solution was combined and concentrated in vacuo with co-evaporation with hexane multiple times to completely remove acetic acid. A white solid intermediate was obtained. This intermediate was pre-mixed with HOBt and DIC in DMF for 5 min, followed by the addition of 1-Naphthylmethylamine. The clear faint yellow solution was stirred at room temperature for 5 h, then water was added to quench the reaction, followed by ethyl acetate (EtOAc) extraction. The organic layer was washed with 1M HCl (×3) then brine (×1), dried over anhydrous Na2SO4 and concentrated by vacuum. The residue was treated with 50% TFA in DCM for 2 h and then the solvent was removed with the N2 flow. The residue was then treated with 50% diethylamine in CH3CN for 30 min to remove Fmoc protecting group, followed by the removal of the solvent. The crude was analyzed and prepared on Waters HPLC system, followed by lyophilization to give the pure product 7.

**Compound 1.** 1H NMR (400 MHz, d6-DMSO) δ 8.26–8.65 (m, 2H), 7.80–8.03 (m, 5H), 7.54–7.79 (m, 6H), 7.37–7.53 (m, 3H), 4.60–4.74 (m, 1.5H), 4.23–4.28 (m, 0.8H), 3.85–4.13 (m, 2H), 3.48–3.66 (brd m, overlapped with H2O, 4.5H), 3.41 (dd, J = 14.8, 9.2 Hz, 0.6H), 3.26 (dd, J = 14.8, 9.2 Hz, 0.5H),
2.61–2.75 (m, 3H), 2.50–2.59 (m, 1H), 2.12–2.80 (m, 0.8H), 1.26–1.75 (m, 6H). HRMS (ESI) C_{34}H_{42}N_{5}O_{3} [M+H]^+ calc’d = 568.3282; found = 568.3271.

**Compound 2.** $^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 8.99 (t, $J = 5.6$ Hz, 0.3H), 8.47–8.52 (m, 1H), 8.10 (brd, 2H), 7.93 (d, $J = 8.0$ Hz, 2H), 7.90 (s, 1H), 7.81 (t, $J = 7.2$ Hz, 2H), 7.77 (brd, 1H), 7.69 (d, $J = 8.0$ Hz, 1.3H), 7.42–7.59 (m, 5H), 7.28 (t, $J = 7.2$ Hz, 0.8H), 7.15 (d, $J = 6.4$ Hz, 0.7H), 4.71–4.83 (m, 0.8H), 4.68 (brd, 1.4H), 4.00–4.39 (m, 2.6H), 3.69 (overlapped, 2H), 2.74–2.83 (m, 2H), 1.21–1.60 (m, 6H). HRMS (ESI) C$_{30}$H$_{35}$N$_{4}$O$_{2}$ [M+H]+ calc’d = 483.2755; found = 483.2756.

**Compound 3.** $^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 8.59 (t, $J = 5.6$ Hz, 0.4H), 8.36–8.41 (m, 1H), 8.22 (d, $J = 8.8$ Hz, 0.6H), 8.14 (brd, 2H), 8.03 (dd, $J = 6.4$, 2.4 H, 1H), 7.93 (td, $J = 5.6$, 1.8 Hz, 1H), 7.80–7.86 (m, 1.8H), 7.6 (brd, 4H), 7.39–7.54 (m, 3.5H), 4.73 (dd, $J = 12.8$, 5.6 Hz, 1.8H), 4.12 (d, $J = 15.2$ Hz, 0.5H), 3.93–4.08 (m, 2H), 3.63–3.72 (m, 3H), 3.32 (dd, $J = 15.2$, 4.8 Hz, 1H), 3.08 (dd, $J = 13.6$, 4.8 Hz, 1H), 1.72–1.93 (m, 4H), 1.47–1.63 (m, 18H), 1.13–1.35 (m, 5H). HRMS (ESI) C$_{37}$H$_{57}$N$_{6}$O$_{3}$ [M+H]+ calc’d = 633.4487; found = 633.4485.

**Compound 4.** $^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 8.34–8.64 (m, 2H), 8.19 (brs, 2.8H), 7.90–7.99 (m, 3.5H), 7.70–7.81 (m, 8H), 7.41–7.58 (m, 4H), 7.26–7.32 (m, 1H), 7.16 (d, $J = 6.8$ Hz, 0.6H), 4.78 (brd, 0.5H), 4.63 (d, $J = 5.2$ Hz, 1.3H), 4.10–4.25 (m, 1.4H), 3.96 (brd, 2H), 3.17 (overlapped, 2H), 3.46–4.39 (m, 2H), 3.24–3.30 (m, 0.6H), 2.56–2.74 (m, 4.4H), 1.51–1.74 (m, 5H), 1.20–1.42 (m, 6H), 1.00–1.14 (m, 1H). HRMS (ESI) C$_{36}$H$_{46}$N$_{6}$NaO$_{3}$ [M+Na]$^+$ calc’d = 633.3524; found = 633.3636.

**Compound 5.** $^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 8.66 (t, $J = 4.4$ Hz, 0.3), 8.44–8.66 (m, 1.5H), 8.16 (brd, 2H), 7.96 (t, $J = 8.0$ Hz, 3H), 7.85 (t, $J = 8.0$ Hz, 2H), 7.76 (brd, 2H), 7.43–7.63 (m, 4.8H), 7.18–7.36 (m, 5H), 6.86–7.09 (m, 1H), 4.47–4.83 (m, 3.8H), 4.12–4.16 (m, 2.8H), 3.96–4.06 (m, 1.6H), 3.06–3.76 (m, 2.5H), 2.78–2.92 (m, 1.5H), 2.56–2.67 (m, 1.7H), 1.31–1.78 (m, 6H). HRMS (ESI) C$_{39}$H$_{44}$N$_{5}$O$_{3}$ [M+H]$^+$ calc’d = 630.3439; found = 630.3424.
Compound 6. $^1$HNMR (400 MHz, d$_6$-DMSO) δ 8.37–8.62 (m, 1.6H), 8.10–8.21 (m, 3H), 7.90–7.97 (m, 3H), 7.80–7.84 (m, 1.6H), 7.66–7.73 (m, 1H), 7.37–7.58 (m, 5H), 7.15–7.32 (m, 6.6H), 6.83–7.01 (m, 1.7H), 4.80–4.92 (m, 0.5H), 4.63–4.68 (m, 1H), 4.39–4.46 (m, 1H), 4.12–4.30 (m, 2H), 3.99 (d, J = 17.2 Hz, 2H), 3.82 (dd, J = 13.6, 6.4 Hz, 0.6H), 3.65–3.69 (m, 1H), 3.39–3.55 (m, 1H), 2.78–3.07 (m, 2H), 2.51–3.07 (m, 0.6H), 0.87–1.73 (m, 4H). HRMS (ESI) C$_{39}$H$_{44}$N$_7$O$_3$ [M+H]$^+$ calc’d = 658.3500; found = 658.3496.

Compound 7. $^1$HNMR (400 MHz, d$_6$-DMSO) δ 8.57–8.63 (m, 1H), 8.32–8.34 (m, 1H), 8.01–8.09 (m, 3H), 7.92 (dd, J = 9.6, 6.0 Hz, 1H), 7.78–1.85 (m, 3H), 7.40–7.53 (m, 4H), 7.14–7.30 (m, 4H), 4.68–4.77 (m, 2H), 4.07–4.21 (m, 2H), 3.83–4.02 (m, 4.8H), 3.66–3.69 (m, 0.9H), 3.47–3.58 (m, 1.2H), 3.37–3.48 (m, 1H), 3.15 (dd, J = 13.6, 7.6 Hz, 0.5H), 2.65–3.91 (m, 3.4H), 2.04–2.15 (m, 0.5H), 1.74–1.92 (m, 4H), 1.45–1.63 (m, 14H), 1.19–1.36 (m, 2.8H). HRMS (ESI) C$_{40}$H$_{54}$N$_5$O$_3$ [M+H]$^+$ calc’d = 652.4221; found = 652.4204.

Compound 8. $^1$HNMR (400 MHz, d$_6$-DMSO) δ 8.57 (d, J = 8.8 Hz, 0.5H), 8.31 (d, J = 8.8 Hz, 0.5H), 8.05 (brd, 2H), 7.77 (brd, 2H), 7.15–7.38 (m, 16H), 4.61–4.67 (m, 0.5H), 4.25–4.57 (m, 5H), 4.13–4.18 (m, 1H), 4.00–4.08 (m, 1H), 3.47–3.64 (m, 4H), 3.12 (dd, J = 9.6, 7.2 Hz, 0.6H), 2.62–2.78 (m, 4H), 2.25 (d, J = 13.6 Hz, 0.6H), 1.76–1.86 (m, 3H), 1.52–1.72 (m, 10H), 1.38–1.47 (m, 3H), 1.11–1.35 (m, 6H). HRMS (ESI) C$_{43}$H$_{58}$N$_5$O$_3$ [M+H]$^+$ calc’d = 692.4534; found = 692.4517.

Compound 9. $^1$HNMR (400 MHz, d$_6$-DMSO) δ 8.51–8.61 (m, 0.8H), 8.29–8.33 (m, 1H), 8.01–8.09 (m, 2.3H), 7.75 (brd, 2H), 7.54–7.61 (m, 3H), 7.42 (t, J = 7.2 Hz, 2H), 7.14–7.34 (m, 7H), 4.09–4.31 (m, 3H), 3.83–4.03 (m, 2H), 3.67 (m, 2H), 3.36–3.44 (m, 2H), 2.17 (dd, J = 13.6, 8.0 Hz, 0.6H), 2.51–2.78 (m, 4H), 1.76–2.13 (m, 4H), 1.43–1.61 (m, 15H), 1.18–1.19 (m, 2H). HRMS (ESI) C$_{42}$H$_{56}$N$_5$O$_3$ [M+H]$^+$ calc’d = 678.4378; found = 678.4365.
**Compound 10.** HNMR (400 MHz, d$_6$-DMSO) δ 8.52–8.70 (m, 1H), 8.31–8.42 (m, 1H), 8.05–8.09 (brdm, 2H), 7.75 (m, 2H), 7.72 (dd, J = 20.0, 8.0 Hz, 2H), 7.43–7.48 (m, 2H), 7.12–7.30 (m, 5H), 4.31–4.32 (m, 2H), 4.09–4.21 (m, 1H), 3.81–4.03 (m, 2H), 3.66–3.68 (m, 1H), 3.36–3.43 (m, 2H), 3.14 (dd, J = 13.6, 8.0 Hz, 0.5H), 2.51–2.78 (m, 4H), 2.11 (J = 13.6 Hz, 0.5H), 1.75–1.93 (m, 4H), 1.47–1.56 (brdm, 15H), 1.12–1.29 (m, 2H). HRMS (ESI) C$_{37}$H$_{51}$F$_3$N$_5$O$_3$ [M+H]$^+$ calc’d = 670.3939; found = 670.3930.

**Compound 11.** HNMR (400 MHz, d$_6$-DMSO) δ 8.43–8.57 (m, 1H), 8.31 (d, J = 12.4 Hz, 0.4H), 8.21 (t, J = 5.6 Hz, 0.6H), 7.99–8.09 (brdm, 2.7H), 7.76 (brd, 2H), 7.12–7.35 (m, 8H), 4.14–4.22 (m, 3H), 3.80–4.05 (m, 3H), 3.65 (m, 1H), 3.35–3.53 (m, 3H), 3.16 (dd, J = 13.6, 8.0 Hz, 0.5H), 2.51–2.91 (m, 4H), 2.10 (d, J = 13.6 Hz, 0.6H), 1.72–1.94 (m, 4H), 1.50–1.58 (brdm, 14H), 1.07–1.37 (m, 12H). HRMS (ESI) C$_{40}$H$_{60}$N$_5$O$_3$ [M+H]$^+$ calc’d = 658.4691; found = 658.4680.

**Compound 12.** HNMR (400 MHz, d$_6$-DMSO) δ 8.77 (dd, J = 12.0, 6.0 Hz, 0.4H), 8.54 (d, J = 8.8 Hz, 0.4H), 8.47 (t, J = 6.0 Hz, 0.6H), 8.31 (d, J = 8.4 Hz, 0.4H), 8.04–8.08 (brdm, 2.7H), 7.97 (s, 0.6H), 7.93 (bri, 2H), 7.75 (brd, 2H), 7.14–7.29 (m, 4.6H), 4.41–4.46 (m, 2H), 4.15–4.20 (m, 1H), 3.81–4.10 (m, 3H), 3.59 (m, 1H), 3.35–3.53 (m, 2.6H), 3.13 (dd, J = 13.6, 8.0 Hz, 0.4H), 2.51–2.85 (m, 4H), 2.08 (d, J = 13.6 Hz, 0.5H), 1.63–1.87 (m, 5H), 1.49–1.58 (brdm, 14H), 1.20–1.31 (m, 2H). HRMS (ESI) C$_{38}$H$_{50}$F$_6$N$_5$O$_3$ [M+H]$^+$ calc’d = 738.3812; found = 738.3815.

**Compound 13.** HNMR (400 MHz, d$_6$-DMSO) δ 8.57 (t, J = 8.4 Hz, 0.4H), 8.34 (t, J = 8.4 Hz, 0.5H), 8.08 (brd, 2H), 7.89–7.94 (m, 0.6H), 7.78 (brd, 2H), 7.55–7.61 (m, 0.4), 7.18–7.27 (m, 4H), 4.12–4.22 (m, 1H), 3.93–4.00 (m, 1.3H), 3.62–3.78 (m, 3.6H), 3.15–3.22 (m, 0.7H), 2.73 (brd, 5.3H), 2.42–2.55 (m, 2.2H), 2.13–2.18 (m, 0.3 H), 1.75–1.92 (m, 7H), 1.52–1.60 (brd, 20H), 1.40 (brd, 7H), 1.20–1.27 (m, 2H). HRMS (ESI) C$_{40}$H$_{62}$N$_5$O$_3$ [M+H]$^+$ calc’d = 660.4847; found = 660.4855.
**Compound 14.** $^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 8.38–8.48 (m, 1H), 8.14 (brd, 2H), 7.88–7.99 (m, 1.4H), 7.82 (brd, 3H), 7.51–7.57 (m, 0.4H), 7.11–7.25 (m, 4.6H), 3.95–3.18 (m, 3H), 3.67–3.76 (m, 1.5H), 3.51–3.52 (m, 2H), 3.33–3.37 (m, 2H), 3.02–3.12 (m, 0.5H), 2.51–2.84 (m, 8H), 2.20 (dd, $J = 13.6, 8.0$ Hz, 0.4H), 1.79–1.97 (m, 7H), 1.44–1.64 (brdm, 23H), 1.20–1.40 (m, 11H), 0.94 (dd, $J = 14.8, 7.2$ Hz, 1H). HRMS (ESI) C$_{46}$H$_{74}$N$_7$O$_4$ [M+H]$^+$ calc’d = 788.5797; found = 788.5797.

**Compound 15.** $^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 8.50–8.66 (m, 1H), 8.20 (brd, 2H), 7.95–8.18 (m, 1.8H), 7.80–7.88 (brd, 3H), 7.51–7.62 (m, 0.5H), 7.11–7.27 (m, 4.7H), 3.90–4.18 (m, 7H), 3.52–3.82 (m, 2.6H), 3.37–3.41 (m, 1H), 2.96–3.96 (m, 0.4H), 2.52–2.87 (m, 8H), 1.78–2.18 (m, 8H), 1.30–1.69 (brdm, 31H), 0.80–0.93 (m, 1H). HRMS (ESI) C$_{45}$H$_{72}$N$_7$O$_4$ [M+H]$^+$ calc’d = 774.5640; found = 774.5651.

**Compound 16.** $^1$HNMR (400 MHz, d$_6$-DMSO) $\delta$ 8.43–8.51 (m, 1H), 8.15–8.21 (m, 0.8H), 8.10 (brd, 2H), 7.86–8.02 (m, 1.2H), 7.73 (brd, 2H), 7.48–7.63 (m, 1.2H), 7.14–7.24 (m, 5H), 3.90–4.23 (m, 3.5H), 3.72–3.76 (m, 1.5H), 3.50–3.55 (m, 3H), 2.87–3.15 (m, 3H), 2.57–2.79 (m, 6H), 1.82–1.95 (m, 7H), 1.10–1.59 (m, 33H). HRMS (ESI) C$_{46}$H$_{74}$N$_9$O$_4$ [M+H]$^+$ calc’d = 816.5858; found = 816.5866.

**Minimum inhibitory concentrations (MICs) against bacteria.** The antimicrobial activity of the compounds was tested on the following six bacteria strains: *K. pneumoniae* (ATCC 13383), *P. aeruginosa* (ATCC27853), *E. coli* (ATCC 25922), vancomycin-resistant *Enterococcus faecalis* (ATCC 700802), Methicillin-resistant *S. aureus* (MRSA, ATCC 33591), Methicillin-resistant *S. epidermidis* (MRSE, RP62A). The procedures were followed as reported previously. The MICs were determined as the lowest concentration that completely inhibits the bacteria growth. The experiments were repeated at least three times with duplicates each time.
**Hemolytic assays.** The freshly drawn, K2EDTA treated human red blood cells (hRBCs) were washed with 1× PBS buffer, and centrifuged at 3500 rpm for 10 min. The step was repeated until the supernatant became clear. The supernatant was removed, and the RBCs were diluted into 5% v/v suspension. 50 µL of the suspension was incubated with 50 µL of compounds of different concentrations at 37 °C for 1 h. The mixture was centrifuged at 3500 rpm for 10 min. Subsequently, to 30 µL of the supernatant 100 µL PBS was added, and the absorbance of the mixture at 540 nm was read on a Biotek Synergy HT plate reader. The positive control was 2% Triton X-100, and the negative control was 1× PBS alone. The hemolysis activity was calculated by the formula % hemolysis = (Abs\text{sample} - Abs\text{PBS})/(Abs\text{Triton} - Abs\text{PBS})×100%. The experiment was repeated at least three times with duplicates each time.

**MTT assays.** Both HK-2 and K562 cells were used to access the cell viability with treatment of compound 5–16 using standard procedure.\textsuperscript{22} Cells were seeded in 96-well plate with 5×10\textsuperscript{4} cells in 100 µL media per well. Control and Blank wells were prepared accordingly. Serial dilutions of compounds at concentrations of 100, 50, 25, 12.5, 6.25 µg/mL were prepared by diluting stock solution with media. After incubation for 12 h, 100 µL aliquots of drugs were added and the plate was incubated for 24 h. All media in cells was then removed and washed with fresh media once, followed by addition of 110 µL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent and then incubation for another 4 h, after which 100 µL of pre-warmed solubilization solution was added. The plate was then incubated at 37 °C for 12 h before absorbance was read at 550 nm. The data was calculated based on the following equation: cell viability % = [ (A − A\text{blank})/ (A\text{control} − A\text{blank})] × 100%. The measurements were repeated at least three times.

**Fluorescence microscopy.** The fluorescence microscopy was used to assess the ability of the compounds to compromise bacterial membranes. Fluorescent dyes propidium iodide (PI) and 4’, 6’-
diamidino-2-phenylindole dihydrochloride (DAPI) were used in the study. DAPI stains bacteria cells irrespective of their viabilities, whereas PI can only permeate bacterial cells and intercalate DNA in order to fluoresce when bacterial cell membranes are damaged membranes. After MRSA grew to mid-logarithmic phase, the compounds 13 or 14 was incubated with the bacteria at 37 °C for 2 h. The mixture was then centrifuged at 5000 rpm for 15 min. The cell pellets were washed with the PBS buffer for three times, and incubated with PI (5 µg/mL) and DAPI (10 µg/mL) for 15 min sequentially on ice in the dark. The mixture was then centrifuged and the pellets were washed with the PBS buffer. Next, 10 µL of the samples were placed on chamber slides and observed under Zeiss Axio Image Zloptical microscope using 100× oil-immersion objective.

**Time kill study.** The kinetics of bacteria killing by the lead compounds 13 and 14 were investigated. Briefly, the bacteria MRSA grew to mid-logarithmic phase in tryptic soy broth (TSB) medium, from which the suspension (10⁶ colony-forming unit per milliliter (CFU/ml)) were made. The suspension was incubated with different concentrations of 13 or 14 for 10 min, 30 min, 1 h and 2 h respectively. The mixtures were diluted by 10² to 10⁴ fold and spread on TSB agar plates. After incubation at 37 °C overnight, the colonies on the plates were counted and plotted against the incubation time.

**Drug resistance study.** The initial MICs of the compounds 13 and 14 for MRSA were obtained as above. Bacteria in wells containing concentration of 1/2 MIC were used to make bacterial suspension (10⁶ CFU/ml) for the next measurement of MICs of 13 and 14. The experiment was repeated each day for 14 passages.

**Inhibition of biofilms.** Overnight grown bacteria were inoculated into fresh 10% of MHII broth at a ratio of 1:100. 100 µL of inoculated culture was incubated with appropriated amount of compound 13 in wells of 96-well plate. The plate was incubated at 37 °C for overnight. Optical density of each wells was recorded at the wavelength of 600 nm and then the biofilm biomass was recorded by the crystal
violet method. Biofilm biomass were presented as CV OD/OD of growth. Relative biofilm biomass values were normalized by the biomass value of control (no addition of compound). Experiments were conducted in triplicate and the data were presented as mean ± STDEV.

**In vivo study**

**Establishment of the rat model of MRSA-induced pneumonia.** All animal experiments were approved by the institutional committee for animal care of Nanjing University and carried out in accordance to the policy of the National Ministry of Health of China. Briefly, male Wistar rats (6–8 weeks, average weight 200 g) were subjected to fast for at least 12 h prior to treatment. Then they were anesthetized with 1 g/kg of urethane administered intraperitoneally. An endotracheal tube with the 16-gage needle front was inserted into the trachea. After the placement of catheter, stethoscope was used to confirm the catheter was right in the trachea. Next, 100 μL PBS containing $2 \times 10^5$ CFU/mL MRSA was injected slowly to induce pneumonia. During the procedure, the rats were ventilated with a constant-volume respirator, and parameters were set as the following: inspired O$_2$ fraction of 1.0; peak airway pressures of 8–12 cm H$_2$O and a 2-cm positive end expiratory pressure (PEEP); as high as 12 mL/kg tidal volume were given every 30 min to avoid potential positional pulmonary atelectasis; breathing frequency was controlled at 70 times/min. The respiratory rate was adjusted to maintain the pressure of CO$_2$ between 35 and 45 mmHg.

**Pathological analysis.** A dose of 10 mg/kg weight of the compound 13 was injected to the tail veins of tested rats intravenously. Time course profiles of histological changes in lungs of rats were assessed post intravenous injection followed by dissection after being euthanized. Sections from lungs were stained with hematoxylin and eosin (H&E) and observed under a light microscope. For pathological investigation of lungs of pneumonia suffering rats at different time points, sections were collected as
above mentioned and existence (indicated by the infiltration of neutrophils) and the extent of possible inflammatory response (indicated by the integrity of the alveolar structure and endothelium cilium) were recorded from five randomly picked sections.

**Bronchoalveolar lavage (BAL) assay.** The assay was conducted to estimate the situation of inflammatory reactions in lungs. Rats were sacrificed and exsanguinated *via* the femoral vessel under sterile condition. Then the thorax was opened, and the 18-gage needle was introduced to the trachea at the cricothyroid membrane. Subsequently, 1 mL of sterilized PBS was administrated and extracted out for six cycles to promote the elution of inflammatory cells. Upon treatment, each specimen was homogenized. The suspension was subjected to enzyme linked immunosorbent assay (Elisa, R&D), and the level of Tumor Necrosis Factor-α (TNF-α) cytokine was investigated.

**4.5 References**


(3) WHO. Antimicrobial resistance: global report on surveillance. 2014.


CHAPTER 5: NOVEL BIS-CYCLIC GUANIDINES AS POTENT MEMBRANE-ACTIVE ANTIBACTERIAL AGENTS WITH THERAPEUTIC POTENTIAL

Note to Reader

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5.1 Introduction

Antimicrobial resistance is an escalating threat in global public health,\textsuperscript{1-3} and requires consistent actions worldwide. Indeed, multidrug-resistant bacterial strains, include Gram-positive bacteria methicillin-resistant \textit{Staphylococcus aureus} (MRSA), methicillin-resistant \textit{Staphylococcus epidermidis} (MRSE), Vancomycin-Resistant \textit{Enterococci} (VRE), and Gram-negative bacteria \textit{Escherichia coli} (E. Coli), \textit{Klebsiella pneumoniae} (KP), and \textit{Pseudomonas aeruginosa} (PA), have emerged to be the major cause of hospital and community-acquired infections.\textsuperscript{4, 5} As such, novel antibiotics that inhibit a panel of multidrug-resistant bacteria is in an urgent need.\textsuperscript{6-8} In recent decade, host-defense peptides (HDPs) have emerged as an alternative approach to combat bacteria resistance.\textsuperscript{9-11} Conventional antibiotics are known to target specific membrane or intracellular components of bacteria, however, HDPs preferentially interact with negatively charged bacterial membranes due to the intrinsic difference between bacterial and mammalian cell membranes,\textsuperscript{4, 12, 13} leading to the destruction of membrane integrity and bacterial cell death. The antimicrobial mechanisms of HDPs are complex, and a few models including barrel stave, carpet, toroidal pores have been proposed.\textsuperscript{4, 14} Nonetheless, all potential membrane-disrupting mechanisms could reduce the risk of resistance development\textsuperscript{15} as these interactions with bacteria are based on physical charge-
charge interaction and lack defined membrane targets. Such mechanisms also confer HDPs with broad-spectrum bactericidal activity.\textsuperscript{4, 16} It should be noted that some HDPs do have defined intracellular targets besides their membrane-disruptive activity, nonetheless, these combined mechanisms of actions could indeed further synergize their antimicrobial activity.\textsuperscript{13} Despite enthusiasm, there are obstacles associated with antibiotic HDPs and HDP-mimicking oligomeric peptidomimetics,\textsuperscript{17-19} including difficulty in scale-up, low cost-effectiveness, potential immunogenicity and systematic toxicity. Therefore, recently there has been considerable interest in the search of small molecules which mimic mechanism of action of HDPs.\textsuperscript{16, 20-22}

Bis-guanidine related compounds such as hexamidine have been used as antiseptics and disinfectant in past decades.\textsuperscript{23, 24} Recently it is suggested that cyclic guanidine compounds may be more potent antibacterial agents than linear guanidines, possibly due to their stronger electrostatic interaction with negatively charged bacterial membranes.\textsuperscript{7} For instance, a series of bis-cyclic guanidine compounds were recently obtained from combinatorial libraries and showed broad-spectrum antibacterial activity, however, their structures lack symmetry and rational design, and thus could face challenge in further optimization.\textsuperscript{25} Building upon these studies, we anticipated that bis-guanidine compounds bearing amphipathic structures could mimic mechanism of action of HDPs. Indeed, amphipathic xanthone derivatives bearing bis-arginine moieties recently demonstrated enhanced membrane selectivity, although they showed potent antimicrobial activity only against Gram-positive bacteria.\textsuperscript{26, 27} Brilacidin, a symmetric bis-guanidine investigational new drug candidate also designed to mimic the mechanism of action of HDPs, possesses an amphipathic structure to replicate the innate function of HDPs.\textsuperscript{28} As such, we envisioned that bis-cyclic guanidine compounds could be rationally designed to adopt cationic amphipathic structures, and thus capable of mimicking HDPs and revive as a promising approach to combat bacterial resistance.
5.2 Results and Discussion

Compared with HDPs which have large molecular weight (MW 1500–3000 Da) and multiple cationic charges and hydrophobic groups, these small bis-cyclic guanidines could be facilely accessed and scaled-up without compromising antimicrobial activity and potential of combating drug resistance, thus they are envisioned to be more promising in antibiotic therapeutic development. We hypothesized that if bis-cyclic guanidine compounds are endowed with the ability of bacterial membrane action by incorporating hydrophobic residues, their overall structures would be amphipathic with positive charges. Therefore, they could interact with bacterial membrane effectively. As shown above, the amphipathic bis-cyclic guanidine compounds could be conveniently designed by using a linker to dimerize the five-membered cyclic guanidine moiety bearing different lipophilicities. The molecules were synthesized in a straightforward manner (Figure 5.5), allowing future optimization and development of this class of compounds at ease. To this end, a new series of bis-cyclic guanidine compounds (MW 600–900 Da) were synthesized (Figure 5.1), and tested against a panel of multidrug resistant bacteria (Table 5.1). As expected, some compounds showed exceptional in vitro and in vivo activity. When R<sub>1</sub> was kept as the phenyl group and R<sub>2</sub> was just proton (no substituent), no activity was detected for 1 and 2 with aliphatic (C<sub>4</sub>H<sub>8</sub>) or aromatic (m-phenylene) linker under the tested condition. We reasoned that compounds without hydrophobic group on cyclic guanidine do not lead to strong hydrophobic interaction with bacteria membranes, even although they could reach on the surface of negatively charged bacteria through electrostatic interactions. We thus hypothesized that appending hydrophobic groups onto the cyclic guanidine ring would enhance the interaction of the compounds to associate with bacterial membranes.
Figure 5.1 The structures of compounds 1–9.

As anticipated, bearing an ethyl group on the cyclic guanidine ring, compounds 3 and 4 started to show excellent activity against Gram-negative bacteria *E. coli* with MICs of 1.5 and 3 μg/mL, respectively.

Replacement of ethyl group in 3 and 4 with the 3-phenylpropyl group led to compounds 5 and 6, respectively. Intriguingly, with more hydrophobic and longer chains which were expected to better span the phospholipid bilayer, both 5 and 6 displayed exciting antibacterial activity with MICs less than 3.0 μg/mL for Gram-negative bacteria (except for *P. aeruginosa*), and less than 3.0 μg/mL against Gram-positive bacteria. It is particularly noted that the MIC of compound 5 was as potent as 0.33 μg/mL, which is better or at least comparable to any known bis-cyclic guanidine compounds. It also
seemed $p$-phenylene and $m$-phenylene spacers do not impact activity intensively, as 3 and 4, and 5 and 6 exhibited similar activity.

Table 5.1 The antibacterial activity of compounds 1–9.

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<tr>
<th>Cpd</th>
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<th>( \text{HC}_{50} ) (µg/mL)</th>
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\[^{[a]}\]ND, Not determined because compounds are not active. \[^{[b]}\]AI, Activity Index, determined \( \text{HC}_{50}/\text{MIC}_{\text{MRSA}} \). \[^{[c]}\]D, Daptomycin, is included for comparison against gram-(+) bacteria. \[^{[d]}\]C, Colistin was included in the test as the positive control against gram-(−) bacteria.
The subsequent studies revealed that the aliphatic chain C₆H₁₃ as the R₂ group (Table 1) could further enhance the antimicrobial activity. Both 7 and 8 exhibited potent and broad-spectrum activity against all tested bacterial strains. It was very encouraging that compound 8 had remarkable MICs of 0.75 μg/mL toward most strains, and MIC of 0.16 μg/mL against MRSA. In addition, both compound 7 and 8 were very selective, as their hemolytic activity are all more than 250 μg/mL, which demonstrated 769 and 1534 folds of selectivity against MIC values of MRSA, respectively. It is known that daptomycin and colistin are last-resort antibiotics and active against Gram-positive and Gram-negative bacteria, respectively. Compound 8 had almost same or even better activity against Gram-positive bacteria compared with daptomycin, meanwhile showed comparable activity against Gram-negative bacteria compared with colistin, suggesting its promising therapeutic potential. Further attempts using more hydrophobic 2-adamantylethyl (compound 9) as the R2 group did not yield compounds with more potent and broad-spectrum activity.

Figure 5.2 Fluorescence micrographs of MRSA and E. coli that were treated or not treated with 10 μg/mL of 8 for 2 h. a1, control, no treatment, DAPI stained; a2, control, no treatment, PI stained. a3, treatment with 8, DAPI stained; a4, treatment with 8, PI stained. Scale bar = 10 μm.

To test the hypothesis that our compounds could possess the mechanism of action of HDPs and interact with bacterial membranes, fluorescence microscopic studies were conducted to evaluate
the ability of most potent compound 8 to compromise membranes of *E. coli* (Gram-negative bacterium) and *S. aureus* (Gram-positive bacterium). As well known, 4',6-diamidino-2-phenylindole (DAPI) could stain membranes of bacterial cells with blue fluorescence regardless of cell viability, whereas the red fluorescence of propidium iodide (PI) due to the DNA intercalation could only be observed in the presence of impaired membranes. As shown in Fig. 5.2, treatment of 8 gave rise to the red fluorescence under the PI channel in both *S. aureus* and *E. coli* groups, indicating that the membranes of these bacteria were disrupted. Moreover, significant aggregation of *S. aureus* occurred in the presence of 8, probably due to the loss of membrane potential upon membrane leakage.18

We next conducted time-kill studies to investigate the bacterial killing kinetics of compounds 7 and 8 by using *E. Coli*. As shown in Figure 5.3a and 5.3b, both compounds could kill *E. Coli* very rapidly. Cell colonies were counted in agar plates at 12.5, 25, and 50 μg/mL. Compound 7 at the concentration of 12.5 or 25 μg/mL could completely remove all bacterial in 2 h, and at 50 μg/mL it could remove bacterial clearly in just 0.5 h (Fig. 3a). Notably, Compound 8 could rapidly kill *E. Coli* completely in 0.5 h even at the concentration of 12.5 μg/mL (Fig. 5.3b). These data supported that 7 and 8 could kill bacteria in a similar way to that of HDPs.

One of the biggest concerns of current antibiotics is the bacterial resistance as described vide supra, while HDPs take advantage of no immediate drug resistance in bacteria due to lack of defined molecular targets as they disintegrate bacterial membranes. To assess the potential emergence of bacterial resistance toward these cyclic guanidines, the compound 8 was employed for further investigation. The compound was incubated with either MRSA or *E. Coli* in the well at the concentration of half-MIC every day and tested for their activity through 14 successive passages. As shown in Fig. 5.3c, MICs of 8 were virtually constant after 14 passages, indicating that they do not readily induce drug resistance in both MRSA and *E. Coli*. These outcomes suggest that our bicyclic
guanidine compounds are not vulnerable in developing drug resistance, which is analogous to the mechanism of action of HDPs.

The bacterial biofilm is another severe problem because bacteria in biofilm generally tolerate antibiotic treatment and thus more difficult to eradicate than planktonic cells.\textsuperscript{31, 32} Moreover, the bacterial biofilm infection could contaminate medical devices,\textsuperscript{33} therefore, organ catheters and implants coated with biofilm-inhibiting antibacterial agents are needed as effective therapeutic methods.\textsuperscript{34} Biofilms formed by MRSA and \textit{E. coli} have frustrated the treatment of persistent bacterial infections.\textsuperscript{35, 36} We thus sought to evaluate the compound 8 for its ability to inhibit biofilm formation of MRSA and \textit{E. coli}. As shown in Fig. 5.3d, at 0.19 μg/mL, 8 could inhibit 85% of biofilm formation of MRSA and 38% of biofilm formation of \textit{E. coli}. At the concentration of 0.39 μg/mL, 8 could completely eradicate biofilm formation of MRSA and almost 90% of biofilm formation of \textit{E. coli}. This analysis revealed that the bis-cyclic guanidine compound is an efficient biofilm formation inhibitor.

\textbf{Figure 5.3} Time-kill curves of 7 (a) and 8 (b) for \textit{E. Coli}. The killing activity was monitored for the first 2 h. (c) Drug-resistance study of 8 toward MRSA and \textit{E. Coli}. (d) Biological activity of 8 on inhibition of biofilm by \textit{E. Coli}.
The development of membrane-active antibacterial peptides for treatment of bacterial infections has been suffered from difficulties with systematic toxicity and tissue distribution, thus few compounds have been reported with in vivo activity and advanced into clinical trials.\textsuperscript{37-39} We envisioned that as small molecules, our bis-cyclic guanidines may possess better therapeutic potential.

The thigh burden model is a widely used animal model for evaluating preclinical antimicrobial activity of compounds.\textsuperscript{17,40} We thus employed the thigh burden model to evaluate the in vivo anti-infective activity of compounds 7 and 8, in which the thigh muscle of neutropenic mice was inoculated with \textit{S. aureus}, followed by intravenous (i.v.) administration of corresponding compounds. As shown in Fig. 5.4, significant activity was observed for both compounds at dose of 5 mg/kg when administered twice with a 6-hour interval between injections. A 3-log\textsubscript{10} decrease in colony-forming unit (CFU) was observed for compound 7, while more significant decrease (5-log\textsubscript{10} CFU) was observed for compound 8, indicating that compound 8 has better efficacy. The result suggested that our compounds provided significant protection against infection with \textit{S. aureus}.

\textbf{Figure 5. 4} In vivo efficacy of the compounds 7 and 8 in thigh-infection mouse model. Neutropenic mice (\textit{n} = 4 per group) were inoculated in the posterior thigh muscles with \textit{S. aureus} ATCC 33591 at $1 \times 10^6$ CFU per thigh and then treated with 7 and 8 (5 mg/kg per dose) by i.v. bolus injection in the tail vein at 1 and 7 h after infection.
5.3 Conclusions

In summary, we have developed a new class of bis-cyclic guanidine-based small molecules (MW 600–900 Da) starting from simply α-Phenylalanine. These molecules exhibit remarkable potency against a panel of multidrug-resistant Gram-positive and Gram-negative bacteria. Although other antimicrobial mechanisms cannot be excluded, our studies suggest that these compounds could kill bacteria rapidly by disrupting bacterial membranes, a mechanism analogous to that of HDPs. The hypothesis is further supported by the fact that the susceptibility of MRSA bacteria to the lead compounds remained nearly unchanged even after 14 passages. Furthermore, antibiotic therapeutic potential of these molecules was confirmed in the MRSA-infected thigh burden mouse model. Our work illustrated the potential of bis-cyclic guanidines for the development of potent antimicrobial molecules with molecular masses in the range of 600–900. Further studies on optimization of activity and selectivity, as well pharmacokinetic assessments are underway in our lab.

5.4 Experimental Section

General information. α-Phenylalanine was purchased from Chem-Impex International, Inc. Solvents and other reagents were purchased from either Sigma-Aldrich or Fisher Scientific and were used without further purification. The final products were purified on a Waters Breeze 2 HPLC system, and lyophilized on a Labcono lyophilizer. The purity of the compounds was determined to be >95% by analytical HPLC (1 mL/min flow, 5% to 100% linear gradient of solvent B (0.1% TFA in acetonitrile) in A (0.1% TFA in water) over 50 min was used). NMR data of compound 7 were collected on a Varian Inova 600 instrument, and others were obtained on a Varian Inova 500 instrument.
Synthesis of desired compounds.

![Synthetic scheme](image)

**Figure 5.5** The general synthetic scheme of cyclic guanidine dimers

The typical procedure for the synthesis of 9 is shown below. The other compounds were synthesized according to the same procedure as compound 9. Different aldehydes were used at the first step to give different compounds with various side chains.

![Synthetic route](image)

**Figure 5.6** Typical synthetic route for compound 9

**Synthesis of the intermediate build block C5.** Compound C1 (TFA salt, 10.8g, 33.6 mmol) was dissolved in MeOH and treated with TFA (5.1 mL, 33.6 mmol) before adding to a solution of 2-((3R,5R,7R)-adamantan-1-yl) acetaldehyde (6g, 33.6mmol) in MeOH and acetic acid (2 mL, 67.2 mmol). After stirring for 10 min under ice/H$_2$O bath, NaBH$_3$CN (3.2g, 50.4 mmol) was added portion
wise. The reaction was stirred for 3 h at room temperature before solvent was removed. The crude mixture was treated with NaHCO₃ (aq.) and extracted with EtOAc, and the organic layer was separated and evaporated to give an oil crude, which was purified by silica gel column chromatography to give 8.2 g of the desired secondary amine. Boc₂O (7g, 32.4 mmol) was added in the THF/H₂O (1:1, v/v) solution of this intermediate containing NaHCO₃ (3.6g, 43.2 mmol) and allowed to react for 5 h, after which EtOAc was added and the organic layer was collected. The solvent was removed in reduced pressure to give the colorless crude, which was purified by flash column chromatography to give 8.5 g of compound C2. Next, compounds C2 was taken in THF and reduced by LiAlH₄ (687 mg, 18 mol) for 30 min at -20 °C, then water was added to quench the reaction. The mixture was extracted with EtOAc, and the organic layer was separated and the solvent was removed in vacuo to give the crude C3, which was used in the next reaction without any further purification. Compound C3 was converted into compound C4 with the same procedure for the synthesis of compound C2. BOC protecting group was attached as the same procedure for attaching BOC onto compound C2, followed by hydrogenation to remove benzyl protecting group in MeOH to give the building block C5 as a white solid after filtration and concentration.

**Synthesis of compound 9.** Building block C5 (300 mg, 0.52 mmol), HOBt (159 mg, 1.04 mmol), DIPEA (129 μL, 1.04 mmol), and m-Phenylenediamine (34 mg, 0.32 mmol) was dissolved in DMF (2 mL) and then DCC (214 mg, 1.04 mmol) was added. The reaction mixture was stirred at room temperature for 24 h. The afforded byproduct DCU was filtered off and the filtration was added into water and extracted with EtOAc (×3). The organic phase was combined and washed with 1M HCl (×2), dried over anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. The crude oil compound was treated with TFA in DCM (1:1, v/v) for 2 h to completely remove BOC protecting groups to yield crude compound C6. Subsequently, C6 was dissolved in acetonitrile (3 mL), to which
CNBr (4 eq.) was added carefully (caution: very toxic). The reaction was stirred for 12 h at room temperature. 1M NaOH solution was added carefully, followed by proper amount of bleach to deactivate excessive CNBr. The mixture was filtered through a millipore filter and purified by HPLC purification on Waters HPLC system, and the desired fraction was lyophilized to give the pure product 9.

**Compound 1.** $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.31–7.34 (m, 4H), 7.22–7.26 (m, 6H), 4.22–4.28 (m, 2H), 3.94, 3.98 (ABq, $J_{AB}$ = 12.0 Hz, 4H), 3.72 (t, $J$ = 9.5 Hz, 2H), 3.42 (dd, $J$ = 9.0, 6.0 Hz, 2H), 3.22 (t, $J$ = 5.5 Hz, 4H), 2.93 (ddd, $J$ = 15.5, 14.0, 7.0 Hz, 4H), 1.54 (dtt, $J$ = 9.0, 6.0, 3.5 Hz, 4H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 167.0, 159.2, 136.1, 128.9 (2C), 128.4 (2C), 126.7, 54.5, 53.2, 46.2, 40.2, 38.7, 26.2. HRMS (ESI) C$_{28}$H$_{39}$N$_8$O$_2$ [M+H]$^+$ calcd = 519.3190; found = 519.3193.

**Compound 2.** $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.05 (s, 1H), 7.31–7.34 (m, 4H), 7.22–7.27 (m, 9H), 4.25–4.30 (m, 2H), 4.14, 4.19 (ABq, $J_{AB}$ = 18.0 Hz, 4H), 3.79 (t, $J$ = 9.0 Hz, 2H), 3.50 (dd, $J$ = 9.5, 5.5 Hz, 2H), 2.93 (ddd, $J$ = 15.0, 13.5, 6.5 Hz, 4H), 1.54 (dtt, $J$ = 9.0, 6.0, 3.5 Hz, 4H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 165.2, 159.3, 138.5, 136.1, 128.9 (2C), 128.4 (2C), 126.7, 115.4, 111.2, 54.6, 53.3, 46.5, 40.3. HRMS (ESI) C$_{30}$H$_{35}$N$_8$O$_2$ [M+H]$^+$ calcd = 539.2877; found = 539.2877.

**Compound 3.** $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.52 (brs, 4H), 7.31–7.33 (m, 4H), 7.22–7.28 (m, 6H), 4.29–4.34 (m, 2H), 4.14, 4.10 (ABq, $J$ = 18.0 Hz, 4H), 3.65 (t, $J$ = 11.5 Hz, 2H), 3.53–3.61 (m, 2H), 3.38–3.45 (m, 4H), 3.21 (dd, $J$ = 13.5, 4.5 Hz, 2H), 2.86 (dd, $J$ = 13.5, 8.5 Hz, 2H), 1.26 (t, $J$ = 7.5 Hz, 6H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 165.1, 157.9, 135.7, 134.3, 128.9 (2C), 128.5 (2C), 126.8, 120.1 (2C), 57.8, 51.8, 37.8, 37.5, 11.2. HRMS (ESI) C$_{34}$H$_{43}$N$_8$O$_2$ [M+H]$^+$ calcd = 595.3503; found = 595.3488.
**Compound 4.** $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.05 (d, $J = 1.5$ Hz, 1H), 7.31–7.34 (m, 4H), 7.23–7.28 (m, 9H), 4.28–4.34 (m, 2H), 4.15, 4.11 (ABq, $J = 17.5$ Hz, 4H), 3.63 (t, $J = 9.5$ Hz, 2H), 3.56 (quintet, $J = 6.5$ Hz, 2H), 3.36–3.44 (m, 4H), 3.21 (dd, $J = 13.5$, 4.5 Hz, 2H), 2.85 (dd, $J = 13.5$, 9.0 Hz, 2H), 1.26 (t, $J = 7.0$ Hz, 6H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 165.2, 157.9, 138.5, 135.7, 129.0 (2C), 128.4 (2C), 126.8, 115.4, 111.2, 57.8, 51.7, 37.8, 37.6, 11.2. HRMS (ESI) C$_{34}$H$_{43}$N$_8$O$_2$ [M+H]$^+$ calcd = 595.3503; found = 595.3490.

**Compound 5.** $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.53 (s, 4H), 7.17–7.32 (m, 20H), 4.22–4.27 (m, 2H), 4.0 (s, 4H), 3.60 (t, $J = 9.5$ Hz, 2H), 3.40 (dd, $J = 9.5$, 5.0 Hz, 2H), 3.33 (dd, $J = 8.5$, 5.5 Hz, 2H), 3.08 (dd, $J = 13.5$, 5.0 Hz, 2H), 2.83 (dd, $J = 13.5$, 8.0 Hz, 2H), 2.61–2.73 (m, 4H), 1.91–2.05 (m, 4H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 165.0, 158.0, 140.9, 135.7, 134.3, 128.9 (2C), 128.4 (2C), 128.2 (2C), 128.0 (2C), 126.8, 125.8, 120.0, 58.0, 51.6, 42.4, 37.4, 32.1 (2C), 28.3. HRMS (ESI) C$_{48}$H$_{55}$N$_8$O$_2$ [M+H]$^+$ calcd = 775.4442; found = 775.4443.

**Compound 6.** $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.05–8.12 (m, 1H), 7.17–7.32 (m, 23H), 4.19–4.25 (m, 2H), 4.12, 4.09 ((ABq, $J_{AB} = 18.0$ Hz, 4H), 3.60 (t, $J = 8.5$ Hz, 2H), 3.52 (quintet, $J = 7.5$ Hz, 2H), 3.39 (dd, $J = 9.5$, 5.0 Hz, 2H), 3.27–3.33 (m, overlapped with CD$_3$OD, 2H), 3.07 (dt, $J = 13.5$ Hz, 5.4 Hz, 2H), 2.82 (ddd, $J = 13.0$, 9.0, 3.0 Hz, 2H), 2.60–2.72 (m, 4H), 1.92–2.02 (m, 4H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 165.2, 158.0, 140.8, 138.5, 135.6, 129.0 (2C), 128.4 (2C), 128.2 (2C), 128.0 (2C), 126.8, 125.8, 115.3, 58.0, 51.6, 42.3, 37.4, 32.1 (2C), 28.3. HRMS (ESI) C$_{48}$H$_{55}$N$_8$O$_2$ [M+H]$^+$ calcd = 775.4442; found = 775.4438.

**Compound 7.** $^1$H NMR (600 MHz, CD$_3$OD) $\delta$ 7.50 (brs, 4H), 7.29–7.32 (m, 4H), 7.22–7.26 (m, 6H), 4.26–4.31 (m, 2H), 4.08 (s, 4H), 3.65 (t, $J = 9.6$ Hz, 2H), 3.45 (ddd, $J = 15.0$, 9.0, 6.6 Hz, 2H), 3.40 (dd, $J = 9.6$, 5.4 Hz, 2H), 3.24–3.27 (m, 2H), 3.14 (dd, $J = 13.8$, 4.8 Hz, 2H), 2.87 (dd, $J = 13.2$, 8.4
Hz, 2H), 1.58–1.69 (m, 4H), 1.32–1.37 (m, 16H), 0.91 (t, $J = 7.2$ Hz, 6H). $^{13}$C NMR (150 MHz, CD$_3$OD) $\delta$ 165.0, 158.0, 135.8, 134.3, 129.0 (2C), 128.4 (2C), 126.7, 120.0 (2C), 57.9, 51.7, 42.8, 37.5, 31.1, 26.6, 25.8, 22.1, 12.9. HRMS (ESI) C$_{42}$H$_{59}$N$_8$O$_2$ [M+H]$^+$ calcd = 707.4755; found = 707.4748.

**Compound 8.** $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.05 (s, 1H), 7.31–7.34 (m, 4H), 7.24–7.28 (m, 9H), 4.27–4.32 (m, 2H), 4.14, 4.10 (ABq, $J = 18.0$ Hz, 4H), 3.65 (t, $J = 8.0$ Hz, 2H), 3.47 (ddd, $J = 15.0$, 9.0, 6.5 Hz, 2H), 3.42 (dd, $J = 9.5$, 5.5 Hz, 2H), 3.24–3.27 (m, 2H), 3.16 (dd, $J = 13.5$, 5.0 Hz, 2H), 2.89 (dd, $J = 13.5$, 8.0 Hz, 2H), 1.59–1.69 (m, 4H), 1.32–1.37 (m, 12H), 0.92 (t, $J = 7.0$ Hz, 6H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 165.2, 158.0, 138.5, 135.8, 129.0 (2C), 128.5 (2C), 126.8, 115.3, 111.1, 62.9, 58.0, 42.9, 37.5, 31.1, 26.6, 25.8, 22.1, 12.9. HRMS (ESI) C$_{42}$H$_{59}$N$_8$O$_2$ [M+H]$^+$ calcd = 707.4755; found = 707.4751.

**Compound 9.** $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 8.12 (t, $J = 1.5$ Hz, 1H), 7.34 (t, $J = 7.5$ Hz, 4H), 7.22–7.29 (m, 9H), 4.28–4.33 (m, 2H), 4.15, 4.12 (ABq, $J = 18.5$ Hz, 4H), 3.70 (t, $J = 9.5$ Hz, 2H), 3.53 (qd, $J = 7.0$, 5.5 Hz, 2H), 3.42 (dd, $J = 10.0$, 5.5 Hz, 2H), 3.16–3.19 (m, 2H), 3.12 (dd, $J = 14.0$, 5.5 Hz, 2H), 2.90 (dd, $J = 14.0$, 7.5 Hz, 2H), 1.95 (brs, 6H), 1.67–1.77 (m, 12H), 1.52–1.54 (m, 12H), 1.44 (td, $J = 13.0$, 4.5 Hz, 2H), 1.35 (td, $J = 12.0$, 5.5 Hz, 2H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$ 165.2, 158.0, 138.6, 136.0, 129.0 (2C), 128.5 (3C), 126.8, 115.3, 111.2, 57.4, 51.9, 41.7 (3C), 39.6, 38.1, 38.0, 36.6 (2C), 31.3, 28.6 (3C), 28.6. HRMS (ESI) C$_{54}$H$_{71}$N$_8$O$_2$ [M+H]$^+$ calcd = 863.5694; found = 863.5670.

**Compound C5.** $^1$H NMR (500 MHz, CD$_3$OD) $\delta$ 7.25–7.29 (m, 2H), 7.19–7.21 (m, 3H), 3.73–3.97 (m, 3H), 3.59 (s, 1H), 3.43–3.50 (m, 1H), 2.95–3.11 (m, 2H), 2.77–2.81 (m, 2H), 1.88 (brd, 4H), 1.71 (brd, 4H), 1.60 (brd, 4H), 1.28–1.52 (m, 21H), 0.87–0.92 (m, 3H). $^{13}$C NMR (125 MHz, CD$_3$OD) $\delta$
171.7, 155.8, 155.7, 128.9 (2C), 128.0 (2C), 126.0, 80.5, 80.3, 79.5, 79.2, 49.3, 42.0 (3C), 41.8, 36.8 (3C), 36.5, 31.2, 28.6 (4C), 28.5, 27.6, 27.5 (2C), 27.4, 27.3, 27.2. HRMS (ESI) C\textsubscript{33}H\textsubscript{51}N\textsubscript{2}O\textsubscript{6} [M+H]\textsuperscript{+} calcd = 571.3742; found = 571.3763.

**Minimum inhibitory concentrations (MICs) against bacteria.** We test the antimicrobial activity of the 8 compounds on six bacteria strains: MRSA (ATCC 33591), MRSE (RP62A), KP (ATCC 13383), PA (ATCC27853), *E. coli* (ATCC 25922), VER (ATCC 700802) according to the same procedures reported previously.\textsuperscript{1} Two-fold serial dilutions of compounds (0.1–25 µg/mL) were used in the assay. The experiments were repeated at least three times with duplicates each time. The absorption at 600 nm wavelength was read on a Biotek Synergy HT microtiter plate reader.

**Hemolytic assays.** The freshly drawn rat red blood cells (hRBCs) were washed with 1× PBS buffer, and centrifuged at 3500 rpm for 10 min. The rest procedures were followed as reported previously.\textsuperscript{1} The experiment was repeated at least three times with duplicates each time. The hemolysis activity was calculated by the formula % hemolysis = (Abs\textsubscript{sample} - Abs\textsubscript{PBS})/(Abs\textsubscript{Triton} - Abs\textsubscript{PBS}) ×100%.

**Fluorescence microscopy.** \textsuperscript{2} The PF assay was used to assess the ability of the compound 8 to compromise bacterial membranes. The compound 8 was incubated with the bacteria at 37 °C for 2 h after MRSA and *E. Coli* grew to mid-logarithmic phase. After centrifuged at 5000 rpm for 15 min, the cell pellets were washed with the PBS buffer for three times, and incubated with DAPI (10 µg/mL) or PI (5 µg/mL) for 15 min sequentially on ice in the dark. The mixture was then centrifuged and the pellets were washed with the PBS buffer. Next, 10 µL of the samples were placed on chamber slides and observed under Zeiss Axio Image Z1 optical microscope using 100× oil-immersion objective.

**Time kill study.** The kinetics of bacteria killing by the lead compounds 7 and 8 were also tested. The bacteria *E. Coli* were grown to mid-logarithmic phase in TSB medium to make the suspension of 10\textsuperscript{6} CFU/ml. The suspension was incubated with different concentrations of 7 or 8 (12.5 µg/mL, 25 µg/mL,
and 50 μg/mL) for 10 min, 30 min, 1 h and 2 h respectively. The mixtures were diluted by $10^2$ to $10^4$ fold and spread on TSB agar plates. After incubation at 37 °C 12 h, the colonies on the plates were counted and plotted against the incubation time.

**Drug resistance study.**³ Bacteria in wells containing concentration of 1/2 MIC of the compounds 7 and 8 were used to make bacterial suspension (10⁶ CFU/ml) for the next measurement of MICs. The experiment was repeated each day for 14 passages.

**Inhibition of biofilms.**⁴ Overnight grown bacteria MRSA and *E. Coil* were inoculated into fresh 10% of MHII broth at a ratio of 1:100. Afterwards, 100 µL of inoculated culture was incubated with appropriated amounts of compounds 7 and 8 in 96-well plate, which was then incubated at 37 °C for overnight. Optical density of each wells was recorded (wavelength 600 nm) and then the biofilm biomass were recorded by the crystal violet (CV) method. Relative biofilm biomass values were normalized by the biomass value of control (no compound added). Data were presented in mean value of three replicates.

**In vivo study of mouse thigh burden infection model.** All protocols and methods associated with animal experiments were approved by University of South Florida (USF) Institutional Animal Care and Use Committee. The in vivo experiment on the mouse model of the thigh burden infection with MRSA was conducted adapted from previously reported protocol.⁵ The CD-1 female mice which were 6 to 8 weeks old and around 25 g in weights were used for the study. Neutropenic Mice were induced by injecting cyclophosphamide (150 mg/kg) intraperitoneally twice at 4 and 1 days before bacterial inoculation. One MRSA colony from trypic soy agar (TSA) cultures was allowed to grow in trypic soy broth (TSB) overnight at 37 °C, then 100 µL culture was withdrawn and diluted with TSB to a total volume of 4 mL, which was subsequently incubated at 37 °C for another 6 h. The bacterial culture was then diluted in sterile PBS to give the final inoculum concentration of approximate 10⁶ CFU/mL.
The thigh burden infection model was established by injecting both posterior thighs of mice with 100 μL of inoculums. Two doses of the compounds 7 and 8 were given at 1 h and 7 h by i.v. bolus injection in the tail vein at 5 mg/kg per dose of drugs after bacterial infection. Thighs were harvested at 25 h for both groups after bacterial inoculation. Thigh muscles were collected in a sterile tared tube, to which 5 mL sterile PBS was added. The mixture was then homogenized with a tissue homogenizer (BioSpec product tissue tearor 985-370) for approximately 30 sec. 100 mL of serial diluted aliquots were plated on tryptic soy agar plates, which were incubated for 24 h at 37 °C. The formed colonies were counted to calculate CFU per thigh.

5.5 References


CHAPTER 6: CONCLUSIONS AND FUTURE DIRECTIONS

In this chapter of the dissertation, we are summarizing the results of our projects and providing an idea for the future direction of our projects in a real-world application. In our projects we have explored the antibacterial activity of polymers, dendrimers and small molecules for their potency, selectivity against a wide range of bacteria.

We have developed a biodegradable polycarbonate by ring opening polymerization technique, which are highly selective and potent against Gram-positive bacteria. These biodegradable molecules can be suitable for in vivo applications. Here we have reported the polymers that can act against a particular strain of bacteria i.e., Gram-positive strain. Further design of polymers can be made by varying the cationic and hydrophobic groups of the polymers, which might result in activity against a broad spectrum of bacteria. By synthesizing the amino acid derived cationic monomers, the cationic nature of the polymer can be potentially enhanced. The activity may be further enhanced by increasing the number of equivalents of the hydrophilic and the hydrophobic monomers used in the polymer synthesis. Apart from targeting the polymers for antibacterial application, they can also be designed for their significant application in targeted drug delivery, anti-coating agents and antifungal agents.

Moving forward, we have synthesized the dendrimers that can act as broad spectrum antibacterial agents. The unimicellar nature of the dendrimers has increased the potential of the molecule to act against a wide range of bacteria. However, the selectivity was comparatively poor than the polymers. Future direction can be laid on designing the groups on the dendrimers in a way that they can potential increase the selectivity and can retain or improve the potency. Due to the presence of the internal voids in the dendrimer, they can also be used as drug delivery agents. The end groups of the dendrimers can
be modified with a ligand and can be tested for various biomedical applications. As dendrimers have found to be highly toxic, the antibacterial activity can also be increased by potential binding of the dendrimers to currently available antibiotics that are in the market. This binding of the dendrimers with the antibiotics may increase the activity by synergistic effect.

Lastly, our research studies have focused on using these polymers and dendrimers as drug delivery agents of the hydrophobic drugs for the treatment of colon cancer. The hydrophobic drugs miRNA lacks activity in the body due to short circulation time. Furthermore, miRNA is rapidly degraded and gets aggregated in the presence of serum proteins. In order to deliver miRNA to the target site for the treatment of the colon cancer PEG-amphiphilic cationic polymeric micelles were used for the encapsulation of the miRNA. The results showed that the polymers were able to successfully encapsulate the miRNA. The preliminary in vivo data on the mice proved that the miRNA encapsulated polymeric micelles has considerably reduced the hepatoma in the mice. These polymeric micelles were observed to be in the size range of 100-200nm which makes them compatible to pass through the blood brain barrier. The factors that further effect the in vivo efficacy of the polymeric nanoparticles is currently underway. The limitation of this study is the polymeric micelles are not target specific and show the activity in the body by passive delivery of the drug in the body. The limitation has further led us to design the nanoparticles that can release the cargo at the targeted location. For this study, we have synthesized a cationic amphiphilic monomer that can bind to the anticancer tanshinone drug and release the drug when there is a change in the pH inside the body. A fluorescent tag was attached to the polymer by covalent binding to keep track of the delivery of the cargo by polymer nanoparticles while conducting the in vivo studies. At this time of writing, we don’t have the complete in vivo data of the polymers that were being used as drug delivery agents. However, we would expect these polymer nanoparticles to be developed as potential drug delivery agents.
APPENDIX A1: $^1$H NMR of Polymers P1-P8 and Monomer 1

Figure S1. $^1$H NMR (400 MHz, CDCl$_3$) of Monomer 1.
Figure S2. $^1$H NMR (400 MHz, CDCl$_3$) of P1.
Figure S3. $^1$H NMR (400 MHz, CDCl$_3$) of P2.
Figure S4. $^1$H NMR (400 MHz, CDCl$_3$) of P3.
Figure S5. $^1$H NMR (400 MHz, CDCl$_3$) of P4.
Figure S6. $^1$H NMR (400 MHz, CDCl$_3$) of P5.
Figure S7. $^1$H NMR (400 MHz, CDCl$_3$) of P6.
Figure S8. $^1$H NMR (400 MHz, CDCl$_3$) of P7.
Figure S9. $^1$H NMR (400 MHz, CDCl$_3$) of P8.
APPENDIX A2. $^1$HNMR spectra of compounds 1–16 and $\gamma$-BB-2

Compound 1

Compound 2
Compound 3

Compound 4
Compound 5

Compound 6
Compound 9

Compound 10
Compound 11

Compound 12
Compound 13

Compound 14
Compound 15

Compound 16
## HPLC analysis of compounds 1–16

### Table S1. HPLC purities and retention time of compounds 1–16

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<th>Retention Time (min)</th>
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HPLC spectra of compounds 1–16
APPENDIX A3: $^1$H NMR (CD$_3$OD) and $^{13}$C NMR (CD$_3$OD) spectra of compounds 1–9, and C5

Compound 1
Compound 2

Compound 2
Compound 6
Compound C5
HPLC analysis of compounds 1–9

Table S1. HPLC purities and retention time of compounds 1–9

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HPLC spectra of compounds 1–9
Compound 3

Compound 4

Compound 5

Compound 6

Compound 7
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Small Antimicrobial Agents Based on Acylated Reduced Amide Scaffold

Author: Peng Teng, Da Huo, Alekhya Nimmagadda, et al

Publication: Journal of Medicinal Chemistry
Publisher: American Chemical Society
Date: Sep 1, 2016

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RESEARCH INTEGRITY AND COMPLIANCE
INSTITUTIONAL ANIMAL CARE & USE COMMITTEE

MEMORANDUM

TO: Chuanhai Cao,

FROM: Farah Moulvi, MSPH, IACUC Coordinator
Institutional Animal Care & Use Committee
Research Integrity & Compliance

DATE: 4/15/2015

PROJECT TITLE: Development of antimicrobial agents 1. Alpha-AApeptides as a novel class of antimicrobial biomaterials

FUNDING SOURCE: National Institutes of Health; USF department, institute, center, etc.

IACUC PROTOCOL #: R IS00001173
PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 4/15/2015:

Mouse: CD-1 (6-8 weeks of age; range 132 of 20-26g in weight; equal number of males and females)

Please take note of the following:

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4/15/2015
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