CHAPTER 1.
INTRODUCTION

Ever since the discovery of antibiotics, they have been our most reliable weapons in fighting off numerous pathogens that cause potentially fatal infections. In the last few decades, resistance development is an unavoidable consequence associated with the use of conventionally available antibiotics. In fact, quite soon after the introduction of antimicrobial drugs, bacteria began to exhibit an accelerated evolution towards resistant strains and the ability to transfer resistance mechanism amongst species [1,2]. In this way, the therapeutic potential of most of the available antibiotics is rather compromised. Moreover, the problem is complicated by the non-appropriate and intensive use of the antibiotics, the increase of immunosuppressed individuals and the spreading of resistant strains by transcontinental travels in modern society [3].

Earlier, infections caused by multi-drug resistant bacterial strains were mostly limited to the nosocomial environment but community acquired resistant strains are now rising in prevalence [4]. Notably, methicillin-resistant *S. aureus* (MRSA) appears to have reached its high point of resistance and has become increasingly difficult to treat in an impressively short time [5]. It is estimated to cause ~19,000 deaths per year in the United States. Apart from their high mortality rate, MRSA infections lead to an estimated $3 billion to $4 billion of additional health care costs per year [6]. In addition, the infections caused by MDR and PDR Gram-negative bacteria are recognized as a severe menace to public health in nations around the globe [7]. The strains of *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* are found to be resistant to most of the antibiotic currently on the market [8]. In particular, *E. coli* garner increasing attention due to their rapid spread of resistance, leaving limited empirical treatment options [9]. Recently, plasmid associated New Delhi metallo-β-lactamase 1 (NDM-1) gene was identified in Gram-negative bacteria, which may be easily transferred to other bacteria through horizontal gene transfer that confers resistance to a number of antimicrobial drugs, such as fluoroquinolones, aminoglycosides, carbapenems and all β-lactams [10].

The phenomenon of resistance is also extended to other kinds of pathogens; fungi, viruses, and parasites. In the past few years, there has been a remarkable increase in the rate of fatal infections caused by opportunistic fungal strains [11]. Systemic fungal infections spread
primarily among the people who are having impaired immune systems resulting from AIDS [12], cancer chemotherapy [13] and organ transplantation [14]. The fungal cells are eukaryotic in nature [15], which further potentiates the difficulties associated with the development of selective antifungal therapeutics. At present, the available antifungal therapies include polyenes and azoles acting through fungal membrane disruption and inhibit biosynthesis of sterol, respectively [16]. However, the high toxicity of polyenes and the development of resistance against azole [17] have intensified the search of new potential antifungal agents with different modes of action.

Simultaneous marked decline in the development of novel anti-infective agents is one of the greatest negative aspects of modern medicine [18]. Only a handful of new antimicrobial drugs has entered the clinic during last few decades [19]. At present, only a small number of the major pharmaceutical companies have R&D programs on anti-infective agents, and the low interest has been defended based on simple economics [20]. In addition, development of new anti-infective agents is associated with high risks of inducing bacterial resistance within a few years, as shown by history, or to be restricted for use as a drug of last resort [21,22].

These trends have emphasized to discover new class of antimicrobial agents possessing novel mode of action as well as different cellular targets compared to existing antibiotics in order to decrease the likelihood of development of resistance. It is now widely recognized that the native antimicrobial peptides could play a promising role in the development of novel anti-infective agents because of their broad spectrum activity and minimal propensity for resistance development [23-25].

1.1. Antimicrobial peptides (AMPs)

AMPs are an abundant group of molecules that are found in virtually all classes of life across the phylogenetic spectrum [25,26]. AMPs are the main elements of the innate immune defense system; a weapons that all multicellular organisms are “born with” to ward off pathogenic microbes in order to survive and thrive on this planet [27,28]. Importantly, peptide mediated innate immunity is recognized as the first host protective barrier. Most of these gene encoded peptides are mobilized shortly after microbial infection and act rapidly to neutralize a broad range of microbes [29]. AMPs were discovered some 30 years ago, initially isolated from insect lymph, the skin of frogs and mammalian neutrophils. Since then, thousands of cationic peptides have been reported from numerous species, isolated from numerous organs and tissues such as eyes, pancreas, oral mucosa and epithelium of respiratory and
gastrointestinal tract in mammalian species [30,31]. In mammals the two major families of AMPs are defensins and cathelicidins [32,33].

Structurally, AMPs were classified in four major classes: (1) $\alpha$-helix, (2) $\beta$-sheet stabilized by two or three disulfide bridges, (3) extended structures with one or more predominant residues (like tryptophan and proline rich) and (4) loop due to the presence of a single disulfide bridge [31,34]. Among these the first two classes being the most abundant in nature [35]. Most of the AMPs exhibit a relatively unstructured conformation in solution, and fold into amphipathic arrangement with hydrophobic and hydrophilic moieties segregating into distinct patches on the molecular surface when interacting with the unique environment of biological membranes [36]. Generally, AMPs are composed of 12 to 50 amino acid residues. Due to the frequent occurrence of lysine and arginine residues in their amino acid sequence, they usually possess a net positive charge (generally $+2$ to $+9$) at physiological pH; although a few negatively charged AMPs have also been found [37]. Such peptides have broad spectra of activity that can encompass bacteria, fungi, viruses and parasites [25]. The potential pharmacologic application and toxicity profile of antimicrobial peptides is mainly determined by their selectivity i.e. the degree to which they differentiate between microbial targets and normal host cells.

1.1.1. Selectivity

The selectivity of antimicrobial peptides is due to fundamental differences, between microbial cells and mammalian host cells and also the microenvironments in which these counterparts convene [38]. The eukaryotic membranes contain zwitterionic components like phosphatidylcholine (PC), phosphatidylethanolamine (PE), sphingomyelin (SM) and sterols such as cholesterol and ergosterol. In contrast, prokaryotic architecture which mainly comprises negatively charged components namely hydroxylated phospholipids, phosphatidylglycerol (PG), cardiolipin (CL) and phosphatidylserine (PS) [39]. Electrostatic interactions between the positively charged AMPs and the negatively charged bacterial phospholipids provide an initial mode of interaction, whereas hydrophobic interactions allow the peptides to penetrate the cell membrane [38,39].

1.1.2. Mode of Action

The mode of action of AMPs is of particular interest, as it is thought to be nonspecific unlike traditional antibiotic drugs (usually directed against a precise cellular receptor) thus, not deriving the development of resistance [40,41]. The mechanism for lytic activity of AMPs
is varied and some significant questions remain still unanswered [42]. The initial contact between the AMPs and the external leaflet of target microorganism would be electrostatic, as most bacterial surfaces are anionic [38, 43]. The linear AMPs re-organize and assume an optimal amphipathic conformation in close proximity of biomembranes. The hydrophilic face interacts with the phospholipid head groups whereas their hydrophobic face is inserted in the bilayer core [43]. Such interactions can lead to structural distortion of the membrane architecture by various possible mechanisms. Three models have been proposed to describe the process of microbial membrane permeation by membrane-active peptides, the carpet model, the barrel-stave model and the toroidal pore model. The details of these models are as follows:

**Figure 1.1:** Schematic representation of the action of AMPs leading toward bacterial membrane permeation and disruption. (A) AMPs adopt amphipathic conformation in the close proximity of biological membrane. (B) Representation of the selectivity of AMPs to bacteria over mammalian cells based on electrostatic attraction. (C) Proposed membrane permeabilization models
1.1.2.1. The carpet model

According to the carpet model, AMPs first bind onto the surface of the target microbial cell membrane and subsequently cover it in a carpet-like manner (Figure 1.1C). The initial interaction between the AMPs and the external leaflet of target microorganism would be electrostatic, as most bacterial surfaces are anionic [44]. In the second step, AMPs reorient themselves such that their hydrophobic face points toward the membrane lipids, and the hydrophilic face toward the phospholipid head-groups. After a threshold concentration has been reached, AMPs cause membrane permeation. It affects the membrane in a detergent-like manner, resulting in the collapse of the membrane packing into fragments with physical dissolution of the cell wall [45]. High local concentration on the surface of the membrane depends upon the type of the target membrane and can occur either after all the surface of the membrane is covered with peptide monomers, or alternatively, antimicrobial peptides that associate on the surface of the membrane can form a local carpet [45,46].

The carpet model describes a situation in which AMPs are in contact with the phospholipid head group throughout the entire process of membrane permeation. The presence of negatively charged lipids in the target membrane architecture is essential for the accumulation of AMPs to form carpet-like structures, as they help to reduce the repulsive electrostatic forces between positively charged peptides. AMPs exerting their antimicrobial action via this mechanism do not require a specific structure, length, or specific amino acids sequence [47]. The carpet model was proposed for the first time to describe the mode of action of dermaseptin S [48], and later on was used to describe the mode of action of other antimicrobial peptides, such as dermaseptin natural analogues [49], cecropins [50], and the human antimicrobial peptide LL-37 [51].

1.1.2.2. The barrel-stave model

The term ‘barrel-stave’ describes the overall topology of a membrane channels/pores formed by the aggregation of AMPs through membrane core (Figure 1.1C). In this model, AMPs are self-associate either in solution prior to binding and insertion into the membrane or alternatively, bind to the membrane followed by peptide oligomerization and insertion. During oligomerization, AMPs orient themselves in such a way that the hydrophobic surfaces of AMPs face outward, toward the acyl chains of the membrane, whereas the hydrophilic surfaces constitute the pore lining [52]. The AMP binding at the outer surface of target membrane, most likely as monomers, is considered as the initiation of this membrane
permeabilization mechanism. AMPs with a large number of cationic charged residues (lysine or arginine) spread along the peptide chain cannot form a transmembrane pore unless its charges become neutralized. This suggests that AMPs acting via this mechanism do not have a high net positive charge. Therefore, in this model the peptide interaction toward phospholipid bilayer is driven predominantly by hydrophobic interactions. As a consequence of these properties the peptides bind to phospholipid membranes irrespective of the membrane charge, and therefore, may be toxic toward both bacterial and mammalian cells [53]. After initial binding on the target membrane, AMPs may undergo a conformational phase transition, forcing displacement of polar-phospholipid head groups which result into localized membrane thinning. It is energetically unfavorable for a single AMP molecule to transverse the membrane as a monomer [52]. Consequently, when membrane bound AMPs reaches a threshold concentration, peptide monomers self-aggregate and insert deeper into the hydrophobic membrane core. Progressive recruitment of additional AMP monomers leads to a further expansion of the membrane pore. Leakage of intracellular components through these pores subsequently causes cell death [45].

1.1.2.3. The toroidal pore model

This model describes the well characterized peptide-membrane interactions. Same as that of barrel-stave model in this model also AMPs exert their antimicrobial action by traverse through the membrane core. The only difference between the toroidal pore and barrel-stave models is that in the former, membrane polar-phospholipid head groups are intercalated in between the AMP molecules in the transmembrane channel (Figure 1.1C) [54]. The aggregation of AMPs through membrane core constitutes a membrane-spanning pore, which is referred as a supramolecular complex. AMPs orient themselves through membrane core in such a way that hydrophobic residues face towards hydrophobic membrane core and polar peptide surfaces as well as phospholipid head groups constitutes the pore lining [55]. The formation of so-called wormholes or toroidal pores was proposed to describe the mode of action of magainin [54,55], protegrin [56], and melittin [57].

1.1.2.4. Alternative mode of action

The barrel stave, the carpet and the toroidal models predict that the killing activity of AMPs occurs due to perturbation of membrane integrity. However, several studies indicate that permeabilization is necessary but may not be enough to explain antimicrobial activity. Several studies investigated the relationship between microbial membrane permeabilization
and cell death revealing that cell killing may proceed with relatively little membrane
disruption and suggesting that AMPs may interact with key intracellular targets [58]. Xiong
and coworkers reported that S. aureus cells remained viable long after rapid membrane
permeabilization induced by tPMPs. These outcomes suggested that non-membranolytic
mechanisms are responsible for cell death. tPMPs exert their microbial killing effect by direct
inhibition of nucleic acid synthesis and the relatively strong negative charge of nucleic acids
is consistent with the hypothesis that cationic peptides bind to and inhibit these molecules
[59]. Kragol et al. reported that insect antibacterial peptides (pyrrhocoricin, drosocin, and
apidaecin) inhibit the bacterial heat shock protein DnaK, and inhibition of this protein is
associated with cell death [60]. Likewise, buforin II has been reported to penetrate microbial
cell membranes and interfere with intracellular functions [58]. It is also believed that the
antimicrobial peptide, microcin B17, specifically target DNA gyrase within E. coli and
subsequently resulted into inhibition of DNA replication. Indolicidin was proposed to inhibit
DNA synthesis leading to filamentation in Escherichia coli [61]. Some AMPs were found to
interfere with the metabolic processes of microbes; an example is the glycine-rich attacins that
were shown to block the transcription of the omp gene in E. coli [62], whereas magainins and
cecropins induce selective transcription of its stress-related genes micF and osmY at non-
bactericidal concentrations [63]. The above observations suggest that AMPs mediated cell
death may occur as a result of several independent mechanisms of action. Furthermore,
peptides may kill the same species via more than one mechanism of action, depending on
individual factors such as growth phase, tissue localization, and the presence or absence of
other immune mechanisms or synergistic exogenous antimicrobial agents. From these
perspectives, AMPs may have multiple and complementary mechanisms of action necessary
to inhibit or kill a wide variety of pathogens in diverse physiologic settings and
simultaneously suppressing the ability of the pathogen to develop resistance.

1.1.3. Resistance to AMPs

Most of the bacteria differ in their intrinsic susceptibility to AMPs, and the relative
resistance of some pathogens to these defense molecules is considered as a part of their
phenotype. The natural mechanisms of resistance development to AMPs are termed as
constructive mechanisms [64]. Though the ability to resist AMP killing appears to be a
formidable challenge for microbial evolution, AMP resistance is increasingly recognized as a
discriminating feature of some important human pathogens. Several bacterial species possess
resistance to AMPs through constructive mechanisms. For example *Serratia*, *Proteus*, *Providencia*, and *Pseudomonas* species due to unusual composition of their membrane can be inherently resistant to AMPs [64,65]. In addition to the constructive mechanisms bacteria have developed inducible mode of resistance in response to the stress generated by AMPs [65]. Similar to pharmaceutical antibiotics, it appears that bacteria exposed to human AMPs have evolved under selective pressure to develop mechanisms of resistance [66]. Diverse inducible mechanisms of bacterial resistance to AMP have been identified which mainly includes altered cell surface charge, active efflux, production of proteases or trapping proteins, and modification of host cellular processes. However, even though these selective pressures have existed for countless centuries, human AMPs still possess a broad spectrum of potent activity against a diverse array of Gram-positive and Gram-negative bacterial species, fungi, as well as certain protozoan parasites and enveloped viruses [65,66].

1.1.4. Therapeutic potential of AMPs

AMPs constitute an attractive class of therapeutic agents having broad antimicrobial spectrum and are effective against pathogens resistant to the conventional antibiotics [35,53]. Moreover, AMPs can complement conventional antibiotic therapy probably by facilitating access into the bacterial cell resulting in a synergistic effect [67]. AMPs may initiate adaptive immune responses by acting as chemokines and/or induce chemokine production, inhibiting lipopolysaccharide (LPS)-induced pro-inflammatory cytokine production and recruiting antigen-presenting cells [68]. AMPs may also possess immunomodulatory activity when involved in the clearance of infection, including the promotion of wound healing [69]. Because of these properties, the term “host defense peptides” has been proposed for AMPs which indicates the real role played by them in the intended bioenvironment [53]. AMPs having non-specific modes of action might indeed decrease pathogens ability to develop resistance [70,71] and consequently boost up their therapeutic potential. In addition, it has been demonstrated that amphiphilic peptides retain their antimicrobial activity when they are covalently bond to a water-insoluble resin [72]. This behavior suggested their use in therapeutic medical devices such as intravenous catheters [35]. The attractive therapeutic features associated with AMPs greatly motivated the researchers to develop them as ideal drug candidates against pathogenic microbes.
1.2. Lipopeptides

Lipopeptides are another class of native antimicrobial agents produced non-ribosomally in the bacteria and fungi during cultivation on various carbon sources [73-74]. They are composed of an aliphatic acid attached to the N-terminus of short cationic or anionic peptidic moiety of six to seven amino acids. Most native lipopeptides have complex cyclic structures [75,76]. The mode of lytic action of some of them is via perturbation of the cell membrane by unknown mechanisms [77-81]. Similarly to that of AMPs, electrostatic interaction between cationic lipopeptides and negatively charged lipopolysaccharide (LPS) of Gram-negative bacteria or lipoteichoic acid of Gram-positive bacteria is the initial step of their bactericidal activity. Further, lipopeptides traverse into the inner core and destabilize the membrane architecture [76,78]. In the fungi lipopeptides bind to the negatively charged membrane phosphatidylinositol (PI) and to the negatively charged terminal sialic acid moieties [82-84]. In addition to this, lipopeptides display broad-spectrum antimicrobial activity against multi-drug resistant bacteria as well as fungi. These clinical features associated with lipopeptides encourage us to develop them as new generation of antibiotics. Conversely, native lipopeptides are non-cell selective and therefore toxic to mammalian cells [81]. Despite this toxicity profile several members of this novel class of antimicrobials including daptomycin (active only toward Gram-positive bacteria) [85], polymyxin B (active only toward Gram-negative bacteria) [86], and echinocandins (β-1,3-D-glucan synthase inhibitors; active only toward fungi) [87] were approved by the Food and Drug Administration (FDA).