Semi-synthetic Glycopeptide Antibiotics: Strategies to Combat Acquired and Intrinsic Bacterial Resistance

A Thesis Submitted for the Degree of

Doctor of Philosophy

By

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Dedicated to My Family and Haldar's Group at JNCASR

Declaration

I hereby declare that the matter embodied in the thesis entitled "***Semi-synthetic Glycopeptide Antibiotics: Strategies to Combat Acquired and Intrinsic Bacterial Resistance***" is the result of the investigations carried out by me at the New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under the supervision of **Dr. Jayanta Haldar** and that it has not been submitted elsewhere for the award of any degree or diploma.

In keeping with the general practice in reporting the scientific observations, due acknowledgement has been made whenever the work described is based on the findings of other investigators. Any omission that might have occurred due to oversight or error in judgement is regretted.

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Mr. Venkateswarlu Yarlagadda

(PhD Student)

Certificate

I hereby certify that the matter embodied in this thesis entitled "***Semi-synthetic Glycopeptide Antibiotics: Strategies to Combat Acquired and Intrinsic Bacterial Resistance***" has been carried out by **Mr. Venkateswarlu Yarlagadda** at the New Chemistry Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India under my supervision and that it has not been submitted elsewhere for the award of any degree or diploma.

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Dr. Jayanta Haldar

(Research Supervisor)

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Preface

Chapter 1 introduces the field of glycopeptide antibiotics and bacterial resistance. The global threat of infectious diseases has been exacerbated by the emergence of multidrug-resistance (MDR) in bacteria. The advent of MDR Gram-positive bacterial pathogens as well as carbapenem-resistant Gram-negative super bugs has rendered most of the frontline antibiotics ineffective. Vancomycin, a natural glycopeptide antibiotic, has been considered as "the antibiotic of last resort" for MDR Gram-positive bacterial infections. However, over the course of time, vancomycin has also been rendered ineffective by vancomycin-resistant bacteria like vancomycin-resistant Enterococci (VRE), vancomycin-intermediate *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA). Additionally, many antibiotics, including vancomycin, are inherently inactive towards Gram-negative bacteria because of their inability to cross the outer membrane of these pathogens. In my research, various strategies have been developed to combat both acquired and inherent resistance of the bacteria towards vancomycin.

Vancomycin binds to D-Ala-D-Ala terminus of peptidoglycan pentapeptide of the bacterial cell wall, thus inhibiting transpeptidase-catalyzed cross-linking and maturation of the bacterial cell wall. Bacteria acquire resistance to vancomycin either by alteration of cell wall precursors from D-Ala-D-Ala to D-Ala-D-Lac (VRE) or by thickening the cell wall (VISA), or sometimes through both the modifications (VRSA). **Chapter 2** describes a simple semi-synthetic strategy aimed at improving the binding efficiency of vancomycin towards the target peptides of resistant bacteria by conjugating various cyclic/acyclic sugar moieties (which have the ability to form extra hydrogen bonding with the target peptides of bacteria) with the C-terminal of vancomycin. An optimized vancomycin-sugar conjugate with additional lipophilicity, exhibited > 150-fold enhanced binding affinity and higher cell wall inhibition with excellent antibacterial activity (> 1000-fold more effective than vancomycin). Further, this compound showed very good efficacy in mice infection models against VISA and VRE, and exhibited improved pharmacological properties. This class of compounds has immense potential to be developed as future antibiotics.

Chapter 3 deals with a rational strategy to impart an additional mechanism of action to vancomycin, namely bacterial membrane disruption to combat acquired resistance. In this

strategy, permanent cationic hydrophobic moiety was conjugated to vancomycin to confer membrane disruptive property to the existing drug. The incorporation of lipophilic moiety into vancomycin along with permanent positive charge makes these compounds unique from other existing glycopeptide derivatives in their ability to cause strong bacterial membrane disruption. Compared to vancomycin, these compounds demonstrated a 40-fold, > 400-fold and 1000-fold greater efficacy against VISA, VRSA and VRE respectively. The optimized compound, in comparison with vancomycin, showed higher cell wall inhibition, better *in-vivo* activity against VISA and VRE in infection models, and exhibited improved pharmacological properties with no observed toxicity. Further, this class of compounds was shown not to trigger the development of bacterial resistance.

Having shown that the conjugation of sugar moiety enhances the binding affinity of the drug and incorporation of permanent cationic hydrophobic moiety imparts membrane disruption properties; **Chapter 4** is aimed at imparting both the properties to the same molecule to effectively tackle drug resistance. An optimized compound showed outstanding activity against VRE, being > 8000-fold more effective than vancomycin. This compound displayed enhanced cell wall inhibition compared to vancomycin and showed excellent *in-vivo* activity against vancomycin-resistant bacterial infection (VRE). Thus, this multipronged approach bears immense potential in the field of antibiotic development for the treatment of vancomycin-resistant bacterial infections.

VanX, VanY and VanXY are some of the key enzymes that are accountable for vancomycin resistance. The common feature of these enzymes lies in their active site bearing zinc ion (Zn^{2+}). In **Chapter 5A**, dipicolyl-vancomycin conjugate (Dipi-van) has been developed which has the ability to complex with Zn^{2+} ion. Dipi-van presumably has the potential to inactivate the enzymes by chelating Zn^{2+} ions and also can bind with the cell wall precursor peptides. Dipi-van showed 350-fold more *in-vitro* activity than vancomycin against VRE. Further, Dipi-van demonstrated high *in-vivo* activity with no observed toxicity.

The acquisition of metallo-β-lactamases (enzymes bearing zinc ions in the active site) in New Delhi metallo-β-lactamase-1 (NDM-1) is the major contributor to the emergence of carbapenem resistance. In **Chapter 5B**, Dipi-van has been shown to restore the activity of meropenem antibiotic against a variety of clinical isolates of NDM-1 producing pathogens.

This compound along with meropenem showed good *in-vivo* activity in mouse sepsis model against NDM-1 expressing super bugs.

Chapter 6 presents overcoming the intrinsic resistance of Gram-negative pathogens (GNPs) towards glycopeptide antibiotics. Glycopeptide antibiotics such as vancomycin are inherently inactive against GNPs because of their inability to cross the outer membrane (OM) of these pathogens. In the present study, for the first time, lipophilic cationic (permanent positive charge) vancomycin analogues were shown to permeabilize the OM of GNPs and inhibit the cell wall biosynthesis. These analogues showed high efficacy against a variety of MDR clinical isolates of GNPs like *E. coli*, *K. pneumoniae*, *P. aeruginosa* and *A. baumanii*. More importantly, the compound showed excellent activity in a carbapenem-resistant *A. baumannii* infected mouse model.

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Chapter 1

An Introduction to Glycopeptide Antibiotics: The Drugs of Last Resort

1.1 Bacterial infectious diseases: A global health concern

Emerging infectious diseases (EIDs) are a significant burden on global economies and public health as they have the potential to cause numerous deaths.[1] According to the recent world health report, published in 2013 by the World Health Organization (WHO), infectious diseases led to more human deaths than any other disease throughout the world.[2] Infectious diseases are caused by mainly the microorganisms like bacteria, viruses, fungi, parasites and so on. Among these all causative agents, bacterial infectious diseases contribute 20 % to overall global infectious disease burden. It has been found that in 2011, about 8.2 million (> 25 % of total) of the total deaths (28.3 million) were directly associated with infectious diseases. Bacterial infections contributed to 6.6 million deaths alone.[2] Indians face a greater risk of being affected by infectious diseases.[3] Recently, The Global Antibiotic Resistance Partnership (GARP) reported that India occupies the highest position in bacterial diseases among the world.[4] *Streptococcus pneumoniae* causes death of 4,10,000 lives each year, which includes many children from economically impaired families.[5,6]

Bacteria are responsible for causing various diseases like respiratory infections, chronic diseases (such as gastric ulcers and gastric cancer), tuberculosis, diarrhoea, sepsis, pneumonia, endocarditis, skin and soft tissue infections etc. The Gram-positive bacteria *Staphylococcus aureus* is responsible for most of the hospital-acquired bacterial infections. Some other Gram-positive bacteria such as *Enterococcus sp.* and *Streptococcus sp.* and Gram-negative bacteria such as *Klebsiella sp.*, *Escherichia sp.*, *Enterobacter sp.*, *Pseudomonas sp.*, *Acenetobacter sp.*; known as 'nosocomial bacteria', are also responsible for hospital associated infections.[7] With the advent of easily transferable New Delhi Metallo-β-lactamase 1 (NDM-1 gene), resistance to β-lactams is rampant.[8,9] Only tigecycline and colistin remain as active drugs against this strain of bacteria.[10] Thus development of novel antibacterial agents against such bacteria is imperative.

1.2 Antibiotics and bacterial resistance

1.2.1 Discovery of antibiotics

Antibiotics are molecules that inhibit bacterial growth or kill them. Antibiotics, agents "against life," can either be natural products or completely man-made synthetic chemicals or semi-synthetic derivatives of natural products designed to block some crucial process selectively in bacterial cells. The discovery of penicillin by Alexander Fleming and its development as an antimicrobial agent

during World War II marked a triumph for human kind over bacteria and heralded a new era of medicine against disease in the 1940s.[11-15] This heralded the "Golden Era" that lasted until the 1960s, during which the most of the antibiotic classes used in the clinic today were discovered (Fig. 1.1).[16]

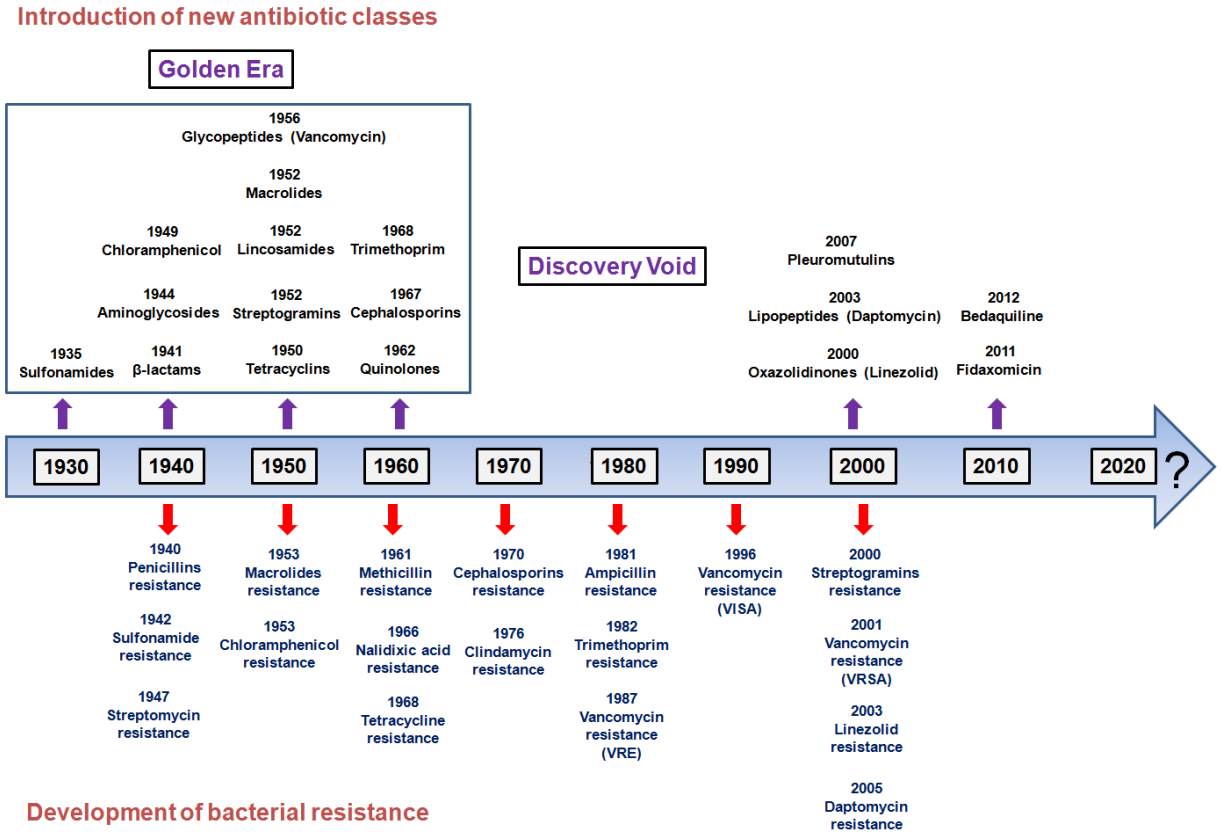


Figure 1.1: Antibiotic pipeline and development of bacterial resistance.

Among the naturally occurring antibiotics, β-lactams were introduced for clinical use in 1941, followed by aminoglycosides in 1944. In the decade that followed, new classes of antibiotics entered in the pipeline in order to combat bacterial infections. Tetracyclines came in 1950, macrolides in 1952 followed by glycopeptides (vancomycin) in 1956. Alongside, man-made synthetic antibiotics like sulfonamide and quinolones were also launched in 1935 and 1962 respectively. After this "Golden Era" of discovery of antibiotics, not a single effective class of

antibiotic entered the antibiotic pipeline until 2000.[14] This gap period, known as 'Discovery Void' in the antibiotics history aggravated the problem. Although there has been a small resurgence in new antibiotic classes launched for the treatment of Gram-positive infections in the last 15 years (linezolid (approved in 2000), daptomycin (2003), retapamulin (2007), fidaxomicin (2011) and bedaquiline (2012)), there is still an urgent need for the development of new antibiotics with activity against drug-resistant bacteria, especially Gram-negative bacteria, which are more difficult to kill.[12,17-21] The antibiotic pipeline is illustrated in Fig. 1.1.

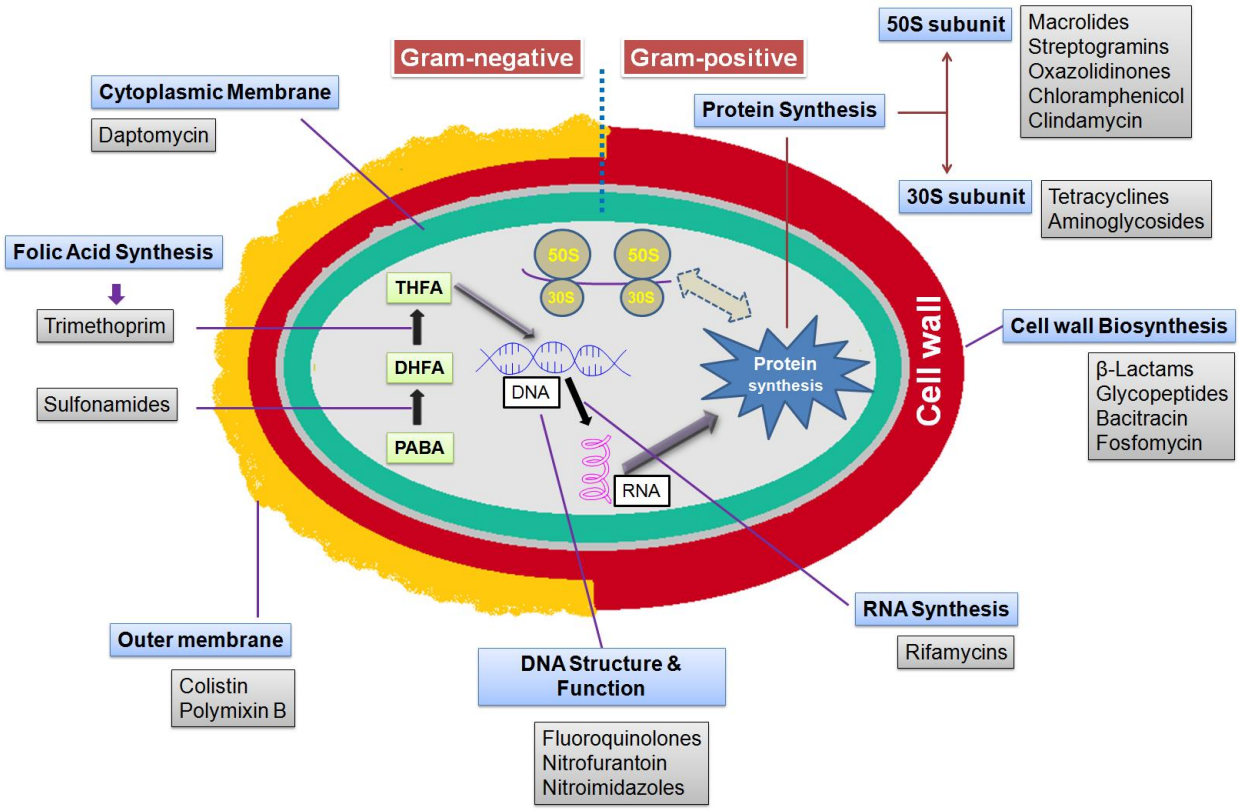


Figure 1.2: Major target sites of antibiotics.

1.2.2 Antibiotics and their targets

Most antibiotics target a specific site in bacteria, and act by either inhibiting bacterial growth (bacteriostatic) or direct killing (bactericidal) mechanism. Five major validated target sites in bacteria (Fig. 1.2) have been discovered.[22] β-lactams, glycopeptides such as vancomycin,

bacitracin and fosfomycin act by targeting bacterial cell wall biosynthesis.[23] Ribosomes are primarily targeted by the aminoglycosides, tetracyclines, macrolides, chloramphenicols, streptogramins and oxazolidinones classes of antibiotics.[24] Nucleic acids such as DNA and RNA are also targeted by various antibiotics such as fluoroquinolones and rifamycins.[25] Another target of antibiotics is the pathway of bacterial folic acid synthesis. Sulfonamide class of antibiotics are one such class, which stop folic acid production in bacteria.[26] Some natural products such as antimicrobial peptides (colistin) and lipopeptides (daptomycin) act by targeting the bacterial cell membrane.[27-29]

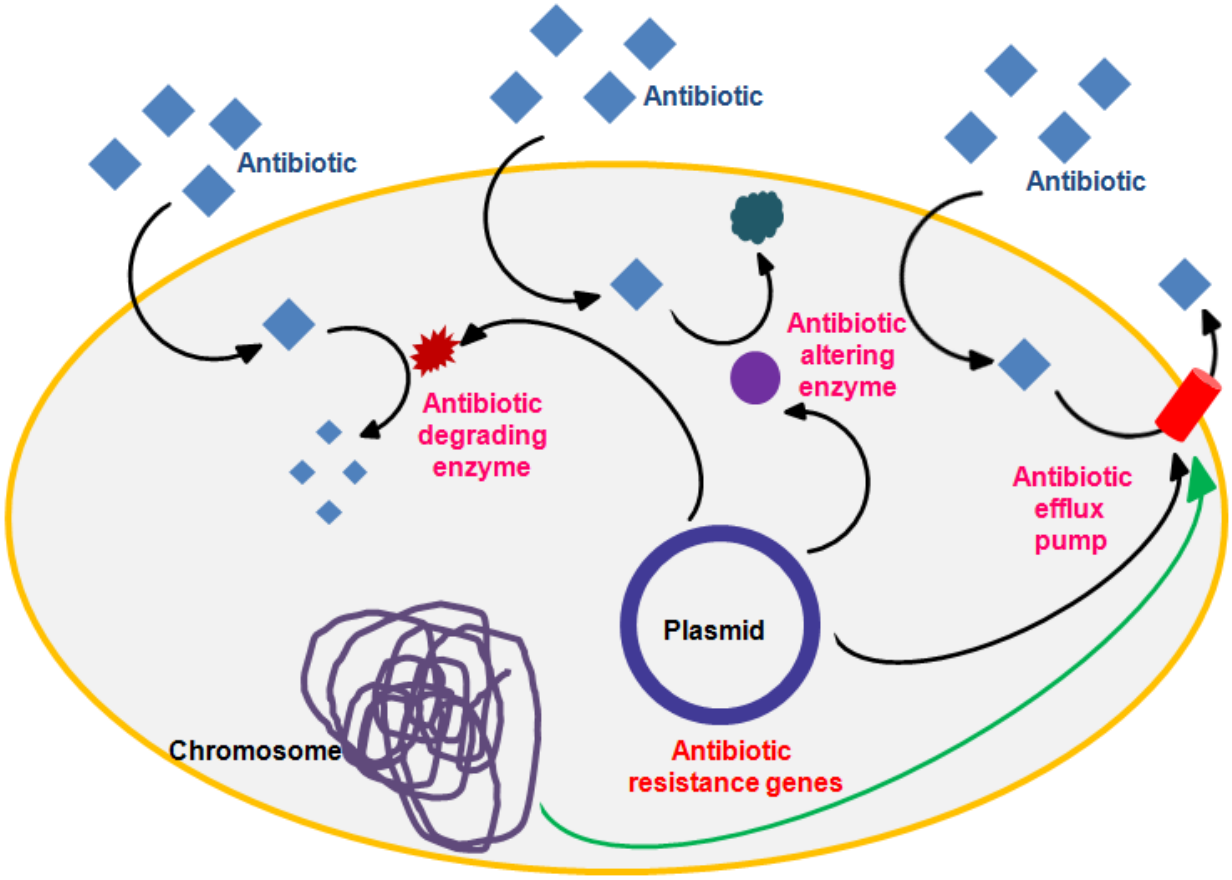


Figure 1.3: Mechanism of resistance to antibiotics.

1.2.3 Bacterial resistance to antibiotics

Although the first generation of antibiotics were called "wonder drugs", with time, bacteria have developed resistance to almost all classes of antibiotics. There are several strategies by which bacteria acquire resistance (Fig. 1.3); first involves targeting the antibiotics themselves. Bacteria produce enzymes for example β-lactamases that degrade the β-lactam (penicillin) class of antibiotics and render them ineffective.[30] Bacteria develop resistance to chloramphenicol and aminoglycosides by producing enzymes that can alter their chemical structure and make them inactive.[30] Another strategy used by bacteria to develop resistance involves pumping out the antibiotics using efflux pumps.[31,32] Bacteria develop resistance to the tetracyclines, chloramphenicol and fluoroquinolones using efflux pumps. Another strategy (not shown in Fig. 1.3) involves the modification of drug binding site. Antibiotics such belonging to the class of glycopeptides (vancomycin), macrolides (azithromycin) lose potency as a result of modified target site.[30]

In addition to the ability of bacteria to "acquire" resistance, they are also intrinsically resistant to different classes of antibiotics; a trait that is universally found within the genome of a bacterial species and is independent of antibiotic selective pressure.[33] Indeed, the conventional example of intrinsic antibiotic resistance is the multi-drug resistant (MDR) phenotype exhibited by Gram-negative bacteria, which are insensitive to many classes of clinically important Gram-positive antibiotics.[33,34] The molecular basis of this phenomenon is the presence of the Gram-negative outer membrane (OM), which is impermeable to many molecules like glycopeptides, and expression of numerous MDR efflux pumps that effectively reduce the intracellular concentration of the given drug.[34]

In the history of antibiotics, generally bacteria acquire resistance towards particular antibiotic within a couple of years of their approval (Fig. 1.1).[30,35] Just a few years after the first antibiotic, penicillin, became widely used in the late 1940s, penicillin-resistant infections emerged that were caused by the bacterium *Staphylococcus aureus*.[35] Methicillin, an example of later generation of β-lactam antibiotics, was introduced in the clinics in 1959, but methicillin-resistant *Staphylococcus aureus* (MRSA) arose in 1961.[35] This particular bacterium was untreatable by all the existing antibiotics except vancomycin. Consequently, between 1970 and 1980, MRSA

associated diseases have resulted in uncountable numbers of deaths around the world. At present the situation is even worse, as MRSA has acquired resistance against the antibiotic of last resort, vancomycin. Vancomycin-resistant *Enterococcus faecium* (VRE) was reported in 1987 followed by vancomycin-intermediate-resistant *Staphylococcus aureus* (VISA) and vancomycin-resistant *Staphylococcus aureus* (VRSA) in 1996 and 2001, respectively.[36,37] At last when the antibiotic pipeline was almost about to dry out, linezolid and daptomycin were launched in the year 2000 and 2003 respectively. However, bacteria had developed resistance to linezolid and daptomycin in 2003 and 2005 respectively.[38,39] Recently two antibiotics fidaxomicin and bedaquiline have been approved by FDA in 2011 and 2012, respectively, but these are not enough to fight against all kinds of bacterial infections.

Glycopeptides (vancomycin), drugs of last resorts for Gram-positive infections, have also been rendered ineffective by rapid emergence of resistance in Enterococci and Staphylococci (VRE, VISA and VRSA). Additionally, glycopeptides, are inherently inactive towards Gram-negative bacteria because of their inability to cross the outer membrane of these pathogens.[40] In our research, we aimed at combating acquired and intrinsic bacterial resistance to glycopeptide antibiotics by various rational strategies. In the following sections of this chapter, glycopeptie antibiotic discovery and their development have been described.

1.3 Glycopeptide Antibiotics

Glycopeptide antibiotics are glycosylated heptapeptides and isolated from soil bacteria 'Actinomycetes'.[22] Vancomycin (**1**, Fig. 1.4), the first member of the glycopeptide class of antibiotics, was discovered in 1956 by Eli Lilly & Co (Indianapolis, IN, USA) and still has an important role in the treatment of Gram-positive bacterial infections caused by MRSA and *Clostridium difficile*.[41,42] Another clinically important natural glycopeptide antibiotic, teicoplanin (Factor A_2-2, **2**, Fig. 1.4) was discovered in the Lepitit Research Center (Milan, Italy) which is also being used for similar infections in Europe since 1980. The first next generation semi-synthetic glycopeptide, telavancin (**3**) was launched in 2009 and followed by dalbavancin (**4**) and

oritavancin (**5**) in late 2014 for the treatment of severe skin and soft tissue infections caused by MRSA (Fig. 1.5).[43]

In the following sections, the discovery, development and the associated resistance of the most important glycopeptide, vancomycin (**1**) and successful semi-synthetic glycopeptides, telavancin (**3**), dalbavancin (**4**) and oritavancin (**5**) are described.

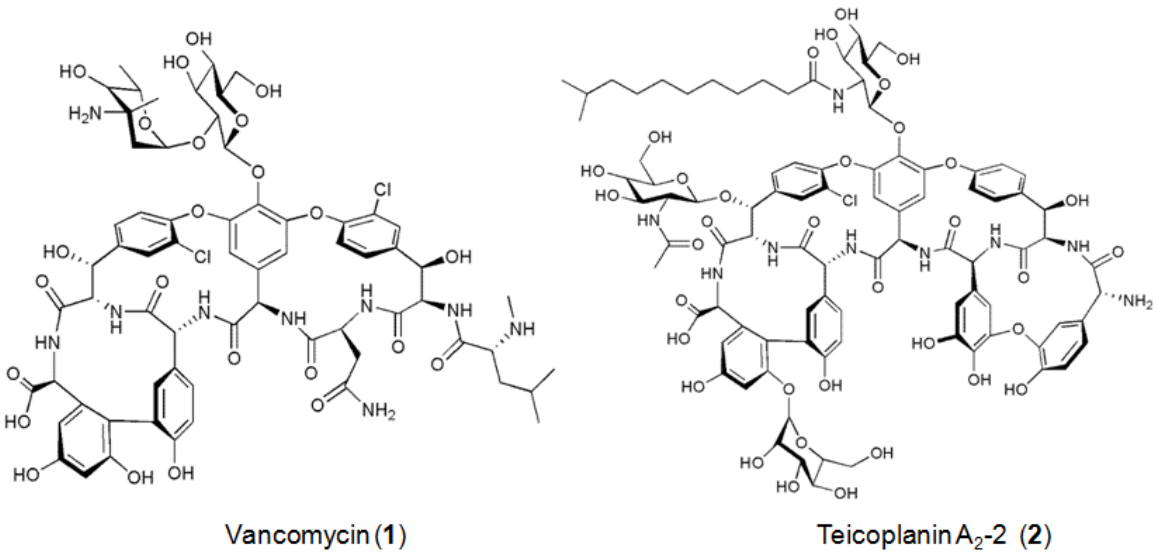


Figure 1.4: Structures of the naturally occurring glycopeptide antibiotics, vancomycin (**1**), teicoplanin A2-2 (**2**).

1.4 Vancomycin

1.4.1 Discovery of vancomycin

In the 1950s, Eli Lilly & Co. started an initiative aiming at discovering antibiotics with activity against penicillin-resistant Staphylococci. In 1952, a missionary led by R. W. Conley in Borneo sent a soil sample to his friend Dr. E. C. Kornfield, an organic chemist at Eli Lilly. An organism, *Streptomyces orientalis* (now called *Amycolatopsis orientalis*) isolated from that sample produced a substance initially called "compound 05865" but was eventually given the generic name

vancomycin, derived from the term "vanquish". This compound was highly active against most Gram-positive organisms, including penicillin-resistant Staphylococci and anaerobes such as Clostridia.[44,45] Two additional strains were isolated from India over the next few years that also produced compound 05865.[46] Subsequent animal experiments demonstrated that compound 05865 might be safe and efficient in humans. The rapid development of penicillin resistance in Staphylococci led to the compound's being fast-tracked for approval by FDA in 1958. Vancomycin hydrochloride was first marketed by Eli Lilly under the trade name "Vancocin®".

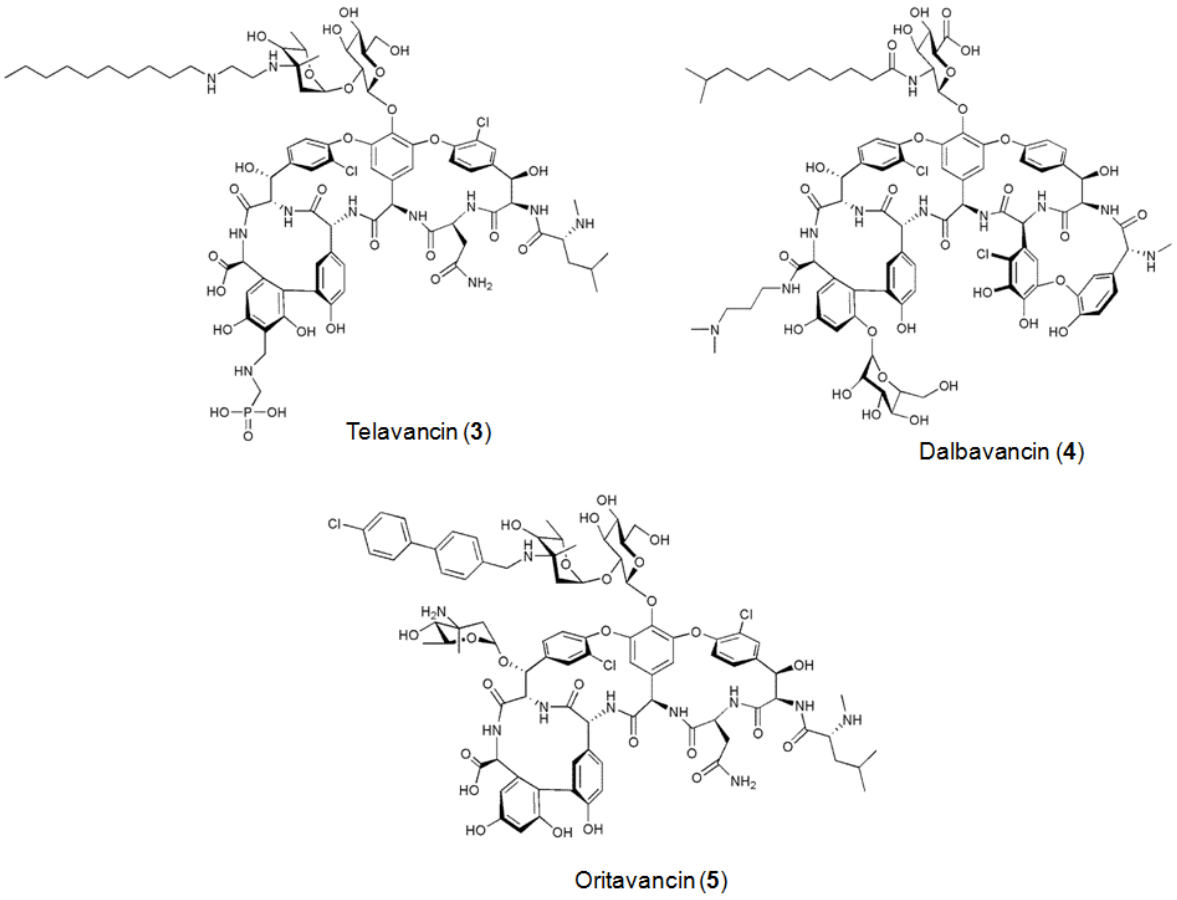


Figure 1.5: Structures of the semi-synthetic glycopeptides telavancin (**3**), dalbavancin (**4**) and oritavancin (**5**).

1.4.2 Mechanism of action of vancomycin

In 1965, Strominger *et al*. performed a series of experiments using crude bacterial membranes comprising peptidoglycan synthesizing enzymes supplemented with radiolabeled cell wall precursors, UDP-*N*-acetyl-muramyl-pentapeptide (UDP-MurNAc-pp) and UDP-*N*-acetyl-glucosamine in which they showed that the glycopeptide antibiotics block peptidoglycan biosynthesis at either transglyoslyation or transpeptidation.[47] Subsequent work by Perkins showed that vancomycin actually binds to peptidoglycan precursors, specifically to the D-Ala-D-Ala terminus of UDP-MurNAc-pp.[48,49] In addition to UDP-MurNAc-pp, other peptidoglycan intermediates that contain the D-Ala-D-Ala dipeptide include lipid I, lipid II and nascent (un-cross-linked) peptidoglycan. Experiments with radioactive vancomycin derivatives confirmed that vancomycin does not enter cells, which indicates vancomycin and other glycopeptides affect the extracellular enzymes that utilize intermediates such as lipid II and nascent peptidoglycan at extracellular surface.[50]

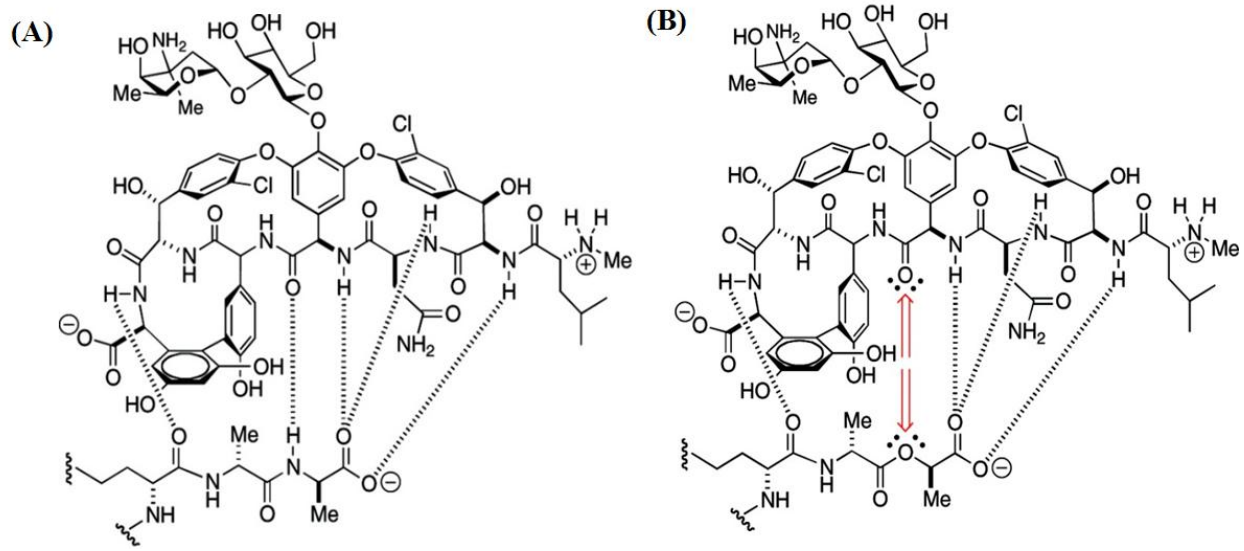


Figure 1.6: (A) Complexation of vancomycin with D-Ala$_4$-D-Ala$_5$ termini of sensitive bacteria (B) Complexation of vancomycin with D-Ala$_4$-D-Lac$_5$ termini of resistant bacteria.

Shortly after the structure of vancomycin was solved in 1980s, the binding interaction between vancomycin and the D-Ala-D-Ala dipeptide was determined by Williams group using NMR studies.[52] Binding between vancomycin and the D-Ala-D-Ala dipeptide was shown to occur through a set of five back bone hydrogen bonding interactions between the D-Ala-D-Ala dipeptide and the amides that line a cleft formed by the heptapeptide of the vancomyicn (Fig. 1.6A).[51,53,54] All other glycopeptides were believed to bind in a similar way. The bound glycopeptide acts as a steric impediment that halts lipid-II and/or the immature glycan chain from being processed further.[55] The net result is the inhibition of the transglycosylation and/or transpeptidation cross-linking steps of peptidoglycan synthesis, which weakens the peptidoglycan layers and leaves the bacterial cell susceptible to lysis due to alterations in osmotic pressure.

1.4.3 Mechanism of resistance to vancomycin

Researchers noticed early on that it is difficult to induce resistance to vancomycin. Ziegler *et al*., compared penicillin and vancomycin, both of which inhibit late stage of peptidoglycan biosynthesis, and found that the MIC of penicillin against *S. aureus* increased by more than 1,00,000-fold after 25 serial passages in antibiotic-containing media.[56] In contrast, the MIC of vancomycin increased by only 8-fold.[56] Furthermore, resistance to the β-lactams appeared almost immediately upon the introduction of penicillin into clinical use, whereas glycopeptide resistance was not observed for a very long time.[35] Hence, it was surmised that bacteria could not alter the target of glycopeptides (the D-Ala-D-Ala terminus of lipid-II and/or the immature glycan) because the process would involve simultaneous modifications to multiple enzymes in the pathway to peptidoglycan synthesis. To corroborate this, in 1986, Cooper and Given noted that "during the three decades in which vancomycin has been in clinical use, there has been no trend toward resistance among organisms usually susceptible", and speculated that the mode of action of glycopeptide antibiotics made the development of high-level resistance almost impossible.[57] One year later, strains of vancomycin-resistant Enterococci (VRE) began to appear in hospitals, and 20 years after that the incidence of VRE in hospitalized patients with Enterococcal infections in the US had spread to 30 %.[58] Some years later, in 1996, the extensive use of vancomycin for MRSA infections resulted in reduced vancomycin susceptibility and led to the emergence of hetero

resistant vancomycin-intermediate *S. aureus* (hVISA).[36] In 2001, the first vancomycin-resistant *S. aureus* (VRSA) was reported, wherein MRSA acquired resistance gene elements from VRE.[36,54]

Resistance to vancomycin in Enterococci is not as a result of spontaneous mutations in clinically relevant microorganisms. They are instead transferred to these pathogens by genes conferring glycopeptide resistance in glycopeptide producing organisms.[54] The mechanism of vancomycin resistance in Enterococci was elucidated by Courvalin and Walsh groups in the 1990s. Subsequent work on glycopeptide resistance in producer organisms has revealed that they consist of the same resistance genes as the resistant Enterococcal strains.[54] The mechanism of vancomycin resistance in Enterococci and Staphylococci is described below.

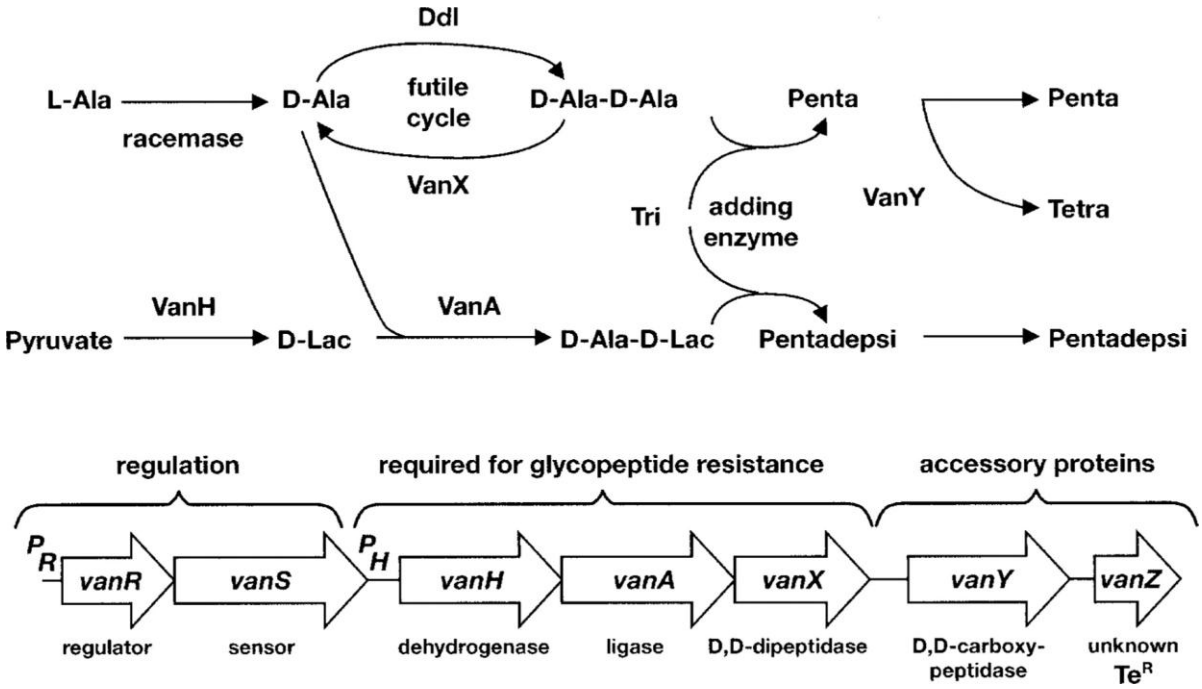


Figure 1.7: The VanA gene cluster that presents vancomycin resistance. Schematic of the five gene *Van RSHAX* cassette in VanA phenotypes of VRE. (Figure was taken from reference 59 with the permission from Oxford University Press).

1.4.3.1 Vancomycin-resistant Enterococci (VRE)

VRE has been categorized into various clinical phenotypes, formerly VanA and VanB (but now including additional variants of these classes such as VanC, VanD, VanE, VanF, VanG, VanM and VanL).[60-62] The VanA and VanB phenotypes are more prominent and initially differentiated by their susceptibility to vancomycin *vs* teicoplanin.[22] The VanA phenotype exhibits 1000-fold increased resistance to both drugs, while VanB VRE isolates have equivalent resistance to vancomycin but remain susceptible to teicoplanin. VanA and VanB isolates of VRE contain five *van* genes, *VanRSHAX*, necessary and sufficient to cause high-level resistance (Fig. 1.7).[59,63]

The five proteins that are encoded from *VanRSHAX* classified into two categories. VanR and VanS together function as a two component regulatory system (TCS), where VanS is a transmembrane receptor histidine kinase.[63] The extra membrane domain senses vancomycin and transfers the information to the cytoplasmic domain, which autophosphorylates on the -His side chain. The phosphor-VanS then transfers the -PO_3 group to an aspartyl side chain in the *N*-terminal domain of VanR. The C-terminal domain of phospho-VanR then acts as a regulator to control the *VanHAX* genes (Fig. 1.7).[59,63-65]

The VanHAX proteins cover the second category. All three are enzymes and coordinately act to reprogram the peptidoglycan termini from *N*-Acyl-D-Ala-D-Ala, a high-affinity target of vancomycin and teicoplanin, as noted above in section 1.4.2, to *N*-acyl-D-Ala-D-lactate (depsipeptide). VanH reduces the common metabolite pyruvate to D-lactate (D-Lac). VanA is a D,D-depsipeptide ligase (Ddl), making D-Ala-D-Lac.[59,63] A critical step in vancomycin resistance involves depletion of D-Ala terminating precursors to prevent interaction of vancomycin with its target. This step is assisted by the D,D-dipeptidase, VanX and the D,D-carboxypeptidase VanY, which hydrolyze D-Ala-D-Ala dipeptide and the C-terminal D-Ala residue from pentapeptide (*N*-acetyl-muramyl-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Ala), respectively.[59,63] In some types of VRE, another accessory protein VanXY is also involved which has both D,D-dipeptidase and D,D-carboxypeptidase activity. D,D-peptidases, VanX, VanY and VanXY are zinc-dependent wherein divalent zinc (Zn^{2+}) exists in the active site. The D-Ala-D-Lac accumulates and gets connected by the MurF ligase to UDP-*N*-acetyl-muramyl-tripeptide to generate the UDP-*N*-acetyl-muramyl-L-Ala-D-γ-Glu-L-Lys-D-Ala-D-Lac that can serve as cross-linking substrate for transpeptidase

action (Fig. 1.7). VanZ confers resistance to teicoplanin by an unknown mechanism in VanA phenotypic vancomycin-resistant bacteria.[59] The 1000-fold resistance in VanA and VanB VRE phenotypes to vancomycin results from the reprogramming of the peptidoglycan termini from D-Ala-D-Ala to D-Ala-D-Lac. The absence of the amide bond in the modified target (D-Ala-D-Lac) results in short of one central hydrogen bond and the presence of ground-state repulsion of the oxygen lone pair, due to which, a 1000-fold loss in binding affinity for vancomycin and teicoplanin to D-Ala-D-Lac (Fig. 1.6B).[66,67]

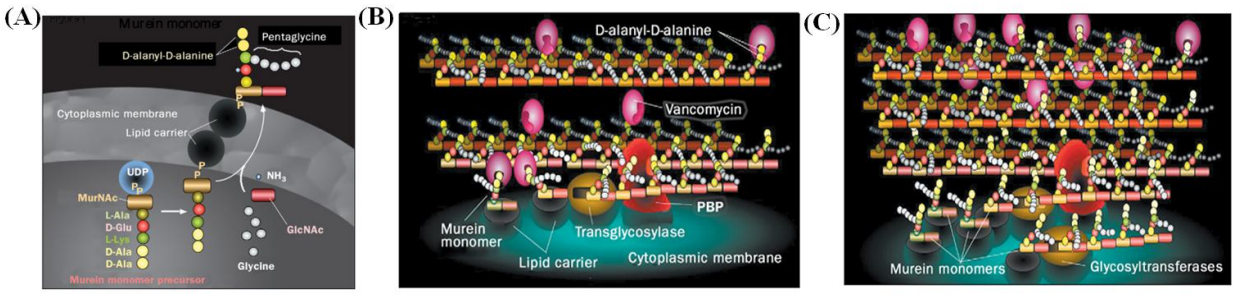


Figure 1.8: (A) Cell wall (peptidoglycan) structure of *Staphylococcus aureus*. (B) Binding of vancomycin to D-Ala-D-Ala residues of peptidoglycan precursor. (C) Thickened cell wall of Mu50. (Figure was taken from reference 36 with the permission from Elsevier Publishing Group).

1.4.3.2 Vancomycin-intermediate-resistant *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA)

Recently, the levels of vancomycin susceptibility among clinical isolates of Staphylococci were low. *S. aureus* with such reduced susceptibility to vancomycin were termed as VISA (vancomycin intermediate *S. aureus*).[68,69] VISA acquired resistance to vancomycin mainly due to thickened cell wall which results in more sites for stoichiometric binding of the drug (Fig. 1.8C).[36] VRSA were first isolated from a dialysis patient who also had a chronic infection with VRE.[70] Genotypic analysis of the VRSA confirmed the same five *VanRSHAX* genes that were found in VRE indicating that the resistance genes transferred from the Enterococcal host to the *S. aureus*.[71] It remains to be seen what the rate of spread of the *VanRSHAX* genes will be into MRSA strains, but

there is no doubt that this only hastens the need for second- and third-generation forms of glycopeptides and lipoglycopeptides. Some types of VRSA acquire resistance to vancomycin due to thickened cell wall (VRSA Mu50, Fig. 1.8C).[36]

1.5 Successful semi-synthetic glycopeptide antibiotics

Lipoglycopeptides such as telavancin (**3**), dalbavancin (**4**) and oritavancin (**5**) demonstrate high activity against multidrug-resistant (MDR) Gram-positive pathogens of Staphylococci, Enterococci and Streptococci, although neither dalbavancin nor telavancin are active against VRE exhibiting the VanA phenotype (Fig. 1.5).[43] A common feature of these lipoglycopeptides is the presence of lipid side chains, which assist in anchoring the molecule to the cell membrane thereby concentrating the drug at the target site and increasing their activity relative to their parent glycopeptide (Fig. 1.9).[72] Compared to vancomycin, all these lipoglycopeptides exhibit lower MIC values for the aforesaid pathogens and demonstrate improved pharmacological properties which allow for once-daily dosing for telavancin, once-weekly dosing for dalbavancin and potentially one dose per treatment course for oritavancin.[43]

1.5.1 Telavancin

Telavancin (**3**, Fig. 1.5) (Vibativ®, TD-6424) is derived from vancomycin (**1**, Fig. 1.4) by conjugation of a decylaminoethyl lipophilic moiety on the vancosamine amino group, and a hydrophilic (phosphomethyl)aminomethyl moiety at the para position of the aromatic ring on the C-terminal dihydroxyphenylglycine residue.[73] The lipophilic group takes part in increasing membrane interactions whereas the hydrophilic group is proposed to promote tissue distribution and clearance with reduced nephrotoxicity.[72,73] Telavancin shows improved activity compared to vancomycin against a range of Gram-positive bacteria, including vancomycin-resistant strains (VISA, VRSA and VRE, VanB phenotype).[43] Telavancin was approved by FDA in 2009 for treatment of complicated skin and skin structure infections caused by MRSA.

Telavancin exhibits a dual mechanism of action against Gram-positive bacteria. Like other glycopeptides, telavancin binds to terminal D-Ala-D-Ala residues and inhibits cell wall synthesis. Due to its ability to bind to bacterial cell membranes, telavancin is more active than vancomycin at

inhibiting both the transglycosylation and the synthesis of peptidoglycan. It also alters cell membrane permeability and dissipates membrane potential (Fig. 1.9A). This second mechanism of action may be the lone reason for its rapid bactericidal activity.[43,74]

1.5.2 Dalbavancin

Amidation of the C-terminal carboxyl group of A40926, a teicoplanin-like glycopeptide with *N*,*N*-dimethylaminopropylamine group produced dalbavancin (**4**, Fig. 1.5) (Dalvance ®, BI-397) and approved in May 2014 for treatment of complicated skin and skin structure infections caused by MRSA.[75-77] The modification led to an extended half-life of ~ 300 h in humans allowing for once weekly dosing.[78] Like telavancin, dalbavancin also demonstrated improved activity compared to vancomycin against a range of Gram-positive bacteria, including vancomycin-resistant strains (VISA, VRSA and VRE VanB phenotype).[43]

Like vancomycin, dalbavancin also inhibits cell wall biosynthesis. In addition, the lipophilic side chain of dalbavancin enhances the antibacterial's binding affinity for D-Ala-D-Ala target site through the formation of dimers and membrane anchoring, leading to a superior potency than its parent glycopeptide.[79] The role of the lipid side chain in perturbation of cell membranes has not been demonstrated for dalbavancin.

1.5.3 Oritavancin

Oritavancin (**5**, Fig. 1.5) (LY333328) is derived from chloroeremomycin (**6**, Fig. 1.10) by the installation of a 4-chlorobiphenylmethylene group, which affords oritavancin.[80,81] Oritavancin exhibits potent activity against Gram-positive bacteria, with an MIC of ~ 1 μg/mL including vancomycin-resistant bacteria and approved in October 2014 for treatment of complicated skin and skin structure infections caused by MRSA.[43]

Like all other glycopeptides, oritavancin inhibits cell wall synthesis by binding to the terminal D-Ala-D-Ala of a nascent peptidoglycan chain and also to the pentaglycine bridge (present in Staphylococci), thus inhibiting transglycosylation and transpeptidation. Oritavancin forms homodimers prior to binding to D-Ala-D-Ala or D-Ala-D-Lac, which increases its binding affinity for the target site.[82] The 4-chlorobiphenyl side chain of oritavancin interacts with the cell

membrane and increases membrane permeability (Fig. 1.9B).[72,82] The dual mechanism of action could also increase the longevity of the drug and reduce the risk of resistance selection. In addition to the aforementioned mechanisms, it has also been shown that oritavancin inhibits RNA synthesis.[83]

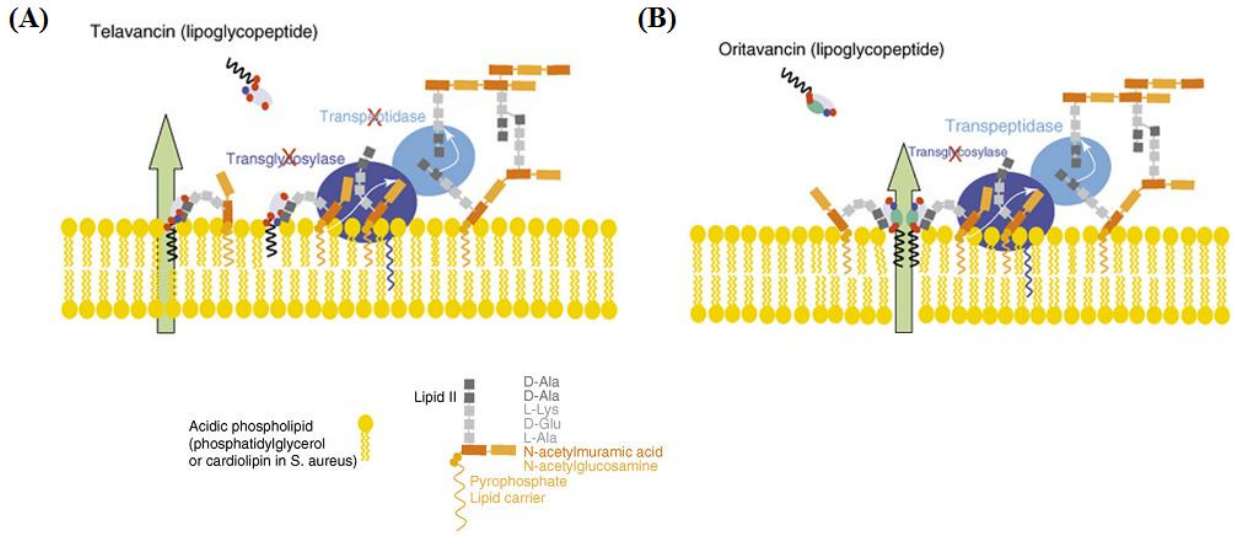


Figure 1.9: Dual mechanism of lipoglycopeptides, Telavancin (A) and Oritavancin (B). (Figure was taken from reference 72 with the permission from Elsevier Publishing Group).

1.6 Recent modifications on vancomycin to combat bacterial resistance to vancomycin

The glycopeptide antibiotics are large, complex molecules with plenty of options for *N*- and *O*-alkylation as well as changes to the amino acids of the heptapeptide. Even the chloro substituents of the triaryl biether backbone can be considered as sites of reactivity.

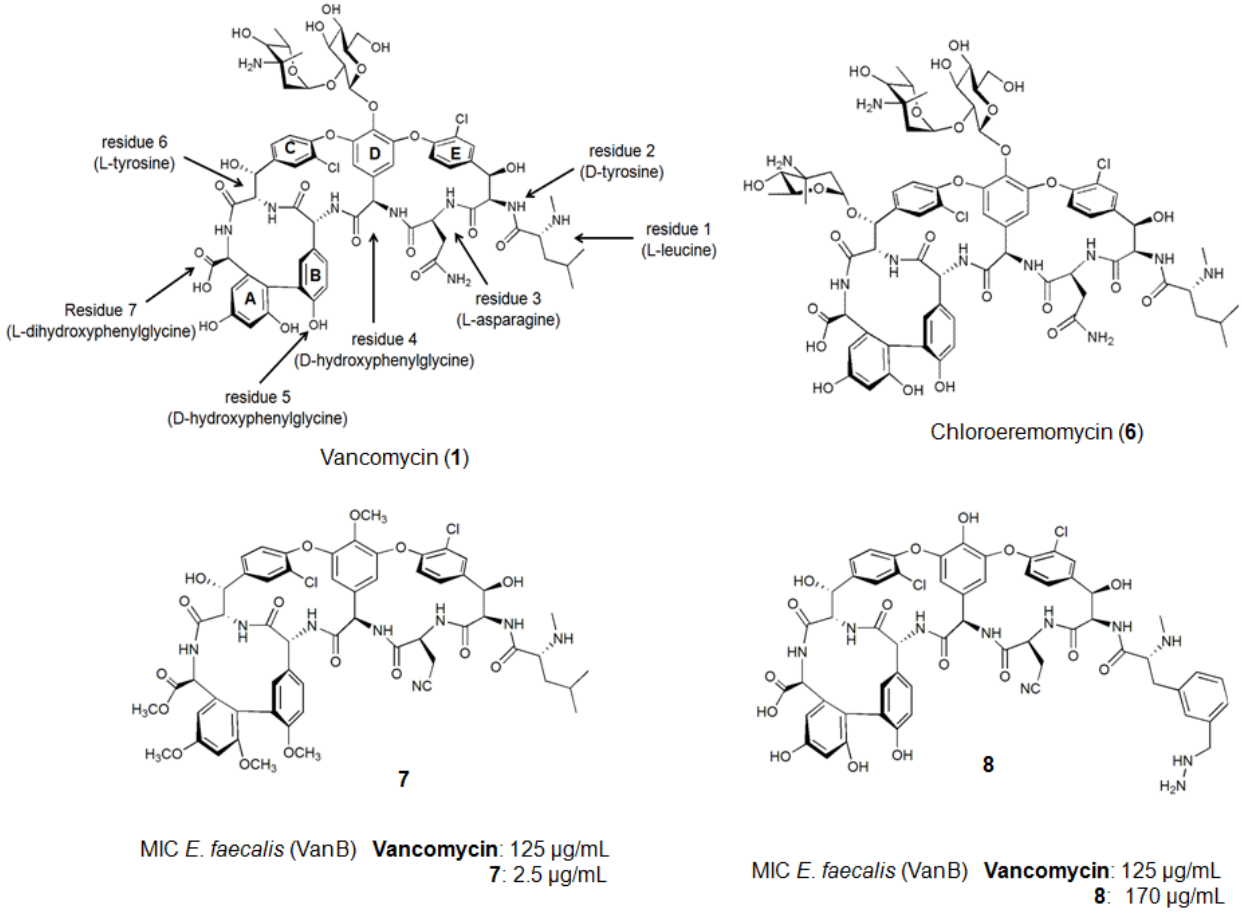


Figure 1.10: Amino acid residues 1-7 of vancomycin (**1**, amino acids of the biosynthetic pathway are labeled in parentheses); structure of chloroeremomycin (**6**), vancomycin aglycon nitrile methyl ether derivative (**7**), vancomycin aglycon benzylhydrazine derivative (**8**).

1.6.1 Modification of the amino acids in vancomycin backbone

Modifications of amino acid residue 3 (Fig. 1.10) were first reported by Boger *et al*. in 2002. Boger group observed that a vancomycin analogue (**7**, Fig. 1.10) containing a nitrile group at residue 3 rather than the carboxamide of asparagine showed increased activity against vancomycin-sensitive and resistant bacteria.[84] Next, the Boger group turned to the *N*-methyl-L-leucine residue of vancomycin (residue 1) with an aim at introducing additional hydrogen bonding to the peptidoglycan terminus away from the binding pocket of the molecule.[85] Further, they envisioned that nucleophilic substituents such as hydrazine might form a covalent attachment to D-

Ala-D-Ala or D-Ala-D-Lac. Keeping this in mind, a series of hydrazine-containing vancomycin derivatives were synthesized and determined their antibacterial activity. Compound **8** (Fig. 1.10) showed similar activity as vancomycin against sensitive bacteria, whereas activity against VRE remained poor.[85]

1.6.1.1 Redesigned glycopeptide

Modification of the vancomycin peptide backbone presents a monumental synthetic challenge, as changes to the backbone may involve a full synthesis of the compound. As discussed earlier, vancomycin binds to the peptide terminus D-Ala-D-Ala through five hydrogen bonds sequesters the substrate from transpeptidase and inhibits cell wall cross-linking. Resistance appeared to vancomycin when this hydrogen bonding network is disturbed by a single atom replacement in the ligand (NH →O in modified target; depsipeptide) that serves to not only remove the essential H-bond but also brings in a ground-state repulsive lone pair/lone pair interaction between the vancomycin residue 4-carbonyl and D-Ala-D-Lac ester oxygens and results in 1000-fold loss in affinity. In an attempt to find out the magnitude of these two effects to the net 1000-fold loss in binding affinity, Boger group provided an experimental estimation by examining the model ligands **9**-**11** (Fig. 1.11A), suggesting that the repulsive lone pair interactions (100-fold) is responsible for the largest share of the lost binding affinity (1000-fold), not the H-bond loss (10-fold) indicating that the designs could primarily focus on eliminating the destabilizing lone pair interaction rather than reintroduction of the lost H-bond.[67] A vancomycin analogue that lacks residue 4 amide carbonyl and replacing it with a methylene group ([Ψ[CH_2NH]Tpg^4]-vancomycin aglycon, **13**, Fig. 1.11B) was developed by Boger *et al*.[86] This analogue demonstrated a 40-fold increase in affinity for D-Ala-D-Lac and a 35-fold reduction in affinity for D-Ala-D-Ala. Also, this derivative exhibited activity against VRE with an MIC of 31 μg/mL, reflecting its improved binding affinity (Fig. 1.11B).[86]

Next, Boger *et al*. introduced an amidine group at residue 4, [Ψ[C(═NH)NH]Tpg^4]vancomycin aglycon (**14**, Fig. 1.11B), that not only binds to the unaltered peptidoglycan D-Ala-D-Ala but also binds to the altered ligand D-Ala-D-Lac by virtue of its ability to serve as either a hydrogen-bond donor or a hydrogen-bond acceptor (Fig. 1.11C).[51,87,88]

This amidine derivative of vancomycin aglycon was equally active against sensitive and resistant bacteria, displaying the MIC of < 0.5 μg/mL. (Fig. 1.11B).

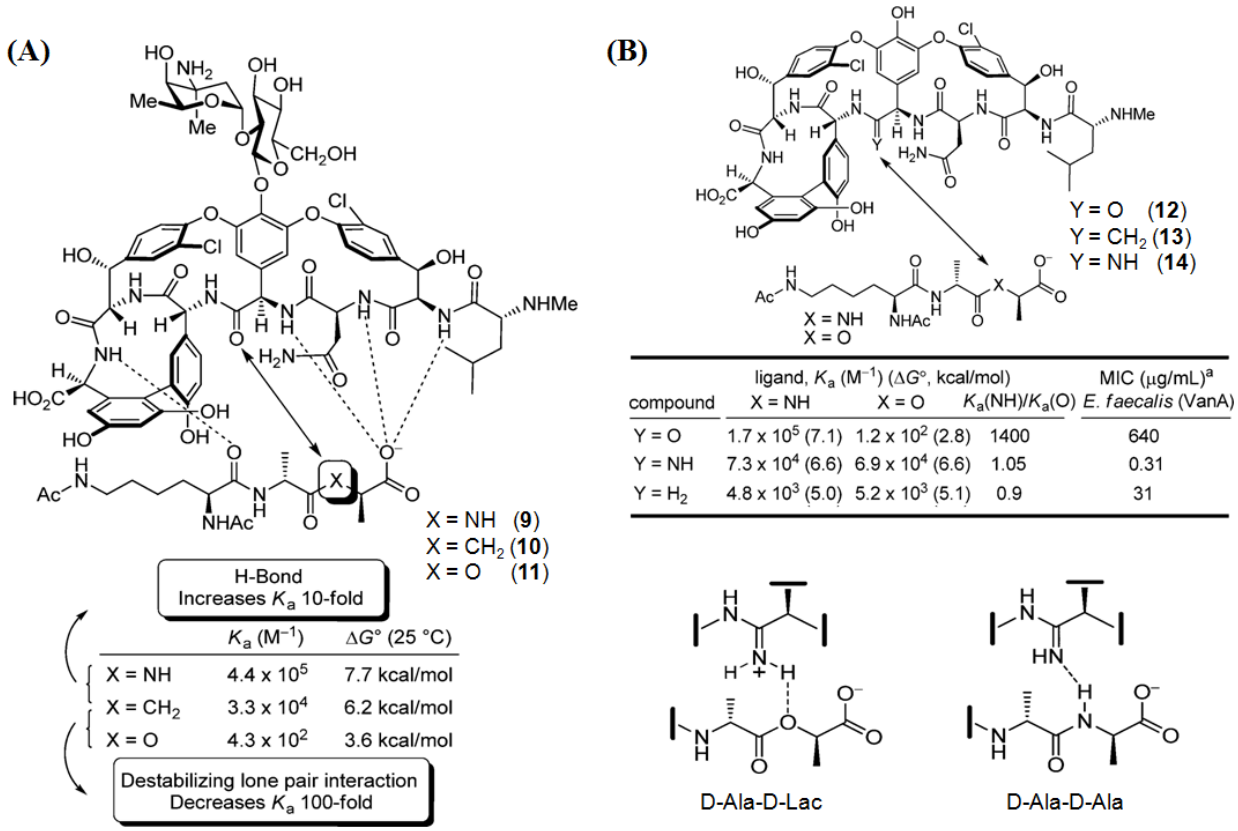


Figure 1.11: (A) Binding affinities of vancomycin with model ligands (**9**-**11**). (B) Structures of vancomycin aglycon (**12**), ([Ψ[CH_2NH]Tpg^4]-vancomycin aglycon (**13**), [Ψ[C(═NH)NH]Tpg^4]vancomycin aglycon (**14**) and their binding affinities with model ligands. (C) Dual binding behaviour of compound **14** toward D-Ala-D-Lac and D-Ala-D-Ala. (Figure was adopted from reference 51 with the permission from American Chemical Society).

1.6.2 Introduction of lipophilicity to the periphery of glycopeptides

Many of the early modifications of vancomycin and the glycopeptide family involved the addition of hydrophobic chains to the molecule as exemplified by telavancin, dalbavancin and oritavancin which have been described already in the previous sections. These derivatives

mimicked the natural hydrophobic character of teicoplanin (**2**), but alkylation of the polar hydroxyl, acid and amino groups can compromise the naturally high aqueous solubility of the parent compound. There are numerous reports on introduction of lipophilicity to glycopeptides which are well documented in literature and not discussed here in detail.[89,90] Here, few important chemical strategies are described which were developed recently, to introduce lipophilicity on vancomycin.

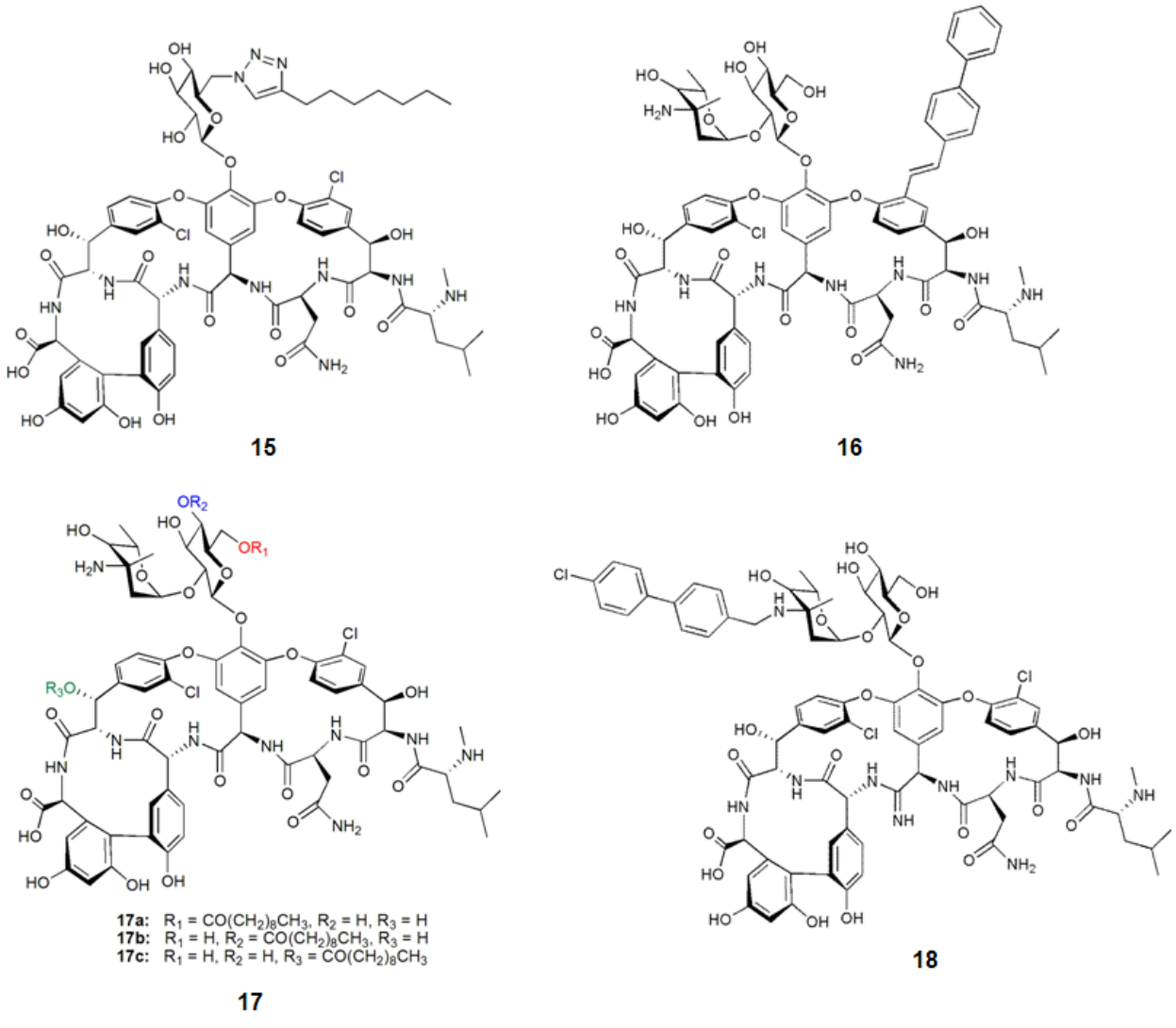


Figure 1.12: Introduction of hydrophobic side chains on vancomycin. Structures of compounds **15-18**.

One of the most versatile and currently widely employed methods of ligation in biological systems is the Huisgen or Click reaction; a copper catalysed 1,3-dipolar cycloaddition reaction between an azide and an alkyne. The application of Click chemistry for glycopeptide modification was first reported in 2005 by Thorson *et al*., who used a chemoenzymatic approach to incorporate 6-azido glucose onto the vancomycin aglycon for subsequent glycorandomization (compound **15**, Fig. 1. 12).[91] Further, compound **15** was found to be more effective than vancomycin against VanB VRE (MIC = 1 µg/mL).

Focusing on amino acid residues 2 and 6 of the aryl halide groups, Arimoto *et al*. reported the application of a Suzuki-Miyaura coupling using *trans*-2-(4-biphenyl)vinylboronic acid as the key coupling agent (compound **16**, Fig. 1. 12).[92] Compound **16** was effective against VanB phenotypic VRE (0.5 µg/mL) whereas it was found to be ineffective against VanA phenotypic VRE.

More recently in 2015, Miller *et al*. developed three regio-selective peptide catalysts that exhibit site-specificity for lipidation of the aliphatic hydroxyls on vancomycin, generating three lipidated vancomycin analogues (compounds **17a**, **17b** and **17c**, Fig. 1. 12).[93] Compounds **17a**-**17c** showed good activity against both VanA and VanB phenotypic VRE (MIC ~ 0.25 µg/mL).

As mentioned previously in section 1.6.1.1, [Ψ[C(=NH)NH]Tpg^4]vancomycin aglycon exhibits balanced binding affinity to both sensitive and resistant bacterial ligands. It has been shown in the previous sections that incorporation of lipophilicity into the glycopeptide scaffold provides favourable additional properties which results in improved bioactivity against vancomycin-resistant Enterococci (VanA and VanB phenotypes of VRE). To have dual binding affinity and favourable hydrophobic interactions in the same molecule, Boger *et al*. developed (4-chlorobiphenyl)methyl derivative of [Ψ[C(=NH)NH]Tpg^4]vancomycin (compound **18**, Fig. 1. 12) that is highly active against vancomycin-resistant bacteria.[94]

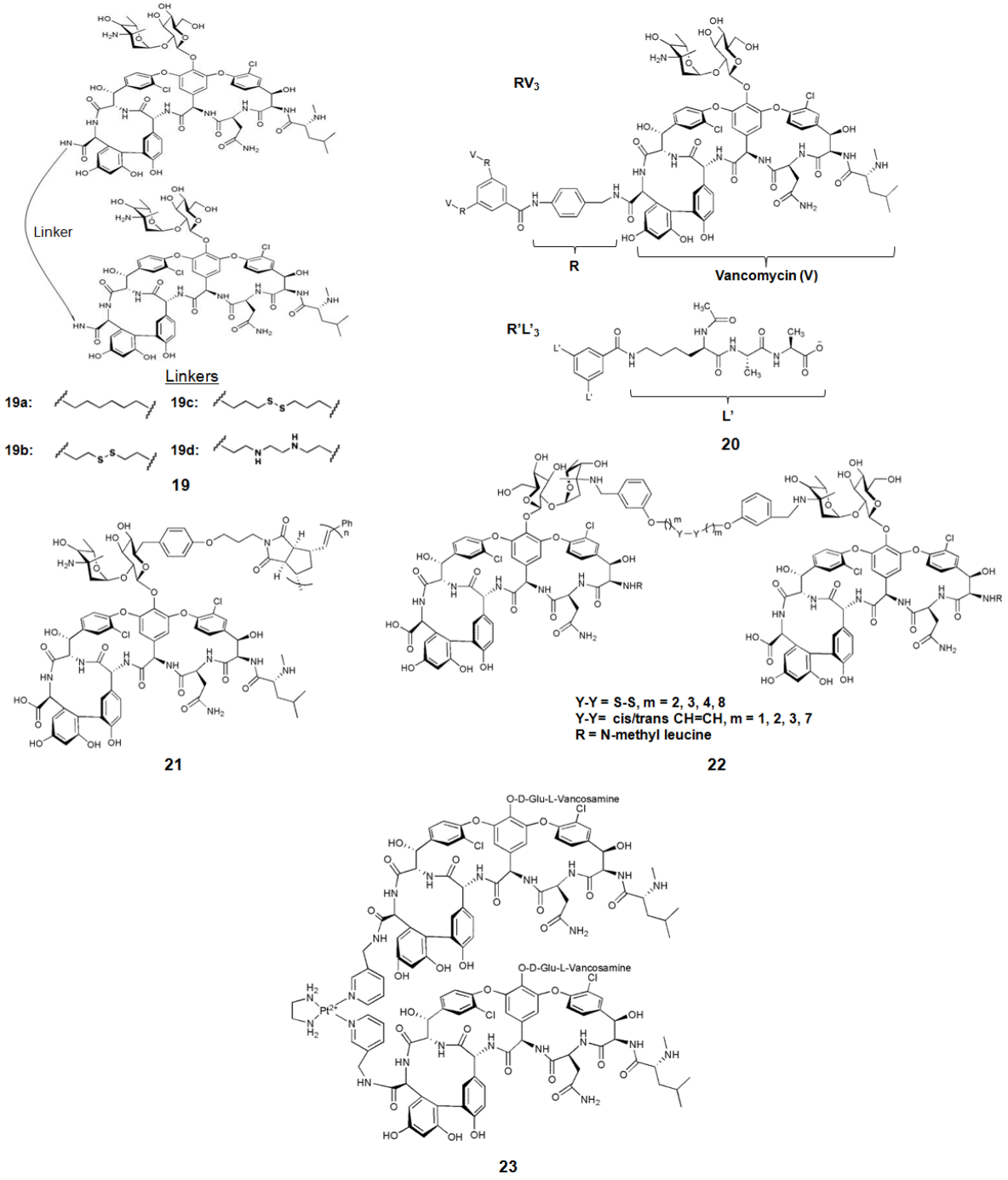


Figure 1.13: Multivalent vancomycin derivatives- Bis(vancomycin)carboxamides developed by Griffin *et al*. (**19**); Structures of the trivalent derivatives of vancomycin, RV_3, and of D-Ala-D-Ala, $R'L'_3$ developed by Whitesides group (**20**); Polymeric vancomycin derivative developed by Arimato *et al*. (**21**); Dimers of vancomycin developed by Nicolaou group (**22**); Structure of divalent vancomycin developed by Bing Xu group (**23**).

1.7 Multivalency approach

Glycopeptide antibiotics such as vancomycin are known to self-associate into homodimers via hydrogen bonding and hydrophobic interactions in both solution and solid states.[95] This noncovalent dimerization is highly favourable and cooperative with the binding of cell wall precursors which could lead to enhancement in antibacterial potency.[96] This observation prompted the scientific community to study the effect of multivalency on antibacterial properties of glycopeptides.

Early on, in 1996, Griffin *et al*. developed a series of bis(vancomycin carboxamides) (**19**, Fig. 1.13) which exhibited improved activity and binding affinity towards VRE compared to monomeric vancomycin.[97] In 1998, Whitesides group introduced tris(vancomycin carboxamide) that binds to a trivalent ligand derived from D-Ala-D-Ala with very high affinity (**20**, Fig. 1.13).[98] Calorimetric measurements for this trivalent system showed favourable thermodynamic properties such as enthalpy relative to the corresponding monomers which illustrated the practicality of designing very high-affinity systems based on polyvalency. In 1999, Arimato group described a multivalent polymer of vancomycin (**21**, Fig. 1.13), synthesized via ring opening metathesis polymerization (ROMP), exhibited significant enhancement in antibacterial activity against VRE.[99] In 2000, Nicolaou group developed a series of vancomycin dimers (**22**, Fig. 1.13) with improved antibacterial activity against drug-resistant bacteria.[100] Bing Xu *et al*. developed dimers of vancomycin (Van), linked by a rigid metal complex, $[Pt(en)(H_2O)_2]^{2+}$ which exhibited potent activities against VRE (**23**, Fig. 1.13).[101]

1.8 An unmet challenge of glycopeptide antibiotics: Intrinsic resistance of Gram-negative pathogens

The frequency of infections caused by Gram-negative pathogens (GNPs) is escalating at an alarming rate posing a serious clinical threat.[18] In addition to acquired resistance in GNPs, a plethora of Gram-positive antibiotics are left unused due to intrinsic resistance displayed by GNPs towards these antibiotics.[33] The additional outer membrane (OM) and multiple efflux pumps appear to be the main contributors to this intrinsic resistance as these effectively hinder

the entry of a variety of drug molecules including glycopeptides antibiotics such as vancomycin.[34] In fact, only two antibacterial agents, which possess good activity against GNPs, have been approved by FDA in the past decade: tigecycline and doripenem.[102,103] The development of Gram-negative antibacterials is impeded by the difficulty associated with identifying molecules that can penetrate OM and do not succumb to the efflux mechanism.[104]

Antibacterial drugs can penetrate the OM mainly by two pathways: porin channel mediated diffusion by hydrophilic antibiotics, and a passive route taken by hydrophobic compounds.[104-107] Glycopeptides are hydrophilic macromolecular compounds with a complex chemical structure and a high molecular weight (1450-2000 Da).[108] Although they are hydrophilic, they are unable to permeate through porins in the OM to reach the cell wall area because of their high molecular weight and size. Since their site of action lies within the cell wall, GNPs are intrinsically resistant to glycopeptides. Significant strategies have been adopted to make vancomycin active against Gram-negative bacteria. In one strategy, Nicolosi *et al.* introduced vancomycin encapsulated fusogenic liposomes to overcome the OM barrier of GNPs, thereby sensitizing GNPs to the composition.[109] In another strategy, Morones-Ramirez *et al.* showed antibacterial activity of vancomycin against GNPs in combination with silver.[110] To date, a covalently modified glycopeptide derivative that could overcome intrinsic resistance of GNPs towards glycopeptides has not been found.

1.9 Scope of the thesis

Presently, the emergence and spread of antibiotic resistance coupled with a diminishing antibiotic pipeline has made it vital to develop novel antimicrobial agents.[111] Vancomycin has been considered as "the drug of last resorts" for the treatment of multidrug-resistant Gram-positive bacterial infections such as MRSA. Vancomycin has also been rendered ineffective due to the emergence of vancomycin resistance in Enterococci and Staphylococci (VRE, VISA and VRSA). At present, only a few drugs such as daptomycin, quinupristin/dalfopristin and linezolid are clinically available for the treatment of infections plagued by these bacteria.[112] Resistance to even these last line antibiotics in bacteria has been reported in clinical settings, which is a matter of

concern.[38,113] In addition to this, many antibacterial agents, including glycopeptide antibiotics such as vancomycin, are inherently inactive towards Gram-negative pathogens because of their inability to cross the outer membrane of these bacteria.[33] Many semi-synthetic approaches have been adopted by various research groups towards the development of novel glycopeptides to combat acquired resistance to vancomycin. Many of them have been promising against vancomycin-resistant VanB strains, but the more virulent VanA strains remained unaffected, and this includes the recently FDA approved semi-synthetic glycopeptides telavancin and dalbavancin which are currently in use to treat MRSA infections.[43] The perennial persistence of vancomycin resistance, calls for an urgent need to develop more potent analogues having additional mode of action, which would not only combat drug-resistant bacteria but also make bacterial resistance difficult to develop. The aim of this thesis is to develop novel semi-synthetic glycopeptides to combat both acquired and intrinsic resistance of the bacteria towards vancomycin.

In Chapter 2, a simple semi-synthetic strategy is described to overcome the acquired resistance which is aimed at enhancing the lost binding efficiency of vancomycin towards the target peptides of resistant bacteria. In Chapter 3, a rational strategy is described to overcome the acquired resistance by imparting an additional mechanism of action to vancomycin, namely, bacterial membrane disruption. In both the strategies, in comparison to vancomycin, the optimized new glycopeptide exhibited > 1000-fold more *in-vitro* activity against VRE and demonstrated improved pharmacological properties in mouse models. Chapter 4 is aimed at imparting both membrane disruption property and improved binding affinity to the same molecule to effectively tackle vancomycin-resistant bacteria. With this combined approach, > 8000-fold higher activity was achieved against VRE compared to vancomycin; the activity being manifold higher compared to that achieved using the individual strategies.

An alternative strategy is described in Chapter 5A to combat vancomycin-resistant bacteria. As described in section 1.6, D,D-peptidases are some of the key enzymes that are responsible for vancomycin resistance. The common feature of these enzymes lies in their active site bearing zinc ion (Zn^{2+}). In Chapter 5A, a vancomycin analogue, dipicolyl-vancomycin conjugate (Dipi-van) has been developed (Dipi-van), which presumably has the ability to complex with Zn^{2+} ion and also have the ability to bind with the cell wall precursor peptides. Dipi-van exhibited high activity against VRE both *in-vitro* and *in-vivo*.

The acquisition of metallo-β-lactamases (enzymes bearing zinc ions in the active site) in New Delhi metallo-β-lactamase-1 (NDM-1) is the major contributor to the emergence of carbapenem resistance in Gram-negative pathogens. In Chapter 5B, Dipi-van has been shown to restore the activity of carbapenem antibiotic against a variety of clinical isolates of NDM-1 producing pathogens both *in-vitro* and *in-vivo*.

Finally, Chapter 6 presents overcoming the intrinsic resistance of Gram-negative pathogens (GNPs) towards glycopeptide antibiotics. For the first time, a chemically modified vancomycin derivative (lipophilic cationic (permanent positive charge) vancomycin analogue) is showed to permeabilize the outer membrane of Gram-negative pathogens and inhibit the cell wall biosynthesis. The compound showed potent activity against a variety of multidrug-resistant clinical isolates of Gram-negative pathogens. The high activity of the compound is attributed to strong membrane disruption properties of the compound.

Chapter 2

Lipophilic-vancomycin-carbohydrate Conjugates Target Vancomycin-resistant Bacteria through Improved Binding Affinity to the Target Peptide

Abstract

Chapter 2 *describes a simple semi-synthetic strategy aimed at improving the binding efficiency of vancomycin derivatives towards the target peptides of resistant bacteria by conjugating various cyclic/acyclic sugar moieties (which have the ability to form extra hydrogen bonds with the target peptides of bacteria) to the C-terminal of vancomycin. The optimized vancomycin-sugar conjugate exhibited > 150-fold increase in binding affinity for N,N'-diacetyl-Lys-D-Ala-D-Lac (resistant bacterial ligand) compared to vancomycin. This improved binding affinity was also reflected in its antibacterial activity, wherein the MIC value was brought down from 750 µM to 36 µM against VRE (VanA phenotype). To further sensitize against VRE, lipophilic alkyl chain (octyl, decyl and dodecyl) was appended to the primary amine group of vancosamine of the optimized vancomycin-sugar conjugate. Lipovancomycin-sugar conjugate comprising decyl chain showed 1000-fold (MIC = 0.7 µM) and 250-fold (MIC = 1 µM) more activity than vancomycin against VanA and VanB strains of VRE respectively. Unlike vancomycin, this lipovancomycin-sugar conjugate is bactericidal and caused improved cell wall biosynthesis inhibition. Further, this compound showed high activity in mice infection models against VISA and VRE and exhibited improved pharmacological properties with no observed toxicity. Therefore, this approach could potentially lead to the development of new generation of glycopeptide antibiotics in order to tackle the vancomycin-resistant bacteria.*

Publications based on this work

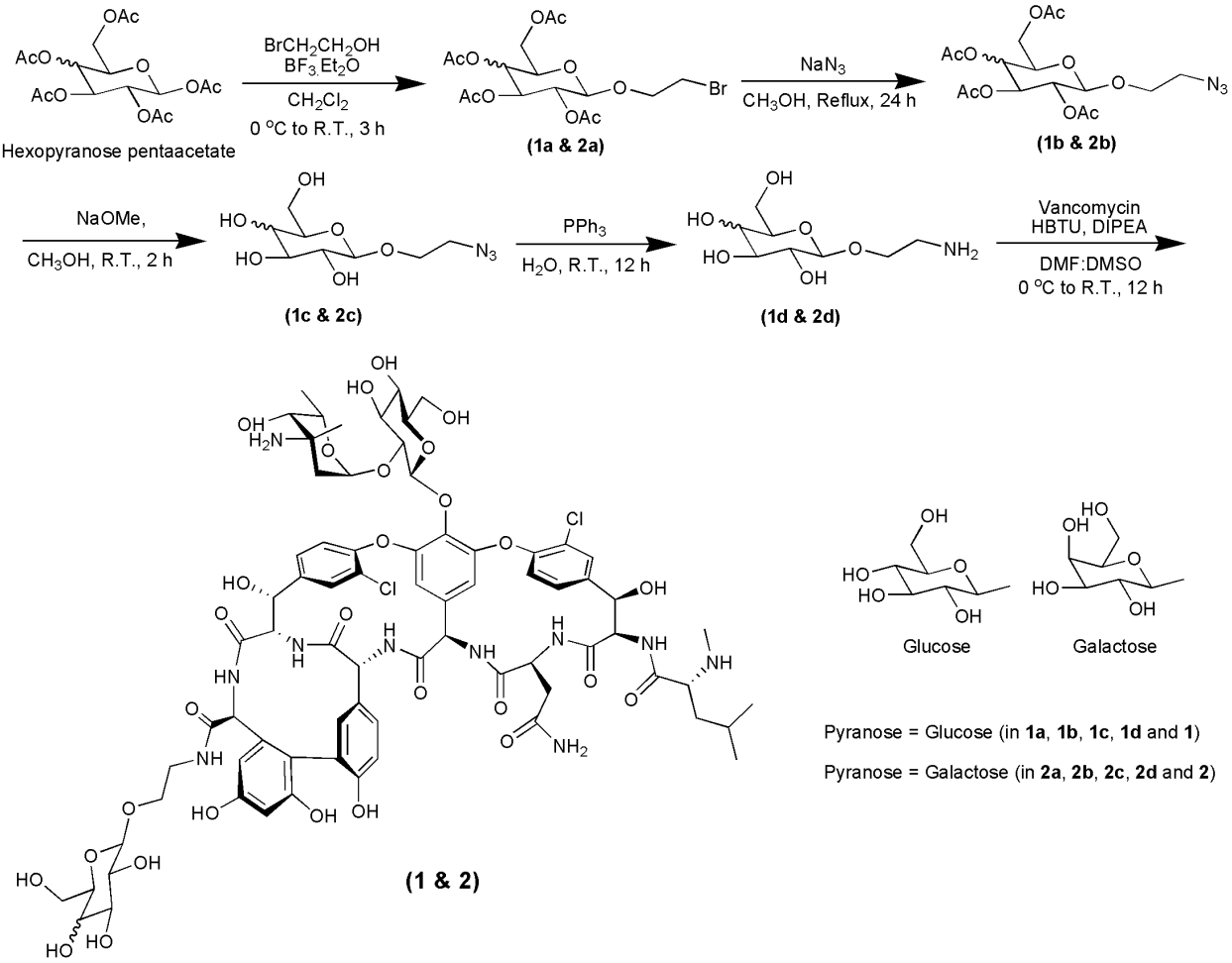
(1) Yarlagadda, V. *et al.* Tackling vancomycin-resistant bacteria with 'lipophilic-vancomycin-carbohydrate conjugates'. *J. Antibiot.* **2015**, *68*, 302. (2) Yarlagadda, V. *et al. In-vivo* efficacy and pharmacological properties of a novel glycopeptide (YV4465) against vancomycin-jntermediate *Staphylococcus aureus* (VISA) *Int. J. Antimicrob. Agents* **2015**, In press.

2.1 Introduction

Vancomycin, the antibiotic of last resort used for hard-to-treat Gram-positive bacterial infections, has also been rendered ineffective by vancomycin-resistant Enterococci (VRE; VanA and VanB phenotypes), vancomycin-resistant *Staphylococcus aureus* (VISA and VRSA). These bacteria developed resistance to the drug either by alteration of cell wall precursor from D-Ala-D-Ala to D-Ala-D-Lac (VRE) or thickening the cell wall (VISA) and sometimes modifying both (VRSA).[35,36,54] The alteration of the cell wall precursor leads to manifold reduction in the binding constant of vancomycin to its target and results in loss of antibacterial activity.[67] Significant strategies have been developed to enhance the lost binding affinity of vancomycin to VRE. Divalent, trivalent, multivalent and polyvalent derivatives of vancomycin were shown to have improved binding affinity towards the ligand D-Ala-D-Lac, which is expressed by resistant bacteria.[97-101] However, these derivatives did not have appreciable activity against more virulent VanA phenotypic VRB. Recently, Boger and coworkers developed vancomycin aglyconamidine, which exhibited dual binding affinity to both D-Ala-D-Ala and D-Ala-D-Lac and the derivative showed potent antibacterial activity against VanA resistant VRE strain.[87,88,94]

According to the observation of Nitanai *et al.*, the carboxylic group of vancomycin and the ligand of the cell wall are bridged by a water molecule in the crystal structure of vancomycin-ligand complex.[114] This suggests that C-terminus modification of the vancomycin to form direct hydrogen bond with the target peptide could stabilize the structure of vancomycin-ligand complex more effectively and leads to higher activity. Here, it was hypothesized that, if the C-terminal of the vancomycin (*N*-hydroxyphenylglycine) is extended with a variety of cyclic (cy) and/or acyclic (acy) sugar moieties (which have the ability to form additional feasible hydrogen bonding with the peptides of peptidoglycan), the overall binding constant of vancomycin derivative with the target peptide of VRE could be increased. These vancomycin-sugar conjugates were developed by simple synthetic methodology as described below. The optimized vancomycin-sugar conjugate appended with a lipophilic alkyl chain displayed increased binding affinity of 2-orders of magnitude and high antibacterial activity against VRE (> 1000-fold more effective than vancomycin). Further, this compound showed better *in-vivo* activity in comparison with vancomycin and linezolid, in the neutropenic mouse thigh infection model against VISA and in kidney infection model against VRE. Furthermore,

pharmacodynamics, pharmacokinetics and acute toxicology studies were performed to validate its safety profile in mice models.



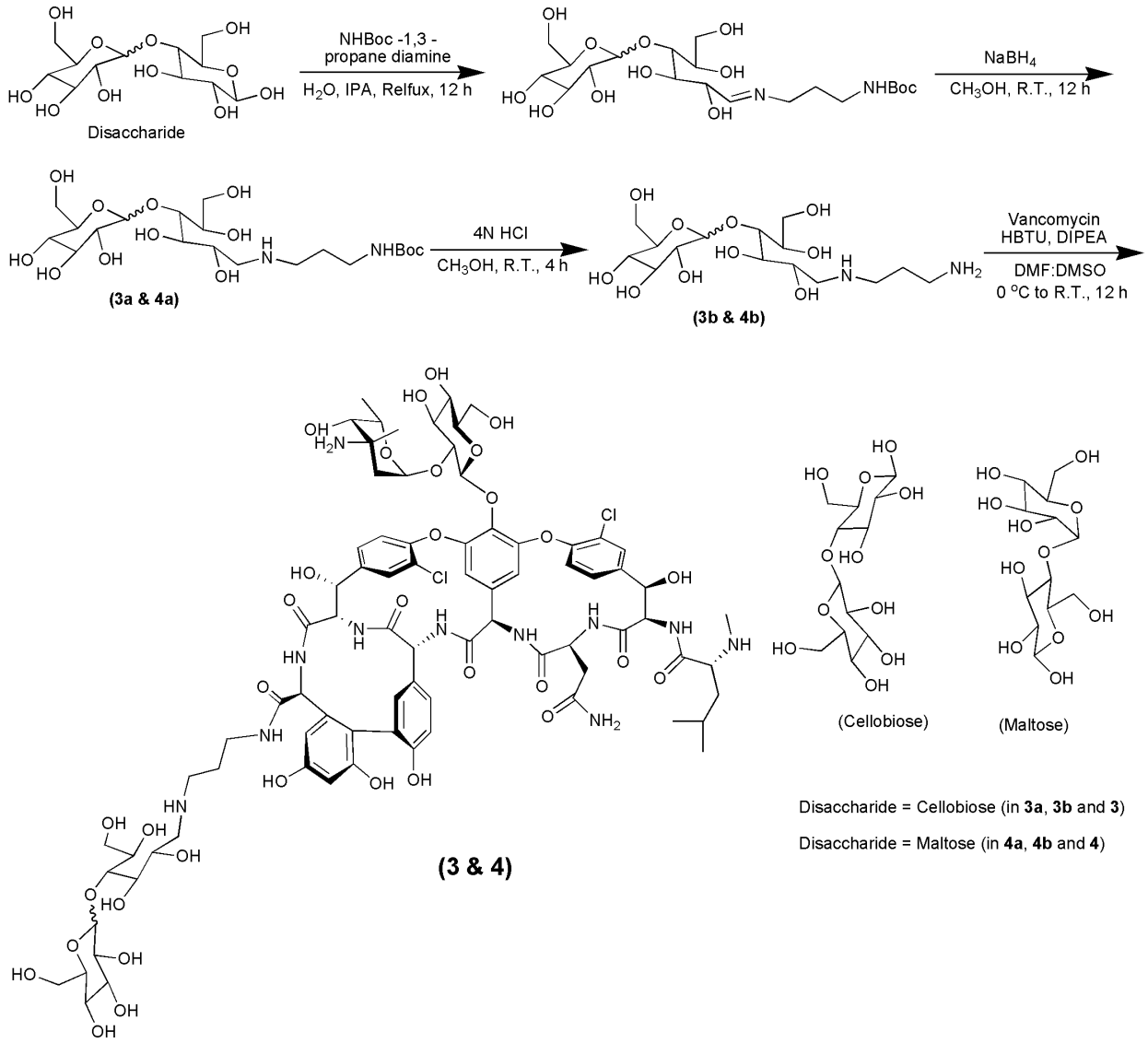
Scheme 2.1: Synthesis of vancomycin-sugar conjugates **1** and **2**.

2.2 Results

2.2.1 Synthesis

In the synthetic strategy used for preparing vancomycin-sugar conjugates, sugar moieties (cyclic or/and acyclic) bearing a linker with a primary amine group (propylene imine or ethylene imine) were coupled to the carboxyl group of vancomycin (compounds **1**-**6**) via amide bond formation

by employing *N,N,N',N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) as coupling reagent. These vancomycin derivatives were purified by reverse-phase HPLC to more than 95 % purity in 70-80 % yield and characterized by ^{1}H-NMR spectroscopy and high resolution mass spectrometry (HR-MS).

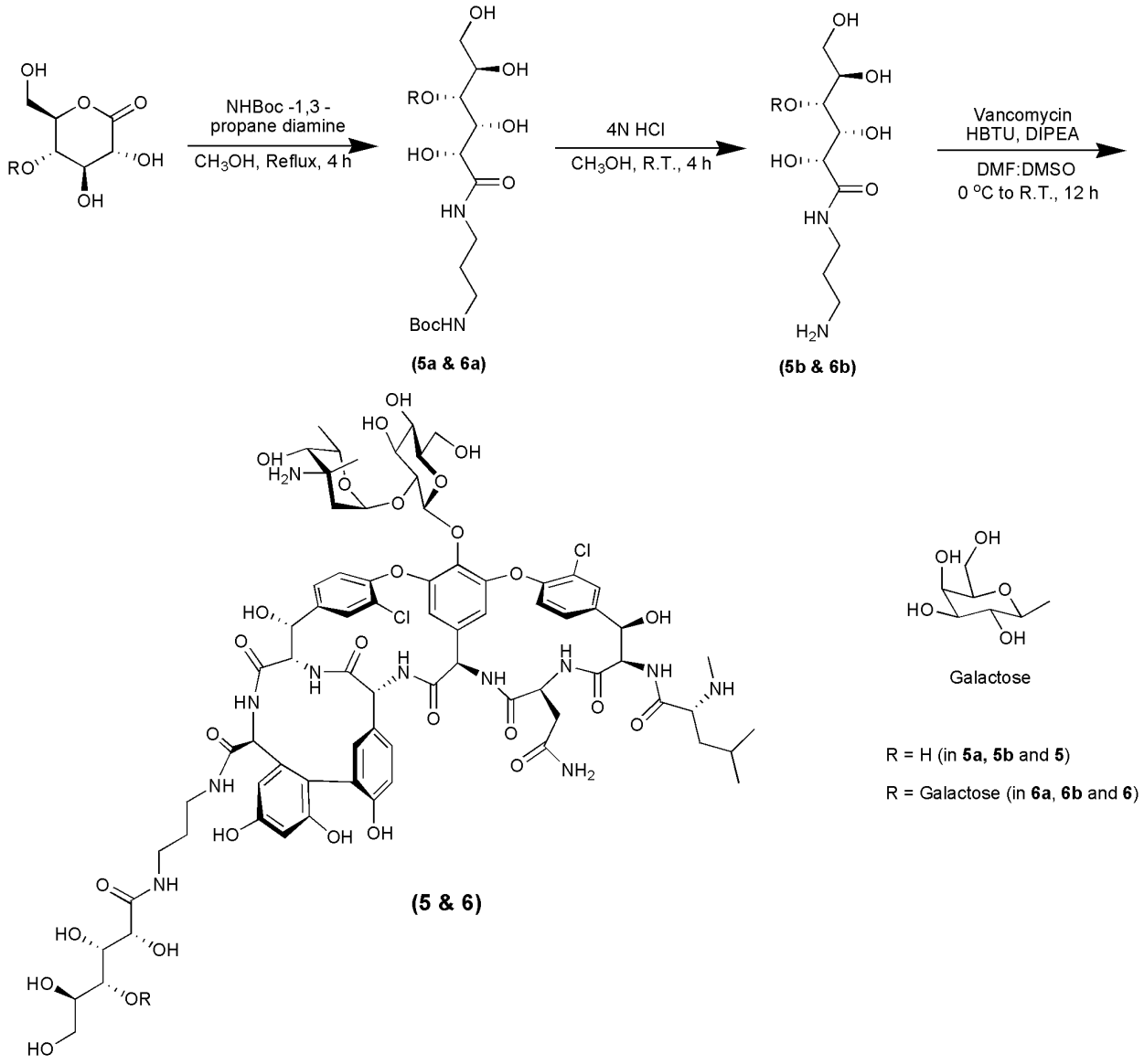


Scheme 2.2: Synthesis of vancomycin-sugar conjugates **3** and **4**.

Firstly, vancomycin-sugar conjugates (compounds **1** and **2**) were synthesized containing cyclic-glucose (cyGlu), and cyclic-galactose (cyGal) where sugar moiety is connected to ethylene imine linker, through ether linkage (Scheme 2.1). To synthesize compounds **1** and **2**, D-hexopyranose pentaacetate was first coupled with 2-bromoethanol in $BF_3.Et_2O$-catalyzed reaction, and then the bromo compound (**1a** or **2a**) was treated with sodium azide in methanol to get azido compound (**1b** or **2b**). After deacetylation, the azido compound (**1c** or **2c**) was subjected to Staudinger reduction to afford 2-aminoethyl D-hexopyranose (**1d** or **2d**). Then 2-aminoethyl D-hexopyranose is coupled to the carboxyl group of vancomycin to give vancomycin-sugar conjugates **1** and **2**.

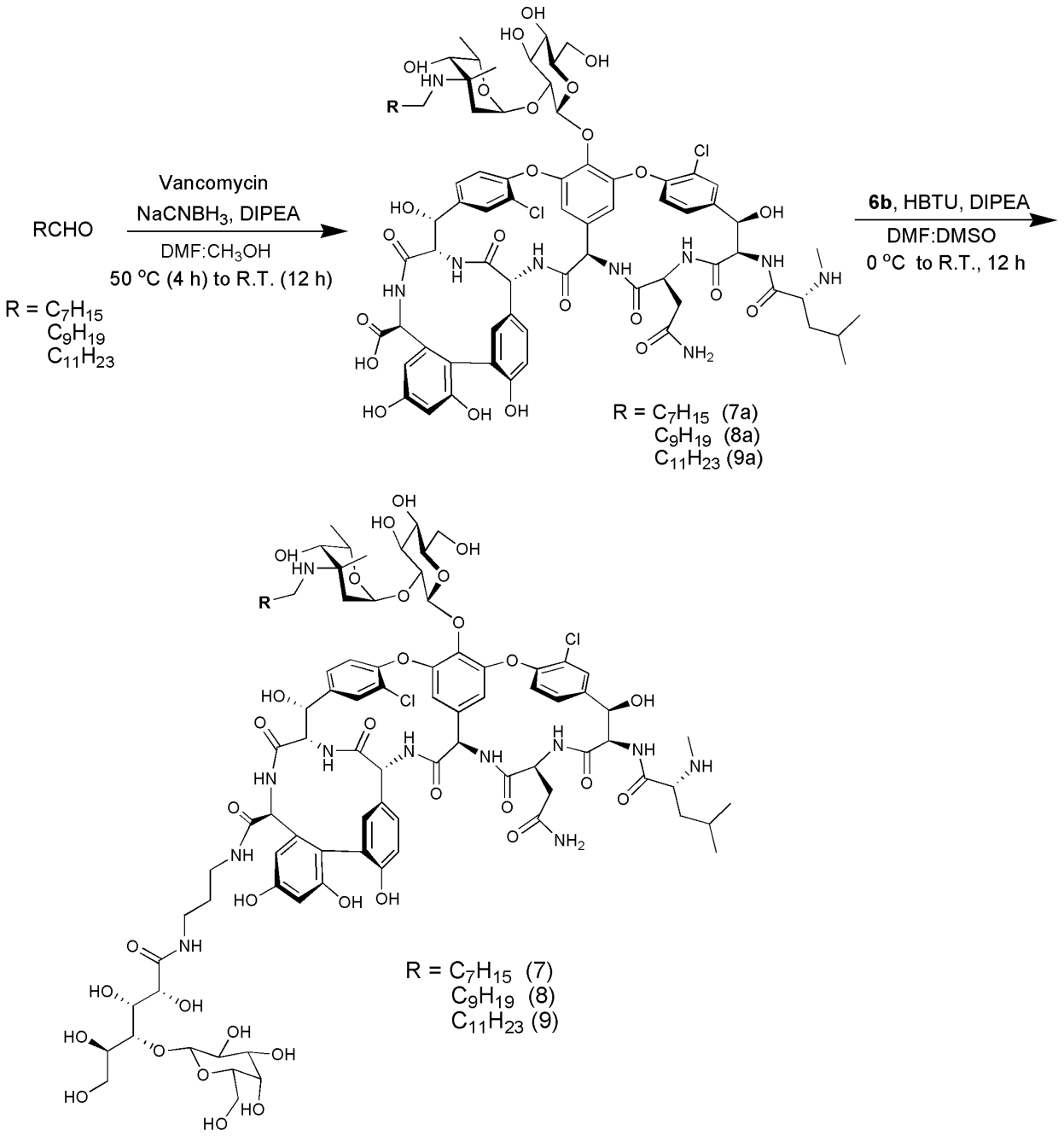
Then, disaccharides such as cellobiose and maltose were incorporated to vancomycin to find whether the number and orientation of hydroxyl groups affects the binding efficiency or not. To do so, compounds **3** and **4** were synthesized which contain both cyclic and acyclic glucose moieties of two different conformations such β1-4 (β-1cyGlu-4acyGlu) and α1-4 (α-1cyGlu-4acyGlu) respectively. Here, Schiff's base formation was performed with *N*-Boc-*1,3*-propanediamine, followed by reduction of imine derivative of disaccharide (cellobiose or maltose) to obtain compounds **3a** and **4a**. Now, compounds **3a** and **4a** were subjected to deprotection in presence of acid to give *N*-Boc free compounds **3b** and **4b**. Then, these compounds were coupled to the carboxylic group of vancomycin to give vancomycin-sugar conjugates **3** and **4** (Scheme 2.2). For both the compounds (**3** and **4**) the acyclic sugar moiety is connected to a propylene imine linker through secondary amine.

Next, compound **5** was synthesized comprising only acyclic glucose moiety (acyGlu) and compound **6** containing a cyclic galactose and an acyclic glucose moieties (β-1cyGal-4acyGlu), wherein the acyclic sugar moiety for both the compounds is connected to a propylene imine linker through amide bond. To synthesize compounds **5** and **6**, δ-Gluconolactone or Lactobionolactone was subjected to nucleophilic ring opening reaction with *N*-Boc-*1,3*-propanediamine which give *N*-Boc-*1,3*-propanediamine derivatized sugar derivatives (**5a** or **6a**) followed by deprotection of *N*-Boc, to give compounds **5b** or **6b** which were finally coupled to vancomycin to give vancomycin-sugar conjugates **5** and **6** (Scheme 2.3).



Scheme 2.3: Synthesis of vancomycin-sugar conjugates **5** and **6**.

Finally, after optimizing the sugar moiety, lipophilicity was incorporated to vancomycin. Here, *N*-alkylation of vancomycin was performed through Schiff's base formation using 1-octanal, 1-decanal and 1-dodecanal followed by reduction to give compounds **7a**, **8a** and **9a**. These *N*-alkylated vancomycin derivatives were coupled to **6b** to give lipophilic-vancomycin-sugar conjugates **7**, **8** and **9** (Scheme 2.4).



Scheme 2.4: Synthesis of lipophilic-vancomycin-sugar conjugates (**7**-**9**).

2.2.2 *In-vitro* antibacterial activities

The antibacterial activities of vancomycin and its derivatives were evaluated by determining the minimum inhibitory concentrations (MICs) against MRSA, vancomycin-resistant strains of

Staphylococci (VISA) and Enterococci (VRE; VanA and VanB phenotypes). The results are summarized in Table 2.1. Against MRSA, all these compounds showed similar or slightly better activity than vancomycin. Compounds **1** (cyGlu) and **2** (cyGal) exhibited much improved antibacterial activity toward VISA (MIC ~ 2 μM) in comparison to vancomycin (MIC of 13 μM). Incorporation of an acyclic moiety and replacing the C-2 oxy spacer with C-3 amine spacer yielded compounds **3** (β-1cyGlu-4acyGlu) and **4** (α-1cyGlu-4acyGlu). Compounds **3** and **4** were ~ 2-fold more active than compounds **1** and **2** against VISA, indicating the importance of disaccharide moieties (additionally the importance of the open form of the sugar) toward antibacterial activity. However, all of these compounds (**1-4**) were found to be inactive against both the strains of VRE.

It was envisioned that incorporation of amide bond might aid in additional hydrogen bonding interactions. Thus compounds **5** and **6** were designed and synthesized. The open monosaccharide analogue, **5**, showed little increase in activity against VISA (MIC of 0.9 μM) in comparison to **3** and **4**. The open disaccharide analogue, **6**, on the other displayed even better activity against VISA with a MIC value of 0.3 μM. In comparison to vancomycin, compounds **5** and **6** showed 15-fold and 40-fold more activity against VISA. When tested against VRE (VanA phenotype, *E. faecium*), however, compounds **5** and **6** exhibited MICs of 54 μM and 36 μM respectively; whereas the MIC for vancomycin was found to be 750 μM (Table 2.1). Compounds **5** and **6**, also showed much improved activity against VanB phenotype of VRE (*E. faecalis*) with the MICs of 60 μM and 30 μM respectively while vancomycin was active at 250 μM. Compound **6** is having the best activity against VRE (VanA phenotype, 36 μM) among compounds **1-6,** turned out to be the highlight of this study.

To sensitize VRE toward such compounds further, lipophilic-vancomycin-sugar conjugates were developed wherein lipophilic alkyl chains (octyl, decyl and dodecyl) were incorporated into compound **6**. The antibacterial activities of lipophilic-vancomycin-sugar conjugates were also evaluated against vancomycin-resistant strains (VRE and VISA). All the compounds showed similar or slightly better activity against VISA in comparison to compound **6** and the best activity was achieved for lipovamcomycin-sugar conjugate containing decyl and dodecyl chains (compounds **8** and **9**). Intermediate compounds (**7a**-**9a**) showed similar or slightly better activity than vancomycin against MRSA. Against VRE (VanA phenotype),

compounds **7a**-**9a** had the MIC values ranging from 6.9 µM to 25 µM, which is 30 to 108-fold more active than vancomycin. Whereas, lipophilic-vancomycin-sugar conjugates (**7**-**9**) exhibited MIC value of 0.7 µM-2 µM, which is 350-fold to > 1000-fold higher than vancomycin (Table 2.1). The MIC_{90} values of telavancin and dalbavancin against VRE (VanA phenotype) were reported to be 4 µM and 18 µM respectively.[115,116]

Table 2.1. *In-vitro* antibacterial activity and binding affinities of the compounds.

	Minimum Inhibitory Concentration (µM)				**Association Constant (K_a, M^{-1})**	
Compound	MRSA	VISA	VREm	VREs	Susceptible	Resistant
Vancomycin	0.6	13	750	250	1.0×10^5	5.0×10^2
1	1.4	2.0	>100	>100	1.4×10^5	9.0×10^2
2	1.2	2.0	>100	>100	1.2×10^5	8.5×10^2
3	1.0	1.0	>100	>100	0.5×10^5	12×10^2
4	1.0	1.0	>100	>100	0.8×10^5	11×10^2
5	0.6	0.9	54	60	2.2×10^5	6.3×10^4
6	0.4	0.3	36	30	2.0×10^5	8.8×10^4
7	0.4	0.3	2.0	6.0	N.D	N.D
8	0.2	0.2	0.7	1.0	6.0×10^5	6.0×10^4
9	0.3	0.2	0.8	1.0	N.D	N.D
7a	0.5	0.4	25	13.0	N.D	N.D
8a	0.3	0.3	14	6.0	N.D	N.D
9a	0.3	0.3	6.9	3.7	N.D	N.D

Abbreviations: MRSA (ATCC 33591), Methicillin-resistant *Staphylococcus aureus*; VISA, Vancomycin-intermediate-resistant *Staphylococcus aureus* was generated from MRSA after treating with vancomycin for 52 passages; VREm, Vancomycin-resistant *Enterococcus faecium* (VanA phenotype, ATCC 51559); VREs Vancomycin-resistant *Enterococcus faecalis* (VanB phenotype, ATCC 51575); Susceptible, *N*,*N*′-diacetyl-Lys-D-Ala-D-Ala (Model ligand for susceptible bacteria); Resistant, *N*,*N*′-diacetyl-Lys-D-Ala-D-Lac (Model ligand for VRE); N.D, Not determined.

2.2.3 Binding affinities

In order, to prove the proposed hypothesis, the binding constants of vancomycin, vancomycin-sugar conjugates **1**-**6** and lipophilic-vancomycin-sugar conjugate **8** were evaluated using UV-difference spectroscopy against both sensitive and resistant model ligands; *N*,*N*′-diacetyl-Lys-D-Ala-D-Ala and *N*,*N*′-diacetyl-Lys-D-Ala-D-Lac respectively and the results are displayed in Table 2.1. The binding affinities of compounds **5** and **6** were found to be 2-fold higher than vancomycin against *N*,*N*′-diacetyl-Lys-D-Ala-D-Ala, whereas compounds **1**-**4** exhibited binding affinities similar to vancomycin. When evaluated against *N*,*N*′-diacetyl-Lys-D-Ala-D-Lac, compounds **1**-**4** displayed low binding affinities similar to vancomycin. The binding affinities of derivatives **5** and **6** against *N*,*N*′-diacetyl-Lys-D-Ala-D-Lac, on the other hand, were 125-fold (6.3×10^4 M^{-1}) and 170-fold (8.8×10^4 M^{-1}) higher than vancomycin (5×10^2 M^{-1}) respectively. This result is a clear proof of initial hypothesis. The binding affinities of lipophilic-vancomycin-sugar conjugate **8** for both sensitive and resistant model ligands were also evaluated. Since the presence of alkyl chain has no effect on the interaction with the peptides, the binding affinities of compound **8** were found to be similar to compound **6** (Table 2.1).

2.2.4 Intracellular accumulation of cell wall (peptidoglycan) precursor

In order to investigate the effect of enhanced binding affinity on peptidoglycan biosynthesis, the accumulation of UDP-linked peptidoglycan precursor, UDP-*N*-acetyl-muramyl-pentadepsipeptide (UDPMurNAc-pp) was determined after treating bacteria (VRE) with new vancomycin derivatives, compounds **6**, **8** and vancomycin at 5 μM. In case of compounds **6** and **8**, a more intense peak was observed at 260 nm compared to vancomycin, which corresponds to accumulation of UDPMurNAc-pp and confirmed by high resolution mass spectrometry (m/z = 1149.94 (cal), 1148.90 (obs) for M^+) (Fig. 2.1A & 2.1B). Further, compound **8** causes more accumulation of cell wall precursor compared to compound **6**. The results suggest compound **8** showed greater cell wall inhibition than vancomycin.

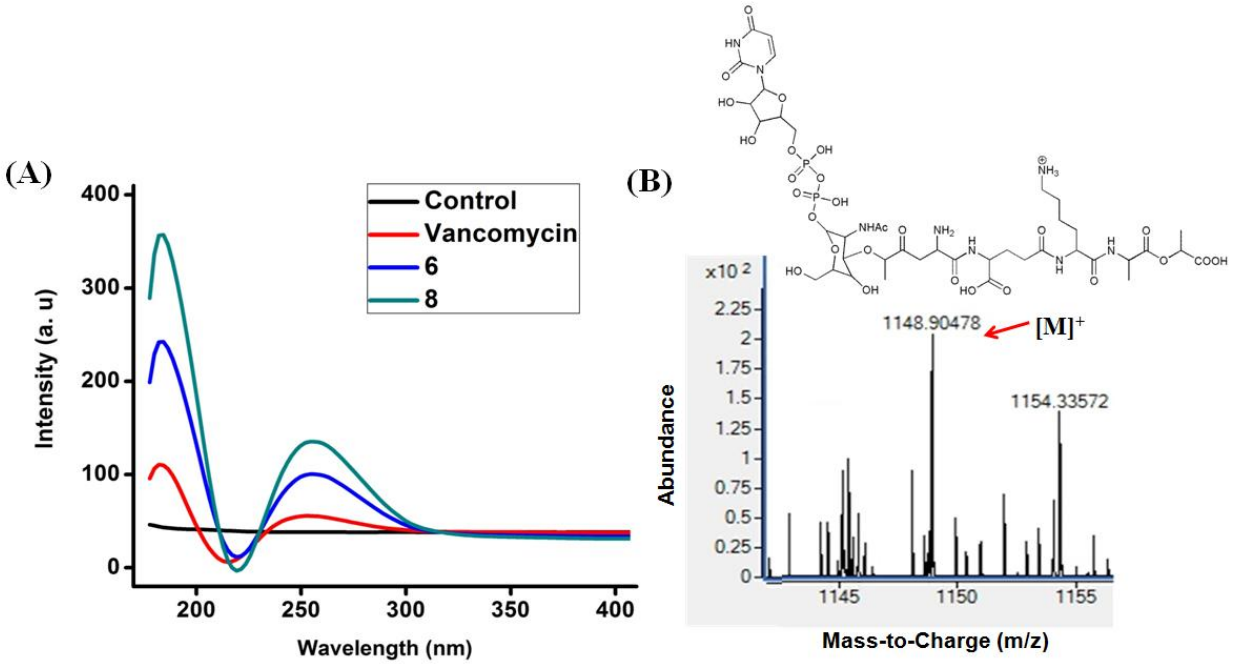


Figure 2.1: Intracellular accumulation of the cell wall precursor UDP-MurNAc-pentadepsipeptide after treatment of VRE with vancomycin, compounds **6** and **8** at 5 µM. Untreated cells were used as control. (A) Identification of intracellular UDP-MurNAc-pentadepsipeptide by monitoring the absorbance at 260 nm wavelength. (B) UDP-MurNAc-pentadepsipeptide was identified by mass spectrometry as indicated by the peak at m/z 1148.90.

2.2.5 Bactericidal activity

Further, *in-vitro* time-kill assay was performed with the most active vancomycin analogue (compound **8**) and vancomycin, against VISA (starting bacterial concentration of ~ 8 $\log_{10}$ CFU/mL), at two different concentrations (2 µM and 4 µM). The results portrayed rapid bactericidal activity of compound **8**, which increased with increasing concentration. Around 3-4 $\log_{10}$ CFU/mL reduction was observed within 3 h of incubation at a concentration of 4 µM and persistent bactericidal activity was observed till 24 h (Fig. 2.2A). On the other hand, vancomycin showed only bacteriostatic effect at 4 µM.

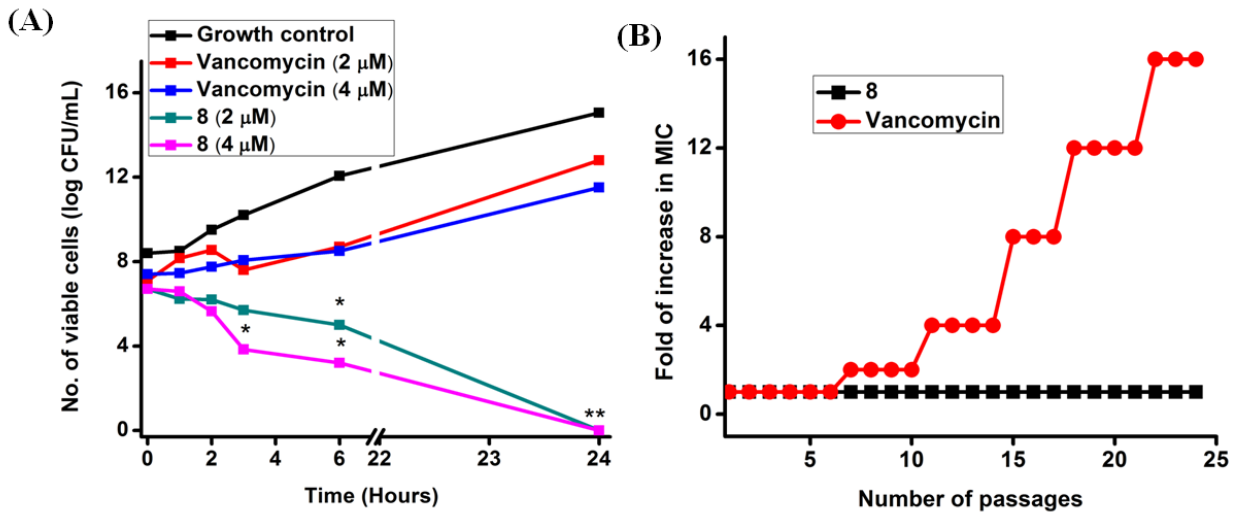


Figure 2.2: (A) Bactericidal properties of vancomycin and compound **8** against VISA. Single stars correspond to reduction of 3 $\log_{10}$ CFU/mL and double stars correspond to < 50 CFU/mL (detection limit). (B) Bacterial resistance studies of vancomycin and compound **8** against MRSA.

2.2.6 Propensity to induce bacterial resistance

The continuous rise of drug resistance in bacteria guided to evaluate the potential emergence of bacterial resistance against this class of compounds. The propensity of bacteria to generate resistance can be evaluated through serial exposure of organisms to antimicrobial agents. To establish whether the lipophilic-vancomycin-sugar conjugate, indeed prevented the development of bacterial resistance, MRSA was exposed to vancomycin and the best new vancomycin derivative, compound **8** for serial passages and monitored the changes in MIC values over a period of 25 days. Even after 25 serial passages, the MIC of compound **8** remained the same. However, in case of vancomycin, the MIC value started increasing after 7 passages and the value increased to 16-fold after 25 passages (Fig. 2.2B). Thus bacterial efforts towards development of resistance against such compounds are futile and this emphasizes the longevity of such compounds in clinics.

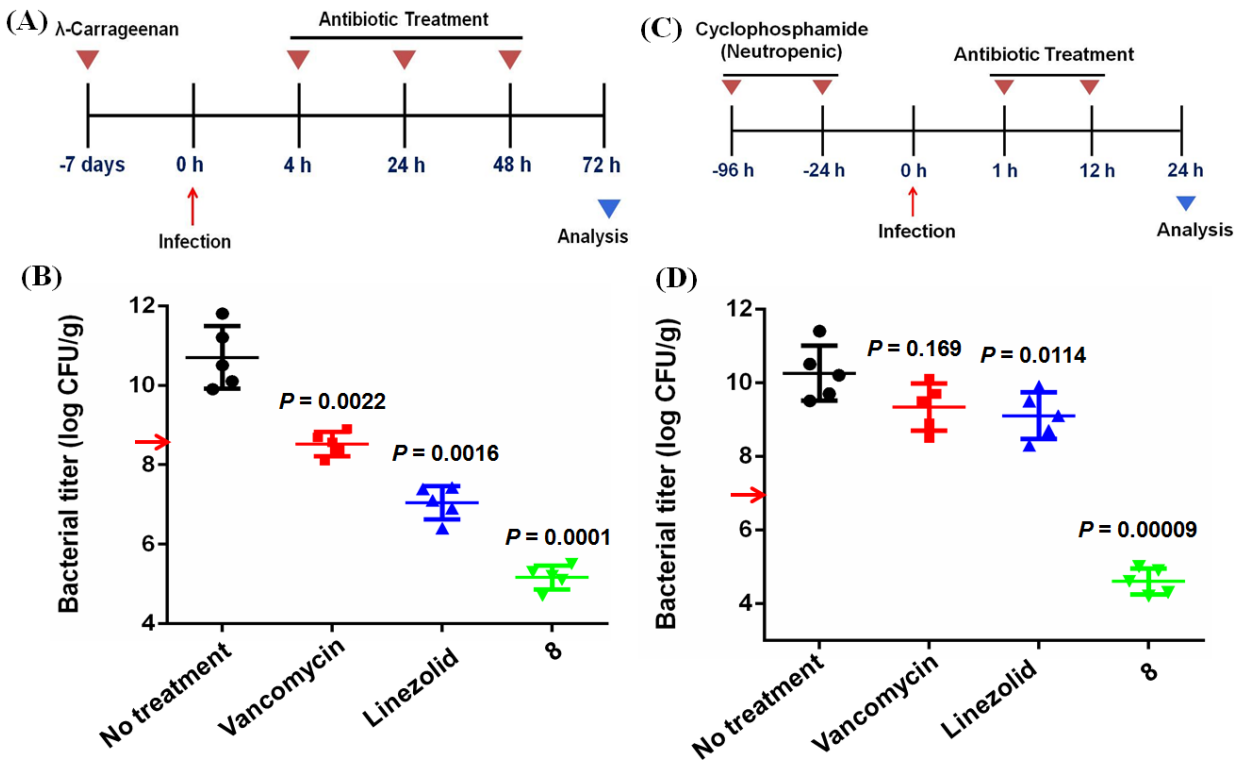


Figure 2.3: *In-vivo* antibacterial activity studies: *In-vivo* activity of vancomycin, linezolid and compound **8** in renal infection model against VRE (n = 5) at 12 mg/kg (A) Experimental design and (B) Experimental data; *In-vivo* activity of compound **8** in comparison with vancomycin and linezolid against VISA (n = 5) at 12 mg/kg (compound **8**, single dose at 1 h post infection) and 24 mg/kg (vancomycin and linezolid, double dose of 12 mg/kg each, at 1 h and 12 h post infection) (C) Experimental design and (D) Experimental data. Differences are considered statistically significant from untreated group with a value of $P < 0.05$. Red arrow in (B) and (D) indicates bacterial pre-treatment titer (~ 8 $\log_{10}$ CFU/g and ~ 7 $\log_{10}$ CFU/g, respectively).

2.2.7 *In-vivo* antibacterial activity

To demonstrate the potential of these compounds for *in-vivo* applications, the most active vancomycin derivative, compound **8** was chosen for further studies. *In-vivo* activity of compound **8** was evaluated in a renal infection model against VRE. Initially, mice were injected intravenously with 0.2 mL of 0.2 % λ-carrageenan to increase their susceptibility to bacterial renal infection. After 7 days, mice were infected with VRE (~ 10^8 CFU/mouse). After 4 h of

infection the mice were treated with three doses (every 24 h interval) of vancomycin (12 mg/kg), linezolid (12 mg/kg), compound **8** (12 mg/kg) and saline (Fig. 2.3A). After 72 h of the initial treatment, antibacterial activity was determined by finding the bacterial titer in the infected kidneys. In comparison to vancomycin, compound **8** and linezolid reduced bacterial titer from the infected kidneys more effectively. Linezolid caused ~ 4 $\log_{10}$ CFU reduction from vehicle treated control (saline) whereas compound **8** produced ~ 5.5 $\log_{10}$ CFU reduction (Fig. 2.3B).

In another study, the *in-vivo* activity of compound **8** was evaluated in a neutropenic mouse thigh infection model against VISA. In this study, mice were infected with VISA (~ 10^7 CFU/mouse) in the thigh. After 1 h of infection the mice were treated with a single dose of compound **8** (12 mg/kg) and a double dose (q12 h) of vancomycin (12 mg/kg), linezolid (12 mg/kg) and, saline being used as control (Fig. 2.3C). After 24h of the initial treatment, antibacterial activity was determined by finding the bacterial titer in the infected thighs. Unlike vancomycin and linezolid, compound **8** showed significantly high activity wherein it produced ~ 6 $\log_{10}$ CFU reduction from saline treated control (Fig. 2.3D).

Table 2.2. Point dose estimates required to achieve different pharmacodynamic end points for compound **8** against VISA thigh infection model.

Bacteria	Pharmacodynamic end points (mg/kg)				
	ED_{50}	ED_{stasis}	$ED_{1\text{-log kill}}$	$ED_{2\text{-log kill}}$	$ED_{3\text{-log kill}}$
VISA (Pretreatment 7.3 $\log_{10}$ CFU/g)	2.7	2.2	4.0	7.3	17.0

Abbreviations: ED_{50}, 50 % effective dose; ED_{stasis}, $\log_{10}$ stasis dose; $ED_{1\text{-log kill}}$, dose required to cause a decrease in titer of 1 $\log_{10}$ CFU/g from the pre-treatment titer (0 h); $ED_{2\text{-log kill}}$, dose required to cause a decrease in titer of 2 $\log_{10}$ CFU/g; $ED_{3\text{-log kill}}$, dose required to cause a decrease in titer of 3 $\log_{10}$ CFU/g.

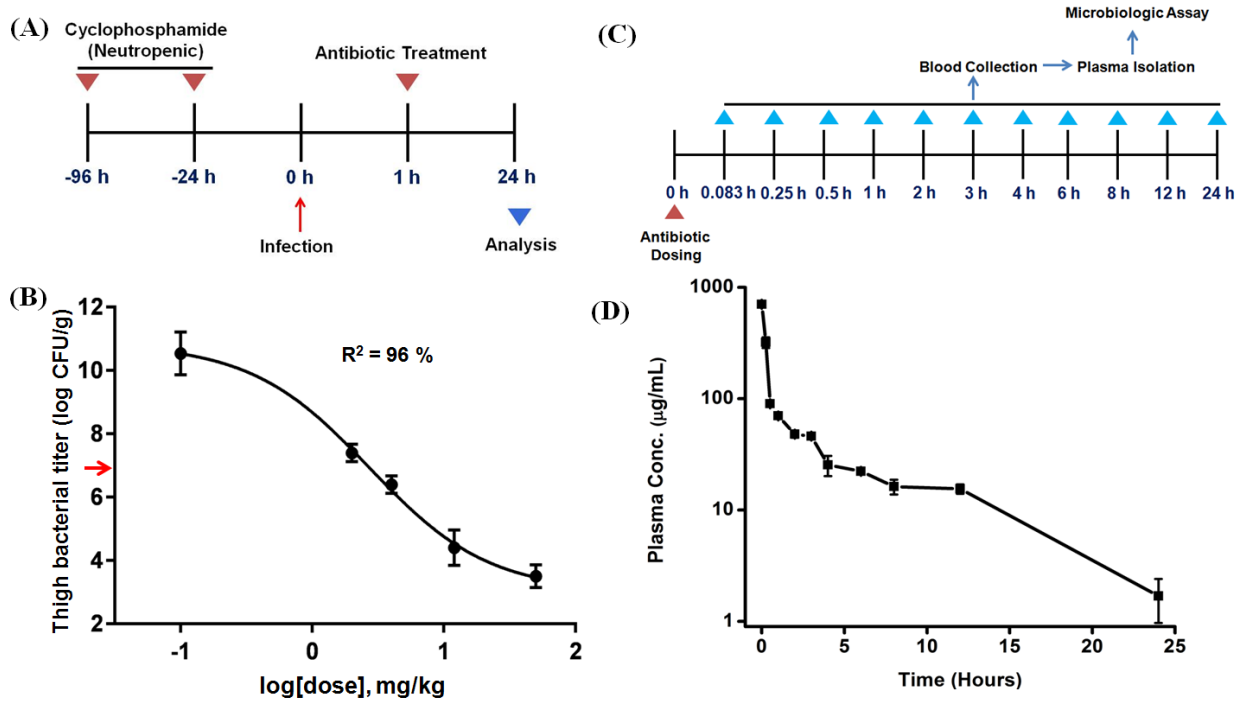


Figure 2.4: Pharmacological studies: Dose-response relationship of compound **8** in thigh infection model against VISA (A) Experimental design and (B) Experimental data. Red arrow in (B) indicates bacterial pretreatment titer (7.3 $\log_{10}$ CFU/g); Single-dose pharmacokinetic study of compound **8** at 12 mg/kg (C) Experimental design and (D) Experimental data.

2.2.8 Dose-response relationship against VISA

The good activity of compound **8** against VISA and VRE infections driven further to examine the effect of dose response on its efficacy. In this study, VISA was chosen as a model organism wherein after 1 h of infection a single dose of compound **8** at different regimens (2, 4, 12and 50 mg/kg) was administered intravenously (Fig. 2.4A). The pretreatment bacterial titer in the thigh was 7.3 ± 0.6 $\log_{10}$ CFU/g. In vehicle treated controls, thigh titer increased to 10.5 ± 0.7 $\log_{10}$ CFU/g within 24 h. Comparable dose dependent reductions were observed in the bacterial titer at each of six dosing regimens. The single dose that resulted in 50 % maximal bacterial killing (ED_{50}) was 2.7 mg/kg (Table 2.2). The dose that resulted in a 24-h colony count similar to the pretreatment count was 2.2 mg/kg (ED_{stasis}). The value of 1-$\log_{10}$ kill dose ($ED_{1\text{-log kill}}$) for

compound **8** was 4.0 mg/kg. Further, compound **8** showed 3-$\log_{10}$ kill ($ED_{3\text{-log kill}}$) at 17 mg/kg (Fig. 2.4B).

2.2.9 Single-dose pharmacokinetics

The pharmacokinetics of i.v. administered compound **8** in mice is shown in Fig. 2.4C & 2.4D. The compound demonstrates increased exposure as measured by area under concentration curve in mice. Time-concentration profiles of plasma for compound **8** are presented in Fig. 2.4D. Peak concentration in plasma was found to be 703 µg/mL. Non-compartmental analysis was used for the determination of pharmacokinetic parameters of compound **8** (Table 2.3). The AUC value in plasma, calculated from 0.083 h to 24 h was 562 µg/mL/h. The plasma half-life ($t_{1/2}$) of compound **8** was found to be 2.76 h with the clearance rate of 0.02 L/h/kg.

Table 2.3. Single-dose pharmacokinetic parameters of compound **8** at 12 mg/kg.

	Pharmacokinetics parameters				
Drug (12 mg/kg)	C_{max} (µg/mL)	C_{min} (µg/mL)	$AUC_{0\text{-}24\,h}$ (µg/mL/h)	$t_{1/2}$ (h)	Clearance (L/h/Kg)
Compound **8**	703	2	562	2.76	0.02

C_{max}, peak plasma concentration; C_{min}, trough plasma concentration; $AUC_{0\text{-}24\ h}$, area under the concentration-time curve from 0-24 h; $t_{1/2}$, half-life.

2.2.10. *In-vivo* toxicology studies

Next, the *in-vivo* systemic toxicity of compound **8** was assessed after single-dose intravenous (i.v.) administration at 100 mg/kg dosing regimen to mice (n = 5). Then, the animals were observed for mortality for a period of 14 days and all the mice were survived at 14 days indicating the high tolerability of compound **8** in animals. Then, the acute toxicity of compound **8** was evaluated to major organs in the body of the mice by evaluating the clinical biochemistry

parameters in the blood of the mice at a concentration of pharmacodynamic point, > $ED_{2\text{-log kill}}$ (dose of 12 mg/kg) after intravenous administration. The levels of the functional parameters of the liver (ALT, Alanine transaminase) and kidney (Urea nitrogen & Creatinine) and the concentrations of electrolytes in the blood (Sodium, Potassium and Chloride) were unchanged after 48 h and 14 days (Table 2.4). These studies indicate that compound **8** did not cause any significant acute damage to liver and kidney functions, nor did it interfere with the balance of electrolytes in the blood.

Table 2.4. *In-vivo* acute toxicology of compound **8** at 12 mg/kg

	Liver (UL^{-1})	**Kidney ($mg\ dL^{-1}$)**		**Electrolytes in the blood ($mmol\ L^{-1}$)**		
Treatment	ALT	Urea Nitrogen	Creatinine	Potassium ion	Sodium ion	Chloride ion
Saline	60 ± 9	22 ± 3	0.3 ± 0.2	9 ± 1.4	143 ± 0.8	108 ± 2
48 h post-treatment	55 ± 5 $P = 0.004$	19 ± 3 $P = 0.06$	0.2 ± 0.1 $P = 0.056$	6 ± 0.8 $P = 0.052$	143 ± 2 $P = 0.83$	113 ± 1.5 $P = 0.005$
14 days post treatment	53 ± 3 $P = 0.02$	24 ± 5 $P = 0.23$	0.2 ± 0.1 $P = 0.054$	6 ± 0.8 $P = 0.053$	143 ± 0.7 $P = 0.095$	111 ± 2 $P = 0.237$
Laboratory range*	63-307	17-35	0.2-0.8	6.3-10	140-150	104-120

The data are expressed as mean ± standard deviation, based on values obtained from 10 mice (n = 10). Statistical analysis was performed using Student's *t*-test. Differences are considered statistically significant with a value of $P < 0.05$. ALT, alanine transaminase. *Source: Charles River laboratories.

2.3 Discussion

In an attempt to develop novel therapeutics to conquer bacterial resistance, much attention has been focused on developing semi-synthetic glycopeptide antibiotics. Successful designs in the field have focused on improving binding affinity of vancomycin analogues to VRB. In this report, a rational strategy was developed using simple chemical approach to enhance binding affinity to the target peptides.

In order to enhance the binding affinity of the compounds with the modified peptidoglycan of resistant bacteria, various sugar moieties were incorporated at the C-terminus of vancomycin backbone. Initially, cyclic glucose and cyclic galactose moieties were incorporated via the anomeric -OH group of the sugars. Significant improvement in activity against VISA was observed independent of the orientation of OH moieties in the sugars. Upon replacement of monosaccharide by a disaccharide, a little improvement in activity was observed. Although it cannot be conclusively said, what brings about this improvement, it is surmised that the additional OH groups or the open structure of the first sugar in the disaccharides bring about some sort of a favorable interaction. But since activity against VRE was not achieved in these compounds, incorporation of amide bonds was envisioned. Significant improvement in antibacterial activity was observed in compounds **5** and **6** (containing the newly incorporated amide bond). The acyclic compound **5** differed from compound **1** in the presence of an amide bond over ether and the presence of a C-3 spacer over C-2 spacer. This small difference was significant in restoring the activity against VRE. This significant improvement in activity might be attributed to the favorable H-bonding interactions provided by the amide bond. On comparing the activity of compounds **3** and **4** with that of compound **5**, it becomes clear that the presence of amide bonds is more important than the presence of additional OH groups. However, the presence of extra OH groups is beneficial after the amide bond has been incorporated, as was concluded upon comparing activity of compound **6** with that of compound **5**. The importance of the amide bond towards increase in activity was well demonstrated in the results portrayed by experiment determining the association constants. Compounds **5** and **6** had binding constants > 100-fold and ~ 150-fold higher than that of compounds **1**-**4** and vancomycin respectively. Similar observations were reported recently by Slusarz *et al.* in a theoretical simulation, wherein vancomycin derivatives modified with non-cyclic sugar moieties not only had more conformational freedom than cyclic sugar vancomycin derivatives, but also moved closer to the peptidoglycan layer to have some favorable interactions.[117]

Additionally, in order to increase the activity against VRE further, a lipophilic aliphatic moiety was incorporated to the optimized vancomycin-sugar conjugate. This appendage brings about an additional property of enhanced bacterial membrane interaction to the molecules. It has been shown in the literature too that inclusion of lipophilicity to glycopeptides leads to

enhancement in antibacterial activity against VRE.[74,82,118-120] *N*-Alkylation of compound **6** through Schiff's base formation using long chain aldehydes (varying from octyl to dodecyl) followed by reduction yielded lipophilic-vancomycin-sugar conjugates. The antibacterial activities of these lipophilic-vancomycin-sugar conjugates were compared to the activities of second generation of glycopeptides such as telavancin and dalbavancin. Compound **8** was 7-fold, 25-fold and > 1000-fold more active against VRE compared to telavancin, dalbavancin and vancomycin respectively. The binding affinity of compound **8** with model ligands simulating the peptides from both sensitive and resistant strains was found to be similar to compound **6**. This was expected as both the compounds bear the same sugar moiety, which aids in binding with the peptides. Further, treatment of VRE with compound **8** resulted in more accumulation of the soluble cell wall precursor undecaprenyl-*N*-acetyl-muramyl-pentadepsipeptide compared to vancomycin, suggesting the enhanced inhibition of peptidoglycan biosynthesis. Additionally, the new installed features of compound **8** had a significant impact on stalling the development of bacterial resistance to the drug.

Infections caused by VRE and VISA have been increasing in frequency, representing an emerging threat to public health.[35,121] Unlike vancomycin, compound **8** and linezolid showed very good activity against VRE in renal infection model. In particular, compound **8** was significantly more active than linezolid. Further, the *in-vivo* activity of compound **8** was determined against VISA in neutropenic mouse thigh infection model. Compound **8** was significantly more potent than comparator drugs vancomycin and linezolid against VISA. These substantial differences in relative *in-vivo* potencies cannot be explained on the basis of MICs alone. For example, the MICs of compound **8** and linezolid varied by a factor of four whereas their *in-vivo* potencies differ by manifold (MIC of linezolid = 1.5 μg/mL). The superior activity of compound **8** in the present study could be explained, in part, by its rapid bactericidal activity against VISA, which might be attributed to its improved binding affinity towards bacterial targets over vancomycin.

Single-dose pharmacokinetic study demonstrated that compound **8** has improved pharmacological properties, which also supported its superior *in-vivo* antibacterial activity. At a dosage of 12 mg/kg, plasma levels of compound **8** remained at 2 μg/mL after 24 h, which also implies its potential *in-vivo* activity due to prolonged drug exposure.

Acute toxicology of compound **8** was performed by determining the biochemical parameters related to liver, kidney and blood. Analysis of biochemical parameters revealed that compound **8** did not induce any significant changes in functional parameters of liver and kidney and did not interfere with the balance of electrolytes in the blood of mice at 48 h post treatment compared to vehicle control and laboratory parameters. These parameters remained almost unchanged even at 14 days post-treatment. All the parameters tested related to the function of major organs like liver, kidney and electrolytes in the blood of mice were found to be well within the acceptable laboratory range. This study showed that compound **8** has low or no *in-vivo* systemic and acute toxicity in mice models and have a good safety profile required for therapeutic applications.

2.4 Conclusions

Various lipophilic-vancomycin-sugar conjugates have been synthesized by simple synthetic strategy to enhance binding affinity to the target peptides of vancomycin-resistant bacteria. This improved binding affinity significantly resulted in the high antibacterial activity of the compounds against VISA and VRE thus successfully overcoming vancomycin resistance. Optimized compound showed good *in-vivo* activity against VISA and VRE and demonstrated improved pharmacological properties with no observed toxicity. The high activity of the compound might be attributed to its intrinsic bactericidal activity unlike vancomycin. Further, no detectable resistance was observed after several serial passages of bacterial exposure to the new compound. These findings suggest that this strategy may have clinical utility for the treatment of vancomycin-resistant bacterial infections.

2.5 Experimental procedure

2.5.1 Materials and Methods

All reagents were purchased from Sigma-Aldrich and SD Fine and used without further purification. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F_{254} (250 μm thickness). Visualization was accomplished

using UV light and Iodine. Column chromatography was performed on silica gel (60-120 Å pore size). All final compounds were purified by reverse phase HPLC using 0.1 % trifluoroacetic acid (TFA) in water/acetonitrile (0-100 %) as mobile phase to more than 95 % purity. HPLC analysis was performed on a Shimadzu-LC 8 Å Liquid Chromatography instrument (C_{18} column, 10 mm diameter, 250 mm length) with UV detector monitoring at 270 nm. Nuclear magnetic resonance spectra were recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. High resolution mass spectra (HR-MS) were obtained using 6538-UHD Accurate Mass Q-TOF LC-MS instrument. UV-absorption measurements were obtained using Thermo-Fisher Scientific UV-10 spectrometer for determination of binding constants. Eppendorf 5810R centrifuge was used. TECAN (Infinite series, M200 pro) Plate Reader was used to measure absorbance. Bacterial strains, MRSA ATCC 33591, Enterococcal strains were obtained from ATCC (Rockville, MD). Tryptic-soy agar media was used for Staphylococci and sheep blood agar plates were used for Enterococci. VISA, Vancomycin-intermediate-resistant *S. aureus* was generated from MRSA (ATCC 33591) after treating with vancomycin for 52 passages.

Animals: Six-week old specific-pathogen free CD-1 female mice weighing 19 to 24 g were used for all studies. Pharmacokinetics and toxicology studies were performed at Jawaharlal Nehru Centre for Advanced Scientific Research (JNCASR) in accordance with institutional ethical guidelines. Infection studies were performed at National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), where six-week old CD-1 female mice were purchased from Centre for Cellular and Molecular Biology, Hyderabad weighing 19 to 24 g. The mice were housed in individually ventilated cages (IVC) maintained with controlled environment as per the standards. The animal experiments were approved by the Institutional Animal Ethics Committee (IAEC) of National Institute of Veterinary Epidemiology and Disease Informatics (Formerly PD_ADMAS), Bengaluru (881/GO/ac/05/CPCSEA) and carried out as per the guidelines of Committee for the purpose of Supervision and Experiments on Animals (CPCSEA), Ministry of Environment and Forests, New Delhi.

2.5.2 Synthesis and Characterization

Synthesis of 2-bromoethyl 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose[122] **(1a):** About 1.0 g of D-glucopyranose pentaacetate was dissolved in 10 mL of dry DCM at 0 ºC. Then 1.3 mL (1.2 equivalents) of $BF_3.Et_2O$ was added to the reaction mixture drop wise followed by 0.22 mL (1.2

equivalents) of 2-bromoethanol. Then reaction mixture was allowed to stir at room temperature for 3 h. After completion of the reaction anhydrous potassium carbonate (0.53 g, 1.5 equivalents) was added and stirring was continued for further 30 min. Then the crude solution was extracted with chloroform and purified through silica gel column chromatography (Ethyl acetate/Hexane 30:70) to get pure **1a** with 79 % yield. ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 4.58-4.56 (d, 1H), 4.28-4.08 (m, 6H), 3.85-3.48 (m, 2H), 3.47-3.44 (m, 2H), 2.03 (s, 12H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 169.5, 100.2, 70.0, 69.3, 68.5, 68.0, 62.0, 61.6, 29.9, 20.9; HR-MS: *m/z* 477.0351 (observed), 477.0372 (calculated for $[M+Na]^+$).

Synthesis of 2-azidoethyl 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose (1b): 0.52 g of **1a** was dissolved in 10 mL of methanol, and then 0.37 g (2.0 equivalents) of sodium azide (NaN_3) was added to the reaction mixture. Now, the reaction mixture was refluxed at 70 ºC for 24 h. Then the crude solution was extracted with chloroform and purified through silica gel column chromatography (Ethyl acetate/Hexane 30:70) to get pure **1b** with 86 % yield. FT-IR (NaCl): 2950 cm^{-1} (-CH_2- asym. str.), 2884 cm^{-1} (-CH_2 sym. str.), 2106 cm^{-1} (-N_3 str.), 1754 cm^{-1} (-OAc C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 4.56-4.49 (d, 1H), 4.24-4.00 (m, 6H), 3.52-3.46 (m, 2H), 3.31-3.26 (m, 2H), 2.02 (s, 12H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 169.3, 99.7, 71.9, 71.0, 70.1, 67.6, 67.4, 60.9, 49.6, 19.7; HR-MS: *m/z* 440.1278 (observed), 440.1281 (calculated for $[M+Na]^+$).

Synthesis of 2-azidoethyl D-glucopyranose (1c): 0.3 g of **1b** was dissolved in 5 mL of methanol, and then 0.165 g (4.0 equivalents) of sodium methoxide (NaOMe) was added to the reaction mixture and kept at room temperature for 2 h with stirring. Then, Dowex resin (strongly acidic) was added to the reaction mixture and pH of the reaction mixture was adjusted to 6. Now the reaction mixture was filtered and the filtrate was evaporated to get **1c** with quantitative yield. FT-IR (NaCl): 3364 cm^{-1} (-OH str.), 2929 cm^{-1} (-CH_2- asym. str.), 2885 cm^{-1} (-CH_2- sym. str.), 2105 cm^{-1} (-N_3 str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 4.19-4.17 (d, 1H), 3.90-3.85 (m, 1H), 3.68- 3.62 (m, 2H), 3.47-3.41 (m, 3H), 3.15-3.08 (m, 2H), 3.04-2.93 (m, 2H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 103.0, 76.9, 76.7, 73.4, 70.1, 67.3, 61.1, 50.4; HR-MS: *m/z* 272.0844 (observed), 272.0859 (calculated for $[M+Na]^+$).

Synthesis of 2-aminoethyl D-glucopyranose (1d): 0.15 g of **1c** was dissolved in water. Then about 0.24 g (1.5 equivalents) of triphenylphosphine was added to the reaction mixture and it

was allowed to stir at room temperature for 12 h. Now the crude solution was extracted with water and dried to get pure **1d** with 75 % yield. FT-IR (NaCl): 3322 cm^{-1} (-OH and -NH_2 asym., sym. str.), 2929 cm^{-1} (-CH_2- asym. str.), 2890 cm^{-1} (-CH_2- sym. str.); ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 4.56-4.55 (d, 1H), 4.20- 4.14 (m, 1H), 3.99-3.95 (m, 1H) 3.87- 3.74 (m, 4H), 3.35-3.30 (m, 2H), 3.19-3.17 (t, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 104.5, 78.3, 77.9, 75.4, 71.9, 68.1, 59.9, 43.6; HR-MS: *m/z* 224.1122 (observed), 224.1134 (calculated for $[M+H]^+$).

Synthesis of 2-bromoethyl 2,3,4,6-tetra-*O*-acetyl-D-galactopyranose (2a): 2.5 g of D-galactose pentaacetate was dissolved in 20 mL of dry DCM at 0 ºC. Then 3.63 mL (1.2 equivalents) of BF_3.Et_2O was added to the reaction mixture drop wise followed by 0.54 mL (1.2 equivalents) of 2-bromoethanol. Then the reaction mixture was allowed to stir at room temperature for 3 h. After completion of the reaction anhydrous potassium carbonate (1.33 g, 1.5 equivalents) was added and stirring was continued for further 30 min. Then the crude solution was extracted with chloroform and purified through silica gel column chromatography (Ethyl acetate/Hexane 30:70) to get pure **2a** with 70 % yield. ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 4.53-4.51 (d, 1H), 4.33-4.31 (t, 1H), 4.30-4.06 (m, 3H), 3.83-3.79 (m, 2H), 3.50-3.43 (m, 4H), 2.06 (s, 12H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 169.7, 100.4, 72.0, 71.2, 69.5, 68.7, 67.2, 61.0, 29.9, 22.1; HR-MS: *m/z* 477.0351 (observed), 477.0372 (calculated for $[M+Na]^+$).

Synthesis of 2-azidoethyl 2,3,4,6-tetra-*O*-acetyl-D-galactopyranose (2b): 1.0 g of **2a** was dissolved in 20 mL of methanol, and then about 0.73 g (2 equivalents) of sodium azide (NaN_3) was added to the reaction mixture. Now, the reaction mixture was refluxed at 70 ºC for 24 h. Then the crude solution was extracted with chloroform and purified through silica gel column chromatography (Ethyl acetate/Hexane 30:70) to get pure **2b** with 60 % yield. FT-IR (NaCl): 2940 cm^{-1} (-CH_2- asym. str.), 2885 cm^{-1} (-CH_2- sym. str.), 2102 cm^{-1} (-N_3 str.), 1742 cm^{-1} (-OAc C=O str.); ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 4.55-4.53 (d, 1H), 4.23-3.90 (m, 6H), 3.51-3.45 (m, 2H), 3.31-3.25 (m, 2H), 2.01 (s, 12H); ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 170.3, 101.3, 71.0, 70.9, 68.7, 68.1, 67.1, 61.4, 50.7, 20.8. HR-MS: *m/z* 440.1274 (observed), 440.1281 (calculated for $[M+Na]^+$).

Synthesis of 2-azidoethyl D-galactopyranose (2c): 0.085 g of **2b** was dissolved in 3 mL of methanol, and then 0.04 g (4.0 equivalents) of sodium methoxide (NaOMe) was added to the reaction mixture and kept at room temperature for 2 h with stirring. Then to the reaction mixture,

Dowex resin (strongly acidic) was added and pH of the reaction mixture was adjusted to 6. Now the reaction mixture was filtered and the filtrate was evaporated to get **2c** with 98 % yield. FT-IR (NaCl): 3394 cm^{-1} (-OH str.), 2923 cm^{-1} (-CH_2- asym. str.), 2885 cm^{-1} (-CH_2- sym. str.), 2105 cm^{-1} (-N_3 str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 4.12-4.11 (d, 1H), 3.88-3.85 (m, 1H), 3.66- 3.63 (m, 2H), 3.45-3.42 (m, 3H), 3.13-3.05 (m, 2H), 3.01-2.93 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d6) δ/ppm: 103.6, 75.3, 73.5, 70.5, 68.0, 67.1, 60.3, 50.5; HR-MS: *m/z* 272.0844 (observed), 272.0859 (calculated for $[M+Na]^+$).

Synthesis of 2-aminoethyl D-galactopyranose (2d): 50 mg of **2c** was dissolved in water. Then 79 mg (1.5 equivalents) of triphenylphosphine was added to the reaction mixture and was allowed to stir at room temperature for 12 h. Now the crude solution was extracted with water and dried to get pure **2d** with 75 % yield. FT-IR (NaCl): 3329 cm^{-1} (-OH, -NH_2 asym. And sym. str.), 2927 cm^{-1} (-CH_2- asym. str.), 2885 cm^{-1} (-CH_2- sym. str.); ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 4.45-4.43 (d, 1H), 4.07-4.01 (m, 1H), 3.95-3.94 (d, 1H), 3.87-3.79 (m, 3H), 3.74-3.66 (m, 2H), 3.58- 3.54 (m, 1H), 3.07-3.04 (t, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 103.8, 76.2, 74.2, 71.1, 69.1, 67.9, 61.3, 51.1; HR-MS: *m/z* 224.1119 (observed), 224.1134 (calculated for $[M+H]^+$).

Synthesis of 3a and 4a[123]: Cellobiose (1 g) or maltose (1 g) was dissolved in 6 mL of water. Then 1.2 equivalents of *N*-Boc-1,3-propanediamine was dissolved separately in 10 mL of isopropanol and added to cellobiose or maltose solution drop wise. The reaction mixture was refluxed for 12 h with stirring. Now the solvent was evaporated to dryness and residue was washed with ethyl acetate followed by chloroform. Finally the solid was dried in high vacuum pump. This residue was dissolved in 5 mL of dry methanol and 1.4 equivalents of sodium borohydride was added to it. The reaction was allowed to stir for 12 h at room temperature. After that the reaction mixture was filtered and the filtrate was evaporated to get the pure 3**a** or **4a** (86 % - 90 %).

3a (Cellobiose derivative): FT-IR (NaCl): 3362 cm^{-1} (-OH str.), 2930 cm^{-1} (-CH_2- asym. str.), 2881 cm^{-1} (-CH_2- sym. str.), 1690 cm^{-1} (-NHBoc C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 4.30-4.28 (d, 1H), 4.12-4.08 (d, 2H), 3.69-3.38 (m, 10H), 3.13-2.94 (m, 6H), 1.67-1.58 (d, 2H), 1.37 (s, 9H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 170.7, 102.8, 76.7, 71.2, 71.1,

70.4, 44.2, 43.9, 36.2, 23.5, 20.6; HR-MS: *m/z* 501.2653 (observed), 501.2659 (calculated for $[M+H]^+$).

4a (Maltose derivative): FT-IR (NaCl): 3354 cm^{-1} (-OH str., -NH- sym. and asym. str.), 2927 cm^{-1} (-CH_2- asym. str.), 2821 cm^{-1} (-CH_2- sym. str.), 1690 cm^{-1} (-NHBoc C=O str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 4.82-4.80 (d, 1H), 4.42-4.38 (d, 2H), 3.60-3.38 (m, 10H), 3.13-2.66 (m, 6H), 1.69-1.55 (m, 2H), 1.37 (s, 9H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.4, 103.1, 77.2, 70.8, 70.1, 68.6, 48.8, 44.5, 36.9, 23.8, 21.1; HR-MS: *m/z* 501.2657 (observed), 501.2659 (calculated for $[M+H]^+$).

Synthesis of 3b and 4b: 3a (1.3 g) or **4a** (1.2 g) was dissolved in 3 mL of methanol, then 5 mL of 4N HCl was added to it. The reaction was allowed to stir at room temperature for 4 h. Now the methanol was removed from the reaction mixture and work up was done with chloroform and water. The aqueous layer was collected and dried by using lyophilizer to get the pure **3b** or **4b** with 75 % yield.

3b (Cellobiose derivative): FT-IR (NaCl): 3329 cm^{-1} (-OH, -NH_2 sym. and asym. str.), 2929 cm^{-1} (-CH_2- asym. str.), 2885 cm^{-1} (-CH_2- sym. str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 5.02-4.98 (d, 1H), 4.80-3.44, (m, 12H), 3.06-2.88 (m, 6H), 2.08-1.96 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 102.3, 76.9, 71.3, 71.1, 70.2, 44.2, 44.1, 36.2, 23.5; HR-MS: *m/z* 401.2159 (observed), 401.2135 (calculated for $[M+H]^+$).

4b (Maltose derivative): FT-IR (NaCl): 3339 cm^{-1} (-OH, -NH_2 sym. and asym. str.), 2928 cm^{-1} (-CH_2- asym. str.), 2886 cm^{-1} (-CH_2- sym. str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 5.02-4.98 (d, 1H), 4.80-3.44 (m, 12H), 3.06-2.88 (m, 6H), 2.08-1.96 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 103.0, 76.5, 71.3, 70.2, 68.5, 49.5, 44.2, 36.2, 23.5; HR-MS: *m/z* 401.2143 (observed), 401.2135 (calculated for $[M+H]^+$).

Synthesis of 5a and 6a: D-Gluconic acid lactone (2 g) or lactobionolactone (1.3 g) was dissolved in 12 mL of methanol, then about 1.2 equivalents of *N*-Boc-1,3-propanediamine was added to the reaction mixture. Now the reaction mixture was refluxed for 4 h. Then methanol was removed by rotary evaporator, the residue was washed with ethyl acetate and finally with chloroform. Then it was kept in high vacuum oven for overnight to get the pure and dry **5a** or **6a**.

5a (Gluconic acid lactone derivative): Yield; 98 %. FT-IR (NaCl): 3329 cm^{-1} (-OH str.), 2933 cm^{-1} (-CH_2- asym. str.), 2882 cm^{-1} (-CH_2- sym. str.), 1687 cm^{-1} (Amide-I C=O str.), 1654 cm^{-1} (Amide-II -NH- ben.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 4.48-3.47 (m, 4H), 4.35-3.57 (m, 2H), 3.92-3.07 (m, 4H), 1.51-1.49 (m, 2H), 1.37 (s, 9H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 173.1, 156.2, 78.2, 73.9, 72.7, 71.8, 70.8, 63.6, 37.5, 36.1, 29.8, 28.6; HR-MS: *m/z* 375.1726 (observed), 375.1743 (calculated for $[M+Na]^+$).

6a (Lactobionolactone derivative): Yield; 72 %. FT-IR (NaCl): 3341 cm^{-1} (-OH str.), 2929 cm^{-1} (-CH_2- asym. str.), 2888 cm^{-1} (-CH_2- sym. str.), 1685 cm^{-1} (Amide-I C=O str.), 1660 cm^{-1} (Amide-II -NH- ben.); ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 4.58-4.56 (d, 1H), 4.41-4.41 (d, 1H), 4.20-4.18 (t, 1H), 4.01-3.55 (m, 10H), 3.31-3.28 (t, 2H), 3.11-3.10 (t, 2H), 1.75-1.68 (m, 2H), 1.44 (s, 9H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 171.9, 170.3, 103.1, 81.2, 73.2, 71.4, 69.1, 68.5, 62.2, 49.7, 36.2, 25.9, 21.0; HR-MS: *m/z* 515.2489 (observed), 515.2452 (calculated for $[M+H]^+$).

Synthesis of 5b and 6b: 5a (2.56 g) or **6a** (1.35 g) was dissolved in 5 mL of methanol and 5 mL of 4N HCl was added to it. Then reaction mixture was kept at room temperature for 4 h with stirring. After completion of the reaction, solvent was removed to get pure and dry **5b** or **6b**.

5b (Gluconic acid lactone derivative): Yield; 96 %. FT-IR (NaCl): 3335 cm^{-1} (-OH, -NH_2 sym. and asym. str.), 2927 cm^{-1} (-CH_2- asym. str.), 2886 cm^{-1} (-CH_2- sym. str.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 4.23-3.53 (m, 4H), 4.12-3.79 (m, 2H), 2.93-2.87 (t, 4H), 1.92-1.88 (m, 2H); ^{13}C-NMR (400 MHz, DMSO-d_6) δ/ppm: 174.3, 80.4, 74.0, 72.6, 69.1, 62.9, 60.3, 36.2, 25.1; HR-MS: *m/z* 253.1381 (observed), 253.1400 (calculated for $[M+H]^+$).

6b (Lactobionolactone derivative): Yield; 89 %. FT-IR (NaCl): 3297 cm^{-1} (-OH, -NH_2 sym. and asym. str.), 2932 cm^{-1} (-CH_2- asym. str.), 2888 cm^{-1} (-CH_2- sym. str.), 1685 cm^{-1} (Amide-I C=O str.), 1648 cm^{-1} (Amide-II -NH- ben.); ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 4.58-4.54 (d, 1H), 4.41-4.40 (d, 1H), 4.19-4.19 (t, 1H), 4.0-3.55 (m, 10H), 3.36-3.4 (t, 2H), 3.28-3.30 (t, 2H), 1.69-1.73 (m, 2H); ^{13}C-NMR (100 MHz, DMSO-d_6) δ/ppm: 172.7, 103.1, 81.3, 73.3, 71.5, 69.1, 68.0, 62.8, 49.6, 36.0, 25.0; HR-MS: *m/z* 415.1901 (observed), 415.1928 (calculated for $[M+H]^+$).

General protocol for the synthesis of 7a, 8a and 9a[124]: Vancomycin hydrochloride (150 mg) was dissolved in dry dimethyl formamide (1 mL) and dry methanol (1 mL). To this one

equivalent of 1-octanal or 1-decanal or 1-dodecanal and 1.2 equivalents of diisopropylethylamine (DIPEA) were added. The reaction mixture was stirred at 50 ºC for 2 h and then allowed to cool to room temperature prior to addition of sodium cyanoborohydride (2.0 equivalents). Then, the reaction mixture was stirred at 50 ºC for additional 2 h and allowed to cool to ambient temperature for overnight. The product was purified by preparative reverse-phase HPLC using 0.1 % trifluoroacetic acid in H_2O/acetonitrile mixture and then lyophilized to afford trifluoroacetate salt of compounds **7a** or **8a** or **9a** in 75-80 % yield.

7a: Yield; 77 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.44 (s, 1H), 9.18 (s, 1H), 9.08 (s, 1H), 8.98 (bs, 1H), 8.88 (bs, 1H), 8.71-8.51 (m, 2H), 8.09 (bs, 1H), 7.81 (bs, 2H), 7.59-7.45 (m, 4H), 7.31-7.1 (m, 3H), 6.78-6.67 (m, 2H), 6.35-6.24 (dd, 2H), 6.0-5.93 (m, 2H), 5.75-5.65 (m, 2H), 5.36-5.2 (m, 6H), 4.91-4.90 (d, 1H), 4.61-4.42 (m, 4H), 4.18-4.08 (m, 4H), 2.67-2.61 (m, 3H), 1.80-1.75 (m, 1H), 1.66-1.51 (m, 4H), 1.24 (m, 13H), 1.09-1.07 (d, 3H), 0.91-0.85 (m, 10H). HR-MS: *m/z* 785.8725 (observed), 785.8578 (calculated for $[M+2H]^{2+}$).

8a: Yield; 80 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.45 (s, 1H), 9.20 (s, 1H), 9.08 (s, 1H), 8.97 (bs, 1H), 8.88 (bs, 1H), 8.71-8.53 (m, 2H), 8.12 (bs, 1H), 7.83 (bs, 2H), 7.59-7.45 (m, 4H), 7.34-7.09 (m, 3H), 6.78-6.67 (m, 2H), 6.38-6.24 (dd, 2H), 5.98-5.93 (m, 2H), 5.75-5.63 (m, 2H), 5.36-5.2 (m, 6H), 4.91-4.90 (d, 1H), 4.63-4.42 (m, 4H), 4.19-4.10 (m, 4H), 2.67-2.61 (m, 3H), 1.80-1.75 (m, 1H), 1.66-1.51 (m, 4H), 1.24 (m, 17H), 1.09-1.07 (d, 3H), 0.92-0.83 (m, 10H). HR-MS: *m/z* 795.7992 (observed), 795.7578 (calculated for $[M+2H]^{2+}$).

9a: Yield; 75 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.41 (s, 1H), 9.20 (s, 1H), 9.12 (s, 1H), 9.01 (bs, 1H), 8.88 (bs, 1H), 8.69-8.53 (m, 2H), 8.25 (bs, 1H), 7.93 (bs, 2H), 7.61-7.45 (m, 4H), 7.33-7.21 (m, 3H), 6.78-6.67 (m, 2H), 6.38-6.24 (dd, 2H), 5.99-5.85 (m, 2H), 5.83-5.63 (m, 2H), 5.36-5.2 (m, 6H), 4.95-4.93 (d, 1H), 4.53-4.42 (m, 4H), 4.21-4.10 (m, 4H), 2.71-2.61 (m, 3H), 1.80-1.77 (m, 1H), 1.66-1.55 (m, 4H), 1.28 (m, 21H), 1.09-1.07 (d, 3H), 0.91-0.86 (m, 10H). HR-MS: *m/z* 809.7417 (observed), 809.7365 (calculated for $[M+2H]^{2+}$).

Synthesis of Vancomycin-sugar conjugates (1-9)[125]

Vancomycin hydrochloride or **7a-9a** (67 µmol) was dissolved in dry dimethyl formamide (1 mL) dry dimethyl sulfoxide (1 mL). To this two equivalents of compounds bearing primary amine

group (**1d**, **2d**, **3b**, **4b**, **5b** and **6b**) in 1 mL of dry dimethylformamide was added. The reaction mixture was cooled to 0 ºC, and 0.22 mL (1.5 equivalents) of 0.45 M HBTU solution in DMF was added followed by 58 µL of DIPEA (5.0 equivalents). The reaction mixture was then allowed to warm to room temperature and stirred for 12 h. The products were purified by preparative reverse-phase HPLC to more than 95 % using 0.1 % trifluoroacetic acid in H_2O/acetonitrile mixture and then lyophilized to afford either bis or tris-(trifluoroacetate) salts of final compounds (47-54 µmol, 70-80 %).

Vancomycin-sugar conjugate (1; Van-cyGlu): Yield; 72 % (48.2 µmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.35-9.34 (d, 1H), 9.07-8.95 (m, 3H), 8.82 (bs, 1H), 8.68 (bs, 1H), 8.45-8.44 (m, 2H), 7.97-7.85 (m, 2H), 7.62-7.44 (m, 6H), 7.33-7.31 (d, 1H), 7.20-7.18 (d, 2H), 7.05-7.04 (m, 1H), 6.77-6.62 (m, 2H), 6.35-6.23 (m, 1H), 5.96-5.87 (m, 1H), 5.76-5.59 (m, 1H), 5.49-5.43 (m, 1H), 5.36-5.02 (m, 6H), 4.99-4.65 (m, 4H), 4.57-4.35 (m, 2H), 4.22-4.02 (m, 2H), 3.69-3.66 (m, 2H), 3.07-2.96 (m, 4H), 2.59 (bs, 2H), 2.19-2.11 (m, 2H), 1.91-1.87 (m, 2H), 1.75-1.74 (m, 4H), 1.07-1.05 (d, 3H), 0.91-0.85 (m, 4H). HR-MS: *mlz* 828.2645 (observed), 828.2436 (calculated for $[M+2H]^{2+}$).

Vancomycin-sugar conjugate (2; Van-cyGal): Yield; 70 % (47 µmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.33 (s, 1H), 9.0-8.97 (d, 2H), 8.68 (bs, 1H), 8.45-8.44 (d, 2H), 7.91-7.86 (t, 2H), 7.61-7.44 (m, 7H), 7.34-7.32 (d, 2H), 7.20-7.18 (t, 2H), 7.04 (bs, 1H), 6.77-6.64 (m, 3H), 6.35-6.27 (dd, 2H), 5.92-5.74 (m, 3H), 5.60 (s, 1H), 5.45-5.08 (m, 9H), 4.90-4.89 (d, 2H), 4.71-4.58 (m, 3H), 4.45-4.38 (m, 3H), 4.22-4.12 (m, 3H), 4.02-4.00 (t, 1H), 3.78-3.43 (m, 8H), 3.18-3.16 (d, 2H), 2.59 (bs, 2H), 2.18-2.12 (m, 2H), 1.91-1.88 (m, 2H), 1.75-1.53 (m, 5H), 1.29 (bs, 3H), 1.07-1.06 (d, 3H), 0.91-0.86 (m, 7H). HR-MS: *m/z* 828.2641 (observed), 828.2436 (calculated for $[M+2H]^{2+}$).

Vancomycin-sugar conjugate (3; Van-β-1cyGlu-4acyGlu): Yield; 78 % (52.3 µmol)**.** ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.36 (s, 1H), 9.06-9.02 (d, 2H), 8.67 (bs, 1H), 8.47-8.30 (m, 3H), 8.09 (bs, 1H), 7.84 (bs, 1H), 7.65-7.45 (m, 7H), 7.34-7.31 (d, 1H), 7.22-7.20 (m, 2H), 7.08 (bs, 1H), 6.77-6.69 (m, 2H), 6.53 (bs, 1H), 6.38 (s, 1H), 6.22 (s, 1H), 5.98-5.57 (m, 3H), 5.59 (s, 1H), 5.49-5.45 (m, 2H), 5.38-5.34 (m, 2H), 5.27-5.10 (m, 6H), 5.02-4.57 (m, 7H), 4.50-4.22 (m, 6H), 4.04-4.01 (t, 2H), 3.88-3.87 (d, 1H), 3.70-3.53 (m, 4H), 3.18-3.12 (m, 3H), 3.08-2.88 (m,

4H), 2.17-2.12 (m, 1H), 1.91-1.53 (m, 7H), 1.30 (s, 3H), 1.26-1.24 (t, 2H), 1.07-1.06 (d, 3H), 0.91-0.85 (m, 7H). HR-MS: *m/z* 916.8140 (observed), 916.8427 (calculated for $[M+2H]^{2+}$).

Vancomycin-sugar conjugate (4; Van-α-1cyGlu-4acyGlu): Yield; 80 % (54 µmol)**.** ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.34 (s, 1H), 9.05-9.02 (d, 2H), 8.64 (bs, 1H), 8.43-8.33 (m, 3H), 8.09 (bs, 1H), 7.82 (bs, 1H), 7.62-7.39 (m, 7H), 7.34-7.31 (d, 1H), 7.22-7.18 (m, 2H), 7.02 (bs, 1H), 6.75-6.63 (m, 2H), 6.53 (bs, 1H), 6.41 (s, 1H), 6.18 (s, 1H), 6.01-5.59 (m, 3H), 5.55 (s, 1H), 5.49-5.45 (m, 2H), 5.35-5.31 (m, 2H), 5.17-5.10 (m, 6H), 5.02-4.57 (m, 6H), 4.45-4.18 (m, 6H), 3.95-3.91 (t, 2H), 3.88-3.84 (d, 1H), 3.65-3.53 (m, 4H), 3.18-3.12 (m, 3H), 3.08-2.92 (m, 4H), 2.17-2.12 (m, 1H), 1.91-1.53 (m, 7H), 1.28 (s, 3H), 1.26-1.24 (t, 2H), 1.07-1.06 (d, 3H), 0.91-0.85 (m, 7H). HR-MS: *m/z* 916.8127 (observed), 916.8427 (calculated for $[M+2H]^{2+}$).

Vancomycin-sugar conjugate (5; Van-acyGlu): Yield; 75 % (50.3 µmol)**.** ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.35 (s, 1H), 9.07-9.00 (m, 3H), 8.68 (bs, 1H), 8.45 (bs, 1H), 7.97-7.85 (m, 2H), 7.62-7.44 (m, 6H), 7.33-7.18 (dd, 2H), 6.77-6.51 (m, 2H), 6.35-6.23 (m, 1H), 5.96-5.87 (m, 1H), 5.76-5.59 (m, 1H), 5.49-5.43 (m, 1H), 5.36-5.04 (m, 6H), 4.99-4.65 (m, 4H), 4.57-4.35 (m, 2H), 4.22-4.01 (m, 2H), 3.69-3.66 (m, 2H), 3.07-2.96 (m, 4H), 2.59 (bs, 2H), 2.19-2.11 (m, 1H), 1.75-1.54 (m, 4H), 1.3 (s, 3H), 1.07-1.05 (d, 2H), 0.91-0.85 (m, 7H). HR-MS: *m/z* 842.7744 (observed), 842.7641 (calculated for $[M+2H]^{2+}$).

Vancomycin-sugar conjugate (6; Van-β-1cyGal-4acyGlu): Yield; 72 % (48.2 µmol)**.** ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.33 (s, 1H), 9.02-8.94 (m, 4H), 8.69 (bs, 1H), 8.53-8.46 (m, 2H), 8.07-8.05 (t, 1H), 7.85 (s, 1H), 7.68-7.45 (m, 10H), 7.33-7.18 (m, 3H), 7.09-7.08 (d, 1H), 6.77-6.66 (m, 3H), 6.48 (bs, 1H), 6.37-6.22 (dd, 2H), 5.94-5.93 (d, 1H), 5.80-5.75 (m, 2H), 5.61 (s, 1H), 5.45-5.43 (d, 1H), 5.34-5.17 (m, 6H), 5.09 (bs, 1H), 4.92-4.91 (d, 1H), 4.68-4.66 (d, 1H), 4.46-4.35 (m, 2H), 4.24-4.21 (d, 2H), 4.02-3.96 (d, 2H), 3.70-3.67 (d, 1H), 3.57-3.44 (m, 3H), 2.9 (bs, 1H), 2.81-2.76 (q, 2H), 2.68-2.62 (m, 4H), 2.15-2.08 (m, 2H), 1.91-1.89 (d, 2H), 1.75-1.55 (m, 7H), 1.30 (s, 3H), 1.07-1.06 (d, 3H), 0.92-0.85 (m, 7H). HR-MS: *m/z* 923.8035 (observed), 923.8346 (calculated for $[M+2H]^{2+}$).

Lipophilic-vancomycin-sugar conjugate (7; VanC_8-β-1cyGal-4acyGlu): Yield; 80 % (54 µmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.33 (s, 1H), 9.03-8.99 (d, 2H), 8.69 (bs, 1H), 8.48-8.46 (d, 2H), 8.14-8.06 (m, 2H), 7.84-7.39 (m, 9H), 7.35-7.06 (m, 4H), 6.78-6.66 (m, 2H),

6.48 (bs, 1H), 6.37-6.22 (dd, 2H), 5.90-5.62 (m, 5H), 5.36-5.10 (m, 8H), 4.91 (bs, 1H), 4.61-4.60 (d, 2H), 4.46-4.45 (d, 2H), 4.37-4.35 (d, 2H), 4.24-4.22 (d, 3H), 4.11-4.08 (t, 3H), 2.79-2.78 (d, 2H), 2.70-2.66 (m, 2H), 2.33-2.31 (m, 2H), 2.19 (bs, 1H), 2.00-1.97 (m, 1H), 1.80-1.65 (m, 5H), 1.59-1.53 (m, 3H), 1.36 (s, 3H), 1.25 (m, 13H), 1.10-1.08 (d, 3H), 0.92-0.84 (m, 10H). HR-MS: *m/z* 979.8707 (observed), 979.9411 (calculated for $[M+2H]^{2+}$).

Lipophilic-vancomycin-sugar conjugate (8; VanC_{10}-β-1cyGal-4acyGlu): Yield; 77 % (51.6 µmol)**.** ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.35 (s, 1H), 9.04-9.00 (d, 3H), 8.68 (bs, 1H), 8.48-8.47 (d, 2H), 8.18-8.06 (m, 3H), 7.72 (bs, 2H), 7.55-7.45 (m, 4H), 6.78-6.65 (m, 3H), 6.38-6.22 (dd, 2H), 5.96-5.75 (m, 3H), 5.67-5.62 (m, 2H), 5.35-5.11 (m, 8H), 4.93-4.92 (d, 1H), 4.64-4.59 (m,1H), 4.46-4.33 (m, 2H), 4.25-4.09 (m, 3H), 3.94 (bs, 1H), 3.71-3.67 (m, 2H), 3.63-3.46 (m, 4H), 2.80-2.78 (m, 3H), 2.62 (bs, 3H), 2.17-1.98 (m, 2H), 1.81-1.54 (m, 8H), 1.36 (s, 3H), 1.27-1.24 (m, 17H), 1.10-1.08 (d, 3H), 0.92-0.84 (m, 10H). HR-MS: *m/z* 993.8801 (observed), 993.9676 (calculated for $[M+2H]^{2+}$).

Lipophilic-vancomycin-sugar conjugate (9; VanC_{12}-β-1cyGal-4acyGlu): Yield; 77 % (51.6 µmol)**.** ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.33 (s, 1H), 9.04-8.99 (d, 2H), 8.69 (bs, 1H), 8.48-8.47 (d, 2H), 8.14-8.05 (m, 2H), 7.84 (s, 2H), 7.67 (bs, 3H), 7.54-7.45 (m, 4H), 7.30-7.21 (m, 3H), 7.07 (bs, 1H), 6.78-6.69 (m, 3H), 6.37-6.22 (dd, 2H), 5.92 (bs, 2H), 5.80-5.75 (m, 3H), 5.63-5.62 (d, 2H), 5.36-5.10 (m, 7H), 4.91-4.90 (d, 1H), 4.61-4.60 (d, 2H), 4.46-4.45 (d, 2H), 4.37-4.35 (d, 2H), 4.24-4.20 (m, 2H), 4.12-4.09 (t, 2H), 3.71-3.66 (m, 4H), 2.81-2.78 (m, 3H), 2.67-2.66 (m, 1H), 2.33-2.32 (m, 2H), 2.00-1.97 (d, 1H), 1.80-1.64 (m, 4H), 1.58-1.53 (m, 3H), 1.36 (s, 3H), 1.24 (m, 21H), 1.09-1.08 (d, 3H), 0.92-0.83 (m, 10H). HR-MS: *m/z* 1007.4024 (observed), 1007.9941 (calculated for $[M+2H]^{2+}$).

2.5.3 Minimum inhibitory concentration (MIC)[126]

Antibacterial activity of the test compounds was measured in broth micro-dilution method following CLSI guidelines. Briefly, the compounds were serially diluted using autoclaved Millipore water. Bacteria, to be tested, were grown for 6 h (~ 10^9 CFU/mL, determined by spread plating method), which was then diluted to 10^5 CFU/mL using suitable media. 50 µL of serially diluted compound was added to a 96 well plate containing 150 µL media containing bacterial solution. Two controls were made; one containing 150 µL of media and 50 µL of compound of

every concentration and the other containing 50 µL of media and 150 µL of bacterial solution. The plates were then incubated at 37 °C for 24 h and then the O. D. value was measured at 600 nm using Plate Reader (TECAN Infinite series, M200 pro). Each concentration had triplicate values and the whole experiment was done at least twice. The MIC was taken at the concentration, at which no growth was observed.

2.5.4 Titration binding assays with model ligands[127]

The binding constants for vancomycin, vancomycin-sugar conjugates (**1**-**6**) and lipophilic-vancomycin-sugar conjugate (**8**) with the model ligands *N*,*N'*-diacetyl-Lys-D-Ala-D-Ala and *N*,*N'*-diacetyl-Lys-D-Ala-D-Lac were determined using UV-absorption difference measurements. UV scans were run with a baseline correction that consisted of 0.02 M sodium citrate buffer (pH = 5.1) and measured the range from 200 to 345 nm. A solution of test compounds (100 µM in 0.02 M sodium citrate buffer) was placed into a quartz UV cuvette (1 cm path length) and the UV spectrum recorded versus a reference cell containing 0.02 M sodium citrate buffer. UV spectra were recorded after each addition of a solution of *N*,*N'*-diacetyl-Lys-D-Ala-D-Ala (0.05 to 5.0 equivalents) or *N*,*N'*-diacetyl-Lys-D-Ala-D-Lac (0.05 to 40.0 equivalents) in 0.02 M sodium citrate buffer. The absorbance value at the λ_{max} (279 nm) was recorded and the running change in absorbance, $\delta A_{x\ equiv}$ ($A_{initial}$-$A_{x\ equiv}$), measured. The number of ligand equivalents was plotted versus δA to afford the ligand binding titration curve. The break point of this curve is the saturation point of the system and its xy coordinates were determined by establishing the intersection of the linear fits of the pre- and post-saturation curves. $\delta A_{saturation}$ was calculated and employed to determine the concentration of free ligand in solution at each titration point at post-saturation. δA was plotted versus δA/free ligand concentration to give a Scatchard plot from which the binding constants were determined.

2.5.5 Intracellular accumulation of UDP-*N*-acetyl-muramyl-pentadepsipeptide[128,129]

Analysis of the cytoplasmic peptidoglycan nucleotide precursor pool was examined using VRE cells grown in 25 mL MHB. Cells were grown to an A_{600nm} (OD_{600}) of 0.6 and incubated with 130 µg/mL of chloramphenicol for 15 min. Then, test compounds vancomycin (5 µM), compounds **6** and **8** (5 µM) were added and incubated for another 60 min. Cells were collected and washed with sterile water to remove the antimicrobial agents and then extracted with boiling

water. The cell extract was then centrifuged and the supernatant lyophilized. Then, the lyophilized powder was dissolved in 2 mL of water and pH was adjusted to 2.0 with 20 % phosphoric acid. Now, the UDP-linked cell wall precursors in the solution were analyzed by RP-HPLC monitoring the UV absorbance peak at 260 nm wavelength and confirmed by HR-MS mass spectrometry.

2.5.6 Bactericidal time-kill kinetics[130]

Briefly, VISA was grown in nutrient broth at 37 °C for 6 h. Test compounds, vancomycin and compound **8** at two different concentrations (2 µM and 4 µM) were inoculated with the aliquots of bacteria, resuspended in fresh media at ~ 1.8×10^8 CFU/mL. After specified time intervals (0, 1, 2, 3, 6, and 24 h), 20 µL aliquots were serially diluted 10-fold in 0.9 % saline, plated on sterile nutrient agar plates and incubated at 37 °C overnight. The viable colonies were counted the next day and represented as $\log_{10}$ (CFU/mL).

2.5.7 Resistance development study[131]

MIC values of the compound **8** and vancomycin were determined against MRSA as described above. For the next day MIC experiment, the bacterial dilution was made by using the bacteria from sub-MIC concentration of the compounds (at MIC/2). Then, the concentration of this bacteria was adjusted to ~ 10^5 CFU/mL based on OD_{600} and subjected to next MIC assay. After a 24 h incubation period, again bacterial dilution was prepared by using the bacterial suspension from sub-MIC concentration of the compound (at MIC/2) and assayed for another MIC experiment. The process was repeated for 25 passages, and the fold increase in MIC was determined. The results indicate the fold of increase in MIC every day.

2.5.8 *In-vivo* antibacterial activity

2.5.8.1 Murine renal infection model[132]

Female CD-1 mice 6 to 8 weeks of age and weighing 20 to 25 g were used in this study. On day -7, all mice were injected intravenously (i.v.) with 0.2 mL of 0.2 % λ-carrageenan to increase their susceptibility to bacterial renal infection. λ-Carrageenan increases mice susceptibility to renal infection following i.v. injection, possibly by forming a lattice structure in renal tissue and

forming a support structure for bacterial attachment and growth. Bacterial strains that fail to cause renal infection in normal mice produce significant renal infections in λ-carrageenan-treated mice. On day 0, all mice were injected i.v. with 0.2 mL of the bacterial culture (VREs ATCC 51575, 10^8 CFU/mouse) through the tail vein. Four hours after the bacterial inoculation, mice were treated intraperitoneally (i.p.) with vancomycin, compound **8** and linezolid at 12 mg/kg of body weight or 0.2 mL of saline (0.9 % NaCl) control (n = 5). All the test compounds or saline were administered once daily for an additional two consecutive days for a total of three doses. All mice were sacrificed on day 3. Both kidneys from each mouse were removed aseptically and homogenized in 10 mL of saline. The dilutions of the homogenate were plated onto sheep blood agar plates, which were incubated overnight at about 37 °C. The bacterial titer was expressed as $\log_{10}$ CFU/g of kidney weight.

2.5.8.2 Mouse neutropenic thigh infection model[133]

About six-week-old, specific-pathogen-free female CD-1 mice (weight, about 24 g) were used for the experiments. The mice were rendered neutropenic (about 100 neutrophils/mL) by injecting two doses of cyclophosphamide intraperitoneally 4 days (150 mg/kg) and 1 day (100 mg/kg) before the infection experiment. About 50 μL of ~ 10^7 CFU/mouse concentration of the bacterial inoculum (VISA) was injected into the thigh. About one hour after inoculation, animals (n = 5) were treated intravenously twice (q12 h) with linezolid (12 mg/kg) and vancomycin (12 mg/kg) whereas compound **8** (12 mg/kg) was injected once at 1 h post infection. At 24 h post first treatment, cohorts of animals were euthanized and the thighs were collected aseptically. The thigh was weighed (about 0.7 g-0.9 g) and placed into about 10 mL of sterile saline and homogenized. The dilutions of the homogenate were plated onto agar plates, which were incubated overnight at about 37 °C. The bacterial titer was expressed as $\log_{10}$ CFU/g of thigh weight.

2.5.8.3 Dose-responsive study (Pharmacodynamics)

A separate single-dose study of compound **8** was performed in neutropenic mice infected in the thigh with VISA (10^7 CFU/mouse). Infected animals were treated intravenously, at 1 h post

infection, with 2, 4, 12and 50 mg/kg (n = 5). At 24 h post infection mice were sacrificed and the thigh tissues were harvested for the bacterial titer as mentioned above.

2.5.9 Single-dose pharmacokinetic study

A single dose pharmacokinetic analysis of compound **8** was performed in CD-1 female mice. Mice were administered a single intravenous dose of 12 mg/kg. Blood samples were collected from mice by retro-orbital aspiration and placed into heparinized tubes at 0.083 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h and 24 h after dosing (n = 3 per data point). The plasma was separated by centrifugation, and drug plasma concentrations were measured by microbiologic assay with *Bacillus subtilis* as the test organism.[134] The lower limit of detection of the assay was 1.25 µg/mL. Pharmacokinetic parameters, including half-life, AUC and C_{max} were calculated by using non-compartmental model.[135] The AUC was estimated up to 24 h and half-life ($t_{1/2}$) was calculated.

2.5.10 *In-vivo toxicology*

2.5.10.1 Systemic toxicity

To evaluate maximum tolerability of the new glycopeptide, systemic toxicity was performed on CD-1 female mice. Each mouse was injected with a 0.2 mL of freshly prepared compound **8** solution in saline. The dose of the compound administered was 100 mg/kg (n = 5). Animals were directly inspected for adverse effects for 4 h, and mortality was observed for 14 days.

2.5.10.2 Acute toxicity[136]

For the evaluation of the acute toxicity, two groups of 10 mice each received intravenous injection of compound **8** at 12 mg/kg (dosage of > $ED_{2\text{-log kill}}$) in 0.2 mL of sterilized saline. 10 mice were sacrificed at 48 h and rest of the mice at 14 days to collect blood samples for analysis of biochemical parameters such as alanine transaminase (ALT), urea nitrogen, creatinine, sodium ion, potassium ion and chloride ion levels. Blood samples were analyzed at Gokula Metropolis clinical laboratory, Bengaluru, India.

2.5.11 *In-vivo* data analysis

Data are expressed as mean ± standard deviation (SD). Dose-response curve is fitted using GraphPad Prism for Microsoft Windows (version 6.05; GraphPad Software) with a four-parameter logistic equation.[137] The equation; $y = Min + (Max - Min)/(1 + 10^{[\log ED50-x]\times Hillslope})$ is used, where x is the logarithm of dose and y is the dose response (in $\log_{10}$ CFU/g). y starts at a maximum (Max) (fixed to the 24-h vehicle control response) and approaches to a minimum (Min) with a sigmoidal shape. The 50 % effective dose (ED_{50}) was defined as the dose required to produce 50 % of the maximum response from the vehicle treated mice (24 h). The $\log_{10}$ stasis dose (ED_{stasis}) was defined as the dose producing no net change in titer compared to pretreatment titer (0 h). The dose required to cause a decrease in titer of 1 $\log_{10}$ CFU/g from pretreatment titer (0 h) was defined 1-$\log_{10}$ kill dose ($ED_{1\text{-log kill}}$). Similarly, the doses required to produce decrease in titer of 2 and 3 $\log_{10}$ CFU/g from pretreatment titer were defined as $ED_{2\text{-log kill}}$ and $ED_{3\text{-log kill}}$.

Pharmacokinetic analysis was performed by microbiologic assay using *Bacillus subtilis* as the test organism. Pharmacokinetic parameters, including half-life, AUC and C_{max} were determined using non-compartmental model.[135] The AUC from the time of dosing to the last measurable concentration was calculated by the linear trapezoidal rule. Biochemical parameters essentially liver function parameters (ALT, Alanine transaminase); kidney function parameters (Urea nitrogen & Creatinine) and electrolytes (Sodium, Potassium and Chloride) levels were analyzed by student's *t*-test.

Chapter 3

Membrane Active Vancomycin Analogues to Overcome Vancomycin Resistance in Bacteria

Abstract

*In **Chapter 3**, a rational strategy has been developed to impart an additional mechanism of action to vancomycin, namely bacterial membrane disruption to combat acquired resistance. Here, permanent positively charged lipophilic vancomycin analogues have been developed using simple synthetic methodology by conjugating the primary amine group of permanent positively charged lipophilic moiety to the carboxylic group of vancomycin to provide increased positive charge in the molecule, thereby enabling better interaction with the negatively charged bacterial membrane. Compared to vancomycin, an optimized compound in the series demonstrated a 40-fold, 400-fold and 1000-fold greater activity against VISA (vancomycin intermediate S. aureus), VRSA (vancomycin-resistant S. aurues) and VRE (vancomycin-resistant Enterococci) respectively. Either the lipophilic cationic quaternary ammonium moiety alone or its physical mixture with vancomycin showed less or no activity against the same bacteria. Significantly, unlike vancomycin, these compounds were shown to be bactericidal at low concentrations, and did not induce bacterial resistance. The incorporation of lipophilic moiety into vancomycin along with installed permanent positive charge makes these compounds distinct from other existing derivatives in their ability to cause strong bacterial membrane disruption (cytoplasmic membrane depolarization, intracellular K^+ ion leakage and cytoplasmic membrane permeabilization). Compared to vancomycin and linezolid, optimized compound showed better in-vivo activity against MRSA, VISA and VRE in infection models and exhibited improved pharmacological properties with no observed toxicity. The potent activity of the compounds is attributed to the incorporation of a new membrane disruption mechanism into vancomycin and opens up a great opportunity for the development of future antibiotics.*

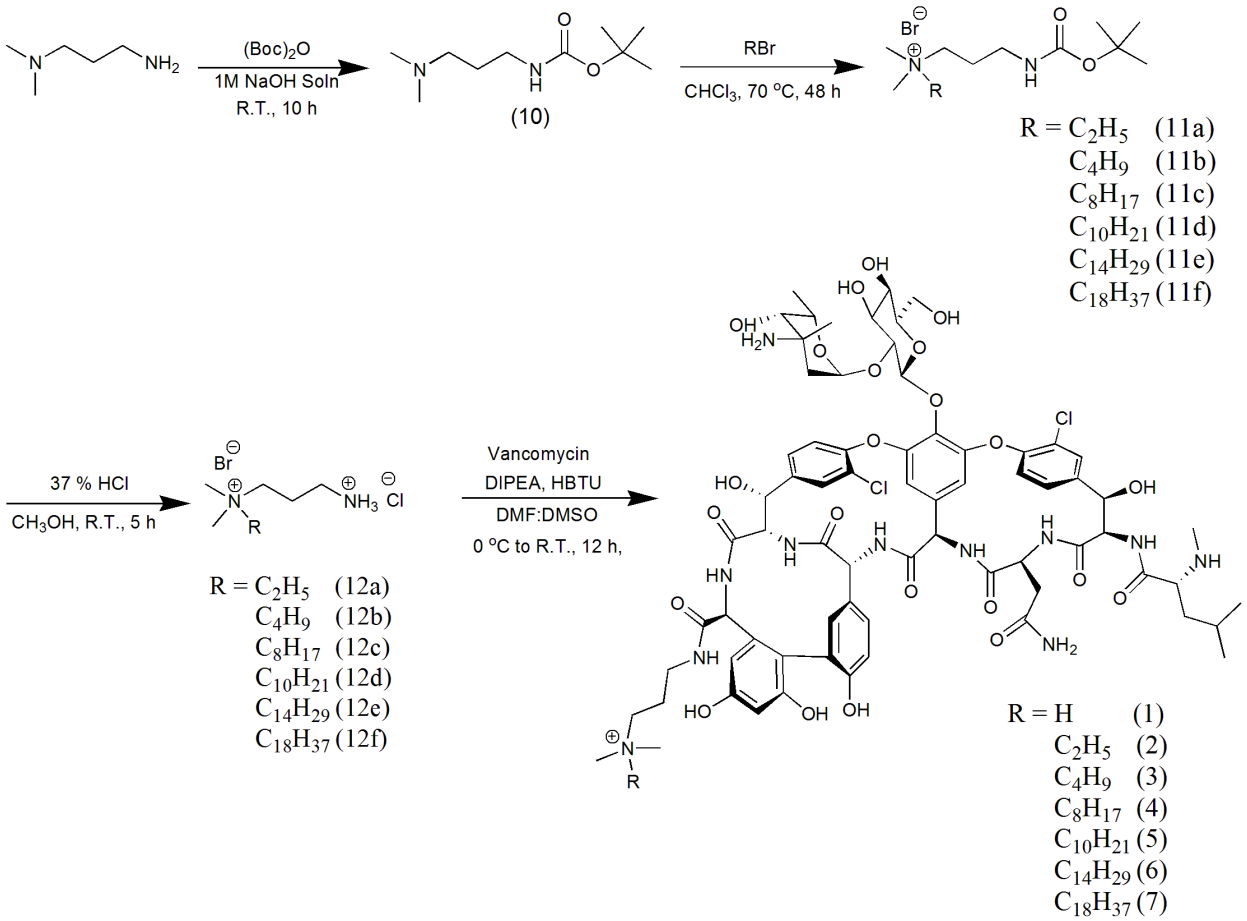
Publications based on this work

(1) Yarlagadda, V. *et al.* Membrane active vancomycin analogues: A strategy to combat bcatreial resistance. *J. Med. Chem.* **2014**, *57*, 4558. (2) Yarlagadda, V. *et al.* In-vivo antibacterial activity and pharmacological properties of membrane active glycopeptide antibiotic YV11455. *Int. J. Antimicrob. Agents* **2015**, *45*, 627.

3.1 Introduction

The perennial persistence of vancomycin resistance, calls for an urgent need to develop more potent analogues having additional mode of action, which would make bacterial resistance difficult to develop. Generally, antimicrobial peptides (AMPs) and antibacterial peptidomimetics kill bacteria by selectively disrupting their membranes through their facial segregation of positive charges and hydrophobic moieties and disrupt the negatively charged bacterial membranes.[138,139] It has also been observed that bacteria are known to develop slow resistance against cell membrane disrupting agents like AMPs and peptidomimetics.[140] Hence, a strategy, which combines the inherent mechanism of action of vancomycin along with membrane disrupting effect is expected to not only possess improved antibacterial potency but also counter the development of bacterial resistance. Semi-synthetic glycopeptide antibiotics such as oritavancin, dalbavancin and telavancin containing hydrophobic groups were shown to have bacterial cell membrane disruption property at high concentrations (10-fold higher than MIC) due to which they exhibit high antibacterial activity against resistant strains. These semi-synthetic glycopeptides have been approved by FDA to treat skin infections caused by MRSA.[43,72,74,82] However, both dalbavancin and telavancin are inactive against the more virulent VanA phenotypes of VRB.[43]

To confer strong membrane disruptive properties to vancomycin, a strategy has been developed wherein, a lipophilic cationic quaternary ammonium moiety is appended to the vancomycin molecule to produce a series of permanent positively charged lipophilic vancomycin analogues (Scheme 3.1). These analogues possess strong, broad-spectrum antibacterial activity and the optimized compound was 1000-fold more effective than vancomycin against VRE. Unlike vancomycin, optimized compounds were found to be bactericidal and did not induce the development of bacterial resistance. This compound, compared to vancomycin, showed higher *in-vivo* antibacterial activity against drug-resistant Staphylococcal thigh infection model (MRSA and VISA) and VRE kidney infection model. Furthermore, pharmacodynamics, pharmacokinetics and acute toxicology studies were performed to validate its safety profile in mice models.



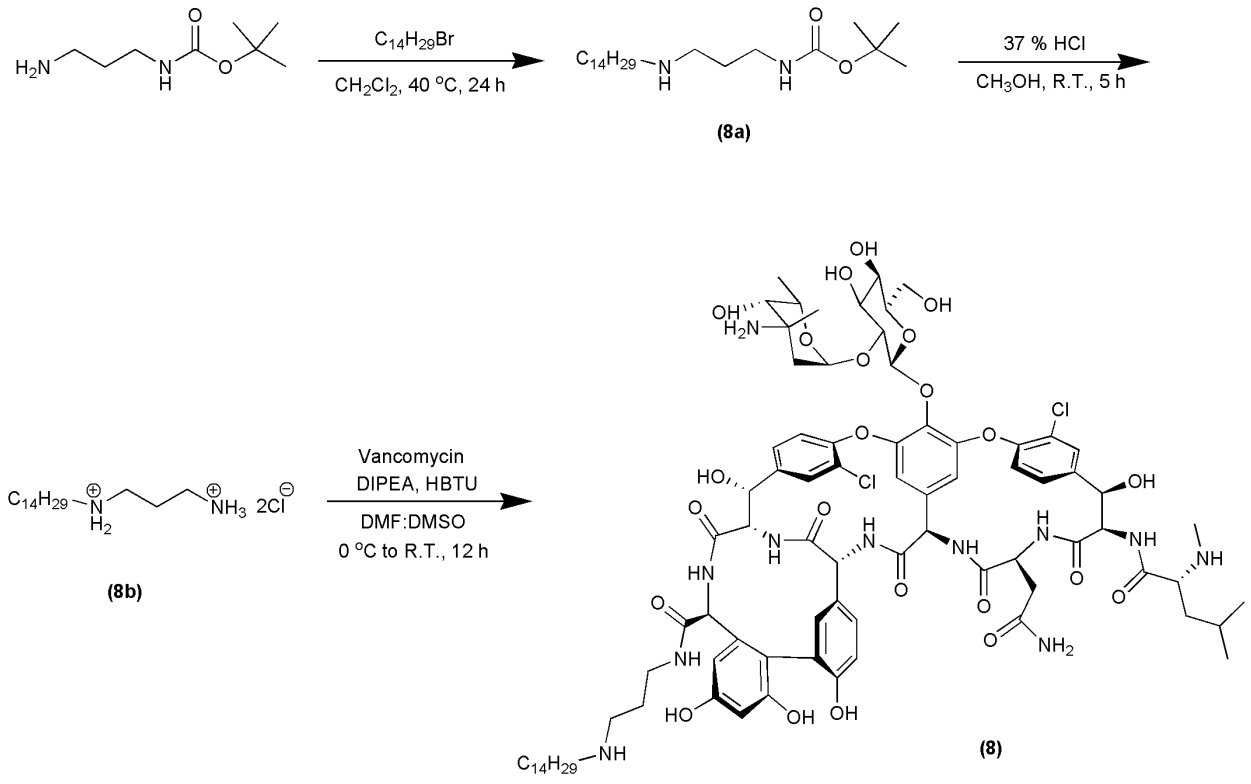
Scheme 3.1: General synthetic scheme for the preparation of new vancomycin analogues (compounds **1**-**7**).

3.2 Results

3.2.1 Synthesis

Permanent positively charged lipophilic vancomycin analogues (**2**-**7**) were prepared by coupling the carboxylic group of vancomycin with lipophilic cationic moieties varying from ethyl to octadecyl through amide coupling using *N*,*N*,*N'*,*N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) (Scheme 3.1) with 70-80 % yield. A vancomycin analogue (compound **1**) that lacked permanent positive charge and lipophilicity was synthesized by conjugating vancomycin with N^1,N^1-dimethylpropan-1,3-diamine. To prepare vancomycin

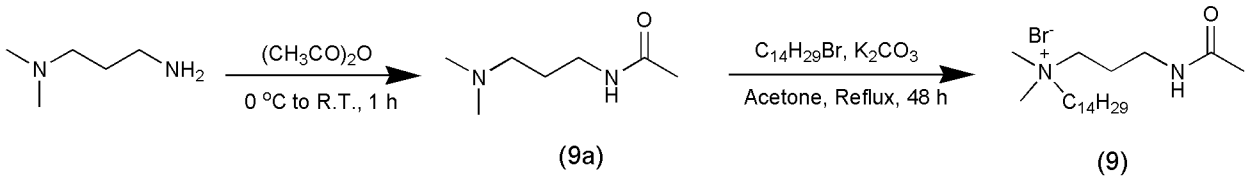
carboxamides (**1-7**), vancomycin was dissolved in 1:1 dry DMF:DMSO and HBTU solution in DMF was added drop wise at 0 ºC. Subsequently the desired amine (**12a-12f**) was added to the vancomycin solution and the reaction mixture was stirred at room temperature for 12 h. All the derivatives of vancomycin were purified by reverse phase HPLC to more than 95 % purity and characterized by ^{1}H-NMR and MALDI-MS. To synthesize the lipophilic cationic moieties (**12a-12f**), initially, N^{1},N^{1}-dimethylpropan-1,3-diamine was protected using Di-*t*-butylpyrocarbonate to give *t*-butyl (3-(dimethylamino)propyl)carbamate (**10**). Then the tertiary amine group of compound **10** was quaternized by various alkyl bromides (ethyl to octadecyl) to yield compounds **11a-11f** followed by deprotection of primary amine group under acidic conditions to afford permanent positively charged lipophilic moieties (Scheme 3.1).



Scheme 3.2: Synthesis of 3-(tetradecylamino)propyl-vancomycin carboxamide (control compound, **8**).

An important vancomycin analogue (compound **8**) was also prepared wherein vancomycin was conjugated to N^1-tetradecylpropan-1,3-diamine which does not have permanent cationic charge (Scheme 3.2). To prepare N^1-tetradecylpropan-1,3-diamine (**8b**), *t*-butyl 3-aminopropylcarbamate was monoalkylated with tetradecyl bromide and followed by deprotection of primary amine group under acidic conditions.

Another important control compound **9** was synthesized comprising lipophilicity and permanent positive charge without vancomycin (Scheme 3.3). To synthesize compound **9**, initially the primary amine group of N^1,N^1-dimethylpropan-1,3-diamine was reacted with acetic anhydride to give compound **9a** which was quaternized to afford compound **9**.



Scheme 3.3: Synthesis of permanent positively charged lipophilic moiety (compound **9**).

3.2.2 *In-vitro* antibacterial activity

The antibacterial activities of vancomycin, its derivatives **1**-**8** and compound **9** were determined against vancomycin-sensitive strains of Staphylococci (MSSA and MRSA) and Enterococci (VSE), as well as against vancomycin-resistant strains of Staphylococci (VISA and VRSA) and Enterococci (VREm; VanA phenotype and VREs, VanB phenotype). The results are summarized in Table 3.1. Against vancomycin-sensitive bacteria, compounds **1**-**7** exhibited similar or slightly better activity than vancomycin. Compounds **4** (octyl chain) and **5** (decyl chain) showed the best activities against MRSA (MIC = 0.3 μM) whereas compound **6** (tetradecyl chain) showed the potent activity against VSE (MIC = 0.1 μM). In case of intermediate-resistant strain VISA, all compounds, **1**-**7** showed better activity (MIC = 0.3-1.5 μM) than vancomycin (MIC = 13 μM). Against this strain too, compounds **4**-**6** were found to be 30 to 40-fold more active than vancomycin, with the MIC being in the range of 0.3-0.4 μM (Table 3.1). Against clinical isolate

of VRSA (VanA and VanB phenotype), compounds **1-7** showed much improved activity with the MIC in the range of 0.2-2.0 μM compared to vancomycin (> 100 μM). Again, compounds **4** and **5** demonstrated the best activity against VRSA with MIC value of 0.2 μM and were about 400-fold more active than vancomycin (Table 3.1).

When tested against pathogenic VREm (VanA phenotype) and VREs (VanB phenotype), these compounds exhibited MIC in the range of 0.7 to > 100 μM. MIC of compound **1** (devoid of lipophilicity and permanent positive charge) was found to be ineffective even at 100 μM against both the VRE. Compounds comprising lower alkyl chain; compound **2** (ethyl chain) and compound **3** (butyl chain) were found to possess moderate activity with the MICs of 85 μM and 41 μM respectively against VREs (VanB phenotype) whereas the same compounds were found to be ineffective against more virulent VREm (VanA phenotype) at 100 μM. Compound **4** consisting of octyl chain displayed significant activity against both the VRE (12.5 μM against VREm and 1.5 μM against VREs) and a gradual increase in activity was observed with increasing chain length, with compound **6** (tetradecyl chain) demonstrating an MIC value of 0.7 μM and 1 μM against VREm and VREs, respectively. However, further increase in lipophilic chain compromised the activity as was observed in octadecyl analogue of vancomycin (compound **7**) wherein the activity was found to be ~ 3-fold lesser than compound **6**. These results suggest that compound **6** possesses an optimum chain length and showed > 1000-fold higher activity than vancomycin against VREm. The MIC_{90} values of telavancin and dalbavancin against VREm (VanA phenotype) were reported to be 4 and 18 μM respectively[115,116] which are less active than compound **6** (Table 3.1).

To assess the role of installed cationic feature (permanent positive charge), the antibacterial activity of compound **8** having a lipophilic moiety bearing secondary amine (which becomes cationic under physiological conditions) was evaluated and compared the results with corresponding vancomycin derivative **6** (bearing quaternary ammonium group having permanent positive charge). The antibacterial activity of compound **8** was found to be similar to compound **6** against sensitive bacteria (Table 3.1). When tested against VREm (VanA phenotype), the activity of compound **6** was found to be 7-fold higher than compound **8** (MIC = 5 μM). These findings suggest that the appended permanent positive charge along with lipophilicity afforded substantial increase in antibacterial activity against VRE.

To evaluate the activity of cationic lipophilic portion alone (without vancomycin), compound **9** was synthesized (Scheme 3.3) as a control. The antibacterial activity of compound **9** was found to be 225-fold lower than the compound **6** against VRE. Further, the activity of a physical mixture of vancomycin and compound **9** was determined and the physical mixture was found to be inactive even up to their individual concentrations of 7 µM against VRE, whereas compound **6** showed MIC of 0.7 µM.

Table 3.1. *In-vitro* antibacterial activity of the compounds.

	Minimum Inhibitory Concentration (µM)						
Compound	MSSA	MRSA	VISA	VRSA	VSE	VREm	VREs
Vancomycin	0.6	0.6	13	>100	0.6	750	250
1	0.6	0.6	1.5	0.5	0.6	>100	>100
2	0.4	2.4	1.3	2.0	0.4	>100	85
3	0.4	0.6	1.1	2.0	0.3	>100	41
4	0.3	0.3	0.4	0.2	0.2	12.5	1.5
5	0.3	0.3	0.3	0.2	0.2	3	1.2
6	1.2	0.6	0.4	1.9	0.1	0.7	1.0
7	5.0	N.D	0.9	N.D	N.D	1.9	N.D
8	0.5	0.6	1.0	N.D	0.4	5	N.D
9	25	47	N.D	N.D	27	>50	N.D

Abbreviations: MSSA, Methicillin-sensitive *Staphylococcus aureus*; MRSA (ATCC 33591), Methicillin-resistant *Staphylococcus aureus*; VISA, Vancomycin-intermediate-resistant *Staphylococcus aureus* was generated from MRSA after treating with vancomycin for 52 passages; VSE, Vancomycin-sensitive *Enterococcus faecium*; VREm, Vancomycin-resistant *Enterococcus faecium* (VanA phenotype, ATCC 51559); VREs Vancomycin-resistant *Enterococcus faecalis* (VanB phenotype, ATCC 51575); N.D, Not determined.

3.2.3 Disruption of bacterial membrane integrity

To confirm proposed hypothesis that these compounds act by disrupting the bacterial cell membrane integrity, experiments were performed to validate the membrane disruption properties of compounds **1**-**9**, vancomycin and physical mixture of vancomycin and compound **9** using fluorescence spectroscopy against VRE (VanA phenotype). Firstly, the abilities of these compounds to depolarize bacterial membranes were examined using the membrane potential sensitive dye $DiSC_3(5)$ ((3, 3'-dipropylthiadicarbocyanine iodide)). In this experiment, the dye was added to bacterial cells, and the change in fluorescence intensity was monitored. As the dye accumulates in the membranes the fluorescence intensity decreases because of self-quenching. Upon disruption of the membrane potential by the compound, an increase in fluorescence is observed due to $DiSC_3(5)$ being displaced into the solution. Dissipation of membrane potential of VRE was observed in less than three minutes by compounds **4** (octyl chain) to **7** (octadecyl chain) at 10 µM. On the other hand compounds **1**-**3** (butyl chain) and vancomycin were ineffective (Fig. 3.1A).

The effect of the new compounds on bacterial membrane integrity was also confirmed by studying the kinetics of bacterial cytoplasmic membrane permeabilization by measuring the uptake of the fluorescent probe, propidium iodide (PI). This dye enters only membrane-compromised cells and fluoresces upon binding to nucleic acids. Unlike vancomycin and compounds **1**-**3**, which did not cause significant membrane permeability, compounds **4**-**7** showed strong ability to permeabilize the cytoplasmic membrane of VRE at a concentration of 10 µM (Fig. 3.1B).

It is known that release of potassium ion from the bacterial cell occurs upon disruption of the membrane potential. An experiment was performed to find out the release of potassium ion caused by the compounds (10 µM) using potassium ion sensitive fluorophore, PBFI-AM against VRE considering valinomycin as a positive control. Compounds **4**-**7** caused significant leakage of intracellular potassium like valinomycin, whereas vancomycin and compounds **1**-**3** were ineffective (Fig. 3.1C). Hence, bacterial exposure to compounds **4**-**7** resulted in increased membrane permeability, perturbation of cell membrane potential and finally leakage of intracellular K^+, while vancomycin and compounds **1**-**3** showed no such effect.

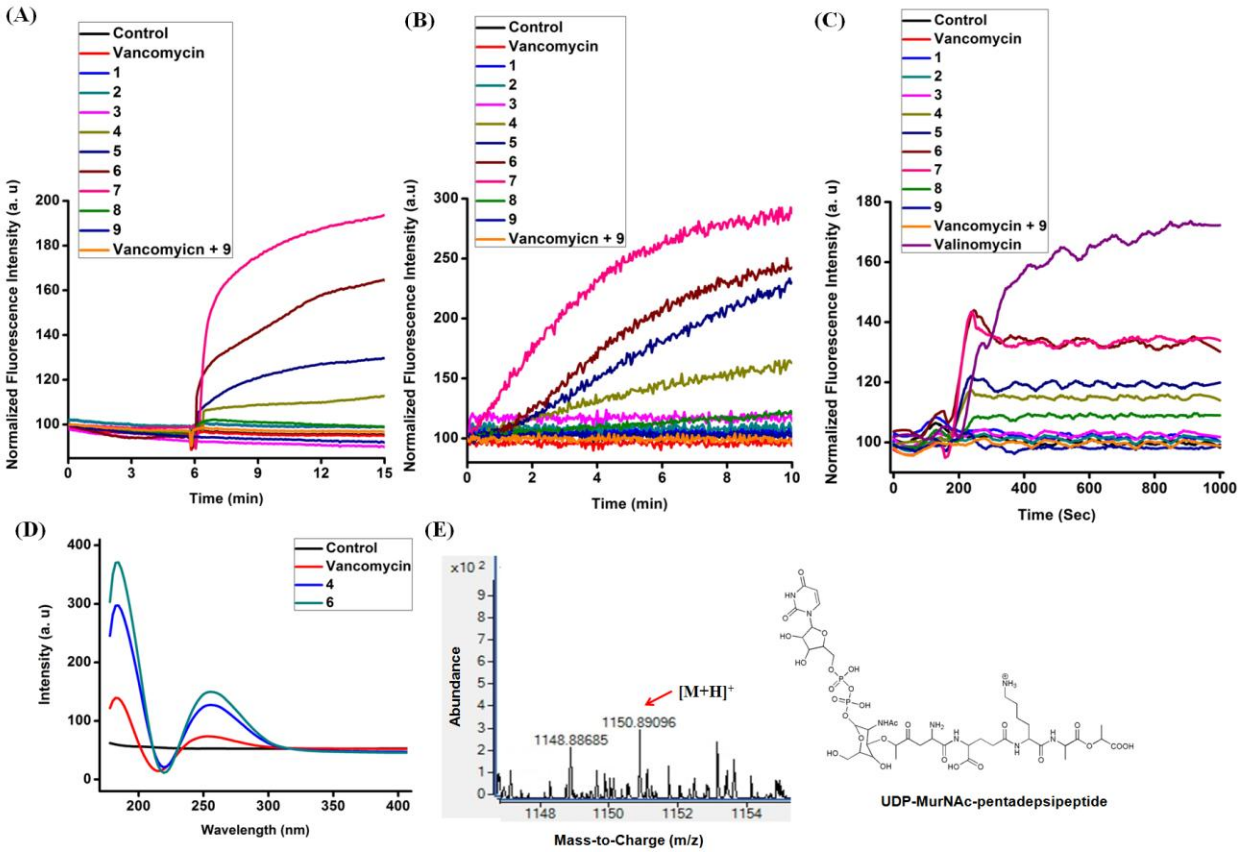


Figure 3.1: Disruption of bacterial cell membrane integrity of vancomycin, compounds **1**-**7**, control compounds **8**, **9** and physical mixture of vancomycin and **9** at 10 µM against VRE, VanA phenotype (3.1A, 3.1B and 3.1C). (A) Cytoplasmic membrane depolarization, (B) Cytoplasmic membrane permeabilization and (C) Intracellular K^+ ion leakage; Intracellular accumulation of the cell wall precursor UDP-MurNAc-pentadepsipeptide after treatment of VRE with vancomycin, compounds **4** and **6** at 5 µM. Untreated cells were used as control (3.1D and 3.1E). (D) Identification of intracellular UDP-MurNAc-pentadepsipeptide by monitoring absorbance at 260 nm wavelength and (E) UDP-MurNAc-pentadepsipeptide was identified by mass spectrometry as indicated by the peak at m/z 1150.89.

To ascertain the role of permanent positive charge, membrane disruption studies were carried out with compound **8** (having secondary amine which becomes cationic at pH 7.4) and the results were compared with corresponding vancomycin derivative **6** (quaternary ammonium

group having permanent positive charge). Compound **8** did not show any membrane disruption effect at 10 μM whereas corresponding vancomycin derivative **6** showed substantial membrane disruption effect at 10 μM against VRE (Fig. 3.1A, 3.1B and 3.1C). This indicates that installed cationic feature (permanent positive charge in compound **6**) along with lipophilicity plays a vital role in producing membrane disruption effect thereby having high antibacterial activity.

Also, membrane disruption studies of compound **9** (cationic lipophilic portion alone) and its physical mixture with vancomycin were carried out against VRE. None of them showed any membrane disruption effect at 10 μM which, was also reflected in their antibacterial activity against VRE (Fig. 3.1A, 3.1B and 3.1C). This implies that lipophilic cationic moiety should be conjugated to vancomycin to impart membrane disruption properties at low concentrations.

3.2.4 Intracellular accumulation of cell wall (peptidoglycan) precursor

In order to investigate whether new vancomycin derivatives interfere with cell wall (peptidoglycan) biosynthesis, the accumulation of UDP-linked peptidoglycan precursor, UDP-*N*-acetyl-muramyl-pentadepsipeptide (UDPMurNAc-pp) was determined after treating the bacteria (VRE) with new vancomycin derivatives, compounds **4**, **6** and vancomycin at 5 μM. In case of compounds **4** and **6**, a more intense peak was observed at 260 nm compared to vancomycin, which corresponds to accumulation of UDPMurNAc-pp and confirmed by high-resolution mass spectrometry (m/z = 1150.94 (cal), 1150.89 (obs) for $[M+H]^+$) (Fig. 3.1D & 3.1E). Further, compound **6** caused more accumulation of cell wall precursor compared to compound **4**. These results suggest that compounds **4** and **6** showed greater cell wall biosynthesis inhibition than vancomycin.

3.2.5 Bactericidal activity

Next, *in-vitro* time-kill assay was performed with compounds **4**, **6** and vancomycin against MRSA (starting bacterial concentration of 8 $\log_{10}$ CFU/mL), at two different concentrations (1 × MIC and 6 × MIC). An impressive and rapid bactericidal activity was observed with compounds **4** and **6**, which increased with increasing concentration. Compound **4** produced ~ 3 $\log_{10}$ CFU/mL reduction in bacterial count at 6 × MIC, whereas at 1 × MIC it was limited to being bacteriostatic (~ 8 $\log_{10}$ CFU/mL). Meanwhile, compound **6** was rapidly bactericidal at 1 × MIC and 6 × MIC (> 5 $\log_{10}$ CFU/mL reduction) and retained its bactericidal activity till 24 h at 6 ×

MIC. Conversely, bacteriostatic effect was observed at 24 h for compound **6** at 1 × MIC. In case of vancomycin, even though concentration dependent action was seen from 1 × MIC to 6 × MIC, but it was restricted to being bacteriostatic unlike compounds **4** and **6** (Fig. 3.2A).

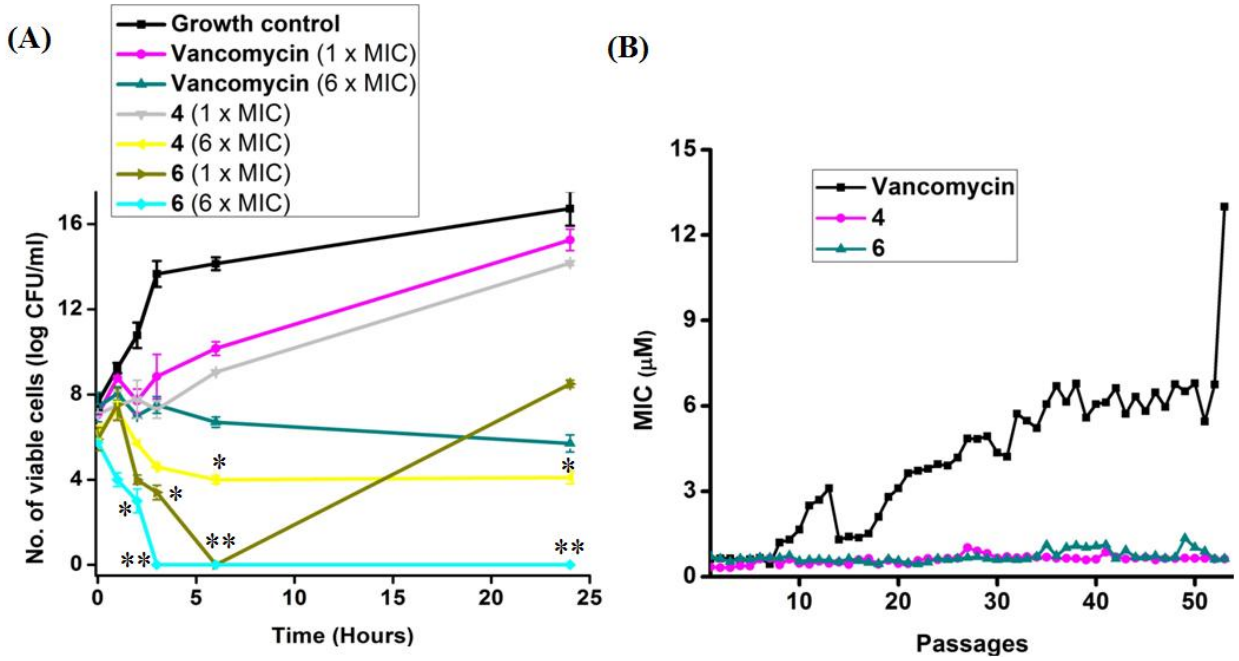


Figure 3.2: (A) Bactericidal properties of vancomycin, compounds **4** and **6** against MRSA. Single stars correspond to reduction of 3 $\log_{10}$ CFU/mL and double stars correspond to < 50 CFU/mL (detection limit). (B) Bacterial resistance studies of vancomycin, compounds **4** and **6** against MRSA.

3.2.6 Propensity to induce bacterial resistance

The escalating rise of drug resistance in bacteria guided to evaluate the possible emergence of bacterial resistance against this new class of compounds. The propensity of bacteria to cause resistance can be evaluated through serial exposure of organisms to antimicrobial agents. MRSA was exposed to vancomycin and compounds **4** and **6** for serial passages and monitored the changes in MIC values over a period of 52 days. Even after 52 serial passages, the MIC of compounds **4** and **6** remained the same. However, in case of vancomycin, the MIC value started increasing after 7 passages and the value increased to 16-fold after 25 passages (Fig. 3.2B). Thus

bacteria are less likely to acquire resistance against this type of compound and this emphasizes the endurance of such compounds in clinics.

Table 3.2. *In-vitro* toxicity of the compounds.

Compound	Toxicity (μM)	
	Hemolytic activity (HC_{50})	Cytotoxicity (CC_{50})
Vancomycin	>1000	>1000
4	>1000	>1000
6	>100	>100
9	125	27

Abbreviations: HC_{50}, 50 % hemolytic concentration; CC_{50}, 50 % cytotoxic concentration.

3.2.7 *In-vitro* toxicity (hemolysis and cytotoxicity)

As these derivatives were shown to disrupt the bacterial cell membrane integrity, the toxicity of compounds **4** and **6** were studied by measuring cytotoxicity (CC_{50}; 50 % cytotoxic concentration) against mammalian cells (HeLa) and hemolytic activity (HC_{50}; 50 % hemolytic concentration) against human RBC. None of the derivatives showed significant toxicity towards either of the cells even up to 100 μM concentration. Notably compound **4** did not show any hemolytic activity even up to 1000 μM (Table 3.2). The selectivity (HC_{50}/MIC) of control cationic lipophilic compound **9** was found to be ~ 5 whereas corresponding vancomycin analogue **6** showed selectivity > 140, which indicates the selective toxicity of compound **6** against bacterial cells compared to control compound **9**.

3.2.8 *In-vivo* antibacterial activity

To demonstrate the potential of these compounds for *in-vivo* applications, the most non-toxic vancomycin derivative, compound **4** was selected for further studies. *In-vivo* activity of

compound **4** was evaluated in a renal infection model against VRE. Initially, mice were injected intravenously with 0.2 mL of 0.2 % λ-carrageenan to increase their susceptibility to bacterial renal infection. After 7 days, mice were infected with VRE (VanB phenotype, ~ 10^8 CFU/mouse). After 4 h of infection the mice were treated with three doses (every 24 h interval) of vancomycin (12 mg/kg), linezolid (12 mg/kg), compound **4** (12 mg/kg) and saline (Fig. 3.3A). After 72 h of the initial treatment, antibacterial activity was determined by finding the bacterial titer in the infected kidneys. In comparison to vancomycin, compound **4** and linezolid reduced bacterial titer from the infected kidneys more effectively. Linezolid produced ~ 4 $\log_{10}$ CFU reduction compared to vehicle treated control (saline) whereas compound **4** produced ~ 5.0 $\log_{10}$ CFU reduction (Fig. 3.3B).

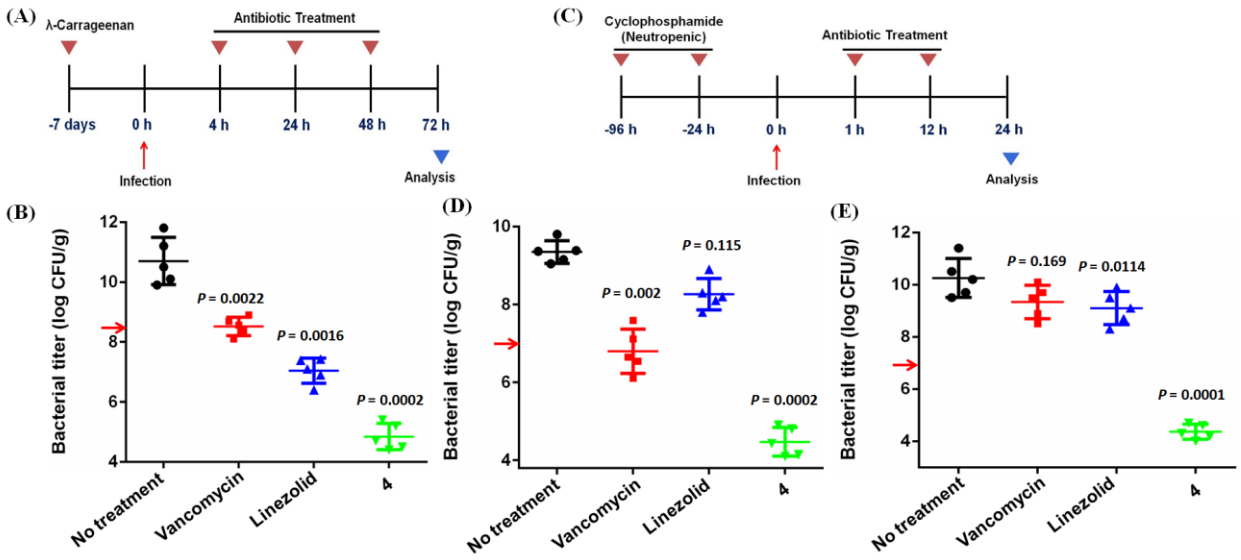


Figure 3.3: *In-vivo* antibacterial studies. *In-vivo* activity of vancomycin, linezolid and compound **4** in renal infection model against VRE (n = 5) at 12 mg/kg. (A) Experimental design for VRE infection and (B) Experimental data; *In-vivo* activity of compound **4** in comparison with vancomycin and linezolid against MRSA and VISA (n = 5) at 12 mg/kg. (C) Experimental design for MRSA or VISA infection, (D) Experimental data for MRSA and (E) Experimental data for VISA Differences are considered statistically significant from untreated group with a value of $P < 0.05$. Red arrow in (B), (D) and (E) indicates bacterial pre-treatment titer (~ 8 $\log_{10}$ CFU/g for VRE kidney infection and ~ 7 $\log_{10}$ CFU/g for VISA and MRSA thigh infection).

In another study, the *in-vivo* activity of compound **4** was evaluated in a neutropenic mouse thigh infection model against MRSA and VISA. In this study, mice were infected with MRSA and VISA (~ 10^7 CFU/mouse) in the thigh. After 1 h of infection the mice were treated with a double dose (q12 h) of compound **4**, vancomycin and linezolid at 12 mg/kg and saline being used as control (Fig. 3.3C). After 24 h of the initial treatment, antibacterial activity was determined by finding the bacterial titer in the infected thighs. Against MRSA infection, vancomycin resulted in no change in bacterial growth from the pre-treatment titer (ED_{stasis}), whereas compound **4** showed high activity, producing 3.0 $\log_{10}$ CFU/g reduction in bacterial count from the initial titer ($ED_{3\text{-log kill}}$). On the other hand linezolid showed a minimal response from the saline treated mice (24 h) against MRSA (Fig. 3.3D). Unlike vancomycin and linezolid, compound **4** showed significantly high activity against VISA infection wherein it produced 4.8 $\log_{10}$ CFU reduction compared to the saline treated control (Fig. 3.3E).

Table 3.3. Point dose estimate required to achieve different pharmacodynamic end points for compound **4** against VISA thigh infection model.

Bacteria	Pharmacodynamic end points (mg/kg)				
	ED_{50}	ED_{stasis}	$ED_{1\text{-log kill}}$	$ED_{2\text{-log kill}}$	$ED_{3\text{-log kill}}$
VISA (Pretreatment ~7 $\log_{10}$ CFU/g)	2.4	5.0	9.4	11.3	N.D

Abbreviations: ED_{50}, 50 % effective dose; ED_{stasis}, $\log_{10}$ stasis dose; $ED_{1\text{-log kill}}$, dose required to cause a decrease in titer of 1 $\log_{10}$ CFU/g from the pre-treatment titer (0 h); $ED_{2\text{-log kill}}$, dose required to cause a decrease in titer of 2 $\log_{10}$ CFU/g; $ED_{3\text{-log kill}}$, dose required to cause a decrease in titer of 3 $\log_{10}$ CFU/g; N.D, Not determined.

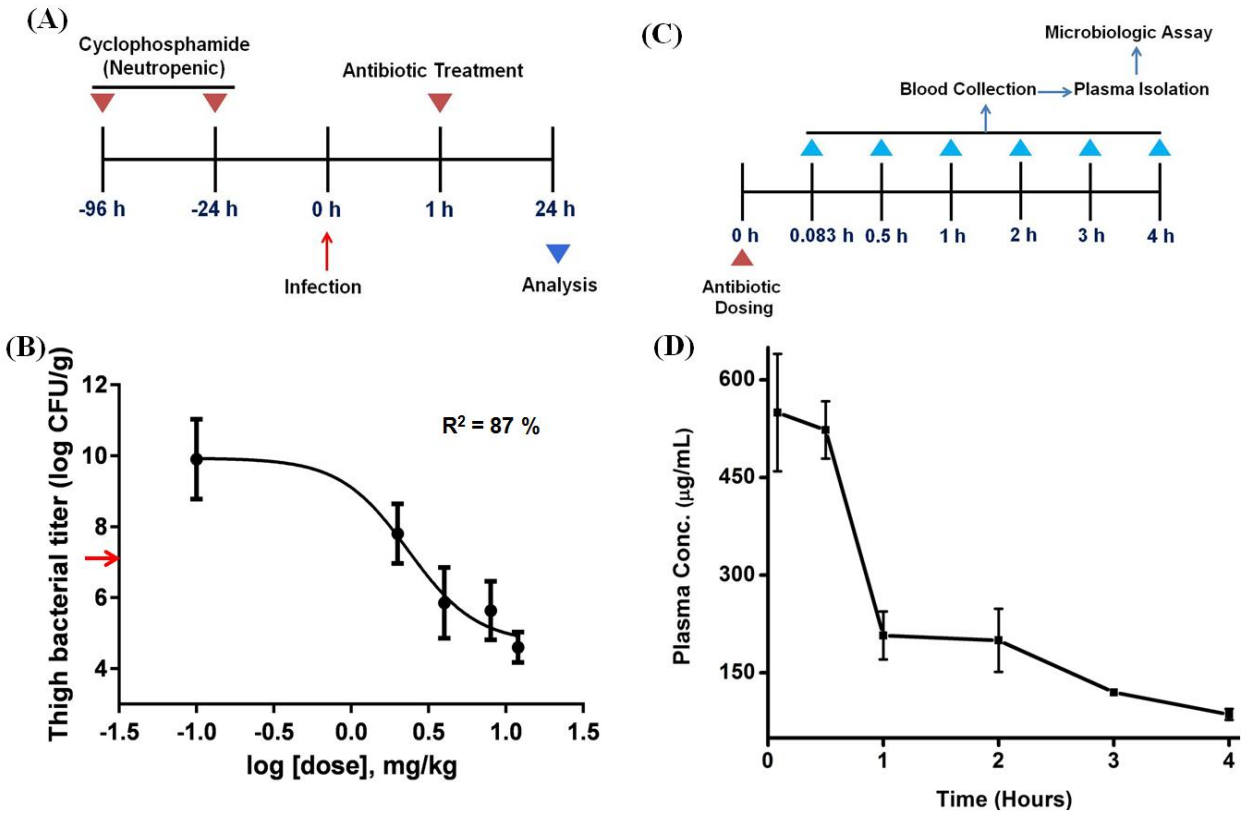


Figure 3.4: Pharmacological studies: Dose-response relationship of compound **4** in thigh infection model against VISA. (A) Experimental design and (B) Experimental data. Red arrow in (B) indicates bacterial pretreatment titer (7.0 $\log_{10}$ CFU/g); Single-dose pharmacokinetic study of compound **4** at 12 mg/kg. (C) Experimental design and (D) Experimental data.

3.2.9 Dose-response relationship against VISA

The potent activity of compound **4** against MRSA, VISA and VRE infections in mice models driven further to examine the effect of dose response on its efficacy. In this study, VISA was chosen as a model organism wherein after 1 h of infection a single dose of compound **4** at different regimens (2, 4, 8 and 12 mg/kg) was administered intravenously (Fig. 3.4A). The pretreatment bacterial titer in the thigh was 7.0 ± 0.9 $\log_{10}$ CFU/g. In saline treated controls, thigh titer increased to 9.1 ± 0.4 $\log_{10}$ CFU/g within 24 h. Compound **4** produced comparable dose dependent reductions in the bacterial titer at each of four dosing regimens (Fig. 3.4B). The single dose that resulted in 50 % maximal bacterial killing (ED_{50}) was 2.4 mg/kg (Table 3.3). The

compound **4** dose that resulted in a 24 h colony count similar to the pretreatment count was 5 mg/kg (ED_{stasis}). The value of 1-log_{10} kill dose ($ED_{1\text{-log kill}}$) for compound **4** was 9.4 mg/kg. At the highest dosing regimen (12 mg/kg), compound **4** showed > $ED_{2\text{-log kill}}$ (Fig. 3.4B).

Table 3.4. Single-dose pharmacokinetic parameters of compound **4** at 12 mg/kg.

Drug	Pharmacokinetics parameters				
	C_{max} (µg/mL)	C_{min} (µg/mL)	$AUC_{0\text{-}4\,h}$ (µg/mL/h)	$t_{1/2}$ (h)	Clearance (L/h/Kg)
Compound **4**	543	90	910	1.6	0.013

C_{max}, peak plasma concentration; C_{min}, trough plasma concentration; $AUC_{0\text{-}4\ h}$, area under the concentration-time curve from 0-4 h; $t_{1/2}$, half-life.

3.2.10 Single-dose pharmacokinetics

The pharmacokinetics of i.v. administered compound **4** in mice is shown in Fig.3.4C & 3.4D. The compound demonstrates increased exposure as measured by area under concentration curve (AUC) in mice. Time-concentration profiles of plasma for compound **4** are presented in Fig. 3.4D. Peak concentration in plasma was found to be 543 µg/mL. Pharmacokinetic parameters of compound **4** were determined by non-compartmental analysis (Table 3.4). The AUC value in plasma, calculated from 0.083 h to 4 h was 910 µg/mL/h. The plasma half-life ($t_{1/2}$) of compound **4** was found to be 1.6 h with a clearance rate of 0.013 L/h/Kg.

3.2.11 *In-vivo* toxicology studies

Next, the *in-vivo* systemic toxicity of compound **4** was assessed after single-dose intravenous (i.v.) administration to mice (n = 5) and the LD_{50} value was determined to be 78 mg/kg. Further, the acute toxicity of compound **4** was investigated to major organs in the body of the mice by

examining the clinical biochemistry parameters in the blood of the mice at a concentration of pharmacodynamic point, > $ED_{2\text{-log kill}}$ (i.v., dose of 12 mg/kg) and here normal saline was used as negative control. The levels of the functional parameters of the liver (ALT, Alanine transaminase) and kidney (Urea nitrogen & Creatinine) and the concentrations of electrolytes in the blood (Sodium, Potassium and Chloride) were unchanged after 48 h and 14 days (Table 3.5). These studies indicate that compound **4** did not cause any significant acute damage to liver and kidney functions, nor did it interfere with the balance of electrolytes in the blood.

Table 3.5. *In-vivo* acute toxicology of compound **4** at 12 mg/kg

	Liver (U L^{-1})	**Kidney (mg dL^{-1})**		**Electrolytes in the blood (mmol L^{-1})**		
Treatment	ALT	Urea Nitrogen	Creatinine	Potassium ion	Sodium ion	Chloride ion
Saline	60 ± 9	22 ± 3	0.3 ± 0.2	9 ± 1	143 ± 0.8	107 ± 2
48 h post-treatment	64 ± 9 $P = 0.47$	22 ± 2 $P = 0.9$	0.3 ± 0.1 $P = 0.32$	8 ± 0.7 $P = 0.075$	144 ± 1 $P = 0.32$	108 ± 2 $P = 0.39$
14 days post treatment	63 ± 24 $P = 0.756$	17 ± 3 $P = 0.45$	0.2 ± 0.1 $P = 0.867$	10 ± 1 $P = 0.78$	137 ± 1 $P = 0.139$	106 ± 1 $P = 0.228$
Laboratory range*	63-307	17-35	0.2-0.8	6.3-10	140-150	104-120

The data are expressed as mean ± standard deviation, based on values obtained from 10 mice (n = 10). Statistical analysis was performed using Student's *t*-test. Differences are considered statistically significant with a value of $P < 0.05$. ALT, alanine transaminase. *Source: Charles River laboratories.

3.3 Discussion

In this report, a permanent cationic (quaternary ammonium group) hydrophobic moiety was appended to vancomycin using a facile synthetic methodology to impart a new mode of action to the existing drug. The antibacterial activities of these derivatives were seen to be dependent on the chain length of the lipophilic moiety as well as on the bacterial strains. In case of vancomycin sensitive strains such as MSSA, MRSA and VSE, presumably the effect of vancomycin

predominates. The compounds have similar to slightly better activity compared to vancomycin, and the differences in activities among the compounds are not very dramatic. However, a more definite trend in activity versus chain length was observed in the case of the resistant strains such as VISA, VRSA and VRE, where the permanent positively charged lipophilic unit contributes more significantly to the antibacterial action. Compounds **1**-**3** had low (VISA and VRSA) to no (VRE) activity, whereas compound **4** onwards the antibacterial activity started to increase thus successfully overcoming the vancomycin resistance. The *in-vitro* antibacterial activities of these new derivatives were comparable to second generation of glycopeptides antibiotics such as oritavancin, telavancin and dalbavancin against sensitive bacteria. However, against VRE, compound **6** showed 7-fold, 25-fold and > 1000-fold more activity than telavancin, dalbavancin and vancomycin respectively. The enhanced antibacterial activity of these cationic lipophilic vancomycin derivatives may be attributed to their increased cationic charge and lipophilicity compared to vancomycin, which facilitates better interaction of the compounds with the negatively charged bacterial membrane. This increased association with the bacterial membranes presumably serves to anchor the drug thereby allowing it to stay for a longer time at the cell wall region and results in enhanced inhibition of cell wall biosynthesis.

The mechanism of antibacterial action of these vancomycin derivatives was evaluated against VRE. The compounds **4**-**7** (bearing octyl chain and above) caused rapid membrane depolarization, showed strong ability to permeabilize the cytoplasmic membrane and displayed significant leakage of intracellular potassium. On the other hand, vancomycin and compounds **1**-**3** (bearing either no alkyl chain or smaller alkyl chain length than octyl) did not cause any membrane disruption. These results show that octyl chain is the minimal hydrophobic moiety required to cause bacterial membrane disruption. It was also found that the membrane disruption action increased with increase in length of alkyl chain from octyl chain onwards. The detailed mechanistic studies substantiate that the conjugation of permanent positively charged lipophilic moieties to vancomycin impart a new mode of action to the drug, namely that of bacterial membrane disruption. This mode of action contributes to the rapid bactericidal activity of these compounds whereas vancomycin showed bacteriostatic action. Most significantly, the introduction of this new mechanism of action to vancomycin had a significant impact on stalling the development of bacterial resistance to the drug because of the complexity in remodeling

bacterial membrane in a way that is compatible with bacterial survival. It was also established the importance of permanent positive charge over soft charge (secondary amine in compound **8**) for potent antibacterial activity and the need to have the lipophilic quaternary ammonium moiety covalently connected to vancomycin as opposed to a physical mixture. Absence of significant *in-vitro* toxicity against hRBC and HeLa cells suggests that these compounds interact specifically to more negatively charged bacterial cells compared to mammalian cells.

Bacterial infections caused by VRE, VISA and MRSA have been increasing in frequency, representing an emerging threat to public health.[35,121] Compound **4** was significantly more potent than comparator drugs, vancomycin and linezolid against VRE in renal infection model. Further, compound **4** was more active than vancomycin and linezolid against MRSA and VISA thigh infections. The effect of dose response of compound **4** produced dose dependent reductions in the bacterial titer against VISA. The superior activity of the compound in the present study could be explained, in part, by the rapid bactericidal activity, which potentially emanated from its additional mechanism of action, namely bacterial membrane disruption. Single-dose pharmacokinetic study demonstrated that compound **4** has improved pharmacological properties, which also supported its superior *in-vivo* antibacterial activity. At a dosage of 12 mg/kg, plasma levels of compound **4** remained at 90 µg/mL after 4 h, which also implies its potential *in-vivo* activity due to prolonged drug exposure.

Then the acute toxicology of compound **4** was performed by determining the biochemical parameters related to liver and kidney. Analysis of biochemical parameters revealed that compound **4** did not induce any significant changes in functional constraints of liver and kidney and did not interfere with the balance of electrolytes in the blood of mice at 48 h post treatment compared to vehicle control (saline) and laboratory parameters. These parameters remained almost unchanged even at 14 days post-treatment. All the parameters tested related to the function of major organs like liver, kidney and electrolytes in the blood of mice were found to be well within the acceptable laboratory range. This study showed that compound **4** has low or no *in-vivo* systemic and acute toxicity in mice models and have a good safety profile required for therapeutic applications.

3.4 Conclusions

The rational strategy described here paves way in the development of antibiotics for the treatment of vancomycin-resistant bacterial infections. The incorporation of lipophilic moiety into vancomycin along with installed permanent positive charge makes these compounds distinct from other existing derivatives in their ability to cause strong bacterial membrane disruption. This is significantly portrayed in the high antibacterial activity of the compounds against VISA, VRSA and VRE and their ability to stall the development of bacterial resistance. An optimized compound showed high *in-vivo* antibacterial activity in mouse infection models against drug-resistant bacteria (MRSA, VISA and VRE) and demonstrated improved pharmacological properties with no observed toxicity. These findings emphasize that this strategy would be a beneficial extension to the therapeutic armamentarium for the treatment of infections compelled by drug-resistant bacteria.

3.5 Experimental procedure

3.5.1 Materials and Methods

All reagents were purchased from Sigma-Aldrich and SD Fine and used without further purification. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F_{254} (250 μm thickness). Visualization was accomplished using UV light and Iodine. Column chromatography was performed on silica gel (60-120 Å pore size). All final compounds were purified by reverse phase HPLC using 0.1 % trifluoroacetic acid (TFA) in water/acetonitrile (0-100 %) as mobile phase to more than 95 % purity. HPLC analysis was performed on a Shimadzu-LC 8 Å Liquid Chromatography instrument (C_{18} column, 10 mm diameter, 250 mm length) with UV detector monitoring at 270 nm. Nuclear magnetic resonance spectra were recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. High resolution mass spectra (HR-MS) were obtained using 6538-UHD Accurate Mass Q-TOF LC-MS instrument. MALDI mass spectra (MALDI-MS) were obtained using Bruker Ultraflex II MALDI/TOF mass spectrometer. Eppendorf 5810R centrifuge was used. TECAN (Infinite series, M200 pro) Plate Reader was used to measure absorbance. Bacterial strains, MRSA ATCC 33591, Enterococcal strains were obtained from ATCC (Rockville, MD). Tryptic-soy agar media

was used for Staphylococci and sheep blood agar plates were used for Enterococci. VISA, Vancomycin-intermediate-resistant *S. aureus* was generated from MRSA (ATCC 33591) after treating with vancomycin for 52 passages. Vancomycin-resistant *S. aureus* MMC-20 (VRSA, VanA and VanB phenotypes) was collected from Midnapore medical college, West Bengal, India.

Animals: The same as described in the section of 2.5.1 in Chapter 2.

3.5.2 Synthesis and characterization

***tert*-Butyl (3-(dimethylamino)propyl)carbamate (10)**: N^1,N^1-Dimethylpropan-1,3-diamine (3.27 g, 31.9 mmol) was dissolved in 1M NaOH solution and two equivalents of $(Boc)_2O$ (27.92 g, 127 mmol) was added to it. The reaction mixture was stirred vigorously at room temperature for 10 h. Then the reaction was stopped and compound **10** was extracted into the organic layer using chloroform. The resultant organic solution was evaporated and dried to afford colourless oily *tert*-butyl (3-(dimethylamino)propyl)carbamate with 70 % yield. ^{1}H-NMR (400 MHz, CD_3OD) δ/ppm: 3.07-3.04 (t, 2H), 2.4-2.33 (q, 2H), 2.26 (s, 6H), 1.69-1.62 (m, 2H), 1.43 (s, 9H).^{13}C-NMR (100 MHz, CD_3OD) δ/ppm: 157.2, 78.43, 57.32, 45.784, 38.8, 26.2.

3-((*tert*-Butoxycarbonyl)amino)-*N*,*N*-dimethyl-*N*-alkylpropan-1-aminium bromide (11a-11f): Compound **10** (1 g, 4.92 mmol) was dissolved in dry chloroform (10 mL) in a sealed tube and appropriate alkyl bromide (9.84 mmol) was added to it. The reaction mixture was refluxed for 48 h. Then the required compounds were purified by column chromatography ($CHCl_3$/CH_3OH) using Silica gel to afford quaternized derivatives, **11a-11f** in 60-65 % yield.

3-((*tert*-Butoxycarbonyl)amino)-*N*,*N*-dimethyl-*N*-ethylpropan-1-aminium bromide (11a): ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.76 (bs, 1H), 3.64-3.59 (m, 4H), 3.43-3.41 (t, 2H), 3.29 (s, 6H), 3.26-3.21 (q, 2H), 2.06-1.99 (t, 3H), 1.38 (s, 9H).

3-((*tert*-Butoxycarbonyl)amino)-*N*,*N*-dimethyl-*N*-butylpropan-1-aminium bromide (11b): ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.78 (bs, 1H), 3.62-3.58 (m, 2H), 3.47-3.40 (t, 4H), 3.29 (s, 6H), 3.24-3.20 (q, 2H), 2.05-1.98 (q, 2H), 1.72-1.64 (m, 2H), 1.39 (s, 9H), 0.97-0.93 (t, 3H).

3-((*tert*-Butoxycarbonyl)amino)-*N*,*N*-dimethyl-*N*-octylpropan-1-aminium bromide (11c): ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.65-5.62 (t, 1H), 3.71-3.67 (t, 2H), 3.44-3.39 (t, 2H), 3.33

(s, 6H), 3.29-3.24 (q, 2H), 2.08-2.01 (m, 2H), 1.73-1.67 (m, 2H), 1.35-1.25 (m, 10H), 1.43 (s, 9H), 0.89-0.86 (t, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 156.2, 64.67, 62.31, 51.31, 31.59, 29.1, 28.98, 28.4, 26.3, 23.5, 22.8, 22.5, 14.01.

3-((*tert*-Butoxycarbonyl)amino)-*N,N*-dimethyl-*N*-decylpropan-1-aminium bromide (11d): ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.78 (bs, 1H), 3.62-3.61 (t, 2H), 3.41-3.37 (t, 2H), 3.29 (s, 6H), 3.24-3.23 (t, 2H), 2.47-2.5 (m, 2H), 2.1-2.06 (m, 2H), 1.68 (m, 2H), 1.39 (s, 9H), 1.28-1.22 (m, 14H), 0.86-0.82 (t, 3H).^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 156.18, 80.79, 64.26, 61.92, 48.51, 45.00, 31.90, 29.63, 29.59, 29.49, 29.38, 29.33, 29.00, 28.29, 26.67, 26.10, 22.66, 14.08.

3-((*tert*-Butoxycarbonyl)amino)-*N,N*-dimethyl-*N*-tetradecylpropan-1-aminium bromide bromide (11e): ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 5.68 (bs, 1H), 3.68-3.64 (t, 2H), 3.43-3.39 (t, 2H), 3.33 (s, 6H), 3.29-3.23 (m, 2H), 2.07-2.00 (m, 2H), 1.71-1.7 (m, 2H), 1.42 (s, 9H), 1.33-1.23 (m, 22H), 0.88-0.84 (t, 3H).^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 156.21, 79.5, 64.57, 62.25, 51.34, 37.52, 31.89, 29.64, 29.61, 29.55, 29.44, 29.36, 29.32, 29.18, 28.41, 26.28, 23.46, 22.77, 22.65, 14.08.

3-((*tert*-Butoxycarbonyl)amino)-*N,N*-dimethyl-*N*-octadecylpropan-1-aminium bromide (11f): ^{1}H-NMR (400MHz, $CDCl_3$) δ/ppm: 5.61-5.59 (t, 1H), 3.72-3.68 (t, 2H), 3.44-3.40 (t, 2H), 3.34 (s, 6H), 3.3-3.25 (q, 2H), 2.09-2.02 (m, 2H), 1.81-1.67 (m, 2H), 1.43 (s, 9H), 1.34-1.25 (m, 30H), 0.89-0.86(t, 3H).^{13}C-NMR (100 MHz, $CDCl_3$) δ/ppm: 156.1, 79.57, 64.65, 62.3, 51.28, 37.57, 31.9, 29.68, 29.66, 29.63, 29.56, 29.44, 29.36, 29.33, 29.18, 28.4, 26.28, 23.47, 22.78, 22.66, 14.08.

3-Amino-*N*-alkyl-*N,N*-dimethylpropan-1-aminium chloride (12a-12f): Quaternized NH-Boc compounds **11a-11f** were dissolved in MeOH and 37 % HCl. The reaction mixture was stirred at room temperature for 5 h and dried in vacuum to afford 3-amino-*N*-alkyl-*N,N*-dimethylpropan-1-aminium chloride derivatives **12a-12f** in quantitative yield.

3-Amino-*N*-ethyl-*N,N*-dimethylpropan-1-aminium chloride (12a): ^{1}H-NMR (400MHz, D_2O) δ/ppm: 3.5-3.45 (m, 6H), 3.15-3.13 (m, 2H), 3.13 (s, 6H), 3.09-3.05 (t, 3H). ^{13}C-NMR (100 MHz, D_2O) δ/ppm: 61.56, 50.9, 37.8, 28.71, 22.2, 8.5. MALDI-MS: *m/z* 130.99 (observed); 131.25 (calculated for M^+).

3-Amino-*N*-butyl-*N,N*-dimethylpropan-1-aminium chloride (12b): ^{1}H-NMR (400 MHz, CD_3OD) δ/ppm: 3.54-3.49 (m, 2H), 3.43-3.38 (m, 4H), 3.17 (s, 6H), 3.11-3.0 (t, 2H), 1.48-1.39 (m, 4H), 1.05-1.01 (t, 3H). ^{13}C-NMR (100MHz, CD_3OD) δ/ppm: 65.8, 61.9, 51.46, 51.42, 37.67, 25.41, 22.07, 20.05, 13.8. MALDI-MS: *m/z* 160.31 (observed); 159.06 (calculated for M^+).

3-Amino-*N*-octyl-*N,N*-dimethylpropan-1-aminium chloride (12c): ^{1}H-NMR (400 MHz, CD_3OD) δ/ppm: 3.61-3.56 (q, 2H), 3.54-3.48 (t, 2H), 3.42-3.38 (t, 2H), 3.18 (s, 6H), 3.3-3.1 (t, 2H), 2.27-2.21 (m, 2H), 1.85-1.8 (m, 2H), 1.39-1.31 (m, 10H), 0.91-0.88 (t, 3H). ^{13}C-NMR (100 MHz, CD_3OD) δ/ppm: 66.1, 62.0, 61.9, 51.4, 45.66, 37.7, 30.2, 27.28, 23.54, 23.52, 22.06, 14.35. MALDI-MS: *m/z* 216.23 (observed); 216.41 (calculated for M^+).

3-Amino-*N*-decyl-*N,N*-dimethylpropan-1-aminium chloride (12d): ^{1}H-NMR (400 MHz, CD_3OD) δ/ppm : 3.48-3.44 (t, 3H), 3.37-3.34 (m, 2H), 3.14 (s, 6H), 3.07-3.04 (t, 2H), 2.14-2.2 (m, 2H), 1.83-1.78 (m, 2H), 1.4-1.29 (m, 16H), 0.91-0.88 (t, 3H). ^{13}C-NMR (100MHz, CD_3OD) δ/ppm: 66.15, 61.99, 51.36, 37.71, 33.01, 30.68, 30.59, 30.54, 30.4, 30.23, 27.39, 23.60, 14.36. MALDI-MS: *m/z* 244.4324 (observed); 244.552 (calculated for M^+).

3-Amino-*N*-tetradecyl-*N,N*-dimethylpropan-1-aminium chloride (12e): ^{1}H-NMR (400MHz, CD_3OD) δ/ppm: 3.51-3.45 (t, 2H), 3.4-3.35 (m, 2H), 3.15 (s, 6H), 3.09-3.05 (t, 2H), 2.22-2.14 (m, 2H), 1.84-1.78 (m, 2H), 1.4-1.28 (m, 22H), 0.91-0.88 (t, 3H). ^{13}C-NMR (100MHz, CD_3OD) δ/ppm: 66.17, 62.02, 51.42, 49.83, 37.83, 37.74, 33.0, 30.72, 30.68, 30.67, 30.59, 30.54, 30.4, 30.22, 27.38, 23.66, 23.62, 22.16, 14.39. MALDI-MS: *m/z* 300.89 (observed); 300.58 (calculated for M^+).

3-Amino-*N*-octadecyl-*N,N*-dimethylpropan-1-aminium chloride (12f): ^{1}H-NMR (400 MHz, CD_3OD) δ/ppm: 3.51-3.46 (t, 2H), 3.4-3.36 (m, 2H), 3.15 (s, 6H), 3.09-3.05 (t, 2H), 2.2-2.16 (m, 2H), 1.84-1.78 (m, 2H), 1.41-1.28 (m, 30H), 0.91-0.88 (t, 3H). ^{13}C-NMR (100MHz, CD_3OD) δ/ppm: 66.17, 62.02, 51.41, 37.74, 33.03, 30.74, 30.71, 30.62, 30.57, 30.42, 30.24, 27.4, 23.69, 23.63, 22.18, 14.41. MALDI-MS: *m/z* 356.53 (observed); 356.68 (calculated for M^+).

***tert*-Butyl 3-(tetradecylaminopropyl) carbamate (8a):** *tert*-butyl 3-aminopropylcarbamate (1 g, 5.73 mmol) was dissolved in dry dichloromethane (10 ml) in a sealed tube and tetradecyl bromide (1.58 g, 5.73 mmol) was added to it. The reaction mixture was refluxed for 24 h. Then

the required compounds were purified by column chromatography ($CHCl_3/CH_3OH$) using Silica gel to afford compound **8a** in 40 % yield. ^{1}H-NMR (400MHz, $CDCl_3$) δ/ppm: 5.77 (bs, 1H), 3.15-3.01 (m, 6H), 2.11-2.0 (m, 2H), 1.65-1.53 (Q, 2H), 1.4 (s, 9H), 1.3-1.28 (m, 22H), 0.85-0.83 (t, 3H). HR-MS: *m/z* 370.36 (observed); 370.61 (calculated for M^+).

3-(Tetradecylamino)propan-1-aminium chloride (8b): Compound **8a** was dissolved in MeOH and 37 % HCl. The reaction mixture was stirred at room temperature for 5 h and dried *in vacuum* to afford 3-(tetradecylamino)propan-1-aminium chloride (**8b**) in quantitative yield. ^{1}H-NMR (400MHz, $CDCl_3$) δ/ppm: 3.18-3.06 (m, 6H), 2.14-2.05 (m, 2H), 1.73-1.66 (Q, 2H), 1.38-1.29 (m, 22H), 0.89-0.86 (t, 3H). HR-MS: *m/z* 343.392 (observed); 343.42 (calculated for M^+).

Vancomycin carboxamides derivatives (1-7 and **8)**[125]**:** Vancomycin hydrochloride (100 mg, 67 µmol) was dissolved in dry dimethyl formamide (1 mL) and dry dimethyl sulfoxide (1 mL). To this two equivalents of compounds bearing primary amine group [N^1,N^1-Dimethylpropan-1,3-diamine); 3-amino-*N*-octadecyl-*N*,*N*-dimethylpropan-1-aminium chloride (**12a-12f**) and 3-(tetradecylamino)propan-1-aminium chloride (**8b**)] in 1 mL of dry dimethylformamide was added. The reaction mixture was cooled to 0 ºC, and 0.22 mL (1.5 equivalents) of 0.45 M HBTU solution in DMF and 58 µL (5.0 equivalents) of diisopropylethylamine (DIPEA) were added to the reaction mixture. The reaction mixture was then allowed to warm to room temperature and stirred for 12 h. The product was purified by preparative reverse-phase HPLC using 0.1 % trifluoroacetic acid in H_2O/acetonitrile mixture and then lyophilized to afford tris-(trifluoroacetate) salts of final compounds with more than 95 % purity (47-54 µmol, 70-80 % yield).

***N*,*N*-Dimethyl-*N*-(3-aminopropyl)-vancomycin carboxamide (1):** Yield; 72 % (48.2 µmol). ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 7.76-7.61 (m, 5H), 7.34-7.0 (m, 6H), 6.61-6.53 (d, 2H), 6.05 (bs, 1H), 5.85 (bs, 1H), 5.58-5.37 (m, 8H), 4.93-4.92 (m, 2H), 4.65 (bs, 2H), 4.3 (s, 2H), 4.17-4.16 (t, 2H), 3.85-3.5 (m, 5H), 2.85 (s, 6H), 2.63 (bs, 1H), 2.16-2.10 (m, 3H), 1.88-1.68 (m, 4H), 1.47-1.37 (m, 4H), 1.26-1.22 (m, 4H), 0.96-0.90 (m, 8H). MALDI-MS: *mlz* 1534.56 (observed), 1534.518 (calculated for $[M+H]^+$).

***N*,*N*-Dimethyl-*N*-(3-(ethylamino)propyl)-vancomycin carboxamide (2):** Yield; 70 % (47 µmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.41 (s, 1H), 9.14-9.03 (d, 2H), 8.73 (bs, 1H),

8.56 (bs, 1H), 7.89 (s, 1H), 7.77-7.48 (m, 8H), 7.42 (s, 1H), 7.3-7.1 (m, 3H), 6.85-6.72 (m, 3H), 6.39-6.38 (d, 1H), 5.9 (bs, 1H), 5.76 (bs, 1H), 5.43-5.18 (m, 9H), 4.85 (s, 1H), 4.68 (s, 1H), 4.45 (s, 3H), 4.19-4.16 (d, 2H), 4.01-4.0 (d, 2H), 3.71-3.67 (m, 2H), 3.41 (s, 6H), 3.2-2.8 (m, 5H), 2.68-2.66 (m, 3H), 2.32 (s, 2H), 1.73-1.65 (m, 4H), 1.29 (s, 3H), 1.08-1.06 (m, 3H), 0.92-0.86 (m, 8H). MALDI-MS: *mlz* 1561.634 (observed), 1561.479 (calculated for M^+).

***N,N*-Dimethyl-*N*-(3-(butylamino)propyl)-vancomycin carboxamide (3):** Yield; 78 % (52.3 µmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.35 (s, 1H), 9.09-9.03 (d, 2H), 8.75 (bs, 1H), 8.56 (bs, 1H), 8.18 (bs, 1H), 7.88-7.83 (m, 4H), 7.72-7.21 (m, 6H), 7.12 (bs, 1H), 6.77-6.71 (m, 2H), 6.52 (bs, 1H), 6.38-6.37 (d, 1H), 6.22 (s, 1H), 5.78 (s, 1H), 5.55-5.45 (d, 1H), 5.43-5.19 (m, 4H), 4.92 (s, 1H), 4.72 (s, 1H), 4.55 (s, 1H), 4.29 (s, 1H), 4.28 (bs, 1H), 4.17 (s, 1H), 3.95 (bs, 1H), 3.72-3.43 (m, 6H), 3.19 (s, 6H), 3.17-3.16 (m, 6H), 3.0-2.86 (m, 3H), 2.69-2.62 (m, 2H), 2.38 (s, 1H), 2.20 (S, 1H), 1.95-1.88 (m, 2H), 1.69-1.65 (m, 5H), 1.31-1.24 (m, 2H), 1.08 (s, 2H), 0.92-0.85 (m, 9H). MALDI-MS: *mlz* 1589.646 (observed), 1589.532 (calculated for M^+).

***N,N*-Dimethyl-*N*-(3-(octylamino)propyl)-vancomycin carboxamide (4):** Yield; 80 % (54 µmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.35 (s, 1H), 9.09-9.03 (d, 2H), 8.75 (bs, 1H), 8.56 (bs, 1H), 8.18 (bs, 1H), 7.88-7.83 (m, 4H), 7.72-7.21 (m, 6H), 7.12 (bs, 1H), 6.77-6.71 (m, 2H), 6.52 (bs, 1H), 6.38-6.37 (d, 1H), 6.22 (s, 1H), 5.78 (s, 1H), 5.55-5.45 (d, 1H), 5.43-5.19 (m, 4H), 4.92 (s, 1H), 4.72 (s, 1H), 4.55 (s, 1H), 4.29 (s, 1H), 4.28 (bs, 1H), 4.17 (s, 1H), 3.95 (bs, 1H), 3.72-3.43 (m, 6H), 3.19 (s, 6H), 3.17-3.16 (m, 4H), 3.0-2.86 (m, 3H), 2.69-2.62 (m, 2H), 2.38 (s, 1H), 2.20 (S, 1H), 1.95-1.88 (m, 5H), 1.69-1.65 (m, 5H), 1.31-1.24 (m, 10H), 1.08 (s, 2H), 0.92-0.85 (m, 9H). MALDI-MS: *mlz* 1645.734 (observed), 1645.638 (calculated for M^+).

***N,N*-Dimethyl-*N*-(3-(decylamino)propyl)-vancomycin carboxamide (5):** Yield; 75 % (50.3 µmol)**.** ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.33 (s, 1H), 9.07-9.0 (d, 2H), 8.75 (bs, 1H), 8.56 (bs, 1H), 8.18 (bs, 1H), 7.82-7.01 (m, 9H), 6.8-6.7 (m, 2H), 6.52 (bs, 1H), 6.38-6.37 (d, 1H), 5.76-5.62 (m, 2H), 5.48-5.45 (d, 1H), 5.43-5.19 (m, 4H), 4.92 (s, 1H), 4.72 (s, 1H), 4.55 (s, 1H), 4.29 (s, 1H), 4.28 (bs, 1H), 4.17 (s, 1H), 3.95 (bs, 1H), 3.72-3.43 (m, 6H), 3.19 (s, 6H), 3.17-3.16 (m, 4H), 3.0-2.86 (m, 3H), 2.69-2.62 (m, 2H), 2.38 (s, 1H), 2.20 (S, 1H), 1.95-1.88 (m, 5H), 1.69-1.65 (m, 5H), 1.31-1.24 (m, 14H), 1.08 (s, 3H), 0.92-0.86 (m, 9H). MALDI-MS: *mlz* 1673.031(observed), 1673.745 (calculated for M^+).

***N*,*N*-Dimethyl-*N*-(3-(tetradecylamino)propyl)-vancomycin carboxamide (6):** Yield; 72 % (48.2 µmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.34 (s, 1H), 9.08-9.02 (d, 2H), 8.75 (bs, 1H), 8.56 (bs, 1H), 8.18 (bs, 1H), 7.88-7.83 (m, 4H), 7.72-7.21 (m, 6H), 7.12 (bs, 1H), 6.77-6.71 (m, 2H), 6.52 (bs, 1H), 6.38-6.37 (d, 1H), 6.22 (s, 1H), 5.78 (s, 1H), 5.55-5.45 (d, 1H), 5.43-5.19 (m, 4H), 4.92 (s, 1H), 4.72 (s, 1H), 4.55 (s, 1H), 4.29 (s, 1H), 4.28 (bs, 1H), 4.17 (s, 1H), 3.95 (bs, 1H), 3.72-3.43 (m, 6H), 3.15 (s, 6H), 3.17-3.16 (m, 4H), 3.0-2.86 (m, 3H), 2.69-2.62 (m, 2H), 2.38 (s, 1H), 2.20 (S, 1H), 1.91-1.63 (m, 8H), 1.31-1.24 (m, 22H), 1.08 (s, 3H), 0.92-0.83 (m, 9H). MALDI-MS: *mlz* 1729.908 (observed), 1730.238 (calculated for M^+).

***N*,*N*-Dimethyl-*N*-(3-(octadecylamino)propyl)-vancomycin carboxamide (7):** Yield; 80 % (54 µmol)**.** ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.36 (s, 1H), 9.09-9.03 (d, 2H), 8.75 (bs, 1H), 8.56 (bs, 1H), 8.18 (bs, 1H), 7.88-7.83 (m, 4H), 7.72-7.21 (m, 6H), 7.12 (bs, 1H), 6.77-6.71 (m, 2H), 6.52 (bs, 1H), 6.38-6.37 (d, 1H), 6.22 (s, 1H), 5.78 (s, 1H), 5.55-5.45 (d, 1H), 5.43-5.19 (m, 4H), 4.92 (s, 1H), 4.72 (s, 1H), 4.55 (s, 1H), 4.29 (s, 1H), 4.28 (bs, 1H), 4.17 (s, 1H), 3.95 (bs, 1H), 3.72-3.43 (m, 6H), 3.19 (s, 6H), 3.17-3.16 (m, 4H), 3.0-2.86 (m, 3H), 2.69-2.62 (m, 2H), 2.38 (s, 1H), 2.20 (S, 1H), 1.95-1.88 (m, 5H), 1.69-1.65 (m, 5H), 1.31-1.24 (m, 30H), 1.08 (s, 3H), 0.92-0.85 (m, 9H). MALDI-MS: *mlz* 1786.214 (observed), 1786.638 (calculated for M^+).

3-(Tetradecylamino)propyl-vancomycin carboxamide (8): Yield; 75 % (50.3 µmol). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.34 (s, 1H), 9.08-9.02 (d, 2H), 8.75 (bs, 1H), 8.56 (bs, 1H), 8.18 (bs, 1H), 7.88-7.83 (m, 4H), 7.72-7.21 (m, 6H), 7.12 (bs, 1H), 6.77-6.71 (m, 2H), 6.52 (bs, 1H), 6.38-6.37 (d, 1H), 6.22 (s, 1H), 5.78 (s, 1H), 5.55-5.45 (d, 1H), 5.43-5.19 (m, 4H), 4.92 (s, 1H), 4.72 (s, 1H), 4.55 (s, 1H), 4.29 (s, 1H), 4.28 (bs, 1H), 4.17 (s, 1H), 3.95 (bs, 1H), 3.72-3.43 (m, 6H), 3.17-3.16 (m, 4H), 3.0-2.86 (m, 3H), 2.69-2.62 (m, 2H), 2.38 (s, 1H), 2.20 (S, 1H), 1.91-1.63 (m, 8H), 1.31-1.24 (m, 22H), 1.08 (s, 3H), 0.92-0.83 (m, 9H). MALDI-MS: *mlz* 1702.03 (observed), 1701.74 (calculated for M^+).

***N*-(3-(Dimethylamino)propyl)acetamide (9a)**: A mixture of N^1,N^1-dimethylpropan-1,3-diamine (4.86 g, 52.8 mmol) and acetic anhydride (19.5 g, 190.9 mmol) was stirred at room temperature for 1 h after which acetic anhydride was removed under reduced pressure and the carboxylate salt was neutralized with sodium hydroxide and extracted into chloroform. The chloroform was removed to yield compound **9a** (8.6 g, 79 %) in the salt form. ^{1}H-NMR (400 MHz, $CDCl_3$)

δ/ppm: 6.54 (bs, 1H), 3.32 (t, 2H), 2.78 (t, 2H), 2.52 (s, 6H), 2.01 (s, 3H), 1.86 (m, 2H).^{13}C-NMR (100MHz, $CDCl_3$) δ/ppm: 170.9, 55.57, 43.3, 36.9, 24.99, 23.0.

***N*-(3-Acetamidopropyl)-*N*,*N*-dimethyltetradecan-1-aminium bromide (9)**: A mixture of **9a** and bromo tetradecane (3.63 g,13.1 mmol) was dissolved in acetone (30 mL). Potassium carbonate (1.68 g) was also added. The entire mixture was refluxed for 48 h. The solution was filtered to remove insoluble potassium carbonate after which the compound was purified by column chromatography. Yield: 40 %. ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 7.9 (bs, 1H), 3.85 (t, 2H), 3.38 (t, 2H), 3.33 (t, 2H), 3.2 (s, 6H), 2.08 (s, 3H), 2.01 (m, 2H), 1.7 (m, 2H) 1.35-1.25 (m, 22H), 0.88 (t, 3H).^{13}C-NMR (100MHz, $CDCl_3$) δ/ppm: 171.7, 64.9, 63.0, 51.08, 36.11, 31.9, 26.29, 22.6-29.65, 14.1.

3.5.3 Minimum inhibitory concentration (MIC)

The same protocol was followed using the test compounds as described in the section of 2.5.3 in Chapter 2.

3.5.4 Bacterial membrane disruption studies

3.5.4.1 Cytoplasmic membrane depolarization Assay[141]

Midlog phase bacterial cells (VRE) were harvested, washed with 5 mM HEPES and 5 mM glucose and resuspended in 5 mM glucose, 5 mM HEPES buffer and 100 mM KCl solution in 1:1:1 ratio (10^8 CFU/mL). Measurements were made in a cuvette containing 2 mL of bacterial suspension and 2 µM $DiSC_3$(5). The fluorescence of the dye was monitored for 10 min at R.T. using Perkin-Elmer spectrofluorometer at excitation wavelength of 622 nm (slit width: 10 nm) and emission wavelength of 670 nm (slit width: 5 nm). Dye uptake, and resultant self quenching, was modulated by the membrane potential. After reaching the maximum uptake of the dye by bacteria, which is indicated by a minimum in dye fluorescence (after 10 min), test compounds **1-9**, vancomycin and physical mixture of vancomycin and compound **9** at 10 µM (**9** + vancomycin) were added to the cells, and the decrease in potential was monitored by the increase in fluorescence for further 10 min.

3.5.4.2 Inner membrane permeabilization assay[142]

Midlog phase (grown for 6 h) bacterial cells (VRE) were harvested (4000 rpm, 4 ºC, 10 min), washed with 5 mM HEPES and 5 mM glucose of pH 7.2, and resuspended in the same buffer. Then test compounds **1**-**9**, vancomycin and physical mixture of vancomycin and compound **9** at 10 µM (**9** + vancomycin) were added to a cuvette containing 2 mL of cells and 10 µM propidium iodide (PI). The fluorescence of the dye was monitored for 10 min at R.T. using Perkin-Elmer spectrofluorometer at excitation wavelength of 535 nm (slit width: 10 nm) and emission wavelength of 617 nm (slit width: 5 nm). The uptake of PI was measured by the increase in fluorescence of PI for 10 min as a measure of inner membrane permeabilization.

3.5.4.3 Potassium ion leakage assay[143]

Midlog phase (grown for 6 h) bacterial cells (VRE) were harvested (4000 rpm, 4 ºC, 10 min), washed twice with 10 mM HEPES (pH 7.2) and 0.5 % glucose and were resuspended in the same amount of 10 mM HEPES (pH 7.2) and 0.5 % glucose. The bacterial suspension (2 mL) was placed in a fluorimeter cuvette. The fluorescence of the bacterial suspension was measured and allowed to stabilize for 60 s at room temperature before the addition of PBFI-AM dye (1 µM). Data were collected for an additional 2 min to establish a baseline signal before the addition of test compounds **1**-**9**, vancomycin, valinomycin and physical mixture of vancomycin and compound **9** at 10 µM (**9** + vancomycin). The fluorescence signals were collected for each sample over 1000 s. The fluorescence of the dye was monitored for 10 min at R.T. using Perkin-Elmer spectrofluorometer at excitation wavelength of 346 nm (slit width: 10 nm) and emission wavelength of 505 nm (slit width: 5 nm).

3.5.5 Intracellular accumulation of UDP-*N*-acetyl-muramyl-pentadepsipeptide

The same protocol was followed using the test compounds **4**, **6** and vancomycin at 5 µM against VRE (VanA phenotype) as described in the section of 2.5.5 in Chapter 2.

3.5.6 Bactericidal time-kill kinetics

The same protocol was followed using the test compounds **4**, **6** and vancomycin against MRSA at two concentrations, 1 × MIC and 6 × MIC as described in the section of 2.5.6 in Chapter 2.

3.5.7 Resistance development study

The same protocol was followed using the test compounds **4**, **6** and vancomycin against MRSA as described in the section of 2.5.7 in Chapter 2.

3.5.8 *In-vitro* toxicology

3.5.8.1 Hemolytic assay[144]

Erythrocytes were isolated from freshly drawn, heparanized human blood and re-suspended to 5 vol % in PBS (pH 7.4). In a 96-well microtiter plate, 150 µL of erythrocyte suspension was added to 50 µL of serially diluted compounds (vancomycin and compound **4** from 1000 µM to 2 µM; compounds **6** and **9** from 250 µM to 0.2 µM). Two controls were made, one without compound and other with 50 µL of 1 vol % solution of Triton X-100. The plate was incubated for 1 h at 37 °C. The plate was then centrifuged at 3,500 *rpm* for 5 min, 100 µL of the supernatant from each well was transferred to a fresh microtiter plate, and A_{540} was measured. Percentage of hemolysis was determined as $(A - A_0)/(A_{total} - A_0) \times 100$, where A is the absorbance of the test well, A_0 the absorbance of the negative controls (without compound), and A_{total} the absorbance of 100 % hemolysis wells (with Triton X-100), all at 540 nm.

3.5.8.2 Cytotoxicity assay[145]

Cytotoxicity of the vancomycin, compounds **4** and **6** was assessed against HeLa cell line. The cells were grown in a 96 well plate in DMEM media supplemented with 10 % Fetal Bovine Serum and 5 % penicillin-streptomycin, till they reached 70-80 % confluency. The cells were then treated with serially diluted compounds (vancomycin and compound **4** from 1000 µM to 2 µM; compounds **6** and **9** from 250 µM to 0.2 µM). Two controls were made, one containing no compound and other with 10 vol % Triton-X 100 solution. Cells were incubated for 24 h at 37 °C under 5 % CO_2 atmosphere. After 24 h the supernatant was carefully removed and 100 µL of 5 mg/mL concentration MTT solution was added to each well. Plate was incubated for 3 h at 37 °C under 5 % CO_2 atmosphere. The cells were then treated with 100 µL DMSO to solubilize formazan crystals. The plate was then read at 570 nm. Percentage of cell survival was calculated using the formula

$(A_{\text{treated cells}} - A_{\text{untreated cells}}) / (A_{\text{triton-X cells}} - A_{\text{untreated cells}}) \times 100$

A plot of % of survival against Concentration of compound was plotted using Origin Pro software.

3.5.9 *In-vivo* antibacterial activity

3.5.9.1 Murine renal infection model

The same protocol was followed using the test compounds **4**, vancomycin and linezolid against VRE (VanB phenotype) at 12 mg/kg as described in the section of 2.5.8.1 in Chapter 2.

3.5.9.2 Mouse neutropenic thigh infection model

The same protocol was followed using the test compounds **4**, vancomycin and linezolid against MRSA and VISA at a total dose of 24 mg/kg (divided into two doses, 12 mg/kg, q12 h) as described in the section of 2.5.8.2 in Chapter 2.

3.5.9.3 Dose-responsive study (Pharmacodynamics)

The same protocol was followed using the test compound **4** against VISA at four different regimens (2, 4, 8 and 12 mg/kg) as described in the section of 2.5.8.3 in Chapter 2.

3.5.10 Single-dose pharmacokinetic study

The same protocol was followed using the test compound **4** at 12 mg/kg as described in the section of 2.5.9 in Chapter 2.

3.5.11 *In-vivo toxicology*

3.5.11.1 Systemic toxicity[136]

Systemic toxicity was examined after i.v injection of compound **4** to four groups of CD-1 female mice. Each mouse was injected with a 0.2 mL of freshly prepared compound solution in saline. The doses of the compound administered per group were 5.5 mg/kg, 17.5 mg/kg, 56.0 mg/kg, and 179.2 mg/kg according to OECD guidelines (OECD, 2005, n = 5). Animals were directly

inspected for adverse effects for 4 h, and mortality was observed for 14 days, thereafter, LD_{50} was determined using Spearman-Karber method.

3.5.11.2 Acute toxicity

The same protocol was followed using the test compound **4** at 12 mg/kg as described in the section of 2.5.10.2 in Chapter 2.

3.5.12 *In-vivo* data analysis

The similar analysis was performed as described in the section of 2.5.11 in Chapter 2.

Chapter 4

Combating Vancomycin-resistant Bacteria through Improved Binding Affinity and Membrane Disruption

Abstract

*In **Chapter 4**, a simple rationalized strategy is described to effectively tackle vancomycin-resistant bacteria (VRB) by restoring the lost binding affinity of vancomycin towards altered peptides of VRB and targeting the bacterial membrane. In this chapter, lipophilic vancomycin-sugar conjugates bearing permanent positive charge have been developed using facile synthetic methodology by conjugating a sugar moiety (which have the ability to form extra hydrogen bonds with the target peptides of bacteria) to the C-terminal of vancomycin and further by appending a permanent positively charged lipophilic moiety to the amine group of vancosamine to enable better interaction with the negatively charged bacterial membrane. An optimized compound in the series was found to be a staggering > 8000-fold more effective than vancomycin against vancomycin-resistant Enterococci (VRE). This compound also displayed good in-vivo antibacterial activity in kidney and thigh infection models against VRB and demonstrated improved pharmacological properties with no observed toxicity (LD_{50} > 100 mg/kg). The high activity of the compound is attributed to their strong membrane disruption properties and enhanced binding affinity of > 150-fold to target peptides of VRB, which resulted in improved peptidoglycan (cell wall) biosynthesis inhibition. Further, no detectable resistance was observed after several serial passages of bacterial exposure to the new compound. Thus, this strategy shows immense potential that can pave a new path of antibiotic development for the treatment of vancomycin-resistant bacterial infections.*

Publication based on this work

(1) Yarlagadda, V. *et al.* Membrane disruption and enhanced inhibition of cell wall biosynthesis: A synergistic approach to tackle vancomycin-resistant bacteria. *Manuscript under review.*

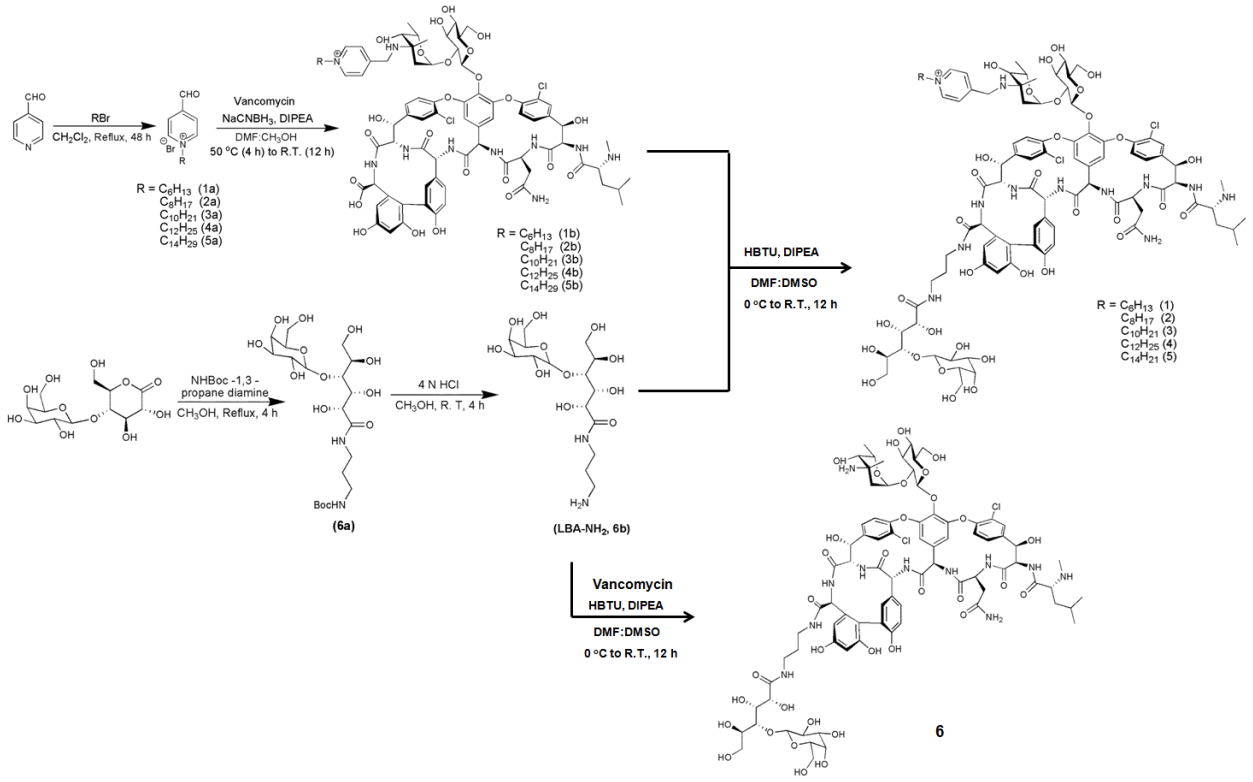
4.1 Introduction

Vancomycin inhibits bacterial cell wall biosynthesis by binding to the peptidoglycan peptide terminus D-Ala-D-Ala found in cell wall precursors, sequestering the substrate from transpeptidase and inhibiting cell wall cross-linking.[54] Vancomycin-resistant bacteria (VRB) sense this antibiotic challenge and remodel their cell wall precursor peptidoglycan terminus from D-Ala-D-Ala to D-Ala-D-Lac (depsipeptide), reducing the binding of vancomycin to its target by manifold and accounting for loss in antimicrobial activity by 1000-fold.[54] To enhance the lost binding affinity of vancomycin towards VRB, in Chapter 2, various vancomycin-sugar conjugates were developed wherein C-terminal of vancomycin was extended with cyclic and acyclic sugar moiety (which have the ability to form extra hydrogen bonding with the peptides of peptidoglycan), to enhance the overall binding constant with the target peptide, thereby reinforcing the activity of the drug against VRB. With this approach, an enhanced binding affinity of two-orders of magnitude was achieved over vancomycin against resistant bacterial ligand.

An alternate approach to tackle vancomycin resistance is to incorporate bacterial membrane disruption properties to vancomycin. In Chapter 3, membrane active vancomycin analogues were reported wherein permanent positively charged lipophilic moiety was appended to the carboxylic group of vancomycin. The appended permanent positively charged lipophilic moiety provides the necessary lipophilicity and electrostatic attraction to interact strongly with the negatively charged bacterial cell membrane at even sub MIC value and the compounds were shown to have good activity against various VRB.

In Chapter 4, permanent positively charged lipophilic vancomycin-sugar conjugates have been developed, wherein the sugar moiety is appended to the carboxylic group of vancomycin and the permanent positively charged lipophilic moiety is conjugated to amine group of vancosamine in order to integrate both enhanced binding affinity towards depsipeptide and membrane disruption properties, with an aim to effectively combat VRB infections. Indeed with this combined approach, ~ 80-fold and > 8000-fold more activity than vancomycin was achieved against VISA and VRE (VanA phenotype) respectively. Unlike vancomycin, these compounds did not induce the development of bacterial resistance. An optimized compound showed high *in-*

vivo antibacterial activity against VRE kidney infection and VISA thigh infection in mouse models and displayed improved pharmacological properties with no observed toxicity.



Scheme 4.1: Synthesis of permanent positively charged lipophilic vancomycin conjugates (**1b**-**5b**), vancomycin-sugar conjugate (**6**) and permanent positively charged lipophilic vancomycin-sugar conjugates (**1**-**5**).

4.2 Results

4.2.1 Synthesis

Permanent positively charged lipophilic vancomycin-sugar conjugates (Scheme 4.1, **1**-**5**) were prepared by conjugating the carboxylic group of permanent positively charged lipophilic vancomycin analogues (Scheme 4.1, **1b**-**5b**) with a sugar moiety bearing a primary amine through amide bond using *O*-Benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyl-uronium-hexafluoro-phosphate (HBTU) as a coupling reagent. Initially, *N*-alkylated pyridinium-4-carboxaldehydes

(**1a**-**5a**) were synthesized wherein *N*-alkylation of 4-pyridine carboxaldehyde was performed with various alkyl (hexyl, octyl, decyl, dodecyl and tetradecyl) bromides. Then, compounds (**1a**-**5a**) were coupled to vancomycin through Schiff's base formation followed by reduction to give permanent positively charged lipophilic vancomycin analogues (**1b**-**5b**). Subsequently, lactobionolactone was subjected to nucleophilic ring opening reaction with *N*-Boc-1,3-propanediamine to give *N*-Boc-1,3-propanediamine derivatized sugar derivative (**6a**) followed by deprotection of *N*-Boc, to give compound **6b** which were finally coupled to compounds **1b**-**5b** using HBTU to give permanent positively charged lipophilic vancomycin-sugar conjugates (**1**-**5**). All the compounds were purified by reverse phase HPLC to more than 95 % purity and characterized by ^{1}H-NMR and HR-MS. A vancomycin-sugar conjugate (compound **6**, Scheme 4.1) was also prepared wherein vancomycin was conjugated to sugar moiety, **6b**, which does not have permanent positively charged lipophilic moiety.

4.2.2 *In-vitro* antibacterial activity

The antibacterial activities of all the compounds were determined against vancomycin-sensitive strains of Staphylococci (Methicillin-sensitive *S. aureus*, MSSA and Methicillin-resistant *S. aureus*, MRSA) and Enterococci (VSE), as well as against vancomycin-resistant strains of Staphylococci (VISA) and Enterococci (VRE) including drug-resistant clinical isolates (*S. aureus*, *S. haemolyticus* and *S. epidermidis*). The results are summarized in Table 4.1 and Table 4.2. All the compounds showed improved activity over vancomycin against vancomycin-sensitive bacteria. Compounds **1b**-**5b** bearing permanent positively charged lipophilic moiety, were found to be 2 to 3-fold more active than vancomycin against vancomycin-sensitive strains with the MICs varying from 0.1-0.3 μM. Amongst compounds **1b**-**5b**, **2b** consisting of octyl chain showed the best activity against MRSA and VSE (MIC = 0.1 μM). In case of resistant strain VISA, most of these compounds were found to be 40 to 80-fold more active than vancomycin, the lowest MIC being 0.1 μM (Table 4.1). When tested against VREm (VanA phenotype) and VREs (VanB phenotype), these compounds exhibited MIC in the range of 0.8-15 μM and 1.0-12.5 μM respectively. The activity increased with increase in chain length of the lipophilic moiety.

Table 4.1. *In-vitro* antibacterial activity of the compounds.

	Minimum Inhibitory Concentration (μM)					
Compound	MSSA	MRSA	VISA	VSE	VREm	VREs
Vancomycin	0.6	0.8	13	0.6	750	250
1b	0.3	0.2	0.3	0.4	14.5	12
2b	0.2	0.1	0.1	0.3	3	6
3b	0.1	0.1	0.1	0.2	1.8	3
4b	0.2	0.2	0.2	0.2	1.0	1.5
5b	0.2	0.1	0.2	0.15	0.7	1
1	0.1	0.1	0.2	0.2	3	2.5
2	0.09	0.09	0.1	0.1	0.09	1.3
3	0.1	0.2	0.09	0.1	0.3	1.3
4	0.2	0.3	0.3	0.1	0.4	1
5	0.2	0.1	0.2	0.15	0.5	0.9
6	0.4	0.4	0.3	0.3	36	30

Abbreviations: MSSA, Methicillin-sensitive *Staphylococcus aureus*; MRSA (ATCC 33591), Methicillin-resistant *Staphylococcus aureus*; VISA, Vancomycin-intermediate-resistant *Staphylococcus aureus* was generated from MRSA after treating with vancomycin for 52 passages; VSE, Vancomycin-sensitive *Enterococcus faecium*; VREm, Vancomycin-resistant *Enterococcus faecium* (VanA phenotype, ATCC 51559); VREs Vancomycin-resistant *Enterococcus faecalis* (VanB phenotype, ATCC 51575).

In previous study (Chapter 2), wherein lactobiono sugar moiety was conjugated to vancomycin, compound **6** exhibited an MIC of 36 μM, which is ~ 25-fold more active than vancomycin against VREm (VanA phenotype). This increase in activity was attributed to higher binding affinity towards target peptide of VRB. Therefore, it was envisioned that incorporation of lactobiono sugar moiety to compounds **1b**-**5b** also might aid in higher binding affinity towards bacterial target peptides. Thus, compounds **1**-**5** bearing both permanent positively charged lipophilic moiety and sugar moiety were synthesized and their antibacterial activities were determined. These compounds showed improved antibacterial activities compared to their

respective homologous compounds **1b**-**5b** against all the bacteria tested. In case of vancomycin-sensitive bacteria, compounds **1**-**5** showed slightly better activity compared to compounds **1b**-**5b** whereas enhanced activity was observed against vancomycin-resistant bacteria. Compound **2** comprising octyl chain showed the best activity against MRSA with the MIC of 0.09 μM. In case of VISA, compounds **2** and **3** displayed good activity with the MIC of ~ 0.1 μM. When checked against VREm (VanA phenotype), compound **2** showed high activity with the MIC of 0.09 μM whereas the MIC of vancomycin was found to be 750 μM (Table 4.1). However, further increase in lipophilic chain length, did not enhance the activity much against VRE as seen for compounds **3**-**5** (MIC = 0.3-0.4 μM). Notably, the MIC values of recently FDA approved semi-synthetic glycopeptides such as oritavancin, telavancin and dalbavancin against VRE (VanA phenotype) were reported as 0.14 μM, 4 μM and 18 μM respectively.[43,115,116] Further, the compounds **1**-**5** also showed good activity against VREs (VanB phenotype) with MIC in the range of 0.9-2.5 μM (Table 4.1). Next, we have evaluated the activities of the new compounds (**1**-**5**) against multidrug-resistant clinical isolates of Staphylococci and the results are presented in Table 4.2. All these new molecules displayed very good activity against these clinical isolates with the MIC of ≤ 0.3 μM. Compound **2** displayed the best activity exhibiting the MIC of ~ 0.1 μM (Table 4.2).

Table 4.2. *In-vitro* antibacterial activity against clinical isolates of Staphylococci.

	Minimum Inhibitory Concentration (μM)		
Compound	MRSA (R3890)	MRSE (N3W)	MRSH (AK6Y)
Vancomycin	0.7	0.7	1.4
1	0.3	0.3	0.4
2	0.1	0.13	0.2
3	0.2	0.15	0.2
4	0.4	0.3	0.2
5	0.3	0.4	0.3

Abbreviations: MRSA, Methicillin-resistant *S. aureus*; MRSE, Methicillin-resistant *S. epidermidis;* MRSH, Methicillin-resistant *S. haemolyticus.*

Compound **6** which bear a sugar moiety and devoid of permanent positively charged lipophilic moiety and compound **2b** comprising permanent positively charged lipophilic (octyl) moiety but lacks sugar moiety showed MIC of 36 μM and 2.9 μM respectively against VRE which are 25 and ~ 300-fold more active than vancomycin (Table 4.1). Compound **2,** on the other hand, (comprising both sugar moiety and permanent positively charged lipophilic moiety) was > 8000-fold more active than vancomycin. This enhanced activity of compound **2** might be attributed to the synergistic action of incorporated sugar moiety and permanent positively charged lipophilic moiety.

Table 4.3. Binding affinities of the compounds.

	Association Constant (K_a, M^{-1})		**Free energy (ΔG, kcal mol^{-1})**	
Compound	Susceptible	Resistant	Susceptible	Resistant
Vancomycin	1.0×10^5	5.0×10^2	6.8	3.7
2b	1.2×10^5	8.5×10^2	6.9	4.0
6	2×10^5	8.8×10^4	7.2	6.7
2	2×10^5	5.7×10^4	7.2	6.5

Where; Susceptible, *N,N'*-diacetyl-Lys-D-Ala-D-Ala (Model ligand for susceptible bacteria); Resistant, *N,N'*-diacetyl-Lys-D-Ala-D-Lac (Model ligand for VRE).

4.2.3 Binding affinities

Then, the binding constants of vancomycin, vancomycin-sugar conjugate (**6**) and the best compound of the series (compound **2**) were evaluated using UV-difference spectroscopy against model ligands which represent the target peptides found in sensitive and resistant bacteria: *N,N'*-diacetyl-Lys-D-Ala-D-Ala and *N,N'*-diacetyl-Lys-D-Ala-D-Lac respectively. The results are displayed in Table 4.3. The binding affinities of compounds **2** and **6** were found to be 2-fold higher than vancomycin against *N,N'*-diacetyl-Lys-D-Ala-D-Ala (Table 4.3). When evaluated against *N,N'*-diacetyl-Lys-D-Ala-D-Lac, the binding affinity of compounds **2** and **6** were > 150-

fold (5.7×10^4 M^{-1} and 8.8×10^4 M^{-1}) higher than vancomycin (5×10^2 M^{-1}). This result suggests that conjugation of sugar moiety improved the binding affinity of the compound towards target peptides and this was irrespective of the presence of the permanent positively charged lipophilic moiety (Table 4.3).

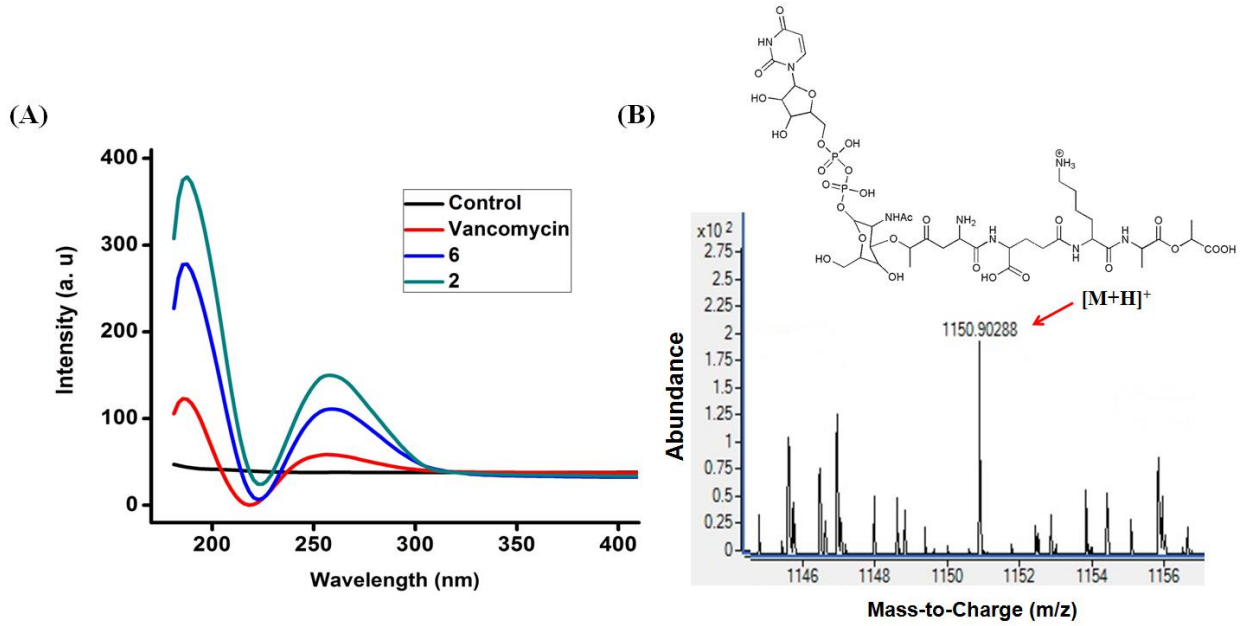


Figure 4.1: Intracellular accumulation of the cell wall precursor UDP-MurNAc-pentadepsipeptide after treatment of VREm (VanA phenotype) with vancomycin, compounds **2** and **6** at 5 μM. Untreated cells were used as control. (A) Identification of intracellular UDP-MurNAc-pentadepsipeptide by monitoring absorbance at 260 nm wavelength (B) UDP-MurNAc-pentadepsipeptide was identified by mass spectrometry as indicated by the peak at m/z 1150.90.

4.2.4 Intracellular accumulation of cell wall (peptidoglycan) precursor

In order to investigate the effect of enhanced binding affinity on inhibition of peptidoglycan biosynthesis, the accumulation of UDP-linked peptidoglycan precursor, UDP-*N*-acetyl-muramyl-pentadepsipeptide (UDPMurNAc-pp) was determined after treating bacteria (VREm, VanA phenotype) with compounds **2**, **6** and vancomycin at 5 μM. On treatment with compounds **2** and **6**, a more intense peak was observed at 260 nm, compared to vancomycin, indicative of greater

accumulation of UDPMurNAc-pp; which was confirmed by high-resolution mass spectrometry (m/z = 1150.94 (cal), 1150.90 (obs) for $[M+H]^+$) (Fig. 4.1A & 4.1B). The higher accumulation of UDPMurNAc-pp indicated increased inhibition of cell wall biosynthesis by compounds **2** and **6** over vancomycin. Further, compound **2** caused greater inhibition of cell-wall biosynthesis compared to compound **6**.

4.2.5 Disruption of bacterial membrane integrity

To support proposed hypothesis that the new compounds also acted by disrupting the bacterial cell membrane integrity, experiments were performed to validate the membrane disruption properties of compounds **1**-**6**, (Fig. 4.2A, 4.2B & 4.2C) and vancomycin using fluorescence spectroscopy against VRE. Firstly, the abilities of these compounds to depolarize VRE membranes were examined using the membrane potential sensitive dye $DiSC_3(5)$ ((3, 3'-dipropylthiadicarbocyanine iodide)). In this study, the dye was added to bacterial cells and the change in fluorescence intensity was observed. The fluorescence intensity decreases as the dye accumulates in the membranes due to self-quenching. Upon dissipation of the membrane potential by the compound, rise in fluorescence was observed due to $DiSC_3(5)$ being displaced into the solution. Unlike in the case of vancomycin and compound **6**, all the new compounds were able to dissipate the bacterial membrane potential in less than three minutes at 5 μM. A gradual increase in depolarization was observed with increase in lipophilic chain length (Fig. 4.2A).

The effect of compounds **1**-**6** (Fig. 4.2B) on bacterial membrane integrity was also confirmed by studying the kinetics of bacterial cytoplasmic membrane permeabilization by measuring the uptake of the fluorescent probe propidium iodide (PI). This dye enters in only membrane-compromised cells and fluoresces upon binding to nucleic acids. Unlike vancomycin and compound **6**, which did not cause significant membrane permeability, compounds **1**-**5** showed strong ability to permeabilize the cytoplasmic membrane of VRE within 5 min at a concentration of 5 μM. Here too, the abilities of membrane permeabilization increased with increase in alkyl chain length.

It is known that release of potassium ion from the bacterial cell occurs upon disruption of the membrane potential. An experiment was performed to find out the release of potassium ion

caused by the compounds (at 5 µM) using potassium ion sensitive fluorophore, PBFI-AM compared to a positive control valinomycin. All new compounds **1**-**5** (Fig. 4.2C) caused significant leakage of intracellular potassium similar to valinomycin, whereas vancomycin and compound **6** were completely ineffective. Hence, bacterial exposure to these new compounds resulted in increased membrane permeability, perturbation of cell membrane potential and finally leakage of intracellular K^+ ion, while vancomycin showed no such effect.

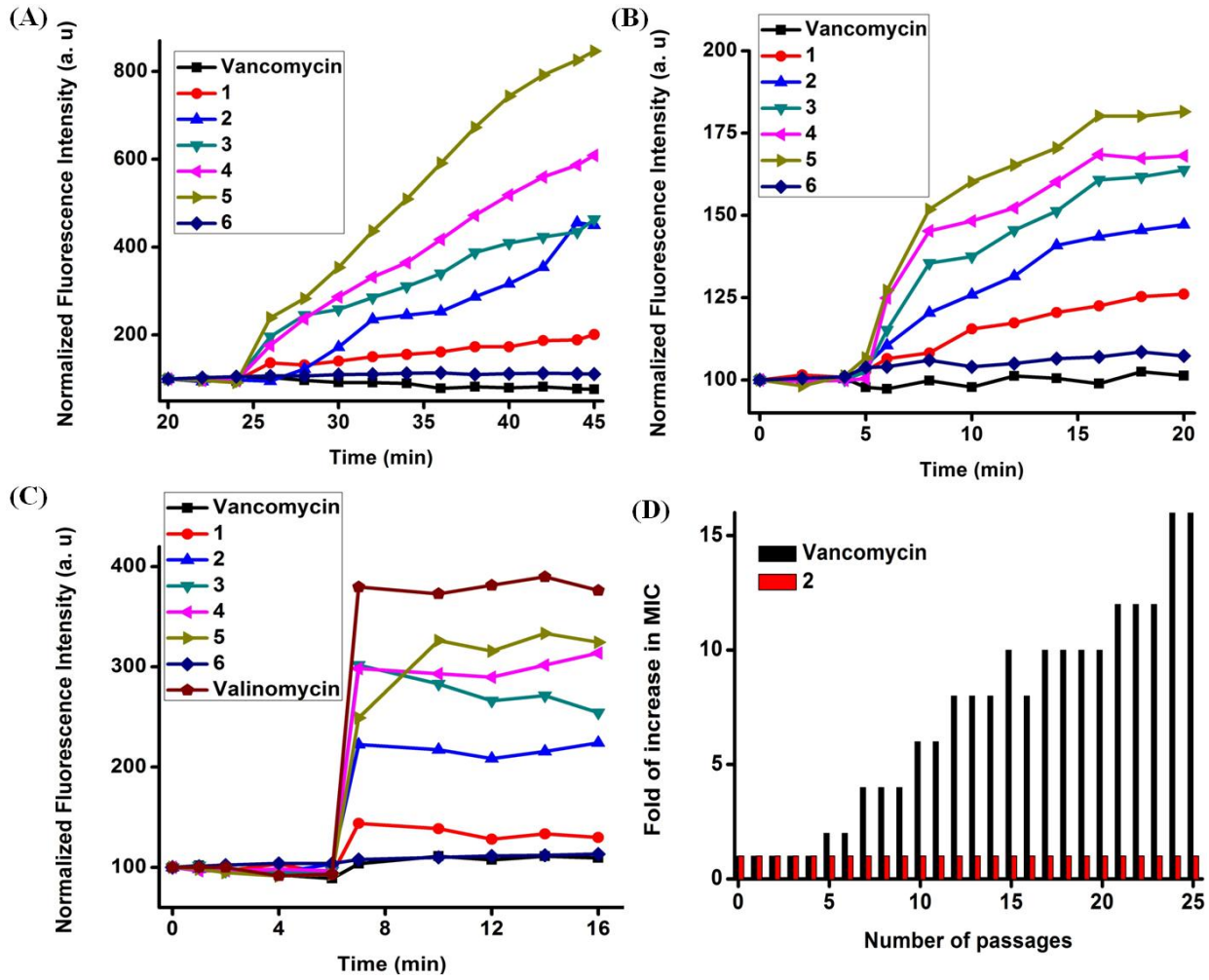


Figure 4.2: Disruption of bacterial cell membrane integrity of vancomycin and compounds **1-6**, at 5 µM against VRE (4.1A, 4.1B and 4.1C). (A) Cytoplasmic membrane depolarization, (B) Cytoplasmic membrane permeabilization and (C) K^+ ion leakage.; Bacterial resistance studies of vancomycin and compound **2** against MRSA (4.2D).

4.2.6 Propensity to induce bacterial resistance

The alarming rise of drug resistance in bacteria guided to evaluate the possibility of emergence of bacteria resistant to this class of compounds. The propensity of bacteria to develop resistance can be evaluated through serial exposure of bacteria to antimicrobial agents. To establish whether these new compounds indeed prevented the development of bacterial resistance, MRSA was exposed to vancomycin and the best active compound **2** for serial passages and monitored the changes in MIC values over a period of 25 days. Even after 25 serial passages, the MIC of compound **2** remained unchanged. However, in case of vancomycin, the MIC value started increasing after 7 passages and the value increased to > 10-fold after 25 passages (Fig. 4.2D). Thus bacteria were futile in acquiring resistance against this type of compound and this emphasizes the long-lasting clinical utility of this class of compounds.

4.2.7 *In-vitro* toxicity (hemolysis)

As these new compounds were shown to disrupt the bacterial cell membrane integrity, the toxicity of compounds **1-5** was evaluated by measuring hemolytic activity (HC_{50}, concentration required for 50 % hemolysis) against human RBC. None of the derivatives showed significant toxicity even up to 100 μM concentration. Notably compounds bearing lipophilic chain below dodecyl did not show any hemolytic activity even up to 1000 μM, which indicates the selective toxicity of these new compounds against bacterial cells over mammalian cells.

4.2.8 *In-vivo* antibacterial activity

To evaluate the potential of this class of compounds for *in-vivo* applications, the activity of compound **2** was determined in a murine renal infection model against VRE. Initially, mice were injected intravenously with 0.2 mL of 0.2 % λ-carrageenan to increase their susceptibility to bacterial renal infection. After 7 days, mice were infected with VRE (~ 10^8 CFU/mouse) through i.v. After 4 h of infection, the mice were treated with three doses (every 24 h interval) of vancomycin (12 mg/kg), linezolid (12 mg/kg), compound **2** (12 mg/kg) and saline (Fig. 4.3A). After 72 h of the initial treatment, antibacterial activity was evaluated by determining the bacterial titer in the infected kidneys. In comparison to vancomycin, compound **2** and linezolid reduced bacterial titer from the infected kidneys more effectively. Linezolid produced ~ 4 $\log_{10}$

CFU reduction from vehicle treated control (saline) whereas compound **2** produced ~ 6 $\log_{10}$ CFU reduction (Fig. 4.3B).

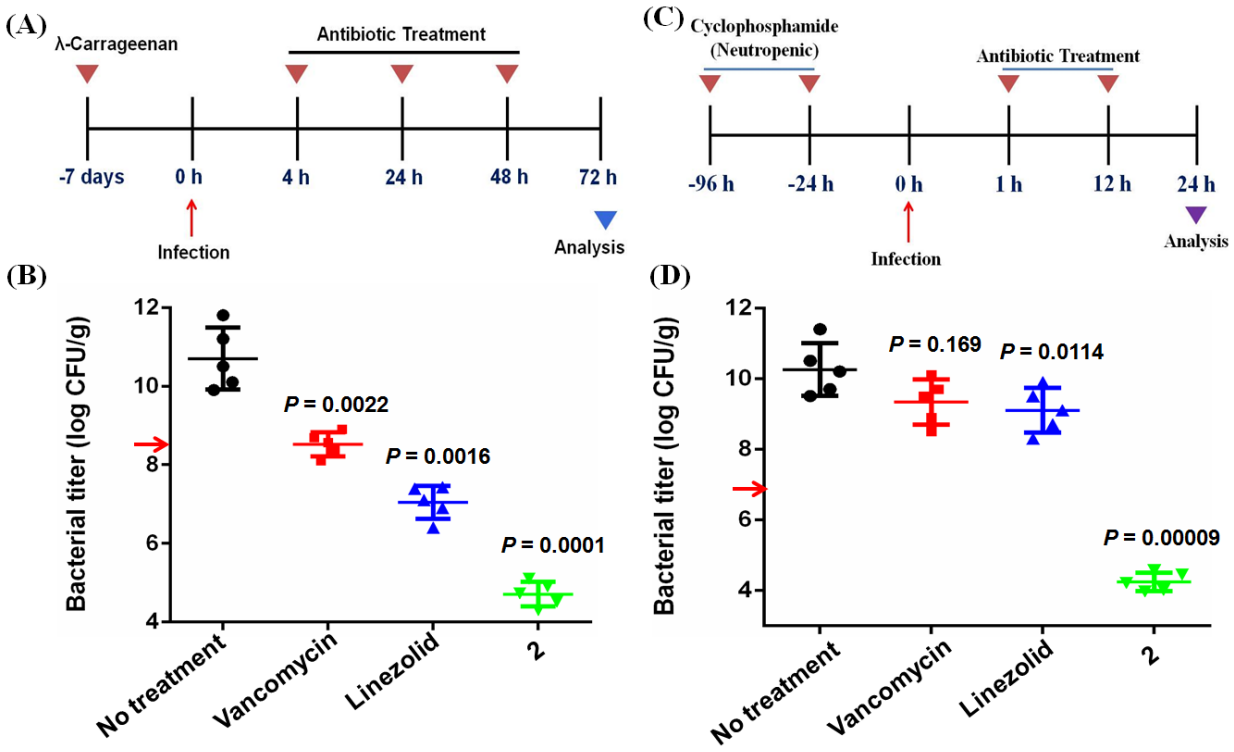


Figure 4.3: *In-vivo* antibacterial activity studies: *In-vivo* efficacy of vancomycin, linezolid and compound **2** in renal infection model against VRE (n = 5) at 12 mg/kg (A) Experimental design and (B) Experimental data; *In-vivo* activity of compound **2** in comparison with vancomycin and linezolid against VISA (n = 5) at 12 mg/kg (compound **2**, single dose at 1 h post infection) and 24 mg/kg (vancomycin and linezolid, double dose of 12 mg/kg each, at 1 h and 12 h post infection) (C) Experimental design and (D) Experimental data. Differences are considered statistically significant from untreated group with a value of $P < 0.05$. Red arrow in (B) and (D) indicates bacterial pretreatment titer (~ 8 $\log_{10}$ CFU/g and ~ 7 $\log_{10}$ CFU/g, respectively).

In another study, the *in-vivo* activity of compound **2** was evaluated in a neutropenic mouse thigh infection model against VISA. In this study, mice were infected with VISA (~ 10^7 CFU/mouse) in the thigh. After 1 h of infection, the mice were treated respectively with saline,

vancomycin, linezolid and compound **2** at 12 mg/kg body weight (n = 5). Again, at 12 h post infection, another dose of vancomycin (12 mg/kg) and linezolid (12 mg/kg) was administered. (Fig. 4.3C). After 24 h of the initial treatment, antibacterial activity was determined by measuring the bacterial titer in the infected thighs. Unlike vancomycin and linezolid, compound **2** showed high activity wherein it caused ~ 6 $\log_{10}$ CFU reduction from saline treated control (Fig. 4.3D).

Table 4.3. Point dose estimates required to achieve different pharmacodynamic end points for compound **2** against VISA thigh infection model.

Bacteria	Pharmacodynamic end points (mg/kg)				
	ED_{50}	ED_{stasis}	$ED_{1\text{-log kill}}$	$ED_{2\text{-log kill}}$	$ED_{3\text{-log kill}}$
VISA (Pretreatment 7.7 $\log_{10}$ CFU/g)	3.3	1.7	3.9	7.8	9.9

Abbreviations: ED_{50}, 50 % effective dose; ED_{stasis}, $\log_{10}$ stasis dose; $ED_{1\text{-log kill}}$, dose required to cause a decrease in titer of 1 $\log_{10}$ CFU/g from the pre-treatment titer (0 h); $ED_{2\text{-log kill}}$, dose required to cause a decrease in titer of 2 $\log_{10}$ CFU/g; $ED_{3\text{-log kill}}$, dose required to cause a decrease in titer of 3 $\log_{10}$ CFU/g.

4.2.9 Dose-response relationship against VISA

The good activity of compound **2** against VISA and VRE infections driven further to examine the effect of dose response on its efficacy. In this study, VISA was chosen as a model bacterium, wherein after 1 h of infection, a single dose of compound **2** at different concentrations (2, 4, 8, 12, 25 and 50 mg/kg) were administered intravenously (Fig. 4.4A). The pretreatment bacterial titer in the thigh was 7.7 ± 0.2 $\log_{10}$ CFU/g. In vehicle treated controls, bacterial titer increased to 10.5 ± 1.2 $\log_{10}$ CFU/g within 24 h. Compound **2** produced a dose dependent reduction in the bacterial titer. The dose that resulted in a 24-h colony count similar to the pretreatment count was

1.7 mg/kg (ED_{stasis}). The value of 1-log_{10} kill dose ($ED_{1\text{-log kill}}$) for compound **2** was 3.96 mg/kg. Further, compound **2** showed 3-log_{10} kill ($ED_{3\text{-log kill}}$) at 9.9 mg/kg (Fig. 4.4B and Table 4.3).

Table 4.4. Single-dose pharmacokinetic parameters of compound **2** at 12 mg/kg.

Drug	Pharmacokinetics parameters				
	C_{max} (µg/mL)	C_{min} (µg/mL)	$AUC_{0\text{-}6\ h}$ (µg/mL/h)	$t_{1/2}$ (h)	Clearance (L/h/Kg)
Compound **2**	556	42	580	1.7	0.03

C_{max}, peak plasma concentration; C_{min}, trough plasma conentration; $AUC_{0\text{-}6\ h}$, area under the concentration-time curve from 0-6 h; $t_{1/2}$, half-life.

4.2.10 Single-dose pharmacokinetics

Pharmacokinetics was determined by intravenously administering compound **2** at 12 mg/kg (> pharmacodynamic end point $ED_{3\text{-log kill}}$) to mice and collecting blood samples at different time intervals after administration (Fig. 4.4C). Plasma samples were subjected to microbiologic assay for the quantification of the compound. Time-concentration profiles of plasma for compound **2** are presented in Fig. 4.4D. Peak concentration in plasma was found to be 556 µg/mL. Pharmacokinetic parameters of compound **2** were determined by using non-compartmental analysis and summarized in Table 4.4. The AUC value for plasma calculated from 0.083 h to 6 h was 580 µg/mL/h. The plasma half-life of compound **2** was found to be 1.7 h with the clearance rate of 0.03 L/h/Kg.

4.2.11 *In-vivo* toxicology studies

Next, the *in-vivo* systemic toxicity of compound **2** was assessed after single-dose intravenous (i.v.) administration at 100 mg/kg dosing regimen to mice (n = 5). Then, the animals were

observed for mortality for a period of 14 days and all the mice were survived at 14 days indicating the high tolerability of compound **2** in animals with LD_{50} > 100 mg/kg.

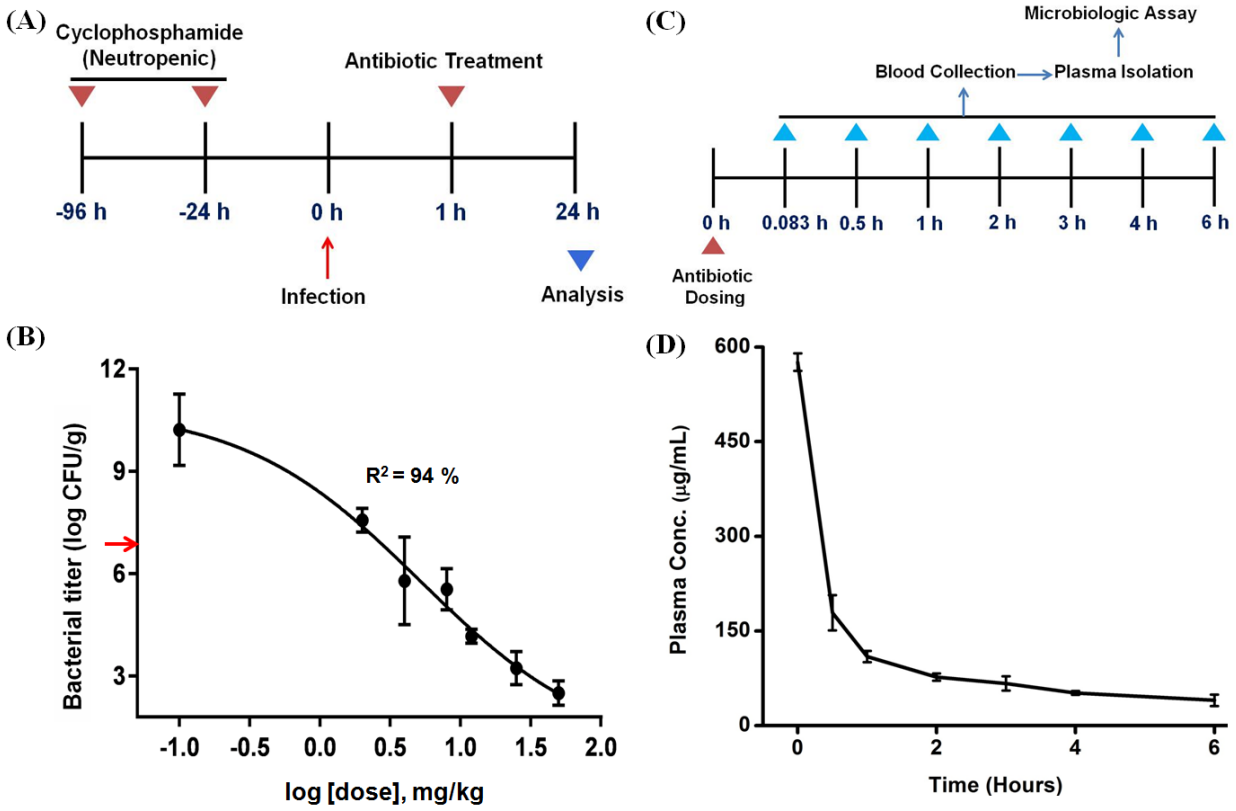


Figure 4.4: Pharmacological studies: Dose-response relationship of compound **2** in thigh infection model against VISA. (A) Experimental design and (B) Experimental data. Red arrow in (B) indicates bacterial pretreatment titer (7.7 log_{10} CFU/g); Single-dose pharmacokinetic study of compound **2** at 12 mg/kg. (C) Experimental design and (D) Experimental data.

4.3 Discussion

Having shown in previous chapters that the conjugation of sugar moiety enhances the overall binding affinity of the drug towards bacterial targets and incorporation of permanent cationic hydrophobic moiety imparts membrane disruption properties; Chapter 4 is aimed at imparting both the properties to the same molecule to effectively tackle drug resistance. In this report, using a facile synthetic methodology, a permanent positively charged lipophilic moiety and sugar

moiety were appended to vancomycin (compounds **1**-**5**) in order to incorporate both membrane disruption property from permanent positively charged lipophilic moiety and improved binding affinity from sugar moiety. The activities of this class of compounds incorporating the dual effects, were found to be much higher compared to compounds incorporating each effect singly. Significantly, an optimized compound (compound **2**) displayed > 8000-fold more activity compared to vancomycin against VRE, whereas vancomycin-sugar conjugate (devoid of permanent positively charged lipophilic moiety) and vancomycin derivative comprising permanent positively charged lipophilic moiety (devoid of sugar unit) were respectively 25 and ~ 300-fold more active than vancomycin against VRE. A strong synergism was observed between the two effects, namely enhanced binding affinity towards target peptide leading to increased inhibition of bacterial cell wall biosynthesis and disruption of bacterial membrane integrity. Significantly, unlike vancomycin, these compounds did not trigger the development of bacterial resistance. Further, this class of compounds was selectively toxic towards bacterial cells compared to mammalian cells and also did not show any systemic toxicity in mice, with high LD_{50} value (> 100 mg/kg).

Next, the *in vivo* activity of the optimized compound (compound **2**) was evaluated in VRE and VISA mouse infection model and compared the activity against vancomycin and linezolid. In particular, compound **2** was significantly more active than linezolid and vancomycin against VRE in renal infection model. Compound **2** was also found to be significantly more potent than vancomycin against VISA in neutropenic mouse thigh infection model. These substantial differences in relative *in-vivo* potencies cannot be explained on the basis of MICs alone. For example, the MICs of compound **2** and linezolid varied by a factor of five whereas their *in-vivo* potencies differ by manifold (MIC of linezolid = 1.5 μg/mL, MIC of compound **2** = 0.25 μg/mL against VISA). The superior activity of compound **2** in the present study can be explained, in part, by its rapid bactericidal activity, which can be attributed to its improved binding affinity and membrane disruption properties towards bacterial targets over vancomycin. Single-dose pharmacokinetic study demonstrated that compound **2** has improved pharmacological properties, which also supported its superior *in-vivo* antibacterial activity.

4.4 Conclusions

The clinical impact of such efficient glycopeptide antibiotics is likely to be enormous, presenting a rational approach forward in the development of antibiotics for the treatment of vancomycin-resistant bacterial infections. The incorporation of membrane disruption properties into vancomycin along with increased binding affinity makes these permanent positively charged lipophilic vancomycin-sugar conjugates distinct from other existing derivatives in their ability to effectively tackle vancomycin-resistant bacteria. This is displayed in the high antibacterial activity of the compounds against MRSA, VISA, VRE and in curbing the development of bacterial resistance. An optimized compound showed high activity against VRE, being > 8000-fold more effective than vancomycin. Further, this class of compounds showed potent *in-vivo* antibacterial activity against VISA and VRE infections with improved pharmacological properties. These findings stress that this multipronged approach bears immense potential and could be a valuable extension to the antibiotic arsenal to combat infections caused by drug-resistant bacteria.

4.5 Experimental procedure

4.5.1 Materials and Methods

All reagents were purchased from Sigma-Aldrich and SD Fine and used without further purification. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F_{254} (250 μm thickness). Visualization was accomplished using UV light and Iodine. Column chromatography was performed on silica gel (60-120 Å pore size). All final compounds were purified by reverse phase HPLC using 0.1 % trifluoroacetic acid (TFA) in water/acetonitrile (0-100 %) as mobile phase to more than 95 % purity. HPLC analysis was performed on a Shimadzu-LC 8Å Liquid Chromatography instrument (C_{18} column, 10 mm diameter, 250 mm length) with UV detector monitoring at 270 nm. Nuclear magnetic resonance spectra were recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. High resolution mass spectra (HR-MS) were obtained using 6538-UHD Accurate Mass Q-TOF LC-MS instrument. UV-absorption measurements were obtained using Thermo-Fisher Scientific UV-10 spectrometer for determination of binding constants. Bacterial strains, *S. aureus* MTCC

737 purchased from MTCC (Chandigarh, India). Methicillin-resistant *S. aureus* (MRSA) ATCC 33591, vancomycin-resistant *E. faecium* (VREm, VanA) ATCC 51559, vancomycin-resistant *E. faecalis* (VREs, VanB) ATCC 51575 were obtained from the American Type Culture Collection (ATCC). VISA, Vancomycin-intermediate-resistant *S. aureus* was generated from MRSA (ATCC 33591) after treating with vancomycin for 52 passages. Methicillin-resistant *S. aureus* R3890 (MRSA R3890) was collected from National Institute of Mental Health and Neuro Sciences (NIMHANS), Bengaluru, India. Methicillin-resistant *S. epidermidis* N3W (MRSE N3W) and methicillin-resistant *S. haemolyticus* AK6Y (MRSH AK6Y) were collected from National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI), Bengaluru, India. Staphylococcal strains were grown in nutrient broth and Enterococcal strains were grown in brain heart infusion broth. Eppendorf 5810R centrifuge was used. TECAN (Infinite series, M200 pro) Plate Reader was used to measure absorbance and fluorescence of 96-well plate. Human RBCs were used for hemolytic assay.

Animals: The same as described in section 2.5.1 in Chapter 2.

4.5.2 Synthesis and characterization

Synthesis of 1-alkyl-4-formyl pyridinium bromides (1a-5a): About 1.0 g of pyridine-4-aldehyde was taken in 15 mL dry chloroform and about 2 equivalents of alkyl bromide (R-Br, R varied from hexyl to tetradecyl) was added to it and then refluxed for 48 h. Then the reaction was allowed to come to room temperature and the solvent was evaporated. The crude product was washed with dry hexane and followed by dry ethyl acetate. The traces of the solvent were removed by using high vacuum pump to afford 1-alkyl-4-formyl pyridinium bromides (**1a**-**5a**) in 35 to 40 % yield.

1-Hexyl-4-formyl pyridinium bromide (1a): Yield; 40 %. ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 10.51 (s, 1H), 9.18-9.09 (dd, 2H), 8.31-8.2 (dd, 2H), 4.91-4.83 (t, 2H), 2.2-2.11 (m, 2H), 1.35-1.21 (m, 10H), 0.89-0.86 (t, 3H).

1-Octyl-4-formyl pyridinium bromide (2a): Yield; 35 %. ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 10.41 (s, 1H), 9.11-9.06 (dd, 2H), 8.31-8.17 (dd, 2H), 4.80-4.75 (t, 2H), 2.17-2.01 (m, 2H), 1.33-1.23 (m, 10H), 0.86-0.83 (t, 3H).

1-Decyl-4-formyl pyridinium bromide (3a): Yield; 34.8 %. ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 10.39 (s, 1H), 9.06-9.00 (dd, 2H), 8.30-8.16 (dd, 2H), 4.73-4.72 (t, 2H), 2.08-2.00 (m, 2H), 1.33-1.23 (m, 14 H), 0.88-0.85 (t, 3H).

1-Dodecyl-4-formyl pyridinium bromide (4a): Yield; 40 %. ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 10.45 (s, 1H), 9.08-9.06 (dd, 2H), 8.30-8.17 (dd, 2H), 4.87-4.75 (t, 2H), 2.16-2.02 (m, 2H), 1.35-1.24 (m, 18H), 0.88-0.85 (t, 3H).

1-Tetradecyl-4-formyl pyridinium bromide (5a): Yield; 37 %. ^{1}H-NMR (400 MHz, $CDCl_3$) δ/ppm: 10.33 (s, 1H), 9.05-9.02 (d, 2H), 8.22-8.15 (d, 2H), 4.67-4.61 (t, 2H), 2.11-2.07 (m, 2H), 1.37-1.25 (m, 22H), 0.89-0.86 (t, 3H).

Synthesis of cationic-lipophilic-vancomycin analogues (1b-5b)[124]**:** Vancomycin hydrochloride (150 mg) was dissolved in dry dimethyl formamide (1 mL) and dry methanol (1 mL). To this 1.5 equivalents of 1-alkyl-4-formyl pyridinium bromides (**1a**-**5a**) and 1.2 equivalents of diisopropylethylamine (DIPEA) were added. The reaction mixture was stirred at 50 ºC for 2 h and then allowed to cool to room temperature prior to addition of sodium cyanoborohydride (2.0 equivalents). Then, the reaction mixture was stirred at 50 ºC for additional 2 h and allowed to cool to ambient temperature for overnight. The purification was done by preparative reverse phase HPLC using 0.1 % trifluoro acetic acid (TFA) in water/acetonitrile (0-100 %) as mobile phase for 20 min. C_{18} column (10 mm diameter, 250 mm length) and UV detector (at 270 nm wave length) were used. The collected fractions, from HPLC were frozen by liquid N_2 and lyophilized in freeze dryer to afford bis-(trifluoroacetate) salts of compounds **1b** to **5b** in 40-55 % yield.

Compound 1b: Yield; 43 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.65 (bs, 1H), 9.38-9.29 (m, 5H), 9.17 (bs, 1H), 8.88-8.71 (m, 3H), 8.37-8.35 (d, 2H), 8.03 (s, 1H), 7.76-7.65 (m, 4H), 7.58-7.36 (m, 3H), 7.23 (bs, 1H), 6.98-6.87 (m, 3H), 6.73 (bs, 1H), 6.58-6.43 (dd, 2H), 6.16 (bs, 2H), 5.95-5.80 (m, 1H), 5.52-5.29 (m, 5H), 5.12 (bs, 1H), 4.91-4.90 (m, 1H), 4.79-4.76 (m, 2H), 4.65-4.56 (m, 3H), 4.40-4.37 (m, 2H), 4.15 (bs, 1H), 3.89-3.87 (m, 2H), 2.83 (bs, 3H), 2.37-2.24 (m, 2H), 2.12-2.06 (m, 3H), 1.89-1.70 (m, 3H), 1.67 (bs, 1H), 1.42 (m, 7H), 1.33-1.31 (d, 3H), 1.1-1.02 (m, 10H). HR-MS: *m/z* 813.2645 (observed), 813.2632 (calculated for $[M+H]^{2+}$).

Compound 2b: Yield; 41 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.46 (s, 1H), 9.19-9.10 (m, 6H), 8.99 (s, 1H), 8.69 (s, 1H), 8.59-8.52 (m, 2H), 8.20-8.16 (d, 2H), 7.85 (s, 1H), 7.57-7.46 (m, 4H), 7.35-7.33 (d, 1H), 7.20-7.17 (m, 2H), 7.04 (bs, 1H), 6.79-6.67 (m, 3H), 6.54 (bs, 1H), 6.40-6.39 (d, 1H), 6.25-6.24 (d, 1H), 5.99 (bs, 2H), 5.77-5.75 (d, 2H), 5.62 (s, 1H), 5.33-5.11 (m, 5H), 4.93 (bs, 1H), 4.72-4.71 (d, 1H), 4.61-4.57 (m, 2H), 4.46-4.37 (m, 4H), 4.21-4.18 (m, 2H), 3.96 9m, 1H), 3.70-3.68 (m, 2H), 2.64 (bs, 3H), 2.21-2.05 (m, 2H), 1.93-1.88 (m, 3H), 1.69-1.56 (m, 3H), 1.48 (m, 3H), 1.27-1.23 (m, 11H), 1.14-1.12 (d, 3H), 0.91-0.83 (m, 10H). HR-MS: *m/z* 827.3086 (observed), 827.2895 (calculated for $[M+H]^{2+}$).

Compound 3b: Yield; 45 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.48 (s, 1H), 9.21-9.11 (m, 4H), 8.72-8.52 (m, 2H), 8.17-8.15 (d, 1H), 7.84 (s, 1H), 7.58-7.06 (m, 6H), 6.79-6.70 (m, 2H), 6.39-6.24 (dd, 2H), 5.99 (bs, 1H), 5.78-5.61 (m, 1H), 5.32-5.1 (m, 4H), 4.72-4.36 (m, 4H), 4.20-4.18 (d, 1H), 3.78-3.75 (d, 2H), 2.88 (bs, 3H), 2.63 (s, 1H), 2.15-2.04 (m, 1H), 1.91-1.80 (m, 3H), 1.67-1.59 (m, 2H), 1.47 (s, 2H), 1.23 (m, 15H), 1.13-1.12 (d, 3H), 0.92-0.83 (10H). HR-MS: *m/z* 841.3186 (observed), 841.3158 (calculated for $[M+H]^{2+}$).

Compound 4b: Yield; 40 %.^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.46 (s, 1H), 9.20-9.10 (m, 4H), 8.71-8.52 (m, 2H), 8.30-7.84 (m, 2H), 7.63-7.07 (m, 5H), 6.78-6.70 (m, 2H), 6.56 (bs, 2H), 6.39-6.24 (d, 1H), 5.98 (bs, 1H), 5.77-5.61 (m, 1H), 5.37-5.09 (m, 4H), 4.72-4.41 (m, 2H), 4.20-3.95 (m, 1H), 3.71-3.55 (m, 3H), 2.63 (s, 2H), 2.16-2.13 (m, 1H), 1.90-1.81 (m, 2H), 1.64-1.45 (m, 3H), 1.26-1.23 (m, 19H), 1.13-1.11 (m, 3H), 0.92-0.83 (m, 10H). HR-MS: *m/z* 855.3396 (observed), 855.3421 (calculated for $[M+H]^{2+}$).

Compound 5b: Yield; 55 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.43 (s, 1H), 9.16-9.06 (m, 5H), 8.67 (bs, 1H), 8.47 (bs, 1H), 8.12-8.10 (d, 2H), 7.79 (s, 1H), 7.53-7.74 (m, 5H), 7.30-7.28 (d, 1H), 7.16-7.09 (m, 2H), 6.74-6.65 (m, 3H), 6.34-6.19 (dd, 2H), 5.73-5.71 (m, 1H), 5.57 (bs, 1H), 5.27-5.25 (m, 2H), 5.14-5.05 (m, 3H), 4.87 (bs, 1H), 4.67-4.65 (d, 1H), 4.55-4.52 (t, 2H), 4.40-4.31 (m, 4H), 4.15-4.13 (m, 2H), 3.65-3.63 (d, 2H), 2.82 (s, 3H), 2.58 (s, 2H), 2.11-1.99 (m, 1H), 1.87-1.82 (m, 3H), 1.60-1.50 (m, 2H), 1.42 (s, 2H), 1.21-1.18 (m, 23H), 1.08-1.07 (d, 3H), 0.87-0.79 (m, 10H). HR-MS: *m/z* 869.8133 (observed), 869.3684 (calculated for $[M+H]^{2+}$).

Synthesis of compound 6a: About 1.3 g of lactobionolactone was dissolved in 5 mL of methanol, then about 0.89 g (1.2 equivalents) of *N*-Boc-1,3-propanediamine was added to the reaction mixture. Now the reaction mixture was refluxed at 70 ºC for 24 h. Then methanol was removed by rotavapour, the residue was washed with ethyl acetate and finally with chloroform. Then it was kept in high vacuum oven for overnight to get the pure and dry compound **6a** with 72 % yield. FT-IR (NaCl): 3341 cm^{-1} (-OH str.), 2929 cm^{-1} (-CH_2- asym. str.), 2888 cm^{-1} (-CH_2- sym. str.), 1685 cm^{-1} (Amide-I C=O str.), 1660 cm^{-1} (Amide-II -NH- ben.). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 4.58 (d, 1H), 4.20-3.58 (m, 12H), 3.30 (t, 2H), 3.12 (t, 2H), 1.72 (Q, 2H), 1.45 (s, 9H). HR-MS: *m/z* 515.2489 (observed); 515.2452 (calculated for $[M+H]^+$).

Synthesis of compound 6b: About 1.35 g of compound **6a** was dissolved in 5 mL of methanol and 5 mL of 4N HCl was added to it. Then it was kept for 5 h at room temperature. Then solvent was evaporated to get pure and dry compound **6b** with 89 % yield. FT-IR (NaCl): 3297 cm^{-1} (-OH and -NH_2 sym., asym. str.), 2932 cm^{-1} (-CH_2- asym. str.), 2888 cm^{-1} (-CH_2- sym. str.), 1685 cm^{-1} (Amide-I C=O str.), 1648 cm^{-1} (Amide-II -NH- ben.). ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 4.57 (d, 1H), 4.41-3.58 (m, 12H), 3.35 (t, 2H), 3.30 (t, 2H), 1.72 (Q, 2H). HR-MS: m/z 415.1901 (observed); 415.1928 (calculated for $[M+H]^+$).

Synthesis of cationic-lipophilic-vancomycin-sugar conjugates (1-5) and vancomycin-sugar conjugate (6)[125]**:** About 1.0 equivalent of cationic-lipophilic-vancomycin analogue (**1b-5b**) or vancomycin was dissolved in 1:1 mixture of dry dimethyl formamide (1 mL) dry dimethyl sulfoxide (1 mL). To this two equivalents of compound **6b** in 1 mL of dry dimethylformamide was added. The reaction mixture was cooled to about 0 ºC, and about 1.5 equivalents of 0.45 M HBTU solution in DMF was added followed by about 5.0 equivalents of diisopropylethylamine (DIPEA). The reaction mixture was then allowed to warm to room temperature and stirred for about 12 h. The product was purified by preparative reversed-phase HPLC using about 0.1 % trifluoroacetic acid in H_2O/acetonitrile mixture and then lyophilized to afford tris-(trifluoroacetate) salts of cationic-lipophilic-vancomycin-sugar conjugates (**1**-**5**) and bis-(trifluoroacetate) salt of vancomycin-sugar conjugate (**6**) with 60-70 % yield. These conjugates were purified and characterized by ^{1}H-NMR and HR-MS. The purification was done by preparative reverse phase HPLC using 0.1 % trifluoro acetic acid (TFA) in water/acetonitrile (0-100 %) as mobile phase for 20 min. C_{18} column (10 mm diameter, 250 mm length) and UV

detector (at 270 nm wave length) were used. The collected fractions, from HPLC were frozen by liquid N_2 and lyophilized in freeze dryer.

Compound 1: Yield; 70 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.37-9.27 (m, 2H), 9.15-8.89 (m, 4H), 8.78-8.57 (m, 2H), 8.45-8.07 (m, 3H), 7.91-7.44 (m, 6H), 7.35-6.66 (m, 6H), 6.34-6.19 (m, 1H), 5.93-5.59 (m, 2H), 5.47-4.95 (m, 6H), 4.69-4.52 (m, 2H), 4.42-4.33 (m, 2H), 4.19-4.03 (m, 3H), 3.65 (bs, 1H), 3.56-3.40 (m, 4H), 3.01 (bs, 3H), 2.79 (m, 7H), 2.57-2.55 (m, 2H), 1.72-1.49 (m, 7H), 1.25-1.21 (m, 7H), 0.96-0.80 (m, 10H). HR-MS: *m/z* 1031.1815 (observed), 1031.0054 (calculated for $[M+K]^{2+}$).

Compound 2: Yield; 63 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.37-9.30 (m, 2H), 9.18-8.92 (m, 3H), 8.72-8.60 (m, 2H), 8.42-8.10 (m, 3H), 7.94-7.47 (m, 6H), 7.38-6.99 (m, 4H), 6.89-6.69 (m, 2H), 6.37-6.22 (m, 1H), 5.96-5.79 (m, 2H), 5.50-5.43 (m, 1H), 5.31-5.08 (m, 5H), 4.72-4.57 (m, 2H), 4.45-4.36 (m, 1H), 4.11-4.06 (m, 2H), 3.68 (bs, 1H), 3.59-3.34 (m, 3H), 3.04 (bs, 3H), 2.93-2.82 (m, 7H), 2.60-2.58 (m, 2H), 1.81-1.66 (m, 5H), 1.57-1.52 (m, 3H), 1.28-1.24 (m, 11H), 0.99-0.83 (m, 10H). HR-MS: *m/z* 1045.8314 (observed), 1045.0317 (calculated for $[M+K]^{2+}$).

Compound 3: Yield; 67 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.33 (s, 1H), 9.03-8.99 (m, 3H), 8.69 (m, 1H), 8.48-8.46 (bs, 1H), 8.14-8.06 (m, 2H), 7.85 (bs, 2H), 7.72-7.67 (m, 3H), 7.56-7.45 (m, 4H), 7.30-7.21 (m, 3H), 7.06 (bs, 1H), 6.78-6.66 (m, 3H), 6.37-6.22 (dd, 2H), 5.90 (bs, 1H), 5.80-5.75 (m, 2H), 5.62 (bs, 2H), 5.36-5.10 (m, 7H), 4.91-4.90 (m, 1H), 4.61-4.60 (m, 1H), 4.46-4.45 (d, 1H), 4.37-4.35 (d, 1H), 4.22 (bs, 2H), 4.11-4.08 (t, 2H), 3.70-3.54 (m, 7H), 2.79-2.78 (bs, 2H), 2.19-2.10 (m, 1H), 2.03-1.90 (m, 1H), 1.80-1.65 (m, 4H), 1.53 (bs, 3H), 1.36 (s, 2H), 1.25 (m, 14H), 1.10-1.08 (d, 2H), 0.92-0.84 (m, 10H). HR-MS: *m/z* 1058.3491 (observed), 1058.5545 (calculated for $[M+K]^{2+}$).

Compound 4: Yield; 65 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.34 (s, 1H), 9.04-8.99 (m, 3H), 8.69 (bs, 1H), 8.48 (bs, 1H), 8.14-8.05 (m, 2H), 7.84 (bs, 2H), 7.67 (m, 3H), 7.54-7.45)m, 4H), 7.30-7.21 (m, 3H), 7.07 (bs, 1H), 6.80-6.66 (m, 3H), 6.37-6.22 (dd, 2H), 5.92 (bs, 1H), 5.80-5.75 (m, 2H), 5.63-5.62 (d, 2H), 5.36-5.10 (m, 7H), 4.92 (bs, 1H), 4.61-4.59 (m, 1H), 4.46-4.35 (m, 3H), 4.24-4.20 (m, 3H), 4.12-4.09 (t, 2H), 3.71-3.61 (m, 4H), 2.81-2.78 (m, 2H), 2.19-

2.12 (m, 1H), 2.03-1.93 (m, 1H), 1.80-1.64 (m, 4H), 1.54 (bs, 3H), 1.36 (s, 3H), 1.27-1.24 (m, 19H), 1.09-1.08 (d, 3H), 0.92-0.84 (m, 10H). HR-MS: *m/z* 1073.8726 (observed), 1073.0843 (calculated for $[M+K]^{2+}$).

Compound 5: Yield; 60 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.29 (s, 1H), 9.00-8.95 (d, 3H), 8.65 (bs, 1H), 8.44-8.42 (m, 2H), 8.09-8.00 (m, 2H), 7.79 (bs, 2H), 7.63 (bs, 3H), 7.49-7.40 (m, 4H), 7.25-7.17 (m, 3H), 7.03 (bs, 1H), 6.73-6.64 (m, 3H), 6.33-6.17 (m, 2H), 5.87 (bs, 1H), 5.76-5.70 (m, 2H), 5.59-5.57 (d, 2H), 5.34-5.06 (m, 7H), 4.86 (bs, 1H), 4.57-4.55 (m, 1H), 4.42-4.40 (d, 1H), 4.32-4.31 (d, 1H), 4.20-4.15 (m, 2H), 4.07-4.05 (t, 2H), 3.66-3.54 (m, 5H), 2.76-2.73 (m, 2H), 2.56 (bs, 1H), 2.14-2.05 (m, 1H), 1.96-1.92 (m, 1H), 1.75-1.60 (m, 4H), 1.49 (bs, 3H), 1.32 (s, 3H), 1.22 (m, 23H), 1.05-1.03 (d, 2H), 0.87-0.80 (m, 10H). HR-MS: *m/z* 1086.9958 (observed), 1087.1106 (calculated for $[M+K]^{2+}$).

Compound 6: Yield; 65 %. ^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.33 (s, 1H), 9.02-8.94 (m, 4H), 8.69 (bs, 1H), 8.53-8.46 (m, 2H), 8.07-8.05 (t, 1H), 7.85 (s, 1H), 7.68-7.45 (m, 10H), 7.33-7.18 (m, 3H), 7.09-7.08 (d, 1H), 6.77-6.66 (m, 3H), 6.48 (bs, 1H), 6.37-6.22 (dd, 2H), 5.94-5.93 (d, 1H), 5.80-5.75 (m, 2H), 5.61 (s, 1H), 5.45-5.43 (d, 1H), 5.34-5.17 (m, 6H), 5.09 (bs, 1H), 4.92-4.91 (d, 1H), 4.68-4.66 (d, 1H), 4.46-4.35 (m, 2H), 4.24-4.21 (d, 2H), 4.02-3.96 (d, 2H), 3.70-3.67 (d, 1H), 3.57-3.44 (m, 3H), 2.9 (bs, 1H), 2.81-2.76 (q, 2H), 2.68-2.62 (m, 4H), 2.15-2.08 (m, 2H), 1.91-1.89 (d, 2H), 1.75-1.55 (m, 7H), 1.30 (s, 3H), 1.07-1.06 (d, 3H), 0.92-0.85 (m, 7H). HR-MS: *m/z* 923.8035 (observed), 923.8346 (calculated for $[M+2H]^{2+}$).

4.5.3 Minimum inhibitory concentration (MIC)

The same protocol was followed using the test compounds as described in the section of 2.5.3 in Chapter 2.

4.5.4 Titration binding assays with model ligands

The same protocol was followed using the test compounds **2**, **2b**, **6** and vancomycin as described in the section of 2.5.4 in Chapter 2.

4.5.5 Intracellular accumulation of UDP-*N*-acetyl-muramyl-pentadepsipeptide

The same protocol was followed using the test compounds **2**, **6** and vancomycin at 5 μM as described in the section of 2.5.5 in Chapter 2.

4.5.6 Bacterial membrane disruption studies

4.5.6.1 Cytoplasmic membrane depolarization Assay[141]

Cytoplasmic membrane depolarization assay was performed against VRE as described previously in the section of 3.5.4.1 in Chapter 3 with slight modifications. Briefly, mid-log phase VRE cells were harvested, washed with 5 mM HEPES and 5 mM glucose and resuspended in 5 mM glucose, 5 mM HEPES buffer and 100 mM KCl solution in 1:1:1 ratio (10^8 CFU/mL). Measurements were made in a 96 well plate containing 0.2 mL of bacterial suspension and 2 μM $DiSC_3(5)$. The fluorescence of the dye was monitored for 20 min at R.T. at excitation wavelength of 622 nm and emission wavelength of 670 nm. Dye uptake, and resultant self quenching, was modulated by the membrane potential. After reaching the maximum uptake of the dye by bacteria, which is indicated by a minimum in dye fluorescence (after 20 min), test compounds (vancomycin and compounds **1**-**6** at 5 μM) were added to the cells, and the decrease in potential was monitored by the increase in fluorescence for further 20 min.

4.5.6.2 Inner membrane permeabilization assay[142]

Inner membrane depolarization assay was performed against VRE as described previously in the section of 3.5.4.2 in Chapter 3 with slight modifications. Briefly, mid-log phase (grown for 6 h) VRE cells were harvested, washed with 5 mM HEPES and 5 mM glucose and resuspended in 5 mM glucose, 5 mM HEPES buffer of pH 7.2 in 1:1 ratio (10^8 CFU/mL). Then test compounds (vancomycin and compounds **1**-**6** at 5 μM) were added to 0.2 mL of bacterial suspension and 10 μM propidium iodide (PI) in a 96 well plate. The fluorescence of the dye was monitored for 15 min at R.T. at excitation wavelength of 535 nm and emission wavelength of 617 nm. The uptake of PI was measured by the increase in fluorescence of PI for 10 min as a measure of inner membrane permeabilization.

4.5.6.3 Potassium ion leakage assay[143]

Intracellular K^+ ion leakage assay was performed against VRE as described previously in the section of 3.5.4.3 in Chapter 3 with slight modifications. Briefly, mid-log phase (grown for 6 h) VRE cells were harvested, washed twice with 10 mM HEPES (pH 7.2) and 0.5 % glucose and were resuspended in the same amount of 10 mM HEPES (pH 7.2) and 0.5 % glucose (10^8 CFU/mL). The bacterial suspension (0.2 mL) was placed in a 96 well plate. The fluorescence of the bacterial suspension was measured and allowed to stabilize for 2 min at room temperature before the addition of PBFI-AM dye (1 µM). Data were collected for an additional 2 min to establish a baseline signal before the addition of test compounds (vancomycin, compounds **1-6** at 5 µM and valinomycin at 10 µM). The fluorescence signals were collected for each sample over 10 min. The fluorescence of the dye was monitored at excitation wavelength of 346 nm and emission wavelength of 505 nm.

4.5.7 Resistance development study

The same protocol was followed using the test compounds **2** and vancomycin against MRSA as described in the section of 2.5.7 in Chapter 2.

4.5.8 Hemolytic assay

The same protocol was followed using the test compounds **1-5** and vancomycin against human RBC as described in the section of 3.5.8.1 in Chapter 3.

4.5.9 *In-vivo* antibacterial activity

4.5.9.1 Murine renal infection model

The same protocol was followed using the test compounds **2**, vancomycin and linezolid against VREs (VanB phenotype) at 12 mg/kg as described in the section of 2.5.8.1 in Chapter 2.

4.5.9.2 Mouse neutropenic thigh infection model

The same protocol was followed using the test compounds **2** (total dose of 12 mg/kg), vancomycin (total dose of 24 mg/kg) and linezolid (total dose of 24 mg/kg) against VISA as described in the section of 2.5.8.2 in Chapter 2.

4.5.9.3 Dose-responsive study (Pharmacodynamics)

The same protocol was followed using the test compound **2** against VISA at six different regimens (2, 4, 8, 12, 25 and 50 mg/kg) as described in the section of 2.5.8.3 in Chapter 2.

4.5.10 Single-dose pharmacokinetic study

The same protocol was followed using the test compound **2** at 12 mg/kg as described in the section of 2.5.9 in Chapter 2.

4.5.11 *In-vivo* toxicology (Systemic toxicity)

The same protocol was followed using the test compound **2** at 100 mg/kg as described in the section of 2.5.10.1 in Chapter 2.

4.5.12 *In-vivo* data analysis

The similar analysis was performed as described in the section of 2.5.11 in Chapter 2.

Chapter 5

5A. An Alternative Approach to Tackle Vancomycin-resistant Bacteria by Dipicolyl-vancomycin Conjugate

Abstract

*In **Chapter 5A**, an alternative strategy has been described to combat acquired resistance of bacteria towards vancomycin. In this chapter, dipicolyl-1,6-hexadiamine (Dipi), which is known to form strong complex with Zn^{2+} ion, is conjugated to C-terminal of vancomycin (Dipi-van). Dipi-van showed 375-fold more in-vitro activity than vancomycin against vancomycin-resistant Enterococci (VRE). Neither Dipi alone nor its physical mixture with vancomycin showed any activity against VRE. Further, Dipi-van showed improved cell wall (peptidoglycan) inhibition and demonstrated high in-vivo activity against VRE in kidney infection model with no observed toxicity. Dipi-van presumably has the ability to complex with Zn^{2+} ion from D,D-peptidases (VanX, VanY, and VanXY, which are the key enzymes responsible for acquired resistance to vancomycin) and bind to the cell wall precursor peptides. These findings stress the importance of the approach to combat vancomycin-resistant bacterial infections.*

Publication based on this work

1) Yarlagadda, V. *et al.* An alternative approach to tackle vancomycin-resistant bacteria by dipicolyl-vancomycin conjugate. *Manuscript under preparation.*

5A.1 Introduction

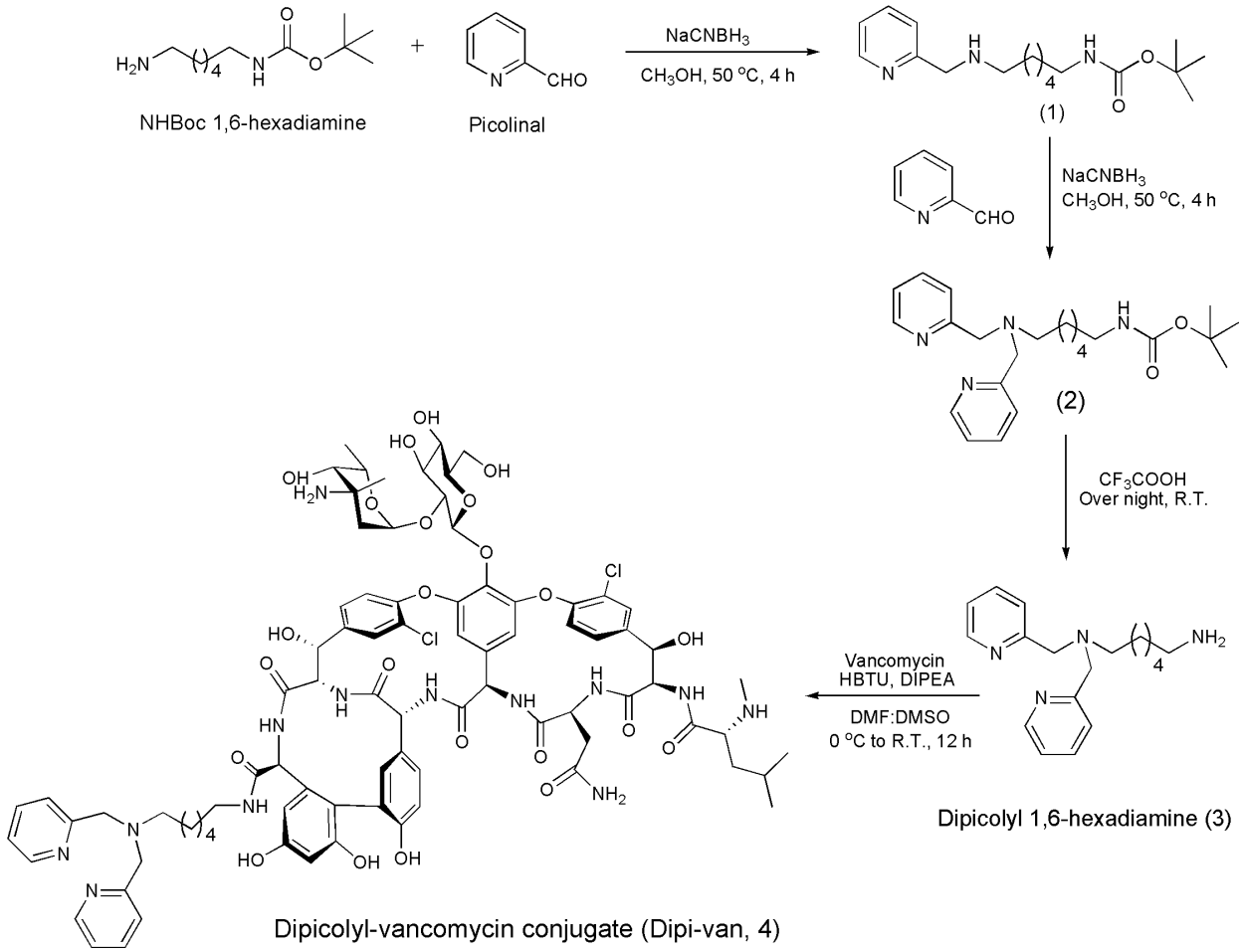
Vancomycin inhibits cell wall biosynthesis of Gram-positive bacteria by specifically binding to cell wall precursors that terminate in D-Ala-D-Ala thus inhibiting transpeptidase-catalyzed cross-linking and maturation of the bacterial cell wall.[54] Bacteria develop resistance to vancomycin by remodeling their cell wall precursors, that terminate in D-Ala-D-Lac (lactate) depsipeptide termini, which significantly reduces vancomycin binding affinity by 1000-fold and results in unimpeded peptide chain cross-linking.[54] D,D-peptidases such as VanX, VanY and VanXY are some of the key enzymes that are responsible for the high level resistance to vancomycin.[59] In all these enzymes divalent zinc ion (Zn^{2+}) lies in the active site.[146] It is known that dipicolylamine moiety has the ability to capture divalent zinc ion (Zn^{2+}) with high selectivity.[147,148] In the present study, dipicolyl-1,6-hexadiamine (Dipi) was conjugated to vancomycin to yield dipicolyl-vancomycin conjugate (Dipi-van). Dipi-van was 375-fold more effective than vancomycin against VRE (VanA phenotype). Unlike vancomycin, Dipi-van did not induce the development of bacterial resistance. This compound, compared to vancomycin, showed higher *in-vivo* antibacterial activity against VRE in kidney infection model with no observed toxicity at the conditions tested.

5A.2 Results

5A.2.1 Synthesis

Dipicolyl-vancomycin conjugate as pentakis-(trifluoroacetate) salt (Dipi-van, **4**) was prepared by coupling the carboxylic group of vancomycin with dipicolyl-1,6-hexadiamine (Dipi, **3**) through amide coupling using *N*,*N*,*N'*,*N'*-tetramethyl-*O*-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) (Scheme 5A.1) with 77 % yield. To prepare Dipi-van, vancomycin was dissolved in 1:1 dry DMF:DMSO and HBTU solution in DMF was added drop wise at 0 ºC. Subsequently the dipicolyl-1,6-hexadiamine was added to the vancomycin solution and the reaction mixture was stirred at room temperature for 12 h. Then, the reaction mixture was purified by reverse phase HPLC to more than 95 % purity and characterized by ^{1}H-NMR and HR-MS. To synthesize dipicolyl-1,6-hexadiamine (**3**), NHBoc-1,6-hexadiamine was reacted twice with 2-picolinal (pyridine-2-aldehyde) to form Schiff's base and followed by reduction

with sodium cyanoborohydride (compounds **1** and **2**). Then the Boc group was deprotected under acidic conditions (Scheme 5A.1) to form dipicolyl-1,6-hexadiamine (**3**) which was directly used in coupling with vancomycin.



Scheme 5A.1: Synthesis of dipicolyl-vancomycin conjugate (**4**).

5A.2.2 *In-vitro* antibacterial activity

The antibacterial activities of vancomycin and compound **4** were evaluated by determining the minimum inhibitory concentrations (MICs) against MRSA, vancomycin-resistant strains of Staphylococci (VISA) and Enterococci (VRE; VanA and VanB phenotypes). The results are summarized in Fig. 5A.1A. Against MRSA, compound **4** showed similar or slightly better

activity than vancomycin. Compound **4** exhibited much improved antibacterial activity toward VISA (MIC ~ 1 µM) in comparison to vancomycin (MIC of 13 µM). When tested against VREm (VanA phenotype, *E. faecium*), compound **4** showed MIC of 2 µM whereas the MIC for vancomycin was found to be 750 µM. Compound **4** also showed much improved activity against VanB phenotype of VREs (*E. faecalis*) with the MIC of 1.5 µM while vancomycin was active at 250 µM (Fig. 5A.1A).

The antibacterial activity of dipicolyl moiety alone (without vancomycin), compound **3** was evaluated against VREm (VanA phenotype) and found to be inactive even at 100 µM. Further, the activity of a physical mixture of vancomycin and compound **3** was determined and the physical mixture was found to be inactive even up to their individual concentrations of 50 µM against VRE, whereas compound **4** showed MIC of 2 µM.

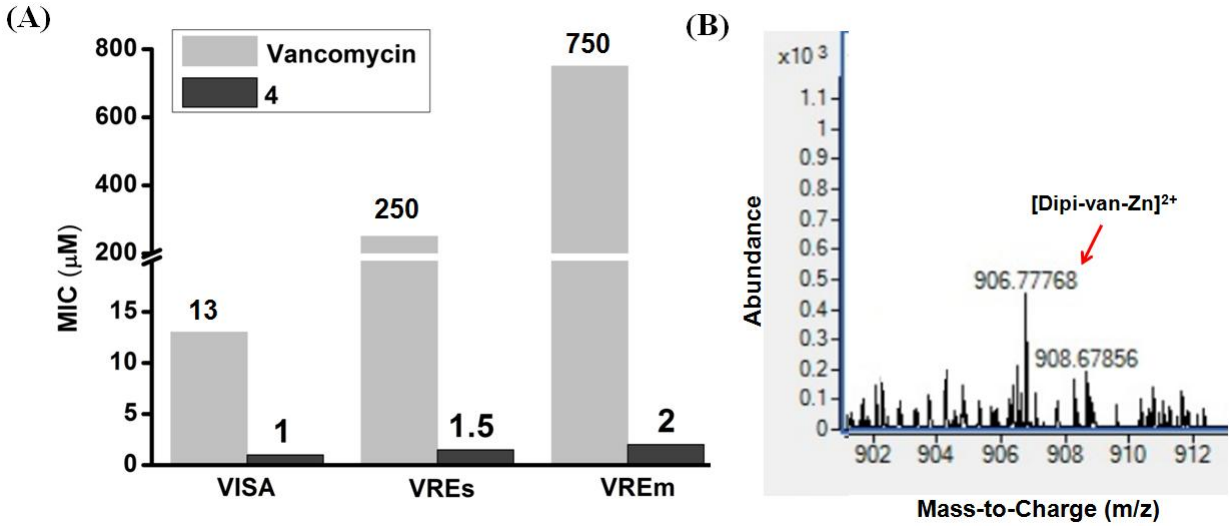


Figure 5A.1: (A) Antibacterial activity of compound **4** (Dipi-van) and vancomycin against vancomycin-intermediate *S. aureus* (VISA), vancomycin-resistant *E. faecalis* (VREs, VanB phenotype) and vancomycin-resistant *E. faecium* (VREm, VanA phenotype). (B) Dipi-van-Zn^{2+} complex confirmed by mass-spectrometry.

Then, the activity of compound **4** was evaluated in presence of external divalent zinc ion (as antagonist) using zinc sulfate ($ZnSO_4$) against VREm. Surprisingly, compound **4** exhibited 2-

fold to 3-fold more activity in presence of external Zn^{2+}. This might be attributed to the formation of Dipi-van-Zn^{2+} complex which can bind to pyrophosphate of lipid II at outer surface of the bacterial membrane. Further, the formation of Dipi-van-Zn^{2+} complex was also confirmed by mass spectrometry (Fig. 5A.1B).

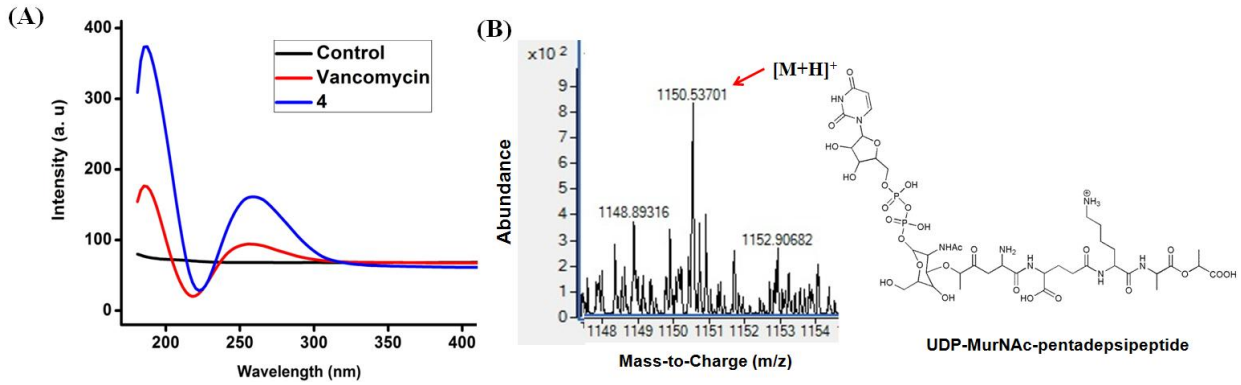


Figure 5A.2: Intracellular accumulation of the cell wall precursor UDP-MurNAc-pentadepsipeptide after treatment of VREm (VanA phenotype) with vancomycin and compound **4** (Dipi-van) at 5 μM. Untreated cells were used as control. (A) Identification of intracellular UDP-MurNAc-pentadepsipeptide by monitoring absorbance at 260 nm wavelength (B) UDP-MurNAc-pentadepsipeptide was identified by mass spectrometry as indicated by the peak at m/z 1150.53.

5A.2.3 Intracellular accumulation of cell wall (peptidoglycan) precursor

In order to investigate whether new vancomycin derivative interfere with cell wall (peptidoglycan) biosynthesis, the accumulation of UDP-linked peptidoglycan precursor, UDP-*N*-acetyl-muramyl-pentadepsipeptide (UDPMurNAc-pp) was determined after treating the bacteria (VRE) with compound **4** and vancomycin at 5 μM (Fig. 5A.2A and 5A.2B). In case of compound **4** a more intense peak was observed at 260 nm compared to vancomycin, which corresponds to accumulation of UDPMurNAc-pp and confirmed by high-resolution mass spectrometry (m/z = 1150.94 (cal), 1150.53 (obs) for $[M+H]^+$). The result suggest that compounds **4** showed greater cell wall biosynthesis inhibition than vancomycin.

5A.2.4 Propensity to induce bacterial resistance

In light of the alarming rise of drug resistance in bacteria, the potential emergence of bacterial resistance against this new compound was evaluated. The propensity of bacteria to generate resistance can be evaluated through serial exposure of organisms to antimicrobial agents. To establish whether the compound **4**, indeed prevented the development of bacterial resistance, MRSA was exposed to vancomycin and compound **4** for serial passages and the changes in MIC values were monitored over a period of 25 days. Even after 25 serial passages, the MIC of compound **4** remained the same. However, in case of vancomycin, the MIC value started increasing after 7 passages and the value increased to 16-fold after 25 passages (Fig. 5A.3A). Thus bacteria were unable in acquiring resistance to this compound and this emphasizes the longevity of such compounds in clinics.

5A.2.5 *In-vitro* toxicity (hemolysis and cytotoxicity)

The toxicity of compound **4** was studied by measuring cytotoxicity (CC_{50}; 50 % cytotoxic concentration) against mammalian cells (HeLa) and hemolytic activity (HC_{50}; 50 % hemolytic concentration) against human RBC. Compound **4** did not show any significant toxicity towards either of the cells even up to 1000 μM concentration, which indicates the selective toxicity of compound **4** against bacterial cells over mammalian cells.

5A.2.6 *In-vivo* antibacterial activity

The *in-vivo* activity of compound **4** was evaluated in a renal infection model against VRE. Initially, mice were injected intravenously with 0.2 mL of 0.2 % λ-carrageenan to increase their susceptibility to bacterial renal infection. After 7 days, the mice were infected with VRE (VanB phenotype, ~ 10^8 CFU/mouse). After 4 h of infection the mice were treated with three doses (every 24 h interval) of vancomycin (12 mg/kg), linezolid (12 mg/kg), compound **4** (12 mg/kg) and saline. After 72 h of the initial treatment, antibacterial activity was determined by finding the bacterial count in the infected kidneys. In comparison to vancomycin, compound **4** and linezolid reduced bacterial titer from the infected kidneys more effectively. Linezolid produced ~ 4 $\log_{10}$ CFU reduction compared to vehicle treated control (saline) whereas compound **4** produced ~ 5.0 $\log_{10}$ CFU reduction (Fig. 5A.3B).

5A.2.7 *In-vivo* toxicology studies

Next, the *in-vivo* systemic toxicity of compound **4** was assessed after single-dose intravenous (i.v.) administration at 100 mg/kg dosing regimen to mice (n = 5). Then, the animals were observed for mortality for a period of 14 days. All the mice were found to survive at 14 days indicating the high tolerability of compound **4** in animals with LD_{50} > 100 mg/kg.

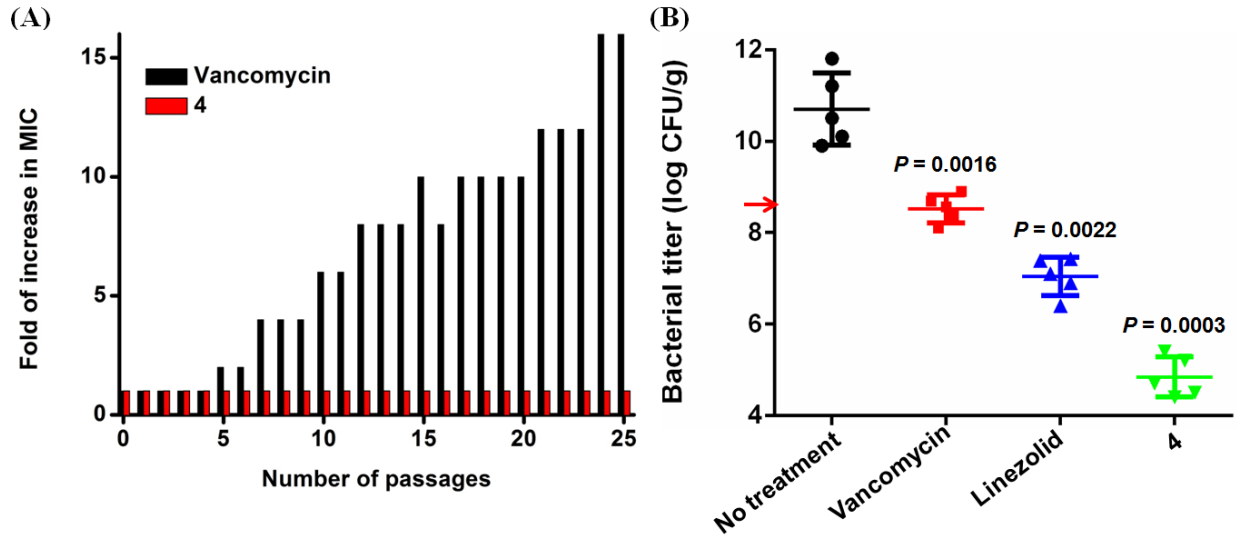


Figure 5A.3: (A) Bacterial resistance studies of vancomycin and compound **4** against MRSA. (B) *In-vivo* antibacterial activity of vancomycin, linezolid and compound **4** in renal infection model against VRE (n = 5) at 12 mg/kg. Red arrow indicates bacterial pre-treatment titer (~ 8.0 log_{10} CFU/g). Five mice were used in each group. Statistical analysis was performed using Student's *t*-test. Differences are considered statistically significant from untreated group with a value of $P < 0.05$.

5A.3 Discussion

In an attempt to develop novel therapeutics to conquer bacterial resistance, much attention has been focused on developing semi-synthetic glycopeptide antibiotics.[54,90] In previous chapters, strategies have been developed to improve the lost binding affinity of vancomycin and to impart

an additional mechanism of action to vancomycin in order to combat vancomycin-resistant bacteria. In this study, an alternative semi-synthetic strategy has been developed to combat VRE.

D,D-Peptidase enzymes such as VanX, VanY and VanXY are zinc (II) dependent and are responsible for high vancomycin resistance in VRE.[146] In this report, dipicolyl-vancomycin conjugate (Dipi-van), which has the ability to complex with Zn^{2+} ion, has been developed using simple synthetic approach. The antibacterial activity of Dipi-van was 375-fold and ~ 160-fold more active than vancomycin against VREm (VanA phenotype) and VREs (VanB phenotype), respectively. Additionally, in presence of external Zn^{2+} ion, the activity of Dipi-van was increased further by ~ 3-fold against VRE. This enhanced activity is attributed to the formation of Dipi-van-Zn^{2+} complex, which is confirmed by mass spectrometry. It has been shown in the literature that dipicolyl-Zn^{2+} complexes have the high affinity to complex with pyrophosphates.[148,149] Following this observation, it is surmised that Dipi-van-Zn^{2+} complex can also bind to pyrophosphate of lipid II and bactoprenol that is accessible at extracellular surface thus halting the role of these lipids in continuous cyclic process of the formation of peptidoglycan layers.

Treatment of whole cells of VRE with Dipi-van resulted in more accumulation of the soluble cell wall precursor undecaprenyl-*N*-acetyl-muramyl-pentadepsipeptide compared to vancomycin, suggesting the enhanced inhibition of peptidoglycan biosynthesis. Additionally, the new installed features of compound **4** had a significant impact on stalling the development of bacterial resistance to the drug.

Infections caused by VRE have been increasing in frequency, representing an emerging threat to public health. Unlike vancomycin, compound **4** and linezolid showed very good activity against VRE in renal infection model. In particular, compound **4** was significantly more active than linezolid. Further, compound **4** was non-toxic to mammalian cells against human RBC and HeLa cells. Moreover, this compound has low or no *in-vivo* systemic toxicity in mice models and has good safety profile required for therapeutic applications.

5A.4 Conclusions

A simple strategy was employed to combat acquired resistance of Gram-positive bacteria towards vancomycin. The new compound showed significantly high activity against vancomycin-resistant bacteria. Further, this compound showed high *in-vivo* activity against VRE compared to linezolid and vancomycin with no observed toxicity. Moreover, no detectable resistance was observed after several serial passages of bacterial exposure to the new compound. These findings suggest that this strategy may have clinical utility for the treatment of vancomycin-resistant bacterial infections.

5A.5 Experimental procedure

5A.5.1 Materials and Methods

All reagents were purchased from Sigma-Aldrich and SD Fine and used without further purification. Analytical thin layer chromatography (TLC) was performed on E. Merck TLC plates pre-coated with silica gel 60 F_{254} (250 μm thickness). Visualization was accomplished using UV light and Iodine. All final compounds were purified by reverse phase HPLC using 0.1 % trifluoroacetic acid (TFA) in water/acetonitrile (0-100 %) as mobile phase to more than 95 % purity. HPLC analysis was performed on a Shimadzu-LC 8 Å Liquid Chromatography instrument (C_{18} column, 10 mm diameter, 250 mm length) with UV detector monitoring at 270 nm. Nuclear magnetic resonance spectra were recorded on Bruker (AV-400) 400 MHz spectrometer in deuterated solvents. High resolution mass spectra (HR-MS) were obtained using 6538-UHD Accurate Mass Q-TOF LC-MS instrument. UV-absorption measurements were obtained using Thermo-Fisher Scientific UV-10 spectrometer for determination of binding constants. Eppendorf 5810R centrifuge was used. TECAN (Infinite series, M200 pro) Plate Reader was used to measure absorbance. Bacterial strains, MRSA ATCC 33591, Enterococcal strains were obtained from ATCC (Rockville, MD). Tryptic-soy agar media was used for Staphylococci and sheep blood agar plates were used for Enterococci. VISA, Vancomycin-intermediate-resistant *S. aureus* was generated from MRSA (ATCC 33591) after treating with vancomycin for 52 passages.

Animals: The same as described in section 2.5.1 in Chapter 2.

5A.5.2 Synthesis and characterization

Synthesis of dipicolyl-1,6-hexadiamine (3): 1 equivalent of picolinal was taken in 15 mL dry methanol and 1.2 equivalents of *N*-Boc-1,6-hexanediamine was added to it and the reaction mixture was kept for stirring at 50 °C for 2 h. Then the reaction was allowed to come to room temperature and 1.5 equivalents of sodium cyanoborohydride ($NaCNBH_3$) was added to it.[124] The reaction mixture was stirred at 50 °C for additional 2 h and then at room temperature for overnight. Then, the crude product was subjected to HPLC and the required product (**1**) was obtained at retention time value of 11.0 min with a yield of 55 %. ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 1.33 (s, 9H), 1.24-1.33 (m, 4H), 1.41-1.69 (m, 4H), 2.90-2.99 (t, 2H), 3.06-3.09 (t, 2H), 4.37 (s, 2H), 7.55-7.62 (m, 2H), 8.01-8.05 (m, 1H), 8.58-8.60 (m, 1H); HR-MS: *m/z* 308.3851 (observed); 308.2372 (calculated for $[M+H]^+$).

The above mentioned procedure was repeated with 1.2 equivalents of picolinal and 1 equivalent of compound **1**. Then product (**2**) was obtained with a yield of 63 %. ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 1.40-1.48 (m, 4H), 1.49 (s, 9H), 1.84-2.02 (m, 4H), 2.91-2.98 (t, 2H), 3.14-3.23 (t, 2H), 4.37 (s, 2H), 7.468-7.554 (m, 4H), 7.90-7.99 (m, 2H), 8.53-8.59 (m, 2H); HR-MS: *m/z* 399.2747 (observed); 399.5571 (calculated for $[M+H]^+$).

Then, compound **2** was dissolved in methanol and 5 mL of trifluoroacetic acid (CF_3COOH) was added to it and the reaction mixture was stirred at room temperature for 4 h. Then the solvent was evaporated and product was obtained with quantitative yield of 95 %. ^{1}H-NMR (400 MHz, D_2O) δ/ppm: 1.372-1.437 (m, 4H), 1.678-1.862 (m, 4H), 3.10-3.14 (t, 2H), 3.24-3.29 (t, 2H), 4.518 (s, 4H), 7.445-7.518 (m, 4H), 7.86-7.957 (m, 2H), 8.58-8.60 (m, 2H); HR-MS: *m/z* 299.2222 (observed); 299.4371 (calculated for M^+).

Synthesis of dipicolyl-vancomycin conjugate (4)[125]: About 150 mg of vancomycin was dissolved in 1:1 mixture of dry dimethyl formamide (1 mL) dry dimethyl sulfoxide (1 mL). To this two equivalents of dipicolyl-1,6-hexadiamine (**3**) in 1 mL of dry dimethylformamide was added. The reaction mixture was cooled to 0 °C, and about 1.5 equivalents of 0.45 M HBTU solution in DMF was added followed by about 5.0 equivalents of diisopropylethylamine (DIPEA). The reaction mixture was then allowed to warm to room temperature and stirred for overnight. The product was purified by preparative reverse-phase HPLC using about 0.1 %

trifluoro acetic acid in H_2O/acetonitrile mixture (0-100 %) as mobile phase and then lyophilized to afford dipicolyl-vancomycin conjugate (**4**) as pentakis (trifluoroacetate) salt with 70 % yield. C_{18} column (10 mm diameter, 250 mm length) and UV detector (at 270 nm wave length) were used. The collected fractions, from HPLC were frozen by liquid N_2 and lyophilized using freeze dryer. This conjugate was characterized by ^{1}H-NMR and HR-MS.

^{1}H-NMR (400 MHz, DMSO-d_6) δ/ppm: 9.43 (s, 1H), 9.14-9.12 (d, 3H), 9.05-9.00 (m, 2H), 8.72 (bs, 2H), 8.52 (bs, 2H), 9.18-8.12 (d, 2H), 7.85 (bs, 1H), 7.56-7.46 (m, 4H), 7.36-7.34 (m, 2H), 7.21-7.09 (m, 2H), 6.79-6.70 (m, 2H), 6.39-6.24 (dd, 2H), 5.95-5.63 (m, 3H), 5.33-4.93 (m, 5H), 4.73-4.58 (m, 2H), 4.46-4.38 (m, 3H), 4.21-4.18 (m, 2H), 3.96 (bs, 2H), 3.29-3.28 (d, 2H), 2.63 (m, 4H), 2.18-2.05 (m, 2H), 1.92-1.90 (m, 3H), 1.67-1.54 (m, 2H), 1.27-1.24 (m, 5H), 0.92-0.83 (m, 8H). HR-MS: *m/z* 865.8226 (observed), 865.8318 (calculated for $[M+2H]^{2+}$).

5A.5.3 Minimum inhibitory concentration (MIC)

The same protocol was followed using the test compounds as described in the section of 2.5.3 in Chapter 2. The combination antibacterial efficacy of compound **3** and vancomycin was measured by using chequerboard assay in the following manner.[10] A solution of 25 μL each of test compounds was added into each well of a 96 well plate followed by 150 μL of bacterial suspension (~ 5.0×10^5 CFU/mL). The plate was then incubated at 37 °C for a period of 24 h and the O.D. value was measured at 600 nm using a Tecan InfinitePro series M200 Microplate Reader. The MIC from chequerboard assay was a result of two independent experiments and each experiment was performed in triplicates. For determination of activity in presence of external zinc sulfate ($ZnSO_4$), Dipi-van and $ZnSO_4$ were mixed in equimolar concentrations (100 μM + 100 μM), then serially diluted and used for MIC experiment.

5A.5.4 Intracellular accumulation of UDP-*N*-acetyl-muramyl-pentadepsipeptide

The same protocol was followed using the test compounds **4** and vancomycin at 5 μM against VRE (VanA phenotype) as described in the section of 2.5.5 in Chapter 2.

5A.5.5 Resistance development study

The same protocol was followed using the test compounds **4** and vancomycin against MRSA as described in the section of 2.5.7 in Chapter 2.

5A.5.6 *In-vitro* toxicology

5A.5.6.1 Hemolytic assay

The same protocol was followed using the compounds **4** and vancomycin (1000 μM to 2 μM) against human RBC as described in the section of 3.5.8.1 in Chapter 3.

5A.5.6.2 Cytotoxicity assay

The same protocol was followed using the compounds **4** and vancomycin (1000 μM to 2 μM) against HeLa cells as described in the section of 3.5.8.2 in Chapter 3.

5A.5.7 murine renal infection model

The same protocol was followed using the test compounds **4**, vancomycin and linezolid against VREs (VanB phenotype) at 12 mg/kg as described in the section of 2.5.8.1 in Chapter 2.

5A.5.8 *In-vivo toxicology*

The same protocol was followed using the test compound **4** at 100 mg/kg as described in the section of 2.5.10.1 in Chapter 2.

Chapter 5

5B. Targeting NDM-1 Gram-negative Bacteria by Combination of Dipicolyl-vancomycin Conjugate and Meropenem

Abstract

The acquisition of metallo-β-lactamases (MBLs, zinc ions in the active site) such as New Delhi metallo-β-lactamase-1 (NDM-1) is the major contributor to the emergence of carbapenem-resistance in Gram-negative pathogens that threatens the use of available antibiotics. To date, clinically approved inhibitor of MBLs that could undo resistance and re-sensitize resistant Gram-negative pathogens to carbapenems has not been found, making these enzymes a serious threat to human health. In ***Chapter 5B****, dipicolyl-vancomycin conjugate (Dipi-van) has been shown to restore the activity of meropenem against a variety of NDM-1 producing Gram-negative pathogens such as Klebsiella pneumoniae and Escherichia coli. Further, in mice infected with NDM-1 expressing K. pneumoniae, Dipi-van efficiently restored meropenem activity in sepsis model. These findings demonstrate that a combination of Dipi-van and meropenem has therapeutic potential to address the clinical challenge of NDM-positive carbapenem-resistant Gram-negative pathogens.*

Publication based on this work

1) Yarlagadda, V. *et al.* Vancomycin analogue restores meropenem activity against NDM-1 Gram-negative pathogens. *Manuscript under preparation.*

5B.1 Introduction

The alarming growth of carbapenem-resistant superbugs such as New Delhi metallo-beta-lactamase-1 (NDM-1) producing pathogens has become a major global health hazard.[150,151] NDM-1 has been identified mostly in *Escherichia coli, Klebsiella pneumoniae* and to a lesser extent in *Pseudomonas* and *Acinetobacter.*[152] The $bla_{\text{NDM-1}}$ gene is located on plasmids harboring multiple resistant determinants, thereby conferring extensive drug resistance. Treatment of these drug-resistant infections is limited by the dearth of effective antibiotics.

The β-lactams remain the most widely used antibiotics and are essential in the treatment of serious Gram-negative bacterial infections.[12] β-Lactam antibiotics inhibit transpeptidases (penicillin-binding proteins, PBPs) involved in cell wall biosynthesis by reacting with a nucleophilic serine residue of PBPs important in catalysis.[153,154] The use of β-lactams is compromised by resistance mechanisms, primarily the production of β-lactamases, which catalyse β-lactam hydrolysis.[155] Four classes of β-lactamases have been identified on the basis of their substrate selectivity and structure. Classes A, C and D are 'serine' β-lactamases (SBLs) and are evolutionarily and mechanistically related to the PBPs.[156,157] Class B β-lactamases are zinc (II) dependent hydrolases, and are mechanistically different.[157]

Inhibitors of Class A, C and D enzymes have been developed and have substantially extended the spectrum of activity of β-lactamas.[158,159] As yet, there are no reports of clinically useful inhibitors of Class B MBLs.[159,160] The potential threat of MBLs is highlighted by NDM-1, which enables resistance to almost all β-lactams, including the latest generations of cephalosporins and all carbapenems, which are often considered as 'last resort antibiotics'.[161] Significant strategies have been reported in recent years for the development of MBL inhibitors but none of them have reached to clinics.[162,163]

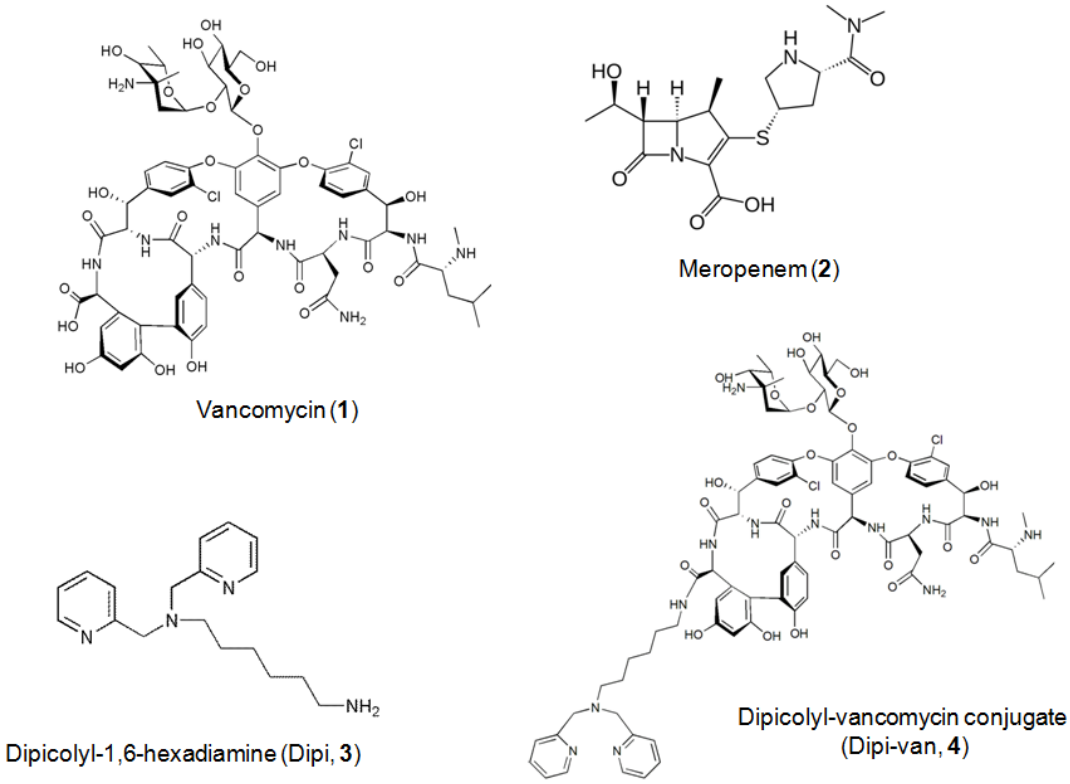


Figure 5B.1: Structures of vancomycin, meropenem, dipicolyl-1,6-hexadiamine, dipicolyl-vancomycin conjugate.

One efficient approach to combating resistance is to use combined treatments consisting of an antibiotic and a compound that potentiates the antibiotic's activity by targeting bacterial resistance mechanisms.[10,164] Recently, King *et al.* reported a naturally occurring fungal compound, aspergillomarasmine A (AMA), can interact with the zinc ions of NDM-1, preventing inactivation of meropenem, which then kills the NDM-1 producing Enterobacteria.[165] Vancomycin, a cell wall biosynthesis inhibitor, has been considered as drug of last resort for Gram-positive pathogens but it is inherently inactive towards Gram-negative pathogens because of its inability to cross the outer membrane of these pathogens.[54] Due to the physical proximity of the principal cellular targets of vancomycin, β-lactams and MBLs, and their sequential role in the cell-wall biosynthetic pathway, it is anticipated that a derivative of vancomycin which can permeabilize the outer membrane of Gram-negative bacteria and also which can detain zinc ions from NDM-1 enzyme, would be able to resensitize meropenem-resistant bacteria (NDM-1

producing pathogens) to meropenem. Due to bacterial specificity of vancomycin, it was reasoned that a derivative of vancomycin might provide enhanced selectivity towards bacterial cell wall associated metalloenzymes (NDM-1). Having shown the ability of dipicolyl-vancomycin conjugate (Dipi-van) to form a complex with Zn^{2+} in Chapter 5A, here, in Chapter 5B, studies were performed to validate its ability to restore meropenem activity against NDM-1 producing pathogens both *in-vitro* and *in-vivo*.

5B.2 Results

5B.2.1 Synthesis

Dipicolyl-vancomycin conjugate (Fig. 5B.1, Dipi-van, **4**) was synthesized as described in section 5A.2.1 in Chapter 5A. Briefly, dipicolyl-1,6-hexadiamine (**3**) was conjugated to the carboxylic group of vancomycin to yield Dipi-van.

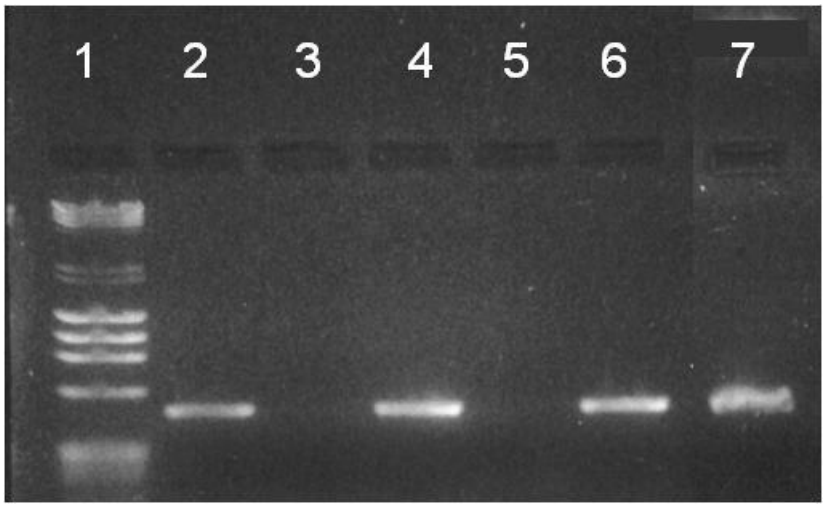


Figure 5B.2: Schematic representation of agarose gel (2%) showing the 475 bp amplified product by conventional polymerase chain reaction. Lane 1, 100 bp DNA ladder; Lane 2, positive control- NDM-1 producing *K. pneumoniae* (ATCC BAA-2146); Lane 3, negative control- *E. coli* (ATCC 25922); Lane 4, *E. coli* R3336 confirm the NDM-1 gene, Lane 5, multi-drug resistant (MDR) *K. pneumoniae* R3421 which was negative for NDM-1 gene; Lane 6, *K. pneumoniae* R3949 and Lane 7, *K. pneumoniae* R3934 confirm the NDM-1 gene.

5B.2.2 Isolation of NDM-1 expressing Gram-negative pathogens

Clinical samples of carbapenem-resistant bacteria (minimum inhibitory concentration, MIC of meropenem > 16 µg/mL) were characterized for NDM-1 gene (475 bp). Conventional polymerase chain reaction (PCR) and gel electrophoresis was used for the identification of NDM-1 gene using primers NDM-F (5′-GGG CAG TCG CTT CCA ACG GT-3′) and NDM-R (5′-GTA GTG CTC AGT GTC GGC AT-3′) (Fig. 5B.2). UV light was used for the visualization of bands and an amplified product corresponding to 475 bp was considered as positive. The NDM-1 gene was confirmed in *K. pneumoniae* R3934, *K. pneumoniae* R3949 and *E. coli* R3336 including the positive control *K. pneumoniae* (ATCC BAA-2146) whereas MDR *K. pneumoniae* R3421 showed negative.

Table 5B.1: Antibacterial activity of conventional antibiotics against NDM-1 expressing clinical isolates

	Minimum Inhibitory Concentration (µg/mL)					
NDM-1 expressing bacteria	Colistin	Tigecycline	Ciprofloxacin	Erythromycin	Kanamycin	Minocycline
K. pneumoniae R3949	0.5	0.5	>250	>250	>250	32
K. pneumoniae R3934	0.5	0.5	>250	>250	>250	62
K. pneumoniae ATCC BAA2146	0.5	0.5	>250	125	>250	62
E. coli R3336	0.5	0.5	125	>250	>250	32

5B.2.3 *In-vitro* antibacterial activities

All the four NDM-1 isolates and the MDR strain are represented here on as R3336, R3934, R3949, ATCC2146 and R3421 respectively. All the isolates were sensitive only to tigecycline and colistin (MIC = 0.5-1 µg/mL) and were highly resistant to other conventional antibiotics (ciprofloxacin, kanamycin, erythromycin, tetracycline, doxycycline and minocycline) and cell-wall directed antibiotics (meropenem) (Table 5B.1). In most of the cases, the MICs of the antibiotics against all the bacteria were > 250 µg/mL, the highest concentration tested. This provides yet another evidence for high level of multi-drug resistance in carbapenem-resistant

bacteria in clinical settings. The antibacterial activity of dipicolyl-vancomycin conjugate (Dipi-van) alone was also evaluated and found to be marginal against all the isolates. Dipi-van displayed MIC of 100 µM against R3336, R3934, R3949, R3421 whereas it showed MIC of 50 µM against ATCC2146 (Table 5B.2).

Table 5B.2: *In-vitro* antibacterial activity of meropenem with or without Dipi-van and FIC index against NDM-1 clinical isolates.

	Minimum Inhibitory Concentration (µg/mL)			
	Meropenem		Dipi-van	FIC index of Dipi-van and meropenem
NDM-1 expressing bacteria	- Dipi-van	+ Dipi-van (12.5 µM)		
K. pneumoniae R3949	>100	3.1	100	0.14
K. pneumoniae R3934	>100	3.1	100	0.14
K. pneumoniae ATCC BAA2146	>100	1.5	50	0.26
E. coli R3336	>100	12	>100	0.14

Table 5B.3: *In-vitro* antibacterial activity of meropenem with or without dipicolyl-1,6 hexadiamine (Dipi, **3**) against NDM-1 clinical isolates.

	Minimum Inhibitory Concentration (µg/mL)	
NDM-1 expressing bacteria	- Dipi	+ Dipi (100 µM)
K. pneumoniae R3949	>100	50
K. pneumoniae R3934	>100	>50
K. pneumoniae ATCC BAA2146	>100	50
E. coli R3336	>100	>50

The combination activity of meropenem and Dipi-van was evaluated against all the four isolates using chequerboard assays. Using the fractional inhibitory concentration (FIC), a combination was called synergistic when combined FIC of both the agents, (the FIC Index; FICI) was ≤ 0.5. In the presence of Dipi-van, the MIC of meropenem (3.1 µg/mL) reduced drastically when tested against the three clinical isolates- R3336, R3949 and R3934, wherein synergistic effect (FICI of ≤ 0.5) of Dipi-van was observed (Table 5B.2).

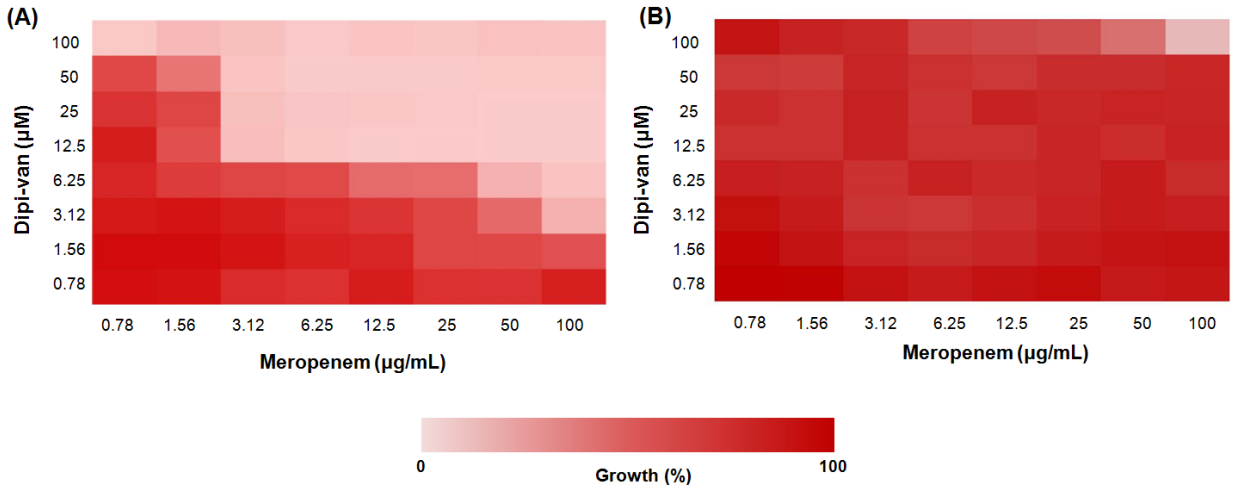


Figure 5B.2: Microdilution chequerboard analysis showing the combined efefct of Dipi-van and meropenem against NDM-1 positive *K. pneumoniae* R3934 (A) and NDM-1 negative carbapenem-resistant *K. pneumoniae* R3921 (B).

Notably, Dipi-van at 12.5 µM, which is 1/8th and 1/4th of its MIC against R3336, R3934, R3949 and ATCC 2146 respectively showed high synergy (FICI as low as 0.14) in combination with meropenem (Table 5B.2 & Fig. 5B.2A). The study also demonstrated that Dipi-van resensitized all the four isolates to meropenem. The CLSI (Clinical & Laboratory Standards Institute) breakpoint for the susceptibility of carbapenem antibiotics is ≤ 4 µg/mL.[166] The MIC of meropenem was brought down to susceptible limits of as low as 1.5-3.1 µg/mL in the presence of Dipi-van (Table 5B.2). Dipi-van at 12.5 µM (1/8th of its MIC) reduced the MIC of meropenem (> 40-fold) to 3.1 µg/mL against R3934 and R3949. Against ATCC 2146, Dipi-van reduced the MIC of meropenem (> 80-fold) to 1.5 µg/mL at 1/8th of its MIC (12.5 µM). Whereas, at 25 µM,

Dipi-van resensitized R3336 meropenem (MIC = 3.1 µg/mL) with the reduction of > 40-fold. Over all, Dipi-van showed synergistic and resensitization profiles at low concentrations with meropenem against all the four NDM-1 expressing clinical isolates (Table 5B.2).

Then, the antibacterial activity of zinc binding motif that is conjugated to vancomycin, dipicolyl-1,6-hexadiamine (Dipi, **3**) and in combination with meropenem was evaluated against all four clinical isolates. Dipicolyl-1,6-hexadiamine did not show any activity up to 100 µM. Also, dipicolyl-1,6-hexadiamine at 100 µM did not resensitize these NDM-1 producing pathogens to meropenem (Table 5B.3) whereas Dipi-van was able to re-sensitize meropenem-resistant bacteria (NDM-1 expressing pathogens) to meropenem at low concentrations.

To partially prove the proposed hypothesis on how Dipi-van restored meropenem activity, the antibacterial activity of meropenem in combination with Dipi-van was evaluated in presence of external divalent zinc ion by using zinc sulphate ($ZnSO_4$) against R3934. The results demonstrate that at 12.5 µM concentrations of $ZnSO_4$ and Dipi-van did not reduce the MIC of meropenem (MIC > 100 µg/mL) (Fig. 5B.3A). Further, Dipi-van at 100 µM did not reduce the MIC of meropenem (MIC > 100 µg/mL) in presence of externally provided Zn^{2+} of 100 µM. This might be attributed to the formation of Dipi-van-Zn^{+2} complex due to which Dipi-van could not remove the zinc ion from NDM-1 enzyme and not able to resensitize NDM-1 pathogens to meropenem (Fig. 5B.3B). The formation of Dipi-van-Zn^{+2} complex was confirmed by mass spectrometry (Fig. 5B.3B).

Also, whether this Dipi-van is only specific against NDM-1 producing pathogens, the MIC of meropenem was evaluated against meropenem-resistant non-metallo-β-lactamase producing clinical isolate R3421. Against this isolate Dipi-van did not show any activity up to 100 µM. Also, Dipi-van did not reduce the MIC of meropenem against NDM-1 negative pathogen, R3421 even at 50 µM (Fig. 5B.2B & Fig. 5B.3C). This observation reveals that Dipi-van restored meropenem activity against NDM-1 expressing pathogens specifically.

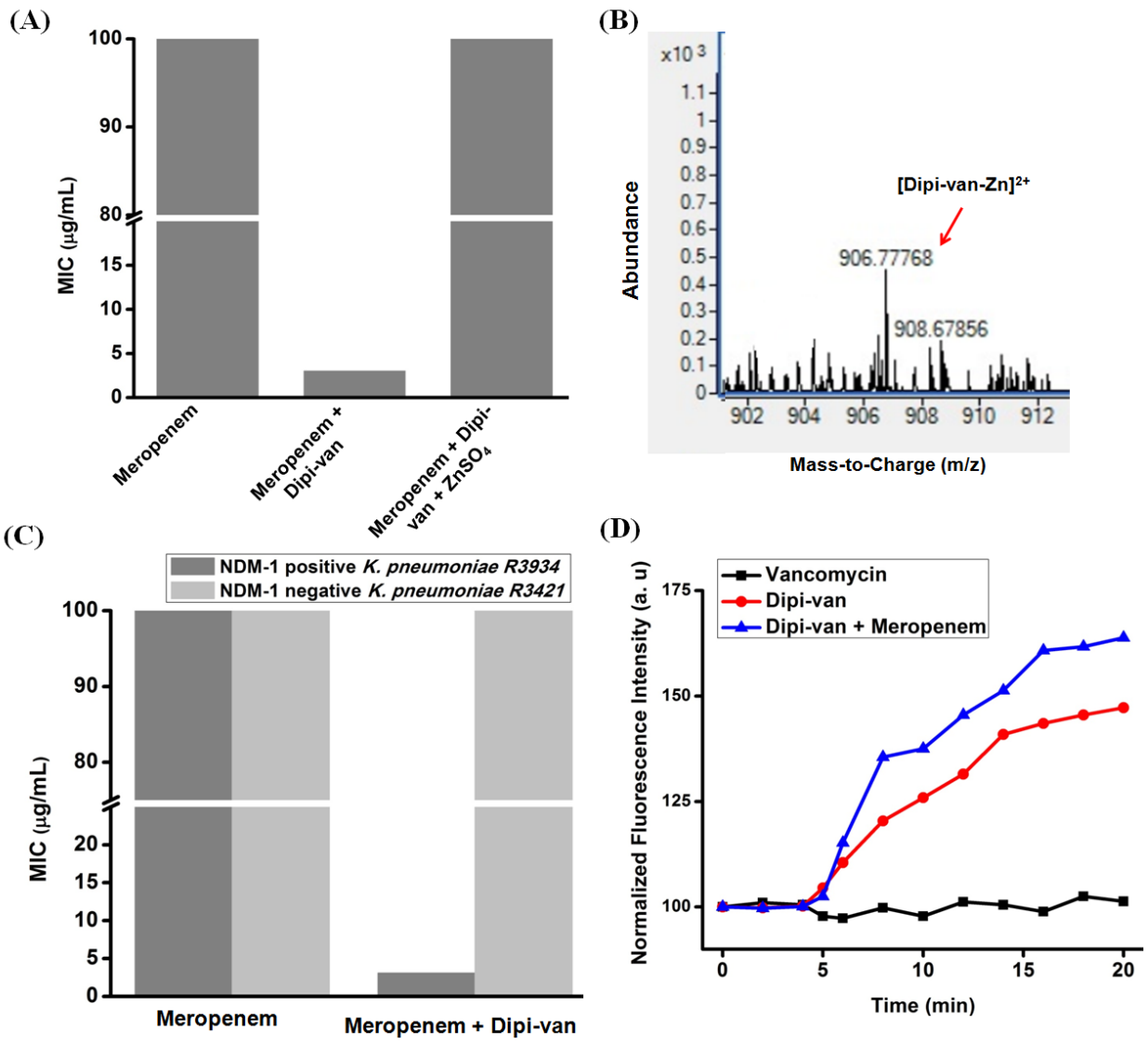


Figure 5B.3: (A) Antibacterial activity of meropenem, combination of meropenem with Dipi-van (at 12.5 µM) and combination of meropenem with Dipi-van (at 12.5 µM) in presence of Zinc sulphate (at 12.5 µM) against NDM-1 positive clinical isolate *K. pneumoniae* R3934 (B) Dipi-van-Zn^{2+} complex confirmed by mass-spectroscopy. (C) Antibacterial activity of meropenem, combination of meropenem with Dipi-van (at 12.5 µM) against meropenem-resistant NDM-1 negative clinical isolate *K. pneumoniae* R3421 and NDM-1 positive *K. pneumoniae* R3934. (D) Outer membrane permeabilization of vancomycin, Dipi-van alone at 15 µM and in combination with meropenem (Dipi-van, 15 µM + meropenem, 5 µM) against NDM-1 positive clinical isolate *K. pneumoniae* R3934.

5B.2.4 Outer membrane permeabilization

Outer membrane (OM) permeabilization refers to disruption of membrane integrity which facilitates the uptake of exogenous molecules. This is an important step in the mode of action of many antibacterial agents as OM plays a vital role in the intrinsic resistance of GNPs. Many antibacterial agents, including glycopeptide antibiotics such as vancomycin, are inherently inactive towards GNPs because of their inability to cross the outer membrane of these pathogens. OM permeabilizing abilities of compounds vancomycin (15 µM), Dipi-van (15 µM) and its physical mixture with meropenem (Dipi-van, 15 µM + meropenem, 5 µM) was studied against R3934. Here, 1-*N*-phenyl-naphthylamine (NPN) was used as a fluorescent probe (NPN fluoresces strongly only in a hydrophobic environment like the interior of a membrane) to identify the kinetic traits of OM permeabilization associated with these compounds. The results suggest that treatment with Dipi-van and its physical mixture with meropenem (Dipi-van + meropenem) caused a time dependent rise in fluorescence intensity due to an increased membrane permeabilization of bacteria and consequent uptake of NPN. The rise in fluorescence signal was rapid and high whereas vancomycin was ineffective (Fig. 5B.3D).

5B.2.5 *In-vivo* activity in sepsis infection model

The resensitization of NDM-1 expressing pathogens *in-vitro* to meropenem in combination with Dipi-van driven further to examine their activity *in-vivo*. *In-vivo* activity experiments were performed against NDM-1 expressing *K. pneumoniae* R3934 in sepsis infection model. Mice were infected with a dose of ~ 10^6 CFU/mouse of *K. pneumoniae* R3934. Then, mice were treated twice at 2 h and 24 h post-infection with monotherapy of meropenem and Dipi-van as well as a combination of Dipi-van with meropenem. Here, colistin and saline were used as positive and negative controls respectively. After 48 h post-infection, organs such as liver, spleen, kidney and lungs were collected to find out the bacterial density.

Bacterial count was found to be more in liver (~ 7.5 log CFU/g) compared to kidneys (~ 6 $\log_{10}$ CFU/g), lungs (~ 6 $\log_{10}$ CFU/g) and spleen (~ 6 $\log_{10}$ CFU/g) in saline treated controls. The bacterial load in organs was unaffected by treatment with Dipi-van alone, and this strain was resistant to meropenem monotherapy. However, combination therapy with Dipi-van and meropenem significantly reduced the bacterial load (3-4 $\log_{10}$ CFU/g) in all the organs compared

to untreated mice (Fig. 5B.4). Significantly, this combination therapy was fairly better than colistin (Fig. 5B.4).

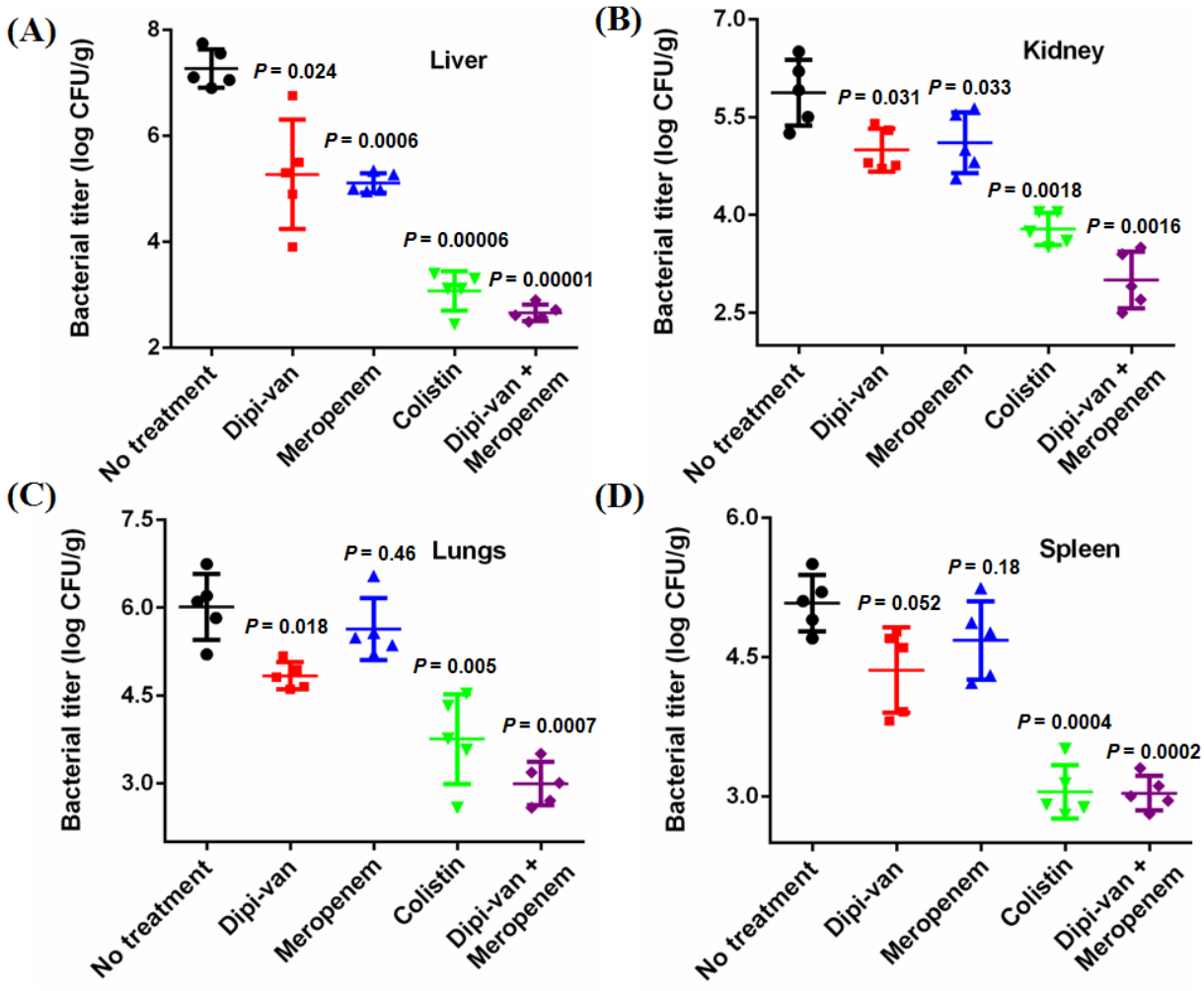


Figure 5B.4: *In-vivo* antibacterial activity of Dipi-van (10 mg/kg), meropenem (10 mg/kg), colistin (5 mg/kg) and combination of meropenem (10 mg/kg) with Dipi-van (10 mg/kg) in sepsis infection model against NDM-1 positive clinical isolate *K. pneumoniae* R3934. Representation of bacterial load in the liver (A), Kidney (B), Lungs (C) and Spleen (D). The data are expressed as mean ± standard deviation, based on values obtained from 5 mice (n = 5). Differences are considered statistically significant from untreated group with probability $P < 0.05$.

5B.3 Discussion

The emergence of carbapenem-resistant Gram-negative bacterial pathogens coupled with the diminishing antibiotic pipeline has resulted in a serious health crisis that will continue to make worse unless new therapeutic measures are soon met.[155,156,111,167] Of particular concern, NDM-1 expressing pathogens are not only recalcitrant to the action of carbapenems but also harbor resistance determinants to an array of additional antibiotics.[155,156] Furthermore, innate resistance offered by the Gram-negative outer membrane makes efforts to identify novel antibacterial compounds exceedingly difficult.[33]

Zinc (II) is required for the activity of metallo-β-lactamases (MBLs) such as NDM-1.[157] Having previously established that dipicolyl-vancomycin conjugate (Dipi-van) was able to complex with Zn^{2+}, in the present study, Dipi-van has been shown to potentiate the activity of a carbapenem antibiotic, meropenem against several clinical isolates of NDM-1 expressing *K. pneumoniae* and *E. coli*. This might be attributed to zinc binding abilities of Dipi-van, which can deplete Zn^{2+} from NDM-1 enzyme thereby resensitizing NDM-1 producing pathogens to the action meropenem. Interestingly, Dipi-van was able to permeate the outer membrane of NDM-1 expressing *K. pneumoniae*. Although it cannot be conclusively said, what brings about the outer membrane permeabilization ability of Dipi-van, it is surmised that the nitrogens that are present in dipicolyl moiety are basic in nature which presumably gets protonated at physiological pH thereby the overall positive charge of the molecule increases and helps in interacting with more negatively charged outer membrane. Further, Dipi-van was not able to resensitize NDM-1 producing *K. pneumoniate* to meropenem in presence of externally provided Zn^{2+}, indicating the formation of Dipi-van-Zn^{+2} complex due to which Dipi-van could not remove the zinc ion from NDM-1 enzyme and not able to restore meropenem activity.

NDM-1 expressing *K. pneumoniae* is among the most dreaded and difficult-to-treat strains of pathogenic bacteria currently plaguing the medical community.[168] Treatment options are severely limited against these pathogens and only tigecycline and colistin are the only drugs of choice for most of the carbapenem-resistant infections.[169] Resistance to even these last line antibiotics is being reported in clinical settings, which is indeed a matter of concern.[170,171] Here, the synergistic activity of Dipi-van with meropenem was evaluated against NDM-1 positive *K. pneumoniae* in sepsis infection model. Sepsis is a serious clinical condition that represents a

patient's response to a severe infection in all major organs and has a very high mortality rate.[172] Dipi-van efficiently reversed NDM-1 mediated resistance to meropenem *in-vivo* and restored clinical efficacy of this antibiotic comparable to last line antibiotic colistin.

5B.4 Conclusions

In summary, dipicolyl-vancomycin (Dipi-van) conjugate presents a non-toxic candidate that can overcome resistance mediated by New Delhi metallo-β-lactamases and resensitize carbapenem-resistant Gram-negative pathogens to carbapenems. Active drug/inhibitor combinations continue to be highly successful in the clinic with inhibitors targeted to serine-β-lactamases. Dipi-van demonstrates promising, *in-vitro* and *in-vivo*, complementary activity against NDM-1 positive bacteria that have become rapidly global and result in significant human morbidity particularly in developing countries. In combination with a carbapenem antibiotic such as meropenem as shown here, resistance can be overcome and antibiotic activity fully restored. Dipi-van is therefore a potent lead for an antibiotic adjuvant co-therapy to address the recent emergence of MBLs in the clinic.

5B.5 Experimental procedure

5B.5.1 Materials and Methods

Antimicrobial agents: Dipicolyl-vancomycin conjugate was synthesized and purified to more than 95 % purity using HPLC as described in the section of 5A.5.2 in Chapter 5A. Vancomycin, meropenem, colistin, tigecycline, minocycline, kanamycin and erythromycin were obtained from Sigma-Aldrich.

Bacterial Strains: Clinical samples were from Department of Neuromicrobiology, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bangalore 560029, India. Bacterial identification was performed by the Vitek 2 Compact 60 system, bioMerieux, France and Gram-negative bacteria were screened for carbapenem resistance using Kirby-Bauer disc diffusion method (data not shown). *E. coli* (ATCC 25922) was purchased from MTCC (Chandigarh, India).

Animals: The same as described in section 2.5.1 in Chapter 2.

5B.5.2 PCR and Gel-electrophoresis

The NDM-1 gene was identified by conventional polymerase chain reaction (PCR) using primers NDM-F (5′-GGG CAG TCG CTT CCA ACG GT-3′) and NDM-R (5′-GTA GTG CTC AGT GTC GGC AT-3′) (Eurofins Genomics India Pvt. Ltd., Bangalore) which amplified an internal fragment of 475 bp using conventional polymerase chain reaction. The conditions included an initial denaturation step of 5 min at 94 ºC, followed by 30 cycles of 30 sec at 95 ºC, 30 sec at 60 ºC and 30 sec at 72 ºC, and then a final extension step of 5 min at 72 ºC. The PCR products were analyzed in 2 % agarose gel, containing 0.05 mg/L ethidium bromide, at 100V for 1 h in 1X Tris Acetate EDTA buffer. A 100 bp DNA ladder was used as a molecular weight marker (SRL Biolit™, Mumbai India). Bands were visualized under UV light and an amplified product corresponding to 475 bp was considered as positive.

5B.5.3 Minimum inhibitory concentration (MIC)

The same protocol was followed using the test compounds as described in the section of 2.5.3 in Chapter 2. The combination antibacterial efficacy of Dipi-van/Dipi and meropenem was measured in nutrient broth using chequerboard assay in the following manner.[10] A solution of 25 μL each of test compounds was added into each well of a 96 well plate followed by 150 μL of bacterial suspension (~ 5.0×10^5 CFU/mL). The plate was then incubated at 37 °C for a period of 24 h and the O.D. value was measured at 600 nm. The MIC from chequerboard assay was a result of two independent experiments and each experiment was performed in triplicates. For determination of activity in presence of external zinc sulfate ($ZnSO_4$), Dipi-van and $ZnSO_4$ were pre-mixed in equimolar concentrations (100 μM + 100 μM), then used for chequerboard assay.

5B.5.4 Outer membrane permeabilization assay[173]

Mid-log phase *K. pneumoniae* R3934 cells were harvested, washed with 5 mM HEPES and 5 mM glucose (10^8 CFU/mL) and resuspended in a 1:1 solution of the same. Measurements were made in a 96 well plate containing 0.2 mL of bacterial suspension using Tecan Plate Reader. To this solution *N*-phenylnaphthylamine dye was added to a final concentration of 10 μM. Now, test compounds (vancomycin, Dipi-van alone at 15 μM and in combination with meropenem (Dipi-van, 15 μM + meropenem, 5 μM)) were added to the bacterial suspension. After addition

fluorescence intensity (excitation wavelength: 350 nm; emission wavelength: 420 nm) was measured for 10 minutes at room temperature.

5B.5.5 Sepsis infection model[174]

About six-week-old, female BALB/c mice (weight, ~ 19-24 g) were used for the experiments. Mice were infected intraperitoneally (i.p.) with a dose of ~ 10^6 CFU/mouse of meropenem-resistant NDM-1 *K. pneumoniae* R3934. Then, mice were treated twice at 2 h and 24 h post-infection with a specified i.p. dose of either saline, meropenem (10 mg/kg), Dipi-van (10 mg/kg), or a combination of compound Dipi-van (10 mg/kg) and meropenem (10 mg/kg). Here, colistin (5 mg/kg) was used as a positive control. Mice were euthanized after 48 h post-infection and then liver, spleen, kidney and lungs were collected to find out the bacterial density in these organs. Organs were placed into 10 mL sterile saline on ice, and then homogenized. The dilutions of the homogenate were plated onto agar plates, which were incubated for 24 h at 37 °C. The bacterial titer was expressed as $\log_{10}$ CFU/g of organ weight.

Chapter 6

Membrane Active Glycopeptide Antibiotic to Overcome the Intrinsic Resistance of Gram-negative Bacteria

Abstract

Chapter 6 *describes a strategy to overcome the intrinsic resistance of Gram-negative pathogens (GNPs) towards glycopeptide antibiotics. Many antibacterial agents, including glycopeptide antibiotics such as vancomycin, are inherently inactive towards GNPs because of their inability to cross the outer membrane of these pathogens. For the first time, vancomycin derivatives bearing a permanently positive charge lipophilic moiety were showed to permeabilize the outer membrane of GNPs and overcoming the inherent resistance of GNPs towards glycopeptides. Unlike vancomycin, an optimized compound showed high activity against a variety of multidrug-resistant clinical isolates of GNPs like E. coli, K. pneumoniae, P. aeruginosa and A. baumannii with the minimum inhibitory concentration (MIC) as low as 1.2 μM. Neither the lipophilic cationic quaternary ammonium moiety alone nor its physical mixture with vancomycin showed any activity against the same bacteria (MIC > 100 μM). More importantly, this compound inhibited the cell wall (peptidoglycan) biosynthesis in GNPs and demonstrated high in-vivo activity against carbapenem-resistant A. baumannii. The notable activity of the compound is attributed to new membrane disruption mechanisms (cytoplasmic membrane depolarization along with outer and inner (cytoplasmic) membrane permeabilization), which also had the impact on stalling bacterial resistance development. These findings emphasize the high potential of this strategy that can be translated clinically for Gram-negative bacterial infections, thus strengthening the antibiotic arsenal.*

Publication based on this work

1) Yarlagadda, V. *et al.* Glycopeptide antibiotic to overcome the intrinsic resistance of Gram-negative bacteria. *Manuscript under review.*

6.1 Introduction

The WHO Global Report on Surveillance of Antimicrobial Resistance 2014 reports that Gram-negative pathogens (GNPs) like *E. coli* and *K. pneumoniae* have developed resistance to more than 50 % of commonly used antibacterial drugs.[168] More importantly, carbapenem-resistant bacteria such as New Delhi metallo-β-lactamase-1 ($bla_{\text{NDM-1}}$) producing GNPs have become resistant to even the last line of antibiotics like colistin.[171] In addition to acquired resistance in GNPs, a plethora of Gram-positive antibiotics are left unused due to intrinsic resistance displayed by GNPs towards these antibiotics.[33] The additional outer membrane (OM) and multiple efflux pumps appear to be the main contributors to this intrinsic resistance as these effectively hinder the entry of a variety of drug molecules including glycopeptides antibiotics such as vancomycin.[33]

The GNPs can be sensitized to a variety of antibacterial agents when the intrinsic resistance elements are compromised or deleted.[175] This observation reveals an alternative approach to conventional drug discovery. If these intrinsic resistance mechanisms are targeted with simple chemical modifications of clinically important Gram-positive antibacterial agents such as vancomycin, then these drugs will become active against GNPs thus expanding the antibacterial spectrum of the drug. Generally, antimicrobial peptides (AMPs) and antibacterial peptidomimetics kill bacteria by selectively disrupting their membranes through their facial segregation of positive charges and hydrophobic moieties and disrupt the negatively charged bacterial membranes.[138-145] Hence, a strategy, which installs membrane disruption properties to vancomycin, could compromise the OM of GNPs and aid in reaching its specific target. In Chapter 3, membrane active vancomycin analogues were developed, which demonstrated good antibacterial activity against vancomycin-resistant Gram-positive bacteria due to the incorporation of novel membrane disrupting mechanisms, which overcome the acquired resistance to vancomycin. In this chapter, these membrane active molecules were showed to have high activity against a variety of multidrug-resistant (MDR) clinical isolates of GNPs, thus overcoming the inherent resistance of GNPs towards vancomycin. The antibacterial activity of these analogues against GNPs is attributed to the installed membrane disruptive properties to

vancomycin in addition to cell wall biosynthesis inhibition. Further, as demonstrated in the present study, this class of compound showed good *in-vivo* activity.

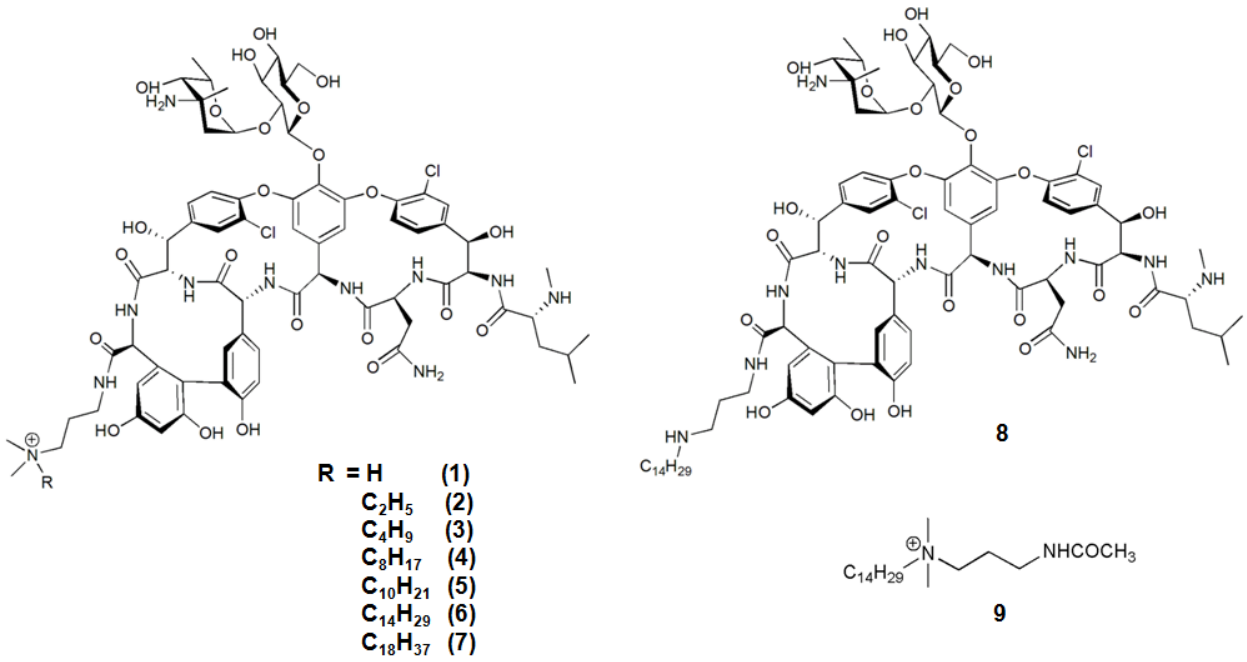


Figure 6.1: Structures of vancomycin analogues (**1**-**8**) and control compound **9**.

6.2 Results

6.2.1 Synthesis

Vancomycin analogues were synthesized as described in section 3.2.1 in chapter 3.

6.2.2 *In-vitro* antibacterial activity

In order to test the activity of the compounds, their antibacterial activity was determined against various ATCC strains and clinical isolates of a variety of clinically relevant GNPs of human importance. Minimum inhibitory concentration (MIC) was determined as the lowest concentration required to completely inhibit bacterial growth. Primarily, the *in-vitro* antibacterial activity of these new compounds was evaluated against *E. coli* ATCC 25922 (Table 6.1 and Fig.

6.2A). MIC of compound **1** (devoid of lipophilicity and permanent positive charge) was found to be 80 µM whereas vancomycin was completely ineffective even at 100 µM. Compounds comprising lower alkyl chain; compound **2** (ethyl chain) and compound **3** (butyl chain) were found to possess moderate activity with the MICs of 50 µM and 21 µM respectively. Compound **4** consisting of octyl chain displayed significant activity against *E. coli* (MIC = 12.5 µM) and a gradual increase in activity was observed with increasing chain length (compound **5**, MIC = 8.5 µM), with compound **6** (tetradecyl chain) demonstrating an MIC value of 4 µM. However, further increase in lipophilic chain compromised the activity, as was observed in octadecyl analogue of vancomycin (compound **7**) wherein MIC was found to be > 100 µM. These results emphasize that lipophilicity from octyl to tetradecyl would be the optimum lipophilic chain length to maintain activity against GNPs.

Table 6.1: Antibacterial activities against various Gram-negative pathogens

Compound	Minimum Inhibitory Concentration (µM)								
	E. coli (ATCC 25922)	*E. coli (R1747)*	*E. coli (ATCC 35218)*	*A. baumannii (R4942)*	*A. baumannii (R676)*	*A. baumannii (R674)*	*E. cloacae (NDM-1 R3921)*	*K. pneumoniae (ATCC 700603)*	*P. aeruginosa (R590)*
Vancomycin	>100	61	>100	54	>100	>100	>100	>100	>100
4	12.5	14.5	14	7	14.5	17	>100	16	25
5	8.5	5.3	8.5	4	13	8	50	14	12.5
6	4.5	1	4.5	3	5.2	5	12.5	9	6
8	75	N.D	>100	N.D	>100	>100	>100	>100	>100
9	>100	N.D	>100	N.D	>100	>100	>100	>100	>100
Vancomycin + 9	50 + 50	N.D	100 + 100	N.D	>100 + >100	>100 + >100	100 + 100	100 + 100	100 + 100
Meropenem	2	>50	2	>50	>50	>50	>50	2	>50
Norfloxacin	1	>50	4	>50	>50	>50	>50	4	>50
Colistin	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5

N.D; Not determined.

Then, the optimized three compounds **4**-**6** were chosen to evaluate their activities against a variety of multidrug-resistant GNPs including carbapenem-resistant clinical isolates. Table 6.1 shows the antibacterial activities of compounds **4**-**6** and control compounds **8** and **9** against an array of GNPs such as *K. pneumoniae* (ATCC 700603), *A. baumannii* (R4942, R674 and R676),

P. aeruginosa (R590), *E. coli* (ATCC 25922, ATCC 35218 and R1747) and *E. cloacae* (*bla*$_{NDM-1}$ R2928). Vancomycin was ineffective even at a concentration of 100 μM against *K. pneumoniae*, *P. aeruginosa*, *E. cloacae*, *A. baumannii* (R674, R676) and *E. coli* (ATCC 25922, ATCC 35218) whereas its MICs were found to be 54 μM and 61 μM against *A. baumannii* R4942 and *E. coli* R1747, respectively. Against all these resistant strains compounds **4**-**6** showed good activity and it was observed that activities increased with increase in the length of the lipophilic chain. Activity against ATCC strain of *E. coli* (ATCC 35218), compound **6** being the most active compound (MIC of 4.5 μM) followed by compound **5** (MIC of 8.5 μM) and compound **4** (MIC of 14 μM). Furthermore, good activity against clinical isolate of *E. coli* R1747 was achieved for compound **6** with the lowest MIC of 1.2 μM. Next, the activity was evaluated against three clinical isolates of carbapenem-resistant *A. baumannii*. Against all the three isolates of *A. baumannii*, again, the best activity was achieved for compound **6** with the MICs ranging from 3-5 μM. Also, it was noticed that compound **6** was 2 to 3-fold more active than its shorter alkyl chain homologues, compounds **4** and **5**. Compound **6** also displayed good activity against MDR clinical isolate of *P. aeruginosa* R590 (MIC = 6 μM). Further, compound **6** demonstrated notable activity against *K. pneumoniae* with the MIC of 9 μM. Next, the activities of these compounds were evaluated against clinical isolate of *bla*$_{NDM-1}$ GNP, *Enterobacter cloacae* R2928. Yet again, compound **6** was found to be the most active in the series exhibiting an MIC of 12.5 μM. All the clinical isolates used in the present study were resistant to meropenem, which is one of the drugs of last resort for multidrug-resistant (MDR) GNPs. Therefore, the good activity of these lipophilic cationic vancomycin analogues against MDR clinical isolates of GNPs is certainly a highlight of this study.

To assess the role of the installed cationic feature (permanent positive charge), the antibacterial activity of compound **8** (Fig. 6.1) having a soft cationic moiety (which is expected to be cationic under physiological conditions) and tetradecyl lipophilic chain was evaluated and compared the results with the corresponding vancomycin derivative compound **6** (having permanent positive charge and tetradecyl lipophilic chain) (Table 6.1). The MIC of compound **8** against *E. coli* ATCC 25922 was found to be 75 μM whereas in case of rest of the GNPs tested its MIC was found to be > 100 μM. In contrast to compound **8**, compound **6** showed high activity with an average MIC of ~ 5 μM against most of the GNPs tested. The results emphasize that it is

the appended permanent positive charge along with lipophilicity in vancomycin that affords the substantial activity against GNPs.

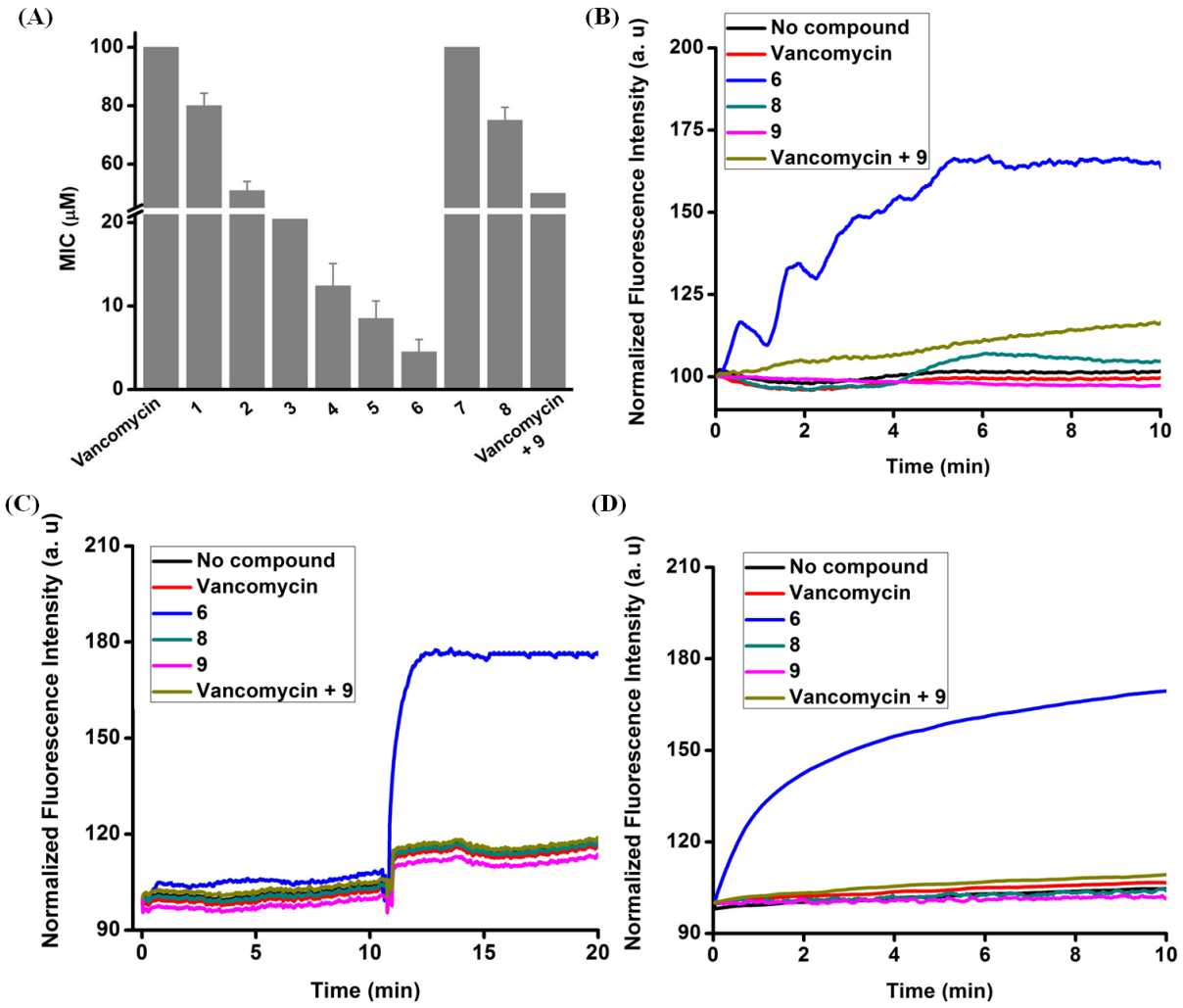


Figure 6.2: (A) Structure-activity relationship study of vancomycin analogues against *E. coli*. (B)-(D) Membrane disruption studies of vancomycin, compounds **6**, **8**, **9** and a physical mixture of vancomycin + **9** against *E. coli* at 5 μM. (B) Outer membrane permeability using 1-*N*-phenyl-naphthylamine (NPN), (C) Inner (cytoplasmic) membrane depolarization using $DiSC_3(5)$ (D) Inner membrane permeability using propidium iodide (PI).

To evaluate the activity of cationic lipophilic portion alone (without vancomycin), compound **9** was synthesized as a control (Fig. 6.1). The MIC of compound **9** was found to be > 100 μM against all GNPs tested. Further, the activity of a physical mixture of vancomycin and compound **9** was determined. Against most of the GNPs tested, the physical mixture showed antibacterial activity either at 100 μM or > 100 μM of their individual concentrations except in case of *E. coli* ATCC 25922 wherein the individual concentrations of 50 μM showed activity (Table 6.1).

6.2.3 Disruption of bacterial membrane integrity

Outer membrane (OM) permeabilization refers to disruption of membrane integrity, which facilitates the uptake of exogenous molecules. This is an important step in the mode of action of many antibacterial agents as OM plays a vital role in the intrinsic resistance of GNPs. The OM permeabilizing abilities were studied for vancomycin, compounds **6**, **8**, **9** and a physical mixture of compound **9** + vancomycin on *E. coli*. Here, 1-*N*-phenyl-naphthylamine (NPN) was used as a fluorescent probe. Generally, OM acts as a permeability barrier, and excludes hydrophobic substances such as NPN but, once damaged, it can allow the entry of NPN to the phospholipid layer, resulting in prominent fluorescence. Hence, this probe could be used to identify the kinetic traits of OM permeabilization associated with new compounds. These results suggest that treatment with compound **6** caused a time dependent rise in fluorescence intensity due to an increased membrane permeabilization of bacteria and consequent uptake of NPN. The rise in fluorescence signal was rapid and high for compound **6** (Fig. 6.2B). However, vancomycin, compounds **8**, **9** and physical mixture (**9** + vancomycin) were ineffective.

To investigate the interactions of these derivatives with the bacterial cytoplasmic membrane, the effect of vancomycin, compounds **6**, **8**, **9** and a physical mixture of compound **9** + vancomycin on the membrane potential of *E. coli* was monitored using $DiSC_3(5)$ assay. In this experiment, the dye was added to bacterial cells, and the change in fluorescence intensity was monitored. It was observed that as the dye accumulated in the membranes, the fluorescence intensity decreased due to self-quenching. Upon disruption of membrane potential, an increase in fluorescence was observed as $DiSC_3(5)$ dye was displaced into the solution. The maximum potential dissipation caused by the compound **6** was rapid and occurred in less than two minutes

after compound addition (the cells were equilibrated for 10 min prior to addition of the test compound), whereas vancomycin and other control compounds **8**, **9** and physical mixture (**9** + vancomycin) remained ineffective (Fig. 6.2C).

The observed effect of vancomycin derivative, compound **6** on membrane potential prompted further to examine the cytoplasmic membrane permeability. Kinetics of bacterial cytoplasmic membrane permeabilization by vancomycin, compounds **6**, **8**, **9** and a physical mixture of compound **9** + vancomycin on *E. coli* was studied by measuring the uptake of the fluorescent probe propidium iodide (PI). This dye enters only membrane-compromised cells and fluoresces upon binding to nucleic acids. Unlike vancomycin, which did not cause membrane permeability, compound **6** showed a strong ability to permeabilize the cytoplasmic membrane within 2 min (Fig. 6.2D). Yet again, the control compounds **8**, **9** and physical mixture (**9** + vancomycin) were ineffective in permeabilizing the cytoplasmic membrane of *E. coli*.

Therefore, these membrane disruption studies suggest that neither vancomycin nor its physical mixture with lipophilic cationic quaternary ammonium moiety (compound **9**) show any effect on bacterial membrane. Also, the lipophilic vancomycin derivative compound **8**, (having a secondary amine which becomes cationic under physiological conditions) did not show any membrane disruption properties. This implies that permanent cationic charge along with lipophilic moiety needs to be chemically conjugated to the carboxylic group of vancomycin in order to impart membrane active properties towards GNPs.

6.2.4 Intracellular accumulation of cell wall (peptidoglycan) precursor

In order to investigate whether new vancomycin derivatives interfere with peptidoglycan biosynthesis, the accumulation of UDP-linked peptidoglycan precursor, UDP-*N*-acetyl-muramyl-pentapeptide (UDPMurNAc-pp) was determined after treating bacteria with new vancomycin derivative (compound **6**) and vancomycin. In case of compound **6**, an intense peak was observed at 260 nm, which corresponds to UDPMurNAc-pp and confirmed by high-resolution mass spectrometry (m/z = 1193.34 (cal), 1193.37 (obs) for M^+). The peak intensity increases with increase in concentration of compound from 20 µM to 40 µM whereas vancomycin (30 µM) showed negligible accumulation of UDPMurNAc-pp (Fig. 6.3). The results imply that unlike

vancomycin, new vancomycin derivative, compound **6** was able to reach the cell wall region and inhibiting cell wall biosynthesis.

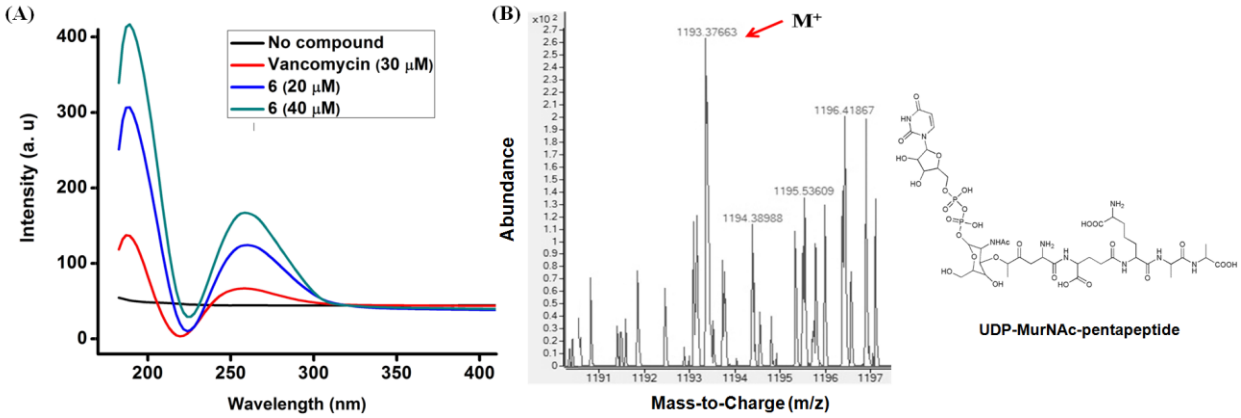


Figure 6.3: Intracellular accumulation of the cell wall precursor UDP-MurNAc-pentapeptide after treatment of *E. coli* with vancomycin and compound **6**. Untreated cells were used as control (No compound). (A) Identification of intracellular UDP-MurNAc-pentapeptide by monitoring absorbance at 260 nm wavelength. (B) UDP-MurNAc-pentapeptide was identified by mass spectrometry as indicated by the peak at m/z 1,193.37.

6.2.5 Propensity to induce bacterial resistance

Emergence of rapid resistance in bacteria to conventional antibiotics is a major problem and is one of the major hurdles for the introduction of new antibiotics in clinical settings. Since the possibility of vancomycin analogues towards the treatment against MDR clinical isolates of GNPs has been explored in this report, it is vital to investigate if the compounds themselves have any tendency to trigger bacterial resistance. In order to evaluate the potential of this class of compounds as long-lasting antibacterial agents, the ability of *A. baumannii* R674 to develop resistance was investigated. Compound **6**, the most active compound was chosen as a model compound for this study. As a positive control colistin, the drug of last resort for MDR Gram-negative bacterial infections, was used. Starting MICs for compound **6** and colistin against *A. baumannii* R674 were found to be 5 µM and 0.5 µM, respectively. The MIC of compound **6**

towards *A. baumannii* did not change even after 20 passages, whereas the MIC of colistin increased by 32-fold (Fig. 6.4A). Thus bacteria are less likely to acquire resistance against compound **6** compared to clinically used antibiotics like colistin.

6.2.6 *In-vitro* toxicity (hemolysis and cytotoxicity)

As these vancomycin analogues were shown to disrupt the bacterial cell membrane integrity, the toxicity of compounds **4** and **6** were studied by measuring their cytotoxicity (CC_{50}) against mammalian cells (HeLa) and hemolytic activity (HC_{50}) against human erythrocytes. None of the derivatives showed significant toxicity towards either of the cells even up to 100 μM (CC_{50}; 50 % cytotoxic concentration and HC_{50}; 50 % hemolytic concentration were found to be > 100 μM) concentration, which is much higher than their corresponding MIC values. Also, the toxicity of permanent positively charged lipophilic compound (**9**) was evaluated and the HC_{50} and CC_{50} values were 125 μM and 27 μM, respectively. The selectivity (HC_{50}/MIC against *E. coli* ATCC 25922) of compound **9** was found to be ≤ 1 whereas corresponding vancomycin analogue compound **6** showed selectivity of > 22, which indicates the selective toxicity of compound **6** against bacterial cells.

6.2.7 *In-vivo* antibacterial activity

A widely used animal model for evaluating antibacterial activity of preclinical compounds is the thigh burden model, in which the thigh muscle of neutropenic mice is inoculated with bacteria, followed by administration of the antibacterial agents. The *in-vivo* activity of compound **6** in comparison with colistin against carbapenem-resistant *A. baumannii* R674 was shown in Fig. 6.4B. In this study, mice were infected with *A. baumannii* in the thigh. After 1 h of infection, the mice were treated with saline, vancomycin (15 mg/kg), colistin (5 mg/kg) and compound **6** (15 mg/kg) and five mice were used in each group. After 24 h of the treatment, antibacterial activity was determined by finding the bacterial titer in the infected thighs. Vancomycin showed ~ 0.9 $\log_{10}$ CFU/g reduction from untreated mice (saline). Whereas, the *in-vivo* activity of compound **6** was found to be comparable to colistin wherein they reduced the bacterial titer by ~ 3 $\log_{10}$ CFU/g compared to control (saline).

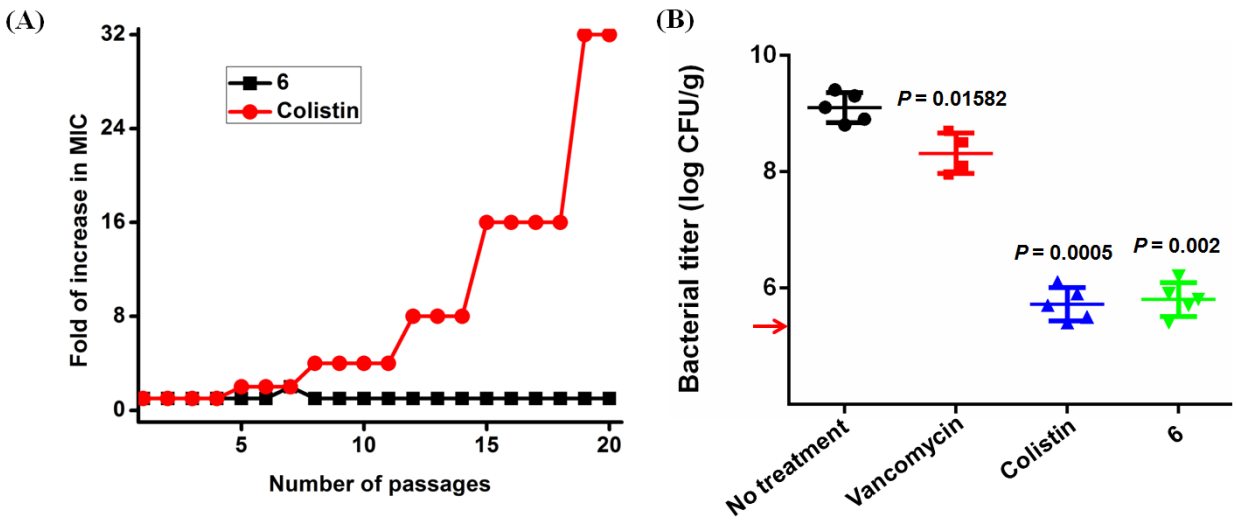


Figure 6.4: (A) Bacterial resistance studies of colistin and compound **6** against *A. baumannii* R674. Starting MIC values for colistin and compound **6** were found to be 0.5 µM and 5 µM, respectively. (B) *In-vivo* antibacterial activity of vancomycin, colistin and compound **6** in thigh infection model against carbapenem-resistant *A. baumannii*. Arrow indicates the bacterial concentration inoculated in the mouse (~ 10^5 CFU/mouse). Five mice were used in each group. Statistical analysis was performed using Student's *t*-test. Differences are considered statistically significant from untreated group with a value of $P < 0.05$.

6.3 Discussion

At present, the development of new antibiotics against Gram-negative bacteria is vital.[111,167] The problem is intensified by the prevalence of extended spectrum β-lactamases (ESBLs) producing Gram-negative bacterial species such as *K. pneumoniae* and *E. coli*.[152] Vancomycin, a glycopeptide antibiotic, is ineffective against Gram-negative pathogens (GNPs) because of its inability to cross the OM of GNPs to reach the cell wall area, which represents its site of action.[54] Significant strategies have been adopted to make vancomycin active against Gram-negative bacteria. In one strategy, Nicolosi *et al.* introduced vancomycin encapsulated fusogenic liposomes to overcome the OM barrier of GNPs, thereby sensitizing GNPs to the composition.[109] In another strategy, Morones-Ramirez *et al.* showed antibacterial activity of vancomycin against

GNPs in combination with silver.[110] Also, there are several reports wherein antibiotic adjuvants have been used to extend the antibiotic activity of Gram-positive antibiotics towards GNPs.[176]

In the present study, glycopeptide antibiotic derivatives have been developed to overcome the intrinsic resistance of GNPs towards glycopeptides using simple rationalized synthetic methodology. This is the first report of covalently modified vancomycin derivatives, which have potent activity against a variety of MDR clinical isolates of GNPs including NDM-1 producing pathogens. In this strategy, permanent cationic charge (quaternary ammonium group) and a lipophilic moiety were attached to vancomycin to impart a new mode of action to the existing drug. It was observed that activity of the compounds varied with the length of the lipophilic moiety. Further, it was established the importance of permanent positive charge over soft charge for potent antibacterial activity and the need to have the lipophilic quaternary ammonium moiety covalently connected to vancomycin as opposed to a physical mixture.

The optimized derivative, compound **6** comprising tetradecyl chain showed antibacterial activity against various clinical isolates of *K. pneumonia*, *E. coli*, *P. aeruginosa*, and carbapenem-resistant *A. baumannii* and NDM-1 producing bacteria *E. cloacae*, without showing any significant *in-vitro* toxicity against mammalian cells. It was anticipated that the appended lipophilic cationic moiety provides the necessary lipophilicity and initial electrostatic attraction to interact with the negatively charged bacterial cell membrane. The compound permeabilized the outer membrane of GNPs and thus was able to overcome the inherent resistance of GNPs. Also, this compound could depolarize and permeabilize the inner (cytoplasmic) membrane of GNPs. Further, treatment of *E. coli* with compound **6** resulted in significant accumulation of the soluble cell wall precursor undecaprenyl-*N*-acetyl-muramyl-pentapeptide, suggesting that peptidoglycan biosynthesis is blocked. In contrast to compound **6**, vancomycin showed negligible inhibition of cell wall biosynthesis. Additionally, the multimodal mechanism of action of the compound had a significant impact on stalling the development of bacterial resistance to the drug because of the complexity in remodeling bacterial membrane in a way that is compatible with bacterial survival. Although leading antibiotic colistin fail to maintain constant activity even till 10 passages, MIC of compound **6** remained intact in 20 passages.

Multidrug-resistant *Acinetobacter* infections have an extremely high mortality rate and occur most frequently in severely ill patients.[177] Treatment options are severely limited and only carbapenems and colistin are the drugs of choice for most of the drug-resistant infections. Compound **6** demonstrated good *in-vivo* activity against carbapenem-resistant clinical isolate of *A. baumannii* R674, which was comparable to colistin.

6.4 Conclusions

Vancomycin analogues have been developed that can overcome the inherent resistance of Gram-negative pathogens (GNPs). The incorporation of lipophilic moiety and permanent positive charge into vancomycin make these compounds distinct from other existing derivatives in their ability to cause strong bacterial membrane disruption thereby overcoming the inherent resistance of GNPs. Optimized compound showed good activity against clinical isolates of carbapenem-resistant GNPs of human importance. Further, this compound demonstrated significant *in-vivo* activity against multidrug-resistant *A. baumannii* with no observed toxicity. Therefore, these findings stress that this strategy can potentially be a beneficial extension to the antibiotic pipeline for the treatment of infections caused by MDR Gram-negative bacteria.

6.5 Experimental procedure

6.5.1 Materials and Methods

Antimicrobial agents: New vancomycin analogues were synthesized and purified to more than 95 % purity using HPLC as described in the section of 3.5.2 in Chapter 3. Vancomycin, meropenem, norfloxacin and colistin were obtained from Sigma-Aldrich.

Bacterial strains: To evaluate the antibacterial activity of new vancomycin analogues against Gram-negative bacteria, we chose the most common human relevant pathogens such as *E. coli*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. cloacae*. *E. coli* ATCC 35218, *K. pneumoniae* ATCC 700603 were obtained from the American Type Culture Collection (ATCC). *E. coli* R1747, *A. baumannii* R492, *A. baumannii* R674, *A. baumannii* R676, *P. aeruginosa* R590

and *E. cloacae* R2928 were isolated from clinical samples by the Department of Neuromicrobiology, National Institute of Mental Health and Neuro Sciences, Hosur Road, Bangalore 560029, India. Bacterial identification was performed by the Vitek 2 Compact 60 system, bioMerieux, France and Gram-negative bacteria were screened for carbapenem resistance using Kirby-Bauer disc diffusion method. *E. coli* ATCC 25922 was procured from MTCC (IMTECH, Chandigarh, India).

Animals: The same as described in section 2.5.1 in Chapter 2.

6.5.2 Minimum inhibitory concentration (MIC)

The same protocol was followed using the test compounds as described in the section of 2.5.3 in Chapter 2. The combination antibacterial activity of vancomycin and compound **9** (vancomycin + **9**) was measured using chequerboard assay as described previously. A two-fold serially diluted solution of 25 μL each of test compounds was added into each well of a 96 well plate followed by 150 μL of bacterial suspension (~ 10^5 CFU/mL). The plate was then incubated at 37 °C for a period of 24 h and the O.D. value was measured at 600 nm. The MIC from chequerboard assay was a result of two independent experiments and each experiment was performed in triplicates.

6.5.3 Bacterial membrane disruption studies

6.5.3.1 Outer membrane permeabilization assay[173]

Mid-log phase *E. coli* ATCC 25922 cells were harvested, washed with 5 mM HEPES and 5 mM glucose and resuspended in a 1:1 solution of the same at concentration of 10^8 CFU/mL. To this solution *N*-phenylnaphthylamine dye was added to a final concentration of 5 μM. Now, test compounds (vancomycin, compounds **6**, **8**, **9** and vancomycin + **9**) were added to the bacterial suspension at the working concentration of 5 μM. After their addition, the fluorescence intensity (excitation wavelength: 350 nm; emission wavelength: 420 nm) was measured for 10 minutes at R.T. using Perkin-Elmer spectrofluorometer.

6.5.3.2 Cytoplasmic membrane depolarization assay

The same protocol was followed using the test compounds (vancomycin, compounds **6**, **8**, **9** and vancomycin + **9**) at 5 μM against *E. coli* ATCC 25922 as described in the section of 3.5.4.1 in Chapter 3.

6.5.3.3 Inner membrane permeabilization assay

The same protocol was followed using the test compounds (vancomycin, compounds **6**, **8**, **9** and vancomycin + **9**) at 5 µM against *E. coli* ATCC 25922 as described in the section of 3.5.4.2 in Chapter 3.

6.5.4 Intracellular accumulation of UDP-*N*-acetyl-muramyl-pentapeptide

The same protocol was followed using the test compounds (Vancomycin at 30 µM, compound **6** at 20 and 40 µM) against *E. coli* ATCC 25922 as described in the section of 2.5.5 in Chapter 2.

6.5.5 Resistance development study

The same protocol was followed using the test compounds (compound **6** and colistin) against *A. baumannii* R674 as described in the section of 2.5.7 in Chapter 2.

6.5.6 *In-vitro* toxicology

The same protocol was followed using the test compounds as described in the section of 3.5.8 in Chapter 3.

6.5.7 *In-vivo* antibacterial activity[178]

About six-week-old, female BALB/c mice (weight, ~ 19-24 g) were used for the experiments. The mice were rendered neutropenic (~ 100 neutrophils/mL) by injecting two doses of cyclophosphamide intraperitoneally 4 days (150 mg/kg) and 1 day (100 mg/kg) before the infection experiment. 50 µL of ~ 10^7 CFU/mL concentration of the bacterial inoculum (*A. baumannii* R674) was injected into the thigh. One hour after inoculation, animals were treated intraperitoneally with saline, colistin at 5 mg/kg, vancomycin and compound **6** at 15 mg/kg (n = 5). At 24 h post treatment, cohorts of animals were euthanized and the thighs were collected aseptically. The thigh was weighed (0.5 g - 0.8 g) and placed into 10 mL of sterile saline and homogenized. The dilutions of the homogenate were plated onto agar plates, which were incubated overnight at 37 °C. The bacterial titer was expressed as $\log_{10}$ CFU/g of thigh weight.

References

1. Morens, D. M.; Folkers, G. K.; Fauci, A. S. The challenge of emerging and re-emerging infectious diseases. *Nature* **2004**, *430*, 242-249.

2. World Health Organization. *World Health Statistics*. **2013**.

3. World Health Organization. *World Health Statistics*. France; **2011**.

4. Global Antibiotic Resistance Partnership (GARP) - India Working Group. Rationalizing antibiotic use to limit antibiotic resistance in India. *Indian J. Med. Res.* **2011**, *134*, 281-294.

5. Mathew, J. L. Pneumococcal vaccination in developing countries: where does science end and commerce begin? *Vaccine* **2009**, *27*, 4247-4251.

6. Levine, O. S.; Cherian, T. Pneumococcal vaccination for Indian children. *Indian Pediatr.* **2007**, *44*, 491-496.

7. Demain, A. L.; Sanchez, S. Microbial drug discovery: 80 years of progress. *J. Antibiot.* **2009**, *62*, 5-16.

8. Kumarasamy, K. K.; Toleman, M. A.; Walsh, T. R.; Bagaria, J.; Butt, F.; Balakrishnan, R.; Chaudhary, U.; Doumith, M.; Giske, C. G.; Irfan, C.; Krishnan, P.; Kumar, A. V.; Maharjan, S.; Mushtaq, S.; Noorie, T.; Paterson, D. L.; Pearson, A.; Perry, C.; Pike, R.; Rao, B.; Ray, U.; Sarma, J. B.; Sharma, M.; Sheridan, E.; Thirunarayan, M. A.; Turton, J.; Upadhyay, S.; Warner, M.; Welfare, W.; Livermore, D. M.; Woodford, N. Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect. Dis.* **2010**, *10*, 597-602.

9. Bonomo, R. A. New Delhi metallo-β-lactamase and multidrug resistance: A global SOS? *Clin. Infect. Dis.* **2011**, *52*, 485-487.

10. Uppu, D. S. S. M.; Manjunath, G. B.; Yarlagadda, V.; Kaviyil, J. E.; Ravikumar, R.; Paramanandham, K.; Shome, B. R.; Haldar, J. Membrane-active macromolecules resensitize

NDM-1 Gram-negative clinical isolates to tetracycline antibiotics. *PLoS ONE* **2015**, *10*, e0119422.

11. Butler, M. S.; Cooper, M. A. Antibiotics in the clinical pipeline in 2011. *J. Antibiot.* **2011**, *64*, 413-425.

12. Butler, S. M.; Blaskovich, M. A.; Cooper, M. A. Antibiotics in the clinical pipeline in 2013. *J. Antibiot.* **2013**, *66*, 571-591.

13. Pucci, M. J.; Bush, K. Investigational antimicrobial agents of 2013. *Clin. Microbiol. Rev.* **2013**, *26*, 792-821.

14. Walsh, C. T.; Wencewic, T. A. Prospects for new antibiotics: a molecule-centered perspective. *J. Antibiot.* **2014**, *67*, 7-22.

15. Fischbach, M. A.; Walsh, C. T. Antibiotics for emerging pathogens. *Science* **2009**, *325*, 1089-1093.

16. Högberg, L. D.; Heddini, A.; Cars, O. The global need for effective antibiotics: challenges and recent advances. *Trends Pharmacol. Sci*. **2010**, *31*, 509-515.

17. Hede, K. Antibiotic resistance: An infectious arms race. *Nature* **2014**, *509*, S2-S3.

18. Boucher, H. W.; Talbot, G. H.; Bradley, J. S.; Edwards, J. E.; Gilbert, D.; Rice, L. B.; Scheld, M.; Spellberg, B.; Bartlett, J. Bad bugs, no drugs: No ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis*. **2009**, *48*, 1-12.

19. Lewis, K. Platforms for antibiotic discovery. *Nat. Rev. Drug Discov*. **2013**, *12*, 371-387.

20. Butler, M. S.; Cooper, M. A. Screening strategies to identify new antibiotics. *Curr. Drug Targets* **2012**, *13*, 373-387.

21. Shlaes, D. M.; Spellberg, B. Overcoming the challenges to developing new antibiotics. *Curr. Opin. Pharmacol.* **2012**, *12*, 522-526.

22. Walsh, C. Antibiotics: Actions, origins, and resistance (ASM Press, Washington DC, USA, **2003**).

23. Bush, K. Antimicrobial agents targeting bacterial cell walls and cell membranes. *Rev. Sci. Tech*. **2012**, *31*, 43-56.

24. Wilson, D. N. Ribosome-targeting antibiotics and mechanisms of bacterial resistance. *Nat. Rev. Microbiol.* **2014**, *12*, 35-48.

25. Wilson, D. N. The A-Z of bacterial translation inhibitors. *Crit. Rev. Biochem. Mol. Biol.* **2009**, *44*, 393-433.

26. Henry, R. J. The mode of action of sulfonamides. *Bacteriol. Rev.* **1943**, *7*, 175-262.

27. Scholar, E. M.; Pratt, W. B. The antimicrobial drugs. 2nd ed. (Oxford University Press, New York, NY, USA, **2000**).

28. Baltz, R. H. Daptomycin: Mechanisms of action and resistance, and biosynthetic engineering. *Curr. Opin. Chem. Biol.* **2009**, *13*, 144-151.

29. Robbel, L.; Marahiel, M. A. Daptomycin, a bacterial lipopeptide synthesized by a nonribosomal machinery. *J. Biol. Chem.* **2010**, *285*, 27501-27508.

30. Levy, S. B.; Marshall, B. Antibacterial resistance worldwide: causes, challenges and responses. *Nat. Med.* **2004**, *10*, S122-S129.

31. Levy, S. B. Active efflux mechanisms for antimicrobial resistance. *Antimicrob. Agents Chemother*. **1992**, *36*, 695-703.

32. Nikaido, H. Multidrug efflux pumps of Gram-negative bacteria. *J. Bacteriol.* **1996**, *178*, 5853-5859.

33. Cox, G.; Wright, G. D. Intrinsic antibiotic resistance: Mechanisms, origins, challenges and solutions. *Int. J. Med. Microbiol*. **2013,** *303*, 287-292.

34. Nikaido, H. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science* **1994**, *264*, 382-388.

35. Taubes, G. The bacteria fight back. *Science* **2008**, *321*, 356-361.

36. Hiramatsu, K. Vancomycin-resistant *Staphylococcus aureus*: A new model of antibiotic resistance. *Lancet Infect. Dis.* **2001**, *1*, 147-155.

37. Rice, L. B. Emergence of vancomycin-resistant Enterococci. *Emerg. Infect. Dis*. **2001**, *7*, 183-187.

38. Gu, B.; Kelesidis, T.; Tsiodras, S.; Hindler, J.; Humphries, R. M. The emerging problem of linezolid-resistant Staphylococcus. *J. Antimicrob. Chemother*. **2013**, *68*, 4-11.

39. Mangili, A.; Bica, I.; Snydman, D. R.; Hamer, D. H. Daptomycin-resistant, methicillin-resistant *Staphylococcus aureus* bacteremia. *Clin. Infect. Dis*. **2005**, *40*, 1058-1060.

40. Barker, K. F. Antibiotic resistance: A current perspective. *Br. J. Clin. Pharmacol*. **1999**, *48*, 109-124.

41. Anstead, G.; Cadena, J.; Javeri, H. Methicillin-resistant *Staphylococcus aureus* (MRSA) Protocols Vol. 1085 Methods in Molecular Biology ed Ji Y., 259-309 Humana Press: New Jersey, (**2014**).

42. Venugopal, A. A.; Johnson, S. Current state of *Clostridium difficile* treatment options. *Clin. Infect. Dis.* **2012**, *55*, S71-S76.

43. Zhanel, G. G.; Calic, D.; Schweizer, F.; Zelenitsky, S.; Adam, H.; Lagacé-Wiens, P. R.; Rubinstein, E.; Gin, A. S.; Hoban, D. J.; Karlowsky, J. A. New lipoglycopeptides: a comparative review of dalbavancin, oritavancin and telavancin. *Drugs* **2010**, *70*, 859-886.

44. Anderson, R. C. G. R, Higgins, H. M. Jr.; Pettinga, C. D. Symposium: How a drug is born. *Cincinnati. J. Med.* **1961**, *42*, 49-60.

45. Geraci, J. E.; Heilman, F. R.; Nichols, D. R.; Ross, G. T.; Wellman, W. E. Some laboratory and clinical experiences with a new antibiotic, vancomycin. *Mayo. Clin. Proc*. **1956**, *31*, 564-582.

46. Griffith, R. S. Vancomycin: continued clinical studies. *Antibiot. Annu.* **1956-1957**, 118-122.

47. Anderson, J. S.; Matsuhashi, M.; Haskin, M. A.; Strominger, J. L. Lipid-phosphoacetylmuramyl-pentapeptide and lipid-phosphodisaccharide-pentapeptide: Presumed membrane transport intermediates in cell wall synthesis. *Proc. Natl. Acad. Sci. USA* **1965**, *53*, 881-889.

48. Perkins, H. R. Specificity of combination between mucopeptide precursors and vancomycin or ristocetin. *Biochem. J.* **1969**, *111*, 195-205.

49. Chatterjee, A. N.; Perkins, H. R. Compounds formed between nucleotides related to the biosynthesis of bacterial cell wall and vancomycin. *Biochem. Biophys. Res. Commun.* **1966**, *24*, 489-494.

50. Bordet, C.; Perkins, H. R. Iodinated vancomycin and mucopeptide biosynthesis by cell-free preparations from *Micrococcus lysodeikticus*. *Biochem. J.* **1970**, *119*, 877-883.

51. James, R. C.; Pierce, J. G.; Okano, A.; Xie, J.; Boger, D. L. Redesign of glycopeptide antibiotics: Back to the future. *ACS Chem. Biol*. **2012**, *7*, 797-804.

52. Williams, D. H.; Williamson, M. P.; Butcher, D. W.; Hammond, S. J. Detailed binding sites of the antibiotics vancomycin and ristocetin A: determination of intermolecular distances in antibiotic/substrate complexes by use of the time-dependent NOE. *J. Am. Chem. Soc.* **1983**, *105*, 1332-1339.

53. Barna, J. C.; Williams, D. H. The structure and mode of action of glycopeptide antibiotics of the vancomycin group. *Annu. Rev. Microbiol.* **1984**, *38*, 339-357.

54. Kahne, D.; Leimkuhler, C.; Lu, W.; Walsh, C. Glycopeptide and lipoglycopeptide antibiotics. *Chem. Rev*. **2005**, *105*, 425-448.

55. Reynolds, P. Structure, biochemistry and mechanism of action of glycopeptide antibiotics. *Eur. J. Clin. Microbiol. Infect. Dis.* **1989**, *8*, 943-950.

56. McGuire, J. M.; Wolfe, R. N.; Ziegler, D. W. Vancomycin, a new antibiotic. II. In vitro antibacterial studies. *Antibiot. Annu.* **1956**, 612-618.

57. Cooper, G. L.; Given, D. B. *Vancomycin, A Comprehensive Review of 30 Years of Clinical Experience*; Park Row Publishers: **1986**.

58. Murray, B. E. Vancomycin-resistant Enterococcal infections. *N. Engl. J. Med.* **2000**, *342*, 710-721.

59. Courvalin, P. Vancomycin resistance in Gram-positive cocci. *Clin. Infect. Dis*. **2006**, *42*, S25-S34

60. Arthur, M.; Courvalin, P. Genetics and mechanisms of glycopeptide resistance in Enterococci. *Antimicrob. Agents Chemother.* **1993**, *37*, 1563-1571.

61. Evers, S.; Quintiliani, R. Jr.; Courvalin, P. Genetics of glycopeptide resistance in Enterococci. *Microb. Drug Resist.* **1996**, *2*, 219-223.

62. Cetinkaya, Y.; Falk, P.; Mayhall, C. G. Vancomycin-resistant Enterococci. *Clin. Microbiol. Rev.* **2000**, *13*, 686-707.

63. Walsh, C. T.; Fisher, S. L.; Park, I. S.; Prahalad, M.; Wu, Z. Bacterial resistance to vancomycin: five genes and one missing hydrogen bond tell the story. *Chem. Biol.* **1996**, *3*, 21-28.

64. Wright, G. D.; Holman, T. R.; Walsh, C. T. Purification and characterization of VanR and the cytosolic domain of VanS: A two-component regulatory system required for vancomycin resistance in *Enterococcus faecium* BM4147. *Biochemistry* **1993**, *32*, 5057-5063.

65. Holman, T. R.; Wu, Z.; Wanner, B. L.; Walsh, C. T. Identification of the DNA-binding site for the phosphorylated VanR protein required for vancomycin resistance in *Enterococcus faecium*. *Biochemistry* **1994**, *33*, 4625-4631.

66. Bugg, T. D.; Wright, G. D.; Dutka-Malen, S.; Arthur, M.; Courvalin, P.; Walsh, C. T. Molecular basis for vancomycin resistance in *Enterococcus faecium* BM4147: Biosynthesis of a depsipeptide peptidoglycan precursor by vancomycin resistance proteins VanH and VanA. *Biochemistry* **1991**, *30*, 10408-10415.

67. McComas, C. C.; Crowley, B. M.; Boger, D. L. Partitioning the loss in vancomycin binding affinity for D-Ala-D-Lac into lost H-bond and repulsive lone pair contributions. *J. Am. Chem. Soc.* **2003**, *125*, 9314-9315.

68. Smith, T. L.; Pearson, M. L.; Wilcox, K. R.; Cruz, C.; Lancaster, M. V.; Robinson-Dunn, B.; Tenover, F. C.; Zervos, M. J.; Band, J. D.; White, E.; Jarvis, W. R. Emergence of vancomycin resistance in *Staphylococcus aureus*. *N. Engl. J. Med.* **1999**, *340*, 493-501.

69. Sieradzki, K.; Tomasz, A. Gradual alterations in cell wall structure and metabolism in vancomycin-resistant mutants of *Staphylococcus aureus*. *J. Bacteriol.* **1999**, *181*, 7566-7570.

70. Chang, S.; Sievert, D. M.; Hageman, J. C.; Boulton, M. L.; Tenover, F. C.; Downes, F. P.; Shah, S.; Rudrik, J. T.; Pupp, G. R.; Brown, W. J.; Cardo, D.; Fridkin, S. K. Infection with vancomycin-resistant *Staphylococcus aureus* containing the vanA resistance gene. *N. Engl. J. Med.* **2003**, *348*, 1342-1347.

71. Weigel, L. M.; Clewell, D. B.; Gill, S. R.; Clark, N. C.; McDougal, L. K.; Flannagan, S. E.; Kolonay, J. F.; Shetty, J.; Killgore, G. E.; Tenover, F. C. Genetic analysis of a high-level vancomycin-resistant isolate of *Staphylococcus aureus*. *Science* **2003**, *302*, 1569-1571.

72. Van Bambeke, F.; Mingeot-Leclercq, M. P.; Struelens, M. J.; Tulkens, P. M. The bacterial envelope as a target for novel anti-MRSA antibiotics. *Trends Pharmacol. Sci*. **2008**, *29*, 124-134.

73. Leadbetter, M. R.; Adams, S. M.; Bazzini, B.; Fatheree, P. R.; Karr, D. E.; Krause, K. M.; Lam, B. M.; Linsell, M. S.; Nodwell, M. B.; Pace, J. L.; Quast, K.; Shaw, J. P.; Soriano, E.; Trapp, S. G.; Villena, J. D.; Wu, T. X.; Christensen, B. G.; Judice, J. K. Hydrophobic vancomycin derivatives with improved ADME properties: Discovery of telavancin (TD-6424). *J. Antibiot.* **2004**, *57*, 326-336.

74. Higgins, D. L.; Chang, R.; Debabov, D. V.; Leung, J.; Wu, T.; Krause, K. M.; Sandvik, E.; Hubbard, J. M.; Kaniga, K.; Schmidt, D. E. Jr.; Gao, Q.; Cass, R. T.; Karr, D. E.; Benton, B. M.; Humphrey, P. P. Telavancin, a multifunctional lipoglycopeptide, disrupts both cell wall

synthesis and cell membrane integrity in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother*. **2005**, *49*, 1127-1134.

75. Candiani, G.; Abbondi, M.; Borgonovi, M.; Romanò, G.; Parenti, F. In-vitro and in-vivo antibacterial activity of BI 397, a new semi-synthetic glycopeptide antibiotic. *J. Antimicrob. Chemother.* **1999**, *44*, 179-192.

76. Malabarba, A.; Goldstein, B. P. Origin, structure, and activity in-vitro and in-vivo of dalbavancin. *J. Antimicrob. Chemother*. **2005**, *55*, ii15-ii20.

77. Malabarba, A.; Ciabatti, R. Glycopeptide derivatives. *Curr. Med. Chem*. **2001**, *8*, 1759-1773.

78. Anderson, V. R.; Keating, G. M. Dalbavancin. *Drugs* **2008**, *68*, 639-648.

79. Cheng, M.; Ziora, Z. M.; Hansford, K. A.; Blaskovich, M. A.; Butler, M. S.; Matthew, A. Anti-cooperative ligand binding and dimerisation in the glycopeptide antibiotic dalbavancin. *Org. Biomol. Chem*. **2014**, *12*, 2568-2575.

80. Cooper, R. D.; Snyder, N. J.; Zweifel, M. J.; Staszak, M. A.; Wilkie, S. C.; Nicas, T. I.; Mullen, D. L.; Butler, T. F.; Rodriguez, M. J.; Huff, B. E.; Thompson, R. C. Reductive alkylation of glycopeptide antibiotics: synthesis and antibacterial activity. *J. Antibiot.* **1996**, *49*, 575-581.

81. Bouza, E.; Burillo, A. Oritavancin: A novel lipoglycopeptide active against Gram-positive pathogens including multiresistant strains. *Int. J. Antimicrob. Agents* **2010**, *36*, 401-407.

82. Allen, N. E.; Nicas, T. I. Mechanism of action of oritavancin and related glycopeptide antibiotics. *FEMS Microbiol. Rev.* **2003**, *26*, 511-532.

83. Belley, A.; Neesham-Grenon, E.; Arhin, F. F.; McKay, G. A.; Parr, Jr. T. R.; Moeck, G. Assessment by time-kill methodology of the synergistic effects of oritavancin in combination with other antimicrobial agents against *Staphylococcus aureus*. *Antimicrob. Agents Chemother*. **2008**, *52*, 3820-3822.

84. McAfee, J. J.; Castle, S. L.; Jin, Q.; Boger, D. L. Synthesis and evaluation of vancomycin and vancomycin aglycon analogues that bear modifications in the residue 3 asparagine. *Bioorg. Med. Chem. Lett*. **2002**, *12*, 1319-1322.

85. Crane, C. M.; Boger, D. L. Synthesis and evaluation of vancomycin aglycon analogues that bear modifications in the *N*-terminal D-leucyl amino acid. *J. Med. Chem*. **2009**, *52*, 1471-1476.

86. Crowley, B. M.; Boger, D. L. Total synthesis and evaluation of [Ψ[CH_2NH]Tpg^4]vancomycin aglycon: Reengineering vancomycin for dual D-Ala-D-Ala and D-Ala-D-Lac binding. *J. Am. Chem. Soc*. **2006**, *128*, 2885-2892.

87. Xie, J.; Pierce, J. G.; James, R. C.; Okano, A.; Boger, D. L. A redesigned vancomycin engineered for dual D-Ala-D-Ala and D-Ala-D-Lac binding exhibits potent antimicrobial activity against vancomycin-resistant bacteria. *J. Am. Chem. Soc.* **2011**, *133*, 13946-13949.

88. Xie, J.; Okano, A.; Pierce, J. G.; James, R. C.; Stamm, S.; Crane, C. M.; Boger, D. L. Total Synthesis of [Ψ[C(═S)NH]Tpg^4]vancomycin aglycon, [Ψ[C(═NH)NH]Tpg^4]vancomycin aglycon, and related key compounds: Reengineering vancomycin for dual D-Ala-D-Ala and D-Ala-D-Lac binding. *J. Am. Chem. Soc.* **2012**, *134*, 1284-1297.

89. R. Nagarajan. Structure-activity relationships of vancomycin-type glycopeptide antibiotics. *J. Antibiot*. **1993**, *46*, 1181-1195.

90. Ashford, P. A.; Bew, S. P. Recent advances in the synthesis of new glycopeptide antibiotics. *Chem. Soc. Rev*. **2012**, *41*, 957-978.

91. Fu, X.; Albermann, C.; Zhang, C.; Thorson, J. S. Diversifying vancomycin via chemoenzymatic strategies. *Org. Lett*. **2005**, *7*, 1513-1515.

92. Nakama, Y.; Yoshida, O.; Yoda, M.; Araki, K.; Sawada, Y.; Nakamura, J.; Xu, S.; Miura, K.; Maki, H.; Arimoto, H. Discovery of a novel series of semisynthetic vancomycin derivatives effective against vancomycin-resistant bacteria. *J. Med. Chem*. **2010**, *53*, 2528-2533.

93. Yoganathan, S.; Miller, S. J. Structure diversification of vancomycin through peptide-catalyzed, site-selective lipidation: A catalysis-based approach to combat glycopeptide-resistant pathogens. *J. Med. Chem*. **2015**, *58*, 2367-2677.

94. Okano, A.; Nakayama, A.; Schammel, A. W.; Boger, D. L. Total synthesis of [Ψ[C(═NH)NH]Tpg(4)]vancomycin and its (4-chlorobiphenyl)methyl derivative: Impact of peripheral modifications on vancomycin analogues redesigned for dual D-Ala-D-Ala and D-Ala-D-Lac binding. *J. Am. Chem. Soc.* **2014**, *136*, 13522-13525.

95. Mackay, J. P.; Gerhard, U.; Beauregard, D. A.; Westwell, M. S.; Searle, M. S.; Williams D. H. Glycopeptide antibiotic activity and the possible role of dimerization: A model for biological signaling. *J. Am. Chem. Soc.* **1994**, *116*, 4581-4590.

96. Cooper, M. A.; Williams, D. H.; Cho, Y. R. Surface plasmon resonance analysis of glycopeptide antibiotic activity at a model membrane surface. *Chem. Commun*. **1997**, *17*, 1625-1626.

97. Sundram, U. N.; Griffin, J. H. Novel vancomycin dimers with activity against vancomycin-resistant Enterococci. *J. Am. Chem. Soc*. **1996**, *118*, 13107-13108.

98. Rao, J.; Lahiri, J.; Isaacs, L.; Weiss, R. M.; Whitesides, G. M. A trivalent system from vancomycin.D-ala-D-Ala with higher affinity than avidin.biotin. *Science* **1998**, *280*, 708-711.

99. Arimoto, H.; Nishimura, K.; Kinumi, T.; Uemura, I. H. D. Multi-valent polymer of vancomycin: Enhanced antibacterial activity against VRE. *Chem. Commun*. **1999**, 1361-1362.

100. Nicolaou, K. C.; Hughes, R.; Cho, S. Y.; Winssinger, N.; Smethurst, C.; Labischinski, H.; Endermann, R. Target-accelerated combinatorial synthesis and discovery of highly potent antibiotics effective against vancomycin-resistant bacteria. *Angew. Chem. Int. Ed.* **2000**, *39*, 3823-3828.

101. Xing, B.; Yu, C.W.; Ho, P.L.; Chow, K. H.; Cheung, T.; Gu, H.; Cai, Z.; Xu, B. Multivalent antibiotics via metal complexes: Potent divalent vancomycins against vancomycin-resistant Enterococci. *J. Med. Chem*. **2003**, *46*, 4904-4909.

102. Yahav, D.; Lador, A.; Paul, M.; Leibovici, L. Efficacy and safety of tigecycline: a systematic review and meta-analysis. *J. Antimicrob. Chemother.* **2011**, *66*, 1963-1971.

103. Livermore, D. M. Doripenem: antimicrobial profile and clinical potential. *Diagn. Microbiol. Infect. Dis.* **2009**, *63***,** 455-8.

104. Pagès, J. M.; James, C. E.; Winterhalter, M. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. *Nat. Rev. Microbiol.* **2008**, *6***,** 893-903.

105. Hancock, R. E. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative Gram-negative bacteria. *Clin. Infect. Dis.* **1998**, *27*, S93-S99.

106. Hancock, R. E.; Brinkman, F. S. L. Function of Pseudomonas porins in uptake and efflux. *Annu. Rev. Microbiol.* **2002**, *56***,** 17-38.

107. Vila, J.; Martí, S.; Sánchez-Céspedes, J. Porins, efflux pumps and multidrug resistance in *Acinetobacter baumannii*. *J. Antimicrob. Chemother.* **2007**, *59*, 1210-1215.

108. Nagarajan, R., Ed. Glycopeptide antibiotics, Marcel Dekker Inc., New York, (**1994**).

109. Nicolosi, D.; Scalia, M.; Nicolosi, V. M.; Piqnatello, R. Encapsulation in fusogenic liposomes broadens the spectrum of action of vancomycin against Gram-negative bacteria. *Int. J. Antimicrob. Agents* **2010**, *35*, 553-558.

110. Morones-Ramirez, J. R.; Winkler, J. A.; Spina, C. S.; Collins, J. J. Silver enhances antibiotic activity against Gram-negative bacteria. *Sci. Transl. Med.* **2013**, *5*, 190ra81.

111. Bush, K.; Courvalin, P.; Dantas, G.; Davies, J.; Eisenstein, B.; Huovinen, P.; Jacoby, G. A.; Kishony, R.; Kreiswirth, B. N.; Kutter, E.; Lerner, S. A.; Levy, S.; Lewis, K.; Lomovskaya, O.; Miller, J. H.; Mobashery, S.; Piddock, L. J.; Projan, S.; Thomas, C. M.; Tomasz, A.; Tulkens, P. M.; Walsh, T. R.; Watson, J. D.; Witkowski, J.; Witte, W.; Wright, G.; Yeh, P.; Zgurskaya, H. I. Tackling antibiotic resistance. *Nat. Rev. Microbiol.* **2011**, *9*, 894-896.

112. Tsai, H. Y.; Liao, C. H.; Chen, Y. H.; Lu, P. L.; Huang, C. H.; Lu, C. T.; Chuang, Y. C.; Tsao, S. M.; Chen, Y. S.; Liu, Y. C.; Chen, W. Y.; Jang, T. N.; Lin, H. C.; Chen C. M.; Shi, Z. Y.; Pan, S. C.; Yang, J. L.; Kung, H. C.; Liu, C. E.; Cheng, Y. J.; Liu, J. W.; Sun, W.; Wang, L. S.; Ko, W. C.; Yu, K. W.; Chiang, P. C.; Lee, M. H.; Lee, C. M.; Hsu, G. J.; Hsueh, P. R. Trends in susceptibility of vancomycin-resistant *Enterococcus faecium* to tigecycline, daptomycin, and linezolid and molecular epidemiology of the isolates: Results from the Tigecycline In Vitro Surveillance in Taiwan (TIST) study, 2006 to 2010. *Antimicrob. Agents Chemother.* **2012**, *56*, 3402-3405.

113. Bertsche, U. Yang, S. J.; Kuehner, D.; Wanner, S.; Mishra, N. N.; Roth, T.; Nega, M.; Schneider, A.; Mayer, C.; Grau, T.; Bayer, A. S.; Weidenmaier, C. Increased cell wall teichoic acid production and D-alanylation are common phenotypes among daptomycin-resistant methicillin-resistant *Staphylococcus aureus* (MRSA) clinical isolates. *PLoS ONE* **2013**, *8*, e67398.

114. Nitanai, Y.; Kikuchi, T.; Kakoi, K.; Hanamaki, S.; Fujisawa, I.; Aoki, K. Crystal structures of the complexes between vancomycin and cell-wall precursor analogs. *J. Mol. Biol.* **2009**, *385*, 1422-1432.

115. Draghi, D. C.; Benton, B. M.; Krause, K. M.; Thornsberry, C.; Pillar, C.; Sahm, D. F. In vitro activity of telavancin against recent Gram-positive clinical isolates: Results of the 2004-05 Prospective European Surveillance Initiative. *J. Antimicrob. Chemother.* **2008**, *62*, 116-121.

116. Biedenbach, D. J.; Bell, J. M.; Sader, H. S.; Turnidge, J. D.; Jones, R. N. Activities of dalbavancin against a worldwide collection of 81,673 Gram-positive bacterial isolates. *Antimicrob. Agents Chemother.* **2009**, *53*, 1260-1263.

117. Slusarz, R.; Szulc, M.; Madaj, J. Molecular modeling of Gram-positive bacteria peptidoglycan layer, selected glycopeptide antibiotics and vancomycin derivatives modified with sugar moieties. *Carbonydr. Res.* **2014**, *389*, 154-164.

118. Allen, N. E.; LeTourneau, D. L.; Hobbs, J. N. Jr.; The role of hydrophobic side chains as determinants of antibacterial activity of semi-synthetic glycopeptide antibiotics. *J. Antibiot.* **1997**, *50*, 677-684.

119. Ge, M.; Chen, Z.; Onishi, H. R.; Kohler, J.; Silver, L. L.; Kerns, R.; Fukuzawa, S.; Thompson, C.; Kahne, D. Vancomycin derivatives that inhibit peptidoglycan biosynthesis without binding D-Ala-D-Ala. *Science* **1999**, *284*, 507-511.

120. Eggert, U. S.; Ruiz, N.; Falcone, B. V.; Branstrom, A. A.; Goldman, R. C.; Silhavy, T. J.; Kahne, D. Genetic basis for activity difference between vancomycin and glycolipid derivatives of vancomycin. *Science* **2001**, *294*, 361-364.

121. Woodford, N.; Livermore, D. M. Infections caused by Gram-positive bacteria: A review of the global challenge. *J. Infect.* **2009**, *59*, S4-S16.

122. Fekete, A.; Borbas, A.; Antus, S.; Liptak, A. Synthesis of 3,6-branched arabinogalactan-type tetra- and hexasaccharides for characterization of monoclonal antibodies. *Carbohydr. Res.* **2009**, *344*, 1434-1441.

123. Blanzat, M.; Perez, E.; Rico-Lattes, I.; Prome, D.; Prome, J. C.; Lattes, A. New catanionic glycolipids. 1. Synthesis, characterization, and biological activity of double-chain and gemini cationic analogues of galactosylceramide (gal$\beta_1$cer). *Langmuir* **1999**, *15*, 6163-6169.

124. Thayer, D. A.; Wong, C. H. Vancomycin analogues containing monosaccharides exhibit improved antibiotic activity: a combined one-pot enzymatic glycosylation and chemical diversification strategy. *Chem. Asian J.* **2006**, *1*, 445-452.

125. Sundram, U. N.; Griffin, J. H. General and efficient method for the solution and solid phase synthesis of vancomycin carboxamide derivatives. *J. Org. Chem.* **1995**, *60*, 1102-1103.

126. Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nat. Protoc*. **2008**, *3*, 163-175.

127. Nieto, M.; Perkins, H. R. The specificity of combination between ristocetins and peptides related to bacterial cell wall mucopeptide precursors.*Biochem. J.* **1971**, *124*, 845-852.

128. Schneider, T.; Kruse, T.; Wimmer, R.; Wiedemann, I.; Sass, V.; Pag, U.; Jansen, A.; Nielsen, A. K.; Mygind, P. H.; Raventós, D. S.; Neve, S.; Ravn, B.; Bonvin, A. M.; De Maria, L.; Andersen, A. S.; Gammelgaard, L. K.; Sahl, H. G.; Kristensen, H. H. Plectasin, a fungal defensin, targets the bacterial cell wall precursor lipid II. *Science* **2010**, *328*, 1168-1172.

129. Ling, L. L.; Schneider, T.; Peoples, A. J.; Spoering, A. L.; Engels, I.; Conlon, B. P.; Mueller, A.; Schäberle, T. F.; Hughes, D. E.; Epstein, S.; Jones, M.; Lazarides, L.; Steadman, V. A.; Cohen, D. R.; Felix, C. R.; Fetterman, K. A.; Millett, W. P.; Nitti, A. G.; Zullo, A. M.; Chen, C.; Lewis, K. A new antibiotic kills pathogens without detectable resistance. *Nature* **2015**, *517*, 455-459.

130. Chin, J. N.; Rybak, M. J.; Cheung, C. M.; Savage, P. B. Antimicrobial activities of ceragenins against clinical isolates of resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother*. **2007**, *51*, 1268-1273.

131. Pollard, J. E.; Snarr, J.; Chaudhary, V.; Jennings, J. D.; Shaw, H.; Christiansen, B.; Wright, J.; Jia, W.; Bishop, R. E.; Savage, P. B. In vitro evaluation of the potential for resistant development to ceragenin CSA-13. *J. Antimicrob. Chemother*. **2012**, *67*, 2665-2672.

132. Alder, J.; Li, T.; Yu, D.; Morton, L.; Silverman, J.; Zhang, X. X.; Critchley, I.; Thorne, G. Analysis of daptomycin efficacy and breakpoint standards in a murine model of *Enterococcus faecalis* and *Enterococcus faecium* renal infection. *Antimicrob. Agents Chemother.* **2003**, *47*, 3561-3566.

133. Andes, D.; Craig, W. A. In vivo pharmacodynamic activity of the glycopeptide dalbavancin. *Antimicrob. Agents Chemother.* **2007**, *51*, 1633-1642

134. Zuluaga, A. F.; Agudelo, M.; Rodriguez, C. A.; Vesga, O. Application of microbiological assay to determine pharmaceutical equivalence of generic intravenous antibiotics. *BMC Clin. Pharmacol*. **2009**, *9*, 1-11.

135. Gabrielsson, J.; Weiner, D. Non-compartmental analysis. *Methods Mol. Biol*. **2012**, *929*, 377-389.

136. Nederberg, F.; Zhang, Y.; Tan, J. P.; Xu, K.; Wang, H.; Yang, C.; Gao, S.; Fukushima, K.; Li, L.; Hedrick, J. L.; Yang, Y. Y. Biodegradable nanostructures with selective lysis of microbial membranes. *Nat. Chem*. **2011**, *3*, 409-414.

137. Hegde, S. S.; Reyes, N.; Wiens, T.; Vanasse, N.; Skinner, R.; McCullough, J.; Kaniga, K.; Pace, J.; Thomas, R.; Shaw, J. P.; Obedencio, G.; Judice, J. K. Pharmacodynamics of telavancin (TD-6424), a novel bactericidal agent, against Gram-positive bacteria. *Antimicrob. Agents Chemother*. **2004**, *48*, 3043-3050.

138. Bragonzi, A. Fighting back: Peptidomimetics as a new weapon in the battle against antibiotic resistance. *Sci. Transl. Med.* **2010**, *2*, 21ps9.

139. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415*, 389-395.

140. Choi, S.; Isaacsb, A.; Clements, D.; Liu, D.; Kim, H.; Scott, R. W.; Winkler, J. D.; DeGrado, W. F. De novo design and in vivo activity of conformationally restrained antimicrobial arylamide foldamers. *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 6968-6973.

141. Uppu, D. S. S. M.; Akkapeddi, P.; Manjunath, G. B.; Yarlagadda, V.; Hoque, J.; Haldar, J. Polymers with tunable side chain amphiphilicity as non-hemolytic antibacterial agents. *Chem. Commun*. **2013**, *49*, 9389-9391.

142. Anantharaman, A.; Sahal, D. Reverse engineering truncations of an antimicrobial peptide dimer to identify the origins of potency and broad spectrum of action. *J. Med. Chem*. **2010**, *53*, 6079-6088.

143. Song, A.; Walker, S. G.; Parker, K. A.; Sampson, N. S. Antibacterial studies of cationic polymers with alternating, random and uniform backbones. *ACS Chem. Biol*. **2011**, *6*, 590-599.

144. Konai, M. M.; Ghosh, C.; Yarlagadda, V.; Samaddar, S.; Haldar, J. Membrane active phenylalanine conjugated lipophilic norspermidine derivatives with selective antibacterial activity. *J. Med. Chem.* **2014**, *57*, 9409-9423.

145. Hoque, J.; Akkapeddi, P.; Yarlagadda, V.; Uppu, D. S. S. M.; Kumar, P.; Haldar, J. Cleavable cationic antibacterial amphiphiles: Synthesis, mechanism of action, and cytotoxicities. *Langmuir*, **2012**, *28*, 12225-122234.

146. Meziane-Cherif, D.; Stogios, P. J.; Evdokimova, E.; Savchenko, A.; Courvalin, P. Structural basis for the evolution of vancomycin resistance D,D-peptidases. *Proc Natl Acad Sci USA.* **2014**, *111*, 5872-5877.

147. Tamilselvi, A.; Nethaji, M.; Mugesh, G. Antibiotic resistance: Mono- and dinuclear zinc complexes as metallo-beta-lactamase mimics. *Chem. Eur. J.* **2006**, *12*, 7797-7806.

148. Ngo, H. T.; Liu, X.; Jolliffe, K. A. Anion recognition and sensing with Zn(II) dipicolylamine complexes. *Chem. Soc. Rev.* **2012**, *41*, 4928-4965.

149. Lin, J. R.; Chu, C. J.; Venkatesan, P.; Wu, S. P. Zinc(II) and pyrophosphate selective fluorescence probe and its application to living cell imaging. *Sensors and Actuators B* **2015**, *207*, 563-570.

150. Bushnell, G.; Mitrani-Gold, F.; Mundy, L. M. Emergence of New Delhi metallo-β-lactamase type 1-producing enterobacteriaceae and non-enterobacteriaceae: Global case detection and bacterial surveillance. *Int. J. Infect. Dis.* **2013**, *17*, e325-e333.

151. Shakil, S.; Azhar, E. I.; Tabrez, S.; Kamal, M. A.; Jabir, N. R.; Abuzenadah, A. M.; Damanhouri, G. A.; Alam, Q. New Delhi metallo-β-lactamase (NDM-1): An update. *J Chemother*. **2011**, *23*, 263-265.

152. Fomda, B. A.; Khan, A.; Zahoor, D. NDM-1 (New Delhi metallo beta lactamase-1) producing Gram-negative bacilli: Emergence & clinical implications. *Indian J. Med. Res*. **2014**, *140*, 672-678.

153. Bush, K.; Jacoby, G. A. Updated functional classification of β-lactamases. *Antimicrob. Agents Chemother.* **2010**, *54*, 969-976.

154. Mattei, P. J.; Neves, D.; Dessen, A. Bridging cell wall biosynthesis and bacterial morphogenesis. *Curr. Opin. Struct. Biol.* **2010**, *20*, 749-575.

155. Walsh, T. R.; Toleman, M. A.; Poirel, L.; Nordmann, P. Metallo-β-lactamases: The quiet before the storm?. *Clin. Microbiol. Rev.* **2005**, *18*, 306-325.

156. Cornaglia, G.; Giamarellou, H., Rossolini, G. M. Metallo-β-lactamases: A last frontier for β-lactams?. *Lancet Infect. Dis.* **2011**, *11*, 381-393.

157. Page, M. I.; Badarau, A. The mechanisms of catalysis by metallo β-lactamases. *Bioinorg. Chem. Appl.* **2008**, 576297.

158. Ehmann, D. E.; Jahic, C.; Ross, P. L.; Gu, R. F.; Hu, J.; Durand-Reville, T. F.; Lahiri, S.; Thresher, J.; Livchak, S.; Gao, N.; Palmer, T.; Walkup, G. K.; Fisher, S. L. Kinetics of avibactam inhibition against class A, C, and D β-lactamases. *J. Biol. Chem.* **2013**, *288*, 27960-27971.

159. Bebrone, C.; Lassaux, P.; Vercheval, L.; Sohier, J. S.; Jehaes, A.; Sauvage, E.; Galleni, M. Current challenges in antimicrobial chemotherapy: focus on β-lactamase inhibition. *Drugs* **2010**, *70*, 651-679.

160. Crowder, M. W.; Spencer, J.; Vila, A. J. Metallo-β-lactamases: novel weaponry for antibiotic resistance in bacteria. *Acc. Chem. Res.* **2006**, *39*, 721-728.

161. Bush, K. Proliferation and significance of clinically relevant β-lactamases. *Ann. NY Acad. Sci.* **2013**, *1277*, 84-90.

162. Drawz, S. M.; Bonomo, R. A. Three decades of β-lactamase inhibitors. *Clin. Microbiol. Rev.* **2010**, *23*, 160-201.

163. Fast, W.; Sutton, L. D. Metallo-β-lactamase: inhibitors and reporter substrates. *Biochim. Biophys. Acta.* **2013,** *1834*, 1648-1659.

164. Ejim, L.; Farha, M. A.; Falconer, S. B.; Wildenhain, J.; Coombes, B. K.; Tyers, M.; Brown, E. D.; Wright, G. D. Combinations of antibiotics and nonantibiotic drugs enhance antimicrobial efficacy. *Nat. Chem. Biol.* **2011,** *7*, 348-350.

165. King, A. M.; Reid-Yu, S. A.; Wang, W.; King, D. T.; De Pascale, G.; Strynadka, N. C.; Walsh, T. R.; Coombes, B. K.; Wright, G. D. Aspergillomarasmine A overcomes metallo-β-lactamase antibiotic resistance. *Nature* **2014**, *510*, 503-506.

166. Bulik, C. C.; Fauntleroy, K. A.; Jenkins, S. G.; Abuali, M.; LaBombardi, V. J.; Nicolau, D. P.; Kuti, J. L. Comparison of meropenem MICs and susceptibilities for carbapenemase-producing *Klebsiella pneumoniae* isolates by various testing methods. *J. Clin. Microbiol*. **2010**, *48*, 2402-2406.

167. Piddock, L. J. The crisis of no new antibiotics-what is the way forward?. *Lancet Infect. Dis.* **2012**, *12*, 249-253.

168. World health Organization. (2014) Antimicrobial resistance: global report on surveillance. World health Organization, Geneva, Switzerland. (http://www.who.int/drugresistance/documents/surveillancereport/en.pdf accessed 9th May 2014).

169. Livermore, D. M.; Warner, M.; Musthaq, S.; Doumith, M.; Zhang, J.; Woodford, N. What remains against carbapenem-resistant Enterobacteriaceae? Evaluation of chloramphenicol, ciprofloxacin, colistin, fosfomycin, minocycline, nitrofurantoin, temocillin and tigecycline. *Int. J. Antimicrob. Agents* **2011**, *37*, 415-419.

170. Stone, N. R.; Woodford, N.; Livermore, D. M.; Howard, J.; Pike, R.; Mushtaq, S.; Perry, C.; Hopkins, S. Breakthrough bacteraemia due to tigecycline-resistant *Escherichia coli* with New Delhi metallo-β-lactamase (NDM)-1 successfully treated with colistin in a patient with calciphylaxis. *J. Antimicrob. Chemother*. **2011**, *66*, 2677-2678.

171. Arpin, C.; Noury, P.; Boraud, D.; Coulange, L.; Manetti, A.; Andre, C.; M'Zali, F.; Quentin, C. NDM-1-Producing *Klebsiella pneumoniae* resistant to colistin in a French community

patient without history of foreign travel. *Antimicrob. Agents Chemother*. **2012**, *56*, 3432-3434.

172. Uppu, D. S.; Ghosh, C.; Haldar, J. Surviving sepsis in the era of antibiotic resistance: Are there any alternative approaches to antibiotic therapy?. *Microb. Pathog*. **2015**, *80*, 7-13.

173. Ghosh, C.; Manjunath, G. B.; Akkapeddi, P.; Yarlagadda, V.; Hoque, J.; Uppu, D. S.; Konai, M. M.; Haldar, J. Small molecular antibacterial peptoid mimics: the simpler the better!. *J. Med. Chem.* **2014**, *57*, 1428-1436.

174. Endimiani, A.; Hujer, K. M.; Hujer, A. M.; Pulse, M. E.; Weiss, W. J.; Bonomo, R. A. Evaluation of ceftazidime and NXL104 in two murine models of infection due to KPC-producing *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **2011**, *55*, 82-85.

175. Liu, A.; Tran, L.; Becket, E.; Lee, K.; Chinn, L.; Park, E.; Tran, K.; Miller, J. H. Antibiotic sensitivity profiles determined with an *Escherichia coli* gene knockout collection: Generating an antibiotic bar code. *Antimicrob. Agents Chemother*. **2010**, *54*, 1393-1403.

176. Cox, G.; Koteva, K.; Wright, G. D. An unusual class of anthracyclines potentiate Gram-positive antibiotics in intrinsically resistant Gram-negative bacteria. *J. Antimicrob. Chemother.* **2014**, *69*, 1844-1855.

177. Maragakis, L. L.; Perl, T. M. *Acinetobacter baumannii:* Epidemiology, antimicrobial resistance, and treatment options. *Clin. Infect. Dis*. **2008**, *46*, 1254-1263.

178. Yokoyama, Y.; Matsumoto, K.; Ikawa, K.; Watanabe, E.; Shigemi, A.; Umezaki, Y.; Nakamura, K.; Ueno, K.; Morikawa, N.; Takeda, Y. Pharmacokinetic/pharmacodynamic evaluation of sulbactam against *Acinetobacter baumannii* in *in-vitro* and murine thigh and lung infection models. *Int. J. Antimicrob. Agents* **2014**, *43*, 547-552.

List of Publications

PhD Thesis Publications

1. **Venkateswarlu Yarlagadda**, Padma Akkapeddi, Goutham B. Manjunath, Jayanta Haldar. Membrane active vancomycin analogues: A strategy to combat bacterial resistance. *J. Med. Chem.* **2014**, *57*, 4558-4568.

2. **Venkateswarlu Yarlagadda**, Mohini M. Konai, Goutham B. Manjunath, Chandradhish Ghosh, Jayanta Haldar. Tackling vancomycin-resistant bacteria with 'lipophilic-vancomycin-carbohydrate conjugates'. *J. Antibiot.* **2015**, *68*, 302-312.

3. **Venkateswarlu Yarlagadda**, Mohini M. Konai, Goutham B. Manjunath, Relekar G. Prakash, Bhuvana Mani, Krishnamoorthy Paramanandham, Shome B. Ranjan, Raju Ravikumar, Subhankari P. Chakraborty, Somenath Roy, Jayanta Haldar. In-vivo antibacterial activity and pharmacological properties of the membrane-active glycopeptide antibiotic YV11455. *Int. J. Antimicrob. Agents* **2015**, *45*, 627-634.

4. **Venkateswarlu Yarlagadda**, Mohini M. Konai, Krishnamoorthy Paramanandham, Venugopal C. Nimita, Bibek R. Shome, Jayanta Haldar. *In-vivo* efficacy and pharmacological properties of a novel glycopeptide (YV4465) against vancomycin-intermediate *Staphylococcus aureus. Int. J. Antimicrob. Agents* **2015**, Just Accepted.

5. **Venkateswarlu Yarlagadda**, Paramita Sarkar, Padma Akkapeddi, Krishnamoorthy Paramanandham, Shome B. Ranjan, Raju Ravikumar, Jayanta Haldar. Glycopeptide antibiotic to overcome the intrinsic resistance of Gram-negative bacteria. *Manuscript under review*.

6. **Venkateswarlu Yarlagadda**, Sandip Samaddar, Krishnamoorthy Paramanandham, Bibek R. Shome, Jayanta Haldar. Membrane disruption and enhanced inhibition of cell wall biosynthesis: A synergistic approach to tackle vancomycin-resistant bacteria. *Manuscript under review*.

7. **Venkateswarlu Yarlagadda**, Jayanta Haldar. An alternative approach to tackle vancomycin-resistant bacteria by dipicolyl-vancomycin conjugate. *Manuscript under preparation*.

8. **Venkateswarlu Yarlagadda**, Jayanta Haldar. Vancomycin analogue restores meropenem activity against NDM-1 Gram-negative pathogens. *Manuscript under preparation*.

Miscellaneous publications

1. **Venkateswarlu Yarlagadda**, Paramita Sarkar, Goutham B. Manjunath, Jayanta Haldar. Lipophilic vancomycin aglycon dimer with high activity against vancomycin-resistant bacteria. *Manuscript submitted*.

2. **Venkateswarlu Yarlagadda**, Jayanta Haldar. Intracellular activity of membrane active glycopeptide antibiotic (YV11455) against MRSA infection. *Manuscript under preparation*.

3. **Venkateswarlu Yarlagadda**, Jayanta Haldar. Vancomycin functionalized carbon nanospheres: A move towards effective nanomedicine. *Manuscript under preparation*.

4. Jiaul Hoque, Padma Akkapeddi, **Venkateswarlu Yarlagadda**, Divakara S. S. M. Uppu, Pratik Kumar, Jayanta Haldar. Cleavable cationic antibacterial amphiphiles: Synthesis, mechanism of action, and cytotoxicities. *Langmuir* **2012**, *28*, 12225-12234.

5. Divakara S. S. M. Uppu, Padma Akkapeddi**,** Goutham B. Manjunath, **Venkateswarlu Yarlagadda**, Jiaul Hoque, Jayanta Haldar. Polymers with tunable side-chain amphiphilicity as non-hemolytic antibacterial agents. *Chem. Commun.* **2013**, *49*, 9389-9391.

6. Chandradhish Ghosh**,** Goutham B. Manjunath, Padma Akkapeddi, **Venkateswarlu Yarlagadda**, Jiaul Hoque, Divakara S. S. M. Uppu, Mohini M. Konai, Jayanta Haldar. Small molecular antibacterial peptoid mimics: The simpler the better!. *J. Med. Chem.* **2014**, *57*, 1428-1436.

7. Jiaul Hoque, Spandhana Gonuguntla, **Venkateswarlu Yarlagadda**, Vinod K. Aswal, Jayanta Haldar. Effect of amide bonds on the self-assembly of gemini surfactants. *Phys. Chem. Chem. Phys.* **2014**, *16*, 11279-11288.

8. Mohini M. Konai, Chandradhish Ghosh, **Venkateswarlu Yarlagadda**, Sandip Samaddar, Jayanta Haldar. Membrane active phenylalanine conjugated lipophilic norspermidine derivatives with selective antibacterial activity. *J. Med. Chem.* **2014**, *57*, 9409-9423.

9. Divakara S. S. M. Uppu, Goutham B. Manjunath, **Venkateswarlu Yarlagadda**, Jyothi E. Kaviyil, Raju Ravikumar, Krishnamoorthy Paramanandham, Bibek R. Shome, Jayanta Haldar. Membrane active macromolecules resensitize NDM-1 Gram-negative clinical isolates to tetracycline antibiotics. *PLoS ONE*, **2015**, *10*, e0119422.

10. Jiaul Hoque, Padma Akkapeddi, Vikas Yadav, Goutham B. Manjunath, Divakara S. S. M. Uppu, Mohini M. Konai, **Venkateswarlu Yarlagadda**, Kaustuv Sanyal, Jayanta Haldar. Broad spectrum antibacterial and antifungal polymeric paint materials: Synthesis, structure-activity relationship, and membrane-active mode of action. *ACS Appl. Mater. Interfaces* **2015**, *7*, 1804-1815.

11. Jiaul Hoque, Mohini M. Konai, Spandhana Gonuguntla, Goutham B. Manjunath, Sandip Samaddar, **Venkateswarlu Yarlagadda**, Jayanta Haldar. Membrane active small molecules show selective broad spectrum antibacterial activity with no detectable resistance and eradicate biofilms. *J. Med. Chem.* **2015**, Just Accepted.

12. Jiaul Hoque, Padma Akkapeddi, Goutham B. Manjunath, **Venkateswarlu Yarlagadda**, Divakara S. S. M. Uppu, Chandradhish Ghosh, Jayanta Haldar. Biodegradable polymeric antimicrobial paint. *Manuscript submitted*.

13. P. Sagar Vijay Kumar, G. V. P. Chandramouli, B. Prasanna, E. Rajeswara Reddy, **Venkateswarlu Yarlagadda**, Jayanta Haldar. Design, synthesis and antimicrobial studies of novel polycyclic fused chromeno-pyrazolo-naphthodiazepines via multi-component reaction: Molecular docking, 2D QSAR and 3D QSAR based approaches. *Manuscript under review*.

Patents

1. Jayanta Haldar, **Venkateswarlu Yarlagadda**, Padma Akkapeddi. "Cationic antibacterial composition". WO 2013 072838 A1. Filed in six countries in national level: India, USA (US 20140308347 A1), Europe (EP 2780359 A1), Australia, Canada (CA 2855753 A1), Korea.

2. Jayanta Haldar, **Venkateswarlu Yarlagadda**, Mohini M. Konai, Goutham B. Manjunath. "Vancomycin-sugar conjugates and uses thereof". WO 2015 040467 A1.

3. Jayanta Haldar, **Venkateswarlu Yarlagadda**. "Glycopeptide derivatives and uses thereof". Patent application no. 6565/CHE/2014.

4. Jayanta Haldar, **Venkateswarlu Yarlagadda**. "Glycopeptide antibiotic derivatives". Patent application no. 605/CHE/2015.

Miscellaneous Highlights of PhD Work

1. **Best Poster Presentation Award** at Chemical Research Society of India (CRSI) meeting, Lucknow, India in 2012.

2. **Best Poster Presentation Award** at 5th International Symposium on Current Trends in Drug Discovery Research (CTDDR), Lucknow, India in 2013.

3. Received travel grant from Department of Science and Technology (DST), India; to present a poster at **Gordon Research Conference** (GRC) on "**New Antibacterial Discovery & Development**", California, USA in 2014.

4. Awarded with **Gandhian Young Technological Innovation Award** (GYTI-2015) under **BIRAC-SRISTI Technological Edge/Strategic Innovation** Category at Rashtrapathi Bhavan, New Delhi, India.

5. Part of the work has been highlighted in leading Indian national news papers-

i) **The Times of India:** "Bangalore scientists break bacteria's resistance to antibiotics" on 10th July, 2014.

ii) **The Indian Express:** "City scientists develop drug to kill resistant bacteria" on 13th July, 2014.

iii) **Scroll.in:** "How a discovery by a team of Bangalore scientists could solve India's antibiotics crisis" on 17th July, 2014.