Chapter 1

Antibiotics and Cationic Antimicrobial Peptides: An Overview

Sir Alexander Fleming (Discovery of penicillin)

Antibiotic resistance

Cecropin A (cationic antimicrobial peptide)

Ceragenins (peptidomimetics)
1.1 Introduction

The first natural protein based antimicrobial agent was discovered by Sir Alexander Fleming almost 90 years ago in the year 1922 which was named lysozyme [1]. Lysozyme is a protein found abundantly in human secretions such as mucus and saliva. Within next 5 years Sir Fleming discovered another antimicrobial agent, this time from a fungus *Penicillium notatum* which was named penicillin [2]. The discovery of penicillin earned him a shared Nobel Prize in Chemistry in the year 1945. After the amazing discovery and industrial production of penicillin, the field of infectious disease medicines i.e. antibiotics was revolutionized and a significant number of scientists devoted research towards discovering novel antibiotics. Antibiotics are simply defined as drugs interfering with some structure or process that is essential to microbial growth or survival without harm to the eukaryotic host.

1.2 Antibacterial drug pipeline

Historically the first antibiotics discovered were sulphonamides. After the market introduction of penicillin as β-lactam, the next novel antibiotic class discovered was aminoglycosides (Figure 1.1).

![Antibiotic pipeline](Figure 1.1: Antibiotic pipeline (Source: www.extendingthecure.org))
The discovery of streptomycin in the 1940’s from the soil bacteria lead to hopes of winning the battle against infectious diseases at that time. In fact the period between the 1950’s and 1970’s was indeed the golden era of discovery of novel antibiotics, as 14 different classes of antibiotics were discovered from natural sources during this period. The antibiotics tetracyclines, choloamphenicols, erythromycin, vancomycin, kanamycin and rifamycin were all discovered within 20 year span as natural or semi-synthetic antibiotics (some of the structures are shown in Figure 1.2). After 1970’s there was a sharp decline in the number of novel classes of antibiotics resulting into a dearth of novel molecules as antimicrobial agents. Near the beginning of this century a completely synthetic antibiotic class oxazolidinone was introduced into clinics with lipopeptides in 2005 being the last novel class of antimicrobial agents introduced in clinics [3,4].

![Figure 1.2: Chemical diversity of antibiotics](image)
1.3 Mode of action of conventional antibiotics

To understand how antibiotics kill the bacteria and why they stop being effective, it is important to know how they work [5]. Antibiotics kill bacteria by either of the following mechanisms (Figure 1.3).

![Figure 1.3: Proven targets for antibiotics](image)

1.3.1 Hampering the cell wall biosynthesis

The layer of the bacterial cell wall that confers strength is the peptidoglycan comprising of N-acetylglucosamine-β-1,4-N-acetylmuramic acid cross-linked to pentapeptide-pyrophosphoryl-undecaprenol (lipid II). The β-lactam class of antibiotics (penicillin and cephalosporins) acts as a substrate to enzyme transglycosylases that act on the glycan strands to extend the sugar chains by incorporation of new peptidoglycan units [6]. The ring opened penicilloylated transpeptidases deacetyl very slowly and so occupies the enzyme active sites, preventing normal cross-linking of peptide chains in the peptidoglycan layer. This leads to inhibition of cell wall biosynthesis making the bacterial cell susceptible to lysis on changes in osmotic pressure.
1.3.2 Interrupting bacterial protein synthesis
There are fundamental differences between prokaryotic and eukaryotic protein synthesis enzyme machinery. Since the process of protein synthesis is complicated and involves a number of steps, antibiotics such as erythromycin, tetracycline and streptomycin target 30S or 50S subunits of ribosome to inhibit bacterial growth [7].

1.3.3 Inhibition of DNA synthesis and functioning
The enzymes involved in DNA replication and repair such as DNA gyrase (the enzyme responsible for uncoiling the intertwined circles of double-stranded bacterial DNA) and DNA topoisomerases (type I or type II) are targets of antibiotics such as ciprofloxacin. Ciprofloxacin act by forming a complex with the enzyme gyrase making a ciprofloxacin-gyrase complex. The gyrase cannot relegate the cleaved DNA and as a consequence, double-strand of DNA breaks accumulates and ultimately set off the SOS repair system that leads to bacterial cell death [8].

1.4 Mechanism of antibiotics resistance in bacteria
Various studies suggest that the mechanisms which render bacteria resistant to antibiotics includes

1.4.1 Destruction of antibiotics
This mechanism involves the destruction of functionalities present at active sites of antibiotics. A well reported example in this case is penicillin which consists of β-lactam ring. The β-lactam ring disrupts the cross-linking of cell wall in bacterial cells so the disruption of ring can render the antibiotic ineffective. Numerous strains of bacteria
now produces β-lactamase (proteins) which hydrolyses this lactam ring and the resultant product cannot function as antibiotic any more [9]. Modification of the chemical structure of active site of antibiotics is another efficient way of rendering them ineffective. The active sites of drug in aminoglycosides are free amines. By producing enzymes that can acetylate, phosphorylate or adenylate these amines the microorganisms were successful at rendering the antibiotic unable to bind the RNA target in ribosomes [10]. The deactivating enzymes which affect this class of antibiotics are acetyl transferases, phosphoryl transferase and adenylyl transferases. These modified products do not bind to RNA effectively so do not interrupt protein synthesis in bacteria.

1.4.2 Removal of antibiotics by efflux pumps

To be effective a proper concentration build up of antibiotics at the target site in host bacterial cells is necessary. Some Gram-positive and Gram-negative bacterial strains produce such membrane proteins which extrude out the drug from the bacterial cell thus act as efflux pumps. For efflux pump to keep the concentration of drug low at target site the pumping out of drug should be faster than diffusing in of the drug for example erythromycin resistant Staphylococci is able to efflux out the drug, preventing its accumulation inside cells [11].

1.4.3 Re-designing of targets

For this mechanism the bacteria does not affect the antibiotic but re-designs the target of the antibiotics. In vancomycin resistant enterococci (VRE) the bacteria re-design its cell wall to decrease the affinity of vancomycin to bind to its target leading to a reduced
affinity of the drug to be effective as an antibiotic. In the cell wall instead of D-Ala-D-Ala the bacterium selects an enzymatic pathway to form D-Ala-D-Lac and then hydrolyses the normal metabolite D-Ala-D-Ala while sparing D-Ala-D-Lac [12]. In this cell, only the D-Ala-D-Lac accumulates and serves as a substrate to be elongated and presented at the termini of the peptidoglycan strands. The reprogramming of peptidoglycan to end in D-Ala-D-Lac rather than the normal D-Ala-D-Ala has no effect on the cross linking efficiency carried out by the trans-peptidating PBPs thus leading to normal cell growth. However, vancomycin has a 1000 fold reduced affinity for D-Ala-D-Lac as compared to D-Ala-D-Ala and thus this reduced affinity leads VRE to grow at 1,000-fold-higher levels of antibiotic.

1.5 Antibiotic resistance: The global threat

Infectious diseases are a major global health concern especially in the present scenario where almost all the clinical bacterial strains have gained resistance against the currently available antibiotics [13,14]. With the withdrawal of major drug companies from antibiotics drug development market there is a prominent need for discovery of novel agents with a potential to combat multi drug resistant (MDR) bacterial strains (Figure 1.4). Multiple drug resistant organisms include hospital and community MDR strains of methicillin resistant *Staphylococcus aureus*, *Enterococcus faecium*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* that render the antibiotic therapy more difficult and costly. Additionally no class of antibiotics is free from the resistance development problem as bacteria are among the fastest growing organisms, which facilitates rapid genetic changes and the selection of traits which facilitates adaptation of bacteria to constantly changing environment.
The emergence of new phenotypes occurs as a result of change in native genes or by transfer of genes among microbes. These processes are often associated with the acquisition of new virulence factors and resistance to currently used antibiotics. Hence, microbes re-invent themselves, leading to re-emerging pathogens that require the development of novel therapeutics for eradication. One possible solution to this end can be design of antimicrobial agents with non specific mode of action. Non specific implies to those agents that do not target a particular site for attack in bacteria. Because the bacteria will endow itself with resistance only when it knows what is being targeted or at what part of the bacterial cell the antibiotic is acting.

1.6 The rise of cationic antimicrobial peptides (CAMPs)

The antibiotic era started and the growing resistance against the antibiotics discovered lead to a drought in clinical drug development pipeline. Meanwhile, revisiting the
discovery of lysozymes by Sir Fleming in 1923, some researchers started focusing on cationic antimicrobial peptides (CAMPs) that are ubiquitously present in almost all forms of life as a first line of defense against microbial invasion. The field of CAMPs got a boost almost three decades ago when in the early 1980’s Boman and co-workers could induce a specific response in form of a 37 residue peptide upon injecting pupae of the *cecropia* moth with a bacterial culture. The induced peptides demonstrated potent antibacterial activity against multiple Gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa* [15]. Later on an abundant class of Cys rich peptides with a fixed intramolecular disulphide bond network were discovered from the human and rabbit granulocytes [16,17]. These cationic, Arg and Cys rich peptides ranged in size from 29 to 34 amino acids and were named defensins. Defensins also showed broad range of activity against Gram-negative and Gram-positive bacterial strains, fungus and even against herpes simplex virus. M. Zasloff and co-workers simultaneously discovered 23 residue cationic peptides named magainins from skin extracts of *Xenopus laevis* [18].

All these pioneering discoveries proved cationic antimicrobial peptides as evolutionarily ancient weapons of innate immunity. Their widespread distribution throughout the animal and plant kingdoms suggests that antimicrobial peptides have served a fundamental role in the successful evolution of complex multicellular organisms therefore they are also called as host defense antimicrobial peptides (HDCPs). The importance of CAMPs to defense of mammals was accepted with the discovery that cathelicidins are released during wound healing and that β-defensins are abundant in psoriatic scale [19].
Although these peptides were first established as important to defense of lower organisms such as plants, insects, and amphibians, cathelicidins and defensins are now valued as important component of innate immunity in higher mammals such as humans. The skin in particular has served best to demonstrate the essential role of CAMPs to mammalian immune protection. Despite their ancient lineage, AMPs have remained effective defensive weapons, confounding the general belief that bacteria, fungi and viruses can and will not develop resistance to any conceivable level against CAMPs as they have dual mode of direct microbial killing and ability for immune modulation (Figure 1.5). Coincident with these observations AMPs have also been identified and characterized in many epithelial structures.

The continuous emergence of bacterial strains resistant to conventional antibiotics has prompted a renewed interest in the use of alternative natural microbial inhibitors such as
antimicrobial peptides. CAMPs vary considerably in sequence and structure, with a few common features such as being small (12-60 amino acids), containing positive charge and an amphipathic structure [20]. CAMPs are amphipathic meaning they possess both a hydrophobic region that interacts with lipids and a positively charged hydrophilic region that interacts with water or negatively charged residues. This feature allows the peptides to interact well with membranes that are composed of amphipathic molecules, especially negatively charged bacterial membranes. Because it is difficult for a microbe to change the phospholipid organization of its membrane, resistance to the CAMPs occurs at levels that are orders of magnitude lower than those observed for conventional antibiotics. Hence these peptides are excellent candidates for development as novel therapeutic agents and complements to conventional antibiotic therapy.

### 1.7 Diversity of CAMPs

Cationic antimicrobial peptides are composed of varied amino acid residues arranged in different primary sequences making it difficult to categorize them except on the basis of their secondary structure [21]. The diversity of sequences is so unique that the same peptide sequence is rarely found in two different species of animal, even those closely related, be they insects, frogs or mammals. Based on secondary structure following are the various classes in which CAMPs can be categorized (Figure 1.6).

#### 1.7.1 CAMPs with α-helical structure

Majority of this class of peptides are short (<60 amino acid residues) and though unstructured in buffer are able to fold into an α-helical structure in the vicinity of helix
inducing environments such as trifluoroethanol (TFE), sodium dodecyl sulphate (SDS) micelles, phospholipids vesicles, liposomes or Lipid A. Formation of helix by linear peptides gives a highly amphipathic structure suitable for interaction with the negatively charged microbial membrane, for example a number of α-helical peptides such as magainin and LL-37 are able to fold into helices in different helix promoting solvents as reviewed by Tossi and co-workers [22].

Figure 1.6: Protein models representing the structural differences of the three classes of antimicrobial peptides. (A) α-helical peptides, (B) peptides composed of a series of β-sheets, (C) peptides that adopt unconventional structures, such as extended boat and loops structure. (Source: All structures were obtained freely from the RCSB Protein Data Bank (PDB) (http://www.pdb.org/)
1.7.2 CAMPs with β-sheet structure

This group contains peptides ranging in size from 17 to 45 residues. The peptides generally contain Cys residues, form disulphide bonds and fold into stable β-sheets. This subgroup includes protegrin from porcine leukocytes (which comprises 16 amino acid residues, including four Cys that are linked by two intra-molecular disulphide bonds) and a diverse family of defensins. Defensins are highly basic, Arg rich peptides consisting of 29-42 residues, including six disulfide-linked Cys. Members of this group include thionin from plants and insect defensins. The plant defensins possesses potent antifungal activity in vitro and the insect defensins are principally active against Gram-positive bacteria [23].

1.7.3 CAMPs with irregular structure

This group adopts a fold which lacks major regions of regular secondary structure. They comprise a high percentage of one or two particular amino acids for example Pro, Trp and Arg residues.

This group includes the bactenecins and PR-39, which are rich in Pro (33-49%) and Arg (13-33%) residues; prophenin, which is rich in Pro (57%) and Phe (19%) residues; indolicidin, rich in Trp and Pro residues and gramicidin with a loop structure and Trp rich sequence. All these peptides lack Cys residues and are linear, although some of them can form extended coils. Some of the antimicrobial peptides isolated from animal and plants are given in table-1.1.
Table 1.1: Overview of antimicrobial peptides from plants and animals

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecropin-1</td>
<td>KWKLFKKIEKVGQNIRDGIKAGPAVAVVGQATQIAK</td>
<td>Silk moth</td>
</tr>
<tr>
<td>Magainin 2</td>
<td>GIGKFLHSAKKFGKAFVGEIMNS</td>
<td>Frog</td>
</tr>
<tr>
<td>Pesiganan</td>
<td>GIGKFLKKAKKFGKAFVKLKK</td>
<td>Synthetic</td>
</tr>
<tr>
<td>Dermaseptin 1</td>
<td>ALWKTMLKKLGTMALHAGKAALGAAADTISQGTQ</td>
<td>Frog</td>
</tr>
<tr>
<td>LL-37</td>
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<td>Human</td>
</tr>
<tr>
<td>Buforin II</td>
<td>TRSSRAQLQFPGVRVHRLLRK</td>
<td>Vertebrate</td>
</tr>
<tr>
<td>Bactenecin 1</td>
<td>RLCRIVVRVCR</td>
<td>Cow</td>
</tr>
<tr>
<td>Thanatin</td>
<td>GSKKPVPVIYCNRRRTGKCQRM</td>
<td>Insect</td>
</tr>
<tr>
<td>Ranalexin</td>
<td>FLGLLIKVPMICAVTKKC</td>
<td>Rana frogs</td>
</tr>
<tr>
<td>Ranateurin 1</td>
<td>SMLSVLKNLGKVGVLGFVACKINKQC</td>
<td>Rana frogs</td>
</tr>
<tr>
<td>Esculentin 1</td>
<td>GIFSKLGRKKIKNLLISGLKNVGEVGMIDVVRTGIDIAG</td>
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</tr>
<tr>
<td>Tachyplesin</td>
<td>RWC\textsubscript{1}FRVC\textsubscript{2}YRGIC\textsubscript{2}YRC\textsubscript{C}R</td>
<td>Horsehoe crab</td>
</tr>
<tr>
<td>Androctonin</td>
<td>RSV\textsubscript{C}1RQIK\textsubscript{C}1R RRGG\textsubscript{C}2YYK\textsubscript{C}21TNRPY</td>
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<td>Protegrin 1</td>
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<td>α-Defensin (HNP3)</td>
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</tr>
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<td>β-Defensin (TAP)</td>
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<td>Cow</td>
</tr>
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<td>γ-Defensin (sapecinA)</td>
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<td>Drosomycin</td>
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<td>Drosophila</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>DTHF\textsubscript{P}1C\textsubscript{I}FC\textsubscript{C}2GC\textsubscript{C}21HR\textsubscript{S}KC\textsubscript{C}2GM\textsubscript{C}2C\textsubscript{A}KT</td>
<td>Human Liver</td>
</tr>
<tr>
<td>Bac 5</td>
<td>RFRP\textsubscript{P}1RPRPPFPYPP\textsubscript{P}1RPRPFP\textsubscript{P}1RPPFP\textsubscript{P}1RPPFP\textsubscript{P}1RPPFP\textsubscript{P}1RPPFP\textsubscript{P}1RPPFP</td>
<td>Cow</td>
</tr>
<tr>
<td>Indolicidin</td>
<td>ILPK\textsubscript{W}1KWWP\textsubscript{P}1WWR</td>
<td>Cow</td>
</tr>
<tr>
<td>Apidaecin</td>
<td>GNRR\textsubscript{P}1VYIPQ\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP</td>
<td>Honeybee</td>
</tr>
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<td>Pyrrhocoricin</td>
<td>VDK\textsubscript{G}1SYLPR\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP\textsubscript{P}1PRP</td>
<td>Insect</td>
</tr>
<tr>
<td>Histatin 5</td>
<td>DSHA\textsubscript{K}1RHHGYKRKFHE\textsubscript{K}1KHS\textsubscript{H}1R\textsubscript{G}1Y</td>
<td>Human</td>
</tr>
</tbody>
</table>
1.8 Determinants of CAMPs activity

The main characteristics of antimicrobial peptides are:

1.8.1 Charge

Antimicrobial peptides display a net positive charge at physiological pH, ranging from +2 to +9, and may contain highly defined cationic domain(s). Cationicity is important for the initial electrostatic attraction of antimicrobial peptides to negatively charged phospholipid membranes of bacteria. The bacterial membranes are rich in the acidic phospholipids such as phosphatidylglycerol (PG), phosphatidylserine (PS) and cardiolipin (CL) that confers overall negative charge. Moreover lipopolysaccharides (LPS) and teichoic/teichuronic acids of Gram-negative and Gram-positive bacteria, impart additional negative charge to the surfaces of these respective organisms. Based on these considerations, it is not surprising that there is a strong correlation between peptide cationicity and antimicrobial activity [24]. However, this relationship is not linear. Within a certain range, increasing peptide cationicity is generally associated with increasing antimicrobial potency.

1.8.2 Hydrophobicity

Peptide hydrophobicity is defined as the percentage of hydrophobic residues present within a peptide and it is approximately 50% for most antimicrobial peptides. Hydrophobicity is an essential feature for antimicrobial peptide-membrane interactions, as it governs the extent to which a peptide can partition into the lipid bilayer. Although hydrophobicity is required for effective membrane permeabilization, increasing levels of hydrophobicity are strongly correlated with mammalian cell toxicity and loss of
antimicrobial specificity [25]. Therefore, many naturally occurring antimicrobial peptides are moderately hydrophobic, such that they optimize activity against microbial cell membranes.

Figure 1.7: Amphipathic design: Clustering of cationic and hydrophobic amino acids into distinct domains in several antimicrobial peptides of different structural classes. Red, positively charged amino acids; green, hydrophobic amino acids. Other amino acids are not shown (Source: reference 20)

1.8.3 Amphipathicity

During interaction with membrane, CAMPs adopts a shape in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete sectors of the molecule which is known as amphipathic arrangement (Figure 1.7). Nearly all antimicrobial peptides form amphipathic structures on interaction with target membranes [20]. Amphipathicity can be achieved via a multitude of protein conformations; however, one of the simplest and perhaps most elegant example is the
amphipathic helix. One quantitative measure of amphipathicity is the hydrophobic moment, $M_{H}$, calculated as the vector sum of individual amino acid hydrophobicity, normalized to an ideal helix [26].

### 1.9. Molecular basis for antimicrobial peptide cell specificity

The most vital property of CAMPs is their cell specificity by which they kill or interact more strongly with microbes but are nontoxic to mammalian cells. The major factors governing cell selectivity are:

- **Charge**: The net positive charge on CAMPs accounts for their preferential binding to the negatively charged outer surface of bacteria, which is different from the predominantly zwitterionic surface of normal mammalian cells.

- **Trans membrane potential**: The usually high, negative inside, trans-membrane potential of bacteria (-130 to -150 mV) acts as a potential driving force for peptide insertion and translocation, whereas a normal mammalian cell exhibits a transmembrane potential ranging from -90 mV to -110 mV.

- **CAMPs as ligands**: Some CAMPs display specific structural affinity for bacterial membrane constituents such as phospholipid head groups or lipid A in LPS [27].

- **Presence of sterols**: Mammalian cells have sterols as a constituent. Sterols are able to influence fluidity of lipid membranes which affects the peptide insertion into cells. It has been reported that cholesterol causes a reduction of the density of the head group in DPPC (mammalian cells) at the interfacial region of the bilayer and an increase in the package of the phospholipid tails in the middle of the bilayer making insertion of peptides less likely (Figure 1.8) [28].
However, recently it was postulated that cholesterol loses its effectiveness in inhibiting CAMPs when incorporated into raft-like domains [29].

1.10 CAMPs as the innate immunity effectors

The innate immune system provides organisms with a rapid, non-specific first line of defense against colonization by pathogenic microorganisms. The components of innate immunity include the barrier function of the skin, reduced pH of the stomach, the sweeping motion of cilia in the airway to remove inhaled pathogens, and chemical defenses which include CAMPs. It is because of their role in immuno modulation that CAMPs are also called as host defense peptides [30]. CAMPs in mammals are expressed throughout the body, both in circulating cells and epithelium within the barrier and delivered to the site by circulating white cells. Fully processed active peptides can be isolated from keratinized epithelial sheets of dry skin and tongue, where
they probably act as epithelial ‘preservatives’ (Figure 1.9). In humans, CAMPs are secreted into a micrometre-thick biofilm directly overlying the epithelium, regulated by a variety of pump and channels to maintain composition and concentration. Also paneth cells of the gastrointestinal tract produce CAMPs. In neutrophils, active peptides are found in primary granules, where they can be used as part of oxygen-independent antibacterial mechanism of these cells. Four peptides of α-defensin family are found in neutrophils. In macrophages and monocytes human β-defensin 2 is induced by cytokines. Both α- and β- defensins have been also been observed in breast milk. CAMPs thus also serve as signals which initiate, mobilize, and amplify adaptive immune host defenses, thus functioning as immune modulatory and immune stimulatory elements [31].
CAMPs can also protect the host against detrimental, potentially lethal effects, resulting from an excessive TLR-induced inflammatory response [32].

### 1.11 Mode of action of CAMPs

The two vital components of the biological membranes of all living organisms are lipids and proteins. In bacteria, in addition to the cytoplasmic membrane, there are outer membranes composed of lipopolysaccharides and lipoteichoic acid. Both Gram-negative as well as Gram-positive bacterial cell has a peptidoglycan layer outside of the cell membrane (Figure 1.10).

![Figure 1.10: Schematic representation of Gram-positive and Gram-negative bacterial cell membranes](image-url)
In Gram-negative bacteria a highly permeable outer membrane that contains lipopolysaccharides (LPS) on its outer leaflet, with the inner leaflet composed mainly of phosphatidylethanolamine and phosphatidylglycerol is present. Gram-positive bacteria have lipoteichoic acid (LTA) adhered to the cell surface membrane as their outer cover. The inner membrane of Gram-positive bacteria is composed mainly of phosphatidylglycerol and cardiolipin.

Generally Gram-negative bacteria contain both anionic and zwitterionic phospholipid, while many Gram-positive bacteria contain predominantly anionic lipids. The basic characteristic of amphipathic arrangement of amino acid residues in CAMPs allow partitioning of peptides into bacterial membranes by the following two types of interactions as a major modulators of mechanism of action.

### 1.11.1 Electrostatic interactions

CAMPs preferentially bind and insert into membranes containing negatively charged head groups such as phosphatidylglycerol (PG) or phosphatidylserine (PS) due to strong electrostatic interactions [33]. The electrostatic interactions between peptides and lipids have been defined based on the Gouy-Chapman theory which considers the negative charge on bacterial membranes as a uniform smear of charge spread throughout the surface [34].

### 1.11.2 Hydrophobic interactions

As lipids are amphiphilic in nature (i.e. charged head group and hydrophobic tail), for effective insertion into bacterial membranes only electrostatic forces are not enough and
hydrophobic interactions are bound to play a role. Hydrophobicity is an essential feature for antimicrobial peptide-membrane interactions, as it governs the extent to which a peptide can partition into the lipid bilayer. Although hydrophobicity is required for effective membrane permeabilization, increasing levels of hydrophobicity are strongly correlated with mammalian cell toxicity and loss of antimicrobial specificity [25].

1.11.3 Proposed mechanisms of action of antimicrobial peptides

The action of antimicrobial peptides induces membrane defects such as phase separation, pore formation, bilayer disruption and depending on the molecular properties of both peptide and lipid translocation inside the cell to target intracellular components.

Following are the proposed models for mechanism of action for CAMP action.

1.11.3.1 Shai-Matsuzaki-Huang model

The model proposes that after initial electrostatic interactions, the peptides tend to insert into the hydrocarbon core. The penetration of peptides disrupts the head group packing in lipids leading to the local induction of curvature strain which after a critical peptide concentration becomes a toroidal pore that is capable of inducing lipid flip flop by lateral diffusion of membranes lipids between two monolayers which are connected by a pore [35]. A toroidal pore is a peptide-lipid supra-molecular complex which may alter the trans-membrane potential in a cell, depriving the cell of its energy source and at the same time may lead to leakage of cellular contents leading to cell death.
Based on Shai-Matsuzaki-Huang model, Shai further refined the model by taking into consideration the orientation of peptides during peptide lipid interactions into three models which are (i) barrel stave model, (ii) toroidal pore model and (iii) carpet model (Figure 1.11) [37].

In Barrel Stave model the peptides forms the staves of the barrel-like pore in which the number of peptides in the pore will determine the size of the channel. This is followed by the progressive recruitment of additional monomers to increase pore size and stability. The pores thus formed are only transient and an increase in the peptide's positive charge reduces pore stability because of enhanced electrostatic repulsion between the side chains of peptides. The model holds good for CAMPs that are too
short to be able to cover the width of the membrane as the pores may be irregular in shape and size.

In the toroidal pore model, insertion of CAMPs into the membrane is envisioned to induce lipids to bend around peptide aggregates until a continuous channel between the outer and inner leaflet is formed which is lined by both peptide and lipid head groups. This model differs from the barrel-stave model in that the peptide remains associated with the lipid head groups in the channel formation which subsequently leads to release of cellular contents.

The detergent-like properties of some CAMPs have been explained using the carpet model. In this model peptide saturates the surface of the cytoplasmic membrane before causing a complete detergent-like disruption of the membrane. A distinct feature of this model is that no distinct pores are formed but rather the membrane integrity is completely disrupted.

The aggregate model has some resemble with toroidal pore model [38]. This model is used for explaining how cationic CAMPs can kill through both membrane permeabilization and internal target attack. After binding to the phospholipid head groups, the peptides insert into the membrane and then cluster into unstructured aggregates that span the membrane. These aggregates are proposed to have water molecules associated with them providing channels for leakage of ions and possibly larger molecules through the membrane.

This model essentially differs from the other three as: only short-lived transmembrane clusters of an undefined nature are formed, which allow the peptides to cross the membrane without causing significant membrane depolarization. Once inside the cell,
the peptides home to their intracellular targets to exert their killing activities (Figure 1.11).

Classical example of peptide following barrel stave mechanism is alamethicin [39]. Magainin 2 and PGLa follow a toroidal pore model for their mode of bacterial killing [40]. Cell lytic hemolytic peptide melittin follows the carpet model of CAMPs action [41]. It is important to recognize that all of these models may be valid under different conditions and for different structural groups of CAMPs.

1.11.3.2. Lipid-clustering model

A more specific mechanism has been suggested for breaching the permeability barrier of membranes by CAMPs that involves the induction and separation of lipid components, resulting in the clustering of anionic lipids (Figure 1.12) and possibly the formation of phase boundary defects between lipid domains [42]. Segregated phases or domains in membranes do not by themselves lead to toxicity, since it is believed that domains are commonly present in biological membranes. However, peptide mediated domain formation gives insufficient time for the membrane to rearrange to accommodate the organization to lipids leading to a membrane tension. Phase boundary defects have been suggested to be responsible for the increased leakage of liposomes content. The importance of induced lateral phase separation has been specifically proposed as a mechanism contributing to the antimicrobial activity of oligo-acyl-lysine (OAK) [43]. Additionally clustering of anionic lipids is not proposed to be the sole mechanism by which these peptides/peptidomimetics act to kill bacteria, but rather it is an additional contributory factor. Proposed factors that would facilitate preferential interaction with anionic lipids and the promotion of phase segregation are:
(a) The presence of multiple cationic groups allowing one molecule of the antimicrobial agent to interact with several anionic lipid molecules.

(b) A conformational flexibility to facilitate the adoption of a conformation in which the distance between positive charges match the distance between anionic lipids in the bacterial membrane after clustering.

(c) Sufficient hydrophobicity to spontaneously partition to a membrane.

Figure 1.12: Diagram of phase separation in a membrane bilayer. Lipids with two different kinds of headgroups (represented by red and green balls), can be induced to phase separate by addition of a substance that interacts preferentially with one of the two lipid components. This will create phase boundary defects between domains enriched in either of the two lipids. (Source: reference 42).

Many methods have been used to demonstrate the clustering of anionic lipids in anionic zwitterionic mixtures by antimicrobial agents, including DSC, NMR, freeze fracture electron microscopy, atomic force microscopy and polarized total internal reflection fluorescence microscopy [44,45].

1.11.3.4 Interfacial activity model

According to this model the ability of a peptide to bind and partition into the membrane-water interface to alter the packing and organization of the lipids interfacial
activity depends mainly on the appropriate balance of hydrophobic and electrostatic interactions between peptides, water, and lipids [46]. This model was recently proposed to bridge the gap between biophysical and biological activity. Upon binding of CAMPs to anionic lipid vesicles through electrostatic and hydrophobic interactions, many types of perturbations can occur, including membrane permeabilization, vesicle aggregation, vesicle fusion and complete solubilization of membranes. Some of these effects may not directly relate to antimicrobial activity and mode of action. According to the model any in vitro membrane permeabilization measurements that show activity only at more than 1 peptide bound per 50 lipids (P:L 1:50) should be viewed with caution. The important features of the model include:

- Interfacial activity requires non amphipathic arrangement of amino acid residues with a proper balance of hydrophobic and polar amino acids.
- Structure acquisition and structural transformations are not prerequisites to show interfacial activity
- Interfacial activity does not require peptide self assembly.
- At low bound peptide to lipid ratios CAMPs will translocate across membranes and lipid translocation, peptide translocation, and membrane leakage will always be coupled.

### 1.12 CAMPs as diverse metabolic inhibitors

Most antimicrobial peptides act directly on lipid bilayers of the target membrane, rather than on a receptor, as discussed above. However, studies with several antimicrobial peptides have shown that CAMPs might target intracellular molecules, such as DNA or
enzymes, since they are capable of spontaneously traversing bacterial outer and inner membranes (Figure 1.13) [47, 48]. For example indolicidin was proposed to inhibit DNA synthesis leading to filamentation in *E. coli*. Accordingly, increasing evidence indicates that antimicrobial peptides could also act through a variety of mechanisms other than disruption of membrane integrity, and that their role in host defense goes well beyond direct killing of microorganisms.

**Figure 1.13: Antimicrobial mode of action of different classes of CAMPs (Source: reference 48)**

Bac5 and Bac7 are CAMPs that inhibit protein and RNA synthesis of *E. coli* and *K. pneumoniae* by inhibition of the respiration in addition to their potential to disturb the membranes of these bacteria [49]. PR-39 binds to the cell membrane of *E. coli* without causing permeabilization, but kills bacteria solely by stopping both DNA and protein synthesis [50]. Similarly, buforin II inhibits the cellular functions of *E. coli* by binding
to DNA and RNA after penetrating the cell membranes [51]. Another emerging view is that many peptides act synergistically with other host molecules with antimicrobial activity to kill microbes. Positive co-perativity has been reported between different antibiotics and CAMPs [52,53].

Overall so far the precise mode of action of CAMPs has not been deciphered as yet owing to the multiple concentration dependent modes of action for different classes of peptides. However, at the heart of the mechanism of action for membrane active CAMPs is the fact that the incorporation of peptides into membranes causes a stress, a destabilization of bacterial membranes that leads to disruption of membrane potential, formation of transient pores or hamper functioning of membrane proteins due to membrane destabilization.

1.13 Design of synthetic mimics/peptidomimetics of antimicrobial peptides

Synthetic mimics/peptidomimetics of CAMPs are based on a different backbone i.e. they are not solely based on $\alpha$-amino acids. The modified backbone or the side chain groups while maintaining a balance between positive charge and hydrophobicity render the compounds protease-resistant improving their bioavailability compared to peptide analogues. Another advantage of peptidomimetics is their small size (2-8 residues) compared to the parent CAMPs (12-60 residues). Thus these molecules overcome two of the hurdles in therapeutic applications of CAMPs i.e. high cost of production and protease stability. Therefore peptidomimetics with a proper therapeutic index are at the verge of entering the clinical trials. Following is a brief introduction of a few clinically relevant classes of peptidomimetics:
1.13.1 Trp-Arg based peptidomimetics

The minimum motif or the pharmacophore for antimicrobial activity was defined by Swendsen and co-workers showing a minimum requirement of two cationic charges and two hydrophobic units to allow a peptidomimetic to interact with bacterial membranes (Figure 1.14) [54]. With the incorporation of unnatural hydrophobic and charged moieties a number of analogues were optimized with potent activity and cell selectivity.

![Figure 1.14: The pharmacophore for shortest antimicrobial peptide mimics](image)

Further many proteolytically stable analogues with *in vivo* efficacy in infection model were optimized using this strategy.

1.13.2 Lipopeptides

Towards design of lipated antimicrobial peptides, keeping in mind the clinically approved lipopeptides daptomycin and polymyxin B, Shai and co-workers reported a new family of ultra-short (4-mer) cationic lipopeptides with representative active
sequences; C_{16}-KAAK, C_{16}-KKKK and C_{12}-KLLK (Figure 1.15) [55]. The short peptides showed potent activity against Gram-positive and Gram-negative bacterial strains and some fungal strains as well. Mode of action studies supported a membranolytic or a detergent like effect.

![Figure 1.15: Representative structure of ultra short lipopeptide C16-KLLK](image)

The most efficient peptide C_{16}-KAAK showed promising in vivo activity in aspergillosis infection model with low toxicity effects and no damage to the treated lung tissues therefore offering a more efficient treatment toward the current standard antifungal therapy than amphotericin B.

### 1.13.3 Arylamide scaffolds

A series of triaryl scaffolds with minimum +2 charge and various hydrophobic functionalities was reported by Tew and co-workers by making use of Suzuki-Miyaura coupling. Amphipathic structures where the backbone and the side chains were systematically varied were synthesized to evaluate the role of conformational stiffness and overall hydrophobicity on the antimicrobial and hemolytic activity (Figure 1.16) [56].
Some of the designed molecules showed potent activity against *S. aureus* with optimum therapeutic index. For this class of molecules, the overall hydrophobicity was shown to have more significant impact on the antimicrobial and hemolytic activity than the conformational stiffness. Further optimization of the series may lead to low molecular weight mimics of CAMPs.

### 1.13.4 Lipo-γ-amino acid peptidomimetics

A series of lipo-γ-AApeptides was designed by Cai and co-workers amalgamating the properties of lipopeptides and non natural amino acid sequences (Figure 1.17) [57]. The designed molecules showed very potent, broad spectrum activity against fungi, and a series of Gram-positive and Gram-negative bacteria, including clinically-relevant pathogens that are resistant to most antibiotics.
A membrane disrupting mode of action based on membrane depolarization abilities of designed conjugates was established for the molecules which allowed no development of resistance against these molecules.

1.14 Comparison of CAMPs and conventional antibiotics

- Antimicrobial peptides have diverse potential application; they can be used as single agent and in combination with conventional antibiotics, for synergistic effect, or as immuno modulatory and/or endotoxin-neutralizing agents.

- Potent antimicrobial peptides usually have broad spectrum activity against most Gram-negative and Gram-positive bacteria and some of them are active against fungi and variety of viruses. On the other hand, conventional antibiotics are often selective.

- Although the potency of these antimicrobial peptides against the more susceptible pathogens is normally not as strong as certain conventional antibiotics, one of their major strengths is their ability to kill multi-drug-resistant bacteria at similar concentrations.

- Compared with conventional antibiotics, the killing of bacteria by peptides is extremely rapid and can involve multiple bacterial cellular targets.

- One substantial advantage of CAMPs over conventional antibiotics is that they have the ability to neutralize sepsis/endotoxemia.

- In addition, some peptides have been demonstrated to have diverse roles in mammalian innate immunity. One of the most important thing about antimicrobial peptide is that they have ability to stimulate the innate immune response while simultaneously reduce the potentially harmful inflammatory response.
1.15 Synergistic activity of antimicrobial peptides

Generally, microbiological and biophysical studies examine the biological activities of individual antimicrobial peptides in isolation to minimize experimental variability. However, it inevitably occurs in nature that a number of CAMPS with different sequences and secondary structure preferences interact together inside microbial pathogens in a variety of ways, including complex mixtures within phagolysosomes or into equally complex extracellular milieus. Therefore, antimicrobial peptides interact with one another, with microorganisms, and with host molecules prior to or at the sites of infection. At present, a number of studies suggest that such peptide interactions may indeed be important to overall antimicrobial activity. For example studies of magainin 2 and PGLa from *Xenopus laevis* skin suggest a synergism in activity i.e the antimicrobial activity of the mixture of these two peptides is better than any of the individual peptide due to favorable interaction between the two peptides. The minimum inhibitory concentration (MIC) for either peptide alone was ~40 µg/mL; however, their minimum inhibitory concentration in combination was reduced by 20-fold [58].

Interestingly, a number of studies have shown that antimicrobial peptides act in synergy with commonly used antibiotics, against multi-drug resistant bacteria [52]. Therefore, even if the peptides cannot be used on their own, they can be administered in combination with antibiotics and improve their potency. The findings discussed above substantiate multiple mechanisms by which antimicrobial peptides affect target cell killing. Undoubtedly, as experimental methodologies become more refined, future studies will more clearly assess the complex interactions among multiple antimicrobial peptides and their targets *in situ*. 
1.16 Clinical experience

Cationic peptides have tremendous structural diversity and an impressive array of clinically meaningful activities. This has provided a huge impetus to the development of new synthetic peptides. Even so, despite nearly three decades of serious design efforts, there has been limited success in the clinic.

Table 1.2: Peptide and peptidomimetic in commercial development

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Application</th>
<th>Trial phase</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pexiganan acetate (MSI 78)</td>
<td>GIGKFLKKAKKFGKAFFV KILKK</td>
<td>Topical antibiotic</td>
<td>III</td>
<td>No advantage demonstrated over existing therapies</td>
</tr>
<tr>
<td>Iseganan (IB-367)</td>
<td>RGGLCYCRGRFCVCGVR</td>
<td>Oral mucositis in patients undergoing radiation therapy</td>
<td>III</td>
<td>No advantage demonstrated over existing therapies</td>
</tr>
<tr>
<td>hLF1-11</td>
<td>GRRRSVQWLCA</td>
<td>Bacteraemia and fungal infections in immuno-compromised haematopoetic stem cell transplant recipients</td>
<td>I/II</td>
<td>Significant efficacy observed in Phase I trials; mechanism of action appears to be immuno-modulatory rather than antibiotic; Phase II trials initiated after a long delay</td>
</tr>
<tr>
<td>XOMA 629</td>
<td>KLFR-(D-naphtho-Ala)-QAK-(D-naphtho-Ala)</td>
<td>Impetigo</td>
<td>I/II</td>
<td>No Phase IIA results available (trial started in July 2008)</td>
</tr>
<tr>
<td>CZEN-002</td>
<td>(CKPV)2</td>
<td>Vulvovaginal candidiasis; anti-inflammatory</td>
<td>IIb</td>
<td>Positive efficacy results announced; Phase IIb trial is a dose-ranging study</td>
</tr>
<tr>
<td>Ghrelin</td>
<td>GSSFSLSEHQRVQQRKES KKPAPKLQPR</td>
<td>Airway inflammation, chronic respiratory infection and cystic fibrosis</td>
<td>II</td>
<td>Peptide hormone that suppresses neutrophil-dominant inflammation in airways of patients with chronic respiratory infection</td>
</tr>
<tr>
<td>PMX-30063</td>
<td>Structure not isclosed</td>
<td>Acute bacterial skin infections caused by <em>Staphylococcus</em> spp.</td>
<td>II</td>
<td>Mimetic rather than peptide; currently in Phase II trials</td>
</tr>
<tr>
<td>Delmitide (RDP58)</td>
<td>RXXXRXXXGY (X = norleucine)</td>
<td>Inflammatory bowel disease</td>
<td>Completed</td>
<td>A protease-resistant, D-amino acid-containing peptide with similar efficacy to asacol; attempting to improve activity through formulation</td>
</tr>
<tr>
<td>Plectasin</td>
<td>GFGC1,NGPWDEDDMQC2 HNHC3,KS1KGYKOGYC1 AKGGFVC2,KC3Y</td>
<td>Bacterial diseases</td>
<td>Pre-clinical</td>
<td>Excellent efficacy demonstrated in animal models</td>
</tr>
<tr>
<td>HB1345</td>
<td>Decanoyl-KFKWPW</td>
<td>Acne; broad-spectrum antibiotic</td>
<td>Pre-clinical</td>
<td>Looks promising as this is a very small lipopeptide</td>
</tr>
</tbody>
</table>
To date, four cationic peptides or proteins have advanced into phase 3 clinical-efficacy trials. Out of these, two of them have been indicated for curing or preventing impetigo and diabetic foot ulcers (the frog magainin derivative MSI-78; Pexiganan) and oral mucositis (the pig protegrin derivative IB-367; Iseganan).

In addition to these peptides, many other molecules are proceeding through discovery, development and clinical trials (Table 1.2) [36].

1.17 Advantages of CAMPs

• CAMPs exhibit broad spectrum of antimicrobial activity including MDR bacterial, fungal and even cancerous cells in concentration ranges that are competitive with even the most potent antibiotics.

• They show a novel membrane lytic mode of action along with rapid bactericidal kinetics.

• They demonstrate a low level of resistance development \textit{in vitro}. Since they operate on the basis of altering the permeability properties of the bacterial plasma membrane and do not interfere with cell wall or macromolecular synthesis as do traditional antibiotics, resistance to the peptide antimicrobials will not likely develop easily.

• They have demonstrated significant synergy with conventional antibiotics.

• Given the relatively small number of building blocks (approximately 20 amino acids on average) the peptides are quite amenable to synthesis and modification and, at the same time, offer tremendous diversity.
1.18 Future perspectives

CAMPs and peptidomimetics can be immobilized via incorporation into a variety of materials or adsorbed to a variety of surfaces where they still retain their ability to bind and kill bacteria. Both of these methods of immobilization have vast applications in medical devices such as implantations and medical equipments to prevent formation of biofilms [59]. Further immobilization may be used into plastics or films to retard spoilage and increase food preservation times. Modified CAMPs, including peptide mimetics, hybrid peptides, peptide congeners, stabilised peptides, peptide conjugates and immobilised peptides have all emerged from natural CAMPs. CAMPs thus have creative medical and industrial application potentials to treat antibiotic-resistant bacterial infections and septic shock, to preserve food or to sanities surfaces both in vitro and in vivo.

1.19 Present work

Antibiotics have been an effective weapon against bacterial infections saving millions of lives worldwide for over 50 years. However, the emergence of multiple drug resistant bacterial strains in nosocomial environments as well as community has lead to a failure of conventional antibiotics. In the search for alternatives therapies, cationic antimicrobial peptides (CAMPs) have received considerable attention since they target the bacterial Achilles’ heel i.e. they compromise the membrane structure of bacterial cells rendering them unable to acquire resistance against CAMPs. A lack of new antibiotics for treatment of bacterial infections combined with emerging multi-drug resistance issues demands an increasing effort to search for antimicrobial agents with new mechanisms of action. The ubiquity and multifaceted role of these peptides in
direct microbicidal activity, wound and burn healing along with a role in modifying innate immunity makes CAMPs suitable candidates for drug development.

Based on this background the main objectives of the present thesis were:

- Design, synthesis and characterization of novel, short antimicrobial peptides.
- To evaluate antimicrobial activity and cell selectivity of synthesized peptides against susceptible and clinically resistant bacterial strains.
- To evaluate the interactions of active peptide sequences with model artificial membranes and intact bacterial cells using biophysical and microscopic approaches to delineate the factors responsible for difference in activity and cell selectivity for these peptides.
- To design and synthesize template based short antibacterial lipo-peptidomimetics and study the structure activity relationship and mode of action of these small peptidomimetics.
- To design peptide based self assembling nanostructures as efficient antibacterial agents with cell selective membrane disruptive mode of action.
1.20 References


