NMR structure and activity relationship of defensin-analogous peptides

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NANYANG
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**NMR STRUCTURE AND ACTIVITY
RELATIONSHIP OF DEFENSIN-
ANALOGOUS PEPTIDES**

BAI YANG
SCHOOL OF BIOLOGICAL SCIENCES
2014

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Abbreviations

AMP	AntiMicrobial Peptides
FDA	Food and Drug Administration
SAR	Structural and Activity Relationship
PABA	Para-AminoBenzoic Acid
DHPS	DiHydroPteroate Synthase
PBP	Penicillin-Binding Proteins
LPS	LiPopolySaccharide
MDR	Multi-Drug Resistance
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
MIC	Minimal Inhibitory Concentration
DPC	DodecylPhosphoCholine
LUV	Large Unilamellar Vesicles
hREMD	Hamiltonian Replica Exchange Molecular Dynamics
RMSD	Root-Mean-Square deviation
POPE	1-Palmitoyl-2-Oleoyl- <i>sn</i> -glycero-3- -PhosphoEthanolamine
POPG	1-Palmitoyl-2-Oleoyl- <i>sn</i> -glycero-3-Phospho-(1'- <i>rac</i> - -Glycerol
POPC	1-Palmitoyl-2-Oleoyl- <i>sn</i> -glycero-3- -PhosphoCholine
DOPG	1,2-DiOleoyl- <i>sn</i> -glycero-3-Phospho-(1'- <i>rac</i> - -Glycerol
DOPE	1,2-DiOleoyl- <i>sn</i> -glycero-3-PhosphoEthanolamine
DOPC	1,2-DiOleoyl- <i>sn</i> -glycero-3-PhosphoCholine
DHPC	1,2-DiHeptanoyl- <i>sn</i> -glycero-3-PhosphoCholine

DMPC	1,2-DiMyristoyl- <i>sn</i> -glycero-3-PhosphoCholine
MM/PBSA	Molecular Mechanics/Generalized Born Surface Area
PRE	Paramagnetic Relaxation Enhancement

Abstract

In recent years, strains of bacteria with resistance to conventional antibiotics have been emerging at an alarming rate leading to the urgency of designing a new generation of antimicrobials. One of the promising avenues has been antimicrobial peptides and peptoids. Despite intensive research, detailed bactericidal and cytotoxic mechanisms of AMPs are still largely unknown. In this thesis, molecular template evolution based on the peptide sequence modifications, biophysical studies using fluorescence labeling and NMR spectroscopy, virtual screening utilizing large scale molecular dynamics simulations are exploited for rational antimicrobial drug discovery. Two main classes of drug candidates are studied, human β -defensin-3 derived linear as well as branched peptides targeting Gram-negative bacteria and peptide mimetics synthesized from cationic charge-modified alpha-mangostin predominantly active against Gram-positive bacteria.

The results show that the binding affinity, partitioning into the lipid bilayer, the ability to oligomerize and form well defined structures upon interactions with biological phases (e.g. bacterial membranes, peptidoglycans and LPS layers) may contribute to condensation of positive charges in AMP macrostructures resulting in membrane remodeling and eventual lysis of bacteria. The interactions between AMPs and negatively charged lipids characteristic to the composition of the bacterial inner membranes can cause redistribution of membrane lipids, which in turn may result in increased membrane permeability and bacterial lysis.

In the case of branched peptide targeting Gram-negative bacteria, a progressive structure accretion is observed in water, LPS, and lipid environments. Despite inducing rapid aggregation of outer membrane lipopolysaccharides, AMPs remain highly mobile in the aggregated lattice. These findings suggest that AMPs possessing both enhanced mobility in the bacterial outer membrane and spatial structure facilitating its interactions with the membrane-water interface may provide excellent structural motifs for developing broad-spectrum new antimicrobials.

For peptoids targeting Gram-positive bacteria, large oligomers formed by strong hydrophobic interactions between xanthone groups were observed in aqueous solution, lipid micelles and liposomes. The peptoid-induced graded leakage of the content of the lipid liposomes used to model bacterium was monitored by both fluorescence study as well as solution state NMR using bacterial membrane mimicking liposomes incorporating fluorescence reporter or paramagnetic molecules inside. A novel AMP membrane targeting mechanism, a patch model, was proposed. Once approaching bacterial membrane, an incoming individual peptoid molecule does not directly bind to the peptoid-free lipid surface but rather preferentially interacts with growing peptoid oligomers (patches) already assembled on the surface. This creates a significant concentration buildup of the disruptors in the limited surface area resulting in the vesicle disruptions only in those liposomes where a sufficiently large patch of AMPs has been formed hence a graded leakage phenomenon in overall liposome ensembles. Fast growing large peptoid patches built on bacterial membrane eventually lead to lipid bilayer deformation and bacterial lysis. Our novel patch model provides explanation for graded leakage observed for many AMPs, inspiring new direction for antimicrobial drug design.

Chapter 1. Introduction

Antibacterial compound is an agent that inhibits bacterial growth or kills bacteria. The term was first introduced by Selman Waksman in 1942, to describe any substance produced by a microorganism that is antagonistic to the growth of other microorganisms [1]. The discovery and introduction of Penicillin into general clinical use in the 1940s represents one of the landmark advances in modern medicine. Since then, a variety of new antibiotics were launched in the drug market. The second half of the 20th century, in particular the period from 1950 to 1960, was truly the golden age of antibiotic drug discovery. One-half of the drugs commonly used today were discovered in that period [2]. However, due to lack of proper control of antibiotics usage, overwhelming dosage applied or misusing of drugs, emerging bacterial antibiotic resistance issue became urgent clinical and public health concern. Despite the successful development of antibiotics, infectious diseases remain the third-leading cause of death in the United States in 1990s [3] and the second-leading cause of death worldwide [4]. The market size for antibiotics was greater than \$25 billion US dollars per year in 2000s [5]. Since 2005, this market was growing at an annual rate of 6.6% until 2011. The demand of antibiotics is expected to reach USD \$44.68 billion by 2016 [6]. Besides the increasing huge requirement for antibiotic drugs, unfortunately the Food and Drug Administration (FDA) approval of new antibacterial agents decreased dramatically. Comparing year of 1998-2002 versus 1983-1987, there are about 56% decline of new drugs approved; meanwhile, drug resistance increased almost 5-8 folds among various pathogens (Figure 1.1).

A. Increase in drug resistance among various pathogens

B. Decrease of new drugs approved by FDA

Figure 1-1. Increased drug resistance among various common pathogens against reduced new drug release in the market. (A) Growing resistance of selected pathogens against the antibiotic drug, Ciprofloxacin. Similar increase of resistance could be seen against most of the commonly used antibiotics. Figure modified from Clinical Microbiology and Infection, 2004, 10 (s4): 1-9 [7]. (B) The number of new antibiotic drugs approved by FDA since 1983 to 2007, with 5 years interval. Figure is based on Nature Biotechnology, 2006, 24(12):1521-1528 [8].

In recent years, world-wide efforts are focused on developing new classes of antibiotics in order to conquer bacterial drug resistance. Several major drug development methods have been explored, like classical screening through chemical libraries; structural modifications of existing antibiotic agents; genome hunting and new route that targeting non-multiplying, latent bacteria. By using these methods, many antibiotic molecules are patented or in clinical trials. One of the promising classes is the antimicrobial peptides (AMP) and peptoids. In this study, we focus on studying the structural and activity relationship (SAR) of several newly designed antibacterial peptides and peptoids, examine their bacterial killing efficacy and selectivity mechanisms, as well as eventually optimize the patented antibiotic molecules for potential clinical applications.

1.1 Introduction to antibiotics

Infectious diseases are very common human health concern which adversely affecting life qualities. Most of the infections are caused by bacteria, which are broadly categorized into two groups according to cell wall structure differences, namely Gram-positive and -negative.

Gram-positive bacteria possess a thick cell wall constituting mainly peptidoglycans to protect the cytoplasmic membrane. Instead, Gram-negative bacteria use lipopolysaccharides made outer membrane for the same protection purpose. Cytoplasmic membranes of Gram-positive and -negative bacteria are similar but very different from mammalian cells. Bacteria membrane contains high level of negatively charged phosphatidylglycerol (about 30%), while the mammalian cell membrane is neutral containing phosphatidylcholine. The differences in bacterial cell wall structures provide antibiotic susceptibility differences; for instance, vancomycin can kill only Gram-positive bacteria and is

ineffective against Gram-negative pathogens

Bacterial infections could be managed and treated by a group of compounds, known as antibiotics. Antibiotics are loosely classified into several groups according to their bacterial targeting spectrum, route of clinical administration, type of activity as well as chemical structure (Figure 1.2). Broad spectrum antibiotics are effective against a broad range of microorganisms whereas for narrow spectrum antibiotics, either Gram-positive or negative bacteria are selected. Bactericidal antibiotics kill the bacteria like vancomycin, fluoroquinolones in comparison to bacteriostatic antibiotics which inhibit the growth of bacteria, for example tetracycline and chloramphenicol.

In this chapter, the history of antibiotics development, common antibiotics in clinical application as well as the future challenges facing the development of new drugs will be briefly reviewed.

Figure 1-2. A common scheme of antibiotics classification. Figure is shown in a horizontal hierarchical layout and colored according to the hierarchical layers.

1.1.1 Historical view of antibiotics development

The word “antibiotic” came from “antibiosis”, which is a term coined in 1889 by Paul Vuillemin. Antibiosis is a biological interaction between two or more organisms that is detrimental to at least one of them or antagonistic association between an antagonism and the metabolic substances produced by another, in another word, a process by which life could be used to destroy other life.

The history of antibiotics can be traced back to the ancient times. In ancient Egyptians, the Chinese, and Indians of central America all used molds to treat infected wounds. In Serbia, moldy bread was traditionally used to treat wounds and infections. Warm soil was used in Russia by peasants to cure infected wounds. Sumerian doctors gave patients beer soup mixed with turtle shells and snake skins. Babylonian doctors healed the eyes using a mixture of frog bile and sour milk. Sri Lankan army used oil cake (sweetmeat) to serve both as desiccant and antibacterial. Although widely used, people at that time did not understand the connection of the antibacterial properties of mold and the treatment of diseases. In the year of 1640, John Parkinson recommended using mold for disease treatment in his pharmacology book. Systematic research of antibiotic science starts in the modern times, 1880s.

In the late 19th century, germ theory of disease became accepted gradually. It said that some infectious diseases are caused by small organisms, which are too small to be seen without magnification (microorganisms), invading the human body.

In 1890s, procyanase, the first antibiotic used in hospitals were discovered by German doctors Rudolf Emmerich and Oscar Low. Unfortunately, the drug did not work equally well on all the patients as

well as quite toxic to humans, therefore it is no longer used today.

In 1928, Sir Alexander Fleming observed that colonies of *Staphylococcus aureus* could be destroyed by fungus *Penicillium notatum* as well as discovered enzyme lysozyme.

In 1935, German chemist Gerhard Domagk discovered Prontosil, the first sulfonamide drug in the market.

Only in the year of 1942, Howard Florey and Ernst Chain invented the manufacturing process of Penicillin G, Procaine. Penicillin, which is probably one of the most successful drugs in the medicinal history, is widely used nowadays. Fleming, Florey, and Chain were awarded Nobel Prize for medicine in 1945 for their great success on Penicillin.

In 1943, American microbiologist Selman Waksman made the drug Streptomycin, which belongs to a new class called aminoglycosides. Streptomycin was heralded as a cure for disease like tuberculosis. The only drawback is that the side effects were sometimes severe.

In 1950s, tetracyclines, a large family of antibiotics were discovered as natural products. Later tetracycline became the most prescribed broad spectrum antibiotic in the United States.

Since then, glycopeptide, quinolones and fluoroquinolones, cephalosporins and its five related generations were discovered and released into drug market. Antibiotic science advanced rapidly.

1.1.2 Traditional organic antibiotics in the market

Until today, there are many antibiotics available in the pharmacy with different bacterial selectivity. The remarkable success of antimicrobial drugs generated a misconception that infectious diseases would be conquered easily. However, bacteria pathogens typical exhibit high mutation rate. A spontaneous or induced genetic mutation may happen

at a rate of about 1 in 10^8 per chromosomal replication. Random genetic mutations lead to alternation of specific bacterial cellular pathways, for example altered site of antibiotics action, by-passed the inhibited metabolic step, reduced intracellular drug concentration, enzymatically inactivate the drug or overproduction of drug targets [5,9-11]. These will result in the development of bacterial resistance. Abused usage of antibiotics serves as environmental pressure which selects resistance. Those survived bacteria could pass the trait to their offspring through reproduction or spread to other individuals through horizontal genetic transfer via conjugation, transduction, or transformation. In this way, the evolution of a fully resistant colony takes place.

In this section, family of commonly used antibiotics, their target and possible bacterial resistance developed are summarized below (Table 1 and Table 2).

- **Sulfonamides**

The sulfonamide is a chemical group which contains a sulfonyl group connected to an amine. Its general formula is RSO_2NH_2 .

Sulfonamides are the first antibacterial drugs discovered in the early 1930s. As a broad spectrum antibiotic, sulfonamides work well against both Gram-positive and –negative bacteria. Since its clinical usage in 1968, sulfonamides are primarily used to treat urinary tract and upper respiratory tract infections. Due to their relatively tolerated side effects and low costs, sulfonamides remain one of the most popular antibiotics in the world [12].

Sulfonamide is bacteriostatic by inhibiting the synthesis of folic acid. It has a chemical structure very similar to para-aminobenzoic acid (PABA). Sulfonamides work as a competitive inhibitor of enzyme dihydropteroate synthase (DHPS), which catalysis the conversion of PABA to

dihydropteroate. The catalyzed reaction is a key step in folic acid synthesis. Reduced folic acid production will lead to decreased bacterial nucleotides quantity and subsequent reduced bacterial growth [13,14].

Resistance to sulfonamides is wide spread among bacterial strains. Resistance might be developed due to the expression of genes encoding alternative forms of the targeting enzyme DHPS [15], production of DHPS in excess amount which will overwhelm sulfa inhibition, or sometimes resuming cell division by bypassing the folic acid synthesis process namely acquiring exogenous supplies of thymidine [16,17].

- **Penicillins and cephalosporins**

Both penicillin and cephalosporins belong to a large group of antibiotics family, β -lactams. β -lactams are the most widely used group of antibiotics. Up to 2003, more than 50% of all commercially available antibiotics in use are belong to the family [18].

The chemical structure of penicillin contains a five-member thiazolidine ring fused with a β -lactam ring; while in cephalosporin, a six-member sulfur-containing dihydrothiazine ring is used together with the β -lactam ring [18]. Both of them are bactericidal by inhibiting bacterial cell wall synthesis. β -lactam ring has a structure analogous to D-alanyl-D-alanine, which is the terminal group of peptidoglycan subunits. DD-transpeptidases, also called penicillin-binding proteins (PBP) due to the high binding affinity to penicillin, catalyzes cross-linking of peptidoglycan precursors forming rigid bacterial cell wall. β -lactam antibiotics competing with D-alanyl-D-alanine bind to PBP, that results in irreversible inhibition of PBP enzymatic activity and disrupted bacterial cell wall [19].

Bacterial resistance to β -lactam antibiotics has risen through different mechanisms. One of the most common one is the destruction of

the β -lactam ring by enzyme β -lactamase [20,21]. β -lactamases covalently react with the β -lactam ring, rapidly hydrolyze it and remove drug activity. Other resistance pathways have also been seen like (1) the reduced penetration of drugs towards PBP target, achieved by reduced porin channels expression in Gram-negative bacterial lipopolysaccharide (LPS) outer membrane (2) enhanced pump out of drugs from the bacterial periplasmic space, facilitated by the endogenous AcrAB multidrug efflux system in *E.coli* and (3) altered PBP binding site leading to reduced drug binding affinity.

- **Aminoglycosides, tetracyclines, chloramphenicols, macrolides, lincosamides and oxazolidonones**

All of the antibiotics listed in this section belong to a superfamily called Protein Synthesis Inhibitors which are bacteriostatic. They bind to different active sites in bacterial ribosome and prevent successful mRNA transcription.

Aminoglycosides share common structure of amino-modified sugar. A six-membered sugar ring with amino group substitutes is called aminocyclitol. Aminoglycosides are named for the glycosidic bonds between the aminocyclitol and two or more sugars with or without amino group. Binding to A site of bacterial 30S ribosomal subunit by aminoglycosides prevents recognition and binding between mRNA codon to anticodon region on tRNA [22,23]. Clinically, aminoglycosides are used for treatment against aerobic, Gram-negative bacteria.

Tetracyclines are derivatives of polycyclic naphthacene carboxamide, which contain four hydrocarbon rings fusing together. They are broad-spectrum antibiotics inhibiting bacterial growth by reversibly binding to the 30S ribosomal subunit of bacteria [24]. The binding has similar effect as aminoglycosides, blocking the association between

tRNA and mRNA-ribosome complex at the acceptance site.

Chloramphenicols are broad-spectrum antibiotics discovered in 1949. It was the first antibiotic which chemical synthesis was economically and technically practical for large-scale production. Chloramphenicols bind to 23S rRNA of the 50S ribosomal subunit, inhibiting the peptidyl transferase activity and preventing protein chain elongation [25].

Macrolides are named by their nucleus structure, a macrolide ring. The macrolide ring is a large macrocyclic lactone ring with one or more deoxy sugars attached. Macrolides also bind to the 23S rRNA of the 50S ribosomal subunit, near the peptidyl transferase center. Different from chloramphenicols which directly interfere with the tRNA binding, macrolides block polypeptide chain elongation through steric blockage [26].

Lincosamides binds similarly as macrolides and chloramphenicols, to the 50S bacterial ribosomal subunit. They cause premature dissociation of the peptidyl-tRNA from the ribosome [27].

Core chemical element of oxazolidinones is a heterocyclic 5-membered organic ring structure containing both nitrogen and oxygen. Different from other protein synthesis inhibitors which bind to 30S or 50S ribosomal subunit, oxazolidinones bind to the interface between 30S and 50S subunit preventing the formation of 70S complex, therefore inhibit the earliest step of bacterial protein synthesis [28]. Linezolid, approved by FDA in 2000, is one of the latest antibiotic new drugs. It effectively targets most of the Gram-positive bacterial pathogens. Unfortunately, resistance to linezolid has been developed quickly since 2004.

Due to the similarities in bacteriostatic mechanism, protein synthesis inhibitors share common bacterial resistance developed. One of the most

common ones is the alteration of target site, methylations or sometimes point mutations on both 16S rRNA (30S ribosomal subunit) and 23S rRNA (50S ribosomal subunit) have been observed. Altered ribosomal active sites prevent antibiotic drugs binding therefore leading to resistance [29,30]. Combinations of decreased bacterial membrane permeability and active efflux of drugs are also seen in bacterial resistance strains, to eventually decrease the antibiotic drug availability to the target [31,32]. LPS layer in Gram-negative bacteria plays an important role to prevent antibiotic drug penetration [33,34]. Besides that bacteria also develop enzymes which will modify antibiotic compounds which results in inactivation of the drug [35-37].

- **Rifamycins**

Rifamycins are broad-spectrum bactericidal agents particularly active against mycobacteria and Gram-positive organisms. Rifamycin derivatives inhibit bacterial DNA-dependent RNA synthesis by inhibiting the β -subunit of DNA-dependent RNA polymerase, which is highly conserved among prokaryotes [38]. Missense mutations in *rpoB* gene cause amino acid substitutions within rifampin-binding pocket of RNA polymerase, which leads to bacterial resistance developed [39].

- **Quinolones**

Quinolones are synthetic bactericidal drugs. The chemical structures of quinolone family share common features of dual ring configuration with a nitrogen at position 1 and a carbonyl group at position 4.

The quinolones inhibit bacterial DNA synthesis which leads to bacterial cells death rapidly. Inhibition of bacterial DNA synthesis is achieved at several levels. The first recognized quinolones target is DNA gyrase, which introduces negative superhelical twists into bacterial DNA. The enzyme is essential for initiation of DNA replication. Another level of

targeting bacterial DNA synthesis is inhibiting the activities of bacterial topoisomerase IV by quinolones. Topoisomerase IV functions to separate two daughter DNA molecules after the replication cycle is complete. Besides that, quinolones are also found to promote the cleavage of DNA when it is in complex with either of the two targeted enzymes [40,41].

Resistance to quinolones is developed mainly by spontaneous mutations in genes encoding either DNA gyrase or topoisomerase IV [42]. Protection of those two enzymes by bacterial protein has also been observed [43]. Another common resistance development scheme is by overexpression of multidrug resistance (MDR) efflux pumps in both Gram-positive and Gram-negative bacteria [44].

Table 1. Organic antibiotic classes, their chemical structure, representative drugs and antibacterial mechanism.

Antibiotics class	Chemical group	Representative compounds	Mechanism of action
Sulfonamides		Prontosil Sulfisoxazole	Inhibiting folate synthesis
Penicillins		Ampicillin Penicillin G	Inhibit bacterial cell wall synthesis
Cephalosporins		Cefalexin Imipenem	
Aminoglycosides		Streptomycin Kanamycin	Inhibit bacterial protein synthesis by binding to ribosome

(Table 1 continued)

Antibiotics class	Chemistry group	Representative drugs	Action mechanism
Tetracyclines		Tetracycline Doxycycline	Inhibit bacterial protein synthesis by binding to ribosome
Chloramphenicol		Chloramphenicol	
Macrolides		Erythromycin Roxithromycin	
Lincosamides		Lincomycin Clindamycin	
Oxazolidonones		Linezolid Radezolid	
Rifamycins		Rifampicin Rifabutin	Inhibit RNA transcription
Quinolones		Norfloxacin Gatifloxacin	Inhibit DNA replication and transcription

Table 2. Major bacterial resistances developed against common organic antibiotics.

	Enzymatic inactivation	Decreased permeability	Active efflux	Altered target site	Overproduction of the target	Protected target site	Bypass inhibited process
Sulfonamides				<i>su1</i> and <i>su2</i> genes coding alternative forms of DHPS	Production of DHPS in excess amount		Acquire exogenous thymidine
Penicillins Cephalosporin	β -lactamase hydrolysis	Reduced porin channels in Gram-negative bacteria	AcrAB multidrug efflux system in <i>E.coli</i>	Altered PBP binding site			

(Continued Table 2.)

	Enzymatic inactivation	Decreased permeability	Active efflux	Altered target site	Overproduction of the target	Protected target site	Bypass inhibited process
Aminoglycosides	N-acetylation; O-nucleotidylation; O-phosphorylation	Altered proton motive force in inner membrane	Mex XY and Amr AB multidrug efflux pumps	Mutation or enzymatic methylation on 16S rRNA			
Tetracyclines	<i>Tet(X)</i> gene	Reduced porin channels in Gram-negative bacteria	Six class of efflux pumps	Mutation in 16S rRNA (<i>Helicobacter pylori</i>)		Ribosomal protection proteins	
Chloramphenicol	3-O-acetylation by chloramphenicol acetyltransferase	Reduced porin channels in Gram-negative bacteria	Multidrug efflux pumps				

(Continued Table 2.)

	Enzymatic inactivation	Decreased permeability	Active efflux	Altered target site	Overproduction of the target	Protected target site	Bypass inhibited process
Macrolides	Phosphotransferases inactivation; Esterase hydrolysis of lactone ring	Lipopolysaccharide outer membrane in Gram-negative bacteria	<i>Mef</i> , <i>msr</i> and <i>mreA</i> gene for Macrolides efflux pump	Dimethylation on 23S rRNA of 50S ribosomal subunit			
Lincosamides	Adenylation	Lipopolysaccharide outer membrane in Gram-negative bacteria		Dimethylation on 23S rRNA of 50S ribosomal subunit			
Oxazolidinones			Efflux pumps; ABC transporters	Point mutation or methylation in 23S rRNA			

(Continued Table 2.)

	Enzymatic inactivation	Decreased permeability	Active efflux	Altered target site	Overproduction of the target	Protected target site	Bypass inhibited process
Rifampicin				Missense mutations in <i>rpoB</i> gene			
Quinolones		Reduced porin channels in Gram-negative bacteria	MDR efflux pumps	Mutations in either DNA gyrase <i>gyrA</i> or topoisomerase IV <i>parC</i> gene		<i>Qnr</i> gene protect DNA gyrase and topoisomerase IV	

1.1.3 Future challenges for new drug development

As shown in table 1 and 2, majority of the antibiotic drug classes are targeting either bacterial cell wall synthesis or essential metabolic pathway (protein, DNA and RNA production). Unfortunately, resistant bacterial pathogen strains have been observed for most of the common antibiotics. Developing new generation drugs become an important and urgent issue for human health concern. Recently, scientists and biotechnological drug companies worldwide have made large effort in antibiotics discovery project. Large number of promising lead compounds have been identified and patented [8]. Several common methods developed for novel antibiotics discovery include:

- **Classic screening**

Classic screening approach means the screening of large numbers of natural and synthetic compound libraries resulting in the identification of a lead compound [45]. Early antibiotics produced in 1940s and 1950s are all discovered by screening through natural products. Chemical synthesis libraries could also be fundamental sources. Oxazolidinones and ketolides are all raised from synthetic chemical libraries. However, for compounds in chemical libraries, toxicity and solubility might be an issue. Inhibitors shown enzymatic activity *in vitro* often could not achieve comparable effect in bacterial cells.

Through classic screening, some natural peptides are shown to be antibiotic. Natural antibiotic peptides have the advantage of low cytotoxicity and high solubility. However, majority of these natural peptides inherit low efficacy and complex secondary structures including disulfide bonds. Chemical modifications are needed for improved antibiotic performance and large scale synthesis. A combination of

natural-product biosynthesis and solid-phase chemistry is needed.

- **Structural changes to existing drugs**

The short-term response from the pharmaceutical companies to observed bacterial resistance is to focus on structural alteration of existing molecules [46]. Many derivatives of currently available antibiotics have been synthesized and tested for antibiotic activity against drug resistance pathogens. Development of five generations of cephalosporins and fluoroquinolones are successful examples. Structural modification strategy has the advantage of fast hit development, but also exhibits the risk of high likelihood of resistance development.

- **Genome hunting**

Genome hunting approach became possible only recently with the advance in genetic sequencing technologies and many bacterial genomes became available. The process includes (1) identification of a target bacterial gene which is essential for bacteria life; (2) clone gene, express and obtain protein atomic structure; (3) identify protein active sites, screen through small molecule libraries for binding partners; (4) chemical modification of lead molecules and optimize binding; (5) finally test on bacterial cell culture [47,48].

Genome hunting process provides the possibility to discover new antibiotics with novel bacterial targets. One of the promising candidates is antisense nucleic acids, which kill bacteria through inhibiting its gene expression. Bacterial cell wall, as a common protection layer shared by many bacterial species, exhibit low mutation rate due to its important functions. It has been interesting drug target for long time. By combining bacterial genetics research together with structural biology, developing new antibiotics targeting bacterial cell wall become more promising.

- **Targeting non-multiplying bacteria**

Majority of current drugs are targeting fast dividing bacteria by inhibiting metabolic pathway. Another group of bacteria has been paid less attention, the non-multiplying bacteria. Although exist in small numbers, non-multiplying bacteria play an important role in the clinical treatment. The slow metabolic rate provides bacteria tolerance to antibiotic drugs and prolonged treatment easily causes emergence of bacterial resistant strains [49,50]. Designing new antibiotics targeting critical bacterial structure rather than metabolic pathway might be very valuable.

1.2 Introduction to AMPs and their derivatives

After years of study, it was found that some naturally occurring peptides contain antimicrobial activity, termed antimicrobial peptides. Antimicrobial peptides are evolutionarily conserved component for the innate immune response and are found among all classes of life. Majority of these peptides contain broad-spectrum antimicrobial activity, actively kill both Gram-positive and Gram-negative bacteria, even fungus.

Due to the suspicion that AMPs target bacteria membrane rather than specific bacterial molecules, chances to develop resistance may be dramatically decreased [51]. In addition, the cytotoxicity of human AMPs is expected to be low due to their nature origin. With these great advantages, AMPs are expected to be promising agents targeting multi-drug resistant pathogens. In order to brand these AMPs into commercial antibiotic drugs, massive studies have been done to improve the peptides bactericidal activity and reduce sequence complexity. Successful peptides with improved drug properties have entered different stages of clinical trial [52,53].

Antimicrobial peptides are unique and diverse group of molecules.

They are generally cationic, containing 12-50 amino acids in length. Secondary structures of AMPs could be α -helical, β -sheet, β -hairpin/loop or extended structure [54]. In this chapter, popular AMPs in market and under research as well as their proposed antibacterial mechanism are briefly reviewed.

1.2.1 Antibiotic drugs composed of AMP and AMP derivatives

After several years of great effort in antimicrobial peptide research, scientists have developed several antibiotic drugs based on AMP or AMP derivatives. Among all those, three have already been approved and used in clinical medicine; more are still under clinical trial or extensive studies.

- **Glycopeptides (Vancomycin)**

Glycopeptides are antibiotics containing glycosylated cyclic or polycyclic non-ribosomal peptides (Figure 1.3). Their main target is Gram-positive bacteria and vancomycin is one of the most successful drugs.

Figure 1-3. Vancomycin structure. Peptidic bonds are highlighted using rounded rectangle.

Vancomycin forms extensive hydrogen bonds with the terminal D-alanyl-D-alanine group in peptidoglycan precursors. This binding prevents the formation of long peptidoglycan polymers also inhibits the cross-linking of peptidoglycan, therefore avoids the proper cell wall synthesis in Gram-positive bacteria.

Gram-negative bacteria are intrinsically resistant to vancomycin since the LPS formed outer membrane is impermeable to large glycopeptides. Resistant Gram-positive bacterial strains also developed recently by producing altered D-alanyl-D-alanine groups which have much lower affinity for vancomycin binding [55].

- **Lipopeptides (Daptomycin)**

Lipopeptides are natural bacterial expressed molecules, which contain aliphatic lipids connected to peptides (Figure 1.4). The designs of lipopeptide drugs are inspired by the idea of peptides kill bacteria through membrane disruption. Attaching hydrophobic lipid tail to peptides may further facilitate AMPs membrane interaction therefore disruption.

Figure 1-4. Daptomycin structure. Peptidic bonds are highlighted using rounded rectangle.

One of the representative drugs in lipopeptide class is deptomycin, which is used for the treatment of systemic and life-threatening infections caused by Gram-positive pathogens. Deptomycin interacts with phosphatidylglycerol in bacterial membrane at high affinity. It aggregates and inserts into the membrane, causing altered membrane curvature and eventually leakage [56].

Bacterial resistance to deptomycin is rarely reported. Targeting bacterial fundamental structure, the cell membrane, results in low possibility of developing resistance by mutation. The discovery of deptomycin provides new knowledge of AMP antibiotics. Phosphatidylglycerol in bacterial membrane appears to be a good drug target, for both its low mutation rate and high specificity (not present in mammalian cell).

- **Polypeptides (Bacitracin and Polymyxin B)**

Polypeptide antibiotics are a chemically diverse class of antibiotics containing non-protein polypeptide chains (Figure 1.5). Representative antibiotics belong to polypeptide group include bacitracin targeting Gram-positive bacteria and polymyxin B against Gram-negative pathogens.

Figure 1-5. Polymyxin B structure. Peptidic bonds are highlighted using rounded rectangle.

Bacitracin is a mixture of related natural cyclic polypeptides. They interact with isoprenyl pyrophosphate, which carries the peptidoglycan building block for bacterial cell wall [57].

Polymyxin B is a mixture of two similar compounds, polymyxin B1 and polymyxin B2. It is bactericidal against most of the Gram-negative bacteria by interacting with both LPS outer membrane and phosphatidylglycerol decorated cytoplasmic membrane. Positively charged polymyxin B binds to negatively charged LPS through strong electrostatic interactions, inducing phase transitions of the latter. LPS aggregation and phase transitions result in increased bacterial outer membrane permeability. The endotoxin effect, caused by the presence of lipid A (part of LPS) in blood circulation, will also be neutralized [58,59]. Once it reaches the inner membrane, the lipid portion of polymyxin B dissolves into the bilayer through hydrophobic interactions, leading to decreased membrane integrity [60,61].

- **Polymedix PMX-30063**

Vancomycin, daptomycin and polymyxin B are the AMP derivative drugs currently available in the market. There are many more AMPs or AMP derivative compounds patented recently. PMX-30063 from PolyMedix Inc. is one of the most promising antibiotic candidates.

Figure 1-6. Structure of PMX-30063. Peptidic bonds are highlighted using rounded rectangle.

PolyMedix a clinical stage biotechnology company developing first-in-class, small molecule drugs for the treatment of serious acute care conditions. Their lead compound PMX-30063 is designed for the treatment of acute bacterial skin and skin structure infections caused by *Staphylococcus aureus*. PMX-30063 is a new molecule which belongs to a novel antibiotic class—host defense protein mimetics (Figure 1.6). Host defense proteins (HDP) function as the first line of defense in host innate immunity against microbe infections. They are evolutionary conserved and could be found in almost all life forms including both eukaryotes and prokaryotes. They are cationic charged amphiphilic proteins targeting bacterial membrane through electrostatic interactions. Although PMX-30063 kills bacteria through membrane disruption, similar as HDPs, its chemical structure is much simpler hence dramatically reduced its production cost. It shows promising antibiotic activity against both Gram-positive and Gram-negative bacteria including drug resistant strains. Since after millions of years of evolution, no widespread resistance to HDPs has been observed, the resistance to HDP mimetic molecules is unlikely to evolve as well. So far, no bacterial resistance has been developed from serial passages of bacteria with PMX-30063 treatment.

PolyMedix announced exciting clinical phase II trial results for PMX-30063 in 2012 and name it as Brilacidin.

- **Lytic Biopharma, LTX-109**

LTX-109 is a peptidomimetic antimicrobial drug developed by Lytic Biopharma (Figure 1.7). Currently it is in clinical trial stage for skin infections and nasal decolonization of MRSA.

Figure 1-7. Structure of LTX-109. Peptidic bonds are highlighted using rounded rectangle.

LTX-109 has demonstrated broad spectrum antimicrobial activity against Gram-positive/-negative bacteria as well as a range of yeast and fungal species. The compound is equally effective against antibiotic resistant species such as methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Enterococci* (VRE) and multi-resistant *Pseudomonas* isolates. The ultra-rapid membrane lysing action of LTX-109 leads to fast bacteria killing effect therefore reduced drug dosage. As a result, a lower propensity to develop bacterial drug resistance could be expected. To date LTX-109 demonstrates no in vitro cross-resistance with other classes of antibiotics.

- **Genaera Crop., MSI-78**

MSI-78, also known as Pexiganon, is a magainin analogous peptide designed by the biotech company Genaera Corporation. It was used in clinical phase III trial against diabetic foot ulcers caused by bacterial infections [62]. Biophysical studies showed that MSI-78 forms amphipathic α -helical structure in solution (Figure 1.8) and it kills bacteria through membrane disruptions.

In lipid micelles, MSI-78 helices were observed to form antiparallel dimers held by phenylalanine-phenylalanine interactions [63].

Self-association of MSI-78 helps it to create positive curvature strains inside of bilayer membranes, thus membrane disruption. Oligomerization of MSI-78 is critical for its toroidal-type bacteria killing mechanism.

Figure 1-8. Structure of MSI-78. 3D structure of MSI-78 is generated using I-Tasser web-based protein structure prediction service [64]. Backbone of the polypeptide chain is shown in ribbon. Heavy side chains of cationic amino acid residues are highlighted in green lines while the hydrophobic ones are labeled in blue.

- **LL-37**

In mammals, the two main families of neutrophil producing antimicrobial peptides are cathelicidins and defensins. They are important effector molecules for innate immunity. LL-37 is the only human cathelicidin has been found so far. The peptide is stored in neutrophil granules in an inactive form and processed extracellularly to the active peptide LL-37 and the propeptide cathelin [65].

LL-37 is an amphipathic, alpha-helical peptide (Figure 1.9). It lays parallel to the bacterial surface and creates positive curvature strains in the bilayer membrane. Toroidal pore mechanism for lipid bilayer disruption is proposed for LL-37 [66]. Unfortunately clinical trials for LL-37 as antibiotics have been hampered by the indications that LL-37 might be toxic for mammalian cells and its antimicrobial activity could be

inhibited by serum. Further modifications of the peptide is needed. LL-37 derived peptide with removed N-terminal hydrophobic amino acids shows decreased cytotoxicity, which might be promising for future drug design [67].

Figure 1-9. Structure of LL-37. Figure is prepared using PDB entry Human LL-37 structure, number 2K6O [68]. Four selected conformers are shown in assemble. Backbone of the polypeptide chain is shown in ribbon. Heavy side chains of cationic amino acid residues are highlighted in green lines while the hydrophobic ones are labeled in blue.

1.2.2 Natural AMPs under extensive research

Antimicrobial peptides were discovered in early 1980s through the identification of cecropin and magainin. To date, thousands of peptides from different natural origin have been identified with bactericidal property. The antimicrobial peptide database (APD) contains 2239 antimicrobial peptides [69,70]. Among them, there are 192 bacteriocin, 294 plant AMPs and 1698 peptides from animal host innate immunity. Besides majority of them kill bacteria through targeting bacterial membranes, their structures are very diverse. Several AMPs and their structures are briefly reviewed.

- **Cecropin**

Cecropin was discovered by Boman's group from silk moths (*Hyalophora cecropia*) [71]. From whence more peptides belong to cecropin family were found from other insects and named as bactericidin, lepisopteran, sarcotoxin etc. Cecropins are small proteins about 31-37 amino acids residues in length targeting both Gram-positive and -negative bacteria. Its structure consists of mainly alpha-helices (Figure 1.10). In vesicles, cecropins form ion channels at low peptide to lipid ratios and large pores are formed at high peptide to lipid ratio allowing probe molecules passing through [72]. When approaching bacterial membrane, cecropins fold into a more compact structure and orient its helical axis parallel to the membrane surface [73]. A "carpet-like" model was proposed that several cecropin monomers bind to membrane in a cooperative manner leading to increased membrane permeability.

Figure 1-10. Structure of cecropin. 3D structure of cecropin A is simulated using I-Tasser [64]. Backbone of the polypeptide chain is shown in ribbon. Heavy side chains of cationic amino acid residues are highlighted in green lines while the hydrophobic ones are labeled in blue.

- **Magainin**

Magainins are a class of antimicrobial peptides found in the African claw footed frog *Xenopus laevis*. They were first reported by Dr Michael Zasloff in 1987 [74]. Magainins are short peptides forming alpha-helices in dodecylphosphocholine (DPC) micelles (Figure 1.11). Structural studies relieved that magainin forms selective ion channels for monovalent cations at critical peptide to lipid ratio of 5:1 [75]. Hence, a toroidal pore model was proposed.

Figure 1-11. Structure of magainin 2. Figure is prepared using PDB entry magainin 2 in DPC micelles, number 2MAG [76]. Ten selected conformers are shown in assemble. Structure of magainin 2 is determined using solution state NMR. Backbone of the polypeptide chain is shown in ribbon. Heavy side chains of cationic amino acid residues are highlighted in green lines while the hydrophobic ones are labeled in blue.

- **Tachyplesin**

Tachyplesin was a cationic peptide isolated from acid extracts of horsehoe crab (*Tachypleus tridentatus*) hemocyte debris [77]. It consists of 17 residues forming beta-sheet structure (Figure 1.12). Tachyplesin showed broad spectrum bactericidal activity against both Gram-positive

and –negative bacteria. It permeabilizes the inner membrane and forms complex with outer membrane lipopolysaccharides in case of Gram-negative bacteria [78].

Figure 1-12. Structure of tachyplesin. Figure is prepared using PDB entry tachyplesin I in DPC micelles, number 1MA5. Structure of tachyplesin I is determined by solution NMR. Backbone of the polypeptide chain is shown in ribbon. Beta-sheet structures are highlighted in cyan and disulfide bond are labeled in orange. Heavy side chains of cationic amino acid residues are highlighted in green lines while the hydrophobic ones are labeled in blue.

- **Defensin**

Defensins are 3-5 kDa cysteine-rich cationic peptides found in both vertebrates and invertebrates [79,80]. They function as host defense peptides through electrostatic interactions with the bacterial membrane, leading to permeability changes or pore formation [81,82]. Defensins are coded by polymorphic genes and based on their expression patterns, defensins are broadly categorized into three classes, α -, β - and θ . α -defensins are primarily expressed in neutrophils while θ -defensins are

rarely found in some leukocytes. β -defensins are most widely distributed in leukocytes and epithelial cells. It plays an important role in human innate immunity. Studies have shown that the antimicrobial activity of mammalian β -defensins mainly depend on the structure and charge distribution (Figure 1.13) but not the primary sequences [83].

Figure 1-13. Structure of human β -defensin 3. Figure is prepared using PDB entry of NMR structure of human β -defensin 3 in solution, number 1KJ6 [84]. For simplicity, one selected conformer instead of 20 is shown. Backbone of the polypeptide chain is shown in ribbon. Beta-sheet structures are highlighted in cyan and disulfide bond are labeled in orange. Heavy side chains of cationic amino acid residues are highlighted in green lines while the hydrophobic ones are labeled in blue.

- **Indolicidin**

Indolicidin is a cationic antimicrobial peptide with unusual high content of tryptophan and proline in its sequence. Within its 13 amino acid residue sequence, there are five tryptophans and three prolines in the middle. Structural studies showed that indolicidin forms extended loop on

membrane-water interface (Figure 1.14), with Trp residues buried into lipid membrane [85]. Insertion of the peptide hydrophobic core into lipid bilayer suggested formation of channel aggregates thus membrane leakage [86]. It was also proposed to kill bacterial through alternative pathways rather than membrane disruption, inhibiting DNA synthesis of *Escherichia coli* [87].

Two antimicrobial peptides drug candidates have been developed based on indolicidin, MX-226 and MX-594AN. MX-226 is a topical antibiotic designed for the prevention of catheter-related infections. In 2005, a confirmatory phase III clinical trial was initiated by Mignenix Inc. MX-594AN is developed for the treatment of acne vulgaris. It was licensed to Cutanea Life Sciences in late 2005.

Figure 1-14. Structure of indolicidin. Figure is prepared using PDB entry indolicidin in DPC micelles, number 1G89 [86]. Structure of indolicidin is determined by solution NMR. 16 selected conformers are shown. Backbone of the polypeptide chain is shown in ribbon. Tryptophan and prolines are labeled in orange. Heavy side chains of cationic amino acid residues are highlighted in green lines while the hydrophobic ones are labeled in blue.

1.2.3 Peptides and Peptoids used in this study

Extensive studies have been done to develop synthetic AMPs into therapeutics. Three major research approaches could be distinguished, namely template-based studies [88], biophysical modeling [89] and virtual screening [90]. Template based studies means modification of known AMP sequences (template) by amino acid substitutions seeking for peptides possessing greater antimicrobial activity or reduced toxicity. The modifications are usually local and the effect of a particular residue substitution is context-dependent. Rigorous biophysical modeling method aims to understand AMP activity and design improved variants by examining peptide structures at atomic level. Choosing proper experimental condition mimicking bacterial membrane is essential. Virtual screening studies [91,92] require less experimental input comparing to biophysical modeling. It provides quantifiable properties of peptides rather than immediate interpretable models. Screening might be limited by computational power if accurate and detailed analyses are needed. In our studies, we explored a combination of all three major AMP development approaches. A large scale dynamic collaboration was built based on multi-group collaborations. AMP design and synthesis was carried out by the organic chemistry group from our collaborator in Singapore Eye Research Institute (SERI), their bactericidal activities and toxicity were tested by the microbiology group also from SERI. Biophysical modeling studies was done in house mainly using NMR spectroscopy method and virtual screenings of AMP-bacterial membrane interactions were simulated by molecular dynamics simulation group from Bioinformatics Institute, A*STAR, Singapore. Two kinds of molecules, defensin analogous peptides targeting Gram-negative

bacteria and alpha-mangostin derivative peptoids against Gram-positive bacteria, were utilized to study AMP bactericidal mechanism as well as discover functional motifs for new generation antibiotics development.

Studies have shown that some linear analogs of human β -defensin 3 (full length) exhibited higher antimicrobial activity while lower cytotoxicity than the native protein [93]. In this study, we demonstrate that three newly designed defensin C-terminal (R36-K45) analogous peptides, C2, Y2, and F2, all displayed minimal cytotoxicity but very different bactericidal efficacies (Figure 1.15). Their 3D structures and bacterial membrane interaction mechanism were studied using NMR spectroscopy and MD simulations.

hBD-3:	RGRKCCRRKK
C2:	RGRKC(Acm)C(Acm)RRKK
F2:	RGRKFFRRKK
Y2:	RGRKYYRRKK

Figure 1-15. Peptide sequences of human β -defensin-3 C-terminal analogous peptides. C2, F2 and Y2 peptides are aligned with original hBD-3 peptide sequence with the substituting amino acids highlighted in green. AcM means acetamidomethyl group ($-\text{CH}_2\text{-NH-CO-CH}_3$) added on Cysteine side chain, to prevent the formation of disulfide bond.

Non-natural multivalent AMPs have been designed by conjugating copies of a peptide monomer to scaffold molecules via naturally occurring intermolecular disulfide bridges or unnatural scaffold linkers [94]. Branched peptides have been shown to have considerable advantages over their monomeric forms, such as maintaining high efficacy under physiological (high salt) conditions [95,96], decreased susceptibility to proteolytic degradation [97], improved antimicrobial

activity [98], and low cytotoxicity [99]. However, the quantitative structure-activity relationships of branched antimicrobial peptides are still poorly understood at the atomic level. Structural studies of C2, Y2 and F2 monomeric peptides showed that these peptides dimerize in the lipid phase or in aqueous solution in a cooperative manner at concentrations corresponding to their minimal inhibitory concentrations (MICs) [100]. A V2 monomer peptide, which had promising activity and was very amenable to large scale production, was chosen as a functional motif to build a covalent dimer peptide. To maintain consistency in the peptide series nomenclature, we identified V2 dimer peptide as B2088 (Figure 1.16).

Comparing with a variety of its analogous peptides and the commonly used antibiotic drug gentamicin, B2088 showed outstanding antimicrobial activity specifically against Gram-negative bacteria. More strikingly, no resistance developed after 15 bacterial serial passages. Neither a cytotoxic effect nor hemolysis was found at a peptide concentration of 2 mg/ml when tested against human conjunctiva epithelial cells. A patent have been issued for the designing of B2088 as a lead compound against common health pathogen *Pseudomonas aeruginosa*. In an attempt to understand both the mechanism of action and the basis of cytotoxicity, spatial structure/dynamics and mobility of the branched peptide, B2088, in various bacterial membrane mimicking media, were studied using environment-sensitive fluorescent probes, electron microscopy, high resolution magic angle spinning (HR-MAS), solution NMR, and molecular dynamics simulations.

Figure 1-16. Primary structure of V2 monomer peptide (A) and B2088 (B). Positively charged amino acids are highlighted in red. Several amino acids are ^{13}C , ^{15}N isotopically labeled for structural determinations by NMR. Those labeled residues are underlined. Branched peptide is built using lysine 9 ϵ NH group. The Lys 9 linker is in blue color.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is an emergent form of *S. aureus* [101,102], which confers resistance to β -lactam antibiotics (oxacillin and ampicillin) [103], vancomycin [104], fluoroquinolones [105], linezolid [106], and daptomycin [107]. It is a predominant source of infections associated with the blood, skin and soft-tissue presenting in US emergency rooms [108,109]. The reduced antibiotic susceptibility is widely associated with the failure of treatment, longer hospital stays and greater health care costs [110]. An urgent and critical need for new therapeutic antibiotics against MRSA has been developed.

Mangosteen (*Garcinia mangostana*) is a tropical evergreen fruit tree from South East Asia. It has a long history of use in the treatment of chronic diarrhea and skin infections [111]. The major bioactive extract, α -Mangostin, has demonstrated potent bactericidal activities against various Gram-positive bacteria including *S. aureus* and MRSA [112,113].

However, lacking of adequate selectivity hindered it from possible therapeutic applications. The strong hydrophobic interactions between the xanthone scaffold and phospholipid membranes as well as the very weak electrostatic attraction towards ionic charged lipids in bacterial membrane lead to the deficiency in the differential disruptions induced by α -Mangostin in mammalian and bacterial membranes.

Although many of the synthetic amphiphilic antimicrobial molecules demonstrate good antibacterial activity, there are several major concerns for using them as drug candidates: the high production cost of peptide synthesis comparing to organic compounds; possible enzymatic hydrolysis of the peptidic bonds leading to decreased AMPs activity; and systematic toxicity caused by AMP or its secondary metabolites. With these considerations, a peptoid molecule, AM052, based on modifying α -Mangostin structures has been developed against MRSA. Two arginines have been incorporated into α -mangostin resulting in the xanthone core flanked by cationic charged arginines (Figure 1.17). Biological studies showed that AM052 has much improved selectivity with very low MIC against MRSA but very high biocompatibility. In attempt to understand the bactericidal mechanism against Gram-positive bacteria, the structure, dynamics and oligomerization state of AM052 in both aqueous solution as well as lipid environment were studied using solution state NMR. The membrane insertion of AM052 and subsequent graded leakage of membranes were monitored by imaging, biophysical method and solution NMR.

Figure 1-17. Structure of AM052. Chemical structure of α -mangosin derivative AM052 is shown. All the protons are numbered. Two Arginine residues are labeled as amino acid nomenclature.

1.2.4 Proposed AMP bactericidal mechanisms

After years of study, verities of peptides possessing anti-microbial functions are found [114]. These peptides could be from nature host (animal or plants) immune system or the analogous peptides, synthetic peptide from combinatorial library, or even from some organic engineered product containing unnatural amino acids [115]. The promising activity of AMPs attracts interest to elucidate their bacterial killing mechanism using both biochemical and biophysical methods. So far, a phenomenological model, Shai-Matsuzaki-Huang (SMH) model, which provides a broad picture of this process has been widely accepted [116-118]. The model emphasizes the importance of AMP induced bacterial membrane disruption via electrostatic interaction. This also explains AMP selectivity since AMP-bacterial membrane interaction is driven by strong electrostatic plus hydrophobic interactions while AMP-mammalian cell membrane interaction is favored by weak hydrophobic interactions only. Provided these understandings, more efforts have been done to study the detail picture of AMP-membrane

interaction. According to AMP membrane insertion structures, a number of models have been proposed.

- **Carpet model**

Carpet model proposes monomeric antimicrobial peptides binds to membrane surface through electrostatic interactions, after that carpet on surface of membrane. Membrane disruption will only occur once high density antimicrobial peptide clusters are formed. A good example of AMPs killing bacteria through carpet model will be cecropin P1 [119].

- **Barrel-stave model**

Different from carpet model which requires a large number of AMPs carpet on the membrane surface, barrel-stave model involves perpendicular insertion and aggregation of a relatively small number of individual peptide, also referred as staves, in a barrel-like ring structure inside the membrane leading to a trans-membrane pore or channel formation [120]. AMPs conformational phase transition occurs through hydrophobic interactions between monomers [121].

Barrel-stave model requires a special class of AMPs which are long enough to transverse through bilayer lipid membrane. For alpha helical peptides minimal length of 22 amino acids is necessary and for beta sheet AMP, at least 8 amino acids in beta sheet region is needed. Alamethicin (Alm), which forms channels in bacterial membrane acting through barrel-stave model [122].

- **Toroidal pore model**

While barrel-stave model emphasizing the channel formation by oligomer of antimicrobial peptides, toroidal pore model suggests the membrane pore created by peptide-lipid interaction [123]. The presence of negatively charged phospholipids in pore-lining reduces the repulsive interactions between antimicrobial peptides developed by their high

positive charges. Peptide and lipid together form well-defined pores and local membrane exhibits positive curvature. In this model, not only peptide concentration but actually lipid-to-peptide ratio determines the AMP effective concentration. Many well studied antimicrobial peptides are categorized into membrane disruption through toroidal pore model, like magainin 2 (MSI-78) [124], mellitin and LL-37 [66].

- **Interfacial activity model**

Recently, a new model of peptide bacteria killing mechanism has been proposed, “interfacial activity model”. The model has been defined as “the ability of a molecule to bind to a membrane, partition into the membrane-water interface, and alter the packing and organization of the lipids” [125,126]. In these papers, people showed that the membrane permeation is the common lethal step for cationic antimicrobial peptides. Based on the observation that antimicrobial peptides exhibit a large variety of structures, Wimley proposed that not a particular secondary structure (e.g. alpha-helical or beta-sheet) but the overall biophysical properties of AMPs (e.g. amphiphilicity, isoelectric point or charge distribution) are critical for AMP bacterial killing activity, and the well-defined membrane local structure may not be necessary [125].

- **Intracellular target, alternative pathways**

All the models mentioned above predict that AMPs kill bacteria through membrane integrity perturbation. However, intracellular targets of AMPs could not be ruled out. Gramicidin S causes membrane depolarization at a concentration much lower than its minimal inhibitory concentrations [127]; buforin II does not permeabilize the cytoplasmic membrane but penetrates it and accumulates in the cytoplasm [128]. These observations indicate that membrane depolarization might not be enough to kill bacteria in the case of some AMPs [129]. An aggregated-pore

model is proposed for AMP translocate into the membrane [130]. The aggregated-pore model says that “peptides insert into the membrane and then cluster into unstructured aggregates allowing the dynamic formation of pores that span the membrane for short peptides” [131]. Dynamic membrane pores could help AMPs to enter bacteria and therefore access to their intracellular target.

Although various AMP bacterial killing models have been suggested, there are still many questions remains to be answered. One of them would be failure to predict the activity of AMP analogous peptide. From combinatorial library screening and our peptide synthesis experience, peptide with very similar sequence could perform very different activity [125,132]. It seems hard to rationalize the role of individual amino acids. Recent literatures suggest that antimicrobial activity is not dependent on specific amino acid sequences [115,133-136]. The activity of designed antimicrobial peptides depend more on its structure. Research showed that replacing one L-Leucine residues by D-Leucine causes severe modulation of peptide activity, changed from broad-spectrum bacterial targeting to only active against limited organisms [125]. Changing from L- to D-amino acids will not alter peptide charge, hydrophobicity, or its length. The only significant change will be peptide structure. This finding shows that not only local secondary structure but the overall 3D fold is important. We explored 3D NMR structures of several AMPs in various bacterial membrane-mimicking media. Structure and dynamics of individual AMP molecule as well as the oligomerization states of AMPs are found to be crucial for bacterial killing activity.

Chapter 2. Materials and Methods

2.1 Materials

2.1.1 Peptide selection and synthesis

A series of human β -defensin-3 (hBD-3) C-terminal (R36-K45) analogues were produced using solid phase peptide synthesis (SPPS), via either chemical modification of two cysteine residues in the original sequence or substitution of them with other amino acids. The series of hBD-3 C-terminal (R36-K45) analogues was derived and named by the two amino acids in the center of the sequences, at the two Cys positions. HPLC and ESI-MS were subsequently performed for peptide purification and identification. Following antibacterial and cytotoxicity assays, C2, F2, and Y2 peptides were selected for structural analyses. The original hBD-3 C-terminal (R36-K45) sequence is RGRKCCRRKK. The C2 sequence is RGRKC(Acm)C(Acm)RRKK (Acm, acetamidomethyl group). The F2 sequence is RGRKFFRRKK. The Y2 sequence is RGRKYYRRKK.

B2088, its analogous variants, and V2 monomer peptide were produced using the solid phase peptide synthesis method. HPLC and electrospray ionization-MS were subsequently performed for peptide purification and identification (29). The sequences were (RGRKVVRR)₂KK for B2088 and RGRKVVRRKK for the monomer, respectively. The secondary chain of B2088 was formed through the Lys9 ϵ -NH-group on the primary chain. The underlined amino acids were ¹⁵N- and ¹³C-double labeled. Lyophilized B2088 powder (10.3 mg) was

dissolved in 219 μ l of Milli-Q water, resulting in 20 mM peptide stock. Lyophilized monomer powder (12.5 mg) was dissolved in 478.3 μ l of Milli-Q water, resulting in 20 mM stock.

α -Mangostin (purified of 99.4% HPLC) was purchased from Chengdu Biopurify Phytochemicals Ltd., Chengdu, China. Amino acid Arginine (H-Arg-OMe.2HCl), and all the other reagents and solvents for chemical synthesis reactions were purchased from Sigma Aldrich. Thin layer chromatograph analyses were carried out with Merck TLC aluminium-backed sheets (silica gel 60 F₂₅₄) to monitor the complete of reaction; final product was purified via HPLC. AM052 in light yellow oil form (12.3 mg) was dissolved in 1.415 ml of Milli-Q water, resulting in 10 mM stock solution for NMR experiments. ¹H NMR (400 MHz, MeOD) δ 6.89 (s, 1H, Ar-H), 6.42 (s, 1H, Ar-H), 5.27-5.20 (m, 2H, 2 $\times$ CH), 4.78 (s, 2H, CH₂), 4.73 (s, 2H, CH₂), 4.61-4.57 (m, 2H, 2 $\times$ CH), 4.11 (d, J=6.4Hz, 2H, CH₂), 3.86 (s, 3H, OCH₃), 3.75 (s, 6H, 2 $\times$ CH₃), 3.40 (d, J=7.4Hz, 2H, CH₂), 3.23-3.17 (m, 4H, 2 $\times$ CH₂), 2.02-1.96 (m, 2H, CH₂), 1.85-1.80 (m, 8H, 2 $\times$ CH₃, CH₂), 1.69-1.62 (m, 10H, 2 $\times$ CH₃, 2 $\times$ CH₂).

Chemical synthesis of various peptides and peptidic mimetics were done by our collaborator in Singapore Eye Research Institute (SERI), Dr Liu Shouping group.

2.1.2 Bacterial culture used for antimicrobial activity, bactericidal kinetics and resistance assay

Bacteria were no more than five passages removed from the original master lot obtained from the American type Culture Collection (ATCC) or clinical isolates.

For AMP antimicrobial activity assay, bacterial cells were grown in Mueller Hinton broth overnight. An aliquot of 1 ml of adjusted inoculum in Mueller Hinton broth was added to 1 ml of each dilution of tested antibiotics to yield a final count of 10^5 to 10^6 cfu/ml bacteria in each tube. The tubes were incubated at 35 °C for 16–20 h and the cloning forming unit (cfu) of surviving bacteria was subsequently counted. A positive control tube containing only the broth and bacteria and a negative control tube containing only the broth were incubated. High salt conditions, 0.9% (w/v) sodium chloride buffer and 6 hours incubation, were also tested on selected defensin analogous peptides.

In AMP bactericidal kinetics assay, standard and gentamicin resistant strains of *P. aeruginosa* (ATCC 9027 and DR 4877/07), standard and clinical isolate stains of Methicillin-resistant *Staphylococcus aureus* (ATCC 43300 and DR 6506) were grown separately on tryptic soy agar plates at 35 °C for 24 h. The colonies were then picked from the plate and re-suspended in United States Pharmacopia (USP) phosphate buffer (pH 7.2). The suspension was adjusted to $10^6/10^7$ cfu/ml with various concentrations of B2088 or AM052 (2.75, 5.5, 11, and 22 μ M) in separate tubes and incubated at 35 °C. Aliquots of 100 μ l samples were withdrawn at 10-, 20-, 30-, and 60-min intervals and plated on tryptic soy agar plates after serial dilution using the same buffer. Simultaneously, the bacteria in USP phosphate buffer without peptide addition was used as a control and plated in a similar fashion. After 48 h, all of the plates were checked, and the colonies were calculated to give the cfu/ml. A time kill curve was plotted with time against the logarithm of the viable count.

AMP resistance assay was conducted in three different sets, one each for B2088, gentamicin, and norfloxacin. The test was initiated by

growing the reference bacteria strain of *P. aeruginosa* (ATCC 27853) in the presence of various concentrations of B2088, gentamicin, or norfloxacin that encompassed their MICs. After 20 h of incubation at 35 °C, the bacteria that showed growth in the highest concentration was re-passaged in a fresh dilution series of B2088 or other antibiotics. The process was repeated every 20 h for up to 18 passages, and the MICs were determined at every passage as specified earlier.

2.1.3 Mammalian cells used for cytotoxicity and hemolysis assay

Cytotoxicity was determined by measuring the amount of ATP generated by viable cells in culture using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI). AMPs were incubated with a human conjunctiva epithelial cell line (IOBANHC) as described previously [137]. The luminescent signal was read by a microplate reader (Tecan GeniosPro, Tecan Asia, Singapore). Blank wells containing only medium were included in each assay, and the reading was subtracted from each sample and control well.

For AMP hemolysis assay, fresh rabbit red blood cells were isolated from the whole blood of New Zealand White rabbits, by centrifugation at 3000 rpm for 10 min. Red blood cells were washed with sterile PBS and diluted to 8% (v/v) stock solution in sterile PBS. AMPs were dissolved in and mixed with red blood cells to prepare the desired concentration (final v/v = 4%). The mixtures were added to 96-well plates and incubated at 37 °C for 60 min. After incubation, the mixtures were transferred into 1.5 ml Eppendorf tubes and centrifuged at 3000 rpm for 3 min. The supernatant (100 µl) was transferred into a clean 96-well plate, and the amount of hemoglobin released was determined by measuring the

absorbance at 576 nm with a TECAN Infinite 200 microplate reader. 2% Triton X-100 and PBS were used as positive and negative control, respectively.

2.1.4 Samples for microscopic imaging

To probe the damaging effect of AMPs on bacterial cells, *S. aureus* DM 4001 was grown on TSA Plates overnight and a suspension of 10^8 cfu/ml was prepared in United States Pharmacopeia Buffer (USP Phosphate buffer). The suspension was centrifuged at 3000 rpm for 20 min and the pellet was washed with the same buffer. An aliquot of the bacterial suspension was incubated with 10 µg/ml of AM052 at 35 °C for 30 min. After incubation, the bacterial suspension was again centrifuged, washed and prefixed in 0.5 ml of a mixed aldehyde fixative (2% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.2) for 24 h. Then the suspension was washed once again in sodium cacodylate buffer (Electron Microscopy Sciences, Washington, USA) and subsequently mounted on poly-L-Lysine coated cover-slips and post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences). Following dehydration in a graded series of ethanol, the samples were critical-point-dried and sputter coated with 10 nm of gold. All samples were viewed and photographed on a Philips XL30-FEG-SEM (FEI Electron Optics BV, Eindhoven, The Netherlands) at an accelerating voltage of 10 kV at the Electron Microscopy Unit, National University of Singapore. Bacteria incubated in USP phosphate buffer without the addition of AM052 was used as a control and processed in the same manner and photographed. Following the same procedure, the destructive effect of B2088 on *P. aeruginosa* was recorded.

A suspension of LPS (extracted from *P. aeruginosa* 10, purchased from Sigma, product L8643) (1 mg/ml) was incubated with B2088 (70 μ M) for 2.5 h. A 1 μ l aliquot of this solution was placed on Formvar-coated copper grids, stained with 1% uranyl acetate, dried, and imaged by a JEOL 100 TEM machine. The same LPS suspension sample without B2088 treatment was used as a control.

A suspension of DOPE-DOPG mixed liposomes (DOPE:DOPG = 3:1, total 30mM) was incubated with AM052 (50 μ M) for 2.5 h. A 10 μ l aliquot of this solution was placed on Formvar-coated copper grids, dried, and imaged by a JEOL 2010 TEM machine operating at 200kV. The same liposome suspension without AM052 treatment was used as a control.

Nano gold particles of 10 nm size (Sigma-Aldrich, Singapore) were covalently linked to streptavidin (Invitrogen, Singapore) and then conjugated with a 1:1 ratio of N-terminal biotinylated B2088. Nano gold particles coated B2088 was mixed with 100 μ L of *P. aeruginosa* for 30 mins. Subsequently the sample was washed and fixed onto Formvar copper grids. A standard TEM sample protocol was performed. Nano gold with nonbiotinylated B2088 was used as a control.

2.1.5 Fluorescent dyes for fluorescence emission studies

Three kinds of fluorescent dyes were used for fluorescence emission assays.

B2088 induced outer membrane permeabilization of Gram-negative bacteria was studied using an *N*-phenyl-1-naphthylamine (NPN) assay as reported by Loh *et al.* [138]. *P. aeruginosa* ATCC 9027 cultured overnight was harvested (3000 rpm, 4 °C). The pellet was washed twice using 5mM HEPES buffer (pH 7.2) and re-suspended in the same buffer

to an A_{600} nm of 0.4. Bacteria were placed in a 10 mm stirred cuvette, and NPN was added to a final concentration of 10 μ M. Appropriate concentrations of B2088 were added, and the increase in fluorescence intensity was monitored on a Quanta Master fluorescence spectrophotometer (Photon Technology International). The excitation and emission wavelengths were set at 350 and 410 nm with slit widths at 2 and 5 nm, respectively.

Live bacteria were used for the depolarization assay of transmembrane potential. Late log phase *P. aeruginosa* ATCC 9027 bacteria were harvested by centrifugation and washed twice with 5 mM HEPES buffer, subsequently re-suspended in the same buffer (A_{600} = 0.4). The membrane-sensitive fluorescence probe 3,3'-dipropylthiadicarbocyanine iodide (diSC3) [139] was added to the bacterial suspension to a concentration of 0.1 μ M and incubated at room temperature for 1 h. The fluorescence emission spectrum was recorded by monitoring the changes in emission intensity at 670 nm (at an excitation wavelength of 622 nm) until no further changes were seen in the intensity values. Time-dependent changes in emission intensities at 670 nm were recorded after adding various concentrations of B2088.

Membrane-permeabilizing activity of AM052 was estimated by the extent of calcein leakage as reported previously [140]. Firstly, lipids with DOPE/DOPG= 80/20 were dissolved in methanol/chloroform (1:2, by volume) and was dried gently under a constant stream of nitrogen gas. The lipid film was then further dried by placing the film under a vacuum for at least 2 hours. The dry film was hydrated with calcein solution (80 mM calcein, 50 mM HEPES, 100 mM NaCl, 0.3 mM EDTA, pH 7.4) to obtain the lipid concentration of 30 mM. Then, the hydrated lipids were freeze-thawed in liquid nitrogen and warm water for 7 cycles. Extrusion

method was used to obtain 100 nm homogeneous LUVs. Column chromatography with Sephadex G-50 was used to separate the calcein-loaded LUVs from the free calcein. Total phosphorus determination assay was used to estimate the concentration of the purified calcein loaded LUVs. AM052 was dissolved in millipore water and added into an aliquot of LUVs to obtain the desired compounds to lipid ratios of 1/2, 1/4 and 1/8, 1/16, 1/32, 1/64, 1/128 and 1/256. The final concentration of lipid was 50 μ M. The release of calcein from the LUVs was fluorometrically monitored using a Photon Technology International Model 814 fluorescence spectrophotometer at an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

2.1.6 Bacterial and mammalian cell membrane mimicking media

Various lipidic media were used to study AMP-membrane interactions. LPS were chosen as a representative for the outer membrane in Gram-negative bacteria, and phospholipid micelles, bicelles and liposomes with various compositions were used to simulate the cytoplasmic membranes of bacterial and mammalian cells (Figure 2.1).

Lipopolysaccharides extracted from *P. aeruginosa* were bought from Sigma (product L8643). LPS extract powder (10 mg) was dissolved in 1 ml of Milli-Q water, resulting in 10 mg/ml stock. The average molecular mass of LPS is 10 kDa, so the stock molar concentration was about 1 mM. Different LPS/peptide ratios were tested, ranging from 1:40 to 1:5. An LPS/peptide molar ratio of 1:20 was finally used to acquire NMR spectra. The final 100 μ l NMR sample (for HR-MAS probe) contained 10 mM B2088 and 5 mg/ml LPS.

DPC (5.3 mg), together with 5.0 mg of POPG (both purchased from Avanti Polar Lipids) was directly dissolved in 0.5 ml of 30 mM phosphate buffer. This resulted in 3:1 DPC-POPG bacterial membrane mimicking micelle solutions (30 mM DPC, 13 mM POPG). Mammalian cell membrane representing micelles were produced using 5.3 mg DPC without POPG.

DMPC (41 mg) (Avanti Polar Lipids) was dissolved in ethanol and dried under a N₂ stream. DHPC (11 mg) (Avanti Polar Lipids) was dissolved in 30 mM phosphate buffer and used to solubilize DMPC. After several cycles of freezing and thawing, the mixture was a 10% (w/v) DMPC/DHPC bicelle solution with a “q” factor of 3 (DMPC:DHPC molar ratio of 3:1). Relatively large bicelles (q=3) were made to provide a large planar region in its discoidal structure and, hence, a better mimic to a membrane bilayer [141,142]. The peptide stock solutions were directly added to the lipid-containing solutions.

Two sets of liposomes were prepared for NMR experiments. To probe the interactions between B2088 and phosphate bilayer membranes, HR-MAS probe was used. POPG (10 mg) together with 28 mg of POPE powder were dissolved in chloroform, dried with N₂, and dissolved in 500 µl of PBS buffer. After several cycles of freezing and thawing, a mixed liposomal medium was prepared. Homogenous unilamellar liposomal vesicles were obtained by passing the liposome medium through a membrane (pore size, 0.4 µm diameter) 10 times. The NMR sample contained 2 mM peptide, 26 mM POPG, and 78 mM POPE. Following the same method, an NMR sample with a low concentration of POPE-POPG mixed liposomes was prepared (4 mM POPG and 6 mM POPE).

To monitor the AM052 induced membrane permeabilization effect, solution-state NMR was utilized. DOPE/DOPG mixed liposomes (DOPE:DOPG = 4:1) with 100 nm in diameter were prepared using similar method. 10 mM, ^{13}C labeled sodium formate solution was used to dissolve liposomes instead of PBS buffer. After large unilamellar vesicles (LUV) are formed, free sodium formate outside of liposomes were removed by overnight dialysis using dialysis bag with 10 kDa cutoff. Final NMR sample contains 10 mM liposomes with sodium formate inside. Paramagnetic probe omniscan (GE Health) 3 μM were added outside of liposomes. A second type of liposomes was prepared using the same method with omniscan inside and 5 mM sodium formate outside of liposomes. ^1H and ^{13}C 1D spectra were recorded at increasing concentrations of AM052 from 100-500 μM .

Figure 2-1. Chemical structures of various lipids and paramagnetic probes used in this study.

2.2 Methods

2.2.1 Methods for AMP activity and resistance determination

Druggabilities of AMPs are generally evaluated by several questions:

1. How well AMPs kill targeting bacterial pathogens (antimicrobial activity assay);
2. How fast AMPs kill bacteria (bactericidal kinetics assay);
3. How toxic are AMPs to mammalian cells (cytotoxicity assay and hemolysis assay);
4. How long the AMPs could be applied in clinical therapeutics (bacterial resistant assay).

In our studies, all of the above questions have been addressed to select possible antibiotic drug candidates among various defensin-analogous peptides and alpha-mangostin derived peptoids.

- **Antimicrobial activity assay**

Pseudomonas aeruginosa and *Staphylococcus aureus* were selected in this study as representatives for Gram-negative and –positive bacteria respectively. They were chosen as common human health threatening pathogens which have prominent clinical impact. The emergency of multidrug resistance strains from clinical isolates adds to a sense of urgency in the development of alternative therapeutic agents [143,144]. Several bacterial strains from both American Type Culture Collection (ATCC) and clinical isolates were used for antimicrobial activity assay.

Minimal inhibitory concentration (MIC) values were determined using the broth macro dilution method modified from that described by the Clinical and Laboratory Institute. The MIC values of defensin analogous

peptides or alpha-mangostin derived peptoid for each clinical isolate or reference bacteria were recorded as the lowest concentration of antibiotics that inhibited 99% visible growth of the test organism.

The antibacterial activity could also be manifested as the logarithmic reduction of the bacteria on exposure to the tested antibiotics. Defensin analogous monomeric peptides that exhibited high bacterial killing activities were subsequently selected and tested in high-salt restricted conditions.

- **Bactericidal kinetics study**

Bactericidal kinetics of antibiotics has a direct impact on therapeutic duration, dosage [145] as well an indication on the bacterial killing mechanism [146]. B2088 and AM052 were tested for bactericidal kinetics. Reductions of bacterial counts after adding in antibiotics were examined for every 10 minutes time interval. A time kill curve was plotted with time against the logarithm of the viable count.

- **Cytotoxicity assay**

The important use of AMPs would be for the eye or other mucosal surfaces such as the lungs and mouth, all of which have an epithelial cell covering [93]. Therefore for the cytotoxicity assays, the primary concern was for the fragile epithelial cells.

Cytotoxicity was determined by measuring the amount of ATP generated by viable cells in culture using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI). Using the luminescence of the untreated wells as 100%, the cell viability was calculated as:

cell viability =

$$\frac{\text{luminescence of control} - \text{luminescence of sample}}{\text{luminescence of control}} \times 100\%$$

Equation 1. Cell viability calculations.

Each condition was assayed in triplicate, and the experiments were repeated three times.

- **Hemolysis assay**

The hemolytic activity of the AMPs was estimated by the amount of hemoglobin released from rabbit erythrocytes [93]. Rabbit erythrocytes were chosen because AMP-induced toxicity easily occurs in these blood cells when compared to human red blood cells [147].

The hemolytic activity of each sample was calculated as:

% hemolysis =

$$\frac{\text{mixture } A_{576nm} - \text{negative control } A_{576nm}}{\text{positive control } A_{576nm} - \text{negative control } A_{576nm}} \times 100\%$$

Equation 2. Hemolysis calculations.

Each condition was assayed in triplicate, and the experiments were repeated three times.

- **Resistant assay**

Serial passage resistant assay was designed to stimulate possible resistance developed in bacteria under AMP treatment. Resistance developed in a reference strain of *P. aeruginosa* (ATCC 27853) was manifested as increased MICs against B2088, gentamicin, and norfloxacin. A resistance developing curve was plotted with bacterial serial passage against the MIC.

Antimicrobial activity assay, bactericidal kinetics studies, AMP cytotoxicity assay, hemolysis assay and bacterial resistance studies were done by the microbiology group in Singapore Eye Research Institute (SERI), leading by Dr. Rajamani Lakshiminarayanan.

2.2.2 Biophysical methods for AMP bactericidal mechanism studies

A variety of biophysical techniques have been used to explore the bacterial killing mechanism of AMPs. Complementary information is provided depending on the strength and limitations of each method. In this study, microscopic imaging, fluorescent emissions, nuclear magnetic resonance spectroscopy and in silicon binding were utilized to assess the interactions between AMPs and bacteria.

- **Microscopic imaging**

High resolution microscopic imaging method could be used to visualize the effects of AMPs on bacterial cells. Scanning electron microscopy (SEM) has been used to demonstrate the damaging effects of B2088 and AM052 on bacteria cells *P. aeruginosa* and *S. aureus*.

Transmission electron microscopy (TEM) could also be utilized to explore the AMP induced phase transitions in various bacterial membrane mimicking media. The effect of B2088 on the morphology of LPS and the topological changes induced by AM052 on liposomes were investigated by transmission electron microscopy. AMP cellular localization was also detected using nano gold particles coated B2088.

Microscopic imaging works were done in Singaproee Eye Research Institute by Ms Sarawathi Pasmanabhan for B2088 branched peptide, and carried in house for AM052 work.

- **Fluorescent emission studies**

Bacterial membrane disruption could be monitored by membrane sensitive fluorescent dyes and the membrane leakage or pore formation could be measured by the release of liposomal entrapped calcein probes.

B2088 induced outer membrane permeabilization of Gram-negative bacteria was studied using an *N*-phenyl-1-naphthylamine (NPN) uptake assay as reported by Loh *et al.* [138]. The extent of *P. aeruginosa* outer membrane disruption was manifested by an increase in fluorescence signal as a function of time. Several fluorescence emission curves were plotted using increasing concentrations of B2088.

Live bacteria were also used for the depolarization assay of transmembrane potential. The membrane-sensitive fluorescence probe 3,3'-dipropylthiadicarbocyanine iodide (diSC3) was added to the bacterial suspension. The fluorescence emission spectrum was recorded by monitoring the changes in emission intensity as a function of time. Similarly to NPN uptake assay, several spectra were recorded using various concentrations of B2088.

AM052 induced membrane disruption was monitored by the release of calcein from the LUVs. 100% leakage was determined by addition of 2% Triton X-100 into the LUVs. The apparent percent leakage value was calculated using the formula:

$$\%L = \frac{I_t - I_0}{I_\infty - I_0} \times 100\%$$

Equation 3. Equation for liposomal dye leakage analysis. I_0 and I_t are intensity before and after addition of AM052 respectively and I_∞ is intensity after addition of 2% Triton X-100.

Fluorescence emission studies were done by Mr Koh Junjie in Singapore Eye Research Institute.

- **Molecular dynamics simulations**

Several sets of molecular dynamics simulations were done to explore the AMP-AMP/AMP-membrane interactions as well as AMP induced membrane deformation.

Six sets of Hamiltonian replica exchange molecular dynamics (hREMD) simulations were conducted for C2, F2, and Y2 peptides with eight replicas each at a temperature of 300 K. The details of this hREMD method can be found in recent work [148]. The novel part of the method is that instead of reducing the intermolecular interactions, the repulsive part of the van der Waals interactions between the peptide atoms were increased. The dissociation enhanced hREMD method decreases the energy barrier between local minima which are mainly stabilized by non-native contacts. Thus, such hREMD simulations helped to sample a broader range of configurations of peptide oligomers. For each peptide dimer, two hREMD simulations were performed with different initial configurations: one begun with the NMR-derived dimer structure and the other started with the two monomers separated. For C2 and F2 peptides, simulations provided converged results by 100 ns, while for Y2 peptides, 150 ns simulations were needed. Twenty of each Y2/F2 dimer conformers reconstructed from NMR data were clustered on the basis of the pairwise RMSD with a cutoff of 1 Å. The center of the largest cluster was selected as a representative conformer of the dimer. The two tyrosine residues of the Y2 model were changed to two cysteine residues to build a model of the C2 dimer. The C2, F2, and Y2 models were separately solvated with explicit water in simulation boxes. The complex is formed when the minimum distance between the two monomers is less than 0.375 nm. The complex structures from the Y2 and C2 dimer

simulations were analyzed to extract binding free energies using the MM/GBSA protocol [149].

AMP-micelle interactions were simulated using the Gromacs package applying the OPLS-AA (all-atom) force field [150]. The micelle structure was modeled using 64 DPC molecules (M64) [151]. A single DPC molecule was extracted from a 65-mer DPC micelle provided by the Tieleman group [152]. The molecule was repeatedly copied, rotated, and translated to generate a 64-mer DPC micelle. Two different initial setups with regard to the relative position of the Y2 dimer within or outside of the micelle were constructed. In these two simulations, distance restraints with a force constant of $3000 \text{ kJ mol}^{-1} \text{ nm}^{-2}$ were imposed on the peptide to maintain the backbone structure of the NMR model while allowing free motion of the side chains. Electrostatic interactions were treated with the particle mesh Ewald method with a cutoff of 9 Å, and a cutoff of 14 Å was used in the calculation of van der Waals interactions.

To study the impact of B2088 on membrane structure, both atomic and coarse grained simulations were performed. We first studied the interactions of B2088 with a model micelle (DPC/POPG = 3:1) using atomic MD simulations. The peptide was modeled using the CHARMM27 force field [153], the lipids were modeled using the CHARMM36 parameter set [154], and the water molecules were described by the TIP3P water model [155]. The coordinates of the micelle were obtained from a 100 ns MD simulations of the self-assembly of DPC-POPG lipid mixtures. The peptide, B2088, was put close to the micelle and subjected to a 200 ns MD simulations. In addition, coarse grained simulations were also performed to study the interaction of B2088 with model membranes. For this purpose, a model bacterial membrane consisting of 384 POPE and 128 POPG lipids was constructed. The Martini force field [156] was

used to model both the peptide and the lipids. Because B2088 is highly positively charged, it is expected that electrostatic interactions will play an important role in peptide-membrane interactions. Thus, we used the polarizable water model recently developed by the Martini group because this water model has been shown to correctly describe long range electrostatic interactions [157]. In both atomic and coarse grained simulations, a cut-off distance of 1.2 nm was used for both the Lennard-Jones and real space electrostatic interactions, and the particle-mesh Ewald algorithm was employed to calculate the long range electrostatic interactions in reciprocal space. During the MD simulations, temperature and pressure were maintained at 300 K and 1 bar, respectively. In the case of coarse grained simulations of the peptide-membrane system, semi-isotropic coupling was used to maintain the pressure.

Molecular dynamics simulations were done by Prof Chandra Verma and Dr Li Jianguo in Bioinformatics Institute (BII) A*STAR, Prof Mu Yuguang and Dr Jiang Ping in School of Biological Sciences NTU.

2.2.3 Structure determination in aqueous solution and different lipid media

The highly mobile structural properties of AMPs limit possible structural determination methods. X-ray crystallography, which requires homogeneously, ordered molecules packed in crystal samples, is not suitable for AMP studies. Solution NMR, in this case, gains the advantage of determining structures of AMPs in near-native, liquid environment, capable to probe AMP-membrane interactions as well as monitor AMP dynamics which is thought to be critical for their function. Structures of Y2, F2 peptides, B2088 and AM052 are determined in

aqueous solution and various lipid media mimicking bacterial membranes (Figure 2.2). NMR experiments were performed using Bruker Avance II 600 or 700 MHz spectrometers equipped with cryoprobes, and summarized in table 3, 4, and 5. Residue specific resonance assignment and assignment for NOESY cross-peaks were performed using CARA (www.nmr.ch). 3D structures of AMPs were reconstructed using CYANA 2.0 [158].

In aqueous solution, structure transitions of AMPs were traced through ^1H chemical shifts changes in a series of one-dimensional (1D) homonuclear experiments with increasing AMP concentrations. DOSY experiments were performed to estimate the oligomerization state of AMPs, complementary to the structure transition information. In DOSY experiments, the pulsed magnetic field gradient strength was calibrated using the ^1H signal in a 99.9% D_2O sample with the self-diffusion coefficient of HDO $[(1.902 \pm 0.002) \times 10^{-9} \text{ m}^2/\text{s}]$. A series of 1D spectra were measured with a diffusion delay of 150 ms and increasing gradient strength in 32 steps from 2 to 95% of G_{max} . Diffusion coefficient D_s is calculated by fitting the curve of signal intensity versus variable gradient strength.

Detergent micelles were first tried for AMP membrane structure characterization. The isotropic, fast tumbling nature of micelles provides well resolved NMR spectra with sharp peaks [159]. Standard NMR samples were prepared with 500 μM AMPs in DPC-POPG micelles at pH 5. Two-dimensional (2D) homonuclear TOCSY, NOESY, and ROESY experiments were conducted with mixing times of 70, 150, and 100ms, respectively. Data were collected for each experiment for 7 hours. 2D ^1H - ^{13}C HSQC experiments using natural abundance ^{13}C in 5 mM Y2 and F2 peptides were also performed. Urea (8M) was added to each sample

subsequently for collection of reference spectra of denatured peptides. Micellar media produce nice NMR spectra, however, the inherent large curvature in micelles as well as the detergent composition in nature, micelles could not be served as ideal membrane mimicking media. Therefore, lipid bicelles are tried for AMP structure determination.

Bicelles are composed of a mixture of detergents and lipids to form disc-shaped aggregates, with a central lipid bilayer and a detergent circumference [160]. The presence of natural lipids and the fact that the surface of the central lipid region is more-or-less flat eliminate the limitations associated with micelles [161]. Bicelles could be produced with varying amounts of lipids and detergents (known as q-factors, $q = [\text{lipids}]/[\text{detergents}]$). Small bicelles with q factor smaller than 0.5 are isotropic and tumbling fast [142], but high lipids content is required for maintaining stable discoidal structures. Therefore in this study, a relatively large q factor 3 is chosen to provide a large planar membrane region. At high temperature, bicelles will undergo phase transition from liquid isotropic phase to gel-crystalline phase aligning along magnetic field [141]. Hence, the low temperature, 10 °C, was used for determining Y2 structure in DHPC/DMPC bicelles. Similar structure determination methods were used as in micelles.

Structural changes of AMPs in aqueous and lipid media were monitored through heteronuclear [^1H , ^{15}N]-HSQC experiments using 500 μM ^{15}N labeled F2, Y2 peptide or B2088.

Figure 2-2. Schematic illustration of micelle, bicelle and lipid membrane. Micelle consisting 64 DPC molecules is shown on left, DMPC/DHPC mixed bicelle is shown in middle and POPC bilayer membrane is shown on the right. Lipids acyl chains are shown in cyan and head groups are shown in pink color.

2.2.4 Molecular dynamics of AMPs in lipid media

Mobility of peptide bond in B2088 was quantitatively monitored by heteronuclear NOE experiment ($[^{15}\text{N}, \{^1\text{H}\}]$ -NOE) and qualitatively represented using longitudinal (T_1) and transverse (T_2) relaxation rates of NH group. T_1 and T_2 were analyzed using 10 $[^1\text{H}, ^{15}\text{N}]$ -HSQC spectra with various delays. For T_1 , the delay time varied from 0.05 to 2.55 s, and for T_2 , the delay time varied from 0.05 to 1 s.

2.2.5 AMP-lipids interactions

Binding of AMPs towards DPC-POPG mixed micelles were directly observed through NOEs in 2D homonuclear NOESY spectra. Line broadening effect of AM052 caused by liposomal binding was also monitored in its 1D ^1H spectra.

DOSY experiments were performed with AMPs in phosphate buffer, DPC micelles (30 mM), and DPC-POPG (30 mM DPC and 13 mM POPG)

mixed micelles [162]. Diffusion coefficient D_S was calculated by fitting the curve of signal intensity *versus* variable gradient strength. The molar fraction of peptide in lipid (F_L) was calculated using Equation 4.

$$D_S = D_L F_L + D_F (1 - F_L)$$

Equation 4. Molar fraction of AMPs in micelles. D_L and D_F are the diffusion coefficients of lipid micelles and free peptides in solution.

H-bonding and special proximity between AMPs and membranes were detected through changes of pK_a in both AMP and lipid atoms. pK_a is determined by plotting chemical shift changes of proton of interests against corresponding increasing/decreasing pH values. pH titration experiments were performed on the sample of B2088 in DPC-POPG mixed micelles, starting from pH 8.77. For each 0.5 pH unit from pH 8 to pH 1, one-dimensional ^1H and two-dimensional ^{15}N - and ^{13}C -HSQC spectra were acquired. Hydrochloride (HCl, 1 M) was used as the titrant, and 16 titration points were obtained in total.

To date, pH titration experiments are widely used in NMR spectroscopy. These experiments provide enormous information about protein folding and dynamics, transient H-bonding and electrostatic interactions between amino acid residues, which is difficult to acquire by other methods. A novel pH titration method was proposed to automate data acquisition in NMR spectroscopy of proteins enabling adding a new pH dimension to existing spectroscopic dimensions (Figure 2.3). In turn this ultra-high resolution of this new pH dimension allows for novel mathematical transformation of pH dimension directly revealing spatial proximity of electrostatically interacting groups. The key elements of this novel method are automatic reading out and tracing of individual proton

chemical shifts at different pH values and novel processing of the high pH resolution dimension. The unique information obtained in this way is extremely valuable for protein structural studies and drug development.

Figure 2-3. Schematic setup of novel pH titration experiment. NMR control words which control the on and off, and the duration of the pumping are written in the pulse sequence. These signal are transferred to NMR spectrometer console, and output through TCU control. Micro-pump is linked to NMR console, and signal go into pump through TTL input. Pump control is achieved by voltage adjustment in TCU unit of NMR console. On signal will be converted to high voltage output and resulted in pumping. On the other hand, off signal will be converted into low voltage output and resulted in pausing. Concentrated hydrochloride acid or sodium hydroxide base will be pumped into NMR sample tube which is rotating inside NMR spectrometer. pH indicators are also inside NMR tube. Whole experiment will be done by controlling pulse sequence only as usual. pH will be changed in well defined manner by

programming via NMR pulse programs; and checked through reading out chemical shift of indicators. In the figure, red and black lines represent electronic wire. Black lines represent thin chromatographic tubing.

2.2.6 Methods for studying AMP membrane insertion and disruption by NMR

Membrane insertion and orientation of AM052 was studied by paramagnetic relaxation enhancement (PRE) experiment. Omniscan, a paramagnetic probe with unpaired electrons in the central metal ions (Gd 3+), was chosen in this study due to its high water solubility, high stability, minimal interactions with AMPs and strong paramagnetism [163]. Even at low concentrations of Omniscan, PRE effect manifested by spectral line broadening could be observed within 10 Å of protons which are accessible to the probe.

The membrane topology of many AMPs and membrane proteins has been studied using quantitative PRE characterization without any chemical perturbations produced [164]. Experimental relaxation enhancements are proportional to the third power of the immersion depth. In this study, 2D homonuclear NOESY spectra of AM052 in DPC-POPG mixed micelles were acquired with increasing concentrations of AM052, from 1 mM to 5 mM. Intensities of resonances stemming from both AM052 and DPC/POPG were recorded and plotted against Omniscan concentrations. Similar pattern of enhanced relaxation rates indicates special proximity between observed protons.

Two sets of liposome media were prepared to trace membrane disruption induced by AM052 (Figure 2.4). One of them contains ¹³C

labeled sodium formate inside of liposomes and Omniscan outside; the other one was prepared with opposite setup. Increasing concentrations of AM052 were added into the liposomal media. ^1H and ^{13}C signal of sodium formate were recorded as a function of AM052 concentrations.

Figure 2-4. Two types of liposome samples used in PRE experiment.

2.2.7 Studying AMP structure and dynamics in heterogeneous material

Aggregation of LPS, POPE-POPG liposome and microbes were observed upon adding in of AMPs. These aggregated materials are anisotropic due to the slow tumbling motion. Traditional solution state NMR for isotropic sample will not be suitable. High-resolution magic angle spinning (HR-MAS) probe was used for structure study in these cases [165]. Unlike the solution state cryo-probe, HR-MAS probe spins at the magic angle (54.7° to the magnetic field B_0) with high speed (about 8-10 kHz). Spinning at magic angle could remove the time-averaged dipolar coupling Hamiltonian in anisotropic sample; therefore reduce the severe line broadening effect. The high spinning rate helps to displace

spinning side bands outside the spectral range of interest [166]. Using the sample, spectra measured in solution state cryo-probe are poorly resolved; with HR-MAS probe, spectra with sharper lines in high resolution were achieved.

$$D_{IS}(\theta) = \frac{\hbar \gamma_I \gamma_S}{4\pi^2 r_{IS}^3} [1 - 3\cos^2\theta]$$

Equation 5. Dipolar coupling constant between *I* and *S* spin. $\hbar$ is the reduced Planck constant, γ_I and γ_S are the gyromagnetic ratios of spin *I* and *S* respectively. r_{IS} is the inter-spin distance. Θ is the angle between the inter-spin vector and the external magnetic field. When Θ equals to 54.7° , dipolar coupling constant D_{IS} equals to zero.

All NMR experiments are summarized in Table 3, 4, and 5.

Table 3. NMR experiments done for C2, Y2, and F2 peptides.

Experiment	Sample
1D ^1H	C2, Y2 and F2 Increasing [C] from 10-500 μM In both aqueous buffer and 30mM DPC
DOSY	C2, Y2 and F2 5 or 500 μM in aqueous buffer 500 μM in 30 mM DPC micelle 500 μM in 30 mM DPC-13 mM POPG micelle
$[^1\text{H}, ^{13}\text{C}]$ - HSQC	5 mM Y2 and F2 peptide 30 mM DPC-13mM POPG micelle
^1H - ^1H TOCSY	500 μM Y2, F2 and C2 peptide Aqueous buffer 30 mM DPC-13mM POPG micelle
^1H - ^1H NOESY	500 μM Y2 and F2 peptide Aqueous buffer 30 mM DPC-13mM POPG micelle 10% (w/v) DMPC-DHPC bicelle (q=3)
^1H - ^1H ROESY	500 μM C2 peptide 30 mM DPC-13mM POPG micelle

Table 4. NMR experiments done for B2088.

Experiment	Sample
1D ^1H	B2088, V2 monomer Increasing [C] from 10-500 μM Aqueous buffer 30mM DPC-13 mM POPG micelle (pH=8.77-1)
DOSY	B2088, V2 monomer 5 or 500 μM in aqueous buffer 500 μM in 30 mM DPC micelle 500 μM in 30 mM DPC-13 mM POPG micelle
[^1H , ^{13}C]- HSQC	500 μM B2088 (^{13}C , ^{15}N double labeled) 1.5 mM POPG-500 μM POPE liposome 30 mM DPC-13mM POPG micelle (pH=8.77-1)
	10 mM B2088 (^{13}C , ^{15}N double labeled) 500 μM LPS micelle (HR-MAS)
[^1H , ^{15}N]- HSQC	500 μM B2088 (^{13}C , ^{15}N double labeled) Aqueous buffer 30 mM DPC-13mM POPG micelle (pH=8.77-1) (T_1 delay 0.05-2.55s, T_2 delay 0.05-1s)
	2 mM B2088 (^{13}C , ^{15}N double labeled) 4 mM POPG-6 mM POPE liposome (HR-MAS) 26 mM POPG-78 mM POPE liposome (HR-MAS)
	10 mM B2088 (^{13}C , ^{15}N double labeled) 500 μM LPS micelle (HR-MAS)
	2 mM V2 monomer (^{13}C , ^{15}N double labeled) 7 $\times 10^9$ cfu/ml bacteria <i>P. aeruginosa</i> (HR-MAS)

$^{15}\text{N} \{^1\text{H}\}$ NOE	500 μM B2088 (^{13}C , ^{15}N double labeled) 30 mM DPC-13mM POPG micelle
	10 mM B2088 (^{13}C , ^{15}N double labeled) 500 μM LPS micelle (HR-MAS)
	2 mM B2088 (^{13}C , ^{15}N double labeled) 4 mM POPG-6 mM POPE liposome (HR-MAS)
^1H - ^1H TOCSY	500 μM B2088 and V2 monomer 30 mM DPC-13mM POPG micelle
^1H - ^1H NOESY	500 μM B2088 and V2 monomer Aqueous buffer 30 mM DPC-13mM POPG micelle
	10 mM B2088 (^{13}C , ^{15}N double labeled) 500 μM LPS micelle (HR-MAS)
^{15}N NOESY (3D)	500 μM B2088 (^{13}C , ^{15}N double labeled) 30 mM DPC-13mM POPG micelle
	10 mM B2088 (^{13}C , ^{15}N double labeled) 500 μM LPS micelle (HR-MAS)
HNCA (3D)	500 μM B2088 (^{13}C , ^{15}N double labeled) 30 mM DPC-13mM POPG micelle
	10 mM B2088 (^{13}C , ^{15}N double labeled) 500 μM LPS micelle (HR-MAS)

Table 5. NMR experiments done for AM052.

Experiment	Sample
1D ^1H	AM052 Increasing [C] from 10 μM -5 mM Aqueous buffer
	500 μM AM052 30 mM DOPC liposome 24 mM DOPE-6 mM DOPG
	AM052 100-500 μM 8 mM DOPE-2 mM DOPG (^{13}C formate inside) 3 mM Omniscan
	AM052 100-500 μM 8 mM DOPE-2 mM DOPG (Omniscan inside) 5 mM ^{13}C formate
DOSY	AM052 10 μM or 5 mM in aqueous buffer
^1H - ^1H NOESY	500 μM AM052 Aqueous buffer 30 mM DPC-13mM POPG micelle (Omniscan 1-5 μM)
	500 μM AM052 24 mM DOPE-6 mM DOPG
TIPSY	1 mM AM052 Aqueous buffer 293-343K, pH 8.5-3

Chapter 3. Results

Defensins are short cationic peptides serving as an important component of the innate immune system [80,167]. The low degree of sequence similarity among members of the mammalian β -defensin family, in addition to the highly conserved structures as well as similarities of charge density, suggests that their antimicrobial activity may depend on the structure and charge distribution rather than their primary sequence [83]. Due to bactericidal activity of AMPs is primarily driven by electrostatic interactions with the membranes [81,82], the C-termini of human β -defensin 3, which has the highest charge density in the peptide, has drawn great attention for AMP research. Several analogous peptides with properties that are appropriate for therapeutic application have been designed [93]. The structural and activity relationship of those peptides has been studied using various chemical-physical methods and results are presented below.

3.1 Structure-dependent charge density as a determinant of antimicrobial activity of peptide analogues of defensin

Our studies started from natural AMPs and their linear peptide analogs. The strategy to design those peptides is described in Introduction section (Chapter 1.2.3) [93]. Three hBD-3 C-terminal (R36-K45) analogues peptides, C2, Y2, and F2, were selected to study their structures and activity relationship using NMR spectroscopy and MD simulations.

Results have shown that the ability of AMPs to accrete a well-defined 3D structure featuring a compact density of positive charges leads to a more efficient bactericidal activity. The work has been published in Biochemistry 2009 [100].

- **Antibacterial Activity Assays**

Various hBD-3 C-terminal (R36-K45) analogous peptides were designed (Table 6) and proved to exhibit differential antibacterial activities against the common human pathogen *P. aeruginosa* ATCC 9027 (Table 7). Studies have shown that AMP bactericidal activity could be hindered by increasing salt concentration [168], presumably through interfering AMP-bacterial membrane interactions [169]. Therefore high salt restricted conditions have been tested on selected peptides as well and results are shown in table 7. The Y2 peptide exhibited high antibacterial activity in the low micro-molar range under both normal and high-salt conditions compared to the others. A dramatic increase in the activity of Y2 was detected at concentrations from 3.2 to 6.3 µg/mL (2.5-5.0 µM) or 12.5 to 25 µg/mL (10-20 µM) in either condition respectively. A structural transition of Y2 in the concentration range of 5-20 µM might result in its remarkably increased activity. In contrast, low antibacterial activities were observed for C2 and F2 peptides. Up to AMP concentration of 100 µM, no detectable transitions to a high-activity state were observed in either peptide. Y2, C2 and F2 peptides were selected for subsequent structural studies.

Table 6. Various hBD-3 C-terminal (R36-K45) analogous peptides synthesized for antimicrobial activity studies.

Peptide	Sequence
Original hBD-3	RGRKCCRRKK ^a
C2	RGRKC(Acm)C(Acm)RRKK ^b
F2	RGRKFFRRKK
H2	RGRKHHRRKK
Y2	RGRKYYRRKK
W2	RGRKWWRRKK

^aModified amino acid residues are highlighted in red. ^bAcm is acetamidomethyl group added to Cysteine thiol to prevent disulfide bond formation.

Table 7. Assays of antimicrobial activity of Defensin's derived peptides against *P. aeruginosa* ATCC 9027 (log reduction)^a

Conc. (μg/ml)	Low salt (4 hours)				High salt (6 hours)	
	C2	F2	H2	Y2	Y2	W2
200	0.41	0.33	0.29			
100	0.57	0.61	0.62		4.19	
50	0.63	0.87		3.07	4.07	1.06
25				2.39	3.31	0.48
12.5				2.10	0.49	0.09
6.25				2.02	0.49	0.08
3.125				0.33		

^aPeptides were incubated with *P. aeruginosa* ATCC 9027 for 4 hours at 22.5 °C. A 0.5 log reduction equals 68% of the bacteria killed, and a 3 log reduction means 99.9% of the bacteria were killed. Error bars constitute 30% of measured value, and therefore not indicated in table.

- **Cytotoxicity and Hemolytic Activity against Mammalian Cells**

Different from bacterial membrane, which constitutes mainly the neutral phospho-ethanolamine (about 70%) and the negatively charged phospho-glycerol (about 30%), mammalian cell membrane is largely neutral made by neutral lipids phospho-choline. Since AMPs are cationic, their binding strengths towards mammalian cell membrane are expected to be lower due to the weaker electrostatic attractions. Both the cytotoxicity and hemolytic activity of hBD-3 analogs were tested on mammalian cells. Up to 200 $\mu\text{g/ml}$ of C2, F2 or Y2 peptide, no significant decrease of viable cell number or cell lysis were observed in contrast to native hBD-3, which is found to be very toxic (Figure 3.1 and Figure 3.2).

Figure 3-1. Cytotoxicity assay of C2 (■), F2 (Δ), and Y2 (●) peptides for human conjunctival epithelial cells. Reported values are means of performed triplicate experiments with variation bars representing the standard deviation.

Figure 3-2. Hemolytic effects of AMPs on rabbit erythrocytes. The hemolytic activities were calculated as a percentage of the total cell lysis. Solid rectangle line represents C2 peptide, solid triangle line represents F2 peptide, hollow square line represents Y2 peptide, and hollow dot line represents hBD-3. The experiments were repeated 3 times and the data presented were the average of all experiments.

- **Lipid Phase Partition of the Peptides**

Translational diffusion coefficients (D_s) of the C2, F2, and Y2 free peptides, peptides in micelle solution and micelles were measured separately using Diffusion Ordered Spectroscopy experiments (DOSY) [170] (Table 8). Both DPC and DPC-POPG mixed micelles were used to represent mammalian and bacterial cell membranes, respectively.

Table 8. Translational diffusion rates of C2, F2, Y2 peptides and lipids measured by DOSY experiments.

$D_s^a (10^{-10} m^2 s^{-1})$	C2	F2	Y2
Free peptide	2.37±0.10	2.31±0.23	2.01±0.03
Peptide in DPC	1.89±0.26	1.92±0.04	1.59±0.06
DPC	0.99±0.10	0.99±0.10	0.99±0.10
Peptide in DPC-POPG	0.90±0.24	0.91±0.24	0.92±0.18
DPC-POPG	0.92±0.25	0.92±0.25	0.92±0.25

^a D_s is the translational diffusion coefficient. In three conditions, D_s of peptides are shown on top, while the lipids diffusion rates are shown in bottom. All samples are measured at peptide concentration of 500 μ M, in 30 mM sodium phosphate buffer, 30 mM DPC micelles or 30 mM DPC, 3 mM POPG mixed micelle solution, pH=5.

Molar fractions of peptides bound to micelles (F_L) were calculated using the measured diffusion coefficients in Table 8 [171]. The results are shown in Table 9. This parameter reflects the affinity of the peptides bind to lipid membranes. For all peptides, partitioning into the mammalian cell membrane mimicking zwitterionic DPC micelles was only ~40%, in contrast to the full localization into the same lipid phase doped with anionic POPG lipids which has similar charge property to the bacterial cells. This observation was consistent with their high antimicrobial activity and low cytotoxicity.

Table 9. Peptide lipophilicity measurement (F_L)

$F_L^a(\%)$	C2	F2	Y2
DPC	35.17	31.25	41.41
DPC-POPG	100	100	100

aF_L is the molar fraction of peptides bound to the lipid phase.

- **Structural Transitions in C2, F2, and Y2**

Diffusion coefficients of C2, F2, and Y2 were measured at both high (500 μM) and low (5 μM) peptides concentrations in phosphate buffer (Table 10). Surprisingly to notice that for all three peptides, the translational diffusion rates at 500 μM peptide concentration was just about half of the rates at 5 μM . Stokes-Einstein equation says the molecule diffusion rate is in reverse proportion to its molecular size if we assume the final structures of the peptides are globular [172,173]. This means at concentrations above 500 μM , all three peptides form stable dimers while at concentrations below 10 μM monomeric forms predominate in aqueous solution.

Table 10. Translational diffusion coefficients of C2, F2 and Y2 measured by NMR.

$D_s^a(10^{-10}\text{m}^2\text{s}^{-1})$	C2	F2	Y2
5 μM	5.32 $\pm$ 0.17	5.5 $\pm$ 0.23	5.17 $\pm$ 0.19
500 μM	2.37 $\pm$ 0.10	2.31 $\pm$ 0.23	2.01 $\pm$ 0.03

aD_s is the translational diffusion coefficient.

To monitor peptide monomer-dimer transition and its structure changes, 1D ^1H spectra of C2, F2, and Y2 were recorded at increasing peptide concentrations from 10 to 500 μM , both in the presence and absence of DPC micelles. Peptides' structural transitions could be reflected by changes in the ^1H chemical shifts. Figure 3.3 shows 1D proton spectra of Y2 and F2 peptides in 30 mM DPC micelles solution. Proton resonance peaks showed gradual changes are labeled in red. At peptides concentration of 20 μM (Figure 3.3 B, F), resonances stemming from both the structure form of lower concentrations (Figure 3.3 A, E) as well as higher concentrations (Figure 3.3 C, G) could be detected, indicating the slow (on the time scale of ^1H chemical shifts) kinetic regime of exchange rate between these two conformations. It also suggests that the structural transitions are likely induced by dimerization of peptides. C2 peptide shows similar spectral change only at concentration of 500 μM , which is much higher than the transition midpoint of Y2 and F2.

1D spectra of Y2 and F2 in water showed structural monomer-dimer transitions as well over the concentration range of 50-100 μM . This indicated that DPC micelles could promote peptide dimerization at lower concentrations than in aqueous solution, presumably stabilizing the peptide dimer at the surface of the lipid membrane. A similar effect was also observed for the C2 peptide. The monomer dimer transition of Y2 correlated with its cooperatively increased activity in the same concentration range. In contrast, the C2 peptide, which exhibits only low antibacterial activity at all the concentrations that were studied, underwent the dimerization transition at much higher concentrations. In addition, F2 dimerizing at low concentrations but lacking high bactericidal

activity suggests that features other than the dimerization ability are also important determinants for the activity of these peptides.

Figure 3-3. 1D ^1H spectra of Y2 (A-D) and F2 (E-H) as a function of concentration in the presence of 30 mM DPC at 25 °C. The concentrations of the peptides are 10 μM (A and E), 20 μM (B and F), 50 μM (C and G), and 500 μM (D and H). ^1H peaks are assigned and labeled. Unknown peaks which could not be assigned are labeled with asterisks. Gradually changing proton resonance peaks are highlighted in red. Spectra in each column share the common axis labeled at the bottom (D and H).

- **3D NMR Studies of F2, Y2 and C2 peptides**

3D structures of Y2 and F2 peptides in DPC-POPG mixed micelles were reconstructed using 2D homonuclear NOESY spectra (Figure 3.4). Two protons in methylene groups of amino acids' side chains showed systematic chemical shift degeneracy in Y2 peptide but not in F2, suggesting more restricted side chain mobility in F2. More short to inter-mediate range NOEs were observed in F2 amide region, whereas only sequential NOEs were shown for Y2 peptide. This indicates F2 peptide adopts more helical structure in contrast to the turn or β -sheet conformation predominantly presents in Y2.

Initially, 63 and 72 NOEs were identified and used for structural calculations of the Y2 and F2 monomers respectively. Subsequently, 14 NOEs for Y2 and 20 NOEs for F2 which could not be fit into monomeric structures were assigned as inter-monomeric constraints and used for dimeric structure calculations.

C2 in DPC-POPG mixed micelles exhibited a significantly overall smaller number of NOEs with remarkably reduced cross-peak intensities in comparison to the diagonal ones. The high dynamic property of C2 prompted us to measure 2D ROESY spectra for structural studies. The 2D ROESY spectrum of C2 unfortunately still shows much reduced structurally significant number of ROEs. For example, the NOEs between $H\beta$ Y6 to ^NH R8 and $H\gamma$ R8 to ^NH F5 highlighted in Figure 3-4 (both A and B) are missing in the case of C2 peptide (Figure 3.5 B). This prevents unambiguous 3D structure reconstruction of the C2 dimer.

Figure 3-5. (A) 2D NOESY spectra of Y2 peptide in the presence of DMPC/DHPC bicelles, and (B), 2D ¹H ROESY spectra of C2 peptide in the presence of DPC-POPG mixed micelles. Only amide to aliphatic proton regions are shown in either spectrum. Spectra were collected using 500 μM peptide sample, pH=5. Spectrum in (A) was collected at 10 °C. The proton specific assignments of individual cross-peaks are indicated.

$\Delta C\alpha$ and $\Delta C\beta$ chemical shifts plots clearly show the differences in secondary structures of Y2 and F2 (Figure 3.6). Both $\Delta C\alpha$ and $\Delta C\beta$ patterns in F2 and Y2 peptides are generally opposite to each other, indicating the prevalence of helical structure in F2 and turn or β -sheet structure in Y2.

Figure 3-6. Peptide secondary chemical shift plot ($C\alpha$ and $C\beta$ region). Both Y2 and F2 peptides' secondary structures in lipids environment (DPC-POPG mixed micelles) were compared to those in urea added as a reference. Panels A and C are plotted for Y2 peptides, while panels B and D are plotted for F2 peptides.

20 selected best fit structures with lowest energy for each Y2 and F2 peptide in dimeric conformation are shown in Figure 3.7. For each peptide, the dimeric structure is built on the antiparallel assembly of two monomeric peptide chains. Y2 peptide is predominantly extended except

for the two turns shown at amino acid residues number 3 and 6. Charged residues at both termini of the peptide are highly mobile, while the hydrophobic tyrosine residues in the middle are constrained serving as the dimeric interface. The F2 dimer is formed by two helix like monomers twisted around each other. Again the monomeric interface was stabilized by the aromatic Phe residues in the central region. Structures of F2 and Y2 peptides are summarized in Figure 3.8 and Table 11.

Figure 3-7. NMR structures of dimeric Y2 and F2 peptides in the presence of DPC-POPG mixed micelles. Panels A and B are super positions of 20 Y2 and F2 conformers, respectively; panels C and D are representative conformers of Y2 and F2 peptides. Dimeric interfaces of F2 and Y2 peptides are indicated by red rectangle. Heavy atom side chains of aromatic and charged amino acid residues are colored in gold and orange. The backbone of individual monomers in the dimeric structure is colored royal blue and dark green and numbered accordingly. Figures were prepared using Molmol [174].

Figure 3-8. Structure summary of Y2 (A) and F2 (B) peptides. J coupling constants are measured in Hz.

Table 11. Structural statistics of F2 and Y2 peptides.

	Y2 dimer	F2 dimer
NMR distance constraints		
Total NOE	128	144
(unambiguous distance restraints)		
Intra-residue	102	108
Inter-residue	12	16
Sequential (i - j = 1)	8	10
Medium (2 ≤ i - j ≤ 4)	4	6
Long range (i - j ≥ 5)		
Intermolecular	14	20
Structure statistics		
Average pairwise r.m.s. deviation ^a (Å) (monomer, residue 3 - 7)		
Backbone (Å)	1.03±0.57	0.93±0.28
Heavy sidechain (Å)	2.81±1.03	2.60±0.50
Average pairwise r.m.s. deviation ^a (Å) (dimer, residue 3 - 7 and 3' - 7')		
Backbone (Å)	1.67±0.66	1.50±0.26
Heavy sidechain (Å)	3.78±0.98	2.92±0.47

^aPairwise r.m.s. deviation was calculated for all 20 final Y2 and F2 dimer structures.

- **Molecular Dynamics simulations**

AMP charge densities were suggested to be important factors for their antibacterial activities [83], local charge distribution and density within C2, Y2 and F2 dimers were analyzed in molecular dynamics simulations. The radius of gyration of charged points (cR_g), which reflects the distribution of charges within the peptides, was calculated (Figure 3.9). Charge densities of the peptides were estimated using Equation 6. The charge density of Y2 shows 30% increase than that of C2 or F2. The charge density of the original hBD-3 C-terminal peptide (R36-K45) (Protein Data Bank entry 1KJ6 [84]) was also calculated and showed comparable result to that of C2 and F2.

$$\rho = \frac{Q}{V} = \frac{Q}{\frac{4}{3\pi} {}^cR_g^3}$$

Equation 6. Equation for the charge density calculations. Q is the total charge of a peptide dimer ($Q=14e$ for all peptides). The average cR_g for C2 and F2 dimers is 14.9 ± 0.25 Å each, and the average cR_g of the Y2 dimer is 11.9 ± 0.28 Å.

Figure 3-7. Distribution function of the radius of gyration for charged points in C2, F2 and Y2 dimers.

Average binding free energies for Y2 and C2 dimers were also calculated (data not shown, refer to [100]). Y2 dimer is more stable. Its lower free energies mainly originate from the π - π stacking of the aromatic side chains of four tyrosine residues resulting in a better packing conformation.

The interactions between Y2 peptide and DPC micelles were also explored by long MD simulations. Strong interactions between charged amino acids and DPC polar head groups were observed. One snapshot obtained at the simulation time of 50 ns demonstrated close contacts between the side chains of positively charged residues (Arg 1, Arg 3, Lys 4, Arg 7, and Arg 8) and the DPC phosphatidylcholine groups (Figure 3.10). Such interactions are expected to be even stronger for bacterial membrane lipids which have negatively charged head groups.

Figure 3-8. Representative snapshot of the Y2-DPC configuration in the 30 ns long MD simulations run. The Y2 dimer is highlighted in ribbon. DPC molecules are labeled according to atoms. This figure was prepared with PyMol [175].

3.2 Progressive structuring of a branched antimicrobial peptide on the path to the inner membrane target

AMP bactericidal mechanism studies using linear hBD-3 peptides suggested that charge density is a determinant for the activity and selectivity of AMPs; meanwhile hydrophobicity is important for AMP-membrane interactions. Among those hBD-3 linear derivatives, V2 and Y2 peptides showed high bacterial killing efficiency but very low cytotoxicity against mammalian cells. V2 was selected as a functional motif due to its higher hydrophobicity. To lower the effective antimicrobial concentrations by improving charge density, two monomer peptides were linked at the K9-NH₂ group, forming the branched peptide B2088.

Progressive structure accretion of B2088 was observed in water, LPS, and lipid environments. Despite inducing rapid aggregation of outer membrane lipopolysaccharides, B2088 remain highly mobile in the aggregated lattice (see below results). These findings suggest that AMPs possessing both enhanced mobility in the bacterial outer membrane and spatial structure facilitating its interactions with the membrane-water interface may provide excellent structural motifs for developing broad-spectrum new antimicrobials. The work has been published in Journal of Biological Chemistry, 2012 [176].

- **Antimicrobial Activities of B2088 and Its Analogous Peptides**

Linearized B2088 (linear V2 dimer), truncated B2088 (truncated V2 dimer), B2088 with modified neutral spacers (V3 dimer and L2 dimer), and modified C termini (V2–1A dimer, V2–1D dimer, and V2–1K-amide dimer) were designed and chemically synthesized. Antimicrobial

activities of those peptides against Gram-negative bacteria strains were tested and compared with B2088 (Table 12).

Table 12. Minimal inhibitory concentrations of B2088 and its analogous variants against Gram-negative ATCC strains.

Peptides	Sequences	Activity (μM) ^a	
		minimal inhibitory concentration	
		<i>P. aeruginosa</i> ATCC 27853	<i>E.coli</i> ATCC 25922
V2 monomer	RGRKVVRKK	>39	39
B2088 (V2 dimer)	(RGRKVVR) ₂ KK	2.75	2.75
Linear V2 dimer	RGRKVVRKKRRVVKRGR	10.91	10.92
Truncated V2 dimer	(KVVR) ₂ KK	>32.22	16.11
V3 dimer	(RGRKVVR) ₂ KK	2.51	2.51
V2-1A dimer	(RGRKVVR) ₂ KA	5.60	5.60
V2-1D dimer	(RGRKVVR) ₂ KD	5.49	5.49
V2-1K-amide dimer	(RGRKVVR) ₂ KK-NH ₂	5.46	5.46
L2-dimer	(RGRKLLR) ₂ KK	2.66	5.33

^aMeasurements have been done in triplicates, with the error bar within 30%.

Besides V3 dimer and L2 dimer, B2088 showed lower MIC values than the rest of the peptides. Peptides with modified charge state (V2–1A dimer and V2–1D dimer) and amphiphilicity (truncated V2 dimer) showed increased MICs, indicating that amino acid composition is important for antimicrobial activity as stated in the interfacial activity model. On the other hand, peptides with similar amino acid composition but different three-dimensional structure also show lowered antimicrobial activity, like

linear V2 dimer. This suggests that exploring peptide structure and peptide-lipid interactions at an atomic level was necessary for understanding the structure-function relationship of this peptide series. V3 dimer and L2 dimer showed comparable activity to B2088. The amphiphilicity and chemical composition of those peptides are similar to those of B2088, suggesting that a similar antimicrobial mechanism could be expected. We selected B2088 for further study as a representative molecule that is also comparatively easy to produce.

The V2 monomer peptide displayed weak antimicrobial activity against *E. coli* and *P. aeruginosa* comparing to B2088 (Table 12 and Table 13). The activity was attributed to the cooperative formation of a non-covalent dimer in aqueous solution and at the lipid-water interface [100]. The antimicrobial activities of B2088 peptides against Gram-negative reference bacteria and clinical isolates were tested and compared to gentamicin, a well-known aminoglycoside targeting Gram-negative bacteria that is used in the ophthalmology clinic (Table 13). B2088 showed lower MIC values than both the monomer peptide and gentamicin. It was also active against gentamicin-resistant clinical isolate *P. aeruginosa* DR4877/07. Importantly, at the concentrations corresponding to the MIC values, B2088 did not undergo oligomerization, as was shown by NMR diffusion experiments.

Table 13. Minimal inhibitory concentration of B2088 against various Gram-negative ATCC and clinical isolates.

<i>Pathogen strains</i>	<i>Minimal inhibitory concentration^a</i>		
	B2088	Monomer	Gentamicin
<i>P.aeruginosa</i> ATCC 9027 ^b	2.75	>39	N.D ^c
<i>P.aeruginosa</i> DR4877/07 Source: sputum	5.5	N.D	837.5
<i>P.aeruginosa</i> DM023104 Source: eye	5.5	N.D	N.D
<i>P.aeruginosa</i> DR15013 Source: wound	2.75	N.D	N.D
<i>P.aeruginosa</i> DU14476/07 Source: urine	2.75	N.D	N.D
<i>E.coli</i> ATCC25922 ^b	2.75	39	N.D
<i>E.coli</i> DU46381R Source: urine	2.75	N.D	N.D
<i>E.coli</i> DB16027 Source: blood	2.75	N.D	1.63

^aUnit of MIC is μM . ^bReference organisms from American Type Culture Collection (ATCC) strains. All the others are clinical isolates with source indicated. ^cN.D is not determined.

In experiments tracking kinetics of antibacterial activity, B2088 displayed rapid killing ability against the reference *P. aeruginosa* ATCC 9027, reducing an inoculum by more than 6 log cfu/ml in less than 10 min at 2 times the MIC value (Figure 3.11 A). Even for the gentamicin-resistant strain *P. aeruginosa* DR4877/07, reduction of $>10^5$ organisms could be achieved using less than 10 μM B2088 within 20 min

(Figure 3.12), suggesting that a membrane-targeted action is likely. Resistance of *P. aeruginosa* ATCC 27853 against B2088 was also tested (Figure 3.11 B). Over more than 17 bacterial passages, the MIC value of B2088 only rises about 4 times higher, whereas for gentamicin, bacteria developed 32 times MIC resistance, and for norfloxacin, more than 64 times higher MIC concentration was needed to inhibit the bacteria growth. Both bacterial killing kinetics and the resistance assay suggest the likelihood of a membrane-targeting action by B2088.

Figure 3-9. A, time kill kinetics of B2088 against *P. aeruginosa* ATCC 9027. B, bacterial resistance assay of B2088 and conventional antibiotics against *P. aeruginosa* ATCC 27853. The x axis represents the bacteria serial passage number, whereas the y axis represents fold increase in MIC. *Black dotted line*, V2 dimer peptide; *red line with square*, Norfloxacin; *blue line with triangle*, Gentamicin.

Figure 3-10. Time-kill kinetics of B2088 against Gentamicin-resistant clinical isolates *P. aeruginosa* DR4877/07.

- **Cytotoxicity of B2088 against Mammalian Cells**

Human conjunctiva epithelial cells were chosen for an *in vitro* cytotoxicity assay (Figure 3.13 A). Even at a peptide concentration of 200 $\mu\text{g/ml}$ (*i.e.* 88 μM), 16–32 times higher than B2088 MIC values for various pathogens, B2088 was found safe (86% cell viability), indicating a high therapeutic index. Hemolytic activity of B2088 was tested using rabbit red blood cells. Up to peptide concentrations of 2 mg/ml, no hemoglobin release was detected (Figure 3.13 B). Applying B2088 to the rabbit eye *in vivo*, no clinically detectable effect was seen on corneal wound healing in a rabbit model (Figure 3.14). Wound healing is a critical issue with the cornea as the presence of a wound increases the likelihood of infection and further visual loss, consequently testing corneal wound healing is an important indicator of biocompatibility of a new therapeutic agent [177].

Figure 3-11. A, cytotoxicity assays using human conjunctiva cells with the monomer or B2088 peptides showed these peptides to be safe. Reported values are means of performed experiments with *error bars* representing the square deviation; **B, hemolysis assay of B2088 against rabbit erythrocytes.**

Figure 3-12. *In vivo* studies of B2088 (1 mg/ml) on rabbit corneal wound healing. No significant effects of B2088 at 1 mg/ml on wound closure were observed, showing a high level of safety.

- **Lipid Phase Partition of B2088 and Monomer Peptide**

To characterize the affinity of B2088 and its monomeric precursors for the lipid phase, translational diffusion coefficients (D_S) for free peptides and peptide micelle complexes were measured using DOSY experiments [170]. Pure DPC and mixed DPC-POPG micelles were used to represent mammalian and bacterial cell membranes, respectively. Table 14 shows the molar fractions of peptides bound to micelles (F_L) calculated using the measured diffusion coefficients [162]. This parameter reflects the lipophilicity and affinity of the peptides for the lipid membranes. Partitioning in zwitterionic DPC micelles was only 50% for all studied peptides, in contrast to the full localization into the same lipid phase doped with anionic POPG lipids. This observation was consistent with their high antimicrobial activity and low cytotoxicity.

Table 14. B2088 and its monomeric peptide lipid phase partitioning.

F_L^a (100%)	Monomer	B2088
DPC	47.65	67.94
DPC-POPG	100	100

^a F_L , molar fraction of peptide that binds to lipid phase.

- **B2088 Interacts with Bacterial Outer and Inner Membranes**

We investigated the interactions of B2088 with bacterial outer and inner membranes using membrane probes. NPN, a membrane-impermeable dye, was used to determine if the peptide acted on the outer membrane of *P. aeruginosa* ATCC 9027. As an impermeable hydrophobic molecule with weak fluorescence in buffer, disruption of the outer membrane allowed the dye to partition toward the inner membrane with enhanced fluorescence [178,179]. Figure 3.15 A shows that the addition of B2088 to the intact bacteria leads to an abrupt increase in the NPN fluorescence intensity. At a concentration of $5.3 \pm 0.1 \mu\text{M}$, B2088 led to a 50% increase (PC_{50}) in NPN fluorescence emission intensity. This concentration corresponded to the previously determined MIC values, implying a correlation between outer membrane disruption and bacterial killing.

We next examined the effect of B2088 on the bacterial inner membrane using the membrane potential-sensitive fluorescent dye diSC₃5. This dye is taken up by the bacteria and self-quenched as the dye concentrates in the cytoplasmic inner membrane. Any disturbance of the membrane potential gradient across the inner membrane will result in the release of the dye and increased fluorescence intensity [180,181]. Figure 3.15 B shows the peptide concentration-dependent fluorescence dequenching of diSC₃5 upon the addition of B2088 to intact bacterial

cells. Below the MIC values, minimal change in the fluorescence intensity was observed. However, above the MIC value, upon the addition of B2088, a significant increase in fluorescence signal was observed immediately followed by a plateau phase. The maximum change in fluorescence intensity was reached in less than 10 min, under which conditions time kill assays showed a 99% reduction in bacteria viability.

Figure 3-13. Effect of B2088 on *P. aeruginosa* ATCC 9027. A, outer membrane permeabilization monitored by NPN uptake. The increase in fluorescence intensity of NPN is plotted against the concentration of B2088. The graph is fit to a sigmoidal function, and PC_{50} was estimated. B, depolarization of inner membrane monitored by diSC₃₅ assay.

- B2088 Structure and Dynamic Studies with Bacterial Outer Membrane LPS Extract**

Lipopolysaccharides are negatively charged molecules decorating the outer leaf of the outer membrane of Gram-negative bacteria. *P. aeruginosa* LPS extract was used to study peptide/bacterial outer membrane interactions. In a wide range of explored concentrations above the MIC values, the addition of B2088 immediately caused turbidity in an otherwise clear and stable solution of LPS micelles. Under TEM, we observed the LPS assembly transformed from a ribbon-like structure into large polymorphous aggregates (Fig. 3.16).

Figure 3-14. The LPS assembly transition induced by adding B2088 was observed by TEM. A, without B2088, LPS formed a ribbon-like assembly, soluble in solution; B, after adding in B2088, LPS formed large amorphous aggregates, which precipitated out from solution.

The three-dimensional structural analysis of B2088 in polymorphous LPS was carried out using NMR. Residual dipole-dipole interactions and magnetic anisotropy of the heterogeneous LPS precipitated by B2088 resulted in broadened and featureless ^1H solution NMR spectra. However, the use of the HR-MAS technique in combination with residue-selective ^{15}N and ^{13}C labeling of B2088 enabled us to obtain the structure of B2088 in complex with LPS. Under these conditions, a $[\text{}^1\text{H}-^{15}\text{N}]\text{-HSQC}$ spectrum of selectively ^{13}C , ^{15}N -labeled B2088 showed four well resolved cross-peaks with chemical shifts displaced from those found in aqueous solution. A minor conformation of the peptide was also detectable in the spectrum (Figure 3.17). The observed resonances were assigned to particular residues using two-dimensional NOESY, three-dimensional HNCA, and ^{15}N -resolved NOESY experiments. The assignment was extended to well resolved ^1H resonances stemming from the unlabeled residues. Three-dimensional structure of the peptide was reconstructed using the program CYANA 3.0.

Figure 3-17. Ensembles of NMR conformers of B2088 in LPS (peptide/LPS molar ratio = 1:20) (A) and in DPC-POPG mixed micelles (peptide/DPC/POPG = 1:30:13) (B). Backbones of primary and secondary chains are labeled in blue and cyan, respectively. The side chains of charged amino acids are shown in orange. The Lys 9 side chain linking the primary and secondary chains is highlighted in gold. $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ - HSQC spectra of B2088 in aqueous solution (C), LPS (D), and DPC-POPG mixed micelles (E). In E, the secondary chain that is more tightly structured is labeled in cyan with a prime. Additional peaks coming from a minor conformation are highlighted in pink. F-H, $[\text{}^1\text{H}, \text{}^{15}\text{N}]$ -HSQC spectra of B2088 peptides in POPG-POPE liposomes (peptide/POPG ratio equals 1:1, 1:12 or 1:13).

Intra-molecular dynamics were assessed via ^{15}N T_1 , T_2 (Table 15), HNOE relaxation and ^{13}C chemical shifts measurements (Figure 3.18) for four backbone amide moieties and a side-chain moiety of Arg 3.

Table 15. ^{15}N backbone relaxation^a of B2088 in LPS.

	Arg 3 ^b	Lys 4	Val 5	Val 6 ^c	Arg sc ^d
T_1 (s)	1.41	1.06	1.06	1.08	3.57
T_2 (s)	0.93	0.50	0.43	0.43	1.41
HNOE	-2.32	-1.01	-0.80	-1.07	-5.92

^a T_1 , T_2 , (^{15}N) and $^1\text{H}/^{15}\text{N}$ heteronuclear NOE experiments performed on a 700 MHz spectrometer. ^bAssignment of cross-peaks is shown in [^1H , ^{15}N]-HSQC (Figure 3.18). ^cThe resonances for the second chain (residues 11-18) in the branched structure are overlap with resonances of the first chain (residues 1-8). ^dArg sc: Arginine side-chain group.

Figure 3-18. Secondary $^{13}\text{C}_\alpha$ chemical shifts (upper panel) and ^1H , ^{15}N HNOE (lower panel) for the ^{15}N backbone residues and Arg 3 side chain of B2088. Left, middle and right panels correspond to the peptide in LPS, in liposomes (B2088/POPE/POPG = 1:3:1), and in DPC-POPG mixed micelles, respectively. Dark and light blue bars represent the backbone of primary and secondary chains of the peptide in DPC-POPG, respectively. In LPS and liposomes, the chemical shifts of two branched chains are spectroscopically unresolved.

In polymorphous large LPS aggregates, B2088 adopted an extended conformation with spectroscopically indistinguishable primary and

secondary chains. Val residues were significantly more dynamically restrained than flanking Arg and Lys residues. Arg side chains showed the highest flexibility, implying that the Arg guanidinium groups were not immobilized by electrostatic interactions with the oppositely charged LPS. Although no direct inter-chain NOEs were detected, it appeared likely that in water and LPS, the peptide exhibited a compact structure mediated by hydrophobic interactions between Val side chains, resulting in restricted backbone dynamics at the central hydrophobic residues. The translational diffusion rates in water indicated that the hydrodynamic radius of B2088 might be in close correspondence to that found for the structurally compact dimer of the non-covalently linked monomer peptides (Table 16) [100]. In addition, the translational diffusion rates of B2088 in water showed no concentration dependence, indicating that B2088 did not further dimerize, presumably due to the absence of surface-exposed hydrophobic groups.

Table 16. Translational diffusion coefficients of B2088 and its monomeric peptide in phosphate buffer.

$D_s^a (10^{-10} m^2 s^{-1})$	Monomer	B2088
5 μ M	5.32 $\pm$ 0.17	2.30 $\pm$ 0.20
500 μ M	2.67 $\pm$ 0.10	2.30 $\pm$ 0.20
Oligomerization states	Dimer	Monomer

^a D_s is the translational diffusion coefficient.

To test whether the aggregation of LPS was only due to the effect of electrostatic neutralization of LPS by countercharged groups of the peptide, calcium chloride was used to induce LPS aggregation. The results showed that to induce the same degree of LPS precipitation, a

300-fold higher concentration of calcium chloride was needed. These findings emphasized the importance of the underlying three-dimensional structure of the branched AMP compared with linear AMPs and hydrated cations.

Although the translational diffusion rates of B2088 in aggregated LPS matrix was of considerable interest, the Z-gradient pulsed field strength available in the commercial HR-MAS probe was not sufficient for quantitative measurements of the rates. Nevertheless, data showed that while maintaining the high rotational diffusion rate, the translational diffusion of the peptide was significantly impeded in LPS but not quenched entirely. We propose that maintaining high mobility in heterogeneous condensed phase enables the peptide to penetrate through the LPS-decorated bacterial outer membrane and diffuse into the bacterial inner membrane, as was shown by environment - sensitive fluorescent assays. Serving as bacterial membrane extensions, LPS molecules effectively enlarge the reactive surface of the bacteria in relationship to the volume; interacting with LPS through strong electrostatic attractions also helps B2088 to achieve effective high concentrations at the bacterial outer membrane.

- **B2088 Structure and Dynamic Studies in Bacterial Inner Membrane Mimics**

To explore conformational transitions and dynamics of the peptide in lipid phases, we used neutral and negatively charged micelles mimicking the lipid composition of mammalian cell membrane as well as bacterial inner membrane, respectively. The binding affinity of B2088 to mixed micelles was explored using DOSY experiments. The peptide showed only weak partitioning into the neutrally charged DPC micelles. Admixture of POPG

(DPC/POPG = 3:1) increased the total negative charge of micelles, causing full partitioning of the peptide into the lipid phase. This was accompanied by accretion of a compact three-dimensional structure of the peptide shown in the HSQC spectrum (Figure 3.17 E) and HNOE data (Figure 3.18 F). Moreover, primary and secondary chains of B2088 manifested different structural associations because the degeneration of resonances stemming from those chains was abolished in the HSQC spectrum (Figure 3.17 E). We determined the detailed three-dimensional structure of B2088 in DPC/POPG micelles (Figure 3.17 B) using NOEs and $^{13}\text{C}_\alpha$ chemical shifts (Figure 3.18 C). In this structure, the primary backbone chain was folded back around the secondary chain with four Val residues occupying the core positions with NOE constraints from Arg 7' (secondary chain) to Val 5 and Arg 8' to Arg 3 proving the spatial proximity between the two chains. Differential structural association between the two chains implied that the covalent branched linkage might provide a new structural quality surpassing just a means to facilitate chemical association.

Several studies have described a strong link between the cationic charge on a peptide and its antimicrobial activity [81]. In addition, the local charge distribution and density within the peptide have been hypothesized to be important factors [83]. We had earlier developed a metric of the peptide charge density as the ratio of the total charge on the peptide and its volume [100]. Our findings suggested a strong correlation between charge density and antimicrobial activity. Thus, we compared the charge density of the monomer and B2088 based on their structures in DPC-POPG micelles (Figure 3.19). Remarkably, B2088 in DPC-POPG micelles had a 30% greater charge density than the monomer, implying a

direct connection between three-dimensional structure and antimicrobial activity.

Figure 3-15. 3D NMR structures of monomer peptide (A,B) and B2088 (C,D) in the presence of DPC-POPG (30 mM DPC and 13 mM POPG) mixed micelles. A and C are the super-position of 20 conformers; B and D are a representative conformer. The N- and C-termini of peptides are indicated. Backbones of individual chains are labeled in light and dark blue and numbered accordingly. Side chains of Val and charged amino acids are highlighted in purple and red. The Lys 9 linker side chain in B2088 is represented in balls and sticks form.

The nature of lipid/peptide interactions was explored by pH scanning experiments using NMR and MD simulations utilizing both atomic and coarse grained membrane models (Figure 3.20). ^1H chemical shifts of the peptides and lipids were recorded as a function of pH in the range from 1 to 9 in a series of one dimensional ^1H and two-dimensional [^1H , ^{15}N]- HSQC spectra. The pK_a values of peptide backbone amide

moieties of individual amino acids and Arg side chains, as well as $^1\text{H}^{\alpha, \beta}$ of DPC/ POPG were estimated via numerical data interpolation and differentiation (Figure 3.20 D, E). We found that the intrinsic basic pK_a of Arg guanidinium groups and acidic pK_a of lipid protons near phosphate groups ($^1\text{H}^{\alpha, \beta}$) were shifted toward each other in the peptide-micelle complex, suggesting electrostatic interactions or hydrogen bonding between these groups. Atomic simulations using peptides in DPC/POPG mixed micelles showed transient hydrogen bonds between peptide Arg/Lys side chains and lipid phosphate groups. Arg manifested greater hydrogen bonding ability than Lys (Figure 3.20 B). This is probably due to the fact that the guanidinium group of Arg can form double hydrogen bonds with PO_4 groups, which is energetically more favorable than the hydrogen bond between Lys side chain and lipids [182,183]. Coarse grained simulations using POPG-doped bacterial membrane models showed that B2088 initially solvated in water, rapidly adsorbed onto the negatively charged membrane surface, and remained at the membrane water interface (Figure 3.20 C). Moreover, B2088 showed clear preferential interactions with anionic POPG rather than zwitterionic DPC and POPE, leading to redistribution of membrane lipids and the emergence of POPG-rich domains (Figure 3.22). These may decrease the membrane stability, eventually resulting in membrane depolarization that is observed in the fluorescent assays.

Figure 3-16. A, chemical groups of B2088 and DPC found in close proximity with the peptide solubilized in DPC/POPG micelles. Dashed red lines represent hydrogen bonds between the DPC PO_4 group and Arg/Lys side chains. An arrow represents NOEs observed in the NOESY spectrum between Val $^1H_\alpha$ and DPC $^1H_\beta$ (Figure 3.21). PO_4 groups are shown in yellow. Arg guanidinium groups are shown in green, and the NH_3 groups of Lys are shown in cyan. **B and C, MD simulations of B2088 in lipid bilayer and DPC/POPG micelles, respectively.** **D and E, chemical shifts as a function of pH for $^1H^N$ of Arg 13 and $^1H^\alpha$ of DPC, respectively.** The fitted titration curves and the numerical first derivatives are shown.

Figure 3-17. A) ^1H - ^1H NOESY spectrum of B2088 in DPC-POPG (fingerprint region). NOE cross-peaks between $^1\text{H}^{\text{N}}$ Valine residues and $^1\text{H}_{\alpha}$ are highlighted in red. B) Illustration of space proximity between valine backbone and DPC $^1\text{H}_{\alpha}$, phosphate group.

Figure 3-18. Initial configuration of the upper leaflet of the bacterial membrane (A and B) and final configuration of the upper leaflet at 2 μs (C and D). Peptide: yellow and green spheres; POPG: red lines;

POPE: blue lines. Blue and red spheres are phosphate beads of the POPE and POPG lipids, respectively.

Retaining high rotational mobility of B2088 in lipids enabled the extension of structural studies of the peptide to POPE/ POPG mixed liposomes. In accordance with other studies of AMPs in lipid vesicles [184], at concentrations above 0.1 mM, B2088 caused immediate turbidity in an otherwise clear solution of mixed liposomes with POPE/POPG = 3:1 used to mimic bacterial anionic membranes. Due to the large size and complex morphology of the resulting magnetically anisotropic and inhomogeneous peptide/liposome solutions, HR-MAS NMR was employed in order to detect resolved NMR spectra. We found that at the stoichiometric ratio of B2088/POPE/POPG = 1:39:13, the peptide exhibits reduced rotational diffusion rates and intra-molecular dynamics comparable with that found in DPC/ POPG micelles (Figure 3.17). With an increase in the relative peptide concentration of B2088/POPE/POPG = 1:3:1, accelerated rotational diffusion of the peptide similar to that found in LPS accompanied by chemical shift changes in the [^1H , ^{15}N]- HSQC spectra was detected (Figure 3.17 F and Table 17). Analysis of the spectra showed that at lower peptide concentrations, two sets of cross peaks stemming from predominantly membrane-bound and membrane-dissociated states of the peptide, respectively, could be detected. Only one set of coalescent resonances was observed at intermediate peptide concentrations (Figure 3.18 G). This indicated the slow to intermediate (in chemical shift time scale) exchange of peptide bound to and dissociated from the lipid surface.

Table 17. ^{15}N backbone relaxation of B2088 in liposome.

	Arg 3 ^b	Lys 4	Val 5	Val 6 ^c	Arg sc ^d
T_1 (s)	1.45	1.25	1.05	1.00	3.70
T_2 (s)	1.08	0.58	0.53	0.48	1.15
HNOE	-2.98	-1.40	-0.93	-1.15	-7.38

^a T_1 , T_2 , (^{15}N) and $^1\text{H}/^{15}\text{N}$ heteronuclear NOE experiments performed on a 700 MHz spectrometer. ^bHNOE is calculated by the peak intensity in the saturated spectrum divided by that in the unsaturated spectrum. ^cThe resonances for the second chain (residues 11-18) in the branched structure are overlap with resonances of the first chain (residues 1-8).

^dArg sc: Arginine side-chain group.

• Comparison of interactions of B2088 with liposomes and bacterial membranes

To compare the interaction affinities of B2088 towards liposomes in biophysical studies and to living bacteria in biological assays, stoichiometric ratios of B2088 binds to lipids in both conditions were calculated and compared. In the NMR experiments studying the structure and dynamics of B2088, peptide concentration of 1.6 mM was used. Saturated B2088 membrane binding was only observed with the total lipid concentration at 104 mM. Prepared liposome medium has homogenized vesicle diameter of 100 nm and the average surface area of lipid head group is about 55 Å. Taking into account that liposome vesicles have bilayer lipid membranes, on average only 0.056 peptide could be found per every 1 nm² of liposome surface. Hence, maximum stoichiometric ratio of B2088 binds to liposomal lipids is 1 to 32.

Biological assays are carried out at bacterial concentration of 106 cfu/ml. We assume that a *P. aeruginosa* cell is on average 2 µm wide and 2-5 µm long in size. Taking into account that the average surface size of

bacterial membrane phospholipids (POPE or POPG) is about 55 Å, the number of lipids is estimated to be in the range of $45\text{-}90 \times 10^{12}$. From bactericidal assay we estimated the B2088 peptide MIC value is 5.5 μM , thus the number of peptide molecules is estimated as 33×10^{14} per 1 ml of bacterial culture. From these data we calculate that, on average, 100 peptide molecules bind to 1 nm^2 of the bacterial surface, or, approximately 80 peptide molecules per 1 lipid molecule constituting the bacteria membrane.

In summary, at a ratio of B2088/POPE/POPG = 1:39:13 about 0.056 peptide molecules were bound to 1 nm^2 of the liposome surface (1 peptide to 32 lipids). This can be compared with 105 peptide molecules/1 nm^2 of the bacterial surface (82 peptides to 1 lipid) estimated from our standard bacterial sterilization experiment. These numbers clearly indicate a significantly lower affinity of B2088 to model liposomes in comparison with the surfaces of live bacteria, which is corroborated the calculations performed in the other studies [115].

3.3 Oligomerization of xanthone derivatives lead to bacterial membrane disruption

Our studies of the linear hDB-3 peptides (see Chapter 3.1) showed that the aggregated charge density might serve as a predicting determinant of the activity of AMPs. The branched dimeric peptide B2088 with the particularly high charge density shows significantly improved bactericidal activity against Gram-negative bacteria. However, AMPs consisting of only genetically encoded amino acids bear inherent bio-instability and highly biodegradable. In particular vulnerable is the peptide bond between Lys and Arg which might be rapidly cleaved by the bacterial and environmental trypsin-type serine proteases, thus inactivating AMP. We directly observed this by adding 2 mM isotopic labeled V2 monomer peptide into 7×10^9 cfu/ml bacteria *P. aeruginosa* in cell suspensions. The time series of ^{15}N -HSQC spectra were acquired to trace the possible changes in chemical structure of the V2 monomer peptide using HR-MAS probe (Figure 3.23). The Arg 3 cross-peak disappeared after one hour, while the Lys 4 cross-peak completely shifted to the new position after two and a half hours. This observation was interpreted as the digestion of peptides by cleaving the amide bond between Arg 3 and Lys 4. To prove the result, after two and a half hours incubation, the molecular weight of the resulting peptides was measured by mass spectrometry. The peptide weight decreased by the amount corresponding to three terminal amino acids verifying the proposed cleavage pattern.

Figure 3-19. ^{15}N -HSQC spectra of V2 monomer peptide in bacteria *Pseudomonas aeruginosa*. Assigned peaks are from ^{15}N -isotopic labeled residues Arg 3, Lys 4, Val 5 and Val 6. Spectra are arranged in time series. Top left spectrum (A) is acquired initially, and bottom right spectrum (F) is acquired after 2.5 hours, leaving half an hour interval between two adjacent spectra. Peaks showing significant change (Arg 3 and Lys 4) are highlighted in red.

To avoid the undesired bio-degradation, AMP mimetics composed of natural amino acids attaching to organic xanthone group were designed. Xanthone scaffolds are known to exhibit anti-microbial properties whereas showing high cytotoxicity at the same time [111,140]. Biophysical studies showed xanthenes form high-order oligomers due to π - π stacking in the aromatic rings [140]. Charged amino acids fused to the xanthone scaffold were chemically attached in the attempt to improve the selectivity of the product [185]. Promising bactericidal activity against

Gram-positive bacteria mainly MRSA as well as dramatically decreased cytotoxicity were observed when two Args were used as the modifying group. The resulting lead compound is named as AM052 for the consistency in our chemically-derived series (chemical structure of AM052 could be found in Introduction section, Chapter 1.2.3). The oligomerization states of AM052, dynamic properties and membrane interactions were studied using NMR spectroscopy, facilitated by fluorescent emission studies, transmission electron microscopy and molecular dynamics simulations.

- **Antimicrobial properties of AM052 against Gram-positive and –negative bacteria**

Antimicrobial properties of AM052 were tested against both MRSA and *P. aeruginosa*, which are the representatives of Gram-positive and –negative bacteria respectively. LTX-109, a peptidomimetic antimicrobial drug developed by Lytic Biopharma which kills bacteria through membrane lysis action, was used for cross comparison. Selectivity of AMPs were calculated using minimal inhibitory concentration (MIC) of AMPs against bacteria divided by hemolysis concentration of AMP on mammalian red blood cells (Table 18 and Table 19).

Table 18. AM052 minimal inhibitory concentration and selectivity against MRSA*.

Antibiotics	MRSA clinical 6506		MRSA ATCC 43300	
	MIC	Selectivity	MIC	Selectivity
AM-052	3.125	75.84	3.125	75.84
LTX-109	4-8	22-44	4-8	22-44

* MRSA: Methicillin-Resistant *Staphylococcus aureus* (MRSA). LTX-109 is a peptidomimetic antimicrobial drug developed by Lytic Biopharma (Figure 1-7).

Table 19. AM052 minimal inhibitory concentration and selectivity against *P. aeruginosa*.

Antibiotics	<i>P.A</i> * clinical 4877		<i>P.A</i> ATCC 14476	
	MIC	Selectivity	MIC	Selectivity
AM-052	25	9.48	25	9.48
LTX-109	4-8	22-44	16	11

* *P.A*: *Pseudomonas aeruginosa*

The results summarized in Tables 15 and 16, AM052 show indeed significantly reduced cytotoxicity and promising bactericidal activity against Gram-positive bacteria MRSA. Its selectivity is even better than that of LTX-109, which is the drug in clinical trials against MRSA. Whereas for Gram-negative bacteria, significantly higher MIC values of AM052 were seen. This could be explained by the reduced molecular dynamics and mobility in the postulated higher order oligomers of AM052

formed by π - π stacking in the aromatic rings. According to our previous studies [176], large oligomers of AMPs might be locked in the lipopolysaccharide layer of the Gram-negative bacterial outer membranes resulting in the decreased accessibility of AMPs to the cytoplasmic membrane showing the markedly decreased bactericidal effect.

- **AM052 induced bacterial cytoplasmic membrane leakage**

To explore the effect of AM052 on bacterial cytoplasmic membranes, both fluorescence emission studies and NMR spectroscopy were utilized. A mimic of the bacterial cytoplasmic membrane was reconstructed using large unilaminar vesicles constituting 80% DOPE mixed with 20% DOPG.

In fluorescence emission studies, a fluorescence dye calcein was encapsulated in liposomes with the minimal fluorescent intensity. Upon the membrane disruption, calcein leaks out from the vesicles showing quantitatively increased fluorescent emission intensity (Figure 3.24 A) which is used to quantify the amount of the disrupted liposomes.

Dye leakage from the LUVs showed that AM052 can induce membrane disruption starting from AM052-to-lipids ratio of 1:64. With the increased AM052 concentrations, calcein leakage increased from 10% to 80% at AM052-to-lipids ratio of 1:8. Upon addition of AM052, an immediate dye leakage event was observed within 50 seconds coincided with its fast bacterial killing kinetics. Interestingly, leakage equilibrium was reached not much longer, on average within 200 seconds upon addition of AM052. No further dye leakage was observed after that regardless of the AM052 concentration added. When the graph of the AM052 concentration added versus the percentage of calcein leakage

reached at equilibrium, a close to linear curve rather than hyperbolic curve was observed (Figure 3.24 B). This means at lower AM052 concentrations, only a fraction of liposomes were disrupted by saturated AM052 binding leaving the rest of vesicles intact presumably caused by non-equal distribution of AM052 binding to liposomes. Liposomes with saturated AM052 binding leaked immediately while some others remained undamaged due to insufficient amount of AM052 molecules bound to the membrane surface to form a membrane disruptive patch (see our proposed patch model of liposomal disruption).

Figure 3-20. AM052 induced leakage of artificial bacterial membrane—calcein leakage assay. A. Percentage of calcein release as monitored through time series with increasing AM052-to-lipids ratio. 0.2% of DMF was used in control experiment. B. Percentage of calcein release at equilibrium plotted against applied AM052 concentration.

The AM052-induced bacterial membrane leakage was explored by NMR spectroscopy using DOPE/DOPG mixed liposomes with encapsulated reporter molecules. Two set of parallel experiments were designed, sodium formate entrapped inside of liposome when the paramagnetic chelator omniscan free in solution or the opposite preparation. Line broadening of the ^1H and ^{13}C signals from sodium formate will be seen once the omniscan got in spacial proximity to the formate group due to membrane disruption.

In the experimental setup with sodium formate encapsulated in the liposomes, peak intensity of the formate resonance started to drop after the concentration of AM052 beyond 100 μM (Figure 3.25). A gradual decrease of the formate signal intensity with the gradual increase of AM052 concentration was observed rather than all-or-none type of spectra change. The results are in good agreement with the fluorescence emission studies, which showed cumulative liposomal leakage with increased AM052 concentration. Graded leakage observed in both NMR and fluorescence emission studies proved unequal binding of AM052 to liposomes.

The other set of NMR experiments using omniscan encapsulated inside of liposome while sodium formate free in solution showed similar results. Gradual line broadening effect was seen from formate signals. Signal from free formate in solution completely vanished after adding 500 μM of AM052. This suggests AM052 induced membrane disruption was large enough to allow bulky molecules like omniscan to leak out. Large amount of omniscan (about 10 mM) released from liposomal vesicles caused severe line broadening beyond detection of any signal.

Figure 3-21. Omniscan-induced line broadening of 1D ^1H spectra (A) and 1D ^{13}C spectra of sodium formate (B) in DOPE/DOPG mixed liposomes. Signal intensity of formate resonances plotted against increasing AM052 concentration.

- **Imaging study showing membrane disruption induced by AM052**

In order to visualize the AM052 induced changes in bacterial cytoplasmic membrane, transmission electron microscopy were utilized using DOPE/DOPG mixed liposomes (Figure 3.26). Intact individual liposome (A) and liposome clusters (B) were observed under TEM as control. Upon addition of 50 μM AM052, liposome membrane disruption was observed. Intact liposomes started to lose their shape from single spot or very closely located spots, suggesting densely localized binding of AM052 rather than even distribution of AM052 all over the membrane.

Figure 3-22. Transmission electron microscopy images showing AM052 induced uneven membrane disruption and liposome aggregation. A. 3 mM liposome as control; B. 30 mM liposome as control; C. 3 mM liposome plus 50 μ M AM052; D. 30 mM liposome plus 50 μ M AM052.

- **Oligomerization states of AM052**

Since membrane patches formed by AM052 aggregation are critical for its bactericidal activity, oligomeric states of AM052 were studied in detail. Both the size of AM052 oligomers as well as the oligomer formation were explored by NMR spectroscopy using DOSY and titration experiments.

The translational diffusion coefficients of AM052 at high (5 mM) and low (50 μ M) concentrations in aqueous solution were measured in DOSY experiments. Results are listed in Table 20. Surprisingly, the diffusion rate of AM052 at 50 μ M was approximately double than that at 5 mM. According to the Stokes-Einstein equation, the peptide diffusion rate is in

the reverse proportion to the molecular size. Studies have shown that the Stokes-Einstein number for planar molecule is about half that found for the group of spherical molecules [186]. Taking into account that the AM052 molecule is planar rather than spherical, this means at concentrations of 5 mM in aqueous solution, AM052 forms stable oligomers of the spherical shape. The longest length of AM052 is about 2.8-3.0 nm in a fully extended conformation, and the inter-planar distance is about 0.35-0.40 nm according to our MD simulations (Figure 3.27). Therefore, oligomers consisting on average of 7 monomers of AM052 are expected which is also in good agreement with the MD simulations.

To monitor the oligomerization transition of AM052, 1D ^1H spectra of AM052 were recorded at increasing concentrations from 10 μM to 5 mM (Figure 3.28). Gradual built up of AM052 oligomers could be reflected by the continuous changes in the ^1H chemical shifts. Resonances from Arg residues displayed prominent changes in 6-9 ppm region and peaks from protons on xanthone rings also show traceable changes in either chemical shifts or broadened lines. These observations suggest slow to intermediate exchange rate (on the time scale of ^1H chemical shifts) of the AM052 oligomerization states. Gradual changes in the 1D ^1H spectra over large AM052 concentrations ranges suggests the formation of AM052 oligomers is heterogeneous and concentration dependent.

Table 20. Translational diffusion coefficients of AM052 in aqueous buffer.

$D_s^a (10^{-10} \text{m}^2 \text{s}^{-1})$	50 μM	5 mM
AM052	3.20 ± 0.12	1.80 ± 0.10

^a D_s is the translational diffusion coefficient.

Figure 3-23. MD simulations of AM052 in aqueous solution.

Molecules of AM052 are shown in lines and colored according to the elements. In total 20 monomers of AM052, 3 major clusters of AM052 oligomers are observed. The average size of an oligomer contains 5 to 7 monomers.

Figure 3-24. 1D ^1H spectra of AM052 as a function of concentration in aqueous solution at 25 °C. ^1H peaks are assigned and labeled. Assignment annotation is according to Figure 3.29. Unknown peaks which could not be assigned are labeled with asterisks. Concentrations of AM052 are highlighted on the right of figures. Spectra share the common axis labeled at the bottom (E).

Aggregation of AM052 monomers packed in different orientations could be observed in the ^1H - ^1H 2D homonuclear NOESY spectrum (Figure 3.29). Sharp NOE cross peaks between both symmetrically and anti-symmetrically stacked AM052 monomers could be clearly distinguished from each other suggesting slow exchange kinetics in AM052 oligomers or co-existence of heterogeneous oligomers consisting of AM052 monomers packed in different orientation. Less NOE cross peaks showing contacts between anti-symmetrical AM052 monomers (for example 9-19, 23-H' and 15-24) were observed comparing to the ones from symmetrical contacts (for example 9-4, 25-H', 15-22 and 22/23-34), and majority of them are weaker in peak intensity compared to the later as well. This might suggest that symmetrical or close-to-symmetrical orientations are relatively predominant contact form in the AM052 oligomers. Protons exhibit inter-monomeric cross-peaks in the NOESY spectrum also displayed most prominent changes in the resonance frequency in the 1D ^1H spectra with the increasing AM052 concentrations, indicating these protons are important contacts from AM052 oligomeric interface.

Observation of the AM052 oligomers by NMR spectroscopy is in a good agreement with the aggregation clusters detected by transmission electron microscopy and fluorescence emission spectroscopy, indicating perhaps the importance of accretion of AM052 patches on the bacterial surface required for membrane disruption. It also proves that the oligomer model built based on NMR constrains and supported by MD simulations might be physiologically relevant.

Figure 3-25. 2D homonuclear NOESY spectrum of AM052 in aqueous solution. ^1H specific assignments of individual NOESY cross-peaks are labeled in A according to the annotation in B. Complete proton resonances assignments were done based on J couplings between chemical groups (2.1.1) as well as NOEs. Inter-molecular NOEs between AM052 monomers are highlighted. NOEs between symmetrically stacked monomers are labeled in red while cross peaks from anti-symmetrical contacts of AM052 monomers are highlighted in blue.

- **Selectivity of AM052 towards bacterial membrane against mammalian cell membrane**

Figure 3-26. 1D ^1H spectra of AM052 in DOPE/DOPG mixed liposomes (A), DOPC liposomes (B) and aqueous solution (C). ^1H resonances from AM052 and lipids are assigned and labeled.

AM052 showed differential chemical shift perturbation in bacterial and mammalian cell membrane mimicking liposomes (Figure 3.30). Comparing to AM052 in aqueous solution, severe line broadening of AM052 in both Arg as well as aliphatic regions were observed in 1D ^1H spectra when mixing with DOPE/DOPG liposome medium. Line width of AM052 becomes comparable to the ones originated from liposomes, meaning the similarly slower tumbling rate of AM052 and liposomes. In contrast, resonances of AM052 ^1H remain sharp and observable in

DOPC liposomes indicating that the majority of AM052 is found free in solution outside of the lipid interface of liposomes. The detected full partition of AM052 into DOPE/DOPG liposomes while free diffusion of AM052 in DOPC liposomes indicate significant difference in the binding affinity. Preferential binding of AM052 to the bacterial membrane over that to the mammalian cell membrane may in part explain its improved selectivity (high antimicrobial activity with low cytotoxicity).

- **AM052 penetrates into bacterial cytoplasmic membrane**

Many studies have shown that AMPs kill bacteria through interacting with the cytoplasmic membranes [187,188], AM052 structures and its membrane interactions were studied in both DPC-POPG mixed micelles and DOPE/DOPG mixed liposomes.

Figure 3.31 and 3.32 show the 2D ^1H - ^1H homonuclear NOESY spectra of AM052 in micelles and liposomes respectively. Numerous NOEs between AM052 and lipids were observed in both spectra (highlighted Figure 3.31 and 3.32). Two arginine residues in AM052 form stable interactions with lipids, in particular the CH_2 groups near phosphorus. These are induced by the strong electrostatic interactions between the positively charged Arg NH_2 side chains and negatively charged phosphorus group in lipids. More NOEs were observed between AM052-POPG/DOPG than AM052-DPC. The observation could be expected since AM052 exhibits stronger electrostatic attraction forces with the negatively charged PG lipid head groups rather than the zwitter-ionic head groups of PC or PEs. The NH_2 terminal groups of Arg side chains (H_ϵ and H_η) displayed strong NOEs with protons close to the lipid head group whereas NH groups of Arg backbone showed NOEs with protons near to the lipid tail. The observed NOE pattern suggests the membrane penetration of AM052, namely the hydrophobic xanthone

groups penetrate deeply into membrane bilayer leaving the terminal group of Arg side chains facing to the membrane-water interface. The driving force of AM052-bacterial membrane interaction is likely to be the combination of hydrophobic and electrostatic interactions. The observed AM052-membrane insertion pattern is different from its initial membrane binding which mainly depends on the electrostatic attractions between Arg side chains and lipid head group. This suggests after binding, while Arg side chains remains interacting with lipids heads, xanthone groups flip from polar water into non-polar lipid membrane bilayer forming strong hydrophobic interactions with lipid aliphatic chains.

Figure 3-27. 2D ^1H - ^1H NOESY spectrum of AM052 in DPC/POPG mixed micelles. Only amide fingerprint region of the NOESY spectrum is shown (A). Proton specific assignment of diagonal peaks and cross-peaks from AM052 are labeled in black; NOEs between AM052 and lipid protons are highlighted in green and cross-peaks between AM052 monomers are shown in red. Assignment is labeled according to

B and C.

Figure 3-28. 2D ^1H - ^1H NOESY spectrum of AM052 in DOPE/DOPG mixed liposomes. Proton specific assignment of cross-peaks are labeled in black; selected NOEs between AM052 and lipid protons are marked (A). Assignment is labeled according to B and C.

To study insertion of AM052 in the mimic membranes at the atomic resolution, the paramagnetic probe omniscan was used. A series of 2D ^1H - ^1H NOESY spectra of AM052 in DPC-POPG mixed micelles was acquired with increasing omniscan concentrations from 0 to 5 mM (Figure 3.33). Paramagnetic Gd^{3+} in solution will enhance the transverse relaxation rate of protons in the proximity to the center of relaxation. Provided sufficiently long inter scan delay in the acquisition of NOESY spectra is employed, the enhanced ^1H $1/T_2$ relaxation rate could be manifested by the resonance line broadening effect and the decreased peak intensities.

Figure 3.33 summarizes signal intensity changes of selected protons

stemming from both AM052 and DPC molecules. AM052 and the corresponding chemical groups in DPC displayed very similar decay rate in signal intensity. The changes in signal intensity clearly show that chemical groups on micelle surface were mostly affected in contrast to protons buried inside which are least affected. The signal intensity of the DPC head groups, a and b, dropped by almost 80% after adding in 5 mM of omniscan. In contrast, proton resonances from the CH₂ groups in DPC aliphatic tail still remain at the level of 60% of the peak intensity at the same omniscan concentration of 5 mM. This means omniscan molecules are free in solution and only protons on membrane-water interface are accessible to its relaxation center. Arg side chains of AM052 (H_η and H_ε) show same trends of dependency of the concentration of the paramagnetic reagent as the DPC head groups (proton a, b and c) (Figure 3.33 D). Though the isoprenol groups on hydrophobic xanthone rings (proton number 1, 2, 22, and 23) display same changing trends as DPC aliphatic tail (proton e and d) (Figure 3.33 A) which is much slower signal decay rate than the ones in Arg side chain. The peak intensity decrease pattern shows good agreement with the NOE pattern observed in NOESY experiment. Both of them suggest when AM052 interacting with cytoplasmic membrane, hydrophobic xanthone groups insert deeply till the aliphatic tail of lipids leaving hydrophilic terminal protons of Arg side chains interacting with the membrane surface.

Figure 3-29. Omniscan induced line broadening of 2D NOESY spectra of AM052 in DPC/POPG mixed micelles. ^1H signal intensity of individual resonance was plotted against paramagnetic probe omniscan concentration. Reference signal intensities were read from 1D ^1H spectra of AM052 in DPC/POPG mixed micelles without adding in omniscan. Intensity changes of resonances from both AM052 and lipids were traced and grouped according to the signal decay rate. Four groups (A, B, C, and D) are shown in the figure (see figure caption).

- **Two-state equilibrium in AM052 oligomers**

Due to the importance of AM052 oligomerization for its bactericidal activity, the dynamics of AM052 oligomers was studied in detail. We expect that the oligomeric state of the compound will be modulated by the temperature, which can be analyzed by NMR. A novel TIPSy (Temperature Induced Projection Spectroscopy) experiment was performed [189]. Series of 1D ^1H spectra of AM052 in aqueous solution were acquired with increasing temperature from 293K to 343K, one

spectrum per each degree. The acquired spectra were then the Radon transformed resulting in a 2D spectrum with the proton resonance frequency in one dimension and temperature coefficient (ppb/K) in the other dimension (Figure 3.34 A).

The TIPSY spectrum showed clearly different chemical shift dependence of AM052 protons on the temperature variation. Protons from Arg residues all show negative temperature coefficients (moving upfield with the increasing temperature) while protons directly attached to xanthone rings (protons number 9 and 15) exhibit positive temperature coefficients (moving downfield) (Figure 3.34 B). Studies have shown that amide protons in H-bonding usually display temperature coefficient more positive than -4.5 ppb/K [190], whereas temperature coefficients from -10 to -6 ppb/K, usually indicative of the lack of intra-molecular hydrogen bonds [191]. Therefore, Arg residues on both sides of AM052 molecule are unlikely involved in hydrogen bonds for oligomer formation and the highly positive temperature coefficients for proton number 9 and 15 are likely due to the strong ring current effect in the xanthone group [192].

More interestingly, protons from the isoprenol groups showed irregular peaks on TIPSY spectrum and when the resonances are overlaid in the 1D spectra, bi-phasic moving of the peaks were observed (Figure 3.34 C). Below 308K, chemical shifts of protons (19, 5 and 24) moved towards downfield but when the temperature increased beyond 308K, they displayed upfield shift. The non-uniform moving of the proton chemical shifts suggests there is one or more states existing at equilibrium with the AM052 oligomers. It is likely that the oligomerization reaction might include multi-state equilibrium.

Based on the different temperature coefficients of AM052 protons, one could suspect that the main driving force for the oligomerization of

AM052 molecules is the hydrophobic interactions from xanthone and its attached isoprenol groups; but not the electrostatic interactions or H-bonds involving Arg residues.

Figure 3-30. TIPSy (Temperature Induced Projection Spectroscopy) spectrum of AM052 in aqueous solution (A). Overlay of the selected 1D ^1H spectra (10K between each spectrum) showing proton resonances movement from 5-9 ppm (B) and 3.9 ppm (C) are also shown.

Chapter 4. Discussion

Defensins are natural AMPs characterized by evolutionally conserved cationic charges and cysteine disulfide bridge patterns. Studies have shown that disulfide bridges are not critical for defensins' antibacterial activity but rather important for their overall structural stability and chemotaxis activity [193]. Similar effects have been found in other antimicrobial peptides, like tachyplesin and polyphemusins [147,194]. Designing AMPs with removed disulfide bonds could minimize the immunogenic propensity of promising drug candidates.

3.1 Charge density is critical for AMP bactericidal selectivity and efficacy

In our study [100], we showed that a human β -defensin-3 C-terminal (R36-K45) analogous peptide, Y2, exhibits higher antibacterial activity against the Gram-negative bacterium *P. aeruginosa* and lower cytotoxicity targeting mammalian cells compared to the native protein. The underlying physicochemical properties distinguishing Y2 from the other peptides sharing high sequence similarities but very low bactericidal activities (F2 and C2) mainly include the difference in propensities to oligomerize on lipid membrane surface and the ability to accrete unique structures upon peptide membrane binding. Molecular dynamics simulations showed that Y2 and F2 dimers are more stable than that of C2. This originates mainly from the π - π stacking and hydrophobic interactions between the aromatic side chains of Tyr and Phe residues. Furthermore, the Tyr residues are also involved in

intra-molecular hydrogen bonds with surrounding polar groups of membrane lipids that further stabilizes the compact configuration. A recent study of MSI-78 showed that dimerization of the MSI-78 peptide enhances its selectivity by increased the electrostatic attractions to bacterial membranes as well as reduced affinity for mammalian cell membranes [63].

The prominent structural difference between Y2 and F2 dimers underlines the difference in their activity. The compact structure of Y2 results in a higher charge density. The strong correlation of antibacterial action and surface charges has been well-studied, and the importance of electrostatic interactions between cationic peptides and bacterial anionic membranes are high-lighted [195-198].

Studies have shown that β -defensins exert their antimicrobial activity by an electrostatic charge-based mechanism [84,199]. A recent study [200] has shown that clustering of anionic lipids in the bacterial membrane might cause a phase boundary defect which is responsible for increased membrane leakage and permeability. Our study of defensin-derived linear peptides confirmed the importance of charge density for AMP activity. High charge density caused strong electrostatic interactions potentially leading to the suggested phase boundary defect in the bacterial cytoplasmic membrane. Structural parameters such as compactness and charge density could be used as filters for designing novel antibacterial peptides or peptide mimetics.

3.2 AMPs targeting Gram-negative bacteria remain high mobile in outer membrane LPS layer

Many AMPs induce rapid membrane depolarization are not effective against Gram-negative bacteria due to the impermeable LPS layer in the

bacterial outer membrane [181]. Branched peptide B2088 showed promising bactericidal activity against *P. aeruginosa*.

In our study, B2088 was used to observe antimicrobial peptide permeation through bacterial outer membrane LPS while inducing LPS phase transition. This was followed by structural transition of the AMP upon interaction with the inner membrane, which resulted in membrane disruption leading to bacterial death. Interestingly, we noticed that this peptide exhibited different binding affinities to bacterial inner and outer membranes. To explore its significance, binding affinities of AMPs toward bacteria cells and bacterial outer/inner membranes were assessed based on B2088 experimental data and compared with previous literature. In order to establish a comprehensive comparison, both the dissociation constant, K_d , and the molecular stoichiometric ratio in the complex were analyzed, providing binding affinity data emphasizing both the “quality” (nature and strength) of binding at equilibrium and their relative quantities (Table 21).

Table 21. Binding affinities of AMP to bacteria-related media.

	Estimated dissociation constant, K_d	Stoichiometric ratio	
		Other work ^b	Our work
AMP/LPS	2 μM	3:1	5:1
AMP/membrane lipids	2 μM	1:50	1:32
AMP/live bacteria	10 nM^a	100:1	82:1

^a Estimated from AMP diffusion tracking [201] by assuming that the apparent concentration of AMP at which a qualitative change in AMP dynamics at the bacterial surface is observed. ^b See reference [126,202]

Estimates of the activities (effective concentrations) of antimicrobial peptides in typical bacterial susceptibility tests showed that AMPs have to accumulate with a tremendous excess of peptides per membrane lipid molecule in order to induce the bactericidal effect [115]. The majority of known potent antimicrobial peptides exhibit bactericidal activity at or above the micro-molar range of concentrations in common bacterial sterilization assays, reaching a peptide/lipid ratio of 100:1. In our assays of B2088, we estimate that an excess of more than 100 peptide molecules/ 1 nm^2 of the bacterial surface (82 peptides to 1 lipid molecule) is needed for the efficient inhibition of bacterial growth followed by cell lysis. The maximum saturation concentrations of AMP on the bacterial membranes are determined by the molecular affinities of AMPs to the interaction surfaces. Those affinities can vary wildly from the absence of interactions in the pH neutral membranes of mammals to highly sticky surfaces presented by bacterial membranes enriched with negatively charged lipids, like phosphatidylglycerol and cardiolipin. In typical membrane binding experiments, there are about 1000 AMPs bound to each large uni-lamellar vesicle, which in turn may contain around 100,000 lipids [126,202]. When using giant uni-lamellar vesicles, a small number of giant uni-lamellar vesicles are exposed to 1–10 μM peptides [203,204]. Even in the stoichiometric excess of AMPs in solution, the saturation (equilibrium) concentration of AMP on the surface does not exceed 1 AMP per 20 lipids [115]. This is supported by our measurements of the equilibrium binding constants of B2088 to liposomes, indicating an average of 0.056 peptide molecules bound for each 1 nm^2 of the liposomal surface (1 peptide to 32 lipids). Apparently, the surface of liposomes or vesicles cannot provide sufficient affinity to

accommodate the tremendous excess of AMPs required to disrupt membrane integrity, even in the presence of negatively charged lipids.

Our experiments suggest that interactions with LPS may provide an additional increase in the affinity of AMPs to the complex bacterial surfaces. The net compounding effect of several concurrent electrostatically mediated weak interactions of AMPs with LPS and negatively charged lipid membranes is a significant increase in net binding affinities and concomitantly equilibrium concentrations of AMPs on the bacterial surfaces as is exemplified in combinatorial chemistry producing higher affinity ligands from lower affinity molecular fragments [205]. Previously, it was estimated that the dissociation constant of AMPs interacting with LPS was in the low micro-molar range [206,207], similar to the affinity level found for the interactions between AMPs and bacterial membrane lipids [72]. However, significantly stronger binding of AMP to live bacteria complex interfaces, typically in the sub-micro-molar range, are estimated from experiments tracing AMP movements in the presence of bacterial surfaces [201]. These smaller dissociation constants can usually be explained through higher stoichiometric binding ratios allowing more AMPs at their recipient surfaces. A combination of interactions with LPS resulting in 3 AMPs per molecule of LPS [208,209] and inner membrane binding resulting in 1 AMP per 50 molecules of lipids [115] might enhance binding of AMPs to the composite surfaces consisting of LPS and lipids delivering a remarkably high equilibrium excess of AMPs per bacterial lipid molecule typically found in bacteria susceptibility tests (100:1). Our experimental observations are in good agreement with these estimates, suggesting that a combination of interactions of B2088 with LPS (peptide/LPS ratio = 5:1) and the inner membrane mimics (peptides/lipids = 1:32) might provide significant enhancement of affinity

to reach the stoichiometric ratios required for the bactericidal effect (Table 21).

Remarkably, upon interaction of AMPs with LPS, liquid expanded domains in the bacterial outer membrane were replaced by liquid condensed domain structures [181,210-212]. In atomic force microscopy, transformation of LPS assembly from ribbon-like structures into large and densely packed multi-lamellar aggregates was seen upon the addition of AMPs [213]. In our experiments, the full absorption of B2088 into the LPS was detected. Interactions with B2088 caused an LPS phase transition from isotropic micelle solution to large insoluble aggregates. AMPs strongly attracted by LPS but locked into the polymorphous outer membrane showed no antimicrobial activity [181,214]. B2088 retains its rotational molecular mobility, slowed down translational diffusion, and three-dimensional structure resembling that found in aqueous solution. The high molecular mobility of B2088 enabled it to permeate through the bacterial outer membrane and accumulate at the surface of the inner membrane at sufficient concentrations. Although LPS can increase total affinity of AMPs to bacterial surfaces, it is not clear how AMPs shall penetrate though oppositely charged medium without being locked. Although “self-promoted” uptake via displacement of LPS-binding divalent cations has been proposed [215,216], LPS phase transition seems critical for providing space to facilitate permeation of AMPs.

These observations support the interfacial activity model, which states that the antimicrobial activity of an antimicrobial peptide depends on “the ability of a molecule to bind to a membrane, partition into the membrane-water interface, and alter the packing and organization of the lipids.” Here we propose to extend this interfacial model to include more complex interfaces, such as LPS/mixed lipids. We showed that B2088

retained translational and rotational mobility at a complex interface. This appears to directly correlate with the peptide's efficacy, helping to achieve significantly higher surface concentrations, which are required for its antimicrobial activity.

Sufficiently high concentrations of AMPs at the bacterial membrane promotes structural compaction in B2088, as observed by NMR, and may facilitate membrane remodeling. Upon association with the bacterial inner membrane, B2088 adopts a significantly more compact structure, facilitating full absorption into the membrane through extensive transient hydrogen bonds. Rather than a highly flexible structure in aqueous solution and LPS, the primary and secondary chains of B2088 show different chemical shifts and tight packing in the inner membrane-mimicking micelles. In our MD simulation studies, such structural accretion helped to increase the strength and number of peptide-membrane lipid interactions, therefore resulting in redistribution of membrane lipids. Other studies reported similar concentration-dependent structural transitions associated with AMP activity [217]. Changes of peptide orientations relative to the membrane were observed in both α -helix-forming [218-220] and β -sheet-forming [221] peptides. A two-state model of the effect of AMPs on bacterial lipid membranes was proposed [121].

In fact all previously proposed models call for sufficiently high concentrations of AMPs on the surface in order to form super-molecular structures (as in the "carpet model" or "barrel stave model") [119,222]. In our own efforts of designing AMPs, we found that covalent linkage of monomeric AMPs contributed to a significant increase of antimicrobial activity [94,223]. Higher covalent oligomerization generally tends to increase affinities to lipid surfaces [224]. However, large peptide

oligomers are prone to be blocked in the LPS condensed phase, whereas a non-aggregated conformation is preferred for membrane-permeating activity [181]. In our hands, higher order AMPs also tend to be more cytotoxic, reflecting their increased interactions with mammalian membranes. B2088 retains its monomeric state in various media, which is in agreement with biophysical observations of other AMPs [63,225,226]. The trend in designing AMPs toward higher covalent order might be limited by increased cytotoxicity and can be counteracted by designing lower order AMPs, maintaining high mobility in LPS and providing higher concentrations at the lipid surfaces of bacteria.

3.3 AMPs targeting Gram-positive bacteria forms high order oligomers via hydrophobic interactions

Many AMPs studies have observed that antimicrobial molecules accumulated on bacterial membrane surface and critical structural transitions of AMPs happened once the concentration reached certain threshold [121,227].

In our studies, phase transition of AM052 structural orientations in bacterial membrane was observed. AM052 selectively binds to bacterial membrane surface through electrostatic interactions between Arg residues and negatively charged bacterial lipids head group. After initial binding, xanthone groups of AM052 penetrate into bacterial membranes due to strong hydrophobic interactions between its aromatic ring and aliphatic lipid tails and probably also facilitated by the membrane disruption effects of Arg side chains.

Oligomerization of AM052 was also directly detected by NMR

spectroscopy in aqueous solution; and indirectly suggested by fluorescence emission spectroscopy, TEM and NMR in bacterial membrane mimicking lipids media. AM052 forms high order oligomer consisting 5 to 7 copies of monomer on average. The oligomers are heterogeneous in both monomer copy number as well as stacking orientations relative to each other. Two-state equilibrium was identified in oligomer formation kinetics, which might correspond to the oligomer heterogeneity or AM052 structural phase transition upon interacting with bacterial membranes. Though no strong inter-molecular H-bonds were observed, AM052 oligomers are mainly stabilized by hydrophobic interactions between xanthone groups.

Distinguished from previously proposed carpet model [54,198,228], non-equilibrium binding of AM052 towards membrane mimicking liposomes was observed indicating a “patch model” which emphasizes the difference in binding affinity. Namely, when AM052 molecule approaches to the bacterial membrane, it exhibits higher binding affinity to preformed membrane patches consisting small AM052 oligomers rather than single monomer copies on bacterial membrane surface. Therefore, a correlation between the increase in liposomal leakage and increased AM052 concentration was observed in both fluorescence emission studies as well as NMR experiments.

Similar leakage kinetics was observed before and two membrane disturbance models were proposed: graded leakage and all-or-none disruption [51,229]. Graded leakage refers to all of the vesicles release portions of their contents [184] whereas all-or-none model suggests a fraction of vesicles release all of their contents leaving the rest remained intact [126]. Studies of vesicle permeabilization by AMPs frequently show a partial transit leakage behavior: upon addition of AMPs, a rapid burst of

the leakage occurs followed by a cessation before all the contents have been released. If adding in more antimicrobials later, a second burst of the leakage will typically occur [115]. This suggests that vesicle permeabilization by most AMPs must be a non-equilibrium event and majority of the proposed AMP bactericidal mechanistic models, which are essentially equilibrium models, fail to fully explain the partial transitive leakage phenomenon. The novel “patch model” qualitatively may explain the observed leakage by non-equilibrium binding of antimicrobials toward lipid membranes and predict the membrane leakage kinetics by affinities of AMPs to form oligomers (AMPs in fast growing oligomers with higher monomer binding affinity disrupts bacterial cytoplasmic membrane faster) (Figure 4.1).

As predicted by many of the previous models, when AMPs bind to the bacterial membrane with equal affinity, the curve plotting vesicle leakage percentage against AMP concentrations should be hyperbolic (Figure 4.1 A). Unfortunately, in reality this type of curves is very rarely seen. If the AMP monomer binding affinity increases in a cooperative manner as the “patch model” expected, the larger AMP oligomer has stronger attraction force, then the curve will be sigmoidal or close to that as observed in most of the experiments using various AMPs (Figure 4.1 B and C).

Figure 4-1. AMPs induced membrane leakage. Percentage of vesicle membrane leakage was plotted against AMP concentrations. A.

predicted curve according to traditional carpet model assuming additional AMPs bind to bacterial membrane with equal affinity; B. experimental observed data from other studies [115]; C. experimental observed data in this study using AM052.

In good agreement with the previous AMP studies, the newly proposed “patch model” foresees the oligomerization of antimicrobials on bacterial membrane surface followed by subsequent necessary structural transitions. Unlike the carpet model, the patch model emphasizes the change in AMP membrane binding affinity hence explains the partial membrane leakage kinetics. This proposed new model is essentially nonlinear, may yield to mathematical analysis and thus provide better quantitative description of the AMP bacterial membrane disruption mechanism offering novel optimization parameters for the antimicrobial drug development.

Conclusions

This study aims to explore the bactericidal mechanism of AMPs hence provides insights for new generation antimicrobial drug discovery. Cationic linear defensin C-terminal analogous peptides were used to study the selectivity and general requirements for AMP bactericidal activity. Branched peptide with unnatural peptide bond, B2088, was used to study AMPs targeting Gram-negative bacteria in detail; and peptide mimetics, AM052, with organic xanthone group attached by Arginine residues was selected for discover AMP bacterial membrane disruption mechanisms in Gram-positive bacteria.

In general, AMPs target bacterial membrane rather than mammalian cell membrane through strong electrostatic interactions. Cationic charged AMP will preferentially bind to bacterial membrane which is heavily decorated by negatively charged PG lipids, but not mammalian cell membrane which mainly consists of zwitterionic phosphatidylcholine. Electrostatic interactions are relatively strong force ensure the selective binding is fast and accurate therefore minimize the possible cytotoxicity.

Once binding to bacterial membranes, most of the AMPs exhibit structural transitions. Accrete well defined structures upon interactions with biological phases contribute to compactization of positive charges within biological membranes. Intensive interactions between AMPs with negatively charged lipids cause the redistribution of membrane lipids, which in turn results in increased membrane permeability and bacterial lysis. Structural parameters such as compactness and charge density could be used as filters in the design of novel antibacterial peptides or small molecule mimics.

In the case of Gram-negative bacteria, complications come from the densely packed lipopolysaccharide in bacterial outer membrane, which serves as an impermeable barrier for antibiotics. Studies have shown that cationic AMPs could cause phase transitions of the LPS layer. Strong binding between AMP and LPS would block large AMP oligomers from penetrating outer membrane thus decrease accessibility of bacterial cytoplasmic membrane by AMPs. Therefore, to target Gram-negative bacteria, possessing enhanced mobility in the bacterial outer membrane becomes critical property for antimicrobial drug development.

For Gram-positive bacteria, which do not have the LPS-rich bacterial outer membrane, accessibility to the cytoplasmic membrane becomes less critical issue for AMP design; but maximizing interactions with membranes comes to be the key concern. Hence, strong and static interactions with membranes are more favorable rather than remaining high mobility and molecular dynamics property for antimicrobials. Van der Waals interactions, which govern the kinetics of AMP oligomerization as well as AMP-membrane contacts, become major parameter to be optimized for developing new drug.

References

1. Waksman SA: **What is an antibiotic or an antibiotic substance?** *Mycologia* (1947) **39**(5):565-569.
2. Davies J: **Where have all the antibiotics gone?** *The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie medicale / AMMI Canada* (2006) **17**(5):287-290.
3. Pinner RW, Teutsch SM, Simonsen L, Klug LA, Graber JM, Clarke MJ, Berkelman RL: **Trends in infectious diseases mortality in the united states.** *JAMA : the journal of the American Medical Association* (1996) **275**(3):189-193.
4. (WHO) WHO: **Deaths by cause, sex and mortality stratum in who regions, estimates for 2001.** *World Health Report-2002* (2002) Geneva: WHO, 2002).
5. Coates A, Hu Y, Bax R, Page C: **The future challenges facing the development of new antimicrobial drugs.** *Nat Rev Drug Discov* (2002) **1**(11):895-910.
6. Transparency Market Research: **Antibiotic market - global industry size, share, trends, analysis and forecast, 2012 - 2018.** *Press Release* (2011): 2011.
7. Livermore DM: **The need for new antibiotics.** *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* (2004) **10 Suppl 4**(1-9.
8. Fox JL: **The business of developing antibacterials.** *Nature biotechnology* (2006) **24**(12):1521-1528.
9. Alanis AJ: **Resistance to antibiotics: Are we in the post-antibiotic era?** *Archives of Medical Research* (2005) **36**(6):697-705.

10. Sefton AM: **Mechanisms of antimicrobial resistance: Their clinical relevance in the new millennium.** *Drugs* (2002) **62**(4):557-566.
11. Mandell GL, Bennett JE, Dolin R: *Mandell, douglas, and bennett's principles and practice of infectious diseases / [edited by] gerald l. Mandell, john e. Bennett, raphael dolin.* Philadelphia, PA : Churchill Livingstone/Elsevier, c2010.
7th ed., (2010).
12. Connor EE: **Sulfonamide antibiotics.** *Primary Care Update for OB/GYNS* (1998) **5**(1):32-35.
13. Landy M, Gerstung RB: **P-aminobenzoic acid synthesis by neisseria gonorrhoeae in relation to clinical and cultural sulfonamide resistance.** *Journal of immunology* (1945) **51**(269-277).
14. Yaniv H, Davis BD: **The relation between sulfonamide resistance and p-aminobenzoic acid requirement.** *Journal of bacteriology* (1953) **66**(2):238.
15. Enne VI, King A, Livermore DM, Hall LM: **Sulfonamide resistance in haemophilus influenzae mediated by acquisition of sul2 or a short insertion in chromosomal folp.** *Antimicrobial agents and chemotherapy* (2002) **46**(6):1934-1939.
16. Maskell R, Okubadejo OA, Payne RH, Pead L: **Human infections with thymine-requiring bacteria.** *Journal of medical microbiology* (1978) **11**(1):33-45.
17. Amyes SG, Smith JT: **R-factor mediated dihydrofolate reductases which confer trimethoprim resistance.** *Journal of general microbiology* (1978) **107**(2):263-271.
18. Elander RP: **Industrial production of beta-lactam antibiotics.** *Applied microbiology and biotechnology* (2003) **61**(5-6):385-392.
19. Spratt BG: **Biochemical and genetical approaches to the mechanism of action of penicillin.** *Philosophical transactions of the Royal Society of London Series B, Biological sciences* (1980) **289**(1036):273-283.

20. Sykes RB, Matthew M: **The beta-lactamases of gram-negative bacteria and their role in resistance to beta-lactam antibiotics.** *The Journal of antimicrobial chemotherapy* (1976) **2**(2):115-157.
21. Medeiros AA: **Evolution and dissemination of beta-lactamases accelerated by generations of beta-lactam antibiotics.** *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* (1997) **24 Suppl 1**(S19-45).
22. Walter F, Vicens Q, Westhof E: **Aminoglycoside-rna interactions.** *Current opinion in chemical biology* (1999) **3**(6):694-704.
23. Lynch SR, Recht MI, Puglisi JD: **Biochemical and nuclear magnetic resonance studies of aminoglycoside-rna complexes.** *Methods in enzymology* (2000) **317**(240-261).
24. Craven GR, Gavin R, Fanning T: **The transfer rna binding site of the 30 s ribosome and the site of tetracycline inhibition.** *Cold Spring Harbor symposia on quantitative biology* (1969) **34**(129-137).
25. Rahal JJ, Jr., Simberkoff MS: **Bactericidal and bacteriostatic action of chloramphenicol against meningial pathogens.** *Antimicrobial agents and chemotherapy* (1979) **16**(1):13-18.
26. Allen N: **Effects of macrolide antibiotics on ribosome function.** In: *Macrolide antibiotics*. Schönfeld W, Kirst H (Eds), Birkhäuser Basel, (2002):261-280.
27. Tenson T, Lovmar M, Ehrenberg M: **The mechanism of action of macrolides, lincosamides and streptogramin b reveals the nascent peptide exit path in the ribosome.** *Journal of molecular biology* (2003) **330**(5):1005-1014.
28. Swaney SM, Aoki H, Ganoza MC, Shinabarger DL: **The oxazolidinone linezolid inhibits initiation of protein synthesis in bacteria.** *Antimicrobial agents and chemotherapy* (1998) **42**(12):3251-3255.
29. Eliopoulos GM, Farber BF, Murray BE, Wennersten C, Moellering

- RC, Jr.: **Ribosomal resistance of clinical enterococcal to streptomycin isolates.** *Antimicrobial agents and chemotherapy* (1984) **25**(3):398-399.
30. Besier S, Ludwig A, Zander J, Brade V, Wichelhaus TA: **Linezolid resistance in staphylococcus aureus: Gene dosage effect, stability, fitness costs, and cross-resistances.** *Antimicrobial agents and chemotherapy* (2008) **52**(4):1570-1572.
31. Williams JB: **Drug efflux as a mechanism of resistance.** *British journal of biomedical science* (1996) **53**(4):290-293.
32. Sutcliffe J, Tait-Kamradt A, Wondrack L: **Streptococcus pneumoniae and streptococcus pyogenes resistant to macrolides but sensitive to clindamycin: A common resistance pattern mediated by an efflux system.** *Antimicrobial agents and chemotherapy* (1996) **40**(8):1817-1824.
33. Labischinski H, Barnickel G, Bradaczek H, Naumann D, Rietschel ET, Giesbrecht P: **High state of order of isolated bacterial lipopolysaccharide and its possible contribution to the permeation barrier property of the outer membrane.** *Journal of bacteriology* (1985) **162**(1):9-20.
34. Takeuchi Y, Nikaido H: **Physical interaction between lipid a and phospholipids: A study with spin-labeled phospholipids.** *Reviews of infectious diseases* (1984) **6**(4):488-492.
35. Bryan LE: *Antimicrobial drug resistance.* Academic Press, (1984).
36. Davies J: **General mechanisms of antimicrobial resistance.** *Reviews of infectious diseases* (1979) **1**(1):23-29.
37. Leclercq R: **Mechanisms of resistance to macrolides and lincosamides: Nature of the resistance elements and their clinical implications.** *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* (2002) **34**(4):482-492.
38. Campbell EA, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst SA: **Structural mechanism for rifampicin inhibition of bacterial rna polymerase.** *Cell* (2001)

104(6):901-912.

39. Williams DL, Waguespack C, Eisenach K, Crawford JT, Portaels F, Salfinger M, Nolan CM, Abe C, Sticht-Groh V, Gillis TP: **Characterization of rifampin-resistance in pathogenic mycobacteria.** *Antimicrobial agents and chemotherapy* (1994) **38**(10):2380-2386.
40. Hooper DC, Rubinstein E: *Quinolone antimicrobial agents.* Amer Society for Microbiology, (2003).
41. Neu HC: **Quinolone antimicrobial agents.** *Annual Review of Medicine* (1992) **43**(1):465-486.
42. Pan XS, Ambler J, Mehtar S, Fisher LM: **Involvement of topoisomerase iv and DNA gyrase as ciprofloxacin targets in streptococcus pneumoniae.** *Antimicrobial agents and chemotherapy* (1996) **40**(10):2321-2326.
43. Tran JH, Jacoby GA: **Mechanism of plasmid-mediated quinolone resistance.** *Proceedings of the National Academy of Sciences of the United States of America* (2002) **99**(8):5638-5642.
44. Murakami S, Nakashima R, Yamashita E, Yamaguchi A: **Crystal structure of bacterial multidrug efflux transporter acrB.** *Nature* (2002) **419**(6907):587-593.
45. Silver L, Bostian K: **Screening of natural products for antimicrobial agents.** *European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology* (1990) **9**(7):455-461.
46. Bax R, Mullan N, Verhoef J: **The millennium bugs--the need for and development of new antibacterials.** *International journal of antimicrobial agents* (2000) **16**(1):51-59.
47. Allsop AE: **New antibiotic discovery, novel screens, novel targets and impact of microbial genomics.** *Current opinion in microbiology* (1998) **1**(5):530-534.
48. Rosamond J, Allsop A: **Harnessing the power of the genome in the search for new antibiotics.** *Science* (2000)

287(5460):1973-1976.

49. Spoering AL, Lewis K: **Biofilms and planktonic cells of pseudomonas aeruginosa have similar resistance to killing by antimicrobials.** *Journal of bacteriology* (2001) **183(23):6746-6751.**
50. Ashby MJ, Neale JE, Knott SJ, Critchley IA: **Effect of antibiotics on non-growing planktonic cells and biofilms of escherichia coli.** *The Journal of antimicrobial chemotherapy* (1994) **33(3):443-452.**
51. Wimley WC, Hristova K: **Antimicrobial peptides: Successes, challenges and unanswered questions.** *J Membr Biol* (2011) **239(1-2):27-34.**
52. Hancock RE, Patrzykat A: **Clinical development of cationic antimicrobial peptides: From natural to novel antibiotics.** *Curr Drug Targets Infect Disord* (2002) **2(1):79-83.**
53. Gordon YJ, Romanowski EG, McDermott AM: **A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs.** *Curr Eye Res* (2005) **30(7):505-515.**
54. Giuliani A, Pirri G, Nicoletto S: **Antimicrobial peptides: An overview of a promising class of therapeutics.** *Central European journal of biology* (2007) **2(1):1-33.**
55. Pootoolal J, Neu J, Wright GD: **Glycopeptide antibiotic resistance.** *Annual review of pharmacology and toxicology* (2002) **42(381-408).**
56. Pogliano J, Pogliano N, Silverman JA: **Daptomycin-mediated reorganization of membrane architecture causes mislocalization of essential cell division proteins.** *Journal of bacteriology* (2012) **194(17):4494-4504.**
57. Stone KJ, Strominger JL: **Mechanism of action of bacitracin: Complexation with metal ion and c 55 -isoprenyl pyrophosphate.** *Proceedings of the National Academy of Sciences of the United States of America* (1971) **68(12):3223-3227.**

58. Warren JR: **Polymyxin b suppresses the endotoxin inhibition of concanavalin a-mediated erythrocyte agglutination.** *Infection and immunity* (1982) **35**(2):594-599.
59. Issekutz AC, Biggar WD: **Effect of methylprednisolone and polymyxin b sulfate on endotoxin-induced inhibition of human neutrophil chemotaxis.** *The Journal of laboratory and clinical medicine* (1978) **92**(6):873-882.
60. Carr C, Jr., Morrison DC: **Mechanism of polymyxin b-mediated lysis of lipopolysaccharide-treated erythrocytes.** *Infection and immunity* (1985) **49**(1):84-89.
61. HsuChen CC, Feingold DS: **The mechanism of polymyxin b action and selectivity toward biologic membranes.** *Biochemistry* (1973) **12**(11):2105-2111.
62. Lamb HM, Wiseman LR: **Pexiganan acetate.** *Drugs* (1998) **56**(6):1047-1052; discussion 1053-1044.
63. Porcelli F, Buck-Koehntop BA, Thennarasu S, Ramamoorthy A, Veglia G: **Structures of the dimeric and monomeric variants of magainin antimicrobial peptides (msi-78 and msi-594) in micelles and bilayers, determined by nmr spectroscopy.** *Biochemistry* (2006) **45**(18):5793-5799.
64. Zhang Y: **I-tasser server for protein 3d structure prediction.** *BMC bioinformatics* (2008) **9**(40).
65. Kai-Larsen Y, Agerberth B: **The role of the multifunctional peptide II-37 in host defense.** *Frontiers in bioscience : a journal and virtual library* (2008) **13**(3760-3767).
66. Henzler Wildman KA, Lee DK, Ramamoorthy A: **Mechanism of lipid bilayer disruption by the human antimicrobial peptide, II-37.** *Biochemistry* (2003) **42**(21):6545-6558.
67. Ciornei CD, Sigurdardottir T, Schmidtchen A, Bodelsson M: **Antimicrobial and chemoattractant activity, lipopolysaccharide neutralization, cytotoxicity, and inhibition by serum of analogs of human cathelicidin II-37.** *Antimicrobial*

- agents and chemotherapy* (2005) **49**(7):2845-2850.
68. Wang G: **Structures of human host defense cathelicidin II-37 and its smallest antimicrobial peptide kr-12 in lipid micelles.** *The Journal of biological chemistry* (2008) **283**(47):32637-32643.
 69. Wang Z, Wang G: **Apd: The antimicrobial peptide database.** *Nucleic acids research* (2004) **32**(Database issue):D590-592.
 70. Wang G, Li X, Wang Z: **Apd2: The updated antimicrobial peptide database and its application in peptide design.** *Nucleic acids research* (2009) **37**(Database issue):D933-937.
 71. Hultmark D, Engstrom A, Bennich H, Kapur R, Boman HG: **Insect immunity: Isolation and structure of cecropin d and four minor antibacterial components from cecropia pupae.** *European journal of biochemistry / FEBS* (1982) **127**(1):207-217.
 72. Silvestro L, Gupta K, Weiser JN, Axelsen PH: **The concentration-dependent membrane activity of cecropin a.** *Biochemistry* (1997) **36**(38):11452-11460.
 73. Silvestro LS: **Function and structure of cecropin a.** In: 9965567. University of Pennsylvania, Ann Arbor (2000):146-146 p.
 74. Zasloff M: **Magainins, a class of antimicrobial peptides from xenopus skin: Isolation, characterization of two active forms, and partial cdna sequence of a precursor.** *Proceedings of the National Academy of Sciences of the United States of America* (1987) **84**(15):5449-5453.
 75. Cruciani RA, Barker JL, Durell SR, Raghunathan G, Guy HR, Zasloff M, Stanley EF: **Magainin 2, a natural antibiotic from frog skin, forms ion channels in lipid bilayer membranes.** *European journal of pharmacology* (1992) **226**(4):287-296.
 76. Gesell J, Zasloff M, Opella SJ: **Two-dimensional 1h nmr experiments show that the 23-residue magainin antibiotic peptide is an alpha-helix in dodecylphosphocholine micelles, sodium dodecylsulfate micelles, and trifluoroethanol/water solution.** *Journal of biomolecular NMR* (1997) **9**(2):127-135.

77. Nakamura T, Furunaka H, Miyata T, Tokunaga F, Muta T, Iwanaga S, Niwa M, Takao T, Shimonishi Y: **Tachyplesin, a class of antimicrobial peptide from the hemocytes of the horseshoe crab (*tachyplesus tridentatus*). Isolation and chemical structure.** *The Journal of biological chemistry* (1988) **263**(32):16709-16713.

78. Ohta M, Ito H, Masuda K, Tanaka S, Arakawa Y, Wacharotayankun R, Kato N: **Mechanisms of antibacterial action of tachyplesins and polyphemusins, a group of antimicrobial peptides isolated from horseshoe crab hemocytes.** *Antimicrobial agents and chemotherapy* (1992) **36**(7):1460-1465.

79. Ganz T: **Defensins: Antimicrobial peptides of innate immunity.** *Nature reviews Immunology* (2003) **3**(9):710-720.

80. Diamond G, Bevins CL: **Beta-defensins: Endogenous antibiotics of the innate host defense response.** *Clinical immunology and immunopathology* (1998) **88**(3):221-225.

81. Hancock RE, Falla T, Brown M: **Cationic bactericidal peptides.** *Advances in microbial physiology* (1995) **37**(135-175).

82. Boman HG: **Peptide antibiotics and their role in innate immunity.** *Annual review of immunology* (1995) **13**(61-92).

83. Raj PA, Dentino AR: **Current status of defensins and their role in innate and adaptive immunity.** *FEMS microbiology letters* (2002) **206**(1):9-18.

84. Schibli DJ, Hunter HN, Aseyev V, Starner TD, Wiencek JM, McCray PB, Jr., Tack BF, Vogel HJ: **The solution structures of the human beta-defensins lead to a better understanding of the potent bactericidal activity of hbd3 against staphylococcus aureus.** *The Journal of biological chemistry* (2002) **277**(10):8279-8289.

85. Schibli DJ, Epand RF, Vogel HJ, Epand RM: **Tryptophan-rich antimicrobial peptides: Comparative properties and membrane interactions.** *Biochemistry and cell biology = Biochimie et biologie cellulaire* (2002) **80**(5):667-677.

86. Rozek A, Friedrich CL, Hancock RE: **Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles.** *Biochemistry* (2000) **39**(51):15765-15774.
87. Subbalakshmi C, Sitaram N: **Mechanism of antimicrobial action of indolicidin.** *FEMS microbiology letters* (1998) **160**(1):91-96.
88. Robinson JA: **Protein epitope mimetics as anti-infectives.** *Current opinion in chemical biology* (2011) **15**(3):379-386.
89. Schneider G, Fechner U: **Computer-based de novo design of drug-like molecules.** *Nat Rev Drug Discov* (2005) **4**(8):649-663.
90. Schneider G, Bohm HJ: **Virtual screening and fast automated docking methods.** *Drug discovery today* (2002) **7**(1):64-70.
91. Schuller A, Suhartono M, Fechner U, Tanrikulu Y, Breitung S, Scheffer U, Gobel MW, Schneider G: **The concept of template-based de novo design from drug-derived molecular fragments and its application to tar rna.** *Journal of computer-aided molecular design* (2008) **22**(2):59-68.
92. Schneider G, Wrede P: **Artificial neural networks for computer-based molecular design.** *Progress in biophysics and molecular biology* (1998) **70**(3):175-222.
93. Liu S, Zhou L, Li J, Suresh A, Verma C, Foo YH, Yap EP, Tan DT, Beuerman RW: **Linear analogues of human beta-defensin 3: Concepts for design of antimicrobial peptides with reduced cytotoxicity to mammalian cells.** *Chembiochem : a European journal of chemical biology* (2008) **9**(6):964-973.
94. Liu SP, Zhou L, Lakshminarayanan R, Beuerman RW: **Multivalent antimicrobial peptides as therapeutics: Design principles and structural diversities.** *International journal of peptide research and therapeutics* (2010) **16**(3):199-213.
95. Tam JP, Lu YA, Yang JL: **Antimicrobial dendrimeric peptides.** *European journal of biochemistry / FEBS* (2002) **269**(3):923-932.

96. Campopiano DJ, Clarke DJ, Polfer NC, Barran PE, Langley RJ, Govan JR, Maxwell A, Dorin JR: **Structure-activity relationships in defensin dimers: A novel link between beta-defensin tertiary structure and antimicrobial activity.** *The Journal of biological chemistry* (2004) **279**(47):48671-48679.
97. Falciani C, Lozzi L, Pini A, Corti F, Fabbrini M, Bernini A, Lelli B, Niccolai N, Bracci L: **Molecular basis of branched peptides resistance to enzyme proteolysis.** *Chemical biology & drug design* (2007) **69**(3):216-221.
98. Liu Z, Deshazer H, Rice AJ, Chen K, Zhou C, Kallenbach NR: **Multivalent antimicrobial peptides from a reactive polymer scaffold.** *Journal of medicinal chemistry* (2006) **49**(12):3436-3439.
99. Pini A, Falciani C, Mantengoli E, Bindi S, Brunetti J, Iozzi S, Rossolini GM, Bracci L: **A novel tetrabranching antimicrobial peptide that neutralizes bacterial lipopolysaccharide and prevents septic shock in vivo.** *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* (2010) **24**(4):1015-1022.
100. Bai Y, Liu S, Jiang P, Zhou L, Li J, Tang C, Verma C, Mu Y, Beuerman RW, Pervushin K: **Structure-dependent charge density as a determinant of antimicrobial activity of peptide analogues of defensin.** *Biochemistry* (2009) **48**(30):7229-7239.
101. David MZ, Daum RS: **Community-associated methicillin-resistant staphylococcus aureus: Epidemiology and clinical consequences of an emerging epidemic.** *Clinical microbiology reviews* (2010) **23**(3):616-687.
102. Deurenberg RH, Stobberingh EE: **The molecular evolution of hospital- and community-associated methicillin-resistant staphylococcus aureus.** *Current molecular medicine* (2009) **9**(2):100-115.
103. Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK, National Healthcare Safety Network T, Participating National Healthcare Safety Network F: **Nhsn annual update: Antimicrobial-resistant pathogens associated with**

- healthcare-associated infections: Annual summary of data reported to the national healthcare safety network at the centers for disease control and prevention, 2006-2007.** *Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America* (2008) **29**(11):996-1011.
104. Otter JA, French GL: **Community-associated meticillin-resistant staphylococcus aureus strains as a cause of healthcare-associated infection.** *The Journal of hospital infection* (2011) **79**(3):189-193.
 105. Rozen DE, McGee L, Levin BR, Klugman KP: **Fitness costs of fluoroquinolone resistance in streptococcus pneumoniae.** *Antimicrobial agents and chemotherapy* (2007) **51**(2):412-416.
 106. Meka VG, Gold HS: **Antimicrobial resistance to linezolid.** *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* (2004) **39**(7):1010-1015.
 107. Hsu LY, Leong M, Balm M, Chan DS, Huggan P, Tan TY, Koh TH, Hon PY, Ng MM: **Six cases of daptomycin-non-susceptible staphylococcus aureus bacteraemia in singapore.** *Journal of medical microbiology* (2010) **59**(Pt 12):1509-1513.
 108. Talan DA, Krishnadasan A, Gorwitz RJ, Fosheim GE, Limbago B, Albrecht V, Moran GJ, Group EMINS: **Comparison of staphylococcus aureus from skin and soft-tissue infections in us emergency department patients, 2004 and 2008.** *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* (2011) **53**(2):144-149.
 109. Skov RL, Jensen KS: **Community-associated meticillin-resistant staphylococcus aureus as a cause of hospital-acquired infections.** *The Journal of hospital infection* (2009) **73**(4):364-370.
 110. Cosgrove SE, Qi Y, Kaye KS, Harbarth S, Karchmer AW, Carmeli Y: **The impact of methicillin resistance in staphylococcus aureus bacteremia on patient outcomes: Mortality, length of stay, and hospital charges.** *Infection control and hospital epidemiology : the official journal of the Society of Hospital*

Epidemiologists of America (2005) **26**(2):166-174.

111. Pedraza-Chaverri J, Cardenas-Rodriguez N, Orozco-Ibarra M, Perez-Rojas JM: **Medicinal properties of mangosteen (garcinia mangostana).** *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association* (2008) **46**(10):3227-3239.
112. Sakagami Y, Iinuma M, Piyasena KG, Dharmaratne HR: **Antibacterial activity of alpha-mangostin against vancomycin resistant enterococci (vre) and synergism with antibiotics.** *Phytomedicine : international journal of phytotherapy and phytopharmacology* (2005) **12**(3):203-208.
113. Iinuma M, Tosa H, Tanaka T, Asai F, Kobayashi Y, Shimano R, Miyauchi K: **Antibacterial activity of xanthones from guttiferaceous plants against methicillin-resistant staphylococcus aureus.** *The Journal of pharmacy and pharmacology* (1996) **48**(8):861-865.
114. Zasloff M: **Antimicrobial peptides of multicellular organisms.** *Nature* (2002) **415**(6870):389-395.
115. Wimley WC: **Describing the mechanism of antimicrobial peptide action with the interfacial activity model.** *ACS Chem Biol* (2010) **5**(10):905-917.
116. Matsuzaki K: **Why and how are peptide-lipid interactions utilized for self-defense? Magainins and tachyplesins as archetypes.** *Biochimica et biophysica acta* (1999) **1462**(1-2):1-10.
117. Shai Y: **Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides.** *Biochimica et biophysica acta* (1999) **1462**(1-2):55-70.
118. Yang L, Weiss TM, Lehrer RI, Huang HW: **Crystallization of antimicrobial pores in membranes: Magainin and protegrin.** *Biophysical journal* (2000) **79**(4):2002-2009.

119. Gazit E, Miller IR, Biggin PC, Sansom MS, Shai Y: **Structure and orientation of the mammalian antibacterial peptide cecropin p1 within phospholipid membranes.** *Journal of molecular biology* (1996) **258**(5):860-870.
120. Breukink E, de Kruijff B: **The lantibiotic nisin, a special case or not?** *Biochimica et biophysica acta* (1999) **1462**(1-2):223-234.
121. Huang HW: **Action of antimicrobial peptides: Two-state model.** *Biochemistry* (2000) **39**(29):8347-8352.
122. Sansom MS: **Alamethicin and related peptaibols--model ion channels.** *European biophysics journal : EBJ* (1993) **22**(2):105-124.
123. Matsuzaki K, Murase O, Fujii N, Miyajima K: **An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation.** *Biochemistry* (1996) **35**(35):11361-11368.
124. Hallock KJ, Lee DK, Ramamoorthy A: **Msi-78, an analogue of the magainin antimicrobial peptides, disrupts lipid bilayer structure via positive curvature strain.** *Biophysical journal* (2003) **84**(5):3052-3060.
125. Rathinakumar R, Walkenhorst WF, Wimley WC: **Broad-spectrum antimicrobial peptides by rational combinatorial design and high-throughput screening: The importance of interfacial activity.** *Journal of the American Chemical Society* (2009) **131**(22):7609-7617.
126. Rathinakumar R, Wimley WC: **Biomolecular engineering by combinatorial design and high-throughput screening: Small, soluble peptides that permeabilize membranes.** *Journal of the American Chemical Society* (2008) **130**(30):9849-9858.
127. Mogi T, Ui H, Shiomi K, Omura S, Kita K: **Gramicidin s identified as a potent inhibitor for cytochrome bd-type quinol oxidase.** *FEBS letters* (2008) **582**(15):2299-2302.
128. Park CB, Yi KS, Matsuzaki K, Kim MS, Kim SC: **Structure-activity analysis of buforin ii, a histone h2a-derived**

- antimicrobial peptide: The proline hinge is responsible for the cell-penetrating ability of buforin ii.** *Proceedings of the National Academy of Sciences of the United States of America* (2000) **97**(15):8245-8250.
129. Park CB, Kim HS, Kim SC: **Mechanism of action of the antimicrobial peptide buforin ii: Buforin ii kills microorganisms by penetrating the cell membrane and inhibiting cellular functions.** *Biochemical and biophysical research communications* (1998) **244**(1):253-257.
130. Wu M, Maier E, Benz R, Hancock RE: **Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of escherichia coli.** *Biochemistry* (1999) **38**(22):7235-7242.
131. Hancock RE, Chapple DS: **Peptide antibiotics.** *Antimicrobial agents and chemotherapy* (1999) **43**(6):1317-1323.
132. Marks JR, Placone J, Hristova K, Wimley WC: **Spontaneous membrane-translocating peptides by orthogonal high-throughput screening.** *Journal of the American Chemical Society* (2011) **133**(23):8995-9004.
133. Hilpert K, Elliott MR, Volkmer-Engert R, Henklein P, Donini O, Zhou Q, Winkler DF, Hancock RE: **Sequence requirements and an optimization strategy for short antimicrobial peptides.** *Chem Biol* (2006) **13**(10):1101-1107.
134. Hilpert K, Volkmer-Engert R, Walter T, Hancock RE: **High-throughput generation of small antibacterial peptides with improved activity.** *Nature biotechnology* (2005) **23**(8):1008-1012.
135. Jin Y, Hammer J, Pate M, Zhang Y, Zhu F, Zmuda E, Blazyk J: **Antimicrobial activities and structures of two linear cationic peptide families with various amphipathic beta-sheet and alpha-helical potentials.** *Antimicrobial agents and chemotherapy* (2005) **49**(12):4957-4964.
136. Mowery BP, Lee SE, Kissounko DA, Epand RF, Epand RM, Weisblum B, Stahl SS, Gellman SH: **Mimicry of antimicrobial**

- host-defense peptides by random copolymers.** *Journal of the American Chemical Society* (2007) **129**(50):15474-15476.
137. Diebold Y, Calonge M, Enriquez de Salamanca A, Callejo S, Corrales RM, Saez V, Siemasko KF, Stern ME: **Characterization of a spontaneously immortalized cell line (ioba-nhc) from normal human conjunctiva.** *Investigative ophthalmology & visual science* (2003) **44**(10):4263-4274.
138. Loh B, Grant C, Hancock RE: **Use of the fluorescent probe 1-n-phenylnaphthylamine to study the interactions of aminoglycoside antibiotics with the outer membrane of pseudomonas aeruginosa.** *Antimicrobial agents and chemotherapy* (1984) **26**(4):546-551.
139. Hancock RE, Sahl HG: **Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies.** *Nature biotechnology* (2006) **24**(12):1551-1557.
140. Koh JJ, Qiu S, Zou H, Lakshminarayanan R, Li J, Zhou X, Tang C, Saraswathi P, Verma C, Tan DT, Tan AL *et al.*: **Rapid bactericidal action of alpha-mangostin against mrsa as an outcome of membrane targeting.** *Biochimica et biophysica acta* (2013) **1828**(2):834-844.
141. Yamamoto K, Soong R, Ramamoorthy A: **Comprehensive analysis of lipid dynamics variation with lipid composition and hydration of bicelles using nuclear magnetic resonance (nmr) spectroscopy.** *Langmuir : the ACS journal of surfaces and colloids* (2009) **25**(12):7010-7018.
142. Whiles JA, Deems R, Vold RR, Dennis EA: **Bicelles in structure-function studies of membrane-associated proteins.** *Bioorganic chemistry* (2002) **30**(6):431-442.
143. Mertens R, Seyler L, Lacor P, Denis O: **Community associated methicillin-resistant staphylococcus aureus.** *Acta clinica Belgica* (2012) **67**(4):235-240.
144. Breathnach AS, Cubbon MD, Karunaharan RN, Pope CF, Planche TD: **Multidrug-resistant pseudomonas aeruginosa outbreaks in two hospitals: Association with contaminated hospital**

- waste-water systems.** *The Journal of hospital infection* (2012) **82**(1):19-24.
145. Bauernfeind A, Eberlein E, Hörl G: **Bactericidal kinetics of various dosages of fleroxacin simulated in bacterial cultures.** *Journal of Antimicrobial Chemotherapy* (1988) **22**(Supplement D):81-89.
146. Gradelski E, Kolek B, Bonner D, Fung-Tomc J: **Bactericidal mechanism of gatifloxacin compared with other quinolones.** *Journal of Antimicrobial Chemotherapy* (2002) **49**(1):185-188.
147. Ramamoorthy A, Thennarasu S, Tan A, Gottipati K, Sreekumar S, Heyl DL, An FY, Shelburne CE: **Deletion of all cysteines in tachyplesin i abolishes hemolytic activity and retains antimicrobial activity and lipopolysaccharide selective binding.** *Biochemistry* (2006) **45**(20):6529-6540.
148. Mu Y: **Dissociation aided and side chain sampling enhanced hamiltonian replica exchange.** *The Journal of chemical physics* (2009) **130**(16):164107.
149. Kollman PA, Massova I, Reyes C, Kuhn B, Huo S, Chong L, Lee M, Lee T, Duan Y, Wang W, Donini O *et al*: **Calculating structures and free energies of complex molecules: Combining molecular mechanics and continuum models.** *Accounts of chemical research* (2000) **33**(12):889-897.
150. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJ: **Gromacs: Fast, flexible, and free.** *Journal of computational chemistry* (2005) **26**(16):1701-1718.
151. Tieleman DP, van der Spoel D, Berendsen HJC: **Molecular dynamics simulations of dodecylphosphocholine micelles at three different aggregate sizes: Micellar structure and chain relaxation.** *The Journal of Physical Chemistry B* (2000) **104**(27):6380-6388.
152. Tieleman DP, Marrink SJ, Berendsen HJ: **A computer perspective of membranes: Molecular dynamics studies of lipid bilayer systems.** *Biochimica et biophysica acta* (1997) **1331**(3):235-270.

153. MacKerell AD, Jr., Banavali N, Foloppe N: **Development and current status of the charmm force field for nucleic acids.** *Biopolymers* (2000) **56**(4):257-265.
154. Klauda JB, Venable RM, Freites JA, O'Connor JW, Tobias DJ, Mondragon-Ramirez C, Vorobyov I, MacKerell AD, Jr., Pastor RW: **Update of the charmm all-atom additive force field for lipids: Validation on six lipid types.** *The journal of physical chemistry B* (2010) **114**(23):7830-7843.
155. Mackerell AD, Jr.: **Empirical force fields for biological macromolecules: Overview and issues.** *Journal of computational chemistry* (2004) **25**(13):1584-1604.
156. Marrink SJ, Risselada HJ, Yefimov S, Tieleman DP, de Vries AH: **The martini force field: Coarse grained model for biomolecular simulations.** *The journal of physical chemistry B* (2007) **111**(27):7812-7824.
157. Yesylevskyy SO, Schafer LV, Sengupta D, Marrink SJ: **Polarizable water model for the coarse-grained martini force field.** *PLoS computational biology* (2010) **6**(6):e1000810.
158. Guntert P: **Automated nmr structure calculation with cyana.** *Methods in molecular biology* (2004) **278**(353-378).
159. Warschawski DE, Arnold AA, Beaugrand M, Gravel A, Chartrand E, Marcotte I: **Choosing membrane mimetics for nmr structural studies of transmembrane proteins.** *Biochimica et biophysica acta* (2011) **1808**(8):1957-1974.
160. Maler L: **Solution nmr studies of peptide-lipid interactions in model membranes.** *Molecular membrane biology* (2012) **29**(5):155-176.
161. Vold RR, Prosser RS, Deese AJ: **Isotropic solutions of phospholipid bicelles: A new membrane mimetic for high-resolution nmr studies of polypeptides.** *Journal of biomolecular NMR* (1997) **9**(3):329-335.
162. Wang J, Schnell JR, Chou JJ: **Amantadine partition and**

- localization in phospholipid membrane: A solution nmr study.** *Biochemical and biophysical research communications* (2004) **324**(1):212-217.
163. Pintacuda G, Otting G: **Identification of protein surfaces by nmr measurements with a paramagnetic gd(iii) chelate.** *Journal of the American Chemical Society* (2001) **124**(3):372-373.
164. Zangger K, Respondek M, Gobl C, Hohlweg W, Rasmussen K, Grampp G, Madl T: **Positioning of micelle-bound peptides by paramagnetic relaxation enhancements.** *The journal of physical chemistry B* (2009) **113**(13):4400-4406.
165. McDermott A: **Structure and dynamics of membrane proteins by magic angle spinning solid-state nmr.** *Annual review of biophysics* (2009) **38**:385-403.
166. Beckonert O, Coen M, Keun HC, Wang Y, Ebbels TM, Holmes E, Lindon JC, Nicholson JK: **High-resolution magic-angle-spinning nmr spectroscopy for metabolic profiling of intact tissues.** *Nat Protoc* (2010) **5**(6):1019-1032.
167. Bevins CL, Martin-Porter E, Ganz T: **Defensins and innate host defence of the gastrointestinal tract.** *Gut* (1999) **45**(6):911-915.
168. Yu HY, Tu CH, Yip BS, Chen HL, Cheng HT, Huang KC, Lo HJ, Cheng JW: **Easy strategy to increase salt resistance of antimicrobial peptides.** *Antimicrobial agents and chemotherapy* (2011) **55**(10):4918-4921.
169. Kandasamy SK, Larson RG: **Effect of salt on the interactions of antimicrobial peptides with zwitterionic lipid bilayers.** *Biochimica et biophysica acta* (2006) **1758**(9):1274-1284.
170. Morris KF, Johnson CS: **Mobility ordered 2d-nmr spectroscopy.** *Journal of the American Chemical Society* (1992) **114**(2):776-777.
171. Wang J, Schnell JR, Chou JJ: **Amantadine partition and localization in phospholipid membrane: A solution nmr study.** *Biochemical and biophysical research communications* (2004) **324**(1):212-217.

172. Martinek TA, Hetenyi A, Fulop L, Mandity IM, Toth GK, Dekany I, Fulop F: **Secondary structure dependent self-assembly of beta-peptides into nanosized fibrils and membranes.** *Angewandte Chemie* (2006) **45**(15):2396-2400.
173. Clark TD, Bartolotti L, Hicks RP: **The application of dosy nmr and molecular dynamics simulations to explore the mechanism(s) of micelle binding of antimicrobial peptides containing unnatural amino acids.** *Biopolymers* (2013) **99**(8):548-561.
174. Koradi R, Billeter M, Wuthrich K: **Molmol: A program for display and analysis of macromolecular structures.** *Journal of molecular graphics* (1996) **14**(1):51-55, 29-32.
175. Schrodinger, LLC: **The pymol molecular graphics system, version 1.3r1.** In: (2010).
176. Bai Y, Liu S, Li J, Lakshminarayanan R, Sarawathi P, Tang C, Ho D, Verma C, Beuerman RW, Pervushin K: **Progressive structuring of a branched antimicrobial peptide on the path to the inner membrane target.** *The Journal of biological chemistry* (2012) **287**(32):26606-26617.
177. Crosson CE, Klyce SD, Beuerman RW: **Epithelial wound closure in the rabbit cornea. A biphasic process.** *Investigative ophthalmology & visual science* (1986) **27**(4):464-473.
178. Falla TJ, Karunaratne DN, Hancock RE: **Mode of action of the antimicrobial peptide indolicidin.** *The Journal of biological chemistry* (1996) **271**(32):19298-19303.
179. Wu M, Hancock RE: **Interaction of the cyclic antimicrobial cationic peptide bactenecin with the outer and cytoplasmic membrane.** *The Journal of biological chemistry* (1999) **274**(1):29-35.
180. Zhang L, Rozek A, Hancock RE: **Interaction of cationic antimicrobial peptides with model membranes.** *The Journal of biological chemistry* (2001) **276**(38):35714-35722.
181. Papo N, Shai Y: **A molecular mechanism for**

- lipopolysaccharide protection of gram-negative bacteria from antimicrobial peptides.** *The Journal of biological chemistry* (2005) **280**(11):10378-10387.
182. Li J, Garg M, Shah D, Rajagopalan R: **Solubilization of aromatic and hydrophobic moieties by arginine in aqueous solutions.** *The Journal of chemical physics* (2010) **133**(5):054902.
183. Li S, Su Y, Luo W, Hong M: **Water-protein interactions of an arginine-rich membrane peptide in lipid bilayers investigated by solid-state nuclear magnetic resonance spectroscopy.** *The journal of physical chemistry B* (2010) **114**(11):4063-4069.
184. Rausch JM, Marks JR, Rathinakumar R, Wimley WC: **Beta-sheet pore-forming peptides selected from a rational combinatorial library: Mechanism of pore formation in lipid vesicles and activity in biological membranes.** *Biochemistry* (2007) **46**(43):12124-12139.
185. Zou H, Koh JJ, Li J, Qiu S, Aung TT, Lin H, Lakshminarayanan R, Dai X, Tang C, Lim FH, Zhou L *et al*: **Design and synthesis of amphiphilic xanthone-based, membrane-targeting antimicrobials with improved membrane selectivity.** *Journal of medicinal chemistry* (2013) **56**(6):2359-2373.
186. Skipp CJ, Tyrrell HJV: **Diffusion in viscous solvents. Part 2.-planar and spherical molecules in propane-1,2-diol at 15, 25 and 35[degree]c.** *Journal of the Chemical Society, Faraday Transactions 1: Physical Chemistry in Condensed Phases* (1975) **71**(0):1744-1753.
187. Papo N, Shai Y: **Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes?** *Peptides* (2003) **24**(11):1693-1703.
188. Yu L, Ding JL, Ho B, Wohland T: **Investigation of a novel artificial antimicrobial peptide by fluorescence correlation spectroscopy: An amphipathic cationic pattern is sufficient for selective binding to bacterial type membranes and antimicrobial activity.** *Biochimica et biophysica acta* (2005) **1716**(1):29-39.

189. Kupce E, Freeman R: **Mapping molecular perturbations by a new form of two-dimensional spectroscopy.** *Journal of the American Chemical Society* (2013) **135**(8):2871-2874.
190. Baxter NJ, Williamson MP: **Temperature dependence of ¹H chemical shifts in proteins.** *Journal of biomolecular NMR* (1997) **9**(4):359-369.
191. Rothmund S, Weisshoff H, Beyermann M, Krause E, Bienert M, Mugge C, Sykes BD, Sonnichsen FD: **Temperature coefficients of amide proton nmr resonance frequencies in trifluoroethanol: A monitor of intramolecular hydrogen bonds in helical peptides.** *Journal of biomolecular NMR* (1996) **8**(1):93-97.
192. Cierpicki T, Otlewski J: **Amide proton temperature coefficients as hydrogen bond indicators in proteins.** *Journal of biomolecular NMR* (2001) **21**(3):249-261.
193. Dhople V, Krukemeyer A, Ramamoorthy A: **The human beta-defensin-3, an antibacterial peptide with multiple biological functions.** *Biochimica et biophysica acta* (2006) **1758**(9):1499-1512.
194. Powers JP, Tan A, Ramamoorthy A, Hancock RE: **Solution structure and interaction of the antimicrobial polyphemusins with lipid membranes.** *Biochemistry* (2005) **44**(47):15504-15513.
195. Prenner EJ, Lewis RN, Kondejewski LH, Hodges RS, McElhaney RN: **Differential scanning calorimetric study of the effect of the antimicrobial peptide gramicidin s on the thermotropic phase behavior of phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol lipid bilayer membranes.** *Biochimica et biophysica acta* (1999) **1417**(2):211-223.
196. Abraham T, Lewis RN, Hodges RS, McElhaney RN: **Isothermal titration calorimetry studies of the binding of a rationally designed analogue of the antimicrobial peptide gramicidin s to phospholipid bilayer membranes.** *Biochemistry* (2005) **44**(6):2103-2112.

197. Dathe M, Wieprecht T: **Structural features of helical antimicrobial peptides: Their potential to modulate activity on model membranes and biological cells.** *Biochimica et biophysica acta* (1999) **1462**(1-2):71-87.
198. Shai Y: **Mode of action of membrane active antimicrobial peptides.** *Biopolymers* (2002) **66**(4):236-248.
199. Sass V, Pag U, Tossi A, Bierbaum G, Sahl HG: **Mode of action of human beta-defensin 3 against staphylococcus aureus and transcriptional analysis of responses to defensin challenge.** *International journal of medical microbiology : IJMM* (2008) **298**(7-8):619-633.
200. Epand RM, Rotem S, Mor A, Berno B, Epand RF: **Bacterial membranes as predictors of antimicrobial potency.** *Journal of the American Chemical Society* (2008) **130**(43):14346-14352.
201. Leptihn S, Har JY, Chen J, Ho B, Wohland T, Ding JL: **Single molecule resolution of the antimicrobial action of quantum dot-labeled sushi peptide on live bacteria.** *BMC biology* (2009) **7**(22).
202. Mani R, Cady SD, Tang M, Waring AJ, Lehrer RI, Hong M: **Membrane-dependent oligomeric structure and pore formation of a beta-hairpin antimicrobial peptide in lipid bilayers from solid-state nmr.** *Proceedings of the National Academy of Sciences of the United States of America* (2006) **103**(44):16242-16247.
203. Tamba Y, Yamazaki M: **Single giant unilamellar vesicle method reveals effect of antimicrobial peptide magainin 2 on membrane permeability.** *Biochemistry* (2005) **44**(48):15823-15833.
204. Tamba Y, Yamazaki M: **Magainin 2-induced pore formation in the lipid membranes depends on its concentration in the membrane interface.** *The journal of physical chemistry B* (2009) **113**(14):4846-4852.
205. Shuker SB, Hajduk PJ, Meadows RP, Fesik SW: **Discovering**

- high-affinity ligands for proteins: Sar by nmr.** *Science* (1996) **274**(5292):1531-1534.
206. Thomas CJ, Surolia N, Surolia A: **Surface plasmon resonance studies resolve the enigmatic endotoxin neutralizing activity of polymyxin b.** *The Journal of biological chemistry* (1999) **274**(42):29624-29627.
207. Zhang L, Benz R, Hancock RE: **Influence of proline residues on the antibacterial and synergistic activities of alpha-helical peptides.** *Biochemistry* (1999) **38**(25):8102-8111.
208. Bland JM, De Lucca AJ, Jacks TJ, Vigo CB: **All-d-cecropin b: Synthesis, conformation, lipopolysaccharide binding, and antibacterial activity.** *Molecular and cellular biochemistry* (2001) **218**(1-2):105-111.
209. Schuerholz T, Brandenburg K, Marx G: **Antimicrobial peptides and their potential application in inflammation and sepsis.** *Critical care* (2012) **16**(2):207.
210. Andra J, Koch MH, Bartels R, Brandenburg K: **Biophysical characterization of endotoxin inactivation by nk-2, an antimicrobial peptide derived from mammalian nk-lysin.** *Antimicrobial agents and chemotherapy* (2004) **48**(5):1593-1599.
211. Brandenburg K, Kusumoto S, Seydel U: **Conformational studies of synthetic lipid a analogues and partial structures by infrared spectroscopy.** *Biochimica et biophysica acta* (1997) **1329**(1):183-201.
212. Snyder S, Kim D, McIntosh TJ: **Lipopolysaccharide bilayer structure: Effect of chemotype, core mutations, divalent cations, and temperature.** *Biochemistry* (1999) **38**(33):10758-10767.
213. Kaconis Y, Kowalski I, Howe J, Brauser A, Richter W, Razquin-Olazarán I, Inigo-Pestana M, Garidel P, Rossle M, Martinez de Tejada G, Gutschmann T *et al*: **Biophysical mechanisms of endotoxin neutralization by cationic amphiphilic peptides.** *Biophysical journal* (2011) **100**(11):2652-2661.

214. Bhunia A, Saravanan R, Mohanram H, Mangoni ML, Bhattacharjya S: **Nmr structures and interactions of temporin-1tl and temporin-1tb with lipopolysaccharide micelles: Mechanistic insights into outer membrane permeabilization and synergistic activity.** *The Journal of biological chemistry* (2011) **286**(27):24394-24406.
215. Bhattacharjya S, Ramamoorthy A: **Multifunctional host defense peptides: Functional and mechanistic insights from nmr structures of potent antimicrobial peptides.** *The FEBS journal* (2009) **276**(22):6465-6473.
216. Hancock RE: **Alterations in outer membrane permeability.** *Annual review of microbiology* (1984) **38**(237-264).
217. Eun SY, Jang HK, Han SK, Ryu PD, Lee BJ, Han KH, Kim SJ: **A helix-induced oligomeric transition of gaegurin 4, an antimicrobial peptide isolated from a korean frog.** *Molecules and cells* (2006) **21**(2):229-236.
218. Ludtke SJ, He K, Wu Y, Huang HW: **Cooperative membrane insertion of magainin correlated with its cytolytic activity.** *Biochimica et biophysica acta* (1994) **1190**(1):181-184.
219. Huang HW, Wu Y: **Lipid-alamethicin interactions influence alamethicin orientation.** *Biophysical journal* (1991) **60**(5):1079-1087.
220. Heller WT, He K, Ludtke SJ, Harroun TA, Huang HW: **Effect of changing the size of lipid headgroup on peptide insertion into membranes.** *Biophysical journal* (1997) **73**(1):239-244.
221. Heller WT, Waring AJ, Lehrer RI, Huang HW: **Multiple states of beta-sheet peptide protegrin in lipid bilayers.** *Biochemistry* (1998) **37**(49):17331-17338.
222. Rapaport D, Shai Y: **Interaction of fluorescently labeled pardaxin and its analogues with lipid bilayers.** *The Journal of biological chemistry* (1991) **266**(35):23769-23775.
223. Zhou L, Liu SP, Chen LY, Li J, Ong LB, Guo L, Wohland T, Tang

- CC, Lakshminarayanan R, Mavinahalli J, Verma C *et al*: **The structural parameters for antimicrobial activity, human epithelial cell cytotoxicity and killing mechanism of synthetic monomer and dimer analogues derived from hbd3 c-terminal region.** *Amino acids* (2011) **40**(1):123-133.
224. Pieters RJ: **Intervention with bacterial adhesion by multivalent carbohydrates.** *Medicinal research reviews* (2007) **27**(6):796-816.
225. Mani R, Tang M, Wu X, Buffy JJ, Waring AJ, Sherman MA, Hong M: **Membrane-bound dimer structure of a beta-hairpin antimicrobial peptide from rotational-echo double-resonance solid-state nmr.** *Biochemistry* (2006) **45**(27):8341-8349.
226. Li P, Wohland T, Ho B, Ding JL: **Perturbation of lipopolysaccharide (lps) micelles by sushi 3 (s3) antimicrobial peptide. The importance of an intermolecular disulfide bond in s3 dimer for binding, disruption, and neutralization of lps.** *The Journal of biological chemistry* (2004) **279**(48):50150-50156.
227. Jung D, Rozek A, Okon M, Hancock REW: **Structural transitions as determinants of the action of the calcium-dependent antibiotic daptomycin.** *Chemistry & biology* (2004) **11**(7):949-957.
228. Shai Y, Oren Z: **From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides.** *Peptides* (2001) **22**(10):1629-1641.
229. Ladokhin AS, Wimley WC, White SH: **Leakage of membrane vesicle contents: Determination of mechanism using fluorescence reuquenching.** *Biophysical journal* (1995) **69**(5):1964-1971.

Appendix

Table 22. Intermolecular NOEs in F2 peptide.

Seq no.	a.a.	Prot	Seq no. ^a	a.a.	Prot	Upl ^b
4	Lys	H α	8'	Arg	H	4.87
2	Gly	H	8'	Arg	Q β	3.61
2	Gly	H	8'	Arg	Q γ	3.81
3	Arg	Q β	9'	Lys	H	4.01
5	Phe	H	8'	Arg	Q γ	4.24
8	Arg	H	10'	Lys	Q ϵ	5.50
4	Lys	H	5'	Phe	Q δ	5.50
6	Phe	H	8'	Arg	H	5.50
3	Arg	Q γ	5'	Phe	H	4.75
3	Arg	H ϵ	5'	Phe	H	5.50
4'	Lys	H α	8	Arg	H	4.87
2'	Gly	H	8	Arg	Q β	3.61
2'	Gly	H	8	Arg	Q γ	3.81
3'	Arg	Q β	9	Lys	H	4.01
5'	Phe	H	8	Arg	Q γ	4.24
8'	Arg	H	10	Lys	Q ϵ	5.50
4'	Lys	H	5	Phe	Q δ	5.50
6'	Phe	H	8	Arg	H	5.50
3'	Arg	Q γ	5	Phe	H	4.75
3'	Arg	H ϵ	5	Phe	H	5.50

^aTwo monomeric peptides in the F2 dimer are annotated differently by using the prime sign; ^bUpper limit distances are used for dimer intermolecular constrains.

Table 23. Intermolecular NOEs in Y2 peptide.

Seq no.	a.a.	Prot	Seq no. ^a	a.a.	Prot	Upl ^b
4	Lys	H	8'	Arg	H ϵ	5.50
6	Tyr	Q ϵ	9'	Lys	H	5.50
6	Tyr	Q δ	8'	Arg	H	5.50
6	Tyr	H	8'	Arg	Q γ	4.31
5	Tyr	H	8'	Arg	Q γ	4.30
5	Tyr	H	8'	Arg	H α	3.77
5	Tyr	H	9'	Lys	H	4.39
4'	Lys	H	8	Arg	H ϵ	5.50
6'	Tyr	Q ϵ	9	Lys	H	5.50
6'	Tyr	Q δ	8	Arg	H	5.50
6'	Tyr	H	8	Arg	Q γ	4.31
5'	Tyr	H	8	Arg	Q γ	4.30
5'	Tyr	H	8	Arg	H α	3.77
5'	Tyr	H	9	Lys	H	4.39

^aTwo monomeric peptides in the F2 dimer are annotated differently by using the prime sign; ^bUpper limit distances are used for dimer intermolecular constrains.

Publications

1. *Structure-Dependent Charge Density as a Determinant of Antimicrobial Activity of Peptide Analogues of Defensin*

Published in Biochemistry, 2009, **48**, 7229-7239

Authors: Yang Bai, Shouping Liu, Ping Jiang, Lei Zhou, Jing Li, Charles Tang, Chandra Verma, Yuguang Mu, Roger W. Beuerman*, and Konstantin Pervushin*

2. *Progressive structuring of a branched antimicrobial peptide on the path to the inner membrane target*

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Authors: Yang Bai, Shouping Liu, Jianguo Li, Rajamani Lakshminarayanan, Padmanabhan Sarawathi, Charles Tang, Duncun Ho, Chandra Verma, Roger W. Beuerman*, Konstantin Pervushin*

3. *Molecular dynamics simulations of a new branched antimicrobial peptide: A comparison of force fields*

Published in the Journal of Chemical Physics, 2012, **137**, 215101

Authors: Jianguo Li, Rajamani Lakshminarayanan, Yang Bai, Shouping Liu, Lei Zhou, Konstantin Pervushin, Chandra Verma, Roger W. Beuerman*

4. *Molecular simulations suggest how a branched antimicrobial peptide perturbs a bacterial membrane and enhances permeability*

Published in Biochimica et Biophysica Acta Biomembranes, 2013, **1828**, 1112-1121

Authors: Jianguo Li, Shouping Liu, Rajamani Lakshminarayanan, Yang Bai, Konstantin Pervushin, Chandra Verma, Roger W. Beuerman*

Poster presentations

1. 3D structures and oligomeric states of human beta-defensin-3 C-terminal analogues

Presented in Cold Spring Harbor-Asia (CSH-Asia) membrane protein conference. On 29th April, 2010, in Suzhou, China.

Authors: Yang Bai, Shouping Liu, Ping Jiang, Lei Zhou, Jing Li, Charles Tang, Chandra Verma, Yuguang Mu, Roger W. Beuerman*, and Konstantin Pervushin*

2. 3D structures and oligomeric states of human beta-defensin-3 C-terminal analogues

Presented in Asia-ARVO (THE Association for Research in Vision and Ophthalmology) conference. On 29th April, 2010, in Suzhou, China.

Authors: Yang Bai, Shouping Liu, Ping Jiang, Lei Zhou, Jing Li, Charles Tang, Chandra Verma, Yuguang Mu, Roger W. Beuerman*, and Konstantin Pervushin*

3. 3D structures and oligomeric states of human beta-defensin-3 C-terminal analogues

Presented in DUKE-NUS scientific congress. On 29th April, 2010, in Suzhou, China.

Authors: Yang Bai, Shouping Liu, Ping Jiang, Lei Zhou, Jing Li, Charles Tang, Chandra Verma, Yuguang Mu, Roger W. Beuerman*, and Konstantin Pervushin*

4. Progressive structuring of a branched antimicrobial peptide on the path to the inner membrane target

Presented in EMBO practical course 2012, Multidimensional NMR in Structural Biology. On August 2012, Berlin.

Authors: Yang Bai, Shouping Liu, Jianguo Li, Rajamani Lakshminarayanan, Padmanabhan Sarawathi, Charles Tang, Duncun Ho, Chandra Verma, Roger W. Beuerman*, Konstantin Pervushin*

5. ***Progressive structuring of a branched antimicrobial peptide on the path to the inner membrane target***

Presented in International Symposium on Nuclear Magnetic Resonance 2013 (ISMAR 2013) conference. On 19th July, 2013, in Rio de Janeiro, Brazil.

Authors: Yang Bai, Shouping Liu, Jianguo Li, Rajamani Lakshminarayanan, Padmanabhan Sarawathi, Charles Tang, Duncun Ho, Chandra Verma, Roger W. Beuerman*, Konstantin Pervushin*

6. ***Detecting oligomeric state/hydrogen bonds in biomolecules by NMR spectroscopy***

Presented in EMBO practical course 2013, Structure, dynamics and function of biomacromolecules by solution NMR. On July 20th-27th 2013, Basel.

Authors: Yang Bai, Konstantin Pervushin*

Invited talks

1. *Why defensins defend us against bacteria: NMR telling the story*

Presented in A*STAR Structural Biology and Molecular Dynamics conference, 30th, March 2008.

2. *Structure-dependent charge density as a determinant of antimicrobial activity of peptide analogues of defensin*

Presented in 3rd Asia-Pacific NMR conference (AP-NMR). 28th Oct, 2009, in Jeju Island, Korea

3. *Structure and Mobility of Branched Antibacterial Peptide in Aggregated Bacteria and Bacterial-related Mimics*

Presented in the Australian and New Zealand Society for Magnetic Resonance (ANZMAG) conference. On 29th Nov, 2011, in Torque Vic, Australia.

Awards

1. 2009, **invited young scientist speaker** for 3rd Asia-Pacific NMR conference (AP-NMR).
Oct, 2009, in Jeju Island, Korea
2. 2011, **young scientist scholar** award from Sigma-Aldrich[®].
3. 2011, **young scientist talk funding support** from Bruker-Biospin[®].
4. 2011, **Student travel bursaries** from ANZMAG conference committee, 2011.
5. 2012, selected to attend **EMBO NMR theory course**.
6. 2013, **Student travel award** from ISMAR 2013 conference committee.
7. 2013, selected to attend **EMBO NMR practical course**.