# Bactericidal Potency and Extended Serum Stability of Stereochemically Diversified Polypeptide Constructs

**A thesis submitted in partial fulfillment of the requirements for the degree of**

**DOCTOR OF PHILOSOPHY**

***by***

**PRAKASH KISHORE HAZAM**

**Department of Biosciences and Bioengineering**
**Indian Institute of Technology Guwahati**
**Guwahati-781039, India**

**April 2018**

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**INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI**

**DEPARTMENT OF BIOSCIENCES AND BIOENGINEERING**

## STATEMENT

I do hereby declare that the research findings of this thesis is the result of research work carried out by me in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati, India, under the supervision of Dr. Vibin Ramakrishnan and Dr. Nitin Chaudhary

As per the general norms of reporting research findings, due acknowledgements have been made, wherever the research findings of other researchers have been cited in this thesis.

**Date:** **Prakash Kishore Hazam**

**INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI**

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## CERTIFICATE

It is certified that the work described in this thesis entitled "**Bactericidal Potency and Extended Serum Stability of Stereochemically Diversified Polypeptide Constructs**" by Mr. Prakash Kishore Hazam for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under our supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, and this work has not been submitted elsewhere for the award of any other degree.

**Dr. Vibin Ramakrishnan (Supervisor)**

**Dr. Nitin Chaudhary (Co-Supervisor)**

## List of Figures

coding of amino acid residues, points to their electrostatic potential with reference to the color bar. The color map shows the potential distribution in KT/e units.

**Figure 4.3**. Starting structure of designed peptide and end structure derived from MD simulation.

**Figure 4.4**. A) RMSD (Root Mean Square Deviation) as a function of time of all the designed peptides from MD simulation in a 10 nanosecond (ns) production run, B) Rg (Radius of Gyration) as a function of time indicating the overall folded nature of the designed peptides.

**Figure 4.5.** Matrix of Hydrogen bonding pattern of designed PDB files. The bonding pattern between N to O has been shown by "Y" in the matrix.

**Figure 4.6.** Matrix of Hydrogen bonding pattern of mean structure of largest cluster from MD simulations. The bonding pattern between N to O has been shown by "Y" in the matrix.

**Figure 4.7.** Bactericidal potency of the peptides 01 and 02 along with their acetylated variants against *Staphylococcus aureus* (Gram-positive) and *Pseudomonas aeruginosa* (Gram-negative) bacteria. A and B show bactericidal activity against Gram-positive bacteria, whereas C and D show activity against Gram-negative bacterial species.

**Figure 4.8.** FE-SEM images of Gram-positive and Gram-negative bacteria, treated with individual peptides, after two hours of incubation. Comparison of untreated Staphylococcus aureus (Gram-positive) and Pseudomonas aeruginosa (Gram-negative) cells against ruptured bacterial membrane treated with 01, 01 Ac,02 and 02 Ac at 100 μM concentration. The scale bars correspond to 200 nm.

**Figure 5.1.** Possible backbone structures for syndiotactic peptides. The left and right handed conformations for designed syndiotactic peptides MS1 (A, B), MS3 (C, D), MS2 (E, F) and MS4 (G, H). The N and C terminal has been shown in this figure.

**Figure 5.2.** Electrostatic potential maps of left handed (LH) and right handed (RH) conformations of designed syndiotactic peptides respectively. MS1 (A, B) and MS2 (C, D) along with MS3 (E, F) and MS4 (G, H). Electrostatic potential was calculated by solving finite difference Poisson-Boltzmann equation using Delphi software, summed for each amino acid side-chain and represented collectively at the chromophoric center of the side-chain. The backbone corresponding to its electrostatic profile with amphiphilic character has been shown (color zone) at the top right of each electrostatic profile map. The N and C terminal for all the figures of electrostatic profile as well as backbone has been shown in the figure. The color scheme represents the distribution of potential in KT/e units.

**Figure 5.3.** Position wise electrostatic potential distribution. The electrostatic potential was calculated by solving finite distance Poisson-Boltzmann equation using DelPhi. Position-wise electrostatic potential is illustrated for A) MS1, B) MS2, C) MS3, D) MS4. All potentials are in kT/e units.

**Figure 5.4.** Clustering analysis and electrostatic potential distribution for designed peptides. All peptides were investigated for their conformational stability by molecular dynamics simulations. A 10 ns run with a 0.2 fs time step was completed, generating 1001 structures per run. These 1001 structures were clustered using g_cluster program from GROMACS package. Mean structures of significant clusters (> 20 structures) were extracted from the trajectory and electrostatic potential at each position was calculated by solving finite distance Poisson-Boltzmann equation using Delphi software. These electrostatic potential distributions were summed for each amino acid side-chain and represented collectively at the chromophoric center of the side chain as per a colour map, with red and

violet indicating highly positive and highly negative values for electrostatic potential respectively. Mean structures for significant clusters of respective peptides are shown in the upper panel and the electrostatic potential distribution is presented in the lower panel (A-O).

**Figure 5.5.** Interaction of peptides with lipid monolayers. The peptides display some preference for negatively-charged lipid.

**Figure 5.6.** Antibacterial activity of the designed peptide systems (MS1 to MS6), against Staphylococcus aureus, Escherichia coli and Gentamicin resistant MRSA. The percent bacterial cell death has been plotted for the designed peptides treated with various peptide concentrations ranging from 0.25 μM - 8 μM. All the peptides show complete killing at 8 μM concentration against the three bacterial cell types. Standard deviation in each case is shown as error bars.

**Figure 5.7.** FE-SEM images indicating membranolytic activity of designed peptides. Membrane deformation as a result of bactericidal action of the designed peptides against (A) Staphylococcus aureus, (B) Escherichia coli and (C) Gentamicin resistant MRSA at their respective minimum inhibitory concentrations. The scale bars correspond to 200 nm.

**Figure 5.8.** Hemolytic assay of peptides against human RBC. Human RBC was treated with buffer and 8 μM concentration of the designed peptides. The experiment was performed in triplicates and the error bars represent standard deviation.

**Figure 6.1.** Design of antimicrobial peptide incorporating D amino acid in the peptide sequence retaining their molecular weight and chemistry, and varying stereochemistry. The design has a pair of LDLD as well as DLDL sequences, with their poly L counterparts, all having identical amino acid composition. The syndiotactic arrangement of the peptide sequences give rise to gramicidin helical structure with alternating L and D residues in peptide sequence.

**Figure 6.2.** Anti-bacterial activity of the designed peptides against *M. smegmatis* and *E. coli* in media and in human serum. The bactericidal potency (percent bacterial cell death) of peptide has been plotted against variable peptide concentration at micro molar concentration (1.56 to 50). The error bars represent standard deviation.

**Figure 6.3.** Field Emission Scanning Electron Microscopic (FE-SEM) images of peptide-membrane interaction. A comparative assessment of untreated specimen with peptide treated sample at their respective MIC can be qualitatively assessed from the extent of deformation of bacterial membrane under each condition. Images have been pseudo-colored using Photoshop CS6. The scale bar resembles to 500 nm.

**Figure 7.1**. Comparative backbone structure of 7 mer peptides (ST1 – ST4) with reeference to their 12 mer model peptide systems (MS1 – MS4). All the peptide sequences are syndiotactic, having alternate L- and D-amino acids in its back bone. The N- and C-terminus of the peptides are shown in each case along with their peptide sequences. Small letter in the sequence indicates D-enantiomer.

**Figure 7.2** Electrostatic profile of 7-mer peptides (ST1 – ST4). The relative orientation of positively charged (yellow) and non-polar (green) side-chain group of the de-novo designed peptide sequence, showing amphipathic character, with distinct regions of polarity. The color scheme represents the distribution of electrostatic potential in KT/e units.

## List of Tables

## Abbreviations

| | |
|---|---|
| ACN | Acetonitrile |
| ADME | Absorption, Distribution, Metabolism and Excretion |
| AMP's | Antimicrobial peptides |
| CDC | Centers for Disease Control and Prevention |
| DAP | Diaminopimelic acid |
| DIPEA | N, N-Diisopropylethylamine |
| DMF | N, N-Dimethylformamide |
| DMSO | Dimethylsulphoxide |
| EDTA | Ethylene diamine tetra acetic acid |
| EDT | 1, 2 Ethane dithiol |
| ESI-MS | Electrospray ionization mass spectrometry |
| FE-SEM | Field Emission-Scanning Electron Microscopy |
| HBTU | N, N, N, N-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| HOBt | 1-Hydroxybenzotriazole |
| K | Lysine |
| LH | left handed |
| M | methionine |
| MALDI | Matrix assisted laser desorption ionization |
| MALDI-TOF | Matrix Assisted Laser Desorption Ionization – Time of Flight |
| MDR | Multi Drug Resistant |
| MD | Molecular Dynamics |
| MIC | Minimal Inhibitory Concentration |
| MDR | Multidrug-resistant |
| MTT | 3 - (4, 5-dimethylthiazol - 2 - yl) -2, 5-diphenyltetrazolium bromide |
| Mtb | *Mycobacterium tuberculosis* |
| ns | Nanosecond |
| PDB | Protein Data Base |
| PDR | Pan drug-resistant |
| POPG | 1- pamlitoyl- 2- oleoyl - sn- glycerol- 3- glycerol |
| (POPC) | 1- pamlitoyl -2 - oleoyl - sn - glycerol- 3- phosphocholine |
| RBCs | Red Blood Cells |
| RH | Right handed |
| RMSD | Root Mean Square Deviation |
| S | Cysteine |
| SPPS | Solid Phase Peptide Synthesis |
| TB | Tuberculosis |
| TFA | Trifluoroacetic acid |
| WHO | World Health Organization |
| XDR | Extensively drug-resistant |
| µM | Micro molar |

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First and foremost, I would like to thank the almighty God for his compassion and I am deeply indebted to my supervisors Dr. Vibin Ramakrishnan and Dr. Nitin Chaudhary whose stimulating motivation and valuable ideas kept me on track during this journey. Moreover, their principles, noble thoughts, politeness and simplicity always influenced me and will always help me in becoming a good person. I would like to express the deepest appreciation to my Doctoral committee chair person Dr. Vishal Trivedi along with other committee members Dr. Kannan Pakshirajan and Dr. Ganesh Natrajan who has continually and convincingly conveyed a spirit of adventure in regard to my research.

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Prakash Kishore Hazam

## ABSTRACT

Conventional peptide design only guarantees functional group constitution by opting specific amino acid sequence, and not their spatial orientation, because peptide molecules are very fluxional and its conformation is subjected to external flux. This is principally due to the isotactic stereochemistry of the peptide chain. Stereo-chemical engineering of peptide main chain offers expansion of design space of a typical sequence, possibly offering greater conformational rigidity by avoiding the 'roughness' of the folding funnel. In this thesis, we experiment with this conceptual possibility by designing amphipathic peptide systems of diversified stereochemistry, which can potentially act as bactericidal agents. We have designed three series of peptides, step-wise investigating the possibilities of such an enquiry, eventually resulting in at least half a dozen peptide molecules, qualified for future development as therapeutic agents. The principal objective of our first series of peptides was to verify, whether we can use the gramicidin helix as a template for AMP design. Gramicidin is a class of penta-deca-peptides isolated from soil bacteria *Bacillus brevis*, but their utility as an antibiotic was limited to topical use due to high levels of hemo-toxicity. Activity profiles of the four *de novo* designed peptide variants designed in the first phase, show better efficiency in treating Gram-positive bacteria than Gram-negative variants. Significantly, our hemolytic assay confirms very low levels of hemo-toxicity for the re-designed peptides, unlike gramicidin. In the next series of peptides in the second phase, we put to test, this conceptual possibility already established in theoretical models, by designing amphipathic peptide systems and experimenting them on Gram-positive, Gram-negative and antibiotic-resistant bacteria. The unusual conformational rigidity and stability of syndiotactic peptides enable them to retain the designed electrostatic environment, while they encounter membrane surface. All the six designed systems exhibited bactericidal activity, pointing to the utility and specificity of stereo-engineered peptide systems for therapeutic applications. This phase of the work provided us important insights and useful directives in designing novel peptide systems with antimicrobial activity, by expanding the design space, incorporating D-

amino acid as an additional design variable. We employed this knowledge-base in the design of next series of eight peptides, with varied electrostatic fingerprints. The phenomenal levels of anti-bacterial potency exhibited by half a dozen peptides in this series, high on specificity and less on toxicity, qualify them for next level of development as an effective therapeutic agent.

Tuberculosis is one of the leading causes of death, with an annual mortality rate of 2 million. The present treatment regimen for Mycobacterium species is strenuous, extending up to 12 months. Even then, rise of antibiotic resistance has limited the prognosis, with increased instances of multidrug resistant (MDR–TB) and extremely drug resistant (XDR–TB) cases reported. We demonstrated the bactericidal potency of cationic amphipathic peptides designed in phase two and three, as effective agents against Mycobacterium smegmatis. Potency of stereo-engineered LDLD or DLDL peptides have retained their potency, while their poly L variants rapidly lost their activity, when the experiment was repeated in human serum. Stability against enzymatic degradation was one of the main reasons for the underutilization of peptides, which could otherwise be a good therapeutic option with minimal side effects. Our assessment with alternating LDLD (or DLDL) stereo-chemical sequence of antimicrobial peptides, suggest that stereo-chemical engineering of designed peptide can address the most significant associated limitation of peptide based treatment regimen against bacterial infections.

# Chapter 1

# Introduction

History of Peptide chemistry started with the synthesis of glycylglycine in 1901, by Emil Fischer and Ernest Fourneau [1]. Peptides are involved in wide variety of functions in a living system, such as antimicrobials, ligands, hormones, signaling molecules, growth factors etc. [2]. Gramicidin, oxytocin, vasopressin and angiotensin were few molecules used in the early years of peptide based therapeutic agents [1]. Discovery of Solid Phase Peptide Synthesis (SPPS) by R. B. Merrifield in 1963, revolutionized this field of science and were once looked upon as a pertinent solution for many diseases. In SPPS, N-terminus protected amino acids are selectively added upon a solid support in a stepwise manner, with step-wise removal of reagents and by-product, resulting in the desired sequence with high yield and purity [1, 3]. However, the progression of peptides as a clinical agent has receded due to the repeated failures in the later phases of clinical trials. Factors like enzymatic degradation, ion susceptibility and low bio-availability were among the principal reasons of such failures [4]. Last few years, we are witnessing a renaissance in peptide drug discovery and usage, with 60 FDA approved peptide drugs and 140 peptide candidates in various phases of clinical trials and over 500 drug candidates in preclinical trials [1, 4]. Globally, peptide drug market is projected to reach US$ 25 billion mark by the end of 2018. Drugs against cancer and metabolic disorders are the most employed therapeutic molecules in this class [2].

Peptides have some interesting features like minimal side effect, high tolerability and selectivity towards specific targets etc. which would help them successfully comply with the stringent safety standards set for clinical trials. Properties like low cell membrane permeability, low oral bio-availability and ion sensitivity are some of the major drawbacks that limits their utility. Low permeability of peptides is a result of extensive hydrogen bonding capacity and imbalanced amphipathicity. A relative hydrophobicity of around 50 percent has been reported to be optimal for antibiotic peptides, resulting in effective peptide-membrane interaction. The reduced bioavailability of the peptide molecule can be attributed to the limited absorption and metabolic factors like pH and enzyme mediated degradation [5]. Nonetheless, the activity of peptide drugs has been retained with multiple

approaches employed by researchers [2, 5]. Modification of N and C terminus by acetylation and amidation have reported to increase the peptide stability. Altering peptide chirality by insertion of D-amino acid, has also resisted degradation of peptides with increased half life. Cyclization through sulfide bridging, lactamization, lactonization, macromolecular conjugation and PEGylation have also increased the stability of peptide drugs. Recently, few groups implanted drug delivery pumps that have proved effective in increasing the bio-availability of peptide drugs [2, 5].

Tacticity is a relative term in stereochemistry, which deals with the arrangement of adjacent chiral centers within a given molecule. Based on the spatial arrangement, a polymer can be isotactic, syndiotactic or heterotactic in nature. An isotactic polypeptide sequence is characterized by amino acids having L- or D-stereochemistry, whereas, a syndiotactic sequence is having a stereo-regular arrangement with alternate L- and D- amino acid or vice versa in succession, and a heterotactic polymer is a random distribution of L and D- amino acids in a sequence [6-8]. The preferential selection of L- over D- enantiomer remains one of the un-answered questions in science, reported in the 125th anniversary edition of the science magazine [8]. The conformational availability of any isotactic (poly-L or poly-D) polymers are restricted to 21 % of a typical Ramachandran plot due to excluded volume effect. Incorporating D amino-acids in the sequence can facilitate the representation of other-wise non-accessible regions in Ramachandran Map [8]. This may result in generating novel architectures, for tailor made applications in protein science (Figure 1.1).

Topology is a key element in structure-activity relationship estimation while designing physiologically-active molecular constructs [9-12]. Peptide design only guarantees functional group constitution by opting specific amino acid sequence, and not their spatial orientation to bind and incite physiological response on chosen targets. This is principally because peptide conformation is subject to external flux, due to the isotactic stereochemistry of the peptide chain. Stereo-chemical

engineering of peptide main chain offers the possibility of multiplying the structural space of a typical sequence to many orders of magnitude, and limiting the otherwise

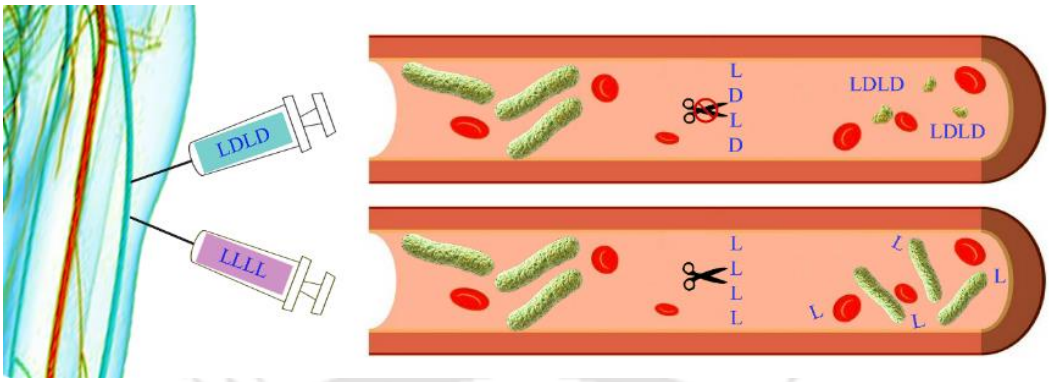



**Figure 1.1.** Schematic illustration of comparative serum life of syndiotactic (LDLD) and isotactic polypeptides (LLLL). LDLD peptides can retain the bactericidal potency in biological fluid, unlike their isotactic poly-L variants.

fluxional non-specific functional group dispensation in space by offering greater conformational rigidity [7, 13, 14]. We put to test, this conceptual possibility already established in theoretical models, by designing amphipathic peptide systems and experimenting them on Gram-positive, Gram-negative and antibiotic-resistant bacteria. The unusual conformational rigidity and stability of syndiotactic peptides enable them to retain the designed electrostatic environment, while they encounter membrane surface. Our work presented in the later sections of this thesis, provides important insights and useful directives in designing novel peptide systems with antimicrobial activity, by expanding the design space, incorporating D-amino acid as an additional design variable [7].

Tuberculosis (TB) is one of the top ten causes of death worldwide, with more than 10 million cases reported every year and among them, approximately 2 million patients succumb to the disease [15, 16]. Increase in drug resistance is a major challenge to the disease control mechanisms presently in place [17]. Even for the drug susceptible TB, the present treatment regimen is onerous, and hence there is an urgent clinical need for the discovery of new chemical entities effective for both drug susceptible and resistant TB [18, 19]. Mechanistic investigations on TB suggests

that, Mycobacterium bacilli are ingested by macrophages in response to bacterial invasion, subsequently resulting in the formation of attenuated phagosomes, creating a friendly intra-phagosomal environment for bacterial proliferation [20]. Even though there is available treatment regimen, the rise of multidrug-resistant superbugs has limited the prognosis [21]. Therefore, we investigated the anti-mycobacterial potential of our reported bactericidal peptides, both *in-vitro* and *in-serum* with Mycobacterium smegmatis, as a representative model for mycobacterium, due to its non-virulence and comparative fastidious nature. Antibacterial potency of isotactic and syndiotactic peptides was evaluated in both conditions (in vitro and in serum). Our results suggest that stereochemically diversified syndiotactic sequences effectively resist enzymatic degradation, while isotactic antibacterial peptides degrade losing their potency [18](Figure 1).

This thesis work has explored the utility of D-amino acid in terms of its stability, and comparative antimicrobial potency as compared to existing AMPs. There has been a clear advantage in employing D-amino acids, that has restored the bactericidal potency in biological fluid, unlike isotactic poly-L peptide variants. All the peptide candidates have shown negligible cytotoxicity towards mammalian Red Blood Cells (RBCs), confirming optimal amphipathicity with selectivity towards microbial cells. Potential activity in the presence of serum reveals negligible reduction of therapeutic potential in presence of biotic factors like enzymatic degradation and ionic interruption [7, 21]. This study may help in enriching the formulation of a comprehensive design algorithm in designing peptide based antimicrobials, which can be potential candidates for clinical evaluation.

# Chapter 2

## Stereo-chemical Engineering of Peptides with Diversified Tacticity and their Potential as future Antibiotics

Antibiotics can be defined as chemical agents that kills or inhibits the growth of microorganisms [22]. Ever since the serendipitous discovery of penicillin by Alexander Fleming in 1929 [23], antibiotics, as a potent therapeutic option was established. But this finding was incomplete until 1940, when Howard Florey and Ernest Chain reported the purification procedure, that could be employed for isolation and clinical testing of penicillin [24]. Much before the utilization of penicillin as a potent antibiotic, Ehrlich started the idea of synthesizing chemical compounds with an antimicrobial property. In 1909, an experiment by Ehrlich, Berthem and Hata led to the discovery of a potent antimicrobial compound against syphilis [25]. Similarly, Domagk introduced the utility of sulfa drugs in 1936 [26] which was employed in the treatment of various diseases. The term "Antibiotic" was coined by Dr. Selman A Waksman in 1941 [27]. He was awarded noble prize in Physiology and Medicine in the year 1952, for the discovery of "streptomycin" [28]. The two decades between 1950 to 1970 can be termed as the golden era of antibiotics [29]. Drugs like Chloramphenicols, Macrolides, Tetracyclines, Rifamycins, Glycopeptides and Quinolones were introduced in fifties and sixties [30]. Few more classes were added in the later years, but the chemotherapeutic agents discovered between 1950 – 1970, were the most effective and predominant till date [30].

Based on mechanism of the action, traditional antibiotics can be classified as Cell wall synthesis inhibitors [31-34], DNA synthesis inhibitors [35, 36], RNA synthesis inhibitors [37] and protein synthesis inhibitors [38-40]. Apart from these, DNA replication intercalators [41-43] and anerobic DNA inhibitors [44, 45] are some other classes of molecules in this class. The use of antibiotics in modern therapy is highly significant. In spite of their utility, a clear reduction of new drug molecules in the market has developed a notable gap between existing pathogens and treatment options. But the scenario is even more alarming with the rise of multidrug-resistant species, reported globally. Contrastingly, there has been a notable 2 to 3-fold decrease of FDA approved antibiotics in recent times [46] (Figure 2.1). Many bacterial species have been causing substantial clinical damage to the population and CDC (Centers for Disease Control and Prevention) has notified them under "concerning

threat" [47]. As per World Health Organization (WHO) a list of "priority pathogens" containing 12 bacterial species have been notified as multi drug resistant bacterial species, causing severe infections with little or no effective treatment regimen available. Among them, carbapenam-resistant *Acinetobacter baumannii is* considered to be the most dreadful bacterial species, with almost no drug based therapy available [48].

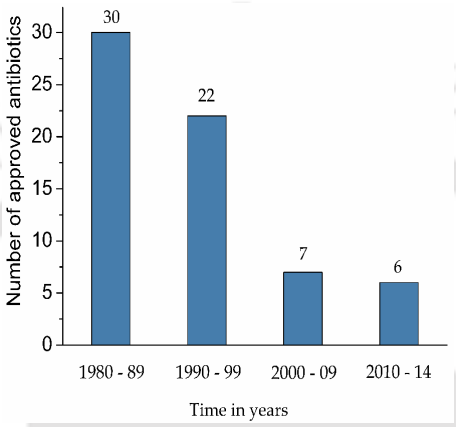



**Figure 2.1.** Number of antibiotics approved in last few decades (up to 2014), suggesting the steady decrease in the discovery and approval of new chemical entities that can potentially be used as antibiotics.

Therefore, the concern shared by the clinical practitioners regarding a steady decrease in the effectiveness of existing antibiotics is noteworthy, and we are in need of newer drug molecules, with potential antimicrobial activity [49]. The fore-sighted Sir Alexander Fleming predicted that the rise of resistant species can happen due to the overuse of antibiotics. The co-existence of resistant bacteria cannot be denied, as they occur in due course of evolution due to the presence of natural antibiotics. Persistent and improper use of antibiotics caused by drug abuse has spared resistant species, which has triggered their multiplication, resulting in increased number of drug resistant species [50]. Apart from drug abuse, over prescription has played an important role in generating microbial superbugs. Incorrect and over prescribed drugs have compromised the beneficial potential, causing the rise of hospital acquired infections, as one of its dreadful outcomes. The

presence of this category of microbes can result in antibiotics induced mutation [51]. Antibiotics also have been overused as an important additive for the safety precaution of the livestock against intimidating infections [52]. Even though this trigger growth with higher yields, ingestion of these drugs will occur while consuming the antibiotic treated food stuff, resulting in the development of drug resistant bacterial species [51, 53, 54]. A brief time line of antibiotics resistance has been summarized below, showing bacterial resistance, against antibiotics [46] (Figure 2.2).

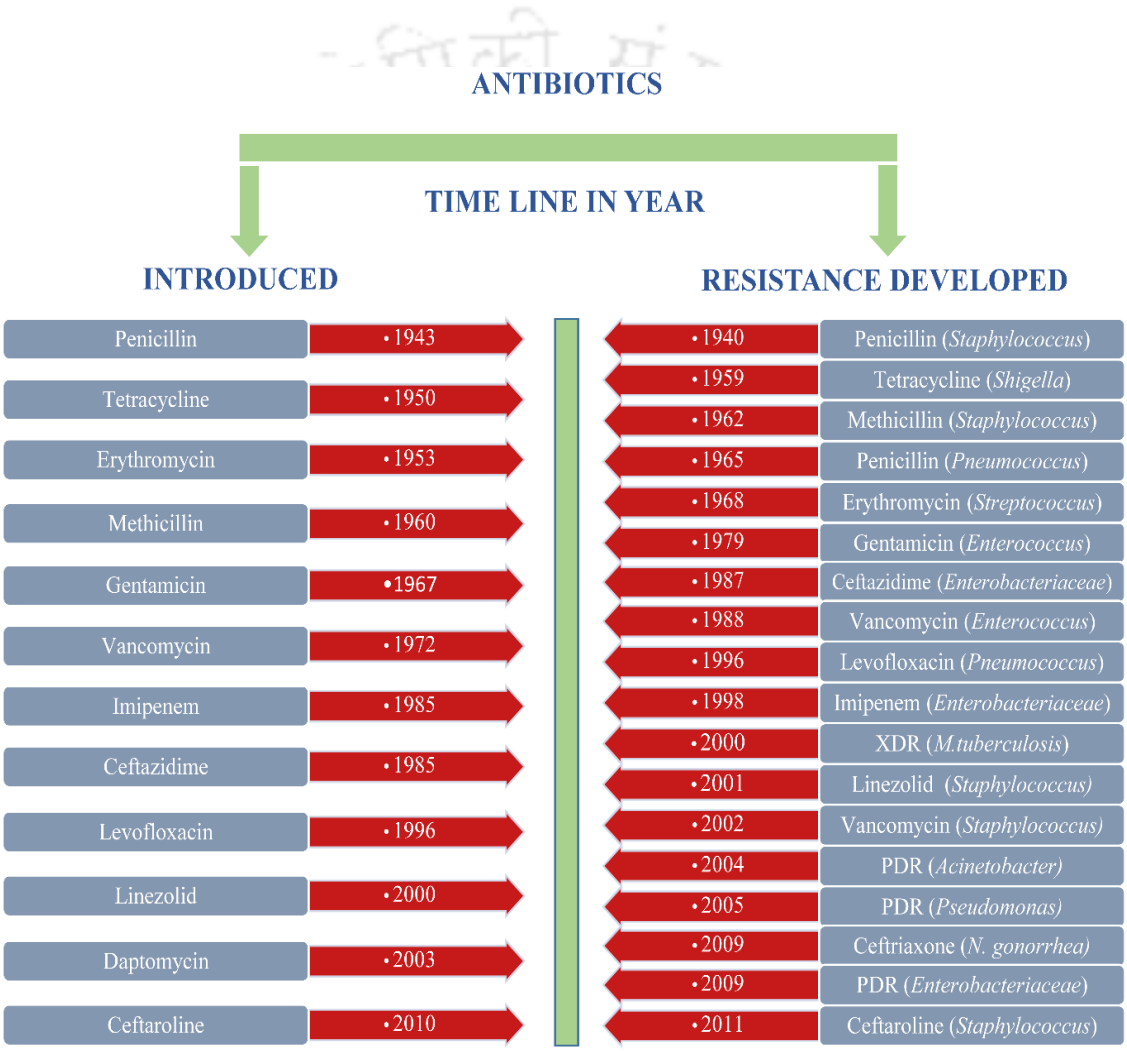



**Figure 2.2.** A brief timeline of the development and introduction of antibiotics, along with the progress of antibiotic resistant bacterial species. (XDR means Extremely Drug Resistant and PDR is Pan Drug Resistant). Penicillin was clinically introduced in 1943, whereas its resistance to Staphylococcus was reported much earlier (in 1940) [29].

The presence of alternative [55] (Table 2.1) has been considered over due course of time, but their limitations have halted their progress as an alternative to existing antibiotics. Despite multiple options available, AMPs are considered as the potential alternatives of current antibiotics, largely owing to their mendable drawbacks.

**Table 2.1.** Probable alternatives for conventional antibiotics

| S No | Alternatives | Activity spectrum |
|---|---|---|
| 1 | Antibodies | Prevent broad spectrum infection. |
| 2 | Lysins | Gram positive infection treatment. |
| 3 | Probiotics | *C.dafficile* and antibiotic associated diarrhea. |
| 4 | Bacteriophages | Treatment against broad spectrum infection. |
| 5 | Immune stimulation | Broad spectrum infection. |
| 6 | Vaccines | Broad spectrum infection. |
| 7 | Antimicrobial peptides (AMPs) | Broad spectrum infection. |
| 8 | Host and Innate defense peptides | Broad spectrum infection. |
| 9 | Anti-biofilm peptides | Broad spectrum infection. |

## 2.1 Antimicrobial peptides

Antimicrobial peptides (AMPs) molecules have been strategically used as a first line of defense [56] against invading pathogens throughout the evolutionary spectrum [57] (Figure 2.3).

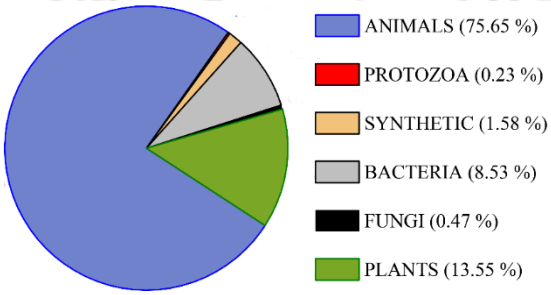
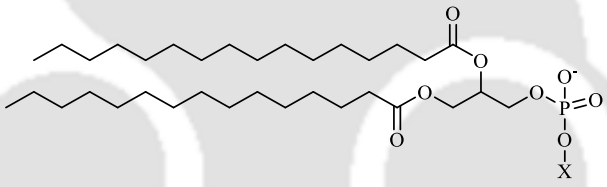


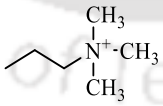
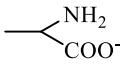
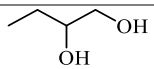
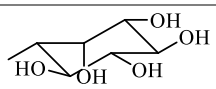
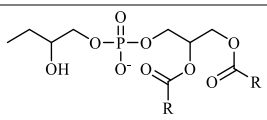

**Figure 2.3.** Source of antimicrobial peptides found in different levels of organisms, in terms of their relative occurrence.

The utilization of these molecules, dates back to early centuries of human evolution and their primary mechanism of activity is based on electrostatic interaction between AMPs and bacterial membranes, followed by membrane disruption. The primary construct of a bacterial membrane is considerably similar to eukaryotic membrane, composing of phospholipid bilayer with imbedded proteins in it.

Even though, membrane similarities are notable, there are differences too, and some of them are very significant while we design novel therapeutic solutions. In general, bacterial membranes are composed of both neutral and anionic phospholipids with higher content of anionic lipids in the outer layer [58, 59] (Table 2.2).

**Table 2.2.** Membrane phospholipids of bacterial membrane with chemical structure



| Name | X | Characteristic |
|---|---|---|
| ethanolamine (PE) |  | zwitterionic |
| choline (PC) |  | zwitterionic |
| serine (PS) |  | anionic |
| glycerol (PG) |  | anionic |
| cardiolipin (CL) |  | anionic |
| inositol (PI) |  | anionic |

Bacterial species can be broadly classified as Gram – positive and Gram – negative, based on their membrane morphology (Figure 2.4). The former possess higher mucopolysaccharide content and teichoic acid, whereas the latter has lipopolysaccharide (LPS) in their respective membrane construct [60]. The presence of net negative charge in bacterial membrane is one of the root cause of antimicrobial property of cationic antimicrobial peptides [61].

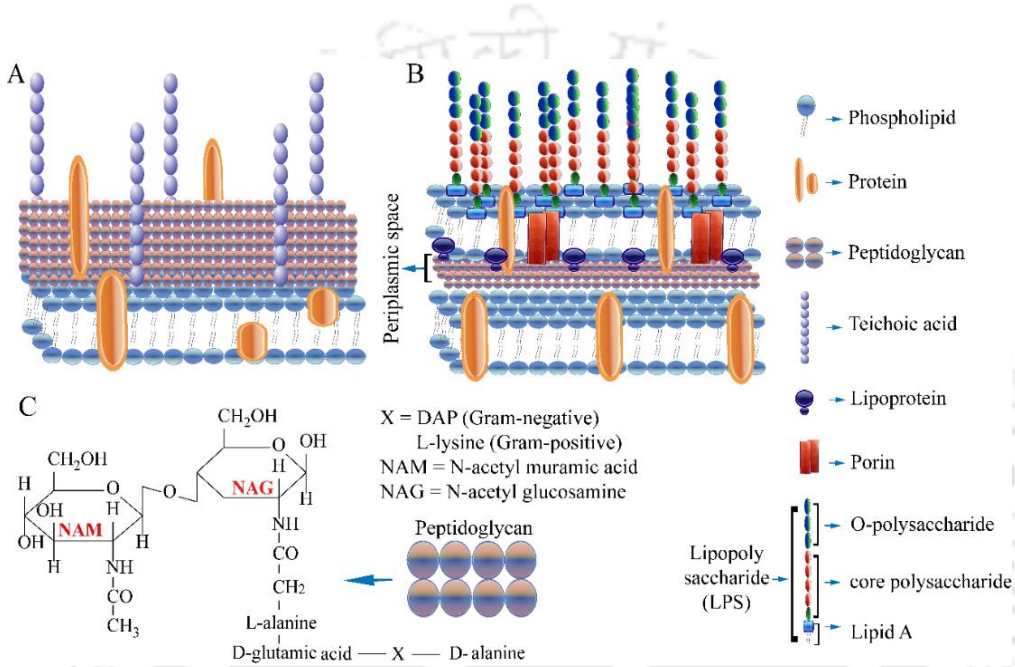



**Figure 2.4.** Morphology of Gram-negative bacteria (A) Gram positive bacteria (B) and chemical structure of bacterial peptidoglycan unit (C). The (X) diaminopimelic acid (DAP) in Gram-negative bacteria, is replaced by L-lysine in the Gram-positive cell envelope. NAM and NAG stands for N-acetylmuramic acid and N-acetylglucosamine respectively.

AMPs are reported to possess various mechanism of actions [59] which also reflects in their bactericidal potency. Even though the membranolytic activity of AMPs seems to be predominant and widely studied, the various pathway mediated bactericidal activities and their long standing side-effects have to be periodically investigated [62-66]. Till date, majority of the AMPs have been identified [56, 67, 68] or designed [69, 70] on the basis of their membranolytic property, but a molecule

designed to perform similar activity by modulating biochemical pathways can be more fruitful, compared to membrane active candidates.

In general, AMPs possess characteristics like net positive charge and amphipathicity as their principle design element [62], but negatively charged AMPs also have been reported in literature [71-74]. There are approximately 5000 reported antimicrobial peptides [57, 75, 76] which varies in length, charge and structure. Mostly, AMPs are found to be 20 – 50 amino acid in length, with a net charge ranging from + 1 to +9 [57, 77]. Amphipathicity of an AMP is an important property, which plays a significant role in peptide membrane interaction. In other words, a relative hydrophobicity of 50 percent [57, 62, 77] in a molecule is desirable in terms of AMP design perspectives. These molecules predominantly acquire α-helical and β-sheet structures in general, which also exists with both or none of these secondary structure in their peptide chain. Apart from these well-defined structures, majority of AMPs acquire uncharacterized 3-dimensional folds upon interaction with membrane [57]. A set of dedicated databases providing wealth of information is available for future AMP designers as a possible starting point (Table 2.3) [57].

**Table 2.3.** List of Antimicrobial peptide databases.

| Names | Links |
|---|---|
| APD [61, 78, 79] | http://aps.unmc.edu/AP/ |
| ANTISTAPHYBASE [80] | https://www.antistaphybase.com/ |
| BACTIBASE [81, 82] | http://bactibase.hammamilab.org/main.php |
| BaAMPs [83] | http://www.baamps.it/ |
| Cybase [84] | http://www.cybase.org.au/ |
| CAMP [85] | http://www.camp.bicnirrh.res.in/ |
| DAMPD [80] | http://apps.sanbi.ac.za/dampd/ |
| Defensins [86] | http://defensins.bii.a-star.edu.sg/ |
| DADP [87] | http://split4.pmfst.hr/dadp/ |
| DBAASP [88] | https://dbaasp.org/home.xhtml |
| DRAMP [89] | http://dramp.cpu-bioinfor.org/ |
| EnzyBase [90] | http://biotechlab.fudan.edu.cn/database/EnzyBase/home.php. |
| InverPep [91] | http://ciencias.medellin.unal.edu.co/gruposdeinvestigacion/prospeccionydisenobiomoleculas/InverPep/public/home_en |
| LAMP [76] | http://biotechlab.fudan.edu.cn/database/lamp. |
| MilkAMP [92] | http://milkampdb.org/home.php |
| PhytAMP [93] | http://www.camp.bicnirrh.res.in/ |
| Peptaibols [94] | http://peptaibol.cryst.bbk.ac.uk/home.shtml |
| THIOBASE [95] | http://dbmml.sjtu.edu.cn/THIOBASE/ |
| YADAMP [96] | http://yadamp.unisa.it/ |

Molecules like lysozyme [97], Gramicidin [98, 99] etc. were the first few antimicrobial agents discovered and characterized. Gramicidin was used to treat wounds and ulcers, as it was the first of its kind to be clinically tested [100]. Since then, there has been a remarkable increase in the number of isolated or designed AMPs [57]. Some of the most widely studied class of AMPs are cathelicidins [101], cecropins [102], defensins [103], sarcotoxins [104], protegrins [105], and dermaseptins [106]. Clinical studies have also started, ever since the consideration of AMPs as potential alternatives for existing antibiotics became popular. Molecules like Gramicidin [107], Daptomycin, Vancomycin [108], Telavancin [109], and Nisin [110] etc. are stablished clinical agents and few of them are in different stages of clinical trials [75] (Table 2.4).

## 2.2 Characteristics of antimicrobial peptides:

Antimicrobial peptides are considered as a potent alternative for conventional antibiotics. These molecules are broad spectrum, biocompatible possessing low toxicity. They are active against bacterial biofilms, Multi Drug Resistant (MDR) species and they can act synergistically with conventional antibiotics.

*Broad spectrum and bactericidal*: A broad spectrum antibiotic molecules are potentially active against Gram-positive and Gram-negative bacterial species, and this ability can be observed in the peptide molecules with antibacterial potency [111, 112]. Even though these primitive molecules are employed by our defense mechanism since ages, their utility in therapeutics came into existence as an alternative for the conventional antibiotics [113]. The broad spectrum activity of AMP's can be attributed to its mode of operation, which is largely *bactericidal* [69] rather than *bacteriostatic* (e.g. Gramicidin [98] , Alamethicin [114] ). The 'cidal' activity is the result of its membrane activity initialized by electrostatic interactions between cationic AMPs and anionic bacterial membrane [114, 115] .

*Biocompatibility*: A therapeutic molecule has to be biocompatible, for its approval as adrug entity. Biocompatibility issue arises when a foreign substance is introduced in a living system resulting in immune response. One of the main advantages ofAMPs in is they are much more 'natural' than any organic molecule,

**Table 2.4.** Antimicrobial peptides in clinical trials

| Name | Description | Medical use |
|---|---|---|
| | **Phase I** | |
| Histatin | Natural histatin from saliva | *P.aeruginosa* |
| hLF1-11 | Human lactoferrin peptide | Antifungal property. |
| Plectasin | Defensin (fungus) | Gram-positive (systemic), streptococcal |
| Opebacan | Peptidomimetic | Anti-infective |
| VIP | Host defense peptide | ARS, sepsis |
| IDR-1 | Peptidomimetics (bovine ) | Immune compromised anti-infective |
| | **Phase II** | |
| P113 | Histatin fragment | HIV |
| MX-594AN | Analogue of indolicidin | Acne (catheter related) |
| PAC113 | Histatin fragment | Oral candidiasis |
| CZEN-002 | α-MSH (synthetic analogue) | Infection caused by *Vulvovaginal candidiasis* |
| EA-230 | Tetramer peptide portion from b-hcg | Sepsis |
| IMX942 | IDR-1 and indolicidin derivatives | Neutropenia, febrile and nosocomial infections. |
| XOMA-629 | AMP derivative | Impetigo |
| RDP58 (Delmitide) | HLA derivative | IBD disease |
| Novexatin (NP213) | AMP (cyclic) | Fungal infection due to dermatophyte |
| PMX-30063 | non-peptide defensin analogue | Staphylococcus cutaneous infection |
| Omiganan (CLS001) | Indolicidin derivative | Inflammatory papules, pustules (rosacea). |
| OP-145 | LL-37 derivative | Ear infection (bacterial) |
| Ghrelin | Synthetic derivative (HDP) | Cystic fibrosis respiratory infection. |
| AP-214 | Alpha MSH derivative | Post-surgical organ failure, sepsis |
| CD-NP | Peptidomimetic (natriuretic peptide) | Organ failure, ADHF |
| Sifuvirtide (SFT) | Based onHIV-1 gp 41 fusogenic core | AIDS, HIV fusion inhibitor |
| | **Phase III** | |
| XMP 629 | Peptide from human | Acne |
| Mycoprex | Peptide from insects | Fungal infections |
| Talactoferrin | hLF mimetic | Diabetic neuropathic ulcers, chemotherapy (NSCLC) |
| Glutoxim (NOV-002) | Glutathione derivative | Tuberculosis, NSCLC |
| BL2060 | Copolymer (lysine and fatty acid) | Lead optimization as anti-infective |
| DP178 | C-terminal ectodomain of gp41 | FDA approved (AIDS) |
| PG-1 | Protegrin | Peritoneal infection (*P.aeruginosa,* Pneumonia) |
| P113D | Histatin 5 fragment | *P. aeruginosa* infections(cystic fibrosis) |

usually administered as antibiotics. Even though proper clinical trial has to be conducted, but their natural origin can be an advantage over natural products and newly designed chemical entities.

*Synergism*: Synergism is termed as increased combinatorial activity while different molecules act together. This property has been identified, when AMPs are employed with classical antibiotics showing additive activity [116, 117]. Some of the well-

known classes of antibiotics like β-lactams, nalidixic acid derivatives and penams etc. have been reported to possess synergistic activity when combined with AMPs [118]. Chimeric peptides along with conventional antibiotics were highly effective against drug resistant *A.baumanii* [119].

*Activity against MDR species*: The utility of AMPs as a potential alternative to conventional antibiotics was predicted due to its prominent bactericidal activity against MDR and biofilm forming resistant bacterial species[119]. These superbugs are one of the major reasons of ever increasing mortality rate of infected patients. In this regard, some of the AMPs have shown their potential activity against multi drug resistant microbial species. Peptide molecules like SMAP-29 have been effective against MDR, VREF and mutated *P.aeruginosa* species [120].

*High selectivity, Low level or mendable toxicity and limited resistance*: An ideal AMP preferentially interacts with anionic microbial membrane [121], unlike neutral mammalian counterpart [122]. The presence of teichoic acid (Gram-positive), lipopolysaccharide (Gram-negative), phosphatidylglycerol, cardiolipin and phosphatidylserine in the prokaryotic membrane imparts relatively larger negative charge at physiological conditions. On the contrary, human cell membrane is composed of phosphatidylcholine and other zwitterionic counterparts resulting in the formation of neutral biological membrane [123]. The selectivity towards anionic membrane is one of the most prominent feature of natural AMPs and the design mainstay of synthetic AMP molecules [123].

*Anti-biofilm activity*: Peptides isolated from Enterococcus have been effective against *S. aureus* biofilms at lower concentrations and there are reports of LL-37 inhibiting the biofilm formation of *S.epedermis* [124]. Some of the peptides like citropin 1.1, melimin, lactoferrin and its conjugated form (with hexapeptidic Titanium-binding peptides) have shown potential broad spectrum anti-biofilm activity when co-administered with classical antibiotics like rifampicin and minocycline in case of medical device infections [124]. The anti-biofilm property of a peptide is a much needed feature because; bactericidal options against biofilm

infections are limited. Even though, antibiotics can easily neutralize planktonic cells during biofilm infections, their inability to penetrate the bacterial biofilms restrict their activity upon sessile bacterial cells. Apart from this, sessile cells in biofilms are metabolically depressed with limited nutrient use, which are least susceptible to many antibiotics. Aggregated microbial colonies can be termed as biofilms. In other words, biofilms are organized microbial clumps which can be functionally compared to tissues of higher organisms. The cells are enclosed with a self-processed matrix, with distributed channels for nutrient supply. The microbial candidates are sessile in the film, whereas dispersed planktonic cells are active and non-sessile in nature. The biofilm matrix is a mixture of polysaccharide, anionic exopolymer and abiotic substances enclosing bacterial cells as a functional unit [125-128].

AMPs can be the potential alternatives of antibiotics due their broad spectrum activity, biocompatibility and minimal cytotoxicity. However, the noted drawbacks such as cost and reduced *in vivo* activity associated with peptides have delayed their progression as a clinical agent.

*Manufacturing cost*: The high cost of production is the prime concern, for the establishment of peptide based entities as a drug molecule [129]. Peptides with longer sequences of amino acid residues correspondingly increase the cost of production, which may be minimized by the design of shorter molecules with conventional synthesis protocol. Industry scale production in cellular expression can be another strategy to reduce production cost of peptide drugs. Haught *et al.* have demonstrated the use of bacterial expression systems for high yield production of therapeutic peptides [130]. Similarly, Pierce *et al.* have reported high yield production of polyphemusin in recombinant bacterial expression system [131]. Some of the reported instances where bacterial and other expression systems have shown high scale production of antimicrobial peptides may be found in the associated references[132-134].

*Reduced activity in in-vivo environment*: Even though there are numerous reports showing the *in-vitro* potential of AMPs, their inferior *in-vivo* activity is a matter of concern [59, 135, 136]. The reduced bioavailability of the peptide molecules can be attributed to their respective pharmacokinetic properties [absorption, distribution, metabolism and excretion (ADME)] [5] (Figure 2.5). Most of these molecules have low permeability through biological membrane, due to excess hydrogen bonding and inadequate lipophilicity [5].

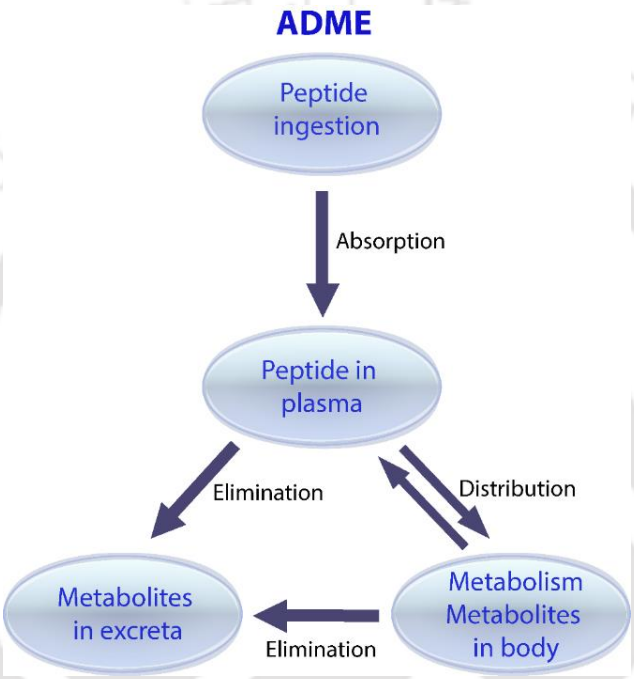



**Figure 2.5.** Pharmacokinetics of peptides in human body

On the other hand, factors associated with metabolism and rapid renal clearance also reduces the bioavailability of peptide-based drug molecules. However, there have been various strategic implants that have improved the pharmacokinetic profile of therapeutic peptides. N-methylation and intramolecular hydrogen bonding have markedly reduced the intermolecular hydrogen bonding potential whereas increased rigidity has been witnessed due to cyclization [5]. Stapled peptides created by bonding two amino acids have shown augmented stability. Peptide

prenylation, reversible lipidation and permeability enhancers[5, 137] have shown to increase permeability resulting in higher bioavailability. The most prominent barrier of peptide stability is enzymatic degradation caused by proteolytic enzymes with in the biological systems. But approaches like C- and N- terminal capping, insertion of D-amino acid, amino acid modification and cyclization have markedly reduced enzymatic degradation of peptide molecules [5, 138]. Rapid renal clearance of peptide molecules has been countered by strategies like peptide conjugation with plasma proteins, covalent linkage to albumin–binding small molecules, conjugation with large polymers, fusion with long half-life proteins etc. All these tactics have increased the pharmacokinetic profile of peptide molecules regarding its bioavailability and stability within an *in-vivo* environment [5].

*Resistance against AMPs*: Resistance towards AMPs are a defense strategy of microbes intended for their survival. Peptide membrane interaction in accordance with antimicrobial activity is mostly mediated through electrostatic attraction due to their opposite charge. Microbial interaction towards AMPs progress through a cascade of pathways. Modification of lipid A (amino arabinose [139], glycosylation [140]), LPS (myrstylation [141]) and lysyl PG [142] formation are some of the profound reasons. Apart from these, the reduction of anionic lipids from their membrane construct is another approach to reduce the potency of antimicrobial peptides. Capsule formation with anionic moieties like glycocalyx [143] and sodium alginate [144] sequesters the AMPs. The antimicrobial potency of peptides may be also affected by protease activity of body fluids. Other defensive measures like RosA and RosB, TrkA and SapG and AacrAB efflux pumps to drain out AMPs have been reported to be another form of defense mechanism [145]. Adaptation of microbes to survive in higher ionic conditions also reduces AMP potency [146] as many peptides are salt sensitive in nature. Even though, bacteria possess multiple pathways for AMP resistance, it's reassuring to know that the basic construct of bacterial bilayer membrane does not allow it to acquire complete resistance against antimicrobial peptides [147].

## 2.3 Antibiotic peptides with diversified tacticity

Tacticity is a relative term in stereochemistry, which deals with the arrangement of adjacent chiral centers within a given molecule. Based on the arrangement, a polymer can be isotactic, syndiotactic or heterotactic in nature [7,8, 148]. An isotactic polypeptide sequence is characterized by having all amino acids in L- and D-stereochemistry, whereas a syndiotactic sequence is a stereoregular arrangement with alternate L- and D- amino acid or vice versa in succession and a heterotactic polymer is a random distribution of L- and D- stereo-isomeric monomers (Figure 2.6).

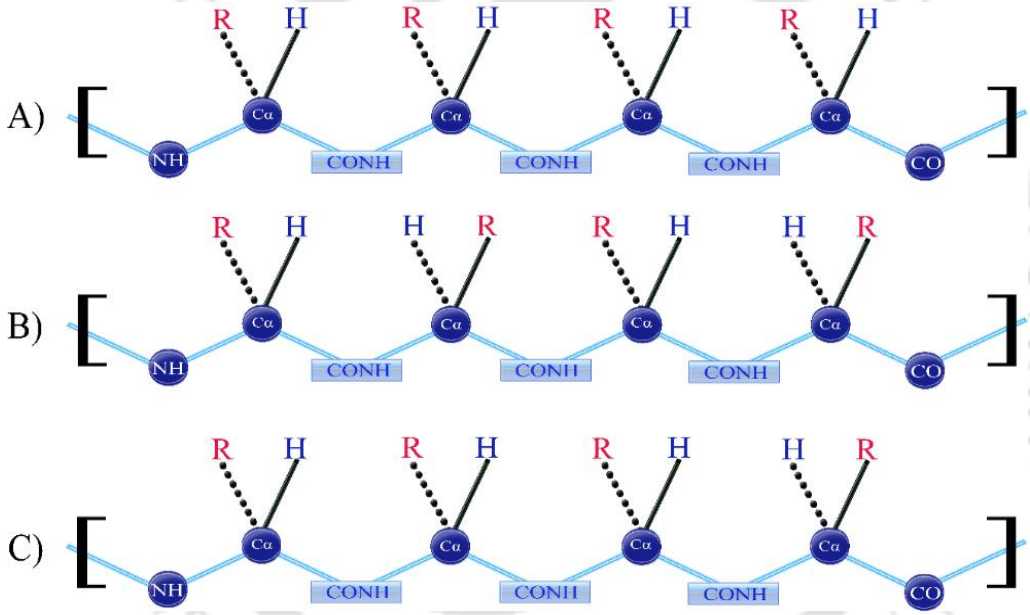



**Figure 2.6.** Arrangement of stereo-centres in a typical polymer chain as A) isotactic, B) syndiotactic and C) heterotactic peptide polymers.

A natural protein or peptide polymer is a polymer of L-amino acids alone and hence, isotactic in nature, with few exceptions. The preferential selection of L- over D-enantiomer remains an enigma, reported in the 125$^{th}$ anniversary edition of science magazine [8]. The conformational availability of any isotactic (poly-L or poly-D) polymers are restricted to 21 % of Ramachandran map due to excluded volume effect [8] or by steric collision, among side-chains[150]. A typical Ramachandran plot is a graphical representation of phi (ϕ) and psi (ψ) dihedral angles showing preferential

conformation of any amino acid (Figure 2.7). Naturally scripted isotactic polypeptide sequence disparity in a Ramachandran plot can be modified and expanded to many folds by insertion of unnatural amino acids (Figure 2.7), as a

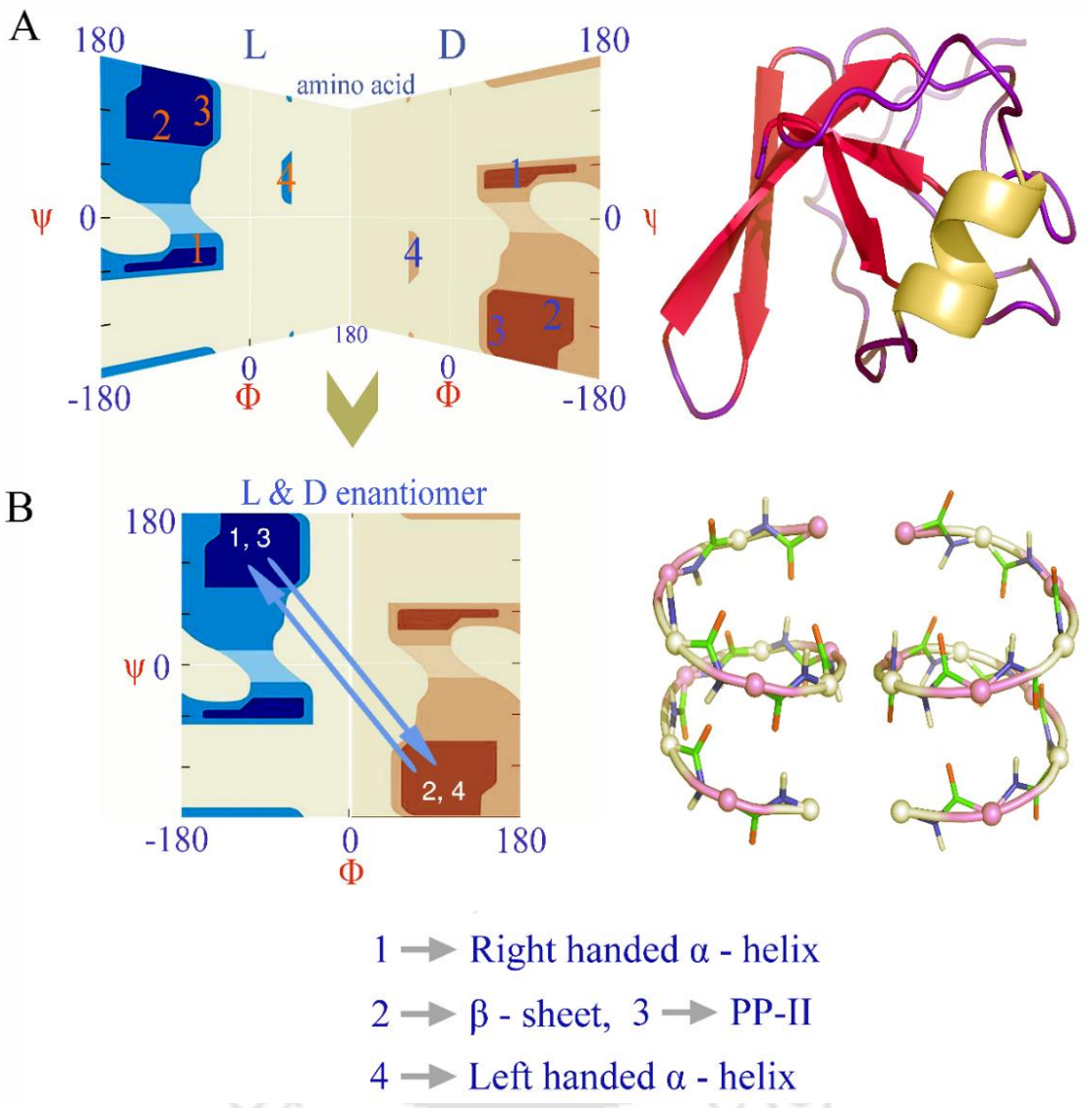



**Figure 2.7.** A) Ramachandran plot showing φ,ψ combinations of a given isotactic poly peptide chain. B) Syndiotactic peptides have approximately two times net access in φ,ψ space.

result of minimized side-chain repulsion. Hence, unusual conformational approach by informed walk across Ramachandran map minimizing local restriction, may result in novel architectures with diversified stereo-chemical conformations suitable for wider range of applications [148, 149, 151, 152].

### 2.3.1 Mechanism of action

It is generally considered that the bacterial membrane is one of the main targets of AMPs [62]. The main models by which antimicrobial peptides are proposed to work against microbes are Barrel stave, Carpet (detergent), Toroidal and Disordered toroidal pore (Figure 2.8) model [153].

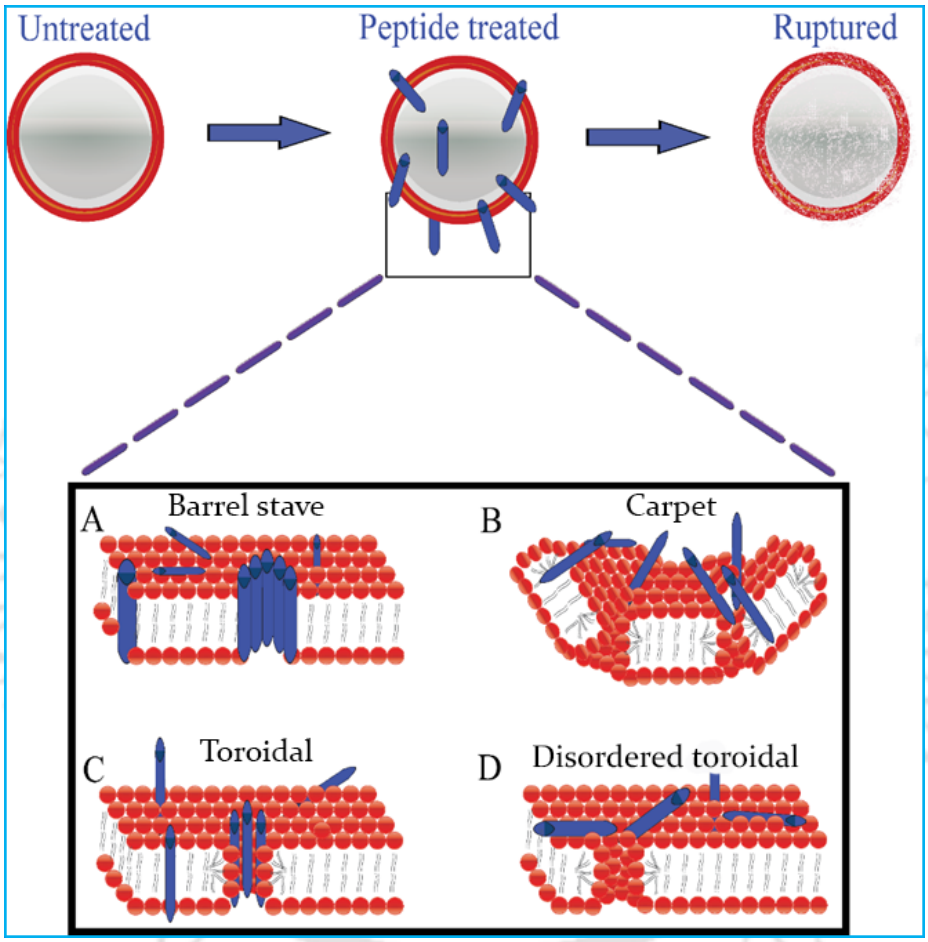



**Figure 2.8.** A schematic representation of commonly cited mechanism of action of anti-microbial peptides.[A)Barrel stave B)Carpet C)Toroidal pore andD)Disordered toroidal]

*Barrel stave Model*: In this model, AMPs first attach to the lipid membrane surface with their axis parallel to it. Because of that arrangement, permanent trans-membrane pores are formed due to alignment of the hydrophobic region of the peptides with the hydrophobic core of the lipid bilayer, and hydrophilic region of the peptide constitute the interior of the trans-membrane pore. So, the formation

of barrel-stave pores requires exact alignment of facial amphipathicity and hydrophobic matching of the AMPs with the bilayer.

*Carpet Model (Detergent)*: Carpet model proposes AMPs getting spread all over the lipid membrane or the peptide molecules align parallel to the membrane. This alignment infuse a disorientation of the stacked lipid molecules followed by disorientation and degeneration into small aggregates. Carpet does not suggest any pore formation during antimicrobial activity.

*Toroidal Model*: This pore formation architecture can be compared to barrel-stave formation, but there is a formation of toroid near the attached peptide. In this case, the lipid molecules fold inwards giving rise to the toroidal pattern. The inner core of the pore is partly aligned by peptide and lipid head.

*Disordered toroidal pore Model*: This mechanism is mostly related to peptide molecules having lesser rigid conformations. Unlike toroidal pore, the inner lumen of the pore is lined by head groups of phospholipids. In other words, the inner core in this model is hydrophilic due to the presence of polar head groups. Apart from these, Hancock and his group reported "aggregate model" for antimicrobial activity of peptides [154]. AMP-lipid interaction with this mechanistic approach can change lateral lipid packing, increased permeability of membrane as well as membrane deformation.

Nature has scripted unusual combination of enantiomers (L/D – amino acids) in achieving very special functions[155-159]. One such prominent example is Gramicidin; an antibacterial (gram positive) peptide discovered by Rene Dubos in the year 1939 from *Bacillus brevis* [98, 160]. It was the first antibiotic agent tested clinically, which was used as an antiseptic in World War II. Gramicidin is a 15-mer syndiotactic antimicrobial peptide with alternate L- and D-enantiomers in its structure. This molecule exists as isoforms with a variation of valine/isoleucine in the first position. Naturally occurring Gramicidin or Gramicidin D is a mixture of Gramicidin A, B and C with a variation at 11$^{th}$ position containing tryptophan, phenylalanine and tyrosine respectively. A Gramicidin helix has syndiotactic arrangement of L- and D- amino

acids. The prime disparity of this molecule is the insertion of D-chiral amino acid, which is unusual in naturally occurring protein sequences [100, 161].

Most of the isotactic (poly-L) sequences that have shown good activity under *in vitro* conditions have failed to repeat the same efficiency in body fluids [59]. Enzymatic degradation of poly-L AMPs are a common phenomenon, which results in reduced activity of peptides. Hammamoto et al [162] performed a comparative analysis of human granulysis peptide and its D-enantiomeric analogues. The antimicrobial potency was comparable in all the cases, but poly-L variants were susceptible to enzymatic degradation. Similarly, Tugyi et al have also reported differential response of poly-L and hetero-chiral sequences against proteases[163]. Similar findings were reported by various other groups [164-166] as well, pointing to the utility of unnatural amino acids in the design of novel therapeutic agents with bactericidal potential.

A 'peptido-mimetic' approach was attempted by our group based on gramicidin structure, for the design of antimicrobial peptides with diversified tacticity [167]. A pair of syndiotactic, dodecamer antimicrobial peptide and their respective acetylated versions were designed for the assessment of their bactericidal potency. All the designed peptides have shown preferential bactericidal activity against Gram-positive species compared to Gram-negative ones, much like gramicidin. Importantly, our peptides displayed negligible cytotoxicity against mammalian RBC, unlike Gramicidin. Toxicity was the principal limiting factor of gramicidin for their use as antibiotics; consequently, their utility was largely limited to topical use.

In another study, we intended to test the effect of interplay between tacticity and chemistry and their consequence in modulating antimicrobial potency [149]. The peptides were systematically constructed, keeping syndiotactic stereochemistry (LDLD or DLDL) variants as the backbone, along with their homo-chiral (poly-L) sequences. The study could successfully demonstrate that stereo-chemical diversification can produce different electrostatic environments while they approach bacterial membrane, by exhibiting differential bactericidal activity. In a follow up study, we have tested the potency of these peptides against

*Mycobacterium smegmatis* both in presence and absence of serum. All the designed peptides were significantly bactericidal in media, but poly-L sequences were substantially less active compared to LDLD and DLDL variants.

### 2.3.2 Modeling Peptide – Membrane Interaction

The interaction of peptide molecules largely varies upon its structure as well as the chemistry of the membrane. The mechanistic study by Garcia and his group [168] has shed some light in to the translocation mechanism of TAT peptide, initiated by its interaction with proximal carbonyl groups and distal phosphate groups. The distal interaction decreases the membrane thickness, where peptide insertion is cooperatively assisted by water molecule (hydration)[168, 169]. Peptide-membrane interaction of an amphiphilic, cationic peptide molecule is initiated by electrostatic interaction between the peptide and anionic lipid bilayer. Amphiphilicity of molecules plays an important role by presenting its hydrophobic segment to the phospholipid chain. The hydrophobic interaction stabilizes the pore formation leading to bacterial cell death [169]. The simulation studies performed by our group largely support the theory of bacterial pore formation during peptide-membrane interaction (Figure 2.9).

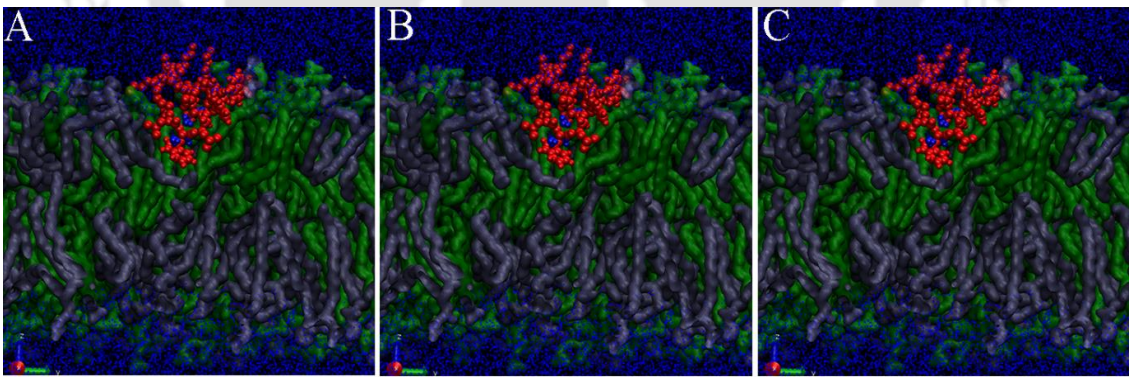



**Figure 2.9.** Snapshots of peptide-membrane interactions from Molecular Dynamics simulation of antimicrobial peptides in a bacterial model membrane, for 200 ns using GROMOS force field.

The stability of the pore is however, concentration dependent, which points to the inability of peptide activity below a threshold level [170, 171]. Usage of unnatural amino-

acids, within a peptide sequence may probably help to increase proteolytic stability [172-175]. Shai *et al*. have reported various instances where attenuated poly-L sequences with D- enantiomers have reduced cytotoxicity to admissible levels [176-179]. Even though cytotoxicity is mainly associated with amphipathicity, increase in percent hydrophobicity can be correlated with reduced cell selectivity. In other words, a peptide with high hydrophobic content is more likely to be a membranolytic peptide [180-182].

### 2.3.2 *Salt sensitivity of Antimicrobial peptides*

There have been certain studies based on the role of unnatural amino acids and their salt-sensitivity profile as well. As per the findings, peptides with D-amino acid were less prone to the solvent [183, 184]; whereas the antimicrobial activity of poly-L variants were diminished in such environments. Despite being innately originated in our physiology and omnipotent against plethora of microbial organisms, these molecules tend to lose their antimicrobial potency in presence of physiological salts. As per the suggested mechanisms, the decrease in antimicrobial potential of AMPs can be attributed to the hindrance caused by ions in terms of the electrostatic interaction between AMP's and microbial membranes. However, retaining conformational rigidity has also been related to the restoration of antimicrobial activity of peptides under high salt concentration. Therefore, we are lack of any conclusion regarding the selective salt sensitivity of AMP in physiological conditions [185].

Through the experiments elaborated in the following chapters in this thesis, we are hopeful of giving critical inputs to enrichthe efforts in framing a successful design strategy in the generation of stereochemically diversified peptide antibiotics with negligible proteolytic enzyme sensitivity, minimal cytotoxicity and salt sensitivity.

# Chapter 3

# Research Design

Short synthetic peptides with amphiphilicity have recently been explored as possible building blocks for the design of effective nano-bio materials, in a variety of applications ranging from antibiotics [186], nanofabrication [187], tissue engineering [188], controlled gene [189] and drug release [190], bio-mineralization [191], and energy generation/storage [192]. Such a range of applications is a consequence of their heavy cross-linking capability coupled with remarkable simplicity and biocompatibility [193].

Amino acids are the fundamental building block of a protein or a peptide. The 20 natural amino acids are diversified in terms of their structure and chemistry. Amino acids may be divided into polar, non-polar, aliphatic, aromatic, negatively or positively charged etc. [194]. In this 'equation' that governs the sequence to structure to function of a polypeptide sequence, the 'variable' is only the side chain, with the main-chain remains 'constant' for all amino acids. In fact, the performance of biological functions by proteins has its roots in this side-chain diversity and their various length-wise and sequence-wise combinations [167]. Formation of secondary and tertiary structures is a result of local interactions and structure complementarity [196]. In a typical protein fold, hydrophobic side chains are buried inside the core with hydrophilic side chains exposed offering favorable interactions with water. The presence of such charged side-chain groups can invoke selective responses in other charged groups and surfaces. Interestingly, such interactions can be tuned by ionic strength or solution pH. This indicates that while designing peptide self-assembly, we need to take into account of four things; hydrogen bonding, electrostatic interactions, hydrophobic interactions and finally the directive nature of the peptide backbone. The first three aspects have been extensively used in the design and fabrication of peptide assemblies [197, 198], but the directive nature of peptide backbone and its effect on structure is yet to be explored to its potential. This thesis work is thus, designed on the concept of stereo-chemical engineering of peptide amphiphilic structural constructs as future antimicrobials.
The role of main-chain structure in selection and stabilization of polypeptide conformation remains a much less explored area in protein science. The chiral

amino acids of a typical protein structure are always L configurational. The origin of this apparent stereo-chemical filtration in protein biogenesis remains unclear; however, its consequence on peptide assemblies can be investigated and explored for future design. Our primary interest is to use the backbone stereo-chemistry as an additional variable for design of novel peptide based structural constructs and design of novel antimicrobial agents.

Principal aim of this thesis work is the design synthesis and characterization of a new genre of anti-microbial peptides incorporating amino acids with L and D stereochemistry. Key objectives may be summarized as follows:

1. Examine the effects of conformational rigidity of peptides in directing anti-microbial activity.
2. Examine the specific effects of structural amphipathicity in peptide membrane interaction.
3. To design a series of peptide molecule those sustains a pore and examine their utility as model template for peptide antibiotics.
4. Synthesis of the designed peptide by Solid Phase Peptide Synthesis.
5. Purification by reverse phase HPLC and primary characterization by mass spectrometry.
6. Estimate the relationship of topology dependent electrostatic profiles of the sequences and their respective membranolytic activity on artificial membranes using Langmuir–Blodgett apparatus.
7. Perform antimicrobial assay against a gram positive and negative bacteria, to verify the individual roles of design element employed and its translational efficacy.
8. Electron microscopic analysis to get a topographical view of peptide-membrane interaction.
9. Estimate the level of toxicity in mammalian cells by performing hemolytic assay.

10. Extend the study to evaluate the designed molecules as a therapeutic agent against Mycobacterium Species.

Enhancement of secondary structure stability can improve the utility of peptides as antimicrobial agents upon interacting with bacterial membranes. According to the currently accepted mechanism of this anti-microbial activity, such peptides should have a very stable fold with amphipathic structure. Stereo-chemically controlled peptide design can significantly improve secondary structure stability, specifically required for membrane binding and lytic activity. The following chapters explain various aspects of this basic paradigm.

# Chapter 4

## Peptido-mimetic Approach in the Design of Syndiotactic Antimicrobial Peptides

## 4.1 Introduction

Higher organisms use peptides as a defense mechanism against invading pathogens [200-203]. Defensins [204], cecropins [205], magainins [206] and cathelicidins [207] constitute major classes of "host defense peptide". There is a remarkable increase in peptide based antibiotics, with nearly two dozen molecules are either completed or under clinical trials as antimicrobial or immunomodulatory agents [208]. However, cost, uncertain toxicology profiles and lability to proteolytic susceptibility hampers the otherwise promising approach, especially in the light of the phenomenal increase in antibiotic resistance against conventional therapeutics [5, 209]. Decreasing the size of peptides and machine learning approach have more or less addressed the first two issues, while the problem of proteolytic degradation may be sorted out by the use of D-stereoisomers and unnatural amino acids in the sequence [162]. Designed antimicrobial peptides, in general, are cationic amphipathic peptide sequences with distinct membrane permeabilizing ability [7, 62]. Though various mechanisms have been proposed like carpet [210], detergent [211], barrel stave [212], toroidal pore [213] etc., a consensus picture is yet to emerge.

We designed two amphipathic peptide sequences and their N-terminal acetylated variants mimicking the stereochemical sequence of Gramicidin, a penta-deca-peptide isolated from soil bacterial species *Bacillus brevis* [161]. Antibiotic profile of Gramicidin reports that they are particularly effective against Gram-positive bacteria. However, their utility is rather limited to topical use as a lotion or ointment in the treatment of infected surface wounds and throat infections [214, 215]. Reported mechanism of action for Gramicidin suggests that it inserts itself into bacterial membranes resulting in membrane disruption and permeabilization, leading to a sequence of events such as the loss of intracellular solutes, dissipation of trans-membrane potential, inhibition of respiration and reduction in ATP pools, culminating in cell death [216]. Naturally-occurring Gramicidin or Gramicidin D exists in isoforms with a variation of valine or isoleucine at first position. Gramicidin

molecule is a mixture of Gramicidin A, B and C with a variation at the 11th position containing tryptophan, phenylalanine and tyrosine respectively [217]. In this work, we present a design philosophy to synthesize cationic, amphipathic peptide molecules incorporating D-amino acids in the design alphabet. To promote the membrane permeabilizing ability, we designed our sequence around Gramicidin with gramicidin helix (6.3 helix) as the target structure. Gramicidins form cation selective trans-membrane ion channel in typical model membranes [218]. Even though, Gramicidin acquires various folding states, two of the major conformations have been predominant in different environments; first is the channel forming single stranded helical dimer and the second type is a non-channel forming intertwined helix [161, 219]. Even though conformations of gramicidin are solvent dependent, single stranded β (6.3) dimer is the thermodynamically most stable structure [219-221].

Like all natural proteins, most of the therapeutic peptides are sequences of asymmetric amino acids having L-chiral stereo-chemical structure. In other words, they are stereo-regular "isotactic" hetero-polymers. The isotactic stereochemistry of the backbone, coupled with planarity of peptide bond and steric effects limits the conformational space of a polypeptide. Even then, the predictive ability of a given amino acid sequence to fix the structure, folding intermediates and pathways are still weak, despite making enormous advancement in the basic understanding of this unsolved problem [222, 223]. This limits the success of a protein *de-novo* design experiment in realizing its translational objectives.

Polymer sequences with alternating L and D-chiral monomers (amino acids) are referred to as syndiotactic polymers and with random distribution of L and D are heterotactic or atactic polymer [6, 224]. Gramicidin is a 15 amino acid long trans membrane helical molecule with alternating L and D-chiral amino acids in its sequence and can hence be termed as a syndiotactic peptide [225]. In this study we tried to develop topologically constrained antimicrobial peptide molecules with

syndiotactic stereo-chemical arrangements after making an objective assessment about their relative stability by molecular dynamics simulations. Acetylated variants of both the designed peptides, were also examined to test their possible differential efficacy, against Gram-positive and Gram-negative bacterial species. The activity profiles were considerably similar, indicating limited significance of acetylation in syndiotactic sequences. The bactericidal potency of the designed peptides was higher against Gram-positive than in Gram-negative bacteria, though the design approach may be further modified in future designs in the generation of broad spectrum antibiotics.

## 4.2 Materials and methods

### 4.2.1. Reagents and chemicals

Rink Amide resin, F-moc amino acids, N, N, N, N-Tetramethyl-O-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU), N, N-Dimethylformamide (DMF), m-Cresol, Glutaraldehyde, Dimethylsulphoxide (DMSO), Chloroform and Ethanol were purchased from Merck. 1-Hydroxybenzotriazole (HOBt), Diethyl ether, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), Sodium Phosphate monobasic anhydrous, Acetonitrile (ACN), Ethylene diamine tetra acetic acid (EDTA) and Piperidine were obtained from SRL laboratories. POPC (1- pamlitoyl - 2 - oleoyl - sn - glycerol- 3- phosphocholine, POPG (1- pamlitoyl- 2- oleoyl - sn-glycerol- 3- glycerol), N, N-Diisopropylethylamine (DIPEA), Thioanisole, 1, 2 Ethane dithiol (EDT), Trifluoroacetic acid (TFA), 3 - (4, 5-dimethylthiazol - 2 - yl) -2, 5-diphenyltetrazolium bromide (MTT), were purchased from Sigma-Aldrich. Nutrient Broth and Agar were purchased from Himedia laboratories. All other reagents used were of the highest purity (≥ 99%).

### 4.2.2. Methodology

#### 4.2.2.1. Modelling and simulation

The peptide molecules used in this study were designed by an in-house software PDB make and a modified version of Ribosome software from G.D. Rose laboratory.

Apart from these, softwares like PyMol, Swiss PDB viewer were used for visualization of (Protein Data Base)PDB files [7, 167, 226]. The backbone integrity of test peptide candidates was examined by Molecular Dynamics (MD) simulation analysis, using GROMACS program suite [228]. GROMACS was used with GROMOS 96 43a1 force field [229] for carrying out MD simulations. The structural stability of the designed peptides was studied at 300 K under standard NVT conditions. The structures were energy minimized by steepest descent algorithm in vacuum in 2000 steps in a simulation box with the size relatively 1.5 nm distant from the peptide in three dimensions. Solvent molecules (SPC, water) were then added and another energy minimization procedure was carried out in water prior to the simulation run. The 10 ns production runs for each peptide were done with an integration step of 2 fs. LINCS algorithm with a geometric accuracy of $10^{-4}$ was used as the bond length constraint. Maxwell distribution was used for initial velocity calculations with 0.1 ps of coupling relaxation step at 300 K. The non-bonded interaction cut off was set at 0.8 nm to 1.1 nm.

#### 4.2.2.2. Electrostatic profiling

Electrostatic potential was calculated at every position by solving Finite Difference Poisson Boltzmann (FDPB) equation, which was summed up for every residue side-chain, represented collectively at the chromophoric center of the side-chain. The hetero atoms of cysteine (S), lysine (K) and methionine (M), C-alpha of glycine and C-zeta of arginine are taken as respective chromophoric centers for electrostatic profiling. On the other hand, C-beta of alanine, valine, isoleucine, serine, and threonine along with C-gamma of asparagine, aspartic acid, leucine, proline, histidine, tyrosine, phenylalanine, and tryptophan are their respective chromophoric centers. Electrostatic profiling was next accomplished by frame-wise comparison of electrostatic potential and structure similarity [7]. The profiles were then plotted in a three dimensional graph using MATLAB

#### 4.2.2.3. Peptide synthesis and characterization

In our study, the peptides were synthesized using standard solid phase peptides synthesis procedure (Fmoc chemistry) [7]. Overnight soaked resin in DMF was treated with 20 % piperidine for a period of 20 minutes to remove Fmoc group. Following that, the resin was washed with DMF to attain neutral pH. An activated amino acid mixture comprised of amino acid, three-fold excess of HBTU, HOBT and six-fold excess of DIPEA was added to resin for amino acid coupling. This process was performed for two cycles of one hour and half hour separately. The cycle of Fmoc removal and amino acid attachment was carried for the entire sequence followed by final Fmoc removal. Post synthesis, the peptides were cleaved from the resin using a cleavage cocktail (m-Cresol: Thioanisole: EDT: TFA :: 2:2:1:20) for 12 hours in dark followed by precipitation in Diethyl ether (ice cold).

The peptides were purified by means of semi-preparative reverse-phase liquid chromatography. A chromatographic run of 10% ACN to 100% ACN with 0.1% TFA, was used for the gradient elution of peptides at a flow rate of 0.5 - 1 mL/min. The eluents were monitored at 210 nm and verified by electrospray ionization mass spectrometry (ESI-MS) and Matrix assisted laser desorption ionization (MALDI) mass spectroscopy [7, 230].

#### 4.2.2.4. Anti-bacterial assay

In our study, mid logarithmic phase bacterial cells were washed and re-suspended in sodium phosphate buffer (10 mM, pH 7.4). The resulting suspension was diluted to a net absorbance value of 0.2 at 600 nm and 50µL of the inoculum was treated with required concentrations of peptide (Net incubation broth is 100 µL), followed by 2 hours of incubation. Post incubation, 100 µL of MTT solution (0.5 mg/mL) was added to the broth and incubated for four hours at 37ºC. Subsequently, the reaction broth was centrifuged and the bacterial pellets were mixed with DMSO. The difference in the absorbance at 570 nm and 660 nm was calculated and percent bacterial cell lysis was reported, relative to untreated bacterial cells [227, 231, 232]. A relative inhibition of 80 % growth with reference to growth control was considered

to be as Minimal Inhibitory Concentration, as per relative reported standard procedure [233].

#### 4.2.2.5. Field Emission-Scanning Electron Microscopy (FE-SEM)

The FE-SEM analysis of bactericidal activity of peptide is a qualitative estimation providing a topographic interpretation of higher magnification, after peptide activity upon bacterial species. For this experiment, the microscopic analysis protocol was similar to antibacterial assay until peptide-bacteria incubation (section 4.2.5). Post incubation the bacterial suspension was mixed with glutaraldehyde up to a net concentration of 4%, followed by incubation at 4°C for 30 minutes. After that, the mixture was washed with buffer and mounted upon a glass slide. The bacterial cells were allowed to attach to the glass surface over a period of 30 minutes. Later, samples were subjected to gradient wash with 30% to 100% alcohol. The samples were dried at room temperature and coated with gold prior microscopy. The membrane active property of peptides was concluded after the comparative study of images obtained from peptide treated and untreated bacterial samples [7, 227, 234].

### 4.2.6. Hemolytic activity

The hemolytic potential of peptides was examined by studying, peptide interaction with human Red Blood Cells (RBC). To conduct this experiment, blood from healthy individual was collected and mixed with 2mg / mL of Ethylene diamine tetra acetic acid (EDTA) to avoid coagulation. Later the blood was washed twice with 5 mM [(4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid, N-(2-Hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid)] HEPES buffer saline and 50 % cell suspension was prepared in the same buffer. 50 µL of the cellular suspensions were mixed with required concentration of peptides and incubated for 2 hours at 37±2 °C. RBC in water was considered as 100 % lysis whereas RBC in buffer as 0% lysis, which was

latter compared with peptide treated RBC's for estimation of hemolytic potential of peptides [7, 227, 235, 236].

## 4.3 Results and Discussion

**4.3.1 Design philosophy:** We designed two pairs of syndiotactic sequences mimicking Gramicidin 6.3 helix as model systems, with a starting (ϕ, ψ) dihedral angle pairs as shown in Table 4.1 Gramicidin has a right handed helical structure with (i to i+7) and (i to i-5) hydrogen bonding pattern [152], and a pitch 4.7 Å [237], with 6.3 residues per turn [161]. Theoretically such helical structures can assume two structural variants depending on their 'handedness'; either right or left handed (Figure 4.1).

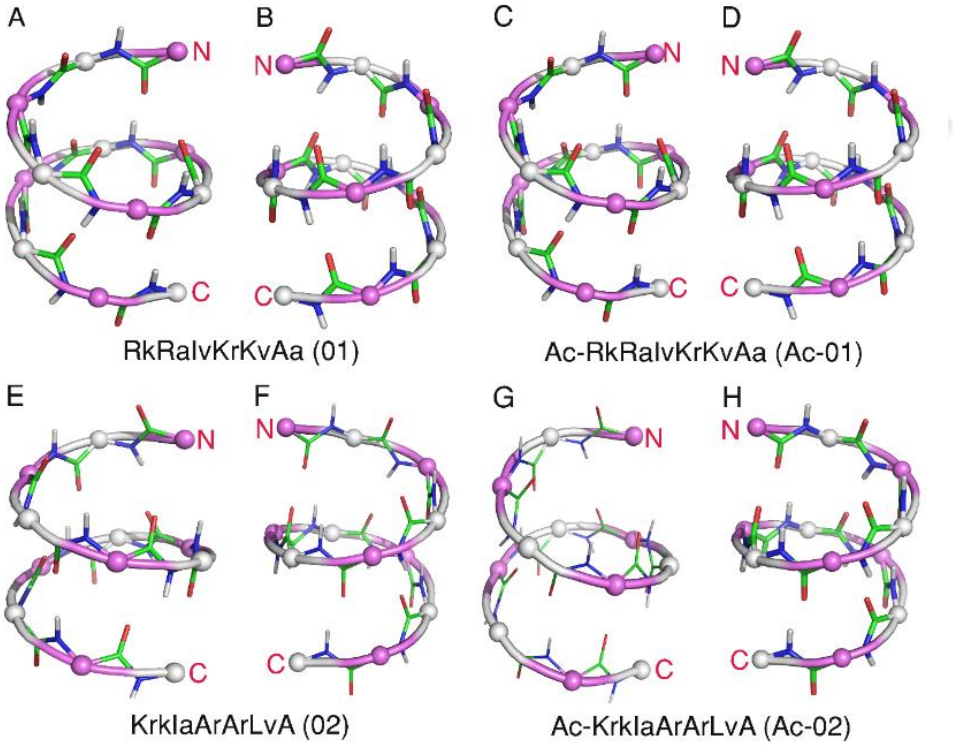



**Figure 4.1.** Backbone structures of the designed hetero-chiral peptides having syndiotactic stereochemistry. All the peptides can either be in left or right handed orientation, resulting in a maximum of eight theoretical backbone combinations possible for four peptides. A (01LH) and B (01RH) are the left handed and right handed back bone of sequence 01, whereas C (01Ac-LH) and D (01Ac-RH) are the left and right handed backbone of 01Ac. Likewise, E (02LH) and F (02RH) are left handed and right handed back bone of sequence 02 along with G (02Ac-LH) and H (02Ac-RH) are left and right handed version of 02Ac.

Therefore, we have designed two variants right handed (RH) and left handed (LH) for each sequence resulting in eight possible variants, though only four variants are synthetically possible. The designed molecules are both cationic and amphipathic, which may play an important role in membrane lysis mediated by electrostatic interactions. Electrostatic potential maps of the designed peptides were generated by solving FDPBE using Delphi software. The topology resulting from the side-chain orientation and the electrostatic potential environment it creates, were visibly distinct in all four models (Figure 4.2). Precisely, the designed molecules display distinct topological and electrostatic fingerprint, which may get translated to their antibacterial potency.

**Table 4.1.** Designed peptides and their sequences. The sequences with N-terminal acetylation have been denoted with a prefix Ac. The dihedral angles used for the peptide sequences has been shown in details. Minimum Inhibitory Concentration of the designed peptides against *S.aureus* and *P.aeruginosa* shows preferential activity of syndiotactic sequences against Gram positive bacteria. The letters in the lower case represents D amino acid.

| Code | Sequence | Handedness | Dihedral angles (L - amino acid) | Dihedral angles (D - amino acid) | Molecular mass (Dalton) | MIC (μM) | |
|---|---|---|---|---|---|---|---|
| | | | | | | *S. aureus* | *P. aeruginosa* |
| **01** | RkRaIvKrKvAa | Right | -120, 140 (ϕ, ψ) | 120, -110 (ϕ, ψ) | 1394.76 | 25 | > 100 |
| | | Left | -110, 120 (ϕ, ψ) | 110, -150 (ϕ, ψ) | | | |
| **01 Ac** | Ac-RkRaIvKrKvAa | Right | -120, 140 (ϕ, ψ) | 120, -110 (ϕ, ψ) | 1436.8 | 25 | > 100 |
| | | Left | -110, 120 (ϕ, ψ) | 110, -150 (ϕ, ψ) | | | |
| **02** | kRkIaArArLvA | Right | -120, 140 (ϕ, ψ) | 120, -110 (ϕ, ψ) | 1351.69 | 12.5 | > 100 |
| | | Left | -110, 120 (ϕ, ψ) | 110, -150 (ϕ, ψ) | | | |
| **02 Ac** | Ac-kRkIaArArLvA | Right | -120, 140 (ϕ, ψ) | 120, -110 (ϕ, ψ) | 1393.73 | 12.5 | > 100 |
| | | Left | -110, 120 (ϕ, ψ) | 110, -150 (ϕ, ψ) | | | |

The stereochemistry and sequences of 01 and 01-Ac pair and 02 and 02-Ac pair are the same, except that 01-Ac and 02-Ac being N-terminal acetylated (Table 4.1). To verify the stability of the designed peptides in a 6.3 helical conformation, similar to gramicidin helix, a 10 ns MD run at 298 K under NVT condition using GROMOS 96

force field, was performed for each structural variant. The cluster analysis of the theoretical models suggest that the structures are quite stable with the percent sampling of most populous structure being more than 95 % in all eight models (Figure 4.3 and Table 4.2). Further, the structural integrity of the molecules was verified by measuring Root Mean Square Deviation (RMSD) from the starting structure (Figure 4.4 A). Radius of Gyration has been measured to verify whether the designed structures remain folded throughout the simulation (Figure 4.4 B). Radius of Gyration has been measured to verify whether the designed structures remain folded throughout the simulation (Figure 4.4 B).

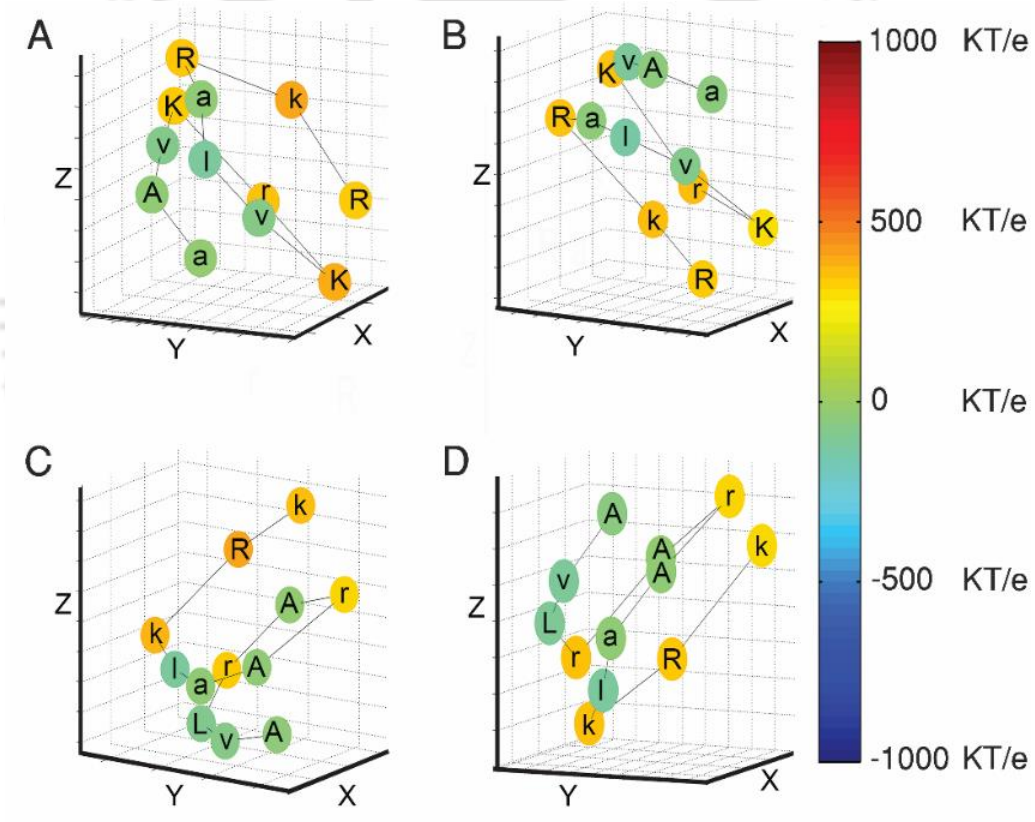



**Figure 4.2.** Electrostatic potential map of the designed peptides, using Delphi Software solving Finite Difference Poisson Boltzmann equation. A, B are the potential maps of left and right handed conformations of the peptide 01. Similarly, C, D are the potential maps of the left handed and right handed peptide 02 respectively. Though two conformations are theoretically possible, only one sequence can be experimentally verified. Differential color coding of amino acid residues, points to their electrostatic potential with reference to the color bar. The color map shows the potential distribution in KT/e units.

Detailed examination of average structure forming largest cluster, however shows that hydrogen bonding pattern has been changed during the course of MD simulations (Figure 4.5 and 4.6, Table 4.3). In our earlier studies, we have shown that gramicidin 6.3 helical structures with alternate L and D stereo-chemical sequence have their short-range and long range backbone electrostatic interactions complementing each other, while in poly L sequences they are in conflict [238-240].

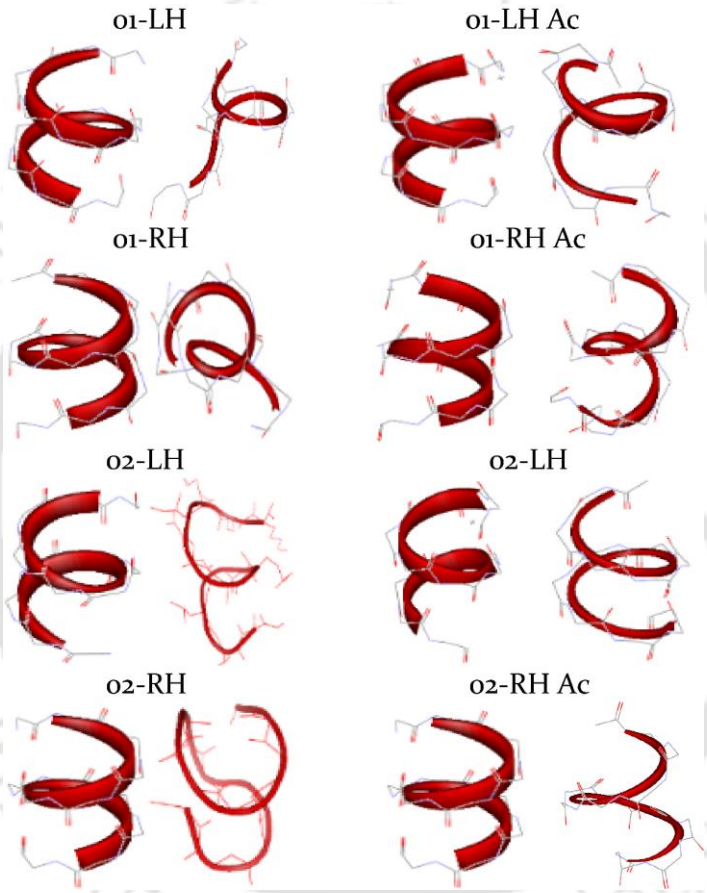



**Figure 4.3.** Starting and end structure structure of designed peptide derived from MD simulation.

Detailed examination of average structure forming largest cluster, however shows that hydrogen bonding pattern has been changed during the course of MD simulations (Figure 4.5 and 4.6, Table 4.3). In our earlier studies, we have shown that gramicidin 6.3 helical structures with alternate L and D stereo-chemical sequence have their short-range and long range backbone electrostatic interactions complementing each other, while in poly L sequences they are in conflict [238-240]. The

resultant modification of energy landscape leading to extra stability due to complimentary local electrostatics is the key for stereo-chemical engineering of peptide sequences. We have further shown that countervailing short range – long range interaction holds the key for side-chain sequences dictating the overall conformation of a given polypeptide sequence.

**Table 4.2.** Number of clusters and percentage occurrence of all the clusters for peptide 01, 01Ac, 02 and 02Ac, obtained from cluster analysis of MD simulation data using GROMACS programme suite. The codes LH and RH denote left and right handed helical structure of all combinatorial possibilities of 4 designed peptide sequences.

| Peptide code | Number of clusters | Size of most populous cluster (% of total structures) |
|---|---|---|
| 01LH | 2 | 99.4 |
| 01RH | 9 | 95.9 |
| 01Ac-LH | 1 | 100 |
| 01Ac-RH | 2 | 99.9 |
| 02LH | 2 | 98.8 |
| 02RH | 1 | 100 |
| 02Ac-LH | 1 | 100 |
| 02Ac-RH | 3 | 99.8 |

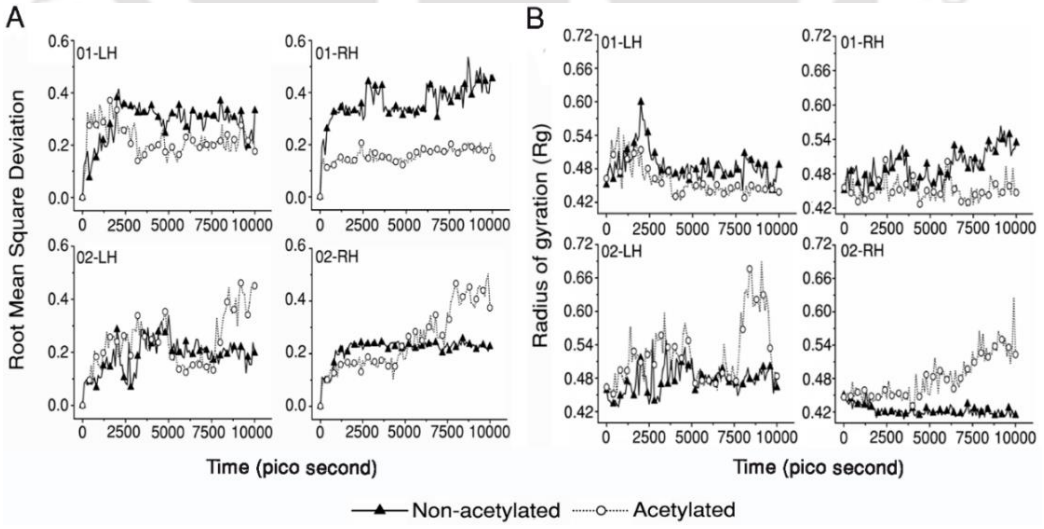



**Figure 4.4**. A) RMSD (Root Mean Square Deviation) as a function of time of all the designed peptides from MD simulation in a 10 nanosecond (ns) production run, B) Rg (Radius of Gyration) as a function of time indicating the overall folded nature of the designed peptides.

Since local electrostatics of gramicidin helical backbone is complimenting and stable, the fold is much more adaptable to varied sequence solutions [152].

| 01LH | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | | Y | |
| 3 | | | | | | | | | Y | | | | | |
| 4 | | | | | | | | | | | | | | |
| 5 | | | | | | | | | | | Y | | | |
| 6 | | | | | | | | | | | | | | |
| 7 | | | | | | | | | | | | | Y | |
| 8 | | | | | | | | | | | | | | |
| 9 | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | |

| 01LH-Ac | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | |
| 2 | | | | | | | | Y | | | | | | |
| 3 | | | | | | | | | | | | | | |
| 4 | | | | | | | | | | Y | | | | |
| 5 | | | | | | | | | | | | | | |
| 6 | | | | | | | | | | | | Y | | |
| 7 | | | | | | | | | | | | | | |
| 8 | | | | | | | | | | | | | | Y |
| 9 | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | |

| 01RH | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | |
| 2 | | | | | | | | Y | | | | | | |
| 3 | | | | | | | | | | | | | | |
| 4 | | | | | | | | | | Y | | | | |
| 5 | | | | | | | | | | | | | | |
| 6 | | | | | | | | | | | | Y | | |
| 7 | | | | | | | | | | | | | | |
| 8 | | | | | | | | | | | | | | |
| 9 | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | |

| 01RH-Ac | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | | | |
| 3 | | | | | | | | | Y | | | | | |
| 4 | | | | | | | | | | | | | | |
| 5 | | | | | | | | | | | Y | | | |
| 6 | | | | | | | | | | | | | | |
| 7 | | | | | | | | | | | | | Y | |
| 8 | | | | | | | | | | | | | | |
| 9 | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | |

| 02LH | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | |
| 2 | | | | | | | | Y | | | | | | |
| 3 | | | | | | | | | | | | | | |
| 4 | | | | | | | | | | Y | | | | |
| 5 | | | | | | | | | | | | | | |
| 6 | | | | | | | | | | | | Y | | |
| 7 | | | | | | | | | | | | | | |
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| 9 | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | |

| 02LH-Ac | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | |
| 2 | | | | | | | | | | | | | | |
| 3 | | | | | | | | | Y | | | | | |
| 4 | | | | | | | | | | | | | | |
| 5 | | | | | | | | | | | Y | | | |
| 6 | | | | | | | | | | | | | | |
| 7 | | | | | | | | | | | | | Y | |
| 8 | | | | | | | | | | | | | | |
| 9 | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | |

| 02RH | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | | | | | | | | | | | | | | |
| 1 | | | | | | | Y | | | | | | | |
| 2 | | | | | | | | | | | | | | |
| 3 | | | | | | | | | Y | | | | | |
| 4 | | | | | | | | | | | | | | |
| 5 | | | | | | | | | | | Y | | | |
| 6 | | | | | | | | | | | | | | |
| 7 | | | | | | | | | | | | | Y | |
| 8 | | | | | | | | | | | | | | |
| 9 | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | |

| 02RH-Ac | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | | | | | | | | | | | | | | |
| 1 | | | | | | | | | | | | | | |
| 2 | | | | | | | | Y | | | | | | |
| 3 | | | | | | | | | | | | | | |
| 4 | | | | | | | | | | Y | | | | |
| 5 | | | | | | | | | | | | | | |
| 6 | | | | | | | | | | | | Y | | |
| 7 | | | | | | | | | | | | | | |
| 8 | | | | | | | | | | | | | | Y |
| 9 | | | | | | | | | | | | | | |
| 10 | | | | | | | | | | | | | | |
| 11 | | | | | | | | | | | | | | |
| 12 | | | | | | | | | | | | | | |
| 13 | | | | | | | | | | | | | | |

**Figure 4.5.** Matrix of Hydrogen bonding pattern of designed PDB files. The bonding pattern between N to O has been shown by "Y" in the structures.

Our MD results on all eight variants support earlier observations and this design strategy. Since amphipathicity and cationicity has been the hallmark of antibacterial activity of natural and designed peptides, we have designed all

peptides retaining amphipathicity, at the same time presenting differential electrostatic potential arising from cationic side-chains.

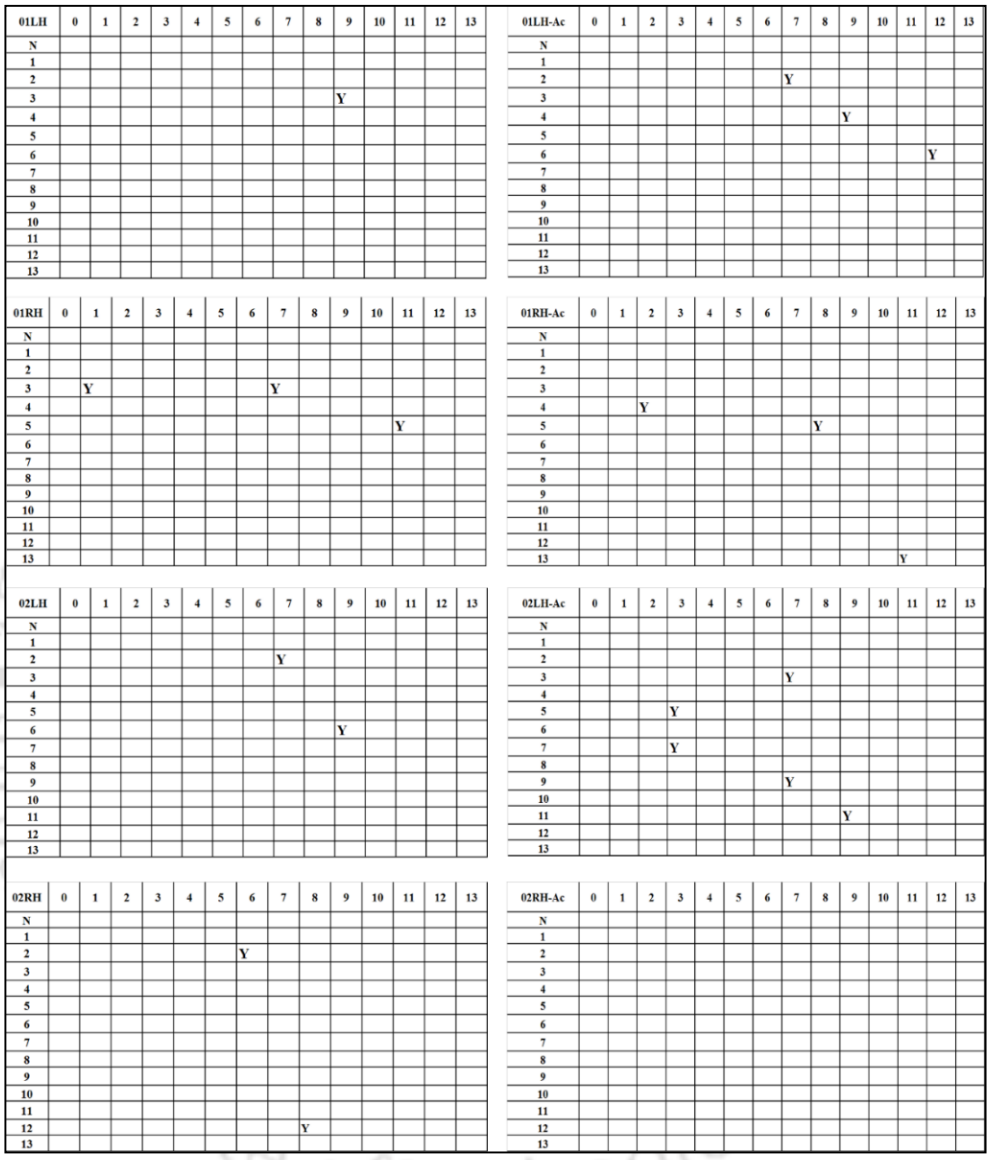


**Figure 4.6.** Matrix of Hydrogen bonding pattern of mean structure of largest cluster from MD simulations. The bonding pattern between N to O has been shown by "Y" in the structures.

**4.3.2 Antibacterial assay and toxicity studies:** The in vitro antimicrobial activity of the synthesized peptides was tested against Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria. All the peptides were showing better activity against Gram-positive bacterium, in comparison with Gram-negative bacteria (Figure 4.7, Table 4.1).

This observation is consistent with the earlier reports on gramicidin. Gramicidin has a reported MIC value of 2.2 μM [241] against *S.aureus* and is inactive against Gram-negative bacteria. Our peptides show similar trend, though the sequence similarity

**Table 4.3.** Hydrogen bond registry of designed peptide and most populous structure.

| Peptide | Designed structure (H-bond registry from N atom to O atom ) | Mean structure of largest cluster (H-bond registry) |
|---|---|---|
| **01LH** | 1(N) to 6(O)<br>3(N) to 8(O)<br>5(N) to 10(O)<br>7(N) to 12(O) | 3(N) to 9(O) |
| **01LH-Ac** | 2(N) to 7(O)<br>4(N) to 9(O)<br>6(N) to 11(O)<br>8(N) to 13(O) | 2(N) to 7(O)<br>4(N) to 9(O)<br>6(N) to 12(O) |
| **01RH** | 2(N) to 7(O)<br>4(N) to 9(O)<br>6(N) to 11(O) | 3(N) to 1(O)<br>3(N) to 7(O)<br>5(N) to 11(O) |
| **01RH-Ac** | 3(N) to 8(O)<br>5(N) to 10(O)<br>7(N) to 12(O) | 4(N) to 2(O)<br>5(N) to 8(O)<br>13(N) to 11(O) |
| **02LH** | 2(N) to 7(O)<br>4(N) to 9(O)<br>6(N) to 11(O) | 2(N) to 7(O)<br>6(N) to 9(O) |
| **02LH-AC** | 3(N) to 8(O)<br>5(N) to 10(O)<br>7(N) to 12(O) | 3(N) to 7(O)<br>5(N) to 3(O)<br>7(N) to 3(O)<br>9(N) to 7(O)<br>11(N) to 9(O) |
| **02RH** | 1(N) to 6 (O)<br>3(N) to 8(O)<br>5(N) to 10(O)<br>7(N) to 12(O) | 2(N) to 6(O)<br>12(N) to 8(O) |
| **02RH-AC** | 2(N) to 7(O)<br>4(N) to 9(O)<br>6(N) to 11(O)<br>8(N) to 13(O) | NA |

between gramicidin and the designed peptides are minimal. Our designed peptides primarily rely on cationicity to induce membranolytic activity. Acetylation of synthesized peptides is aimed at lowering the net positive charge by blocking the free N-terminus. We, however also could not find any notable difference in antimicrobial potency between acetylated and non-acetylated peptide variants.

**4.3.3 Hemolytic activity:** Hemolytic assay against mammalian RBC was performed to estimate the extent of toxicity. All four peptides exhibited negligible cytotoxicity against mammalian cells at 100 μM concentration following standard protocols.

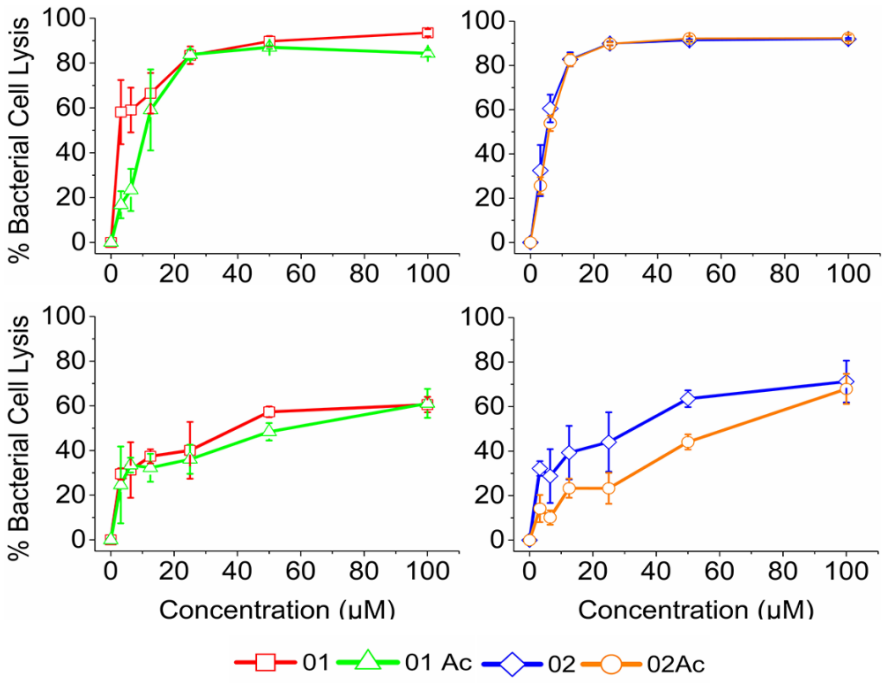


**Figure 4.7.** Bactericidal potency of the peptides 01 and 02 along with their acetylated variants against *Staphylococcus aureus* (Gram-positive) and *Pseudomonas aeruginosa* (Gram-negative) bacteria. A and B show bactericidal activity against Gram-positive bacteria, whereas C and D show activity against Gram-negative bacterial species.

Gramicidin has a reported toxicity estimate of 50 % hemolysis at 5 µM concentration [214]. Unlike gramicidin, toxicity levels of all four reported peptides are well within the accepted safe threshold of 2% lysis at a very high concentration of 100 µM; and three out of the four peptide variants are showing a value less than 1% (Table 4.3).

**4.3.4 Microscopic analysis:** A qualitative evaluation of membrane rupturing potential of designed antimicrobial peptide against bacteria was performed using FE-SEM analysis. The cationic peptides caused significant membrane damage which was inferred after a comparative analysis between peptide treated (ruptured) and untreated (intact) bacterial cells.

**Table 4.3.** Hemolytic assay of designed peptides against mammalian Red Blood Cells (RBC) at 100 µM peptide concentration.

| S No | Peptide code | % RBC lysis | Standard Deviation |
|---|---|---|---|
| 1 | 01 | 0.5 | 0.02 |
| 2 | 01 Ac | 2 | 0.05 |
| 3 | 02 | 0.7 | 0.03 |
| 4 | 02 Ac | 0.09 | 0.02 |

The bacterial cells were treated with 100 µM peptide resulting in deformed bacterial membrane architecture. (Figure 3.4).

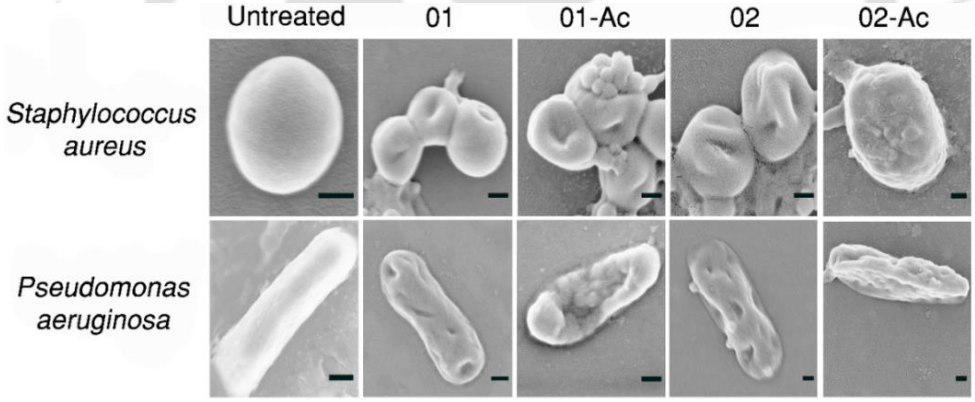


**Figure 4.8.** FE-SEM images of Gram-positive and Gram-negative bacteria, treated with individual peptides, after two hours of incubation. Comparison of untreated Staphylococcus aureus (Gram-positive) and Pseudomonas aeruginosa (Gram-negative) cells against ruptured bacterial membrane treated with 01, 01 Ac,02 and 02 Ac at 100 µM concentration. The scale bars correspond to 200 nm.

Qualitative pictures indicate that the membrane rupturing ability of the amphipathic cationic peptides are responsible for the desired activity profile of the designed peptides, though detailed mechanism investigation is beyond the scope of this study.

## 4.4 Conclusion

Almost all natural proteins are isotactic with chain stereochemistry (poly L). An isotactic peptide molecule roughly acquires 21 % allowed conformational space in Ramachandran plot. If the stereochemical sequence is a variable, it increases the designable space manifold, resulting in an informed walk across Ramachandran ϕ, ψ space. We employed a peptido-mimetic approach, modeling around Gramicidin adopting the simplest stereo-chemical sequence with syndiotactic stereochemistry. Gramicidin is a natural antibiotic peptide with Syndiotactic backbone (alternating L and D) having good bactericidal activity against Gram-positive bacteria, but could only be used topically because of very high levels of toxicity. We attempt to re-design the amino acid sequence (side-chain sequence) space retaining its stereo-chemical (main-chain) sequence, with an objective to minimize its toxicity, while retaining antimicrobial activity. The two designed cationic amphipathic peptides with alternating L and D chiral amino acid sequences, and their acetylated variants exhibit negligible levels of toxicity, well within the acceptable limits. Our best peptide sequence is showing an MIC value of 12.5 μM against a reported value of 2.2 μM [241] for Gramicidin D. Importantly, this design strategy offers an effective means to develop possible sequence variation in future designs. Incorporation of stereochemistry as an additional design variable significantly expands the design space of a peptide sequence. In a previous study [7], we have systematically varied stereo-chemistry of the designed peptides resulting in different structure and electrostatic interaction profiles while they approach a target. However, our efforts to directly relate the structure and electrostatic profiles in defining activity and toxicity of a given peptide, is not yet conclusive.

Role of acetylation in naturally occurring proteins has not been precisely deduced but studies indicate that it improves the potency of peptides, as it mimics the native protein itself [242]. As indicated in literature, it enhances the overall potency, metabolic stability and resistance against enzymatic degradation [165, 243]. In our study, we could not find any significant difference in activity or toxicity profiles of our designed peptides, which was also an objective of this investigation.

Apart from increasing the overall design space considerably, incorporation of D amino acids effectively negates the possibility of proteolytic degradation. This point to the potential utility of de novo designed peptides with diversified stereochemistry as a promising new approach in the generation of antibiotic peptides. Such attempts are important to effectively manage the broad antibiotic spectrum and pharmacological profile with increased instances of antibiotic resistance reported in recent times.

# Chapter 5

## Effect of tacticity-derived topological constraints in bactericidal peptides

## 5.1 Introduction

Topology is a key element in structure-activity relationship. Topology, also plays a key role in the geometrical evolution of a typical protein fold [9-12]. Topological variation in a molecule with identical chemical structure can be provided by differences in their stereochemistry [8]. Ever since the historical discovery by Louis Pasteur in 1847, stereochemical variations and their possibilities are abundantly utilized while designing molecules at atomistic scale. Despite being the principal functional molecule in a living system, nature did not adopt stereochemistry as a variable in protein design [6].

All proteins are isotactic polymeric molecule, exclusively with L-chiral amino acids, as monomeric units. Tacticity refers to the relative stereochemistry of successive stereocenters in a polymeric macromolecule. If any given polymer chain has all stereocenters of exclusively L-(S) or D-(R) configurational-type, they are referred to as 'isotactic' polymers. Syndiotactic molecules have stereoregular L- and D-stereocenters in alternate succession, and if the stereocenters are randomly distributed in a stereoirregular fashion, it is 'heterotactic' [224].

Approximately 7000 naturally-occurring peptides with potential physiological effects have been discovered so far, which include hormones, neurotransmitters, growth factors, ion channel ligands, etc. High selectivity and attractive pharmacological profile qualifies them to be identified as potential therapeutic agents, with 140 different peptide molecules currently being evaluated in clinical trials [244, 245]. Almost all therapeutic peptides are made of asymmetric amino acids, with L-chiral stereochemical structure, thus identified as stereoregular 'isotactic' hetero-polymer [246-248]. This stereo-specificity points to the possibility of diversifying the basic alphabet of amino acid sequence by including D-enantiomers [156]. With addition of D amino acids at each sequence positions, number of stereoisomeric diversity increases exponentially from $1^N$ to $2^N$, where N is the number of amino acids in the sequence; for a 12 amino acid long peptide, this number would be from 1 ($1^{12}$) to 4096 ($2^{12}$).

Peptides and protein structures are not only sensitive to mutations in their own sequences, but also to even minimal perturbations in their environment. Earlier reports suggest that the stereochemical mutation of polypeptide sequences can transform the structure from a condition of extreme sensitivity to extreme insensitivity to its environment [152, 238]. A pioneering analysis along this line was done by Brant and Flory, where they calculated the limiting values of the characteristic ratio for random copolymers of L-Ala and Gly and L and D-Ala [249]. Characteristic ratio corresponds to the chain stiffness of a polymer chain. For a typical Poly isotactic peptide chain, this value is between 9.0 and 9.5, whereas for poly (Gly), it reduces to 2.0, quite similar to typical polymeric chains, owing to the lack of stereocenter in Glycine [250]. In an interesting experiment, Flory further calculated the characteristic ratios in random L-Alanine, D-Alanine copolymers for different mole ratios. He found a significant reduction in characteristic ratio to a value of 2 for 50% mole ratio of L- and D- enantiomers.

Characteristic ratio progressively increased upon increased mole ratio of one enantiomer; with both Poly-L chiral and Poly-D chiral isotactic isomers attain a value of 9.0. This nearly four and a half times reduction was proposed to be a consequence of the "harmonious" nature of short and intermediate range electrostatics, an observation later corroborated by follow up investigations with chiral engineering of polypeptide sequences [251]. Chiral engineering was first utilized effectively by Ghadiri and coworkers, while designing nano-level molecular assemblies with D- and L-alpha-amino acids adopting ring-shaped conformations forming hollow tubular structures [252]. This was followed by a series of de novo designed artificial peptide constructs of diversified tacticity incorporating L- and D-amino acids resulting in unusually-stable structures.

Peptide based molecular constructs with adaptive modulation for targeted functions have been identified as a futuristic option while designing antimicrobial agents, protein transduction domains and tumor homing molecular devices [253-255]. Effective interaction with cell membrane is central in all these molecules for its functional fulfillment. Antimicrobial peptide database (APD) with 2687 molecular

entries [256], Tumor homing peptide (TumorHoPe) with 744 [257] and cell penetrating peptide database (CPPSite) with 1700 unique sequences [258] deposited and validated against various cell, pathogen and tumor types underlines their potential utility. Despite the above described variations it can potentially generate, option for steriochemically-diversified peptide sequences remain largely unexplored. Ghadiri and co-workers have also made use of cyclic D, L-alpha-peptide framework for designing broad spectrum antibacterial and antiviral agents [259, 260]. Metzler-Nolte and coworkers have reported 4-8 fold decrease in MIC values by adopting specific combination of L and D amino acids [261]. Diversity of biological membranes and adaptation by antibiotic resistant bacterial species, such as gentamicin-methicillin-resistant *Staphylococcus aureus* (gentamicin-resistant MRSA) demands a systematic approach with molecular models resulting in key design directives for future molecular generation and development. In this work, we investigate the physiological effects of stereochemical mutation, manifested through the topological variations it offers, by systematically designing a possible antimicrobial sequence.

Structure-activity studies of peptide-based antimicrobial agents suggest incorporation of two key design elements; a cationic charge [262] and an induced amphipathic conformation [77, 263]. Chain stereochemistry has not been a design element in such investigations, with few exceptions [13, 14, 176, 259-261, 264-278]. In this study we have retained the amino acid content and consequently their hydropathy index, but experimented with their chain stereochemistry to generate different topology. Conformational locking or topological fixation have never been a prime concern for antimicrobial peptide design and probably because of the lack of a consensus mechanistic model and large number of conformational variations a peptide sequence can adopt while they encounter a membrane. Imposing a constraint on peptide conformational flexibility by cyclization of peptide chain was reported to be a successful strategy to improve broad spectrum activity and also specifically effective against resistant species [13, 14].

Through this study, we aim to investigate the following objectives; (i) utility of peptide molecular systems with diversified tacticity as antimicrobial agents and (ii) effect of topological variation and subsequent chain stiffness and resultant restrictions reflected on the antimicrobial activity. We investigate these two fundamental questions by measuring the relative efficacy of stereochemical cum topological variants of designed peptides against Gram-positive, Gram-negative and antibiotic-resistant bacteria, with constant amphipathicity and cationicity as design elements [263]. We observed that systematic design of heterotactic backbone induce topological restrictions, addressing the issue of non-specific distribution and consequent toxicity [265].

## 5.2 Materials and methods

Modelling and simulation, Electrostatic profiling, Peptide synthesis and characterization, Field Emission-Scanning Electron Microscopy (FE-SEM) and Hemolytic activities were performed as explained in Chapter 4 section 4.2.

### 5.2.1 Peptide-lipid interaction

Monolayer studies or Langmuir films have been often used to imitate bacterial membranes. To perform this experiment, test peptide at saturated concentration in buffer (10 mM, pH 7.4) is monitored in terms of its interaction with model phospholipid monolayers. The magnitude of interaction is measured with respect to the change in surface pressure (mN $m^{-1}$) of the system.

*Estimation of saturated peptide concentration*: Surface pressure was monitored by the Wilhelmy method, using a paper plate and a custom made polytetrafluoroethylene (PTFE) Langmuir trough (volume approximately 15 cm3) carried out on a Langmuir instrument (Biolin Scientific, UK). Peptides were injected into 10 mL sub-phase (10 mM phosphate buffer, pH 7.4) and changes in surface pressure was monitored until saturation was obtained. The objective of this study was to find the saturated peptide concentration in buffer, in order to accomplish further study of peptide - monolayer interaction [279-281].

*Peptide interaction with lipid monolayers*: Monolayers were prepared from phospholipid systems i.e. POPC and POPC: POPG :: 7:3 in chloroform solutions, which were spread onto

the air/buffer interface up to a final stable surface pressure of 30 ± 2 mN m-1. After evaporation of the chloroform and stabilization of the pressure, peptides with saturated concentration were steadily injected into the sub-phase (10 mM phosphate buffer, pH 7.4) via the vertical hole, in order to cause minimal disturbance to the lipid layer. To ensure homogenous distribution of peptide in the sub-phase, the peptide mixing was done using magnetic-stirrer. Peptide monolayer interactions were recorded as changes in monolayer surface pressure and the resultant data was plotted as change in surface pressure (mN $m^{-1}$) against time (minutes) [7, 279-281]

### 5.2.2 Anti-bacterial assay

In this methodology, overnight incubated bacterial suspension in nutrient broth (NB) were sub-cultured to mid-log phase, followed by washing with buffer (10 mM phosphate, pH 7.4). Later, the bacterial suspension was adjusted to a net OD of 0.02 and 50 µL of the same was mixed with peptides to make required concentrations, and the net reaction volume was adjusted to 100 µL with buffer if required. The peptide-bacterial broth was incubated for 2 hours at 37 ± 2 °C. Subsequently, the incubated broth was diluted to tenfold and 20 µL of diluted broth was spread on NB agar plate, followed by an incubation of 12 to 16 hours at 37 ± 2°C. The bactericidal potency of the peptides was calculated by comparison with untreated bacterial plates [7, 282].

## 5.3 Results and Discussion

### 5.3.1 Modeling, simulation and electrostatic profiling:

As described earlier, a 12 amino acid long polypeptide can have 4096 stereochemical variants and poly-L is only one among them. Since it is impossible to evaluate all 4096 stereochemical variants and its sequence variants, we opted to exhaustively evaluate the next possible variant of chiral variation; an alternating LDLD sequence. LDLD sequence naturally adopts a structure similar to gramicidin helix. Depending on the handedness (right or left) and sequence, eight LDLD variant sequences are theoretically possible (Figure 5.1). Handedness is a result of differential phi and psi angle combinations, a particular syndiotactic backbone adopts, while folding to a given structure. Since we don't have any control on the handedness of the synthesized peptide, out of the eight topological variants, only four can be

synthetically achieved; LDLD and DLDL stereochemical sequences with a given sequence and sequence reversed (Table 5.1).

**Table 5.1.** Amino acid sequence and stereochemical sequence of designed and synthesized model molecular systems 1 to 6 for biophysical studies and bactericidal activity. Number of clusters resulted from 10 ns Molecular Dynamics simulations, indicating the number of microstates at approximate equilibrium. Syndiotactic sequences are far more rigid; largely retain the designed topology, whereas poly-L sequence can adopt a range of degenerate conformational states. Small letter in the sequence indicates D-amino acid.

| Model System (MS) | Amino Acid Sequence | Stereochemical sequence | Number of clusters |
|---|---|---|---|
| **MS1** | KrKiFlRtKiLv | LDLDLDLDLDLD | 5 |
| **MS2** | kRkIfLrTkIlV | DLDLDLDLDLDL | 1 |
| **MS3** | vLiKtRlFiKrK | DLDLDLDLDLDL | 1 |
| **MS4** | VlIkTrLfIkRk | LDLDLDLDLDLD | 3 |
| **MS5** | KRKIFLRTKILV | LLLLLLLLLLL | 22 |
| **MS6** | VLIKTRLFIKRK | LLLLLLLLLLL | 74 |

More precisely, compared to model system 1 (MS1), model system 2 (MS2) has its stereochemistry reversed and model system 4 (MS4) is sequence-reversed, model system 3 (MS3) is both stereochemically- and sequence-reversed. Model systems 5 (MS5) and 6 (MS6) are poly L (isotactic) variants of MS1 and MS3 to estimate their activity against the bacterial species (Table 5.1). More precisely, compared to model system 1 (MS1), model system 2 (MS2) has its stereochemistry reversed and model system 4 (MS4) is sequence-reversed, model system 3 (MS3) is both stereochemically- and sequence-reversed. Model systems 5 (MS5) and 6 (MS6) are poly L (isotactic) variants of MS1 and MS3 to estimate their activity against the bacterial species (Table 5.1).

The relative difference between two molecular systems in any of these six peptides are different either in sequence or stereochemistry or both; with invariable cationicity and amphipathicity in order to investigate specific role that stereochemistry imparts, manifested through their topology during membrane

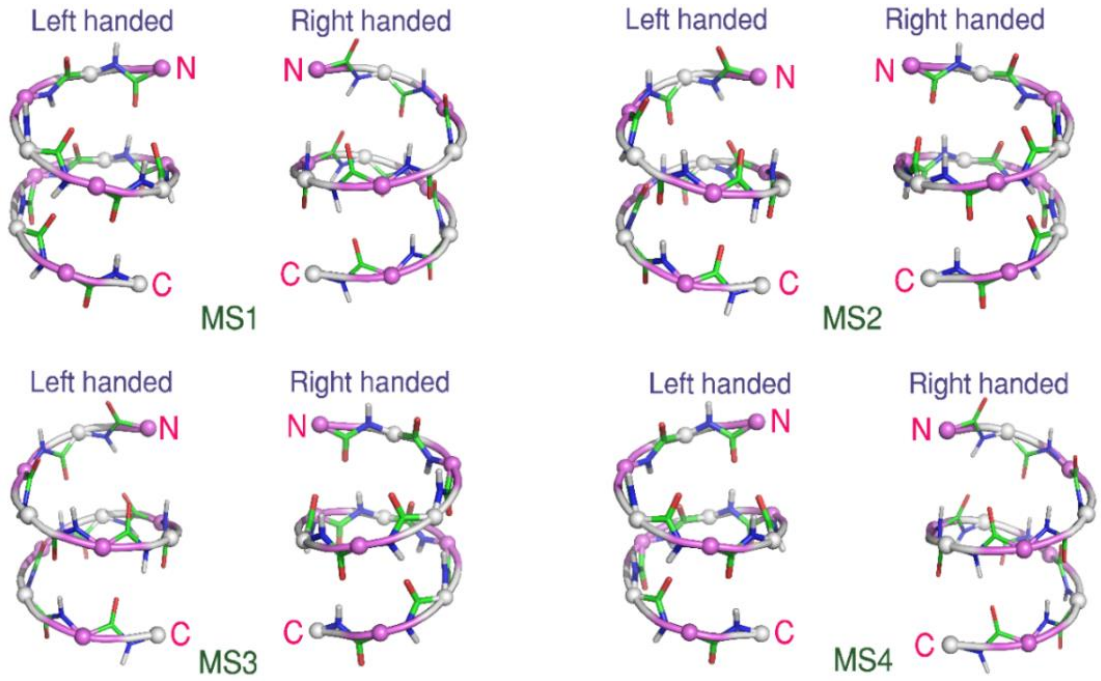


**Figure 5.1.** Possible backbone structures for syndiotactic peptides. The left and right handed conformations of designed syndiotactic peptides MS1 (A, B), MS3 (C, D), MS2 (E, F) and MS4 (G, H).

activity. Several models have been proposed to mechanistically explain membranolytic activity that principally arise from amphipathicity and cationicity of interacting molecule. Pore forming and non-pore forming mechanistic models, suggested for peptide-based membrane activity is essentially manifested through electrostatic interactions of amphipathic cationic peptide and its oppositely charged membrane counterpart. We use the finite difference Poisson-Boltzmann method as implemented in DelPhi [283, 284], to compute the electrostatic potential of all designed sequences on all theoretical possibilities of an alternating LDLD sequence, both in terms of its handedness and diastereomeric sequence. Electrostatic cum topology based molecular fingerprints of all eight variants, theoretically possible with our designed systems were examined (Figure 5.2).

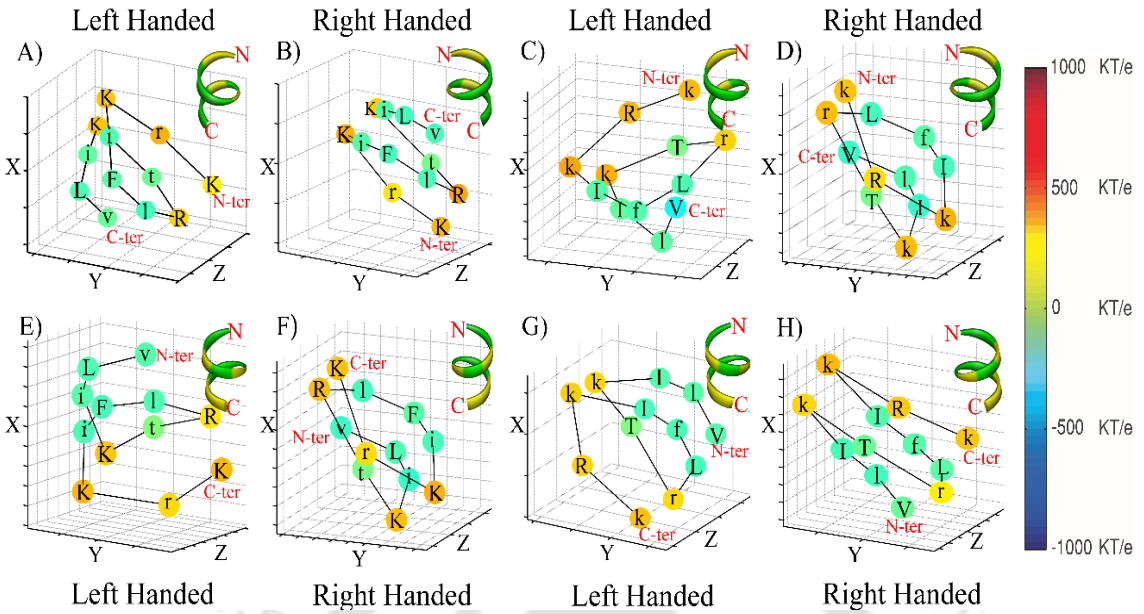


**Figure 5.2.** Electrostatic potential maps of left handed (LH) and right handed (RH) conformations of designed syndiotactic peptides respectively. MS1 (A, B) and MS2 (C, D) along with MS3 (E, F) and MS4 (G, H). Electrostatic potential was calculated by solving finite difference Poisson-Boltzmann equation using Delphi software, summed for each amino acid side-chain and represented collectively at the chromophoric center of the side-chain. The backbone corresponding to its electrostatic profile with amphiphilic character has been shown (color zone) at the top right of each electrostatic profile map. The N and C terminus for all the figures of electrostatic profile as well as backbone has been shown in this figure. The color scheme represents the distribution of potential in KT/e units.

The spatial disposition with charged and neutral groups in a peptide system was found to be distinct and unique in each case, while the syndiotactic polypeptide chain assumes its most stable gramicidin helical conformation. Since handedness in design cannot be implemented on synthetic sequences, our sample set will be reduced to four LDLD, DLDL, LDLD-sequence reversed and DLDL-sequence reversed. Their potencies are compared with both isotactic poly-L sequences thus forming a complete set of designed test systems to study the combined effect of topology and electrostatics of peptidic chromophoric groups on Gram-positive, Gram-negative and antibiotic resistant bacteria. Syndiotactic peptides are

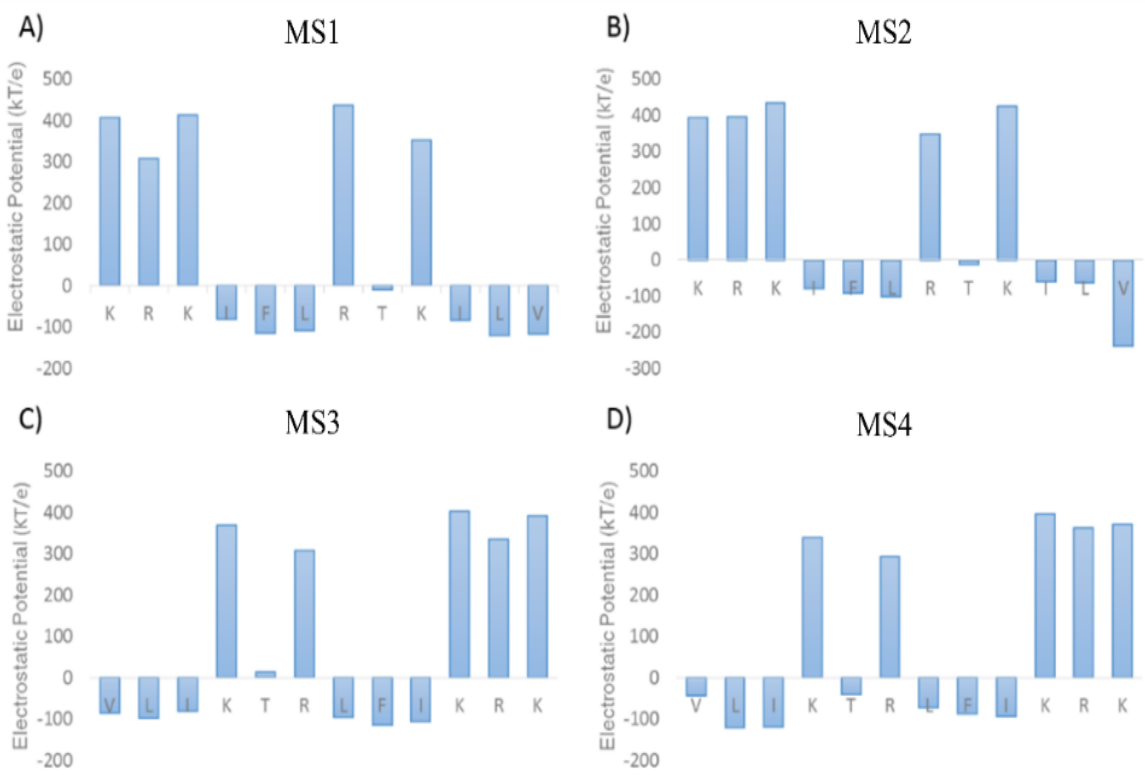


**Figure 5.3.** Position wise electrostatic potential distribution. The electrostatic potential was calculated by solving finite distance Poisson-Boltzmann equation using DelPhi. All potentials are in kT/e units.

amphiphilic by design, as can be verified from their electrostatic potential maps (Figure 5.2 & 5.3).

We performed molecular dynamics simulations of syndiotactic and isotactic variants at 298 K for 10 ns under NVT conditions using GROMACS program suite [285] with gramicidin helical conformation as the starting structure for syndiotactic peptides. The number of clusters for each structure has been shown in Table 5.1 and Figure 5.4. Syndiotactic peptides are quite stable and insensitive to its environment and in general agreement with earlier reports [238]. MD results

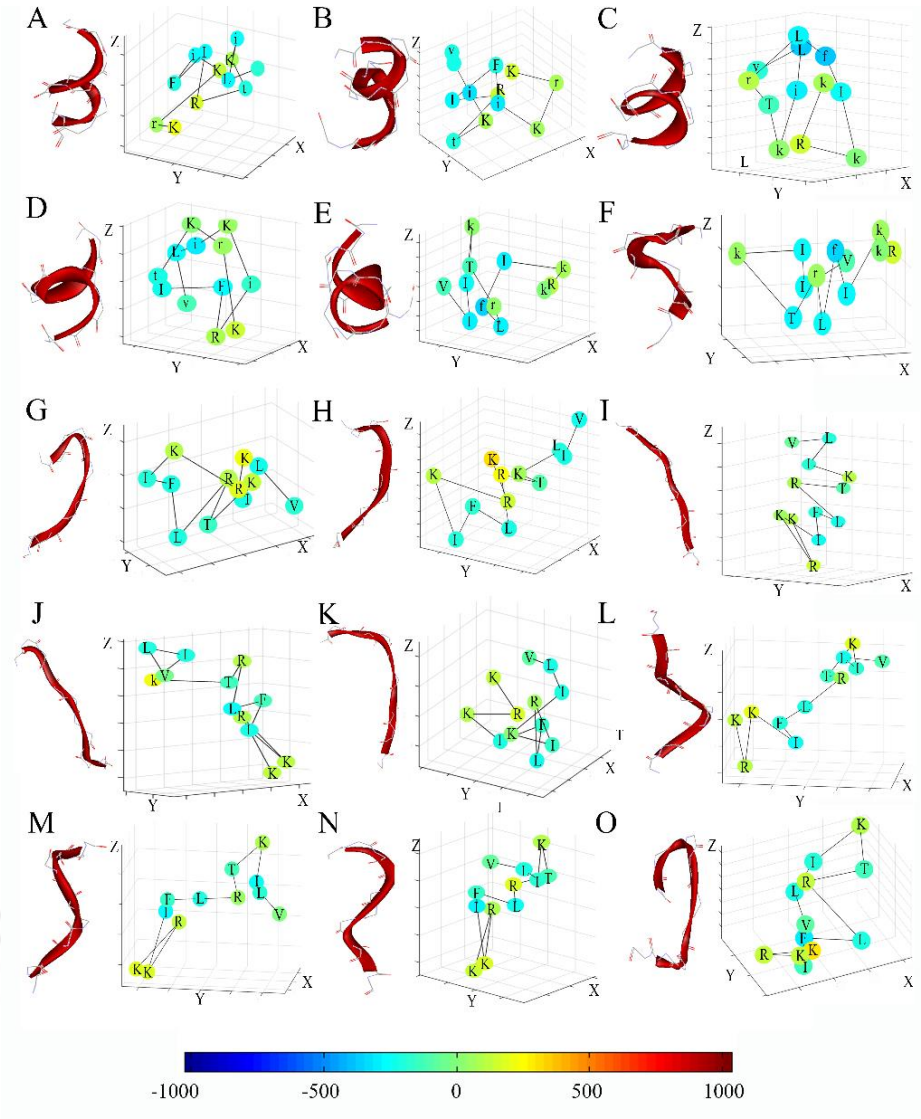


**Figure 5.4.** Clustering analysis and electrostatic potential distribution of designed peptides. All peptides were investigated for their conformational stability by molecular dynamics simulations. A 10 ns run with a 0.2 fs time step was completed, generating 1001 structures per run. These 1001 structures were clustered using g_cluster program from GROMACS package. Mean structures of significant clusters (> 20 structures) were extracted from the trajectory and electrostatic potential at each position was calculated by solving finite distance Poisson-Boltzmann equation using Delphi software. These electrostatic potential distributions were summed for each amino acid side-chain and represented collectively at the chromophoric center of the side chain as per a colour map, with red and violet indicating highly positive and highly negative values. Mean structures for significant clusters of respective peptides and the electrostatic potential distribution are shown in the panels (A-O).

point to two important observations; i) syndiotactic structures are exceptionally stable under ambient conditions in gramicidin helical conformation, whereas poly L peptides generate conformational ensemble forming a very rugged energy landscape and ii) this distinct topological manifestation may translate to different activity profiles, while they encounter membranes of different cell types.

**5.3.2 Peptide synthesis and characterization:** The designed peptide sequences were synthesized, characterized by reversed phase HPLC and MALDI-TOF mass spectrometry. All the peptide was further purified using semi-preparative reversed phase HPLC (Appendix).

**5.3.3 Peptide-lipid interaction:** Differential topology dependent electrostatic profiles of the sequences give insight to test whether they differ in their membranolytic activity; both on artificial membranes and natural systems. Interaction of the peptides was studied with monolayers made up of POPC (zwitterionic lipid) and 7:3 POPC: POPG (negatively-charged). The lipids were spread as monolayers to achieve a surface pressure of 30±2 mN $m^{-1}$. A surface pressure of ~30 mN $m^{-1}$ in lipid monolayer corresponds to the packing of lipids in the biological membranes [279, 286]. Injection of the peptides in the sub-phase caused an increase in the surface pressure. The peptides MS1, MS4, MS5, and MS6 had a sub-phase concentration of 9 µM while MS2 and MS3 had sub-phase concentrations of 6 µM and 12 µM, respectively. The increase in surface pressure was by and large more for the negatively charged lipid monolayer (Figure 5.6). Even though the peptides display only slight preference towards negatively charged lipids, they exhibited excellent antibacterial activities without any hemolysis as discussed in the subsequent sections. Syndiotactic peptides induce surface pressure changes upon peptide insertion ranging from 3.1 to 7.9 mN $m^{-1}$ compared to 3.1 to 3.9 mN $m^{-1}$ for isotactic poly L peptides, against negatively-charged membranes. For zwitterionic membranes, this value drops to 2.1 – 4.9 $mNm^{-1}$ and 1.5 – 2.2 mN $m^{-1}$ for syndiotactic and isotactic peptides, respectively. This points to the fact that electrostatic interaction map of all six peptide systems are distinct and different, inducing different activity profiles against different model membranes. Overall results are in

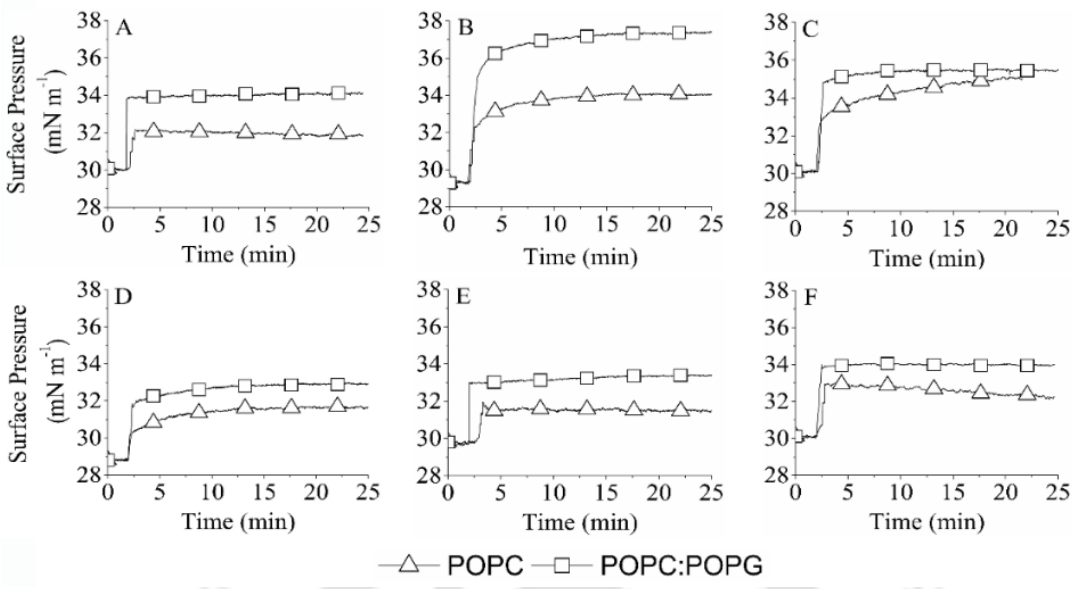


**Figure 5.6.** Interaction of peptides with lipid monolayers. The peptides display some preference for negatively-charged lipid.

good agreement with earlier theoretical investigation on the membrane permeability of alternating L, D peptides by Chipot and coworkers [287].

**5.3.4 Antibacterial assay:** The *in vitro* anti-bacterial activity of the peptides were evaluated against *S. aureus*, *E. coli* and antibiotic-resistant bacterial species. All the peptides exhibited good antibacterial activity, even at micro-molar concentration range (Figure 5.7 and Table 5.2). However, the results do not indicate any specific advantage in syndiotactic series (LDLD or DLDL) in comparison to the poly-L

**Table 5.2.** Minimal Inhibitory Concentration (MIC) of designed peptides. All MIC values are in micro-molar concentration.

| Bacteria | Peptides | | | | | |
|---|---|---|---|---|---|---|
| | MS1 | MS2 | MS3 | MS4 | MS5 | MS6 |
| *S. aureus* | 8 | 8 | 6 | 8 | 3 | 8 |
| *E. coli* | 0.5 | 4 | 1 | 4 | 2 | 3 |
| Gentamicin-resistant MRSA | 4 | 6 | 8 | 8 | 3 | 4 |

sequences, except that MS1 and MS3 have shown to be highly effective against Gram-negative bacteria. From a therapeutic point of view, this lead information

may be further optimized to be included in the treatment regimens designed to address Gram-negative bacteria [288].

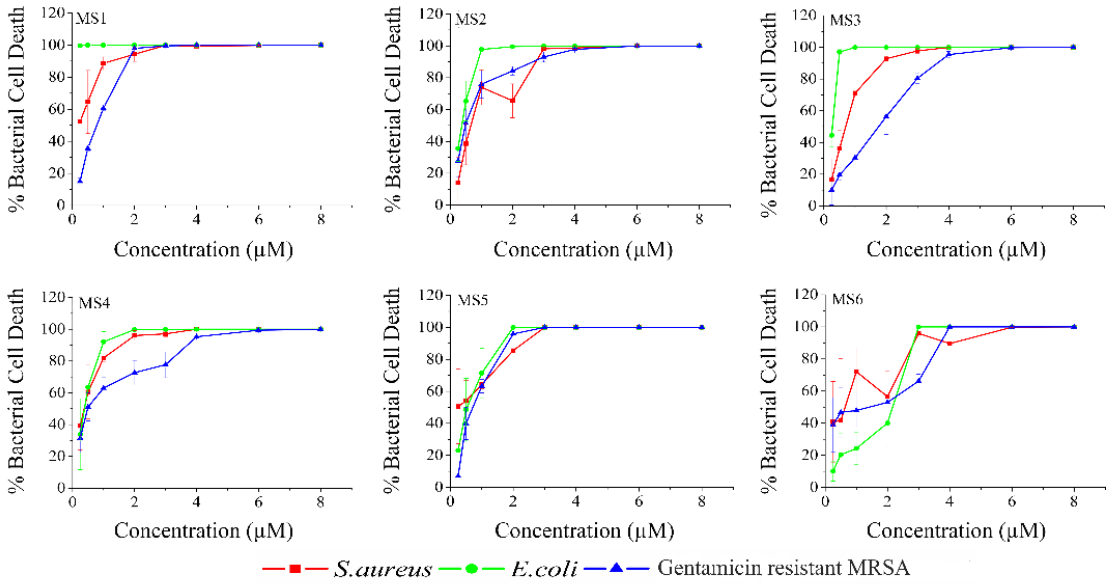


**Figure 5.7.** Antibacterial activity of the designed peptide systems (MS1 to MS6), against *Staphylococcus aureus*, *Escherichia coli* and Gentamicin resistant MRSA. The percent bacterial cell death has been plotted for the designed peptides treated with various peptide concentrations ranging from 0.25 μM - 8 μM. All the peptides show complete killing at 8 μM concentration against the three bacterial cell types. Standard deviation in each case is shown as error bars.

**5.3.5 Field Emission Scanning Electron Microscopy (FE-SEM):** To provide a topographical view of the bacterial membrane perturbation by the designed peptides, FE-SEM was performed. All peptides were assayed against Gram-positive, Gram-negative and antibiotic resistant bacterial species at their respective MIC values and the images were taken at 2-3 kV. Ruptured (treated) and intact (untreated) membrane bacterial samples were observed, qualitatively confirming the membranolytic activity of the designed peptides (Figure 5.8). Even though it is evident that the peptides possess membranolytic activity, it does not eliminate the possibility of a receptor mediated action against the bacterial cells.

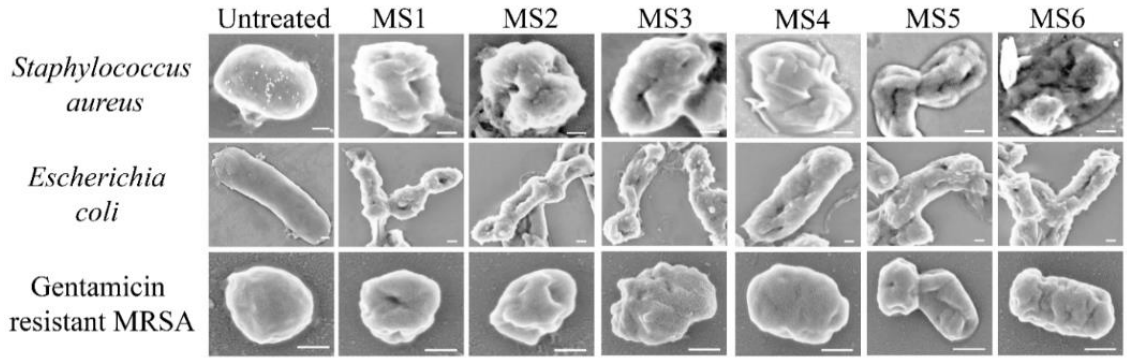


**Figure 5.8.** FE-SEM images indicating membranolytic activity of designed peptides. Membrane deformation as a result of bactericidal action of the designed peptides against (A) *Staphylococcus aureus*, (B) *Escherichia coli* and (C) Gentamicin resistant MRSA at their respective minimum inhibitory concentrations. The scale bars correspond to 200 nm.

**5.3.6 Hemolytic assay**: To evaluate whether the designed peptides had any potential toxicity to mammalian cells, hemolytic assay was performed. RBCs (Red Blood Cells) were incubated with 8 μM peptides for 2 hours at room temperature. RBC lysis was determined by assaying the hemoglobin content in the medium, spectroscopically at 540 nm. None of the peptides caused any notable hemolytic activity (Figure 5.9) [289, 290]. The results support the notion that the designed peptides have the capability of selectively destabilizing bacterial membrane but not typical mammalian cell membranes.

## 5.4 Conclusion

Antibiotic resistance seriously hampers effective broad spectrum therapeutic solutions, which demands generation of new chemical entities periodically. Peptides can be a desirable option for therapeutics, principally due to their high specificity and low toxicity profile [247, 248]. Incorporation of D amino acids in peptide design has been a distinct possibility for designing novel folds but untapped so far to its true potential. The significant reduction in characteristic ratio of polypeptide

backbone having both L and D chiral amino acids offers a possibility to design novel stable folded constructs with minimal chain length.

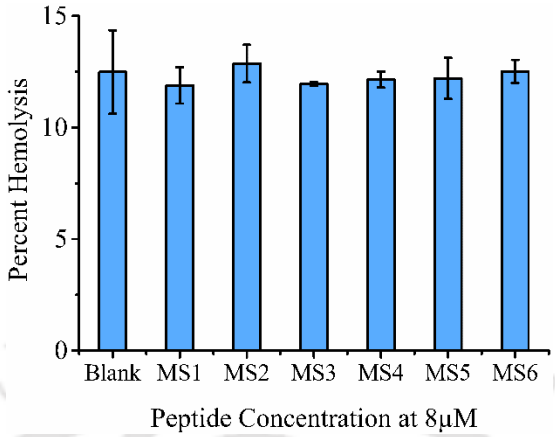


**Figure 5.9.** Hemolytic assay of peptides against human RBC. Human RBC was treated with buffer and 8 μM concentration of the designed peptides. The experiment was performed in triplicates and the error bars represent standard deviation.

Incorporation of D amino acids in peptide design has been a distinct possibility for designing novel folds but untapped so far to its true potential. The significant reduction in characteristic ratio of polypeptide backbone having both L and D chiral amino acids offers a possibility to design novel stable folded constructs with minimal chain length. We tried to examine the potential to expand the peptide design space manifold, by adopting the simplest designed system, LDLD chiral polypeptide to verify its possible structural and resultant physiological effects.

Examination of peptide backbone at atomistic scale for folded and extended conformations of poly-L and alternating-L, D structure reveal that short range and long range electrostatics are either conflicting or harmonious depending upon the chain stereochemistry. The stereochemical transformation of inter peptide electrostatics from a condition of conflict to one of harmony respectively for poly-L and alternating-L, D explains why peptides differ in properties such as “stiffness” and solvent sensitivity. Our molecular dynamics simulation results further underline this earlier observation. The big difference in the number of clusters

among syndiotactic and isotactic variants points to the rugged energy landscape of poly-L sequences compared to a more facile one in LDLD (Table 1, Figure 5.5).

Since the sequences are identical, this extra stability may have realized through favorable short range and long range electrostatics as explained by Ramakrishnan et al., in an earlier published work [238]. As a consequence of this, gramicidin helical conformation of LDLD peptides is more or less insensitive to external stimulus, whereas multiple conformational possibilities of relatively similar energy in their energy landscape allows poly L variants to switch from one conformation to other, an observation similar to earlier reported studies from Ghadiri and Gellman's group, with unnatural amino acids [252, 259-261, 267, 268, 274]. Therefore, amphipathicity in LDLD is clearly defined (Figure 5.2), well-crafted and conformationally retained, independent of external stimulus.

Side-chains with appreciable cationicity such as arginine and lysine cannot be designed to be co-localized on an isotactic poly L peptide chain, due to short range electrostatics, because the resultant repulsive forces are not compensated by favorable backbone interactions. In LDLD diastereomers, short range and long range electrostatics are favorable in a gramicidin helical conformation, so that side-chain interactions between positive charges of and between lysine and arginine can be compensated. The conflicting interactions at side-chain may be balanced by LDLD backbone electrostatics while they assume gramicidin-helical conformation. Poly-L backbone, on the other hand, remains extended thus generating sequence dependent structures of relatively similar energy profiles. The relatively large number of clusters in poly-L ensemble in our MD simulations supports this assumption. Bacterial cell systems comprise of a negatively charged plasma membrane, where the complexity of the membrane varies from a Gram-positive to Gram-negative bacteria. The Gram-positive cell wall consists of a 20 - 80 nm thick layer of peptidoglycans and is rich in lipoteichoic acid and its derivatives, with teichoic acid endowing majority of the negative charge to the cell surface. Cell wall for Gram-negative bacteria is however, more complex than others, comprising of 2 - 7 nm thick layer of peptidoglycans surrounded by 7-8 nm thick outer membrane.

In contrast to lipoteichoic acid of Gram-positive bacteria, the negative charge on Gram-negative bacterial surface is due to the presence of lipopolysaccharide.

As per the prediction of Sir Alexander Fleming, rise of resistant species can be attributed to the overuse of antibiotics. Persistent use of antibiotics caused by drug abuse, spared resistant species which triggered their number due to bacterial multiplication and mutation through gene transfer [291-296]. Such a biochemical adaptation provides a shielding effect against traditional antibiotics. The designed peptides show better activity against antibiotic resistant Gram-positive species as compared to the native Gram-positive one.

The stiffness of an LDLD diastereomeric backbone, thus facilitates the maintenance of the designed amphiphilicity, while the poly-L backbone undergoes major changes in conformations owing to same charge repulsions and dipole moment. Despite having the same amino acid content and only two sequence variations, the six designed peptide systems are distinct by themselves and have unique structure dependent electrostatic fingerprints (Figure 5.2), and therefore have differential membranolytic potential. Significantly, all designed systems are showing comparable or better efficiency compared to typical molecules reported as broad spectrum peptide antibiotics (Appendix).

For Gram-positive bacteria, the potency is comparable across designed systems, but all LDLD peptides are showing better activity compared to their poly L variants in Gram-negative bacteria with MIC value as low as 0.5 μM, possibly owing to the unique charge disposition of negative species on Gram-negative bacteria. The poly-L backbone in our model systems may be adopting a complementary structure to the membrane surface resulting in much better MIC values against antibiotic resistant species. This may not be the case always as there could be situations wherein, the poly-L version may not adapt a suitable amphiphilic profile, crucial for bacterial membrane lysis. The hetero-chiral versions (LDLD and DLDL) on the other hand are expected to retain their designed amphiphilicity independent of the any modulation in the membrane surface charge. At 4 μM, both poly-L peptides

have 100% killing efficiency, as against 6-8 μM for 3 out of 4 LDLD sequences. However, more elaborate study is necessary to fully verify this hypothesis.

The MIC values for peptides against Gram-negative bacteria are, lower against other species, indicating better performance in lysing bacterial cells. The designed peptide MS1 (Table 5.2) is showing excellent activity against Gram-negative bacteria, emerging as an important molecule for further verification as a potential drug molecule. This can again be attributed to the retention of designed amphiphilicty of the syndiotactic variants and relatively fluxional nature of homochiral sequences. Adaptation of diastereomeric variants by changing the stereochemical sequence thus offers a unique possibility to lock the topology of the designed peptide, thus modulating its specificity and activity. Receptor based design of peptide drugs has traditionally been facing issues of the lack of specificity, principally due to the degenerate conformational variants offered by poly L diastereomeric sequences. Conformational locking by stereochemical engineering offer possibility of fixing the structure and therefore imparts good degree of specificity to the designed constructs. Differential activity profiles of six designed systems against three different bacterial membranes tested and constituent cells in human blood underlines this distinct possibility.

# Chapter 6

## Bactericidal potency and extended serum life of stereo-chemically engineered peptides against Mycobacterium

## 6.1 Introduction

Tuberculosis (TB) is one of the top 10 causes of death [297], with more than 10 million cases reported every year and among them, approximately 2 million patients succumb to the disease [15, 16]. *Mycobacterium tuberculosis* (Mtb), is the causative agent, which parasitizes human macrophages for its sustainability and proliferation [20]. Increase in drug resistance is a major challenge to the disease control mechanisms presently in place [17]. Even for the drug susceptible TB, the present treatment regimen is onerous [19], and hence there is an urgent clinical need for the discovery of new chemical entities effective for both drug susceptible and resistant TB.

Peptide based antimicrobials have been recognized as a safe and effective option, with increased selectivity and tolerance [201, 298-302]. But usage of peptides as a therapeutic agent was limited by short plasma half-life [303] and negligible oral bio-availability [5]. The shorter half-life is due to numerous peptidases and excretory mechanisms that inactivate and clear peptides from the system [5, 304]. Several techniques for extending plasma half-life have been developed, such as stapling and clipping of peptide sequences, cyclization, binding to albumin and Polyethylene glycol (PEGylation) etc. [2]. Limitations of endogenous peptides have motivated researchers to focus on the design of peptide analogs, short sequence oligopeptides and innovative delivery systems. Recently Henry et. al reported a subcutaneous implantable osmotic pump system that delivers peptide continuously up to one year for the treatment of type 2 diabetes [305].

Serum represents the intact concoction of human body fluid, which conclusively correlates to its ionic as well as proteome bio-reserve [306, 307]. Human serum proteases [135, 308-311], and their microbial counter parts form an invasive microflora which reduces the therapeutic activity of peptides manifold [304, 312-316]. Apart from this, higher ionic environment has also been reported to reduce bactericidal potency of peptides [317, 318]. Therefore, it is necessary to ascertain the metabolic stability and therapeutic potential of a peptide drug in serum to verify it's in vivo activity. Natural proteins and peptides are polymers 20 amino-acids in varying length and

sequences, with almost all amino-acids exclusively L chiral [8, 148]. In polymer science, such an arrangement is termed as isotactic. If we can have an amino-acid sequence with alternating L and D chirality, it is syndiotactic and random L,D combinations are hetero-tactic [224]. From a design point of view, incorporation of D-amino acid at each position will hugely expand the design space. For example, a ten residue peptide can have $2^{10}$ stereochemical variations possible. However, steriochemically-diversified peptide sequences have not been explored to its potential. Shai and coworkers have reported the utility of D-amino acids in therapeutic peptide sequences for resisting enzymatic degradation [176, 319, 320]. Peptides with D - enantiomers have also displayed much reduced ionic sensitivity and cytotoxicity against mammalian cell systems [183]. Self-assembling organic nanotubes with alternating L and D amino-acids were first reported from Ghadiri's laboratory and interestingly, the same group has designed cyclic D, L-alpha-peptide framework as potential broad spectrum antibacterial and antiviral agents [252, 260]. Metzler-Nolte and coworkers have made use of specific combinations of L and D amino acids to achieve 4 to 8 fold decrease in MIC values [261].

Side-chain derived cationic charge and a designed amphipathic conformation are the two principal elements incorporated in the sequence of peptide-based antimicrobial agents [62, 77]. So far, chain stereochemistry has not been systematically used as a design variable in most of these studies, with few exceptions [7]. In an earlier study, we have retained the amino acid content, but varied their chain stereochemistry to generate different topological variants with same chemical species, and tested their bactericidal potential against gram positive, negative and resistant bacteria. Conformational locking or topological fixation by stereo-chemical engineering has been systematically experimented to verify its differential effects against bacterial membranes[7]. In this work, we extend this study by testing the relative efficacy of isotactic and syndiotactic variants of a peptide sequence in both directions by retaining its chemical constitution.

Mechanistic investigations on TB suggests that, Mycobacterium bacilli are ingested by macrophages in response to bacterial invasion, subsequently resulting in the formation of attenuated phagosomes, creating a friendly intra-phagosomal

environment for bacterial proliferation [17, 20]. Even though there is available treatment regimen, the rise of multidrug-resistant superbugs has limited the prognosis [321-323]. Therefore, we investigated the anti-mycobacterial potential of our reported bactericidal peptides, both in vitro and in serum with *Mycobacterium smegmatis* as a representative model for mycobacterium, due to its non-virulence and comparative fastidious nature.

We made an objective comparison of antibacterial potency of isotactic and syndiotactic peptides in both conditions. We found that stereochemically diversified syndiotactic sequences effectively resist enzymatic degradation, while isotactic antibacterial peptides degrade losing their potency. Since mycobacterium is basically a gram positive bacteria, we further verified our results, we further extended the study to a gram negative bacterium *E. Coli*. In this study, we have designed six model systems, systematically and exhaustively varying the stereo-chemical and amino-acid sequence resulting in six variant topological models. The designed molecules were synthesized and further verified for the effect of chain stereochemistry in formulating their bactericidal potency, hemolysis and plasma life.

## 6.2 Materials and methods

Modelling and simulation, Electrostatic profiling, Peptide synthesis and characterization, Field Emission-Scanning Electron Microscopy (FE-SEM) and Hemolytic activities were performed as explained in Chapter 4 section 4.2.

### 6.2.1 Antimicrobial assay (Micro-dilution method)

Broth micro-dilution method is the most common and standard technique used to test bacterial susceptibility against antibiotics in most of standard laboratories worldwide [324]. In our experiment, *Mycobacterium smegmatis* (ATCC® 607) bacterial strain was cultured in M7H9 media, overnight at 37°C. The bacterial suspension was diluted to $2 \times 10^5$ cfu/mL. Subsequently, 50µL of the diluted inoculum was mixed with fixed concentrations of peptides and incubated at 37° C for 72 hours. The bactericidal potency of the peptides was estimated by comparison with untreated

bacterial cells at 600 nm [325]. Similarly, *E.coli* MG1655 was diluted to 2 × $10^6$ CFU/mL in Nutrient broth (NB) media. 50 µL of the bacterial suspension ($10^5$ cells per well) was mixed with peptides at required concentration, followed by incubation at 37 ° C for 12 to 16 hours. The percent bacterial cell death was calculated by a comparative assessment with untreated bacterial suspension (negative control) at 600 nm [326].

### 6.2.2. Serum sensitive assay

Serum sensitivity assay of peptide based drug molecules are performed to ensure their stability in representative biological fluid. In general, peptide molecules are prone to enzymatic degradation and ion sensitivity, that markedly reduces their therapeutic potential. Therefore, it is important to check the stability of these kind of molecules in *in-vivo* mimicking condition [306, 307].

To test the serum stability of peptides, the anti-bacterial (micro-dilution method section 6.2.1) activity was performed in presence of 50 % human serum [327]. The percent bacterial cell death was calculated by a comparative assessment with untreated bacterial suspension (negative control) at 600 nm [326].

## 6.3 Results and Discussion

### 6.3.1. Design and Synthesis of Syndiotactic Antimicrobial Peptides

We have designed a series of 12 amino acids long peptides incorporating residues with cationic and neutral side-chains. A typical 12 amino acid long polypeptides can theoretically have $2^{12}$ (4096) stereo-chemical variants possible. Naturally occurring, isotactic Poly-L sequence is only one among these, while the remaining 4095 are hetero-chiral. When we systematically evaluate stereo-chemical effects in extending the plasma life of the designed peptides, it is impossible to evaluate all 4096 stereo-chemical variants and further its sequence variants. Therefore, as a design strategy, we opted to exhaustively design the next theoretically possible chiral sequence variation, which is an alternating LDLD peptide sequence (Figure 6.1).

Incidentally, Gramicidin, a naturally occurring polypeptide obtained from soil bacteria *Bacillus brevis* [217], has an alternating L- and D-amino acid sequence [151]. The

helical structure it naturally adopts is known as gramicidin helix. Stereo-chemical sequence LDLD and its reverse DLDL can adopt both right handed and left handed conformations, and with regards to the handedness, one can design eight variants (Figure 6.1).

Differential handedness is achieved by using different phi and psi angle combinations in each syndiotactic sequence. However, out of eight, only four variants are synthetically possible (Figure 6.1, Table 6.1), because handedness is adopted by the peptide, post synthesis. Therefore, out of the eight topological variants, two LDLD and its two mirror sequence DLDL has been synthesized (Table 6.1).

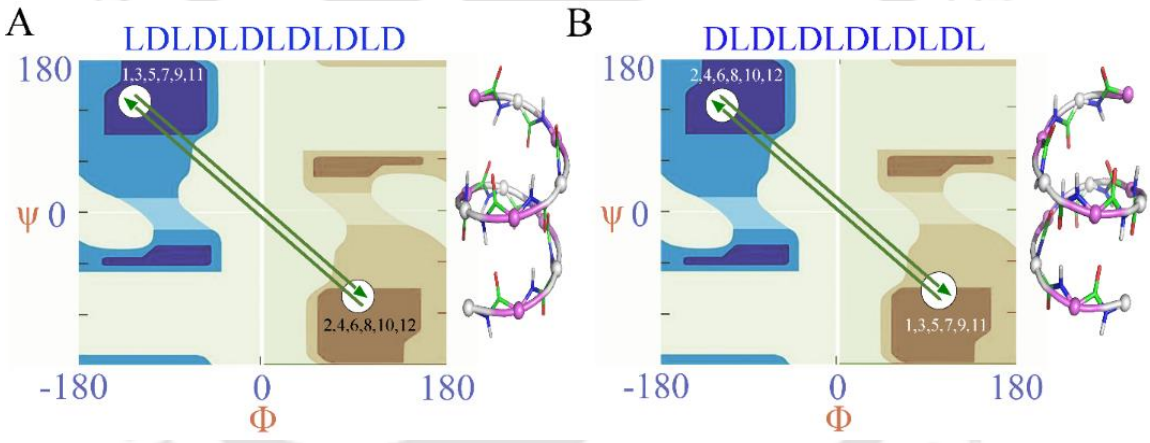


**Figure 6.1.** Design of antimicrobial peptide incorporating D amino acid in the peptide sequence retaining their molecular weight and chemistry, and varying stereochemistry. The design has a pair of LDLD as well as DLDL sequences, with their poly L counterparts, all having identical amino acid composition. The syndiotactic arrangement of the peptide sequences give rise to gramicidin helical structure with alternating L and D residues in peptide sequence.

More precisely, model system MS2 is the stereochemically reversed sequence of model 1 (MS1), MS4 is sequence reversed MS1, and MS3 is both sequence and stereochemically reversed model of MS1.

**Table 6.1.** Designed antimicrobial peptides and their respective amino acid sequences. The letters in uppercase represent L – amino acid, whereas letters in lower case denotes D – amino acid. Comparative activity assessment of antimicrobial activity of the designed peptides calculated as Minimal Inhibitory Concentration (MIC) of peptides against both *M.smegmatis and E.coli* in human serum and media alone. The MIC values are expressed in micro molar (µM) unit.

| Peptide Code | Sequence | *M. smegmatis* | *M.smegmatis* (serum) | *E.coli* | *E.coli* (serum) |
|---|---|---|---|---|---|
| **MS1** | KrKiFlRtKiLv | 6.35 | 12.5 | 3.13 | 6.35 |
| **MS2** | kRkIfLrTkIlV | 3.13 | 3.13 | 3.13 | 3.13 |
| **MS3** | vLiKtRlFiKrK | 6.35 | 12.5 | 6.35 | 6.35 |
| **MS4** | VlIkTrLfIkRk | 6.35 | 12.5 | 6.35 | 6.35 |
| **MS5** | KRKIFLRTKILV | 6.35 | > 50 | 3.13 | > 50 |
| **MS6** | VLIKTRLFIKRK | 12.5 | > 50 | 6.35 | > 50 |

With this we could achieve all the stereochemical and topological possibilities of a given sequence in syndiotactic stereochemistry (Table 6.1). MS5 and MS6 are isotactic (poly L) variants of MS1 and MS3 for an objective comparison of their relative potential against *Mycobacterium smegmatis*, in conditions with and without serum. The design philosophy and their activity against gram positive, gram negative and resistant bacteria (MRSA) have described elsewhere. Two molecules in this series, differs either by stereo-chemistry or sequence or both. Amino acid content, and consequently molecular weight, cationicity, amphipathicity and hydrophobic index of all peptides are the same, which provides an objective assessment of the effect of stereo-chemistry manifested through their topology during membrane activity and protease and peptidase enzyme induced degradation under *in vivo* conditions. All the 6 peptide sequences were synthesized using standard solid phase peptide synthesis employing Fmoc chemistry. All the peptides were purified using reverse phase liquid chromatography followed by characterization using mass spectrometry (MALDI-TOF).

### 6.3.2 Bactericidal potency of the designed cationic peptides against *M. Smegmatis*

The bactericidal potency of the peptide molecules was performed by the use of Broth micro-dilution method (Figure 6.3).

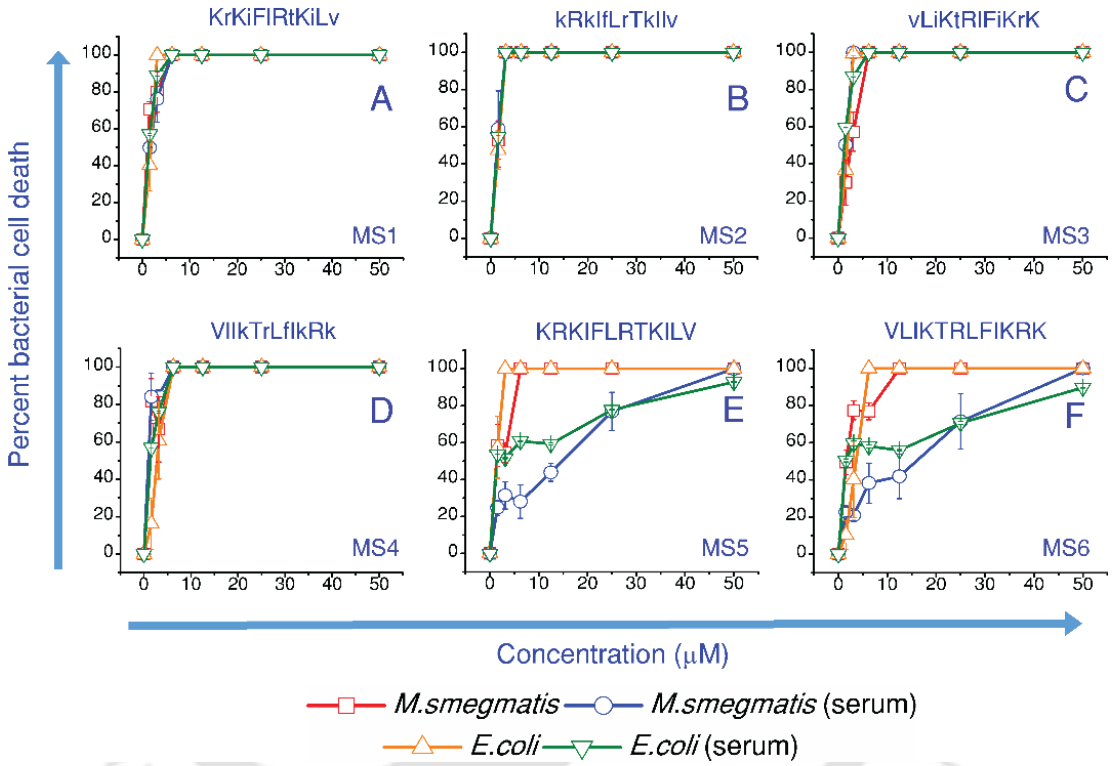


**Figure 6. 2.** Anti-bacterial activity of the designed peptides against *M. smegmatis* and *E. coli* in media and in human serum. The bactericidal potency (percent bacterial cell death) of peptide has been plotted against variable peptide concentration at micro molar concentration (1.56 to 50). The error bars represent standard deviation.

All the designed peptides exhibited good antibacterial activity against *M. smegmatis*, even at micro-molar concentration range under in vitro conditions in media. The syndiotactic and poly-L peptides have shown comparable antibacterial potencies, which indicates that the designed peptides were membrane active in nature (Table 6.1). This observation was supported by a comparative study of

isotactic and syndiotactic peptides with field emission scanning electron microscopy (FE-SEM). The untreated bacterial sample possessed intact membrane architecture, while bacterial membranes treated with peptide have been deformed significantly (Figure 6.3). Anti-tubercular peptides [328] identified in last few decades have shown to possess multiple advantages such as low immunogenicity, selective affinity, and divergent mechanisms of action. Available reports suggest that they bind multiple targets simultaneously showing both anti-mycobacterial as well as immune-modulatory properties. To verify the potential toxicity of the designed peptides to mammalian cells, hemolytic assay was performed with RBC's, incubating with 8 µM and 16 µM peptides for 2 hours

Extend of RBC lysis can be estimated spectroscopically at 540 nm, by measuring the hemoglobin content in the medium. All the peptides were found to have negligible hemolytic activity (Table 6.2).

**Table 6.2.** Percent hemolysis of peptide model systems (MS1 to MS6) in comparision with buffer (5 mM HEPES in 150 mM NaCl) at 8 µM, 16 µM concentration against human Red blood cells (RBCs).

| Peptide | Percent hemolysis at 8 µM | Percent hemolysis at 16 µM |
|---|---|---|
| **Blank** | 11.10 - 11.74 | 10.62 - 14.36 |
| **MS1** | 10.75 - 12.34 | 11.07 - 12.70 |
| **MS2** | 11.63 - 13.47 | 12.02 - 13.71 |
| **MS3** | 11.83 - 12.00 | 11.86 - 12.03 |
| **MS4** | 11.65 - 12.37 | 11.79 - 12.51 |
| **MS5** | 11.35 - 12.03 | 11.28 - 13.12 |

### 6.3.3 Effect of stereo-chemical engineering of the designed peptides in extending plasma half life

To verify the comparative performance of syndiotactic and Poly L peptides, we repeated the experiment in mammalian serum. All the four syndiotactic peptides in LDLD and DLDL stereo-chemical sequence showed remarkable difference in their activity compared to their Poly L counter-parts. Syndiotactic peptides MS1, MS3 and MS4, more or less retained their antimicrobial property, with a relatively lesser extent in their membrane activity, while MS2 retained its bactericidal potency even

in serum. Expectedly, for poly-L peptides, the MIC values have shown a phenomenal decrease from 12.5 μM and 6.35 μM to a value greater than 50 μM

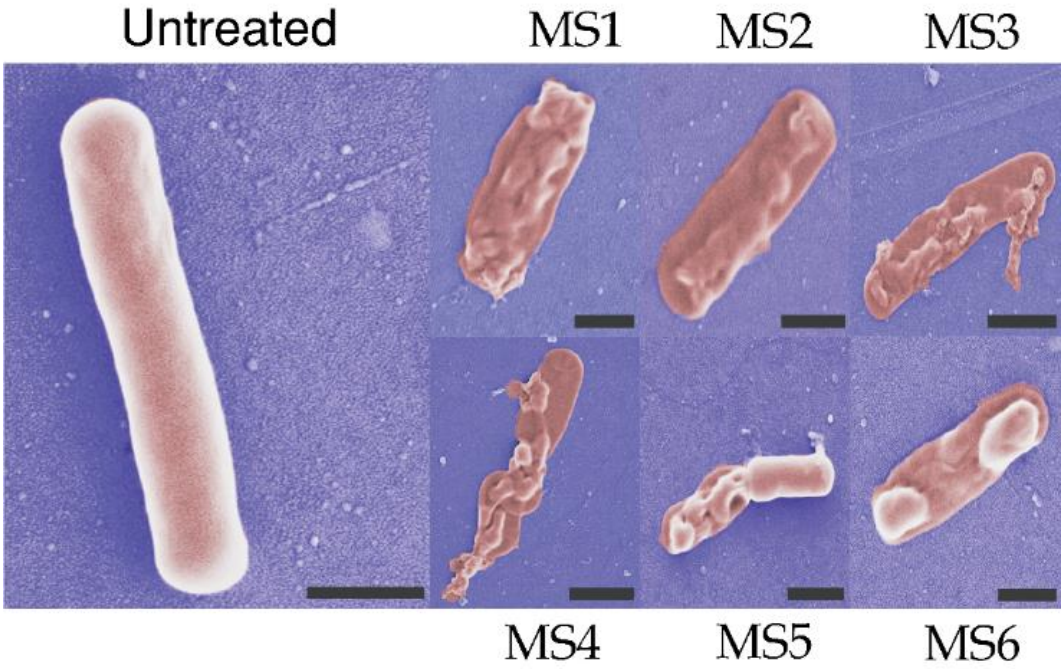


**Figure 6.3.** Field Emission Scanning Electron Microscopic (FE-SEM) images of peptide-membrane interaction. A comparative assessment of untreated specimen with peptide treated sample at their respective MIC can be qualitatively assessed from the extent of deformation of bacterial membrane under each condition. Images have been pseudo-colored using Photoshop CS6. The scale bar resembles to 500 nm.

(Figure 6.2, Table 6.1). The much studied enzymatic degradation of poly-L peptides was evident from the activity profiles. Since serum is a cocktail of innate proteins and ions [307], its effect on therapeutic peptides in known and well demonstrated [310]. The observation that syndiotactic peptides could resist enzymatic degradation can be an important lead in the future designs of peptide based therapeutics. *Mycobacterium smegmatis* is a gram positive bacterium [329]. To further verify our observation, we have repeated the experiment with gram negative bacteria, *E. Coli*. Overall results were found to be a repeat of *M. Smegmatis* experiment, with syndiotactic peptides more or less retaining its activity in serum, while poly L peptides degraded. Interestingly, MS2 was found to an important lead compound

for future development as a very potent broad spectrum antibiotic, showing exceptional activity against both bacterial species of contrasting membrane architecture (Figure 6.2). Mycomembrane is very rigid compared to even gram negative bacteria [330], which explains the general trend of comparatively lesser MIC values of the designed peptides in Gram-negative bacteria, supporting earlier studies with comparative estimates.

## 6.4 Conclusion

Antibiotic resistance limits the sustained usage of broad spectrum therapeutic solutions, thereby demanding a robust pipeline of newer lead compounds getting into clinical trials [323, 331]. The present treatment regimen of tuberculosis is complicated, demanding long-term use of multiple antibiotics up to 12 months, and not devoid of side-effects [332]. Recent reports suggest that BCG (*Mycobacterium bovis* bacille Calmette-Guérin) vaccine, widely used for children can fail to prevent pulmonary TB in adults [333]. Further, multidrug-resistant resistance (MDR), an inevitable consequence of noncompliance with the full therapeutic regimen, is leading to the emergence of resistant strains of *Mycobacterium* [334]. Recent reports of rising resistance to age old drugs like isoniazid [335] and rifampin [336], points to the immediate necessity of new line of solutions to this disease.

Peptides serve as the first line of immune defense and probably the only immune effector for most organisms [203, 337]. The basic molecular construct of physiologically active peptides is mostly composed of poly-L amino acids [227]. AMP's are utilized as a viable option against microbial invasions by physiological systems across different levels of organization [338]. Stability against enzymatic degradation was one of the main reasons for their underutilization [304], which could otherwise be a good therapeutic option with minimal side effects. Our assessment with alternating stereo-chemical sequence (LDLD or DLDL) of antimicrobial peptides, suggest that stereochemical engineering of designed peptide can address the most significant associated limitation of peptide based treatment regimen against bacterial infections, especially Mycobacterium.

# Chapter 7

## Effect of chain length in Modulating bactericidal potential in short syndiotactic peptides

## 7.1 Introduction

Microbial resistance against traditional antibiotics has been a global threat to public health. Monitoring agencies like World Health Organization (WHO), US Center for Disease Control and Prevention (CDC) etc. have raised serious concerns about this growing menace in recent times. The rapid and widespread development of drug resistant pathogens and reduced approval rate of new antibiotic agents have resulted in increased mortality rate globally [46, 339, 340]. As per the reports, nearly 25000 deaths per annum in Europe has been recorded due to antibiotic-resistant bacterial infections. Similar number of causalities have been reported from United States as well [3414] and mortality rate in developing and third world countries is even worst. Overall, an annual death toll of 7 lakh has been reported, which is projected to touch 10 million by 2050 [342].

Antibiotic-resistant bacterial species can be multidrug-resistant (MDR), extensively drug-resistant (XDR) or pan drug-resistant (PDR) [343]. Carbapenem-resistant *Acinetobacter baumannii* is reported to be the most resistant bacterial species (PDR) till date [342]. WHO published a list of 12 MDR bacterial species and families, suggesting an urgent need for discovery of newer antibiotics against them. Other than the drug resistant species, bacterial biofilms [344] are also known to be the most resistant form, where no antibiotics have shown significant bactericidal activity [345, 346]. However, there have been reported instances of antimicrobial peptides (AMPs) showing significant activity against multidrug-resistant pathogens and bacterial biofilms as well [347]. Increased attention to AMP research is primarily hinging around this hope.

Antimicrobial peptides (AMPs) are natural molecules of innate immunity that counters microbial invasion [348, 349]. Peptides like defensins, cathelicidins, cecropins, magainins etc. are some of the most widely studied classes of antimicrobial peptides (AMPs) [227]. Despite having varied sequence, structure and amino acid composition, these molecules share common characteristic features like cationicity and amphipathicity in their primary molecular construct, which exhibits broad spectrum antimicrobial activity [57, 62]. Gramicidin; a syndiotactic peptide antibiotic

from *Bacillus brevis* is the first ever clinically tested molecule of its kind [107]. We have described in an earlier chapter (chapter -6) of this thesis, about how systematic variation in main-chain stereochemistry or tacticity can multiply the design space of antimicrobial peptides, its efficacy and utility as a therapeutic agent.

Through this work, we aim to investigate the influence of polypeptide chain length on the bactericidal potency of syndiotactic peptides, by synthesizing a sequence subset of an earlier studied peptide sequence [7]. The earlier model systems were comprised of syndiotactic peptides (MS1 – MS4), along with their poly – L counter parts (MS5 and MS6). All the peptides were identical, in terms of their amino acid content. The entire peptide model systems were active against Gram-positive, Gram-negative and resistant bacterial species [7]. A detailed account of their activity, serum sensitivity and biophysical properties were presented in chapter 5 and 6 [19]. We designed and synthesized an approximate sequence subset of these peptides with seven amino acids. Shorter sequences are relatively easy to synthesize in laboratory and are therefore more economical. We have retained the basic design protocol and to a great extent sequence, so that we can make an objective assessment of its comparative performance while reducing its size. We observed a significant reduction in antimicrobial potency of peptides (ST1 – ST4) upon chain length reduction, with varied bactericidal activity profiles.

## 7.2 Materials and methods

Modelling and simulation, Electrostatic profiling, Peptide synthesis and characterization, Field Emission-Scanning Electron Microscopy (FE-SEM) and Hemolytic activity were performed as explained in Chapter 4 section 4.2. The antimicrobial assay was performed as described in Chapter 5, section 5.2.2.

## 7.3 Results and Discussion

We designed four 7-mer syndiotactic peptide sequences (Table 7.1) showing single turn Gramicidin helical structure for making objective comparison of their bactericidal potency with previously reported 12-mer peptide sequences [7].

**Table 7.1.** Designed 7-mer peptide sequence, code and their respective molecular mass. Number of clusters found after 10 ns Molecular Dynamics simulation. The letters in the uppercase represent L – amino acid, whereas letters in lower case denotes D – amino acid.

| Peptide code | Sequence | Molecular mass (g/mol) |
|---|---|---|
| **MS1** | KrKiFlRtKiLv | 1513.02 |
| **ST1** | KrKiFaL | 873.58 |
| **MS2** | kRkIfLrTkIlV | 1513.02 |
| **ST2** | kRkIfAl | 873.58 |
| **MS3** | vLiKtRlFiKrK | 1513.02 |
| **ST3** | lAflkRk | 873.58 |
| **MS4** | VlIkTrLflkRk | 1513.02 |
| **ST4** | LaFiKrK | 873.58 |

The 12-mer sequences were reported to have stable Gramicidin helical structure as per their modeling and simulation studies, explained in chapter 5 [7]. However, these 7-mer sequences may retain single turn like structure, because of their syndiotactic backbone (Figure 7.1). Among the designed sequences, all the peptides were varying either by stereochemistry or sequence within themselves, despite having similar amino acid composition. Specifically, peptide ST2 is the stereochemically reversed model of ST1, ST3 is a sequence and stereochemistry reversed ST1, and ST4 is sequence reversed model of ST1.

### 7.3.1 Electrostatic profiling and MD simulation of peptide sequences

The designed peptide molecules are amphipathic in nature, due to the presence of both cationic (hydrophilic) and hydrophobic moieties within the sequence. These characteristics are known to play a significant role in selective membrane (bacterial) interaction due to assisted electrostatic interactions followed by lysis causing bacterial cell death. The amphipathic nature of the *de-novo* designed peptides was confirmed by electrostatic potential mapping of the designed peptides (Figure 7.2).

All the electrostatic potential maps were generated by solving Finite Difference Poisson Boltzmann (FD-PBE) equation using Delphi software. The variable electrostatics within a peptide sequence can be attributed to its polar and non-polar

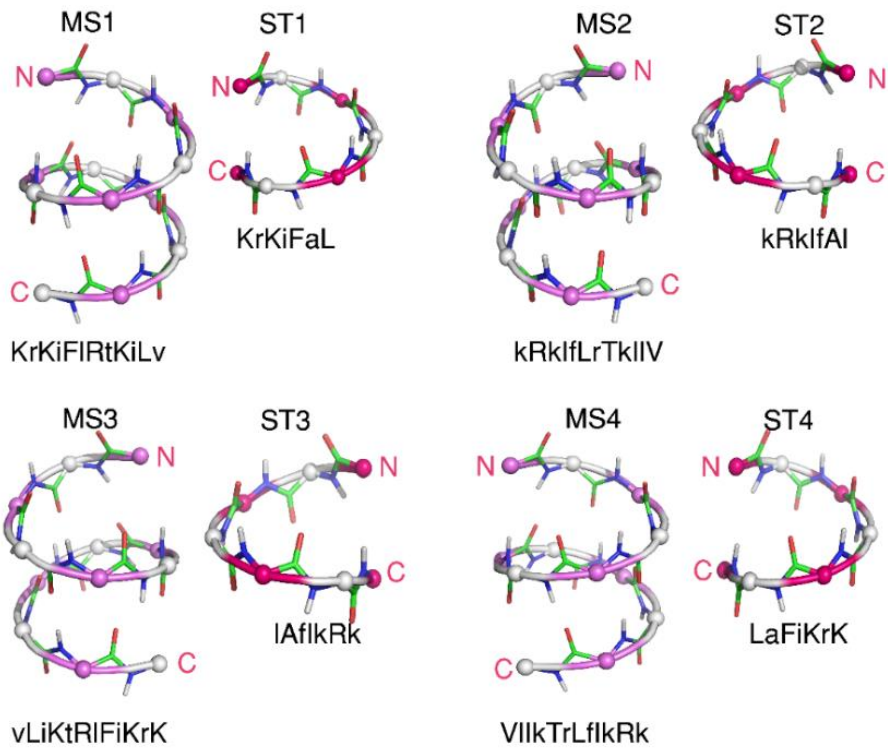


**Figure 7.1**. Comparative backbone structure of *7 mer* peptides (ST1 – ST4) with reference to their respective *12 mer* model peptide systems (MS1 – MS4). All the peptide sequences are syndiotactic, having alternate L- and D-amino acids in its back bone. The N- and C-terminus of the peptides are shown in each case along with their peptide sequences. Small letter in the sequence indicates D-enantiomer.

side chains, which was apparent in all the designed sequences. In general, optimum amphipathicity is one of the principal design element of AMPs with significant bactericidal potency as well as minimal mammalian cytotoxicity [182]. Hence, designed peptides with amphipathic electrostatic fingerprint can be expected to yield the desired physiological activity. The designed peptide sequences are made of similar amino acid composition; peptide variants were created as a result of altered stereochemistry and sequence reversal. No two peptides within the set have similar sequence or stereochemistry (Table 7.1). To check the stability of the peptide candidates, a 10 ns MD simulation run was performed under NVT conditions using GROMOS 96 force field. Cluster analysis were performed with an RMSD cut-off of 0.5 nm. All the peptides were reasonably stable roughly maintaining the designed structure.

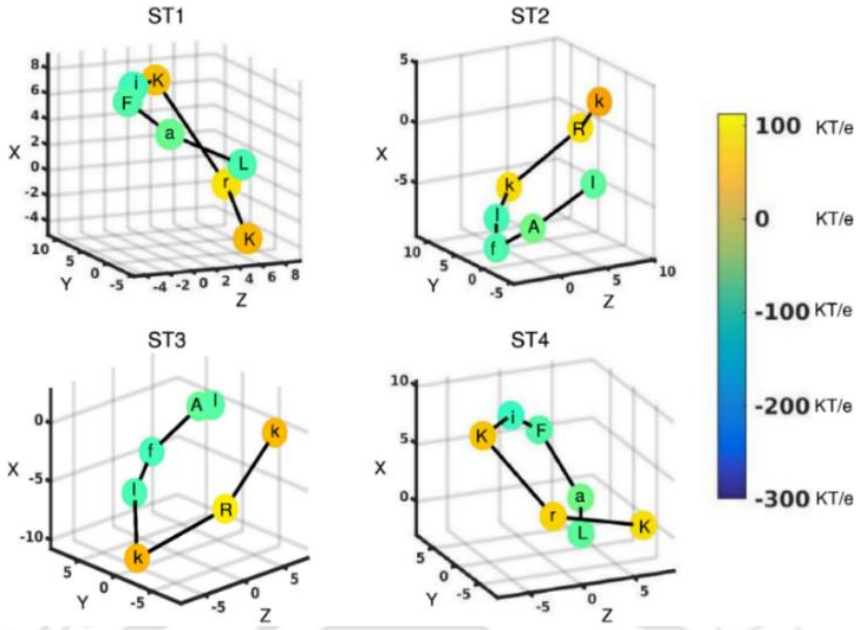


**Figure 7.2**. Electrostatic profile of 7-mer peptides (ST1 – ST4). The relative orientation of positively charged (yellow) and non-polar (green) side-chain group of the de-novo designed peptide sequence, showing amphipathic character, with distinct regions of polarity. The color scheme represents the distribution of electrostatic potential in KT/e units.

The structural stability was further assessed by measuring the Root Mean Square Deviation (RMSD) (Figure 7.3 A), and Radius of Gyration (Rg) with reference to the starting structure (Figure 7.3 B). In the earlier reported studies [7, 238-240], syndiotactic peptides with alternate L- & D- amino acid composition or vice versa have shown to have short range and long range electrostatic interactions (in peptide backbone) in perfect harmony, unlike Poly L peptides. The relatively greater probability of syndiotactic helical structures retain their gramicidin helical conformation is principally due to this mutually complementing short-range and long range electrostatics.

### 7.3.2 Antimicrobial assay and Hemolytic activity

The *in-vitro* antibacterial potency of the synthesized peptides was tested against *Staphylococcus aureus* (Gram-positive), *Escherichia coli* (Gram-negative) and

Gentamicin resistant MRSA (resistant bacterial species). The bactericidal potency of the peptide sequences was found to have markedly reduced (**Figure 7.4, Table 7.2**) compared to highly active, 12 mer peptides.

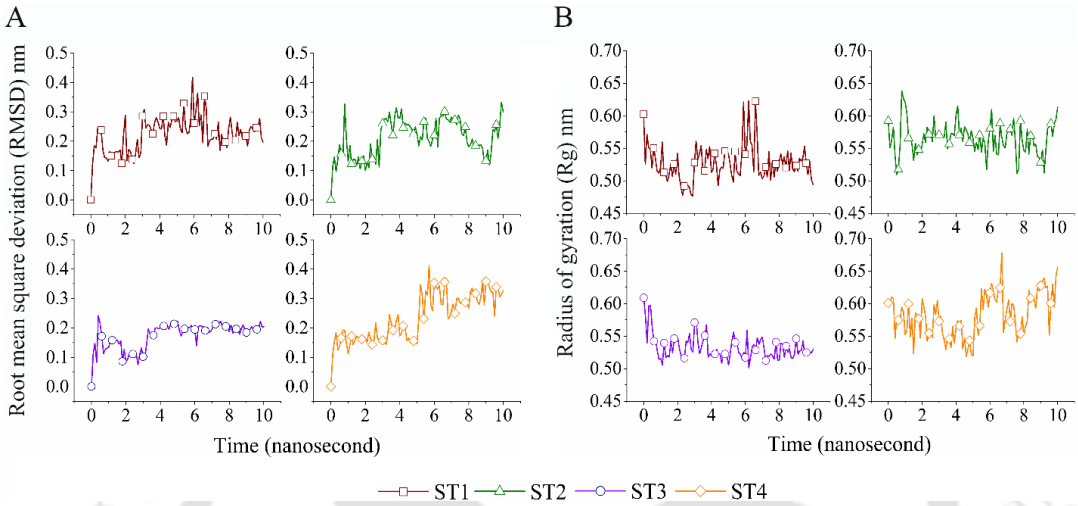


**Figure 7.3.** A) Root Mean Square Deviation (RMSD) and B) Radius of Gyration (Rg) as a function of time in a 10 nanosecond MD simulation production run.

The hemolytic activity of AMP's is an important parameter to establish their selective membranolytic potential against bacterial cells. To assess the toxicity of the designed AMPs against mammalian cells, we performed hemolytic activity assay of peptides against mammalian Red Blood Cells (RBC). A 100 µM concentration of peptides were incubated with 5% RBC at room temperature for 2 hours.

**Table 7.2.** Inhibitory Concentration 50 (IC 50) values of peptides at micro-molar(µM) concentration.

| Peptide code | MS1 | ST1 | MS2 | ST2 | MS3 | ST3 | MS4 | ST4 |
|---|---|---|---|---|---|---|---|---|
| ***S.aureus*** | 1.1 | 13.7 | 1.3 | 3.3 | 0.7 | 5.4 | 0.7 | 5.07 |
| ***E.coli*** | 2.2 | 15.2 | 0.5 | 8.3 | 0.4 | 3.8 | 0.5 | 6.5 |
| **Gentamicin resistant MRSA** | 3.1 | 8.1 | 0.7 | 6.2 | 1.9 | 5.2 | 1.2 | 6.8 |

The percent hemolysis was calculated by comparative evaluation with 0% and 100% RBC lysis in buffer and water respectively.

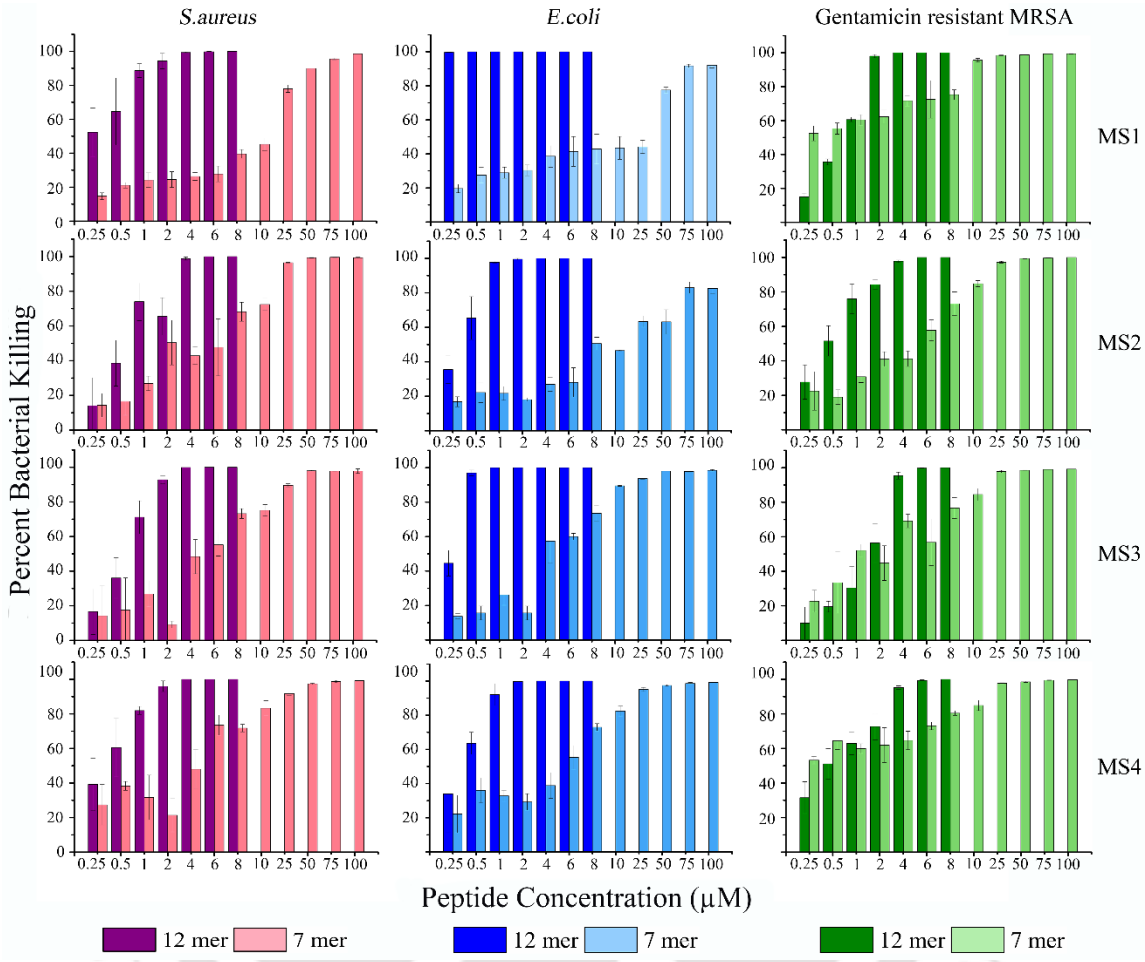


**Figure 7.4**. Comparative antimicrobial activity of peptides (MS1 to MS4 and ST1 to ST4) against *Staphylococcus aureus*, *Escherichia coli* and Gentamicin resistant MRSA. The standard deviation has been shown as error bars in the graph itself.

All the designed peptides are well within 3 % safety limit compared to the blank. As per earlier reports, insertion of D-amino acid has been reported to reduce the extent of hemolysis [182, 320].

### 7.3.3 Microscopic analysis (FE-SEM)

The microscopic analysis (FE-SEM) provides a qualitative pictorial overview of deformed membrane architecture upon treatment with bactericidal agents. The

bacterial cells (*S.aureus*, *E.coli* and Gentamicin resistant MRSA) were treated with their respective MIC values followed by their fixation and imaging. The images were taken at 50-75 KX using 2-3 kV.

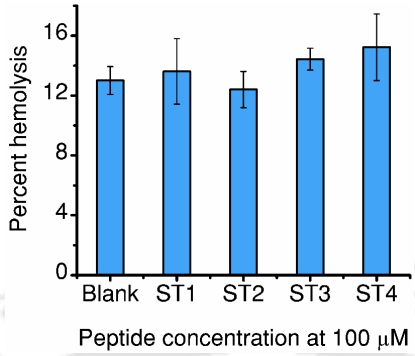


**Figure 7.5.** Percent hemolysis of antimicrobial peptides against human RBC at 100 μM. All the peptides showed negligible hemolysis suggesting minimal cytotoxicity as the blank alone showed 13 % RBC lysis. The error bars represent standard deviation.

The comparative qualitative analysis of peptide treated bacterial cells showing deformed membrane architecture in comparison with the untreated bacterial cells, having non-deformed membrane morphology supports their probable membranolytic activity, though of a lesser extent in comparison with the 12 mer sequences.

## 7.4 Conclusion

Antibiotic resistance reduces the therapeutic activity of antibiotics which results in need of newer drug entities [46]. Recurrent rise in antibiotic resistant superbugs and drying up of new lead candidates has been an issue of major concern [46]. AMPs are projected to be a useful option, due to its broad spectrum activity [57], selectivity towards microbial cells and negligible mammalian cytotoxicity [350]. However, there are notable limitations that have withheld its progression. Two major drawbacks are susceptibility to proteolysis and sensitivity to salt. Strategies like the incorporation of D-amino acid within the peptide sequences have considerably reduced these limitations, resulting in improved bactericidal activity [5].

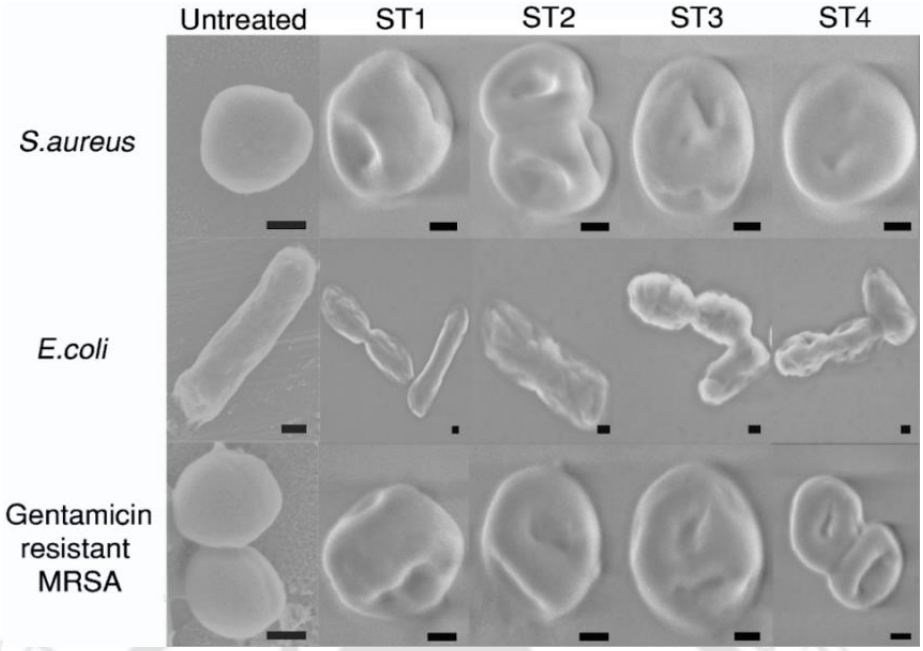


**Figure 7.6**. Field Emission Scanning Electron Microscopy (FE-SEM) of bacterial cells treated with peptides (ST1 – ST4) at 100 μM concentration and untreated cells as control. Bacterial cells of *Staphylococcus aureus, Escherichia coli* and Gentamicin resistant MRSA were used in this study and all the peptide treated cell were seen to develop deformed membrane texture as compared to untreated control cells. The scale bars correspond to 200 nm.

The experiments reported in this chapter suggest two major design directives; i) bactericidal activity of syndiotactic sequence is chain length and structure dependent, and ii) bactericidal activity is a function of the electrostatic environment generated by the stereo-chemical cum amino acid sequence and their resultant topology.

# Chapter 8

## Optimization of bactericidal potential with designed electrostatic gradients

## 8.1 Introduction

Antibiotics have increased the average life expectancy of the global population by effectively countering microbial infections, ever since the discovery of penicillin [351]. Over the years, evolution of resistant bacterial species has reduced the efficacy of antibiotics; with varieties of resistant bacterial forms like Multi-Drug Resistant (MDR), Extensively Drug Resistant (XDR) and Pan Drug Resistant (PDR) species evolving [46]. "ESKAPE", a coterie of bacterial organisms are largely responsible for drug resistance and hospital acquired infections [352]. Many of these organisms can form biofilms; a highly resistant form without effective solution from present treatment regimen [126, 353]. The increasing mortality rate may reach up to additional 10 million deaths annually by 2050 [354]. Such a projected estimate would welcome all options that can be made available as futuristic solutions and peptides are the most promising ones due to their prominent antimicrobial activity in our physiological systems [355].

AMPs are defense molecules of the innate immune system that combats microbial infection within our biological systems [356]. The primary characteristic of these molecules are reported to be amphipathic as well as cationic in nature [77]. Some of the well-known molecules of this class are Cecropins [357], Magainin [358], and Defensins [359] with potential antimicrobial activity. Gramicidin, the first ever clinically used antibiotic is an AMP isolated from *Bacillus brevis* [360]. Certain molecules like Daptomycin, Telavancin, and Vancomycin are some of the well-known clinical molecules of its class. There are about two dozen AMPs in various phases of clinical trials [76, 89]. AMPs have shown potential antimicrobial activity against drug resistant pathogens and biofilms [361]. There have been reported instances of synergistic activity of AMPs with conventional antibiotics [362]. Despite their desirable characteristics, the clinical utility of these class of molecules has not been up to the expectations [174]. Factors like poor pharmacokinetic profile and the high cost of production have restricted their progression as clinical molecules. However, strategies like end capping, cyclization, conjugation with large polymers and proteins, prenylation, lipidation, insertion of D–amino acids and use of permeability enhancers have addressed some of the existing issues of adopting

peptides as lead candidates [2, 5, 363]. Unlike natural proteins, hetero-chiral peptides have more Ramachandran space accessible, when we consider peptide as a singular unit. In hetero-tactic and syndiotactic proteins, short range and long range interactions may be more optimized[8, 151, 152]. Peptide backbone with diversified stereochemistry can negate enzymatic degradation, mammalian cytotoxicity and to a considerable extent ion sensitivity [18, 77, 176, 182, 183, 319, 320].

In this chapter, we present first investigations of a third series of peptides, designed on the basis of design directives and knowledge base created from the first two series of peptides elaborated in the first seven chapters of this thesis. Out of eight model systems designed, seven are having syndiotactic backbone with LDLD stereochemistry and the remaining one is an LLDD peptide. The principal objective of this investigation is to design various combinations of zones with varying degrees of polarity, such that, we may get important clues about specific combinations better optimized against gram positive, negative or Mycobacterium species. All peptides were synthesized and characterized against three different bacterial species in media and serum. As per our expectations, differential electrostatic profiles have their physiological effects translated to their bactericidal potential, with few candidates showing exceptional broad spectrum anti-bacterial activity even in serum.

## 8.2 Materials and methods

Modelling, Peptide synthesis, characterization, Field Emission-Scanning Electron Microscopy (FE-SEM) and Hemolytic activities were performed as explained in Chapter 4 section 4.2. The antimicrobial assay and serum sensitive assay was performed as described in Chapter 6, section 6.2.1 and 6.2.2.

## 8.3 Results and Discussion

### 8.3.1 Design and Synthesis of Antimicrobial Peptides

The antimicrobial peptides reported in this work was designed with a modified version of Ribosome, and PDB make software packages (Table 8.1).

**Table 8.1:** Detailed peptide sequence along with their molecular weight. The letter in lower case denotes D-enantiomers of the respective amino acid.

| Peptide Code | Sequence | Mol. Wt. (Da) |
|---|---|---|
| **AS-01** | R k R w W v V k R k W w V | 1911.126 |
| **AS-02** | R k R w L w L k R k W l W | 1953.173 |
| **AS-03** | K k K w W v K k K w W v V | 1827.108 |
| **AS-04** | W w K k K v W v K k K w W | 1914.119 |
| **AS-05** | K k K k K k K v V v V v W | 1595.102 |
| **AS-06** | K k K v V v K k K v V w K | 1595.102 |
| **AS-07** | V K k v V K k v V V k v W | 1566.075 |
| **AS-08** | W l W l W l W v K k A k A k K | 1982.203 |

All the peptides were amphipathic in nature. However, the electrostatic fingerprint and amino acid composition were varying in each model system. The peptides AS01 –AS04, AS06 and AS07 had longitudinal division of hydrophobic (grey) and cationic (blue) zones, whereas AS05 and AS08 were designed to have hydrophobic and cationic zones in vertical divisions (Figure 8.1 and 8.2). The designed peptide sequences were synthesized using F-moc chemistry. Post-synthesis, all the peptides were purified using reverse-phase high performance liquid chromatography. The purified peptides were characterized by Matrix Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) mass spectrometry.

### 8.3.2. Antibacterial activity and serum sensitivity of designed Antimicrobial Peptides.

The antibacterial activity of peptides was performed against *Escherichia coli*, Methicillin-resistant MRSA and *Mycobacterium smegmatis*. All the peptides except AS05 and AS06 were showing exceptional antimicrobial activity in micro-molar scale (Table 8.2, Figure 8.3). The prominent activity against the selected model organisms is noteworthy as they are among the most difficult organisms to kill and

among the frequent disease producing genus within the microorganism's community.

**Table 8.2.** Peptide code and their respective MIC values against *E.coli*, Gentamicin resistant MRSA and *M.smegmatis* in presence and absence of serum. The MIC values are expressed in micro-molar (μM) unit.

| Peptide code | *E.coli* | | Gentamicin resistant MRSA | | *M.smegmatis* | |
|---|---|---|---|---|---|---|
| | Medium | 50 % serum | Medium | 50 % serum | Medium | 50 % serum |
| **ASo1** | 3.13 | 6.25 | 6.25 | 6.25 | 6.25 | 6.25 |
| **ASo2** | 6.25 | 3.13 | 3.13 | 6.25 | 3.13 | 3.13 |
| **ASo3** | 6.25 | 3.13 | 3.13 | 6.25 | 6.25 | 6.25 |
| **ASo4** | 3.13 | 6.25 | 1.6 | 1.6 | 6.25 | 6.25 |
| **ASo5** | 25 | 25 | 50 | 50 | 50 | 25 |
| **ASo6** | 25 | 12.5 | 50 | 50 | 25 | 25 |
| **ASo7** | 6.25 | 12.5 | 3.13 | 6.25 | 3.13 | 6.25 |
| **ASo8** | 6.25 | 6.25 | 6.25 | 3.13 | 6.25 | 6.25 |

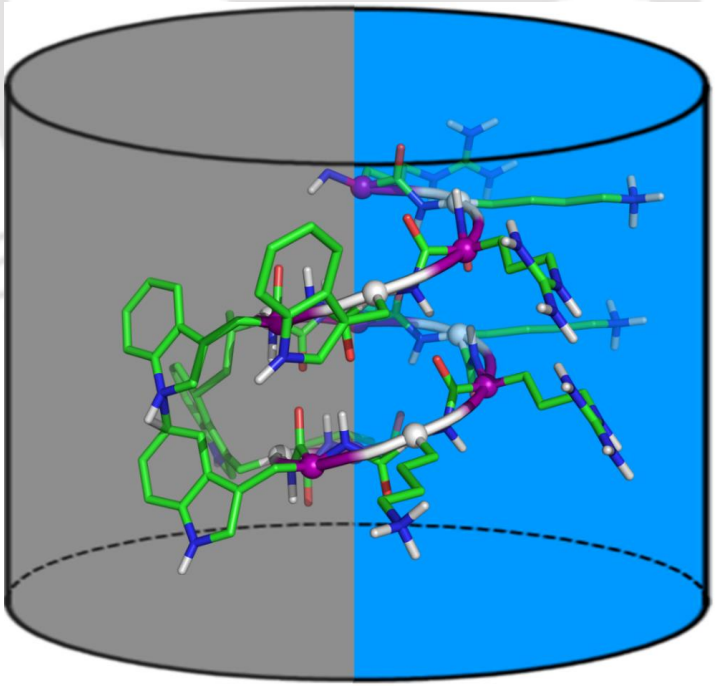

**Figure 8.1**. Design of syndiotactic peptides with distinct polar (cationic, blue) and non-polar (hydrophobic, grey) zones.

The antibacterial activity of the peptides was also tested in presence of 50 percent serum. The serum is a representative bio-fluid comprised of multiple factors responsible for reduced therapeutic activity [306, 307]. Insertion of D-enantiomers within a peptide sequence has been reported to address issues like enzymatic degradation, ion sensitivity and mammalian cytotoxicity. The heterochiral peptide sequences were shown to retain their antibacterial potency in serum as reported in previous studies [26-30]. All the peptides were showing very impressive activity profile in micro-molar range (Table 8.2, Figure 8.3). However, the peptides AS05 and AS06 were comparatively poor performers. We are attempting to develop an evolving knowledgebase based on electrostatic fingerprint for peptide design against specific bacterial species, and the third series of peptides described in this chapter is one step closer to that objective. The antimicrobial potential of the peptide sequences was retained in the presence of serum, indicating their stability and retention of therapeutic potential in physiological conditions[2, 5, 176, 183].

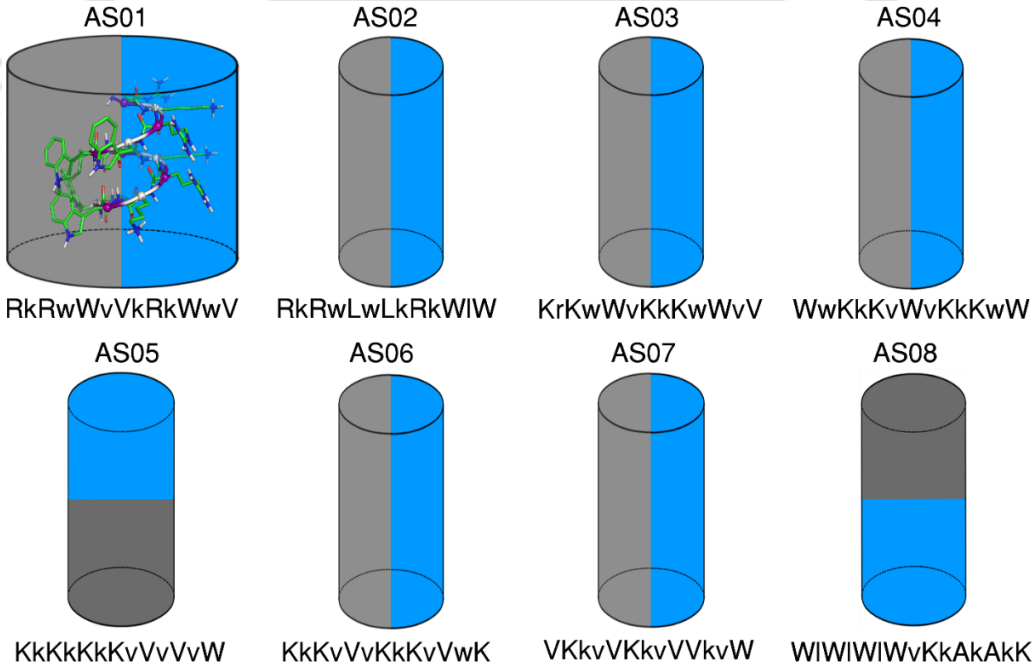


**Figure 8.2**. Backbone structure of peptide series (AS01 – AS08). The blue color shows the sequences with cationic side-chain, whereas, the grey color displays non-polar (hydrophobic) side-chains. Small letter in the sequence represents D-enantiomers of respective amino acids. All the cylinders are having N terminus on top and C- terminus at the bottom.

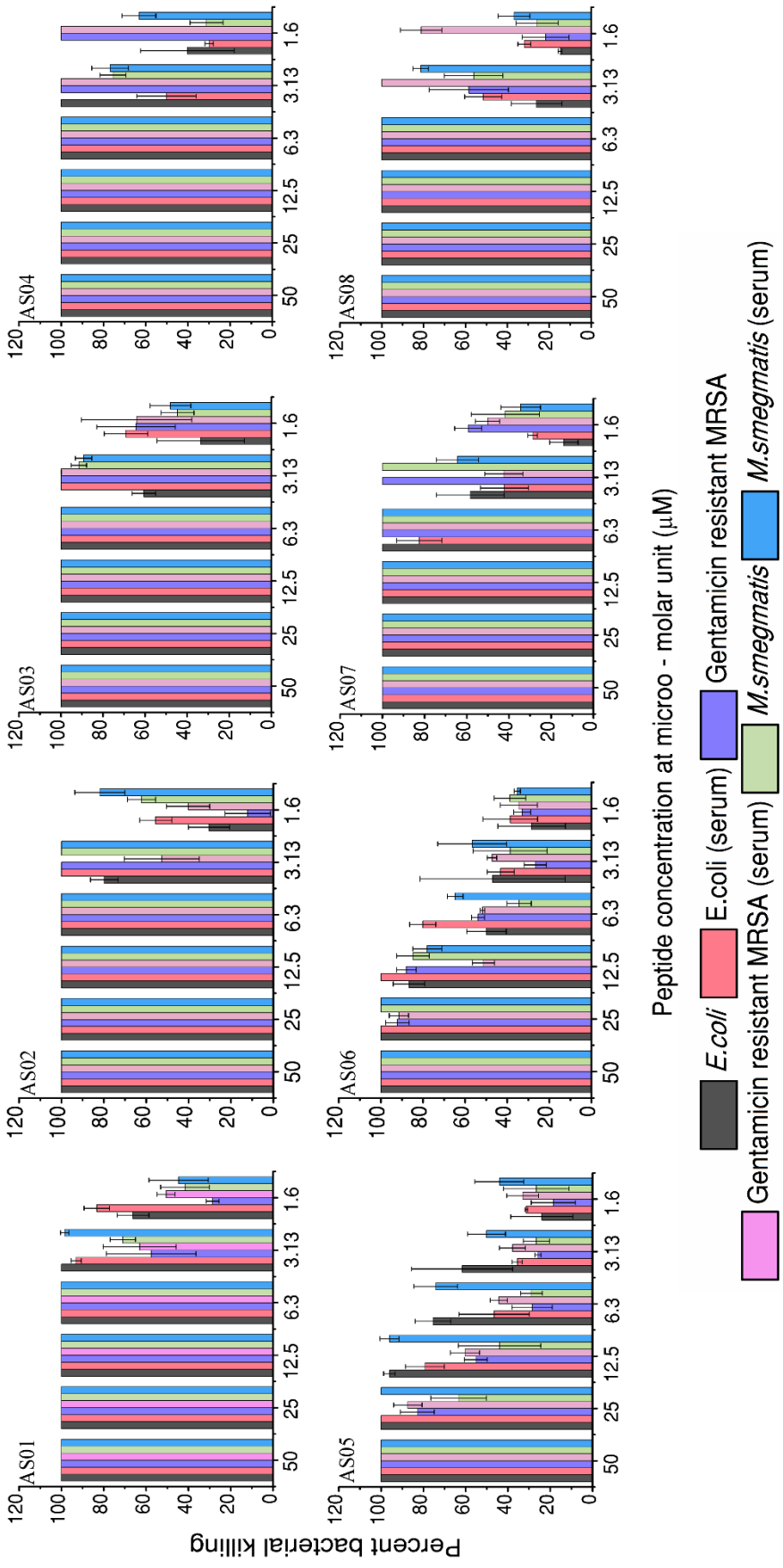


**Figure 8.3**. Antimicrobial activity of designed peptides (AS01 – AS08) against *E. coli*, Gentamicin resistant MRSA and *M.smegmatis* in media and serum containing media. The error bars represent standard deviation.

## 8.4 Conclusion

Increasing numbers of drug-resistant microbial species and limited antibiotic candidates have resulted in increased mortality rate and treatment regimen is becoming more complex and difficult [365]. In general, proteins and peptides of biological origin are polymers of L-amino acids [148]. They are prone to enzymatic degradation and ion sensitivity, apart from other ADME disadvantages [2, 5]. Apart from extended stability, D enantiomers increase the design possibilities many fold by accessing otherwise excluded areas of Ramachandran Map [7, 227]. All the designed peptides were good in terms of their antimicrobial activity except AS05 and AS06, and may be considered as potential candidates for further development and clinical trials.

# Chapter 9

# Conclusion and Future Work

We are witnessing a renaissance in the research of peptide based therapeutics, with 60 FDA approved peptide drugs and 140 peptide candidates in various phases of clinical trials. Market for peptide drugs is projected to reach US$ 25 billion mark by the end of 2018. Peptides are effectors in the innate immune response of eukaryotes. Minimal side effect, high tolerability and selectivity towards specific targets would help a peptide molecule successfully comply with the stringent safety standards set for clinical trials. However, low cell membrane permeability, low oral bio-availability and ion sensitivity are some of the factors that are working against their potential utility as a therapeutic agent. Along with a fairly large number of modifications possible on peptide based molecular systems, altering peptide chirality by designing peptides incorporating D-amino acid is probably the simplest one. Earlier reports suggest that hetero-chiral peptides have resisted proteolytic degradation with increased half-life.

We have designed, synthesized and characterized three series of peptides, asking three different levels of design questions. The principal objective of our first series of peptides explained in chapter 4 was to verify, whether we can use the gramicidin helix template for AMP design. The successful design of alternating LDLD peptide systems like gramicidin stereo-chemical sequence, but not having its higher levels of toxicity provided the confidence to design six model systems experimenting with all possible stereo-chemical options of syndiotactic stereochemistry. The impressive levels of bactericidal potential, especially against gram negative and resistant bacteria prompted us to extent the study to *Mycobacterium* species. All the peptide candidates have shown negligible cytotoxicity towards mammalian Red Blood Cells (RBCs). All peptide candidates to a varied extend retained their activity in serum, confirming its utility as a potent therapeutic agent in the presence of biotic factors like enzymatic degradation and ionic interruption. The third series of peptides comprising of eight model systems, display the usage of the knowledgebase acquired by us after designing the first two series into practice. The phenomenal levels of anti-bacterial potency exhibited by half a dozen peptides in this series, high

on specificity and less on toxicity, qualify them for next level of development as an effective therapeutic agent.

Despite having a fairly long list of therapeutic options available for anticancer treatment, there is always an enquiry of a new line of investigation in developing therapeutic agents with a new mode of action, primarily because of resistance by cancer cells towards currently available drugs. Some cationic antimicrobial peptides have already reported showing significant anti-cancer activities. This would be one future objective, this project can actively pursue.

Bacteria can attach themselves on to a surface, and grow as an assemblage forming a biofilm. They are mostly immune to the currently employed antibiotics. Reduced antibiotic susceptibility will result in the persistence of biofilm infections, especially on implanted devices. We would like to also extend our study to peptide based treatment of biofilm infections, in future projects.

1. Sachdeva, S. Peptides as 'Drugs': The Journey so Far. *International Journal of Peptide Research and Therapeutics* **23**, 49-60 (2017).
2. Fosgerau, K. & Hoffmann, T. Peptide therapeutics: current status and future directions. *Drug Discovery Today* **20**, 122-128 (2015).
3. Chandrudu, S., Simerska, P. & Toth, I. Chemical Methods for Peptide and Protein Production. *Molecules* **18**, 4373 (2013).
4. Loffet, A. Peptides as Drugs: Is There a Market? *Journal of Peptide Science* **8**, 1-7 (2002).
5. Di, L. Strategic Approaches to Optimizing Peptide ADME Properties. *The AAPS Journal* **17**, 134-143 (2015).
6. Durani, S. Protein design with L- and D-alpha-amino acid structures as the alphabet. *Accounts of chemical research* **41**, 1301-1308 (2008).
7. Hazam, P.K., Jerath, G., Kumar, A., Chaudhary, N. & Ramakrishnan, V. Effect of tacticity-derived topological constraints in bactericidal peptides. *Biochimica et biophysica acta. Biomembranes* **1859**, 1388-1395 (2017).
8. Kumar, A. & Ramakrishnan, V. Creating novel protein scripts beyond natural alphabets. *Systems and Synthetic Biology* **4**, 247-256 (2010).
9. Bollen, Y.J.M. & van Mierlo, C.P.M. Protein topology affects the appearance of intermediates during the folding of proteins with a flavodoxin-like fold. *Biophysical Chemistry* **114**, 181-189 (2005).
10. Jerath, G., Hazam, P.K., Shekhar, S. & Ramakrishnan, V. Mapping the Geometric Evolution of Protein Folding Motor. *PLOS One* **11**, e0163993 (2016).
11. Mucherino, A., Costantini, S., Serafino, D., Apuzzo, M.D., Facchiano, A & Colonna, G. Understanding the role of the topology in protein folding by computational inverse folding experiments. *Computational Biology and Chemistry* **32**, 233-239 (2008).
12. Ramakrishnan, V., Srinivasan, S.P., Salem, S.M., Matthews, S.J., Colón, W & Zaki, M. Geofold: Topology-based protein unfolding pathways capture the effects of engineered disulfides on kinetic stability. *Proteins: Structure, Function, and Bioinformatics* **80**, 920-934 (2012).
13. Michaud, G., Visini, R., Bergmann, M., Salerno, G., Bosco, R., Gillon, E., Richichi, B., C, Nativi., Imberty, A., Stocker, A., Darbare & T., Reymond, J.L. Overcoming antibiotic resistance in Pseudomonas aeruginosa biofilms using glycopeptide dendrimers. *Chemical Science* **7**, 166-182 (2016).
14. Wu, H., Niu, Y., Padhee, S., Wang, R. E., Li, Y., Qiao, Q., Bai, G., Cao, C & Cai, J. Design and synthesis of unprecedented cyclic [gamma]-AApeptides for antimicrobial development. *Chemical Science* **3**, 2570-2575 (2012).
15. Beg, S., Gaur, P. & Mishra, S. New. Drugs And Vaccines For Tuberculosis. *Recent patents on anti-infective drug discovery* (2017).
16. Sandhu, G. Tuberculosis: Current situation, challenges and overview of its control programs in India. *Journal of Global Infectious Diseases* **3**, 143-150 (2011).
17. Podinovskaia, M., Lee, W., Caldwell, S. & Russell, D.G. Infection of macrophages with Mycobacterium tuberculosis induces global modifications to phagosomal function. *Cellular Microbiology* **15**, 843-859 (2013).
18. Hazam, P.K., Singh, A., Chaudhary, N. & Ramakrishnan, V. Bactericidal Potency and Extended Serum Life of Stereo-Chemically Engineered Peptides Against Mycobacterium. *International Journal of Peptide Research and Therapeutics*, 1-8 (2018).

19. Zuniga, E.S., Early, J. & Parish, T. The future for early-stage tuberculosis drug discovery. *Future microbiology* **10**, 217-229 (2015).
20. Estrella, J., Kan-Sutton, C., Gong, K., Eissa, T.N., Rajagopalan, M., Lewis, D., Hunter, R. and Jagannath, C. A Novel in vitro Human Macrophage Model to Study the Persistence of Mycobacterium tuberculosis Using Vitamin D3 and Retinoic Acid Activated THP-1 Macrophages. *Frontiers in Microbiology* **2** (2011).
21. Paul, T. & Beveridge, T. Reevaluation of envelope profiles and cytoplasmic ultrastructure of mycobacteria processed by conventional embedding and freeze-substitution protocols. Journal of bacteriology 174, 6508-6517 (1992).
22. Hoffman, L.R., D'argenio, D.A., MacCoss, M.J., Zhang, Z., Jones, R.A. and Miller, S.I. Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* **436**, 1171-1175 (2005).
23. Ligon, B.L. Penicillin: its discovery and early development. *Seminars in pediatric infectious diseases* **15**, 52-57 (2004).
24. Chain, E., Florey, H. W., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J., Sanders, A. G & Peltier, L. F. The Classic: Penicillin as a chemotherapeutic agent. 1940. *Clinical orthopaedics and related research* **439**, 23-26 (2005).
25. Ehrlich, P. & Hata, S. Die experimentelle Chemotherapie der Spirillosen:(Syphilis, Rückfallfieber, Hühnerspirillose, Frambösie). (1910).
26. Domagk, G. Chemotherapie der streptokokken-infektionen. *Journal of Molecular Medicine* **15**, 1585-1590 (1936).
27. Clardy, J., Fischbach, M. & Currie, C. The natural history of antibiotics. *Current biology : CB* **19**, R437-R441 (2009).
28. Woodruff, H.B. Selman A. Waksman, Winner of the 1952 Nobel Prize for Physiology or Medicine. *Applied and Environmental Microbiology* **80**, 2-8 (2014).
29. Aminov, R.I. A Brief History of the Antibiotic Era: Lessons Learned and Challenges for the Future. *Frontiers in microbiology* **1**, 134 (2010).
30. Lewis, K. Platforms for antibiotic discovery. *Nature reviews. Drug discovery* **12**, 371-387 (2013).
31. Frere, J.M., Ghuysen, J.M., Reynolds, P.E. & Moreno, R. Binding of beta-lactam antibiotics to the exocellular DD-carboxypeptidase-transpeptidase of Streptomyces R39. *The Biochemical journal* **143**, 241-249 (1974).
32. Dalhoff, A., Janjic, N. & Echols, R. Redefining penems. *Biochemical Pharmacology* **71**, 1085-1095 (2006).
33. Fuchs, P., Jones, R. & Barry, A. In vitro antimicrobial activity of tigemonam, a new orally administered monobactam. *Antimicrobial agents and chemotherapy* **32**, 346-349 (1988).
34. Hsu Chen, C.C. & Feingold, D.S. Mechanism of polymyxin B action and selectivity toward biologic membranes. *Biochemistry* **12**, 2105-2111 (1973).
35. Hooper, D.C. Mode of action of fluoroquinolones. *Drugs* **58** Suppl 2, 6-10 (1999).
36. Henry, R.J. The mode of action of sulfonamides. *Bacteriological Reviews* **7**, 175-262 (1943).
37. Wehrli, W. & Staehelin, M. Actions of the rifamycins. *Bacteriological Reviews* **35**, 290-309 (1971).
38. Chopra, I. & Roberts, M. Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews* **65**, 232-260 (2001).
39. Kotra, L.P., Haddad, J. & Mobashery, S. Aminoglycosides: Perspectives on Mechanisms of Action and Resistance and Strategies to Counter Resistance. *Antimicrobial Agents and Chemotherapy* **44**, 3249-3256 (2000).

40. Mazzei, T., Mini, E., Novelli, A. & Periti, P. Chemistry and mode of action of macrolides. *Journal of Antimicrobial Chemotherapy* **31**, 1-9 (1993).
41. Szulawska, A. & Czyz, M. Molecular mechanisms of anthracyclines action. *Postepy higieny i medycyny doswiadczalnej (Online)* **60**, 78-100 (2006).
42. Koba, M. & Konopa, J. Actinomycin D and its mechanisms of action. *Postepy higieny i medycyny doswiadczalnej (Online)* **59**, 290-298 (2005).
43. Demain, A.L. & Sanchez, S. Microbial drug discovery: 80 years of progress. *Journal of Antibiotibiotics* **62**, 5-16 (2009).
44. Herrlich, P. & Schweiger, M. Nitrofurans, a group of synthetic antibiotics, with a new mode of action: discrimination of specific messenger RNA classes. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 3386-3390 (1976).
45. Wong, Weng R., Oliver, Allen G. & Linington, Roger G. Development of Antibiotic Activity Profile Screening for the Classification and Discovery of Natural Product Antibiotics. *Chemistry & Biology* **19**, 1483-1495 (2012).
46. Ventola, C.L. The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *Pharmacy and Therapeutics* **40**, 277-283 (2015).
47. Lushniak, B.D. Antibiotic resistance: a public health crisis. *Public health reports (Washington, D.C. : 1974)* **129**, 314-316 (2014).
48. Willyard, C. The drug-resistant bacteria that pose the greatest health threats. *Nature* **543**, 15 (2017).
49. Gould, I.M. Who's winning the war? *Journal of Antimicrobial Chemotherapy* **62**, 1113-1116 (2008).
50. Read, A.F. & Woods, R.J. Antibiotic resistance management. *Evolution, medicine, and public health* **2014**, 147 (2014).
51. Bartlett, J.G., Gilbert, D.N. & Spellberg, B. Seven ways to preserve the miracle of antibiotics. *Clinical infectious diseases* **56**, 1445-1450 (2013).
52. Control, C.f.D. & Prevention (2015).
53. Golkar, Z., Bagasra, O. & Pace, D.G. Bacteriophage therapy: a potential solution for the antibiotic resistance crisis. *Journal of infection in developing countries* **8**, 129-136 (2014).
54. Sengupta, S., Chattopadhyay, M.K. & Grossart, H.P. The multifaceted roles of antibiotics and antibiotic resistance in nature. *Frontiers in microbiology* **4**, 47 (2013).
55. Czaplewski, L. et al. Alternatives to antibiotics-a pipeline portfolio review. *The Lancet. Infectious diseases* **16**, 239-251 (2016).
56. Melo, M.N., Ferre, R. & Castanho, M.A. Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations. *Nature reviews. Microbiology* **7**, 245-250 (2009).
57. Wang, G., Li, X. & Wang, Z. APD3: the antimicrobial peptide database as a tool for research and education. *Nucleic Acids Research* **44**, D1087-D1093 (2016).
58. Sohlenkamp, C. & Geiger, O. Bacterial membrane lipids: diversity in structures and pathways. *FEMS Microbiology Reviews* **40**, 133-159 (2016).
59. Yeaman, M.R. & Yount, N.Y. Mechanisms of antimicrobial peptide action and resistance. *Pharmacological reviews* **55**, 27-55 (2003).
60. Silhavy, T.J., Kahne, D. & Walker, S. The Bacterial Cell Envelope. *Cold Spring Harbor Perspectives in Biology* **2**, a000414 (2010).
61. Wang, Z. & Wang, G. APD: the Antimicrobial Peptide Database. *Nucleic Acids Research* **32**, D590-D592 (2004).
62. Wimley, W.C. Describing the Mechanism of Antimicrobial Peptide Action with the Interfacial Activity Model. *ACS Chemical Biology* **5**, 905-917 (2010).

63. Rathinakumar, R., Walkenhorst, W.F. & Wimley, W.C. Broad-spectrum antimicrobial peptides by rational combinatorial design and high-throughput screening: the importance of interfacial activity. *Journal of the American Chemical Society* **131**, 7609-7617 (2009).
64. Rathinakumar, R. & Wimley, W.C. Biomolecular engineering by combinatorial design and high-throughput screening: small, soluble peptides that permeabilize membranes. *Journal of the American Chemical Society* **130**, 9849-9858 (2008).
65. Rathinakumar, R. & Wimley, W.C. High-throughput discovery of broad-spectrum peptide antibiotics. *The FASEB Journal* **24**, 3232-3238 (2010).
66. Yesylevskyy, S., Marrink, S.-J. & Mark, A.E. Alternative mechanisms for the interaction of the cell-penetrating peptides penetratin and the TAT peptide with lipid bilayers. *Biophysical Journal* **97**, 40-49 (2009).
67. Liu, X., Cao, R., Wang, S., Jia, J. & Fei, H. Amphipathicity determines different cytotoxic mechanisms of lysine-or arginine-rich cationic hydrophobic peptides in cancer cells. *Journal of Medicinal Chemistry* **59**, 5238-5247 (2016).
68. Sahoo, B.R. et al. Mechanistic and structural basis of bioengineered bovine Cathelicidin-5 with optimized therapeutic activity. *Scientific Reports* **7**, 44781 (2017).
69. Tew, G.N., Liu, D., Chen, B., Doerksen, R. J., Kaplan, J., Carroll, P. J., Klein, M. L & DeGrado, W. F. De novo design of biomimetic antimicrobial polymers. *Proceedings of the National Academy of Sciences* **99**, 5110-5114 (2002).
70. Hamuro, Y., Schneider, J.P. & DeGrado, W.F. De novo design of antibacterial β-peptides. *Journal of the American Chemical Society* **121**, 12200-12201 (1999).
71. Brogden, K.A., Ackermann, M. & Huttner, K.M. Small, anionic, and charge-neutralizing propeptide fragments of zymogens are antimicrobial. *Antimicrob Agents Chemother* **41**, 1615-1617 (1997).
72. Steffen, H., Reig, S., Wiedemann., Kalbacher, H., Deeg, M., Sahl, H. G, Peschel, A., Gotz, F., Garbe, C & Schittek, B. Naturally processed dermcidin-derived peptides do not permeabilize bacterial membranes and kill microorganisms irrespective of their charge. *Antimicrob Agents Chemother* **50**, 2608-2620 (2006).
73. Lai, R., Liu, H., Hui Lee, W. & Zhang, Y. An anionic antimicrobial peptide from toad Bombina maxima. *Biochemical and biophysical research communications* **295**, 796-799 (2002).
74. Lai, Y., Villaruz, A. E., Li, M., Cha, D. J., Sturdevant, D. E., & Otto, M.. The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci. *Molecular Microbiology* **63**, 497-506 (2007).
75. Fan, L., Sun, J., Zhou, M., Zhou, J., Lao, X., Zheng, H., & Xu, H. (2016). DRAMP: a comprehensive data repository of antimicrobial peptides. Scientific reports, 6, 24482.
76. Zhao, X., Wu, H., Lu, H., Li, G. & Huang, Q. LAMP: A Database Linking Antimicrobial Peptides. *PLoS ONE* **8**, e66557 (2013).
77. Mishra, B. & Wang, G. Ab Initio Design of Potent Anti-MRSA Peptides Based on Database Filtering Technology. *Journal of the American Chemical Society* **134**, 12426-12429 (2012).
78. Wang, G., Li, X. & Wang, Z. APD2: the updated antimicrobial peptide database and its application in peptide design. *Nucleic acids research* **37**, D933-D937 (2008).
79. Wang, G., Li, X. & Wang, Z. APD3: the antimicrobial peptide database as a tool for research and education. *Nucleic acids research* **44**, D1087-D1093 (2016).
80. Seshadri Sundararajan, V. et al. DAMPD: a manually curated antimicrobial peptide database. *Nucleic acids research* **40**, D1108-D1112 (2011).

81. Hammami, R., Zouhir, A., Hamida, J.B. & Fliss, I. BACTIBASE: a new web-accessible database for bacteriocin characterization. *Bmc Microbiology* **7**, 89 (2007).
82. Hammami, R., Zouhir, A., Le Lay, C., Hamida, J.B. & Fliss, I. BACTIBASE second release: a database and tool platform for bacteriocin characterization. *Bmc Microbiology* **10**, 22 (2010).
83. Di Luca, M., Maccari, G., Maisetta, G. & Batoni, G. BaAMPs: the database of biofilm-active antimicrobial peptides. *Biofouling* **31**, 193-199 (2015).
84. Wang, C.K., Kaas, Q., Chiche, L. & Craik, D.J. CyBase: a database of cyclic protein sequences and structures, with applications in protein discovery and engineering. *Nucleic acids research* **36**, D206-D210 (2007).
85. Thomas, S., Karnik, S., Barai, R.S., Jayaraman, V.K. & Idicula-Thomas, S. CAMP: a useful resource for research on antimicrobial peptides. *Nucleic acids research* **38**, D774-D780 (2009).
86. Seebah, S. et al. Defensins knowledgebase: a manually curated database and information source focused on the defensins family of antimicrobial peptides. *Nucleic acids research* **35**, D265-D268 (2006).
87. Novković, M., Simunić, J., Bojović, V., Tossi, A. & Juretić, D. DADP: the database of anuran defense peptides. *Bioinformatics* **28**, 1406-1407 (2012).
88. Gogoladze, G. et al. DBAASP: database of antimicrobial activity and structure of peptides. *FEMS microbiology letters* **357**, 63-68 (2014).
89. Fan, L. et al. DRAMP: a comprehensive data repository of antimicrobial peptides. *Scientific Reports* **6**, 24482 (2016).
90. Wu, H., Lu, H., Huang, J., Li, G. & Huang, Q. EnzyBase: a novel database for enzybiotic studies. *BMC microbiology* **12**, 54 (2012).
91. Gómez, E.A., Giraldo, P. & Orduz, S. InverPep: A database of invertebrate antimicrobial peptides. *Journal of global antimicrobial resistance* **8**, 13-17 (2017).
92. Théolier, J., Fliss, I., Jean, J. & Hammami, R. MilkAMP: a comprehensive database of antimicrobial peptides of dairy origin. *Dairy Science & Technology* **94**, 181-193 (2014).
93. Hammami, R., Ben Hamida, J., Vergoten, G. & Fliss, I. PhytAMP: a database dedicated to antimicrobial plant peptides. *Nucleic acids research* **37**, D963-D968 (2008).
94. Whitmore, L. & Wallace, B. The Peptaibol Database: a database for sequences and structures of naturally occurring peptaibols. *Nucleic Acids Research* **32**, D593-D594 (2004).
95. Li, J. et al. ThioFinder: a web-based tool for the identification of thiopeptide gene clusters in DNA sequences. *PLoS One* **7**, e45878 (2012).
96. Piotto, S.P., Sessa, L., Concilio, S. & Iannelli, P. YADAMP: yet another database of antimicrobial peptides. *International journal of antimicrobial agents* **39**, 346-351 (2012).
97. Fleming, A. On a remarkable bacteriolytic element found in tissues and secretions. *Proceedings of the Royal Society of London B: Biological Sciences* **93**, 306-317 (1922).
98. Dubos, R.J. Studies on a bactericidal agent extracted from a soil bacillus: I. Preparation of the agent. Its activity in vitro. *The Journal of experimental medicine* **70**, 1 (1939).
99. Dubos, R.J. Studies on a bactericidal agent extracted from a soil bacillus : ii. protective effect of the bactericidal agent against experimental pneumococcus infections in mice. *The Journal of Experimental Medicine* **70**, 11-17 (1939).
100. Van Epps, H.L. René Dubos: unearthing antibiotics. *The Journal of Experimental Medicine* **203**, 259-259 (2006).

101. Zanetti, M., Gennaro, R. & Romeo, D. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. *FEBS letters* **374**, 1-5 (1995).
102. Steiner, H. Secondary structure of the cecropins: antibacterial peptides from the moth Hyalophora cecropia. *FEBS letters* **137**, 283-287 (1982).
103. Ganz, T. Defensins: antimicrobial peptides of innate immunity. *Nature reviews immunology* **3**, 710-720 (2003).
104. Okada, M. & Natori, S. Primary structure of sarcotoxin I, an antibacterial protein induced in the hemolymph of Sarcophaga peregrina (flesh fly) larvae. *Journal of Biological Chemistry* **260**, 7174-7177 (1985).
105. Kokryakov, V. N., Harwig, S. S., Panyutich, E. A., Shevchenko, A. A., Aleshina, G. M., Shamova, O. V., & Lehrer, R. I. Protegrins: leukocyte antimicrobial peptides that combine features of corticostatic defensins and tachyplesins. *FEBS Letters* **327**, 231-236 (1993).
106. Amiche, M., Seon, A.A., Pierre, T.N. & Nicolas, P. The dermaseptin precursors: a protein family with a common preproregion and a variable C-terminal antimicrobial domain. *FEBS Letters* **456**, 352-356 (1999).
107. Kelkar, D.A. & Chattopadhyay, A. The gramicidin ion channel: A model membrane protein. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1768**, 2011-2025 (2007).
108. Andersson, D.I., Hughes, D. & Kubicek-Sutherland, J.Z. Mechanisms and consequences of bacterial resistance to antimicrobial peptides. *Drug Resistance Updates* **26**, 43-57 (2016).
109. Damodaran, S.E. & Madhan, S. Telavancin: A novel lipoglycopeptide antibiotic. *Journal of Pharmacology & Pharmacotherapeutics* **2**, 135-137 (2011).
110. Hansen, J.N. Nisin as a model food preservative. *Critical reviews in food science and nutrition* **34**, 69-93 (1994).
111. Takahashi, H., Caputo, G.A., Vemparala, S. & Kuroda, K. Synthetic Random Copolymers as a Molecular Platform To Mimic Host-Defense Antimicrobial Peptides. *Bioconjugate Chemistry* **28**, 1340-1350 (2017).
112. Kang, H.K., Kim, C., Seo, C.H. & Park, Y. The therapeutic applications of antimicrobial peptides (AMPs): a patent review. *Journal of microbiology (Seoul, Korea)* **55**, 1-12 (2017).
113. Seo, M.-D., Won, H.-S., Kim, J.-H., Mishig-Ochir, T. & Lee, B.-J. Antimicrobial peptides for therapeutic applications: a review. *Molecules* **17**, 12276-12286 (2012).
114. Epand, R. M & Vogel, H. Diversity of antimicrobial peptides and their mechanisms of action. Biochimica et Biophysica Acta (BBA) - Biomembranes **1462**, 11 - 28 (1999).
115. Thankappan, B., Jeyarajan, S., Hiroaki, S., Anbarasu, K., Natarajaseenivasan, K., & Fujii, N. Antimicrobial and antibiofilm activity of designed and synthesized antimicrobial peptide, KABT-AMP. *Applied biochemistry and biotechnology* **170**, 1184-1193 (2013).
116. Rajasekaran, G., Kim, E.Y. & Shin, S.Y. LL-37-derived membrane-active FK-13 analogs possessing cell selectivity, anti-biofilm activity and synergy with chloramphenicol and anti-inflammatory activity. *Biochimica et Biophysica acta-Biomembranes* **1859**, 722-733 (2017).
117. Wu, X., Li, Z., Li, X., Tian, Y., Fan, Y., Yu, C., Zhou, B., Liu, Y., Xiang, R. and Yang, L. Synergistic effects of antimicrobial peptide DP7 combined with antibiotics against multidrug-resistant bacteria. *Drug design, development and therapy* **11**, 939-946 (2017).

118. Giacometti, A., Cirioni, O., Kamysz, W., Silvestri, C., Licci, A., Riva, A., Łukasiak, J. and Scalise, G. In vitro activity of amphibian peptides alone and in combination with antimicrobial agents against multidrug-resistant pathogens isolated from surgical wound infection. *Peptides* **26**, 2111-2116 (2005).
119. Gopal, R., Kim, Y.G., Lee, J.H., Lee, S.K., Chae, J.D., Son, B.K., Seo, C.H. and Park, Y. Synergistic effects and antibiofilm properties of chimeric peptides against multidrug-resistant Acinetobacter baumannii strains. *Antimicrobial Agents and Chemotherapy* **58**, 1622-1629 (2014).
120. Skerlavaj, B., Benincasa, M., Risso, A., Zanetti, M. & Gennaro, R. SMAP-29: a potent antibacterial and antifungal peptide from sheep leukocytes. *FEBS Letters* **463**, 58-62 (1999).
121. Nguyen, L.T., Haney, E.F. & Vogel, H.J. The expanding scope of antimicrobial peptide structures and their modes of action. *Trends in biotechnology* **29**, 464-472 (2011).
122. Travkova, O.G., Moehwald, H. & Brezesinski, G. The interaction of antimicrobial peptides with membranes. *Advances in colloid and interface science* (2017).
123. Teixeira, V., Feio, M.J. & Bastos, M. Role of lipids in the interaction of antimicrobial peptides with membranes. *Progress in Lipid Research* **51**, 149-177 (2012).
124. Chung, P.Y. & Khanum, R. Antimicrobial peptides as potential anti-biofilm agents against multidrug-resistant bacteria. *Journal of Microbiology, Immunology and Infection* (2017).
125. Costerton, J.W., Stewart, P.S. & Greenberg, E.P. Bacterial biofilms: a common cause of persistent infections. *Science (New York, N.Y.)* **284**, 1318-1322 (1999).
126. Donlan, R.M. Biofilm Formation: A Clinically Relevant Microbiological Process. *Clinical Infectious Diseases* **33**, 1387-1392 (2001).
127. Flemming, H.-C. & Wingender, J. The biofilm matrix. *Nature reviews. Microbiology* **8**, 623-633 (2010).
128. Fux, C., Costerton, J.W., Stewart, P.S. & Stoodley, P. Survival strategies of infectious biofilms. *Trends in microbiology* **13**, 34-40 (2005).
129. Hancock, R.E. & Sahl, H.-G. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. *Nature biotechnology* **24**, 1551-1557 (2006).
130. Haught, C., Davis, G.D., Subramanian, R., Jackson, K.W. & Harrison, R.G. Recombinant production and purification of novel antisense antimicrobial peptide in Escherichia coli. *Biotechnology and bioengineering* **57**, 55-61 (1998).
131. Pierce, J.C., Maloy, W.L., Salvador, L. & Dungan, C.F. Recombinant expression of the antimicrobial peptide polyphemusin and its activity against the protozoan oyster pathogen Perkinsus marinus. *Molecular marine biology and biotechnology* **6**, 248-259 (1997).
132. Zorko, M., Japelj, B., Hafner-Bratkovič, I. & Jerala, R. Expression, purification and structural studies of a short antimicrobial peptide. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1788**, 314-323 (2009).
133. Zorko, M. & Jerala, R. Production of recombinant antimicrobial peptides in bacteria. *Antimicrobial Peptides: Methods and Protocols*, 61-76 (2010).
134. Lee, J.H., Kim, J.H., Hwang, S.W., Lee, W.J., Yoon, H.K., Lee, H.S. and Hong, S.S Lee, J. et al. High-level expression of antimicrobial peptide mediated by a fusion partner reinforcing formation of inclusion bodies. *Biochemical and Biophysical Research Communications* **277**, 575-580 (2000).
135. Nguyen, L.T., Chau, J.K., Perry, N.A., De Boer, L., Zaat, S.A. and Vogel, H.J. Serum Stabilities of Short Tryptophan- and Arginine-Rich Antimicrobial Peptide Analogs. *PLOS One* **5**, e12684 (2010).

136. Knappe, D., Henklein, P., Hoffmann, R. & Hilpert, K. Easy Strategy To Protect Antimicrobial Peptides from Fast Degradation in Serum. *Antimicrobial Agents and Chemotherapy* **54**, 4003-4005 (2010).
137. Renukuntla, J., Vadlapudi, A.D., Patel, A., Boddu, S.H.S. & Mitra, A.K. Approaches for Enhancing Oral Bioavailability of Peptides and Proteins. *International Journal of Pharmaceutics* **447**, 75-93 (2013).
138. Biron, E. et al. Improving oral bioavailability of peptides by multiple N-methylation: somatostatin analogues. *Angewandte Chemie (International ed. in English)* **47**, 2595-2599 (2008).
139. Breazeale, S.D., Ribeiro, A.A., McClerren, A.L. & Raetz, C.R.H. A Formyltransferase Required for Polymyxin Resistance in Escherichia coli and the Modification of Lipid A with 4-Amino-4-deoxy-l-arabinose: Identification And Function Of Udp-4-Deoxy-4-Formamido-L-Arabinose. *Journal of Biological Chemistry* **280**, 14154-14167 (2005).
140. Banemann, A., Deppisch, H. & Gross, R. The lipopolysaccharide of Bordetella bronchiseptica acts as a protective shield against antimicrobial peptides. *Infection and immunity* **66**, 5607-5612 (1998).
141. Fathy Mohamed, Y., Hamad, M., Ortega, X.P. & Valvano, M.A. The LpxL acyltransferase is required for normal growth and penta-acylation of lipid A in Burkholderia cenocepacia. *Molecular Microbiology* **104**, 144-162 (2017).
142. Rehal, R.P., Marbach, H., Hubbard, A.T., Sacranie, A.A., Sebastiani, F., Fragneto, G. and Harvey, R.D. The influence of mild acidity on lysyl-phosphatidylglycerol biosynthesis and lipid membrane physico-chemical properties in methicillin-resistant Staphylococcus aureus. *Chemistry and physics of lipids* **206**, 60-70 (2017).
143. Pálffy, R., Gardlík, R., Behuliak, M., Kadasi, L., Turna, J. and Celec, P. On the Physiology and Pathophysiology of Antimicrobial Peptides. *Molecular Medicine* **15**, 51-59 (2009).
144. Chan, C., Burrows, L.L. & Deber, C.M. Alginate as an auxiliary bacterial membrane: binding of membrane-active peptides by polysaccharides*. *The Journal of Peptide Research* **65**, 343-351 (2005).
145. Cole, J.N. & Nizet, V. Bacterial Evasion of Host Antimicrobial Peptide Defenses. *Microbiology spectrum* **4**, 10.1128/microbiolspec.VMBF-0006-2015 (2016).
146. Heimlich, D.R., Harrison, A. & Mason, K.M. Host antimicrobial peptides in bacterial homeostasis and pathogenesis of disease. *Antibiotics* **3**, 645-676 (2014).
147. Bahar, A.A. & Ren, D. Antimicrobial Peptides. *Pharmaceuticals* **6**, 1543-1575 (2013).
148. Durani, S. Protein Design with l- and d-α-Amino Acid Structures as the Alphabet. *Accounts of Chemical Research* **41**, 1301-1308 (2008).
149. Hazam, P.K., Jerath, G., Kumar, A., Chaudhary, N. & Ramakrishnan, V. Effect of tacticity-derived topological constraints in bactericidal peptides. *Biochimica et Biophysica Acta -Biomembranes* **1859**, 1388-1395 (2017).
150. Kuznetsova, I.M., Zaslavsky, B.Y., Breydo, L., Turoverov, K.K. & Uversky, V.N. Beyond the excluded volume effects: mechanistic complexity of the crowded milieu. *Molecules* **20**, 1377-1409 (2015).
151. Kumar, A., Ranbhor, R., Patel, K., Ramakrishnan, V. & Durani, S. Automated protein design: Landmarks and operational principles. *Progress in Biophysics & Molecular Biology* **125**, 24-35 (2017).
152. Ramakrishnan, V., Ranbhor, R. & Durani, S. Simulated folding in polypeptides of diversified molecular tacticity: implications for protein folding and de novo design. *Biopolymers* **78**, 96-105 (2005).

153. Melo, M.N., Ferre, R. & Castanho, M.A. Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations. *Nature reviews. Microbiology* **7**, 245 (2009).
154. Haney, E.F., Mansour, S.C. & Hancock, R.E.W. in Antimicrobial Peptides: Methods and Protocols. (ed. P.R. Hansen) 3-22 (Springer New York, New York, NY; 2017).
155. Blaskovich, M.A. Unusual Amino Acids in Medicinal Chemistry. *Journal of Medicinal Chemistry*. **59**, 10807-10836 (2016).
156. Huo, L. & van der Donk, W.A. Discovery and Characterization of Bicereucin, an Unusual d-Amino Acid-Containing Mixed Two-Component Lantibiotic. *Journal of American Chemical Society* **138**, 5254-5257 (2016).
157. Genchi, G. An overview on d-amino acids. *Amino Acids* **49**, 1521-1533 (2017).
158. Martinez-Rodriguez, S., Martinez-Gomez, A.I., Rodriguez-Vico, F., Clemente-Jimenez, J.M. & Las Heras-Vazquez, F.J. Natural occurrence and industrial applications of D-amino acids: an overview. *Chemistry & biodiversity* **7**, 1531-1548 (2010).
159. Baumann, T., Nickling, J.H., Bartholomae, M., Buivydas, A., Kuipers, O.P. and Budisa, N. Prospects of In vivo Incorporation of Non-canonical Amino Acids for the Chemical Diversification of Antimicrobial Peptides. *Frontiers in microbiology* **8**, 124 (2017).
160. Dubos, R.J. Studies on a bactericidal agent extracted from a soil bacillus: II. Protective effect of the bactericidal agent against experimental Pneumococcus infections in mice. *The Journal of experimental medicine* **70**, 11 (1939).
161. Urry, D. The gramicidin A transmembrane channel: a proposed π (L, D) helix. *Proceedings of the National Academy of Sciences United States of America* **68**, 672-676 (1971).
162. Hamamoto, K., Kida, Y., Zhang, Y., Shimizu, T. & Kuwano, K. Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions. *Microbiology and immunology* **46**, 741-749 (2002).
163. Tugyi, R., Uray, K., Iván, D., Fellinger, E., Perkins, A. and Hudecz, F. Partial D-amino acid substitution: Improved enzymatic stability and preserved Ab recognition of a MUC2 epitope peptide. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 413-418 (2005).
164. Miller, S.M. et al. Comparison of the proteolytic susceptibilities of homologous L-amino acid, D-amino acid, and N-substituted glycine peptide and peptoid oligomers. *Drug Development Research* **35**, 20-32 (1995).
165. Strömstedt, A.A., Pasupuleti, M., Schmidtchen, A. & Malmsten, M. Evaluation of Strategies for Improving Proteolytic Resistance of Antimicrobial Peptides by Using Variants of EFK17, an Internal Segment of LL-37. *Antimicrobial Agents and Chemotherapy* **53**, 593-602 (2009).
166. Li, X., Du, X., Li, J., Gao, Y., Pan, Y., Shi, J., Zhou, N. and Xu, B. Introducing d-Amino Acid or Simple Glycoside into Small Peptides to Enable Supramolecular Hydrogelators to Resist Proteolysis. *Langmuir* **28**, 13512-13517 (2012).
167. Hazam, P.K., Jerath, G., Chaudhary, N. & Ramakrishnan, V. Peptido-mimetic Approach in the Design of Syndiotactic Antimicrobial Peptides. *International Journal of Peptide Research and Therapeutics*, 1-9 (2017).
168. Herce, H.D. & Garcia, A.E. Molecular dynamics simulations suggest a mechanism for translocation of the HIV-1 TAT peptide across lipid membranes. *Proceedings of the National Academy of Sciences United States of America* **104**, 20805-20810 (2007).
169. Lundberg, P. & Langel, Ü. A brief introduction to cell-penetrating peptides. *Journal of Molecular Recognition* **16**, 227-233 (2003).

170. Huang, H.W., Chen, F.-Y. & Lee, M.-T. Molecular mechanism of peptide-induced pores in membranes. *Physical review letters* **92**, 198304 (2004).
171. Huang, H.W. Action of antimicrobial peptides: two-state model. *Biochemistry* **39**, 8347-8352 (2000).
172. Giuliani, A., Pirri, G. & Nicoletto, S. Antimicrobial peptides: an overview of a promising class of therapeutics. *Open Life Sciences* **2**, 1-33 (2007).
173. Hunter, H.N. et al. The interactions of antimicrobial peptides derived from lysozyme with model membrane systems. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1668**, 175-189 (2005).
174. Aoki, W. & Ueda, M. Characterization of Antimicrobial Peptides toward the Development of Novel Antibiotics. *Pharmaceuticals* **6**, 1055-1081 (2013).
175. Zasloff, M. Antimicrobial peptides of multicellular organisms. *nature* **415**, 389-395 (2002).
176. Shai, Y. & Oren, Z. Diastereomers of Cytolysins, a Novel Class of Potent Antibacterial Peptides. *Journal of Biological Chemistry* **271**, 7305-7308 (1996).
177. Shai, Y. Mode of action of membrane active antimicrobial peptides. *Peptide Science* **66**, 236-248 (2002).
178. Oren, Z. & Shai, Y. Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: structure– function study. *Biochemistry* **36**, 1826-1835 (1997).
179. Avrahami, D., Oren, Z. & Shai, Y. Effect of multiple aliphatic amino acids substitutions on the structure, function, and mode of action of diastereomeric membrane active peptides. *Biochemistry* **40**, 12591-12603 (2001).
180. Hong, J., Oren, Z. & Shai, Y. Structure and organization of hemolytic and nonhemolytic diastereomers of antimicrobial peptides in membranes. *Biochemistry* **38**, 16963-16973 (1999).
181. Jelokhani-niaraki, M., Kondejewski, L.H., Farmer, S.W., Hancock, R.E. & Hodges, R.S. Diastereoisomeric analogues of gramicidin S: structure, biological activity and interaction with lipid bilayers. *Biochemical Journal* **349**, 747-755 (2000).
182. Oren, Z., Hong, J. & Shai, Y. A Repertoire of Novel Antibacterial Diastereomeric Peptides with Selective Cytolytic Activity. *Journal of Biological Chemistry* **272**, 14643-14649 (1997).
183. Olli, S., Rangaraj, N. & Nagaraj, R. Effect of selectively introducing arginine and D-amino acids on the antimicrobial activity and salt sensitivity in analogs of human beta-defensins. *PloS one* **8**, e77031 (2013).
184. Lai, J.R., Epand, R.F., Weisblum, B., Epand, R.M. & Gellman, S.H. Roles of salt and conformation in the biological and physicochemical behavior of protegrin-1 and designed analogues: correlation of antimicrobial, hemolytic, and lipid bilayer-perturbing activities. *Biochemistry* **45**, 15718-15730 (2006).
185. Bastos, P., Trindade, F., da Costa, J., Ferreira, R. & Vitorino, R. Human Antimicrobial Peptides in Bodily Fluids: Current Knowledge and Therapeutic Perspectives in the Postantibiotic Era. *Medicinal research reviews* (2017).
186. Mor, A. Peptide-based antibiotics: A potential answer to raging antimicrobial resistance. *Drug Development Research* **50**, 440-447 (2000).
187. Park, H. et al. Nanofabrication and microfabrication of functional materials for tissue engineering. *Tissue engineering* **13**, 1867-1877 (2007).
188. Shin, H., Jo, S. & Mikos, A.G. Biomimetic materials for tissue engineering. *Biomaterials* **24**, 4353-4364 (2003).

189. Kuipers, O.P., de Ruyter, P.G.G.A., Kleerebezem, M. & de Vos, W.M. Quorum sensing-controlled gene expression in lactic acid bacteria. *Journal of Biotechnology* **64**, 15-21 (1998).
190. Nagai, Y., Unsworth, L.D., Koutsopoulos, S. & Zhang, S. Slow release of molecules in self-assembling peptide nanofiber scaffold. *Journal of Controlled Release* **115**, 18-25 (2006).
191. Schweizer, S. & Taubert, A. Polymer-Controlled, Bio-Inspired Calcium Phosphate Mineralization from Aqueous Solution. *Macromolecular Bioscience* **7**, 1085-1099 (2007).
192. Zelzer, M. & Ulijn, R.V. Next-generation peptide nanomaterials: molecular networks, interfaces and supramolecular functionality. *Chemical Society Reviews* **39**, 3351-3357 (2010).
193. Sanborn, T.J., Messersmith, P.B. & Barron, A.E. In situ crosslinking of a biomimetic peptide-PEG hydrogel via thermally triggered activation of factor XIII. *Biomaterials* **23**, 2703-2710 (2002).
194. Poluri, K.M. & Gulati, K. in Protein Engineering Techniques: Gateways to Synthetic Protein Universe. (eds. K.M. Poluri & K. Gulati) 1-25 (Springer Singapore, Singapore; 2017).
195. Jerath, G., Hazam, P.K., Shekhar, S. & Ramakrishnan, V. Mapping the Geometric Evolution of Protein Folding Motor. **11**, e0163993 (2016).
196. Yang, Y. & Zhou, Y. Ab initio folding of terminal segments with secondary structures reveals the fine difference between two closely related all-atom statistical energy functions. *Protein science* **17**, 1212-1219 (2008).
197. Dill, K.A. & MacCallum, J.L. The protein-folding problem, 50 years on. *science* **338**, 1042-1046 (2012).
198. Neidigh, J.W., Fesinmeyer, R.M. & Andersen, N.H. Designing a 20-residue protein. *Nature structural biology* **9**, 425-430 (2002).
199. Dahiyat, B.I. & Mayo, S.L. Protein design automation. *Protein Science* **5**, 895-903 (1996).
200. Dosler, S. & Karaaslan, E. Inhibition and destruction of Pseudomonas aeruginosa biofilms by antibiotics and antimicrobial peptides. *Peptides* **62**, 32-37 (2014).
201. Ravichandran, G. et al. Pellino-1 derived cationic antimicrobial prawn peptide: Bactericidal activity, toxicity and mode of action. *Molecular immunology* **78**, 171-182 (2016).
202. Czihal, P. & Hoffmann, R. Mapping of apidaecin regions relevant for antimicrobial activity and bacterial internalization. *International journal of peptide research and therapeutics* **15**, 157-164 (2009).
203. Saikia, K., Sravani, Y.D., Ramakrishnan, V. & Chaudhary, N. Highly potent antimicrobial peptides from N-terminal membrane-binding region of E. coli MreB. *Scientific reports* **7**, 42994 (2017).
204. Lehrer, R.I. Primate defensins. *Nature Review Microbiology* **2**, 727-738 (2004).
205. Moore, A.J., Beazley, W.D., Bibby, M.C. & Devine, D.A. Antimicrobial activity of cecropins. *The Journal of antimicrobial chemotherapy* **37**, 1077-1089 (1996).
206. Berkowitz, B.A., Bevins, C.L. & Zasloff, M.A. Magainins: a new family of membrane-active host defense peptides. *Biochemical pharmacology* **39**, 625-629 (1990).
207. Zanetti, M. The role of cathelicidins in the innate host defenses of mammals. *Current Issues in Molecular Biology* **7**, 179-196 (2005).
208. Felício, M.R., Silva, O.N., Gonçalves, S., Santos, N.C. & Franco, O.L. Peptides with dual antimicrobial and anticancer activities. *Frontiers in chemistry* **5**, 5 (2017).

209. Koh, J.-J. et al. Amino Acid Modified Xanthone Derivatives: Novel, Highly Promising Membrane-Active Antimicrobials for Multidrug-Resistant Gram-Positive Bacterial Infections. *Journal of Medicinal Chemistry* **58**, 739-752 (2015).
210. Téné, N. et al. Biochemical and biophysical combined study of bicarinalin, an ant venom antimicrobial peptide. *Peptides* **79**, 103-113 (2016).
211. Bechinger, B. & Lohner, K. Detergent-like actions of linear amphipathic cationic antimicrobial peptides. *Biochimica et Biophysica Acta-Biomembranes* **1758**, 1529-1539 (2006).
212. Chang, W.K., Wimley, W.C., Searson, P.C., Hristova, K. & Merzlyakov, M. Characterization of antimicrobial peptide activity by electrochemical impedance spectroscopy. *Biochimica et biophysica acta* **1778**, 2430-2436 (2008).
213. Brogden, K.A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nature reviews. Microbiology* **3**, 238-250 (2005).
214. Wang, F., Qin, L., Pace, C.J., Wong, P., Malonis, R. and Gao, J. Solubilized gramicidin A as potential systemic antibiotics. *Chembiochem* **13**, 51-55 (2012).
215. Wishart, D.S. et al. DrugBank: a knowledgebase for drugs, drug actions and drug targets. *Nucleic Acids Research* **36**, D901-D906 (2008).
216. Burkhart, B.M., Li, N., Langs, D.A., Pangborn, W.A. & Duax, W.L. The conducting form of gramicidin A is a right-handed double-stranded double helix. *Proceedings of the National Academy of Sciences United States of America* **95**, 12950-12955 (1998).
217. Burkhart, B.M. et al. Gramicidin D conformation, dynamics and membrane ion transport. *Biopolymers* **51**, 129-144 (1999).
218. Hladky, S.B. & Haydon, D.A. Ion transfer across lipid membranes in the presence of gramicidin A. I. Studies of the unit conductance channel. *Biochimica et biophysica acta- Biomembranes* **274**, 294-312 (1972).
219. LoGrasso, P.V., Moll, F. & Cross, T.A. Solvent history dependence of gramicidin A conformations in hydrated lipid bilayers. *Biophysical Journal* **54**, 259-267 (1988).
220. Killian, J.A., Prasad, K.U., Hains, D. & Urry, D.W. The membrane as an environment of minimal interconversion. A circular dichroism study on the solvent dependence of the conformational behavior of gramicidin in diacylphosphatidylcholine model membranes. *Biochemistry* **27**, 4848-4855 (1988).
221. Kelkar, D.A. & Chattopadhyay, A. The gramicidin ion channel: a model membrane protein. *Biochimica et Biophysica Acta -Biomembranes* **1768**, 2011-2025 (2007).
222. Ramakrishnan, V., Srinivasan, S.P., Salem, S.M., Matthews, S.J., Colon, W. and Zaki, M. GeoFold: Topology-based protein unfolding pathways capture the effects of engineered disulfides on kinetic stability. *Proteins* **80**, 920-934 (2012).
223. Khoury, G.A., Smadbeck, J., Kieslich, C.A. & Floudas, C.A. Protein folding and de novo protein design for biotechnological applications. *Trends in biotechnology* **32**, 99-109 (2014).
224. Billmeyer, F.W. Textbook of polymer science. (1971).
225. Nanda, V., Andrianarijaona, A. & Narayanan, C. The role of protein homochirality in shaping the energy landscape of folding. *Protein Science : A Publication of the Protein Society* **16**, 1667-1675 (2007).
226. Tien, M.Z., Sydykova, D.K., Meyer, A.G. & Wilke, C.O. PeptideBuilder: A simple Python library to generate model peptides. *PeerJ* **1**, e80 (2013).
227. Legrand, B. et al. Selectivity Modulation and Structure of α/aza-$\beta^3$ Cyclic Antimicrobial Peptides. Chemistry (2018).
228. Pronk, S. et al. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics* **29**, 845-854 (2013).

229. Christen, M., Hünenberger, P.H., Bakowies, D., Baron, R., Bürgi, R., Geerke, D.P., Heinz, T.N., Kastenholz, M.A., Kräutler, V.. The GROMOS software for biomolecular simulation: GROMOS05. *Journal of computational chemistry* **26**, 1719-1751 (2005).
230. Chaudhary, N. & Nagaraj, R. Impact on the replacement of Phe by Trp in a short fragment of Aβ amyloid peptide on the formation of fibrils. *Journal of Peptide Science* **17**, 115-123 (2011).
231. Piaru, S.P., Mahmud, R. & Perumal, S. Determination of antibacterial activity of essential oil of Myristica fragrans Houtt. Using tetrazolium microplate assay and its cytotoxic activity against vero cell line. *International Journal of Pharmacology* **8**, 572-576 (2012).
232. Rufián-Henares, J.A. & Morales, F.J. Microtiter plate-based assay for screening antimicrobial activity of melanoidins against E. coli and S. aureus. *Food Chemistry* **111**, 1069-1074 (2008).
233. Wu, X. et al. In vitro and in vivo activities of antimicrobial peptides developed using an amino acid-based activity prediction method. *Antimicrobial agents and chemotherapy* **58**, 5342-5349 (2014).
234. Abd-El-Aziz, A.S., Agatemor, C., Etkin, N., Overy, D.P., Lanteigne, M., McQuillan, K. & Kerr, R.G. Antimicrobial Organometallic Dendrimers with Tunable Activity against Multidrug-Resistant Bacteria. *Biomacromolecules* **16**, 3694-3703 (2015).
235. Balaji, S.N. & Trivedi, V. Extracellular Methemoglobin Mediated Early ROS Spike Triggers Osmotic Fragility and RBC Destruction: An Insight into the Enhanced Hemolysis During Malaria. *Indian journal of clinical biochemistry : IJCB* **27**, 178-185 (2012).
236. Lee, M. R., Raman, N., Gellman, S.H., Lynn, D.M. & Palecek, S.P. Hydrophobicity and Helicity Regulate the Antifungal Activity of 14-Helical β-Peptides. *ACS Chemical Biology* **9**, 1613-1621 (2014).
237. Katsaras, J., Prosser, R.S., Stinson, R.H. & Davis, J.H. Constant helical pitch of the gramicidin channel in phospholipid bilayers. *Biophysical Journal* **61**, 827-830 (1992).
238. Ramakrishnan, V., Ranbhor, R., Kumar, A. & Durani, S. The link between sequence and conformation in protein structures appears to be stereochemically established. *The journal of physical chemistry. B* **110**, 9314-9323 (2006).
239. Kumar, A., Ramakrishnan, V., Ranbhor, R., Patel, K. & Durani, S. Homochiral stereochemistry: the missing link of structure to energetics in protein folding. *The Journal of Physical Chemistry. B* **113**, 16435-16442 (2009).
240. Ranbhor, R., Ramakrishnan, V., Kumar, A. & Durani, S. The interplay of sequence and stereochemistry in defining conformation in proteins and polypeptides. *Biopolymers* **83**, 537-545 (2006).
241. Xiong, Y.Q., Mukhopadhyay, K., Yeaman, M.R., Adler-Moore, J. & Bayer, A.S. Functional Interrelationships between Cell Membrane and Cell Wall in Antimicrobial Peptide-Mediated Killing of Staphylococcus aureus. *Antimicrobial Agents and Chemotherapy* **49**, 3114-3121 (2005).
242. Drazic, A., Myklebust, L.M., Ree, R. & Arnesen, T. The world of protein acetylation. *Biochimica et Biophysica Acta - Proteins and Proteomics* **1864**, 1372-1401 (2016).
243. Di, L. Strategic approaches to optimizing peptide ADME properties. *The AAPS journal* **17**, 134-143 (2015).
244. Suat, K. & Jois, S.D. Design of β-turn based therapeutic agents. *Current pharmaceutical design* **9**, 1209-1224 (2003).
245. Schmidt, N.W., Mishra, A., Lai, G.H., Davis, M., Sanders, L.K., Tran, D., Garcia, A., Tai, K.P., McCray Jr, P.B., Ouellette, A.J. and Selsted, M.E. Criterion for amino acid

composition of defensins and antimicrobial peptides based on geometry of membrane destabilization. *Journal of the American Chemical Society* **133**, 6720-6727 (2011).

246. Wang, C.K., King, G.J., Conibear, A.C., Ramos, M.C., Chaousis, S., Henriques, S.T. and Craik, D.J. Mirror Images of Antimicrobial Peptides Provide Reflections on Their Functions and Amyloidogenic Properties. *Journal of the American Chemical Society* **138**, 5706-5713 (2016).
247. Otvos, L., Jr. Peptide-based drug design: here and now. *Methods in molecular biology (Clifton, N.J.)* **494**, 1-8 (2008).
248. Saikia, K., Sravani, Y.D., Ramakrishnan, V. & Chaudhary, N. Highly potent antimicrobial peptides from N-terminal membrane-binding region of E. coli MreB. *Scientific reports* **7**, 42994 (2017).
249. Brant, D.A. & Flory, P.J. The Configuration of Random Polypeptide Chains. I. Experimental Results. *Journal of the American Chemical Society* **87**, 2788-2791 (1965).
250. Brant, D.A. & Flory, P.J. The Configuration of Random Polypeptide Chains. II. Theory. *Journal of the American Chemical Society* **87**, 2791-2800 (1965).
251. Flory, P.J. Principles of polymer chemistry. (Cornell University Press, 1953).
252. Ghadiri, M.R., Granja, J.R., Milligan, R.A., McRee, D.E. & Khazanovich, N. Self-assembling organic nanotubes based on a cyclic peptide architecture. *Nature* **366**, 324-327 (1993).
253. Liu, D. & DeGrado, W.F. De Novo Design, Synthesis, and Characterization of Antimicrobial β-Peptides. *Journal of the American Chemical Society* **123**, 7553-7559 (2001).
254. Myrberg, H., Zhang, L., Mäe, M. & Langel, Ü. Design of a Tumor-Homing Cell-Penetrating Peptide. *Bioconjugate Chemistry* **19**, 70-75 (2008).
255. Pasqualini, R. et al. Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis. *Cancer research* **60**, 722-727 (2000).
256. Wang, G., Li, X. & Wang, Z. APD2: the updated antimicrobial peptide database and its application in peptide design. *Nucleic acids research* **37**, D933-937 (2009).
257. Kapoor, P., Singh, H., Gautam, A., Chaudhary, K., Kumar, R. and Raghava, G.P. TumorHoPe: A Database of Tumor Homing Peptides. *PLOS One* **7**, e35187 (2012).
258. Agrawal, P., Bhalla, S., Usmani, S.S., Singh, S., Chaudhary, K., Raghava, G.P. and Gautam, A. CPPsite 2.0: a repository of experimentally validated cell-penetrating peptides. *Nucleic acids research* **44**, D1098-1103 (2016).
259. Fernandez-Lopez, S. et al. Antibacterial agents based on the cyclic d,l-[alpha]-peptide architecture. *Nature* **412**, 452-455 (2001).
260. Horne, W.S., Wiethoff, C.M., Cui, C., Wilcoxen, K.M., Amorin, M., Ghadiri, M.R. and Nemerow, G.R. Antiviral cyclic D,L-alpha-peptides: targeting a general biochemical pathway in virus infections. *Bioorganic & medicinal chemistry* **13**, 5145-5153 (2005).
261. Albada, H.B., Prochnow, P., Bobersky, S., Bandow, J.E. & Metzler-Nolte, N. Highly active antibacterial ferrocenoylated or ruthenocenoylated Arg-Trp peptides can be discovered by an L-to-D substitution scan. *Chemical Science* **5**, 4453-4459 (2014).
262. Ravipati, A.S., Henriques, S.T., Poth, A.G., Kaas, Q., Wang, C.K., Colgrave, M.L. and Craik, D.J. Lysine-rich Cyclotides: A New Subclass of Circular Knotted Proteins from Violaceae. *ACS chemical biology* **10**, 2491-2500 (2015).
263. Gautam, S., Kim, T., Lester, E., Deep, D. & Spiegel, D.A. Wall teichoic acids prevent antibody binding to epitopes within the cell wall of Staphylococcus aureus. *ACS chemical biology* **11**, 25-30 (2016).

264. Stach, M., Siriwardena, T.N., Köhler, T., Van Delden, C., Darbre, T. and Reymond, J.L. Combining topology and sequence design for the discovery of potent antimicrobial peptide dendrimers against multidrug-resistant Pseudomonas aeruginosa. *Angewandte Chemie (International ed. in English)* **53**, 12827-12831 (2014).
265. Chen, H., Liu, C., Chen, D., Madrid, K., Peng, S., Dong, X., Zhang, M. and Gu, Y. Bacteria-Targeting Conjugates Based on Antimicrobial Peptide for Bacteria Diagnosis and Therapy. *Molecular Pharmaceutics* **12**, 2505-2516 (2015).
266. Dartois, V., Sanchez-Quesada, J., Cabezas, E., Chi, E., Dubbelde, C., Dunn, C., Granja, J., Gritzen, C., Weinberger, D., Ghadiri, M.R. and Parr, T.R. Systemic antibacterial activity of novel synthetic cyclic peptides. *Antimicrobial agents and chemotherapy* **49**, 3302-3310 (2005).
267. Ghadiri, M.R., Granja, J.R. & Buehler, L.K. Artificial transmembrane ion channels from self-assembling peptide nanotubes. *Nature* **369**, 301-304 (1994).
268. Kim, H.S., Hartgerink, J.D. & Ghadiri, M.R. Oriented Self-Assembly of Cyclic Peptide Nanotubes in Lipid Membranes. *Journal of the American Chemical Society* **120**, 4417-4424 (1998).
269. Papo, N. & Shai, Y. Effect of Drastic Sequence Alteration and d-Amino Acid Incorporation on the Membrane Binding Behavior of Lytic Peptides. *Biochemistry* **43**, 6393-6403 (2004).
270. Benkirane, N., Friede, M., Guichard, G., Briand, J.P., Van Regenmortel, M.H. and Muller, S. Antigenicity and immunogenicity of modified synthetic peptides containing D-amino acid residues. Antibodies to a D-enantiomer do recognize the parent L-hexapeptide and reciprocally. *The Journal of biological chemistry* **268**, 26279-26285 (1993).
271. Fisher, P.J., Prendergast, F.G., Ehrhardt, M.R., Urbauer, J.L., Wand, A.J., Sedarous, S.S., McCormick, D.J. and Buckley, P.J. Calmodulin interacts with amphiphilic peptides composed of all D-amino acids. *Nature* **368**, 651 (1994).
272. Wade, D., Boman, A., Wåhlin, B., Drain, C.M., Andreu, D., Boman, H.G. and Merrifield, R.B. All-D amino acid-containing channel-forming antibiotic peptides. *Proceedings of the National Academy of Sciences* **87**, 4761-4765 (1990).
273. Zhang, L. & Falla, T.J. Cationic antimicrobial peptides - an update. *Expert Opinion on Investigational Drugs* **13**, 97-106 (2004).
274. Raguse, T.L., Porter, E.A., Weisblum, B. & Gellman, S.H. Structure-activity studies of 14-helical antimicrobial beta-peptides: probing the relationship between conformational stability and antimicrobial potency. *Journal of the American Chemical Society* **124**, 12774-12785 (2002).
275. Reymond, J.-L. & Darbre, T. Expanding the topological space of bioactive peptides. *CHIMIA* **67**, 864-867 (2013).
276. Findlay, B., Zhanel, G.G. & Schweizer, F. Cationic amphiphiles, a new generation of antimicrobials inspired by the natural antimicrobial peptide scaffold. *Antimicrobial agents and chemotherapy* **54**, 4049-4058 (2010).
277. Holtzel, A., Jack, R.W., Nicholson, G.J., Jung, G., Gebhardt, K., Fiedler, H.P. & Sussmuth, R.D. Streptocidins AD, Novel Cyclic Decapeptide Antibiotics Produced by Streptomyces sp. Tü 6071. *The Journal of antibiotics* **54**, 434-440 (2001).
278. Dahiyat, B.I. & Mayo, S.L. De novo protein design: fully automated sequence selection. *Science* **278**, 82-87 (1997).
279. Dennison, S.R., Baker, R.D., Nicholl, I.D. & Phoenix, D.A. Interactions of cell penetrating peptide Tat with model membranes: a biophysical study. *Biochemical and Biophysical Research Communications* **363**, 178-182 (2007).

280. Dennison, S.R., Kim, Y.S., Cha, H.J. & Phoenix, D.A. Investigations into the ability of the peptide, HAL18, to interact with bacterial membranes. *European Biophysics Journal* **38**, 37 (2008).
281. Paczesny, J., Binkiewicz, I., Janczuk, M., Wybrańska, K., Richter, Ł. and Hołyst, R. Langmuir and Langmuir–Blodgett Films of Unsymmetrical and Fully Condensed Polyhedral Oligomeric Silsesquioxanes (POSS). *The Journal of Physical Chemistry C* **119**, 27007-27017 (2015).
282. Varkey, J. & Nagaraj, R. Antibacterial activity of human neutrophil defensin HNP-1 analogs without cysteines. *Antimicrobial agents and chemotherapy* **49**, 4561-4566 (2005).
283. Li, L., Li, C., Sarkar, S., Zhang, J., Witham, S., Zhang, Z., Wang, L., Smith, N., Petukh, M. and Alexov, E. DelPhi: a comprehensive suite for DelPhi software and associated resources. *BMC Biophysics* **5**, 9 (2012).
284. Li, C., Petukh, M., Li, L. & Alexov, E. Continuous development of schemes for parallel computing of the electrostatics in biological systems: Implementation in DelPhi. *Journal of computational chemistry* **34**, 1949-1960 (2013).
285. Pronk, S., Páll, S., Schulz, R., Larsson, P., Bjelkmar, P., Apostolov, R., Shirts, M.R., Smith, J.C., Kasson, P.M., Van Der Spoel, D. and Hess, B. GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. *Bioinformatics (Oxford, England)* **29**, 845-854 (2013).
286. Dennison, S.R., Kim, Y.S., Cha, H.J. & Phoenix, D.A. Investigations into the ability of the peptide, HAL18, to interact with bacterial membranes. *European biophysics journal* **38**, 37-43 (2008).
287. Comer, J., Dehez, F.o., Cai, W. & Chipot, C. Water conduction through a peptide nanotube. *The Journal of Physical Chemistry C* **117**, 26797-26803 (2013).
288. Tamma, P.D., Cosgrove, S.E. & Maragakis, L.L. Combination therapy for treatment of infections with gram-negative bacteria. *Clinical microbiology reviews* **25**, 450-470 (2012).
289. Rigano, M.M., Romanelli, A., Fulgione, A., Nocerino, N., D'Agostino, N., Avitabile, C., Frusciante, L., Barone, A., Capuano, F. and Capparelli, R. A novel synthetic peptide from a tomato defensin exhibits antibacterial activities against Helicobacter pylori. *Journal of peptide* **18**, 755-762 (2012).
290. Scott, K.L., Lecak, J. & Acker, J.P. Biopreservation of red blood cells: past, present, and future. *Transfusion medicine reviews* **19**, 127-142 (2005).
291. Ventola, C.L. The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and Therapeutics* **40**, 277 (2015).
292. Zur Wiesch, P.A., Kouyos, R., Engelstädter, J., Regoes, R.R. & Bonhoeffer, S. Population biological principles of drug-resistance evolution in infectious diseases. *The Lancet infectious diseases* **11**, 236-247 (2011).
293. Roy, H. & Ibba, M. RNA-dependent lipid remodeling by bacterial multiple peptide resistance factors. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 4667-4672 (2008).
294. Kumariya, R., Sood, S.K., Rajput, Y.S., Saini, N. & Garsa, A.K. Increased membrane surface positive charge and altered membrane fluidity leads to cationic antimicrobial peptide resistance in Enterococcus faecalis. *Biochimica et biophysica acta-Biomembranes* **1848**, 1367-1375 (2015).
295. Maria-Neto, S., de Almeida, K.C., Macedo, M.L.R. & Franco, O.L. Understanding bacterial resistance to antimicrobial peptides: From the surface to deep inside. *Biochimica et Biophysica Acta - Biomembranes* **1848**, 3078-3088 (2015).

296. Sahl, H.-G. & Shai, Y. Bacterial resistance to antimicrobial peptides. *Biochimica et Biophysica Acta - Biomembranes* **1848**, 3019-3020 (2015).
297. Carranza-Rosales, P. et al. Modeling tuberculosis pathogenesis through ex vivo lung tissue infection. *Tuberculosis* **107**, 126-132 (2017).
298. Paterson, D.J., Tassieri, M., Reboud, J., Wilson, R. & Cooper, J.M. Lipid topology and electrostatic interactions underpin lytic activity of linear cationic antimicrobial peptides in membranes. *Proceedings of the National Academy of Sciences* **114**, E8324-E8332 (2017).
299. Jaskiewicz, M. et al. Rapid screening of antimicrobial synthetic peptides. *International journal of peptide research and therapeutics* **22**, 155-161 (2016).
300. Arakha, M., Borah, S.M., Saleem, M., Jha, A.N. & Jha, S. Interfacial assembly at silver nanoparticle enhances the antibacterial efficacy of nisin. *Free Radical Biology and Medicine* **101**, 434-445 (2016).
301. Kaur, K.J., Sarkar, P., Nagpal, S., Khan, T. & Salunke, D.M. Structure–function analyses involving palindromic analogs of tritrypticin suggest autonomy of anti-endotoxin and antibacterial activities. *Protein Science* **17**, 545-554 (2008).
302. Koh, J.J., Lin, S., Aung, T.T., Lim, F., Zou, H., Bai, Y., Li, J., Lin, H., Pang, L.M., Koh, W.L. and Salleh, S.M. Amino acid modified xanthone derivatives: novel, highly promising membrane-active antimicrobials for multidrug-resistant Gram-positive bacterial infections. *Journal of medicinal chemistry* **58**, 739-752 (2014).
303. Molchanova, N., Hansen, P. & Franzyk, H. Advances in Development of Antimicrobial Peptidomimetics as Potential Drugs. *Molecules* **22**, 1430 (2017).
304. Yeaman, M.R. & Yount, N.Y. Mechanisms of antimicrobial peptide action and resistance. *Pharmacological reviews* **55**, 27-55 (2003).
305. Henry, R.R., Rosenstock, J., Logan, D., Alessi, T., Luskey, K. & Baron, M.A. Continuous subcutaneous delivery of exenatide via ITCA 650 leads to sustained glycemic control and weight loss for 48 weeks in metformin-treated subjects with type 2 diabetes. *Journal of Diabetes and its Complications* **28**, 393-398 (2014).
306. Greene, C.H., Power, M.H. & Bledsoe, W.t.t.a.o.M.S. The distribution of electrolytes between serum and the in-vivo dialysate. *Journal of Biological Chemistry* **91**, 183-202 (1931).
307. Anderson, L. Candidate-based proteomics in the search for biomarkers of cardiovascular disease. *The Journal of Physiology* **563**, 23-60 (2005).
308. Moncla, B.J., Pryke, K., Rohan, L.C. & Graebing, P.W. Degradation of naturally occurring and engineered antimicrobial peptides by proteases. *Advances in bioscience and biotechnology* **2**, 404-408 (2011).
309. Stefanini, A.C.B., da Cunha, B.R., Henrique, T. & Tajara, E.H. Involvement of Kallikrein-Related Peptidases in Normal and Pathologic Processes. *Disease Markers* **2015**, 946572 (2015).
310. Starr, C.G. & Wimley, W.C. Antimicrobial peptides are degraded by the cytosolic proteases of human erythrocytes. *Biochimica et Biophysica Acta - Biomembranes* **1859**, 2319-2326 (2017).
311. Jenssen, H. & Aspmo, S.I. in Peptide-Based Drug Design. (ed. L. Otvos) 177-186 (Humana Press, Totowa, NJ; 2008).
312. Kooi, C. & Sokol, P.A. Burkholderia cenocepacia zinc metalloproteases influence resistance to antimicrobial peptides. *Microbiology* **155**, 2818-2825 (2009).
313. Fjell, C.D., Hiss, J.A., Hancock, R.E.W. & Schneider, G. Designing antimicrobial peptides: form follows function. *Nat Rev Drug Discov* **11**, 37-51 (2012).

314. Joo, H.-S., Fu, C.-I. & Otto, M. Bacterial strategies of resistance to antimicrobial peptides. *Philosophical Transactions of the Royal Society B: Biological Sciences* **371** (2016).
315. Nizet, V. Antimicrobial peptide resistance mechanisms of human bacterial pathogens. *Current issues in molecular biology* **8**, 11-26 (2006).
316. Brannon, J.R., Thomassin, J.-L., Gruenheid, S. & Le Moual, H. Antimicrobial Peptide Conformation as a Structural Determinant of Omptin Protease Specificity. *Journal of Bacteriology* **197**, 3583-3591 (2015).
317. Park, I.Y., Cho, J.H., Kim, K.S., Kim, Y.B., Kim, M.S. and Kim, S.C. Helix Stability Confers Salt Resistance upon Helical Antimicrobial Peptides. *Journal of Biological Chemistry* **279**, 13896-13901 (2004).
318. Bals, R., Goldman, M.J. & Wilson, J.M. Mouse β-Defensin 1 Is a Salt-Sensitive Antimicrobial Peptide Present in Epithelia of the Lung and Urogenital Tract. *Infection and Immunity* **66**, 1225-1232 (1998).
319. Shai, Y. Mode of action of membrane active antimicrobial peptides. *Biopolymers* **66**, 236-248 (2002).
320. Oren, Z. & Shai, Y. Selective Lysis of Bacteria but Not Mammalian Cells by Diastereomers of Melittin: Structure–Function Study. *Biochemistry* **36**, 1826-1835 (1997).
321. Phyu, S. et al. Drug-Resistant Mycobacterium tuberculosis among New Tuberculosis Patients, Yangon, Myanmar. *Emerging Infectious Diseases* **9**, 274-276 (2003).
322. Fofana, M.O. et al. A Multistrain Mathematical Model To Investigate the Role of Pyrazinamide in the Emergence of Extensively Drug-Resistant Tuberculosis. *Antimicrobial Agents and Chemotherapy* **61**, e00498-00416 (2017).
323. Falzon, D. et al. World Health Organization treatment guidelines for drug-resistant tuberculosis, 2016 update. *The European Respiratory Journal* **49**, 1602308 (2017).
324. Tenover, F.C. in Encyclopedia of Microbiology (Third Edition) 67-77 (Academic Press, Oxford; 2009).
325. Chung, G.A., Aktar, Z., Jackson, S. & Duncan, K. High-throughput screen for detecting antimycobacterial agents. *Antimicrob Agents Chemother* **39**, 2235-2238 (1995).
326. Tenover, F. Antibiotic susceptibility testing. (2009).
327. Xu, W., Zhu, X., Tan, T., Li, W. & Shan, A. Design of Embedded-Hybrid Antimicrobial Peptides with Enhanced Cell Selectivity and Anti-Biofilm Activity. *PLoS One* **9**, e98935 (2014).
328. Khusro, A., Aarti, C. & Agastian, P. Anti-tubercular peptides: A quest of future therapeutic weapon to combat tuberculosis. *Asian Pacific Journal of Tropical Medicine* **9**, 1023-1034 (2016).
329. Mogi, T., Murase, Y., Mori, M., Shiomi, K., Ōmura, S., Paranagama, M.P. and Kita, K. Polymyxin B identified as an inhibitor of alternative NADH dehydrogenase and malate: quinone oxidoreductase from the Gram-positive bacterium Mycobacterium smegmatis. *Journal of biochemistry* **146**, 491-499 (2009).
330. Gutsmann, T. Interaction between antimicrobial peptides and mycobacteria. *Biochimica et Biophysica Acta (BBA) - Biomembranes* **1858**, 1034-1043 (2016).
331. Sommer, M.O.A., Munck, C., Toft-Kehler, R.V. & Andersson, D.I. Prediction of antibiotic resistance: time for a new preclinical paradigm? **15**, 689 (2017).
332. Lienhardt, C., Nahid, P., Rich, M.L., Bansbach, C., Kendall, E.A., Churchyard, G., González-Angulo, L., D'Ambrosio, L., Migliori, G.B. and Raviglione, M. Target

regimen profiles for treatment of tuberculosis: a WHO document. *European Respiratory Journal* **49** (2017).

333. Verreck, F.A., Tchilian, E.Z., Vervenne, R.A., Sombroek, C.C., Kondova, I., Eissen, O.A., Sommandas, V., van der Werff, N.M., Verschoor, E., Braskamp, G. and Bakker, J. Variable BCG efficacy in rhesus populations: Pulmonary BCG provides protection where standard intra-dermal vaccination fails. *Tuberculosis* **104**, 46-57 (2017).
334. Li, S., Tang, B. & He, H. An Imbalanced Learning based MDR-TB Early Warning System. *Journal of medical systems* **40**, 164 (2016).
335. Rueda, J., Realpe, T., Mejia, G., Zapata, E. & Robledo, J. GenoType MTBDR plus 1.0(R) for the detection of cross-resistance between isoniazide and ethionamide in isolates of multidrug-resistant Mycobacterium tuberculosis. *Biomedica* **35**, 541-548 (2015).
336. Kohlmorgen, B., Elias, J. & Schoen, C. Improved performance of the artus Mycobacterium tuberculosis RG PCR kit in a low incidence setting: a retrospective monocentric study. **7**, 14127 (2017).
337. Dosler, S., Karaaslan, E. & Alev Gerceker, A. Antibacterial and anti-biofilm activities of melittin and colistin, alone and in combination with antibiotics against Gram-negative bacteria. *Journal of Chemotherapy* **28**, 95-103 (2016).
338. Jenssen, H., Hamill, P. & Hancock, R.E.W. Peptide Antimicrobial Agents. *Clinical Microbiology Reviews* **19**, 491-511 (2006).
339. Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., Cohen, J., Findlay, D., Gyssens, I., Heure, O.E. & Kahlmeter, G. The global threat of antimicrobial resistance: science for intervention. *New Microbes and New Infections* **6**, 22-29 (2015).
340. Thaker, H.D., Som, A., Ayaz, F., Lui, D., Pan, W., Scott, R.W., Anguita, J. & Tew, G.N. Synthetic Mimics of Antimicrobial Peptides with Immunomodulatory Responses. *Journal of the American Chemical Society* **134**, 11088-11091 (2012).
341. Colomb-Cotinat, M., Lacoste, J., Brun-Buisson, C., Jarlier, V., Coignard, B. & Vaux, S. Estimating the morbidity and mortality associated with infections due to multidrug-resistant bacteria (MDRB), France, 2012. *Antimicrobial Resistance and Infection Control* **5**, 56 (2016).
342. Willyard, C. The drug-resistant bacteria that pose the greatest health threats. *Nature News* **543**, 15 (2017).
343. Magiorakos, A.P. et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical microbiology and infection* **18**, 268-281 (2012).
344. Costerton, J.W., Stewart, P.S. & Greenberg, E.P. Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318-1322 (1999).
345. Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S. & Ciofu, O. Antibiotic resistance of bacterial biofilms. *International Journal of Antimicrobial Agents* **35**, 322-332 (2010).
346. Stewart, P.S. Mechanisms of antibiotic resistance in bacterial biofilms. *International Journal of Medical Microbiology* **292**, 107-113 (2002).
347. Chung, P.Y. & Khanum, R. Antimicrobial peptides as potential anti-biofilm agents against multidrug-resistant bacteria. *Journal of Microbiology, Immunology and Infection* **50**, 405-410 (2017).
348. Diamond, G., Beckloff, N., Weinberg, A. & Kisich, K.O. The Roles of Antimicrobial Peptides in Innate Host Defense. *Current pharmaceutical design* **15**, 2377-2392 (2009).
349. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389 (2002).

350. Javadpour, M.M., Juban, M.M., Lo, W.C.J., Bishop, S.M., Alberty, J.B., Cowell, S.M., Becker, C.L. and McLaughlin, M.L. De novo antimicrobial peptides with low mammalian cell toxicity. *Journal of Medicinal Chemistry* **39**, 3107-3113 (1996).
351. Adedeji, W.A. The treasure called antibiotics. *Annals of Ibadan Postgraduate Medicine* **14**, 56-57 (2016).
352. Chin, W. et al. A macromolecular approach to eradicate multidrug resistant bacterial infections while mitigating drug resistance onset. *Nature Communications* **9**, 917 (2018).
353. Bjarnsholt, T. The role of bacterial biofilms in chronic infections. *APMIS.*, 1-51 (2013).
354. Abat, C., Raoult, D. & Rolain, J.M. Are we living in an antibiotic resistance nightmare? *Clinical Microbiology and Infection* (2018).
355. Spencer, J.J., Pitts, R.E., Pearson, R.A. & King, L.B. The effects of antimicrobial peptides WAM-1 and LL-37 on multidrug-resistant Acinetobacter baumannii. *Pathogens and Disease* **76**, fty007-fty007 (2018).
356. Park, C.H., Valore, E.V., Waring, A.J. & Ganz, T. Hepcidin, a urinary antimicrobial peptide synthesized in the liver. *Journal of biological chemistry* **276**, 7806-7810 (2001).
357. Christensen, B., Fink, J., Merrifield, R. & Mauzerall, D. Channel-forming properties of cecropins and related model compounds incorporated into planar lipid membranes. *Proceedings of the National Academy of Sciences* **85**, 5072-5076 (1988).
358. Ludtke, S.J., He, K., Heller, W.T., Harroun, T.A., Yang, L. and Huang, H.W. Membrane pores induced by magainin. *Biochemistry* **35**, 13723-13728 (1996).
359. Ganz, T. Defensins: antimicrobial peptides of innate immunity. *Nature reviews immunology* **3**, 710 (2003).
360. Ageitos, J.M., Sánchez-Pérez, A., Calo-Mata, P. & Villa, T.G. Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria. *Biochemical Pharmacology* **133**, 117-138 (2017).
361. Haney, E.F., Brito-Sánchez, Y., Trimble, M.J., Mansour, S.C., Cherkasov, A. and Hancock, R.E. Computer-aided Discovery of Peptides that Specifically Attack Bacterial Biofilms. *Scientific Reports* **8**, 1871 (2018).
362. Feng, Q., Huang, Y., Chen, M., Li, G. & Chen, Y. Functional synergy of alpha-helical antimicrobial peptides and traditional antibiotics against Gram-negative and Gram-positive bacteria in vitro and in vivo. *European journal of clinical microbiology & infectious diseases* **34**, 197-204 (2015).
363. Böttger, R., Hoffmann, R. & Knappe, D. Differential stability of therapeutic peptides with different proteolytic cleavage sites in blood, plasma and serum. *PLOS One* **12**, e0178943 (2017).
364. Käser, M., Ruf, M.-T., Hauser, J., Marsollier, L. & Pluschke, G. Optimized method for preparation of DNA from pathogenic and environmental mycobacteria. Applied and environmental microbiology 75, 414-418 (2009).
365. Van Duin, D. & Paterson, D. Multidrug Resistant Bacteria in the Community: Trends and Lessons Learned. *Infectious disease clinics of North America* **30**, 377-390 (2016).

# Patent and Publications

## Patent Filed

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## Publications

1. Hazam, P. K., Goyal, R & Ramakrishnan, V. (2018). Peptide based Antimicrobials: Design Strategies and Therapeutic Potential. **Progress in Biophysics and Molecular Biology**. doi.10.1007/s10989-018-9690-0.
Publisher: Elsevier

2. Hazam, P. K., Singh, A., Chaudhary, N., & Ramakrishnan, V. (2018). Bactericidal potencyand extended serum life of stereo-chemically engineered peptides against Mycobacterium.**International Journal of Peptide Research and Therapeutics**. doi.org/10.1007/s10989-018- 9690-0.
Publisher: Springer

3. Hazam, P. K., Jerath, G., Kumar, A., Chaudhary, N., & Ramakrishnan, V. (2017). Effect of tacticity-derived topological constraints in bactericidal peptides. **Biochimica et Biophysica Acta** (BBA) – Biomembranes. 1859(8), 1388-1395. doi: 10.1016/j.bbamem.2017.05.002.
Publisher: Elsevier

4. Hazam, P. K., Jerath, G., Chaudhary, N., & Ramakrishnan, V. (2017). Peptido-mimeticApproach in the Design of Syndiotactic Antimicrobial Peptides. **International Journal ofPeptide Research and Therapeutics**, 1-9. doi: org/10.1007/s10989-017-9615-3.
Publisher: Springer

5. Sasidharan, S., Hazam, P. K., & Ramakrishnan, V. (2017). Symmetry-Directed Self-Organization in Peptide Nanoassemblies through Aromatic π–π Interactions. **The Journal of Physical Chemistry B**, 121(2), 404-411. doi: 10.1021/acs.jpcb.6b09474.
Publisher: American Chemical Society

6. Jerath, G., Hazam, P. K., Shekhar, S., & Ramakrishnan, V. (2016). Mapping the GeometricEvolution of Protein Folding Motor. **PLOS one**, 11(10), e0163993. doi:10.1371/journal.pone.0163993.
Publisher: PLOS

7. Jerath, G., Hazam, P. K., & Ramakrishnan, V. (2014). bPE toolkit: toolkit for computational protein engineering. **Systems and Synthetic Biology**, 8(4), 337-341. doi:10.1007/s11693-014-9156-4.
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# Bactericidal Potency and Extended Serum Life of Stereo-Chemically Engineered Peptides Against *Mycobacterium*

**Prakash Kishore Hazam[1] · Anjali Singh[1] · Nitin Chaudhary[1] · Vibin Ramakrishnan[1]**

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**Abstract**
Tuberculosis is one of the leading causes of death, with an annual mortality rate of 2 million. The present treatment regimen for *Mycobacterium* species is strenuous, extending up to 12 months. Even then, rise of antibiotic resistance has limited the prognosis, with increased instances of multidrug resistant (MDR–TB) and extremely drug resistant (XDR–TB) cases reported. Peptide based antibiotics can be an effective solution due to their low toxicity, biocompatibility and predictable metabolism, but has not been employed due to their short plasma half life. In this brief communication, we demonstrate the bactericidal potency of cationic amphipathic peptides as an effective bactericidal agent against *Mycobacterium smegmatis*. Potency of stereo-engineered LDLD or DLDL peptides have been retained their potency, while their poly L variants rapidly lost their activity, when the experiment was repeated in human serum. To establish this as a design strategy, we further verified the results by repeating the experiment in a gram negative bacteria *E. coli*. One of the designed peptides were showing a minimum inhibitory concentration (MIC) value as low as 3.13 μM, suggesting the possibility of future development as a therapeutic peptide.

**Keywords** Chain stereochemistry · Plasma half-life · Antimicrobial peptide · Minimum inhibitory concentration · Mycobacterium · *E. coli*

## Introduction

Tuberculosis (TB) is one of the top 10 causes of death (Carranza-Rosales et al. 2017), with more than 10 million cases reported every year and among them, approximately 2 million patients succumb to the disease (Sandhu 2011; Beg et al. 2017). *Mycobacterium tuberculosis* (Mtb), is the causative agent, which parasitizes human macrophages for its sustainability and proliferation (Estrella et al. 2011). Increase in drug resistance is a major challenge to the disease control mechanisms presently in place (Podinovskaia et al. 2013). Even for the drug susceptible TB, the present treatment regimen is onerous (Zuniga et al. 2015), and hence there is an urgent clinical need for the discovery of new chemical entities effective for both drug susceptible and resistant TB.

Peptide based antimicrobials have been recognized as a safe and effective option, with increased selectivity and tolerance (Paterson et al. 2017; Jaskiewicz et al. 2016; Arakha et al. 2016; Ravichandran et al. 2016; Kaur et al. 2008; Koh et al. 2015). But usage of peptides as a therapeutic agent was limited by short plasma half-life (Molchanova et al. 2017) and negligible oral bio-availability (Di 2015). The shorter half-life is due to numerous peptidases and excretory mechanisms that inactivate and clear peptides from the system (Di 2015; Yeaman and Yount 2003). Several techniques for extending plasma half-life have been developed, such as stapling and clipping of peptide sequences, cyclization, binding to albumin and Polyethylene glycol (PEGylation) (Fosgerau and Hoffmann 2015) etc. Limitations of endogenous peptides have motivated researchers to focus on the design of peptide analogs, short sequence oligopeptides and innovative delivery systems. Recently Henry et al. reported a subcutaneous implantable osmotic pump system that delivers peptide continuously up to one year for the treatment of type 2 diabetes (Henry et al. 2014).

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✉ Vibin Ramakrishnan
vibin@iitg.ernet.in

[1] Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781 039, India

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Serum represents the intact concoction of human body fluid, which conclusively correlates to its ionic as well as proteome bio-reserve (Greene and Power 1931; Anderson 2005). Human serum proteases (Moncla et al. 2011; Stefanini et al. 2015; Starr and Wimley 2017; Nguyen et al. 2010; Jenssen and Aspmo 2008) and their microbial counter parts form an invasive microflora which reduces the therapeutic activity of peptides manifold (Kooi and Sokol 2009; Yeaman and Yount 2003; Fjell et al. 2012; Joo et al. 2016; Nizet 2006; Brannon et al. 2015). Apart from this, higher ionic environment has also been reported to reduce bactericidal potency of peptides (Park et al. 2004; Bals et al. 1998). Therefore, it is necessary to ascertain the metabolic stability and therapeutic potential of a peptide drug in serum to verify it's in vivo activity.

Natural proteins and peptides are polymers 20 amino-acids in varying length and sequences, with almost all amino-acids exclusively L chiral (Durani 2008; Kumar and Ramakrishnan 2010). In polymer science, such an arrangement is termed as isotactic. If we can have an amino-acid sequence with alternating L and D chirality, it is syndiotactic and random L,D combinations are hetero-tactic (Billmeyer 1971). From a design point of view, incorporation of D-amino acid at each position will hugely expand the design space. For example, a ten residue peptide can have $2^{10}$ stereochemical variations possible. However, steriochemically-diversified peptide sequences have not been explored to its potential. Shai and Oren, along with few others have reported the utility of D-amino acids in therapeutic peptide sequences for resisting enzymatic degradation (Shai and Oren 1996; Shai 2002; Oren and Shai 1997; Lan et al. 2014; Jiang et al. 2011). Peptides with D-enantiomers have also displayed much reduced ionic sensitivity and cytotoxicity against mammalian cell systems (Olli et al. 2013). Self-assembling organic nanotubes with alternating L and D amino-acids was first reported from Ghadiri's laboratory and interestingly. The same group has designed cyclic D, L-alpha-peptide framework as potential broad spectrum antibacterial and antiviral agents (Ghadiri et al. 1993; Horne et al. 2005). Metzler-Nolte and coworkers have made use of specific combinations of L and D amino acids to achieve 4–8-fold decrease in MIC values. (Albada et al. 2014).

Side-chain derived cationic charge and a designed amphipathic conformation are the two principal elements incorporated in the sequence of peptide-based antimicrobial agents (Mishra and Wang 2012; Wimley 2010). So far, chain stereochemistry has not been systematically used as a design variable in most of these studies, with few exceptions reported (Hazam et al. 2017b). In an earlier study, we have retained the amino acid content, but varied their chain stereochemistry to generate different topological variants with same chemical species, and tested their bactericidal potential against gram positive, negative and resistant bacteria. Conformational locking or topological fixation by stereochemical engineering has been systematically experimented to verify its differential effects against bacterial membranes (Hazam et al. 2017b). In this work, we extend this study by testing the relative efficacy of isotactic and syndiotactic variants of a peptide sequence in both directions by retaining its chemical constitution.

Mechanistic investigations on TB suggests that, Mycobacterium bacilli are ingested by macrophages in response to bacterial invasion, subsequently resulting in the formation of attenuated phagosomes, creating a friendly intra-phagosomal environment for bacterial proliferation (Estrella et al. 2011; Podinovskaia et al. 2013). Even though there is available treatment regimen, the rise of multidrug-resistant superbugs has limited the prognosis (Phyu et al. 2003; Fofana et al. 2017; Falzon et al. 2017). Therefore, we investigated the anti-mycobacterial potential of our reported bactericidal peptides, both in vitro and in serum with *Mycobacterium smegmatis* as a representative model for mycobacterium, due to its non-virulence and comparative fastidious nature. We made an objective comparison of antibacterial potency of isotactic and syndiotactic peptides in both conditions. We found that stereochemically diversified syndiotactic sequences effectively resist enzymatic degradation, while isotactic antibacterial peptides degrade losing their potency. Since mycobacterium is basically a gram positive bacteria, we further verified our results, we further extended the study to a gram negative bacterium *E. Coli*. In this study, we have designed six model systems, systematically and exhaustively varying the stereo-chemical and amino-acid sequence resulting in six variant topological models. The designed molecules were synthesized and further verified the effect of chain stereochemistry in formulating their bactericidal potency, hemolysis and plasma life.

## Materials and Methods

### Design Tools

PDBMake an in-house designed software was used for the design of peptides. Pross, Ribosome, SPDB Viewer, Pymol and tools available in APD database have been used for further analysis.

### Peptide Synthesis and Characterization

Peptide synthesis using *N*-9-fluorophenylmethoxy-carbonyl (F-MOC) strategy was employed over a solid support (rink amide resin). The peptides were cleaved from the resin using a cleavage cocktail as described earlier (Hazam et al. 2017a). A reverse phase liquid chromatography using C-18 semi-preparative coloumn with a gradient run of 10–100%

acetonitrile (ACN), at a flow rate of 0.5 mL/min was performed at 210 nm for separation. The eluents were characterized by mass spectrometry (MALDI-TOF) using HCCA (alpha-Cyano-4-hydroxycinnamic acid) matrix (Chaudhary and Nagaraj 2011; Hazam et al. 2017b).

### Anti-bacterial and Serum Sensitive Assay

The bactericidal activity of peptides was carried out using micro-dilution method. *M.smegmatis* (ATCC® 607) strains was cultured in M7H9 media, overnight at 37 °C. The bacterial suspension was diluted to $2\times10^5$ cfu/mL. Subsequently, 50 µL of the diluted inoculum was mixed with fixed concentrations of peptides, incubated at 37 °C for 72 h. The bactericidal potency of the peptides was estimated with an objective comparison with untreated bacterial cells at 600 nm (Chung et al. 1995). Similarly, *E.coli* MG1655 was diluted to $2\times10^6$ CFU/mL in Nutrient broth (NB) media. 50 µL of the bacterial suspension was mixed with peptides at required concentration, followed by incubation at 37 °C for 12 to 16 h. The percent bacterial cell death was calculated by a comparative assessment with untreated bacterial suspension (negative control) at 600 nm. Serum sensitive activity of the peptides against *M. smegmatis* and *E. coli* was similar to that of anti-bacterial assay in 50% serum environment (Xu et al. 2014). The MIC values were reported, where no microbial growth was observed at 600 nm.

### Field Emission-Scanning Electron Microscopy (FE-SEM)

All the peptides were assayed at their respective MIC values as explained. Later, the inoculum was mixed with glutaraldehyde (4%) and incubated at room temperature for 30 min. Post incubation, the cells were mounted over a glass slide and a gradient washing (30–100%) with alcohol was performed. The sample was dried and gold coated before microscopic analysis (Hazam et al. 2017b).

## Result and Discussion

### Design and Synthesis of Syndiotactic Antimicrobial Peptides

We have designed a series of 12 amino acids long peptides incorporating residues with cationic and neutral side-chains. A typical 12 amino acid long polypeptide can theoretically have $2^{12}$ (4096) stereo-chemical variants possible. Naturally occurring, isotactic poly-L sequence is only one among these, while the remaining 4095 are hetero-chiral. When we systematically evaluate stereo-chemical effects in extending the plasma life of the designed peptides, it is impossible to evaluate all 4096 stereo-chemical variants and further its sequence variants. Therefore, as a design strategy, we opted to exhaustively design the next theoretically possible chiral sequence variation, which is an alternating LDLD peptide sequence. Incidentally, Gramicidin, a naturally occurring polypeptide obtained from soil bacteria *Bacillus brevis* (Burkhart et al. 1999), has an alternating L- and D-amino acid sequence (Kumar et al. 2017). The helical structure it naturally adopts is known as gramicidin helix. Stereo-chemical sequence LDLD and its reverse DLDL can adopt both right handed and left handed conformations, and with regards to the handedness, one can design eight variants. (Supplementary Fig. 1). Differential handedness is achieved by using different phi and psi angle combinations in each syndiotactic sequence. However, out of eight, only four variants are synthetically possible (Fig. 1; Table 1), because handedness is adopted by the peptide, post synthesis.

Therefore, out of the eight topological variants, two LDLD and its two mirror sequence DLDL has been synthesized (Table 1). More precisely, model system MS2 is the stereochemistry reversed sequence of model 1 (MS1), MS4 is sequence reversed MS1, and MS3 is both sequence and stereochemistry reversed model of MS1. With this we could achieve all the stereochemical and topological possibilities of a given sequence in syndiotactic stereochemistry. (Table 1). MS5 and MS6 are isotactic (poly L) variants of MS1 and MS3 for an objective comparison of their relative potential against *Mycobacterium smegmatis*, in conditions with and without serum.

The design philosophy and their activity against gram positive, gram negative and resistant bacteria (MRSA) have described elsewhere. Two molecules in this series, differs either by stereo-chemistry or sequence or both. Amino acid content, and consequently molecular weight, cationicity, amphipathicity and hydrophobic index of all peptides are the same, which provides an objective assessment of the effect of stereo-chemistry manifested through their topology during membrane activity and protease and peptidase enzyme induced degradation under in vivo conditions.

All the six peptide sequences were synthesized using standard solid phase peptide synthesis employing Fmoc chemistry. All the peptides were synthesized and purified using reverse phase liquid chromatography. A semi-preparative liquid chromatography was performed, followed by characterization using mass spectrometry (MALDI-TOF).

### Bactericidal Potency of the Designed Amphiphilic Cationic Peptides Against *M. smegmatis*

All the designed peptides exhibited good antibacterial activity against *M. smegmatis*, even at micro-molar concentration range under in vitro conditions in media. The syndiotactic and poly-L peptides have shown comparable

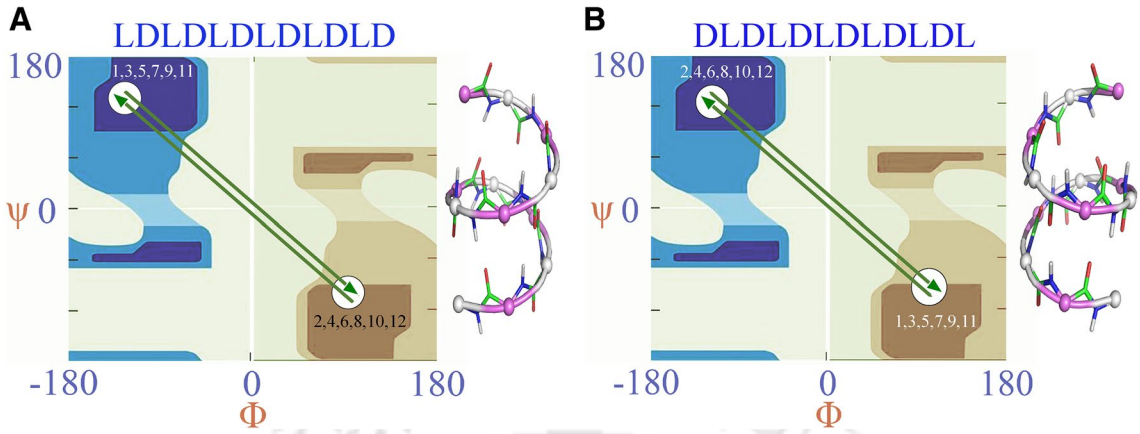


**Fig. 1** Design of antimicrobial peptide incorporating D amino acid in the peptide sequence retaining their molecular weight and chemistry, and varying stereochemistry. The design has a pair of LDLD as well as DLDL sequences, with their poly L counterparts, all having identical amino acid composition. The syndiotactic arrangement of the peptide sequences give rise to gramicidin helical structure with alternating L and D residues in peptide sequence

**Table 1** Designed antimicrobial peptides and their respective amino acid sequences

| Peptide code | Sequence | *M. smegmatis* | *M. smegmatis* (serum) | *E. coli* | *E. coli* (serum) |
|---|---|---|---|---|---|
| MS1 | KrKiFlRtKiLv | 6.25 | 12.5 | 3.13 | 6.25 |
| MS2 | kRkIfLrTkIlV | 3.13 | 3.13 | 3.13 | 3.13 |
| MS3 | vLiKtRlFiKrK | 6.25 | 12.5 | 6.25 | 6.25 |
| MS4 | VlIkTrLfIkRk | 6.25 | 12.5 | 6.25 | 6.25 |
| MS5 | KRKIFLRTKILV | 6.25 | > 50 | 3.13 | > 50 |
| MS6 | VLIKTRLFIKRK | 12.5 | > 50 | 6.25 | > 50 |

The letters in uppercase represent L—amino acid, whereas letters in lower case denotes D—amino acid. Comparative assessment of antimicrobial activity of the designed peptides calculated as Minimal Inhibitory Concentration (MIC) of peptides against both *M. smegmatis* and *E. coli* in human serum and media alone. The MIC values are expressed in micro molar (μM) unit

antibacterial potencies, which indicates that the designed peptides were membrane active in nature (Fig. 2; Table 1).

This observation was supported by a comparative study of isotactic and syndiotactic peptides with field emission scanning electron microscopy (FE-SEM). The untreated bacterial sample possessed intact membrane architecture, while bacterial membranes treated with peptide have been deformed significantly (Fig. 3).

Anti-tubercular peptides (Khusro et al. 2016) identified in last few decades have shown to possess multiple advantages such as low immunogenicity, selective affinity, and divergent mechanisms of action. Available reports suggest that they bind multiple targets simultaneously showing both anti-mycobacterial as well as immune-modulatory properties. To verify the potential toxicity of the designed peptides to mammalian cells, hemolytic assay was performed with RBC's, incubating with 8 and 16 μM peptides for 2 h. Extend of RBC lysis can be estimated spectroscopically at 540 nm, by measuring the hemoglobin content in the medium. All the peptides were found to have negligible hemolytic activity (Supplementary Table 1).

## Effect of Stereo-Chemical Engineering of the Designed Peptides in Extending Plasma Half Life

To verify the comparative performance of syndiotactic and poly L peptides, we repeated the experiment in mammalian serum. All the four syndiotactic peptides in LDLD and DLDL stereo-chemical sequence showed remarkable difference in their activity compared to their poly L counter-parts. Syndiotactic peptides MS1, MS3 and MS4, more or less retained their antimicrobial property, with a relatively lesser extent in their membrane activity, while MS2 retained its bactericidal potency even in serum. Expectedly, for poly-L peptides, the MIC values have shown a phenomenal decrease from 12.5 and 6.25 μM to a value greater than 50 μM (Fig. 2; Table 1). The much

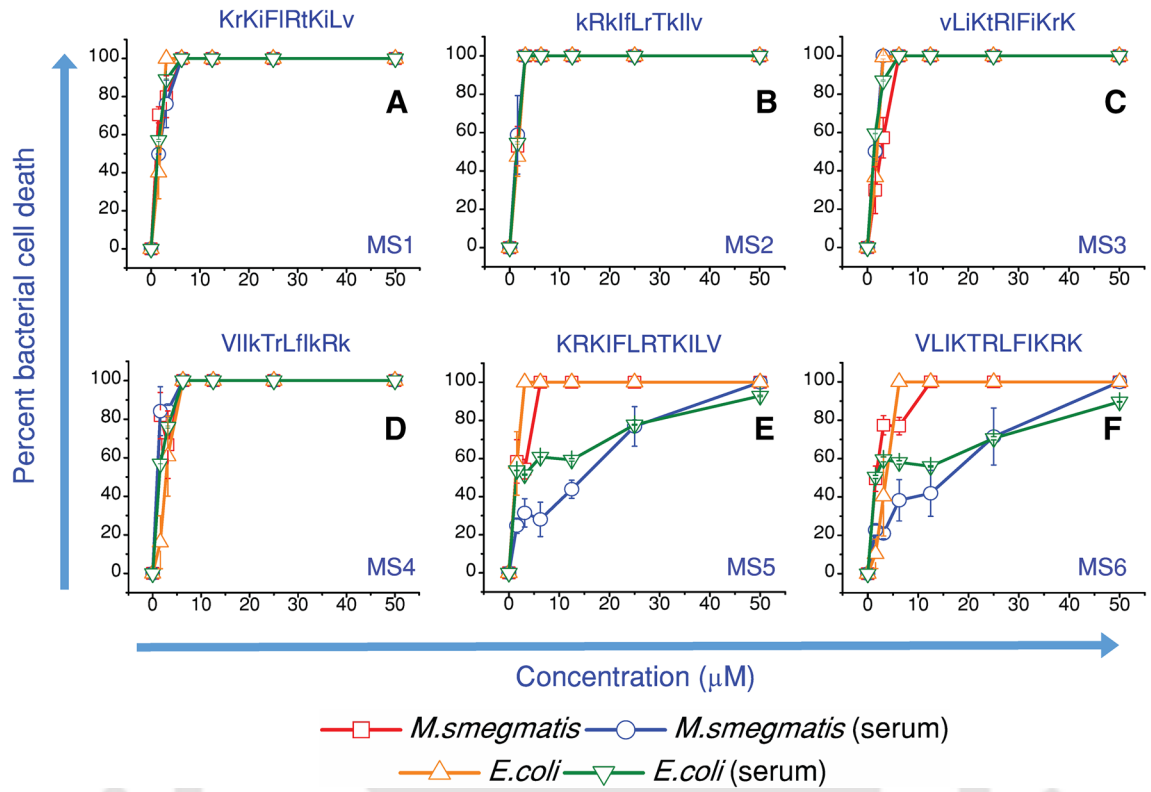


**Fig. 2** Anti-bacterial activity of the designed peptides against *M. smegmatis* and *E. coli* in media and in human serum. The bactericidal potency (percent bacterial cell death) of peptide has been plotted against variable peptide concentration at micro molar concentration (1.56–50). The error bars represent standard deviation

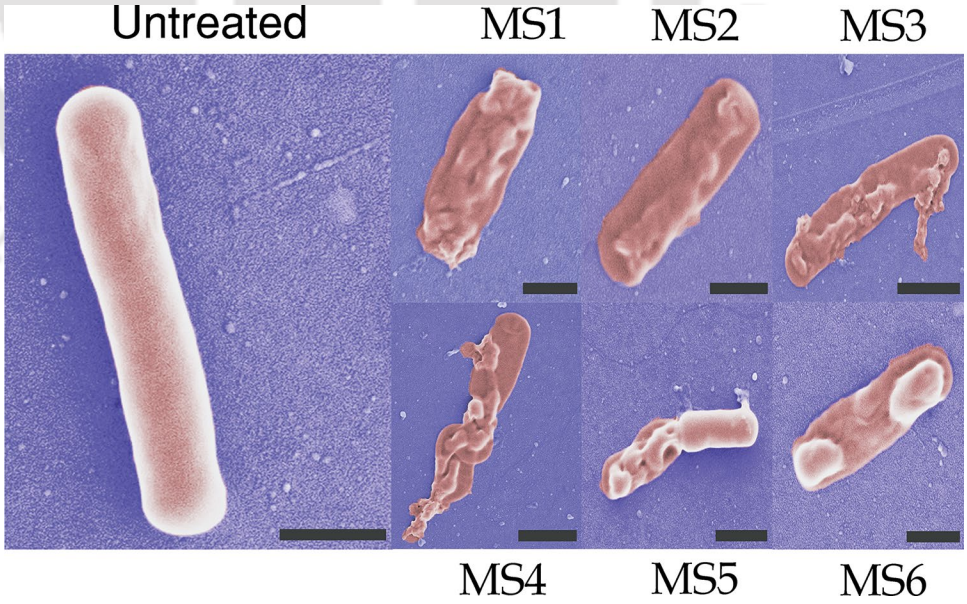


**Fig. 3** Field emission scanning electron microscopic (FE-SEM) images of peptide-membrane interaction. A comparative assessment of untreated specimen with peptide treated sample at their respective MIC can be qualitatively assessed from the extent of deformation of bacterial membrane under each condition. Images have been pseudo-colored using Photoshop CS6. The scale bar resembles to 500 nm

studied enzymatic degradation of poly-L peptides was evident from the activity profiles. Since serum is a cocktail of innate proteins and ions (Anderson 2005), its effect on therapeutic peptides in known and well demonstrated (Starr and Wimley 2017). The observation that syndiotactic peptides could resist enzymatic degradation can be an important lead in the future designs of peptide based therapeutics.

*Mycobacterium smegmatis* is a gram positive bacterium (Mogi et al. 2009). To further verify our observation, we have repeated the experiment with gram negative bacteria, *E. Coli*. Overall results were found to be a repeat of *M. Smegmatis* experiment, with syndiotactic peptides more or less retaining its activity in serum, while poly L peptides degraded. Interestingly, MS2 was found to an important lead compound for future development as a very potent broad spectrum antibiotic, showing exceptional activity against both bacterial species of contrasting membrane architecture (Fig. 2). Mycomembrane is very rigid compared to even gram negative bacteria (Gutsmann 2016), which explains the general trend of comparatively lesser MIC values of the designed peptides in Gram-negative bacteria, supporting earlier studies with comparative estimates.

## Conclusion

Antibiotic resistance limits the sustained usage of broad spectrum therapeutic solutions, thereby demanding a robust pipeline newer lead compounds getting into clinical trials. (Falzon et al. 2017; Sommer et al. 2017). The present treatment regimen of tuberculosis is complicated, demanding long-term use multiple antibiotics up to 12 months, and not devoid of side-effects (Lienhardt et al. 2017). Recent reports suggest that BCG (*Mycobacterium bovis* bacille Calmette-Guérin) vaccine, widely used for children can fail to prevent pulmonary TB in adults (Verreck et al. 2017). Further, multi-drug-resistant resistance (MDR), an inevitable consequence of noncompliance with the full therapeutic regimen, is leading to the emergence of resistant strains of Mycobacterium (Li et al. 2016). Recent reports of rising resistance to age old drugs like isoniazid (Rueda et al. 2015) and rifampin (Kohlmorgen et al. 2017), points to the immediate necessity of new line of solutions to this disease.

Peptides serve as the first line of immune defense and probably the only immune effector for most organisms (Saikia et al. 2017; Dosler et al. 2016). The basic molecular construct of physiologically active peptides is mostly composed of poly-L amino acids (Hazam et al. 2017a). AMP's are utilized as a viable option against microbial invasions by physiological systems across different levels of organization (Jenssen et al. 2006). Stability against enzymatic degradation was one of the main reasons for their underutilization (Yeaman and Yount 2003), which could otherwise be a good therapeutic option with minimal side effects. Our assessment with alternating LDLD (or DLDL) stereo-chemical sequence of antimicrobial peptides, suggest that stereochemical engineering of designed peptide can address the most significant associated limitation of peptide based treatment regimen against bacterial infections, especially *Mycobacterium*.

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## Compliance with Ethical Standards

**Conflict of interest** The authors declare no competing financial interest.

**Ethical Approval** All the testing and experiment related to human blood was performed as per the norms of ethical guidelines approved by ethical committee of Indian Institute of Technology Guwahati.

**Informed Consent** All the subjects were informed and an informed consent was obtained as per the requirement.

## References

Albada HB, Prochnow P, Bobersky S, Bandow JE, Metzler-Nolte N (2014) Highly active antibacterial ferrocenoylated or ruthenocenoylated Arg-Trp peptides can be discovered by an l-to-d substitution scan. Chem Sci 5:4453–4459

Anderson L (2005) Candidate-based proteomics in the search for biomarkers of cardiovascular disease. J Physiol 563:23–60

Arakha M, Borah SM, Saleem M, Jha AN, Jha S (2016) Interfacial assembly at silver nanoparticle enhances the antibacterial efficacy of nisin. Free Radic Biol Med 101:434–445

Bals R, Goldman MJ, Wilson JM (1998) Mouse β-defensin 1 is a salt-sensitive antimicrobial peptide present in epithelia of the lung and urogenital tract. Infect Immun 66:1225–1232

Beg S, Gaur P, Mishra S (2017) New drugs and vaccines for tuberculosis. Recent Pat Anti-Infect Drug Discov. https://doi.org/10.2174/1574891X12666171006105921

Billmeyer FW (1971) Textbook of polymer science. Wiley, New York

Brannon JR, Thomassin J-L, Gruenheid S, Le Moual H (2015) Antimicrobial Peptide conformation as a structural determinant of omptin protease specificity. J Bacteriol 197:3583–3591

Burkhart BM, Gassman RM, Langs DA, Pangborn WA, Duax WL, Pletnev V (1999) Gramicidin D conformation, dynamics and membrane ion transport. Biopolymers 51:129–144

Carranza-Rosales P et al (2017) Modeling tuberculosis pathogenesis through ex vivo lung tissue infection. Tuberculosis 107:126–132

Chaudhary N, Nagaraj R (2011) Impact on the replacement of Phe by Trp in a short fragment of Aβ amyloid peptide on the formation of fibrils. J Pep Sci 17:115–123

Chung GA, Aktar Z, Jackson S, Duncan K (1995) High-throughput screen for detecting antimycobacterial agents. Antimicrob Agents Chemother 39:2235–2238

Di L (2015) Strategic approaches to optimizing peptide ADME properties. AAPS J 17:134–143

Dosler S, Karaaslan E, Alev Gerceker A (2016) Antibacterial and antibiofilm activities of melittin and colistin, alone and in combination with antibiotics against Gram-negative bacteria. J Chemother 28:95–103

Durani S (2008) Protein design with L- and D-α-amino acid structures as the alphabet. Acc Chem Res 41:1301–1308

Estrella J et al (2011) A novel in vitro human macrophage model to study the persistence of *Mycobacterium tuberculosis* using

vitamin D3 and retinoic acid activated THP-1 macrophages. Front Microbiol 2:1–16
Falzon D, Schünemann HJ, Harausz E, González-Angulo L, Lienhardt C, Jaramillo E, Weyer K (2017) World Health Organization treatment guidelines for drug-resistant tuberculosis, 2016 update. Eur Respir J 49:1602308
Fjell CD, Hiss JA, Hancock REW, Schneider G (2012) Designing antimicrobial peptides: form follows function. Nat Rev Drug Discov 11:37–51
Fofana MO, Shrestha S, Knight GM, Cohen T, White RG, Cobelens F, Dowdy DW (2017) A multistrain mathematical model to investigate the role of pyrazinamide in the emergence of extensively drug-resistant tuberculosis. Antimicrob Agents Chemother 61:e00498–e00416
Fosgerau K, Hoffmann T (2015) Peptide therapeutics: current status and future directions. Drug Discov Today 20:122–128
Ghadiri MR, Granja JR, Milligan RA, McRee DE, Khazanovich N (1993) Self-assembling organic nanotubes based on a cyclic peptide architecture. Nature 366:324–327
Greene CH, Power MH (1931) The distribution of electrolytes between serum and the in vivo dialysate. J Biol Chem 91:183–202
Gutsmann T (2016) Interaction between antimicrobial peptides and mycobacteria. Biochim Biophys Acta Biomembr 1858:1034–1043
Hazam PK, Jerath G, Chaudhary N, Ramakrishnan V (2017a) Peptido-mimetic approach in the design of syndiotactic antimicrobial peptides. Int J Pept Res Ther. https://doi.org/10.1007/s10989-017-9615-3
Hazam PK, Jerath G, Kumar A, Chaudhary N, Ramakrishnan V (2017b) Effect of tacticity-derived topological constraints in bactericidal peptides. Biochim Biophys Acta Biomembr 1859:1388–1395
Henry RR, Rosenstock J, Logan D, Alessi T, Luskey K, Baron MA (2014) Continuous subcutaneous delivery of exenatide via ITCA 650 leads to sustained glycemic control and weight loss for 48 weeks in metformin-treated subjects with type 2 diabetes. J Diabetes Complicat 28:393–398
Horne WS, Wiethoff CM, Cui C, Wilcoxen KM, Amorin M, Ghadiri MR, Nemerow GR (2005) Antiviral cyclic D,L-alpha-peptides: targeting a general biochemical pathway in virus infections. Bioorg Med Chem 13:5145–5153
Jaskiewicz M, Orlowska M, Olizarowicz G, Migon D, Grzywacz D, Kamysz W (2016) Rapid screening of antimicrobial synthetic peptides. Int J Pept Res Ther 22:155–161
Jenssen H, Aspmo SI (2008) Serum stability of peptides. In: Otvos L (ed) Peptide-based drug design. Humana Press, Totowa, pp 177–186
Jenssen H, Hamill P, Hancock REW (2006) Peptide antimicrobial agents. Clin Microbiol Rev 19:491–511
Jiang Z, Higgins MP, Whitehurst J, Kisich KO, Voskuil MI, Hodges RS (2011) Anti-tuberculosis activity of alpha-helical antimicrobial peptides: de novo designed L- and D-enantiomers versus L- and D-LL-37. Protein Pept Lett 18:241–252
Joo H-S, Fu C-I, Otto M (2016) Bacterial strategies of resistance to antimicrobial peptides. Philos Trans R Soc B 371:20150292
Kaur KJ, Sarkar P, Nagpal S, Khan T, Salunke DM (2008) Structure–function analyses involving palindromic analogs of tritrypticin suggest autonomy of anti-endotoxin and antibacterial activities. Protein Sci 17:545–554
Khusro A, Aarti C, Agastian P (2016) Anti-tubercular peptides: a quest of future therapeutic weapon to combat tuberculosis. Asian Pac J Trop Med 9(11):1023–1034
Koh JJ, Lin S, Aung TT, Lim F, Zou H, Bai Y, Li J, Lin H, Pang LM, Koh WL, Salleh SM, Lakshminarayanan R, Zhou L, Qiu S, Pervushin K, Verma C, Tan DT, Cao D, Liu S, Beuerman RW (2015) Amino acid modified xanthone derivatives: novel, highly promising membrane-active antimicrobials for multidrug-resistant Gram-positive bacterial infections. J Med Chem 58:739–752
Kohlmorgen B, Elias J, Schoen C (2017) Improved performance of the artus *Mycobacterium tuberculosis* RG PCR kit low incidence setting: a retrospective monocentric study. Sci Rep 7:14127
Kooi C, Sokol PA (2009) Burkholderia cenocepacia zinc metalloproteases influence resistance to antimicrobial peptides. Microbiology 155:2818–2825
Kumar A, Ramakrishnan V (2010) Creating novel protein scripts beyond natural alphabets. Syst Synth Biol 4:247–256
Kumar A, Ranbhor R, Patel K, Ramakrishnan V, Durani S (2017) Automated protein design: landmarks and operational principles. Prog Biophys Mol Biol 125:24–35
Lan Y, Lam JT, Siu GKH, Yam WC, Mason AJ, Lam JKW (2014) Cationic amphipathic D-enantiomeric antimicrobial peptides with in vitro and ex vivo activity against drug-resistant *Mycobacterium tuberculosis*. Tuberculosis 94:678–689
Li S, Tang B, He H (2016) An imbalanced learning based MDR-TB early warning system. J Med Syst 40:164
Lienhardt C et al (2017) Target regimen profiles for treatment of tuberculosis: a WHO document. Eur Respir J 49:1602352
Mishra B, Wang G (2012) Ab initio design of potent anti-MRSA peptides based on database filtering technology. J Am Chem Soc 134:12426–12429
Mogi T, Murase Y, Mori M, Shiomi K, Omura S, Paranagama MP, Kita K (2009) Polymyxin B identified as an inhibitor of alternative NADH dehydrogenase and malate: quinone oxidoreductase from the Gram-positive bacterium *Mycobacterium smegmatis*. J Biochem 146:491–499
Molchanova N, Hansen P, Franzyk H (2017) Advances in development of antimicrobial peptidomimetics as potential drugs. Molecules 22:1430
Moncla BJ, Pryke K, Rohan LC, Graebing PW (2011) Degradation of naturally occurring and engineered antimicrobial peptides by proteases. Adv Biosci Biotechnol 2:404–408
Nguyen LT, Chau JK, Perry NA, de Boer L, Zaat SAJ, Vogel HJ (2010) Serum stabilities of short tryptophan- and arginine-rich antimicrobial peptide analogs. PLoS ONE 5:e12684
Nizet V (2006) Antimicrobial peptide resistance mechanisms of human bacterial pathogens. Curr Issues Mol Biol 8:11–26
Olli S, Rangaraj N, Nagaraj R (2013) Effect of selectively introducing arginine and D-amino acids on the antimicrobial activity and salt sensitivity in analogs of human beta-defensins. PLoS ONE 8:e77031
Oren Z, Shai Y (1997) Selective lysis of bacteria but not mammalian cells by diastereomers of melittin: structure—function study. Biochemistry 36:1826–1835
Park IY, Cho JH, Kim KS, Kim Y-B, Kim MS, Kim SC (2004) Helix stability confers salt resistance upon helical antimicrobial peptides. J Biol Chem 279:13896–13901
Paterson DJ, Tassieri M, Reboud J, Wilson R, Cooper JM (2017) Lipid topology and electrostatic interactions underpin lytic activity of linear cationic antimicrobial peptides in membranes. Proc Natl Acad Sci 114:E8324–E8332
Phyu S et al (2003) Drug-Resistant *Mycobacterium tuberculosis* among new tuberculosis patients, Yangon, Myanmar. Emerg Infect Dis 9:274–276
Podinovskaia M, Lee W, Caldwell S, Russell DG (2013) Infection of macrophages with *Mycobacterium tuberculosis* induces global modifications to phagosomal function. Cell Microbiol 15:843–859
Ravichandran G, Kumaresan V, Arasu MV, Al-Dhabi NA, Ganesh M-R, Mahesh A, Dhayalan A, Pasupuleti M, Arockiaraj J (2016) Pellino-1 derived cationic antimicrobial prawn peptide: bactericidal activity, toxicity and mode of action. Mol Immunol 78:171–182

Rueda J, Realpe T, Mejia G, Zapata E, Robledo J (2015) GenoType MTBDR plus 1.0(R) for the detection of cross-resistance between isoniazide and ethionamide in isolates of multidrug-resistant *Mycobacterium tuberculosis*. Biomedica 35:541–548

Saikia K, Sravani YD, Ramakrishnan V, Chaudhary N (2017) Highly potent antimicrobial peptides from N-terminal membrane-binding region of *E. coli* MreB. Sci Rep 7:42994

Sandhu G (2011) Tuberculosis: current situation, challenges and overview of its control program in India. J Global Infect Dis 3:143–150

Shai Y (2002) Mode of action of membrane active antimicrobial peptides. Biopolymers 66:236–248

Shai Y, Oren Z (1996) Diastereomers of cytolysins, a novel class of potent antibacterial peptides. J Biol Chem 271:7305–7308

Sommer MOA, Munck C, Toft-Kehler RV, Andersson DI (2017) Prediction of antibiotic resistance: time for a new preclinical paradigm? Nat Rev Microbiol 15:689

Starr CG, Wimley WC (2017) Antimicrobial peptides are degraded by the cytosolic proteases of human erythrocytes. Biochim Biophys Acta Biomembr 1859:2319–2326

Stefanini ACB, da Cunha BR, Henrique T, Tajara EH (2015) Involvement of Kallikrein-related peptidases in normal and pathologic processes. Dis Markers 2015:946572

Verreck FAW et al (2017) Variable BCG efficacy in rhesus populations: Pulmonary BCG provides protection where standard intra-dermal vaccination fails. Tuberculosis 104:46–45

Wimley WC (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. ACS Chem Biol 5:905–917

Xu W, Zhu X, Tan T, Li W, Shan A (2014) Design of embedded-hybrid antimicrobial peptides with enhanced cell selectivity and antibiofilm activity. PLoS ONE 9:e98935

Yeaman MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacogn Rev 55:27–55

Zuniga ES, Early J, Parish T (2015) The future for early-stage tuberculosis drug discovery. Future Microbiol 10:217–229

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# Effect of tacticity-derived topological constraints in bactericidal peptides

Prakash Kishore Hazam [a], Gaurav Jerath [a], Anil Kumar [b], Nitin Chaudhary [a], Vibin Ramakrishnan [a,*]

[a] Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781 039, India
[b] Biological and Organic Chemistry, University of Toronto, Ontario M5S 3H6, Canada

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ABSTRACT

Topology is a key element in structure-activity relationship estimation while designing physiologically-active molecular constructs. Peptides may be a preferred choice for therapeutics, principally due to their biocompatibility, low toxicity and predictable metabolism. Peptide design only guarantees functional group constitution by opting specific amino acid sequence, and not their spatial orientation to bind and incite physiological response on chosen targets. This is principally because peptide conformation is subject to external flux, due to the isotactic stereochemistry of the peptide chain. Stereochemical engineering of the peptide main chain offers the possibility of multiplying the structural space of a typical sequence to many orders of magnitude, and limiting the otherwise fluxional non-specific functional group dispensation in space by offering greater conformational rigidity. We put to test, this conceptual possibility already established in theoretical models, by designing amphipathic peptide systems and experimenting with them on Gram-positive, Gram-negative and antibiotic-resistant bacteria. The unusual conformational rigidity and stability of syndiotactic peptides enable them to retain the designed electrostatic environment, while they encounter the membrane surface. All the six designed systems exhibited bactericidal activity, pointing to the utility and specificity of stereo-engineered peptide systems for therapeutic applications. Overall, we hope that this work provides important insights and useful directives in designing novel peptide systems with antimicrobial activity, by expanding the design space, incorporating D-amino acid as an additional design variable.



## 1. Introduction

Topology is a key element in structure-activity relationship. Topology, also plays a key role in the geometrical evolution of a typical protein fold [1–4]. Topological variation in molecules with identical chemical structure can be provided by differences in their stereochemistry [5]. Ever since the historical discovery by Louis Pasteur in 1847, stereochemical variations and their possibilities are abundantly utilized while designing molecules at atomistic scale. Despite being the principal functional molecule in a living system, nature did not adopt stereochemistry as a variable in protein design [6]. All proteins are isotactic polymeric molecules, exclusively with L-chiral amino acids, as monomeric units. Tacticity refers to the relative stereochemistry of successive stereocenters in a polymeric macromolecule. If any given polymer chain has all the stereocenters of an exclusively L-(S) or D-(R) configurational-type, it is referred to as an 'isotactic' polymer. Syndiotactic molecules have stereoregular L- and D-stereocenters in alternate succession, and if the stereocenters are randomly distributed in a stereoirregular fashion, they are 'heterotactic' [7]. Approximately 7000 naturally-occurring peptides with potential physiological effects have been discovered so far, which include hormones, neurotransmitters, growth factors, ion channel ligands, etc. High selectivity and attractive pharmacological profile qualifies them to be identified as potential therapeutic agents, with 140 different peptide molecules currently being evaluated in clinical trials [8,9]. Almost all therapeutic peptides are made of asymmetric amino acids, with L-chiral stereochemical structure, thus they are identified as stereoregular 'isotactic' hetero-polymers [10–12]. This stereo-specificity points to the possibility of diversifying the basic alphabet of the amino acid sequence by including D-enantiomers [13]. With the addition of D amino acids at each sequence position, the number of stereoisomeric diversity increases exponentially from $1^N$ to $2^N$, where N is the number of amino acids in the sequence; for a 12 amino acid long peptide, this number would be from 1 ($1^{12}$) to 4096 ($2^{12}$). Peptides and protein structures are not only sensitive to mutations in their own sequences, but also even to minimal perturbations in their environment. Earlier reports suggest that the stereochemical mutation of polypeptide sequences can transform the structure from a condition of extreme sensitivity to extreme insensitivity to its environment [14,15]. A pioneering analysis along this line was done by Brant and Flory, where they calculated the limiting values of the characteristic ratio for random copolymers of L-Ala and Gly and L and D-Ala [16]. Characteristic ratio corresponds to the chain stiffness of a polymer chain. For a typical poly isotactic peptide chain, this value is between 9.0 and 9.5, whereas

* Corresponding author.
*E-mail address:* vibin@iitg.ernet.in (V. Ramakrishnan).

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for poly (Gly), it reduces to 2.0, quite similar to typical polymeric chains, owing to the lack of a stereocenter in glycine [17]. In an interesting experiment, Flory further calculated the characteristic ratios in random L-alanine, D-alanine copolymers for different mole ratios. He found a significant reduction in characteristic ratio to a value of 2 for the 50% mole ratio of L- and D-enantiomers. Characteristic ratio progressively increased upon an increase in mole ratio of one enantiomer; with both poly-L chiral and poly-D chiral isotactic isomers attaining a value of 9.0. This nearly four and a half times reduction was proposed to be a consequence of the "harmonious" nature of short and intermediate range electrostatics, an observation later corroborated by follow up investigations with chiral engineering of polypeptide sequences [18]. Chiral engineering was first utilized effectively by Ghadiri and coworkers, while designing nano-level molecular assemblies with D- and L-alpha-amino acids adopting ring-shaped conformations forming hollow tubular structures [19]. This was followed by a series of de novo designed artificial peptide constructs of diversified tacticity incorporating L- and D-amino acids resulting in unusually-stable structures. Peptide based molecular constructs with adaptive modulation for targeted functions have been identified as a futuristic option while designing antimicrobial agents, protein transduction domains and tumor homing molecular devices [20–22]. Effective interaction with the cell membrane is central in all these molecules for their functional fulfillment. Antimicrobial peptide database (APD) with 2687 molecular entries [23], Tumor homing peptide (TumorHoPe) with 744 [24] and cell penetrating peptide database (CPPSite) with 1700 unique sequences [25] deposited and validated against various cell, pathogen and tumor types underline their potential utility. Despite the above described variations that it can potentially generate, the option for steriochemically-diversified peptide sequences remains largely unexplored. Ghadiri and co-workers have also made use of cyclic D, L-alpha-peptide framework for designing broad spectrum antibacterial and antiviral agents [26,27]. Metzler-Nolte and coworkers have reported a 4–8 fold decrease in MIC values by adopting a specific combination of L and D amino acids [28]. Diversity of biological membranes and adaptation by antibiotic resistant bacterial species, such as gentamicin-methicillin-resistant *Staphylococcus aureus* (gentamicin-resistant MRSA) demand a systematic approach with molecular models resulting in key design directives for future molecular generation and development. In this work, we investigate the physiological effects of stereochemical mutation, manifested through the topological variations it offers, by systematically designing a possible antimicrobial sequence.

Structure-activity studies of peptide-based antimicrobial agents suggest incorporation of two key design elements; a cationic charge [29] and an induced amphipathic conformation [30,31]. Chain stereochemistry has not been a design element in such investigations, with few exceptions [26–28,32–51]. In this study we have retained the amino acid content and consequently their hydropathy index, but experimented with their chain stereochemistry to generate different topologies. Conformational locking or topological fixation has never been a prime concern for antimicrobial peptide design probably because of the lack of a consensus mechanistic model and a large number of conformational variations, a peptide sequence can adopt while it encounter a membrane. Imposing a constraint on peptide conformational flexibility by cyclization of a peptide chain was reported to be a successful strategy to improve broad spectrum activity and also specifically effective against resistant species [33,34].

Through this study, we aim to investigate the following objectives; (i) utility of peptide molecular systems with diversified tacticity as antimicrobial agents and (ii) effect of topological variation and subsequent chain stiffness and resultant restrictions reflected on the antimicrobial activity. We investigate these two fundamental questions by measuring the relative efficacy of stereochemical cum topological variants of designed peptides against Gram-positive, Gram-negative and antibiotic-resistant bacteria, with constant amphipathicity and cationicity as design elements [30]. We observed that a systematic design of heterotactic backbone induces topological restrictions, addressing the issue of non-specific distribution and consequent toxicity [35].

## 2. Materials and methods

### 2.1. Modeling and simulation

The PDB files were prepared using an in-house software called PDB make. Our earlier studies on protein and peptide design indicate that short range and long range electrostatic interactions of the peptide main chain will be complementing each other for LDLD and DLDL peptides while forming gramicidin helical conformation [10,52]. This would provide added stability to conformation, which may be utilized to fix the topology and relative orientation of side-chains in space. The amino acid side-chains were designed to create distinct polar and hydrophobic zones, while they assume gramicidin helical conformation. The amino acid choice was also limited, with seven residues frequently reported in studies [23,31]. GROMACS [53], a molecular dynamics simulation suite was used with GROMOS 96 43a1 force field [54] for carrying out molecular dynamics simulations. The structural stability of the designed peptides was studied at 300 K under standard NVT conditions. The structures were energy minimized by steepest descent algorithm in vacuum in 2000 steps in a simulation box with the size relatively 1.5 nm distant from the peptide in three dimensions. Solvent molecules (SPC, water) were then added and another energy minimization procedure was carried out in water prior to the simulation run. The 10 ns production runs for each peptide were done with an integration step of 2 fs. LINCS algorithm with a geometric accuracy of $10^{-4}$ was used as the bond length constraint. Maxwell distribution was used for initial velocity calculations with 0.1 ps of coupling relaxation step at 300 K. The non-bonded interaction cut off was set at 0.8 nm to 1.1 nm.

### 2.2. Electrostatic profiling

The electrostatic potential of peptides was calculated at every position by solving the finite difference Poisson-Boltzmann equation using DelPhi software [55,56], which was summed up for every residue side-chain. Electrostatic profiling was next accomplished by frame-wise comparison of electrostatic potential and structure similarity. The profiles were then plotted in a three dimensional graph using MATLAB [57].

### 2.3. Solid-phase peptide synthesis and characterization

Peptides were synthesized using Fmoc chemistry on the solid phase (Rink amide resin). Peptide constructs were cleaved from the resin by addition of a deprotection mixture involving trifluoroacetic acid (TFA), ethanedithiol, m-cresol and thioanisole. Subsequently, the, peptides were precipitated in ice-cold diethyl ether. Reversed-phase liquid chromatography (C18 column) was used to purify synthesized peptides by running a gradient of 10% acetonitrile in water to 100% acetonitrile and 0.1% TFA while detecting at 210 nm. Peptide fractions were characterized by MALDI-TOF (matrix ionization-time of flight) mass spectrometry [58–60].

### 2.4. Peptide-lipid interaction

#### 2.4.1. Peptide surface activity

Surface pressure was monitored by the Wilhelmy method, using a paper plate and a custom made polytetrafluoroethylene (PTFE) Langmuir trough (volume approximately 15 $cm^3$) carried out on a Langmuir instrument (Biolin Scientific, UK). Peptides were injected into a 10 mL sub-phase (10 mM phosphate buffer, pH 7.4) and changes in surface pressure were monitored until surface pressure saturation.

*2.4.2. Lipid monolayers*

Monolayers were prepared from phospholipid systems i.e. POPC (1-pamlitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine) and POPC:POPG (1-pamlitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol):: 7:3 in chloroform solutions, which were spread onto the air/buffer interface up to a final stable surface pressure of 30 ± 2 mN $m^{-1}$. After evaporation of the chloroform and stabilization of the pressure, the saturation concentration of peptides, determined from the previous experiment, were injected into the sub-phase (10 mM phosphate buffer, pH 7.4). Peptides were steadily injected via the vertical hole, directly into the sub-phase in order to cause minimal disturbance to the lipid layer. To ensure homogenous distribution of the peptide in the sub-phase, the peptide was mixed using magnetic-stirring. Peptide monolayer interactions were recorded as changes in monolayer surface pressure and the resultant data was plotted as change in surface pressure (mN $m^{-1}$) against time (minutes) [61–63].

*2.5. Antibacterial assay*

Antibacterial assays were carried out by spread plate method for visual verification of peptide activity. *Staphylococcus aureus* NCTC 8530, *Escherichia coli* MG1655 and Gentamicin resistant MRSA (ATCC 33592) were cultured in 1.3% (*w*/*v*) nutrient broth at 37 °C overnight. The mid logarithmic phase of a microorganism was confirmed by recording the absorbance at 600 nm, which should fall between 0.4 and 0.7. A second subculture was done by mixing 100 μL of inoculum to 10 mL of fresh nutrient broth and incubated until it reaches mid-log phase (O.D of 0.4 to 0.7). The cells were washed and re-suspended in sodium phosphate buffer (10 mM, pH 7.4). The resulting suspension was diluted until the net absorbance value of 0.02 at 600 nm was achieved. The inoculum (50 μL) was treated with required concentrations of peptide and volume adjusted to 100 μL with buffer if required, and incubated for 2 h (net incubation volume is 100 μL). 20 μL of the reaction broth was added to 180 μL of the re-suspension buffer. 20 μL of this diluted sample was plated while keeping untreated bacterial suspension of similar dilution as negative control [64–66].

*2.6. Field emission-scanning electron microscopy (FE-SEM)*

The initial part of the assay protocol up to bacterial incubation with the peptide for 2 h is similar to that of an antibacterial assay. Later, the reaction broth was treated with glutaraldehyde (2.5% glutaraldehyde concentration), followed by incubation for 30 min at 37 °C. The broth was centrifuged at 5000*g* for 5 min and supernatant was discarded, leaving behind 20 μL of the volume. The remaining sample was mounted over a glass cover slip, washed with increasing concentrations of ethyl alcohol (30 to 100%) and dried at room temperature. The sample was coated with gold, prior to imaging [67].

*2.7. Hemolytic activity*

2 mL of blood was collected from a healthy individual and 2 mg/mL of ethylene diamine tetra acetic acid disodium dihydrate salt (EDTA) was dissolved in it. Later, it was centrifuged at 800*g* force for 5 min. The pellet was collected and re-suspended in 5 mM HEPES buffered-saline, pH 7.4 and washed in a similar manner multiple times. A 10% hematocrit was prepared using the cells and the buffer. Peptides at required concentrations were incubated with 5% hematocrit for 2 h at 37 °C. Following incubation, the hematocrit was centrifuged (800*g* for 5 min), followed by the collection of the supernatant and recording the absorbance at 540 nm [68,69]. Complete lysis was observed when the hematocrit was prepared with deionized water instead of re-suspension buffer.



## 3. Results

*3.1. Modeling, simulation and electrostatic profiling*

As described earlier, a 12 amino acid long polypeptide can have 4096 stereochemical variants and poly-L is only one among them. Since it is impossible to evaluate all 4096 stereochemical variants and its sequence variants, we opted to exhaustively evaluate the next possible variant of chiral variation; an alternating LDLD sequence. The LDLD sequence naturally adopts a structure similar to a gramicidin helix. Depending on the handedness (right or left) and sequence, eight LDLD variant sequences are theoretically possible (Supplementary Fig. 1).

Handedness is a result of differential phi and psi angle combinations, which a particular syndiotactic backbone adopts, while folding to a given structure. Since we don't have any control on the handedness of the synthesized peptide, out of the eight topological variants, only four can be synthetically achieved; LDLD and DLDL stereochemical sequences with a given sequence and sequence reversed (Table 1).

More precisely, compared to model system 1 (MS1), model system 2 (MS2) has its stereochemistry reversed, model system 4 (MS4) is sequence-reversed, and model system 3 (MS3) is both stereochemically- and sequence-reversed (Table 1). Model systems 5 (MS5) and 6 (MS6) are poly L (isotactic) variants of MS1 and MS3 to estimate their activity against the bacterial species. The relative difference between two molecular systems in any of these six peptides are different either in sequence or stereochemistry or both, with invariable cationicity and amphipathicity in order to investigate the specific role that stereochemistry imparts, manifested through their topology during membrane activity.

Several models have been proposed to mechanistically explain membranolytic activity that principally arises from amphipathicity and cationicity of the interacting molecule. Pore forming and non-pore forming mechanistic models, suggested for peptide-based membrane activity is essentially manifested through electrostatic interactions of an amphipathic cationic peptide and its oppositely charged membrane counterpart.

We use the finite difference Poisson-Boltzmann method as implemented in DelPhi [55,56], to compute the electrostatic potential of all designed sequences on all theoretical possibilities of an alternating LDLD sequence, both in terms of its handedness and diastereomeric sequence. Electrostatic cum topology based molecular fingerprints of all eight variants, theoretically possible with our designed systems were examined. The spatial disposition with charged and neutral groups in a peptide system was found to be distinct and unique in each case, while the syndiotactic polypeptide chain assumes its most stable gramicidin helical conformation (Fig. 1).

Since handedness in design cannot be implemented on synthetic sequences, our sample set will be reduced to four sequences: LDLD, DLDL, LDLD-sequence reversed and DLDL-sequence reversed. Their potencies are compared with both isotactic poly-L sequences thus forming a complete set of designed test systems to study the combined effect of

**Table 1**
Amino acid sequence and stereochemical sequence of designed and synthesized model molecular systems 1 to 6 for biophysical studies and bactericidal activity. Number of clusters resulted from 10 ns molecular dynamics simulations, indicating the number of microstates at approximate equilibrium. Syndiotactic sequences are far more rigid; they largely retain the designed topology, whereas the poly-L sequence can adopt a range of degenerate conformational states. Small letters in the sequence indicate D-amino acid.

| Model system (MS) | Amino acid sequence | Stereochemical sequence | Number of clusters |
|---|---|---|---|
| MS1 | KrKiFlRtKiLv | LDLDLDLDLDLD | 5 |
| MS2 | kRklfLrTklIV | DLDLDLDLDLDL | 1 |
| MS3 | vLiKtRlFiKrK | DLDLDLDLDLDL | 1 |
| MS4 | VlIkTrLflkRk | LDLDLDLDLDLD | 3 |
| MS5 | KRKIFLRTKILV | LLLLLLLLLLLL | 22 |
| MS6 | VLIKTRLFIKRK | LLLLLLLLLLLL | 74 |

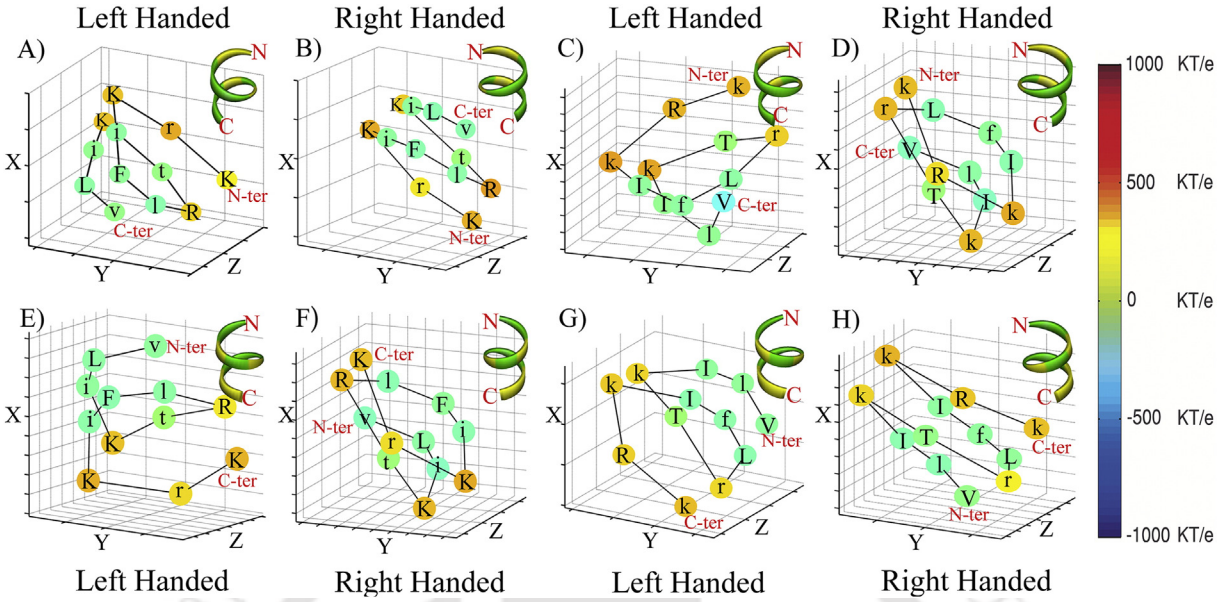


**Fig. 1.** Electrostatic potential maps of left handed (LH) and right handed (RH) conformations of designed syndiotactic peptides respectively. MS1 (A, B) and MS2 (C, D) along with MS3 (E, F) and MS4 (G, H). Electrostatic potential was calculated by solving the finite difference Poisson-Boltzmann equation using DelPhi software, summed for each amino acid side-chain and represented collectively at the chromophoric center of the side-chain. The hetero atoms of cysteine (S), lysine (K) and methionine (M), C-alpha of glycine and C-zeta of arginine are their respective centers. On the other hand, C-beta of alanine, valine, isoleucine, serine, and threonine along with C-gamma of asparagine, aspartic acid, leucine, proline, histidine, tyrosine, phenylalanine, and tryptophan are their respective chromophoric centers. The backbone corresponding to its electrostatic profile with an amphiphilic character has been shown (color zone) at the top right of each electrostatic profile map. The N and C terminals for all the figures of the electrostatic profile as well as the backbone have been shown in this figure. The color scheme represents the distribution of potential in KT/e units.

topology and electrostatics of peptidic chromophoric groups on Gram-positive, Gram-negative and antibiotic resistant bacteria.

Syndiotactic peptides are amphiphilic by design, as can be verified from their electrostatic potential maps (Fig. 1, Supplementary Fig. 2). Distinct polar and non-polar zones in overall structure may be achieved only while they all assume the gramicidin helical conformation similar to gramicidin A, a naturally occurring syndiotactic peptide. We performed molecular dynamics simulations of syndiotactic and isotactic variants at 298 K for 10 ns under NVT conditions using the GROMACS program suite [53] with gramicidin helical conformation as the starting structure for syndiotactic peptides.

The number of clusters for each structure has been shown in Table 1 and Supplementary Fig. 3. Syndiotactic peptides are quite stable and insensitive to its environment and in general agreement with earlier reports [15]. MD results point to two important observations; i) syndiotactic structures are exceptionally stable under ambient conditions in gramicidin helical conformation, whereas poly L peptides generate a conformational ensemble forming a very rugged energy landscape, with multiple structures of a relatively small energy difference (Supplementary Fig. 4) and ii) this distinct topological manifestation may translate to different activity profiles, while they encounter membranes of different cell types.

### 3.2. Peptide synthesis and characterization

The designed peptide sequences were synthesized, characterized by reversed phase HPLC and MALDI-TOF mass spectrometry. All the peptides were further purified using semi-preparative reversed phase HPLC (Supplementary Figs. 5 and 6).

### 3.3. Peptide-lipid interaction

Differential topology dependent electrostatic profiles of the sequences give insight to test whether they differ in their membranolytic activity; both on artificial membranes and natural systems. Interaction of the peptides was studied with monolayers made up of POPC (zwitterionic lipid) and 7:3 POPC:POPG (negatively-charged). The lipids were spread as monolayers to achieve a surface pressure of 30 ± 2 mN $m^{-1}$. A surface pressure of ~30 mN $m^{-1}$ in a lipid monolayer corresponds to the packing of lipids in the biological membranes [61,62]. Injection of the peptides in the sub-phase caused an increase in the surface pressure. The peptides MS1, MS4, MS5, and MS6 had a sub-phase concentration of 9 μM while MS2 and MS3 had sub-phase concentrations of 6 μM and 12 μM, respectively. The increase in surface pressure was by and large more for the negatively charged lipid monolayer (Fig. 2). Even though the peptides display only slight preference towards negatively charged lipids, they exhibited excellent antibacterial activities without any hemolysis as discussed in the subsequent sections.

Syndiotactic peptides induce surface pressure changes upon peptide insertion ranging from 3.1 to 7.9 mN $m^{-1}$ compared to 3.1 to 3.9 mN $m^{-1}$ for isotactic poly L peptides, against negatively-charged membranes. For zwitterionic membranes, this value drops to 2.1–4.9 mN $m^{-1}$ and 1.5–2.2 mN $m^{-1}$ for syndiotactic and isotactic peptides, respectively. This points to the fact that electrostatic interaction map of all six peptide systems are distinct and different, inducing different activity profiles against different model membranes. Overall results are in good agreement with earlier theoretical investigation on the membrane permeability of alternating L, D peptides by Chipot and coworkers [70].

### 3.4. Antibacterial assay

The in vitro anti-bacterial activity of the peptides were evaluated against *S. aureus*, *E. coli* and antibiotic-resistant bacterial species. All the peptides exhibited good antibacterial activity, even at micro-molar concentration range (Fig. 3 and Table 2).

However, the results do not indicate any specific advantage in syndiotactic series (LDLD or DLDL) in comparison to the poly-L sequences, except that MS1 and MS3 have shown to be highly effective against Gram-negative bacteria. From a therapeutic point of view, this lead information may be further optimized to be included in the treatment regimens designed to address Gram-negative bacteria [71].

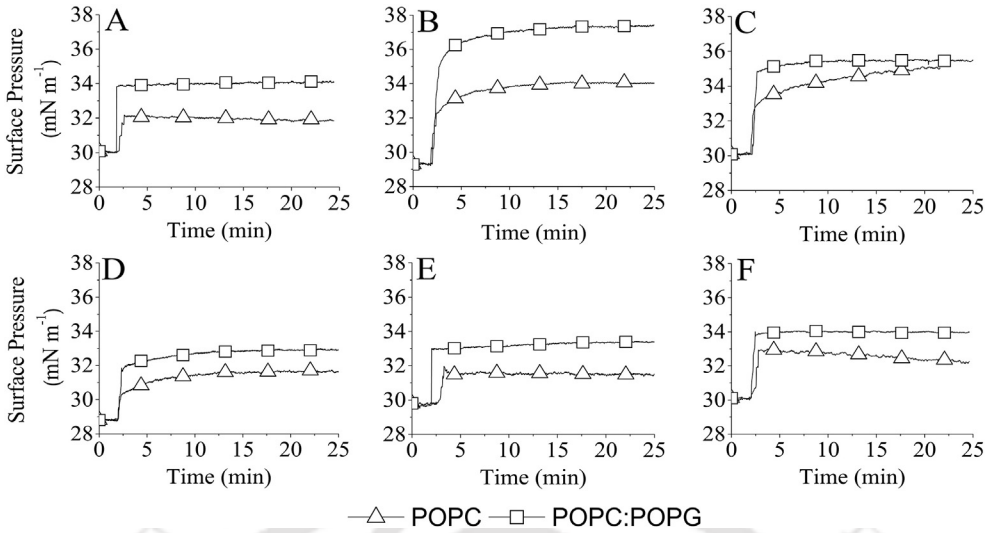


**Fig. 2.** Interaction of peptides with lipid monolayers. The peptides display some preference for a negatively-charged lipid.

### 3.5. Field emission scanning electron microscopy

To provide a topographical view of the bacterial membrane perturbation by the designed peptides, field emission scanning electron microscopy (FE-SEM) was performed.

All peptides were assayed against Gram-positive, Gram-negative and antibiotic resistant bacterial species at their respective MIC values and the images were taken at 2–3 kV. On comparison, ruptured (treated) and intact (untreated) membrane bacterial samples were observed, qualitatively confirming the membranolytic activity of the designed peptides (Fig. 4). Even though it is evident that the peptides possess membranolytic activity, it does not eliminate the possibility of a receptor mediated action against the bacterial cells.

### 3.6. Hemolytic assay

To evaluate whether the designed peptides had any potential toxicity to mammalian cells, hemolytic assay was performed. RBCs (red blood cells) were incubated with 8 μM peptides for 2 h at room temperature. RBC lysis was determined by assaying the hemoglobin content in the medium, spectroscopically at 540 nm. None of the peptides caused any notable hemolytic activity (Fig. 5) [72,73]. The results support the notion that the designed peptides have the capability of selectively destabilizing bacterial membrane but not typical mammalian cell membranes.

## 4. Discussion and conclusion

Antibiotic resistance seriously hampers effective broad spectrum therapeutic solutions, which demands generation of new chemical entities periodically. Peptides can be a desirable option for therapeutics, principally due to their high specificity and low toxicity profile [11,12]. Incorporation of D amino acids in peptide design has been a distinct possibility for designing novel folds but untapped so far to its true potential. The significant reduction in characteristic ratio of polypeptide backbone having both L and D chiral amino acids offers a possibility to design novel stable folded constructs with minimal chain length. We

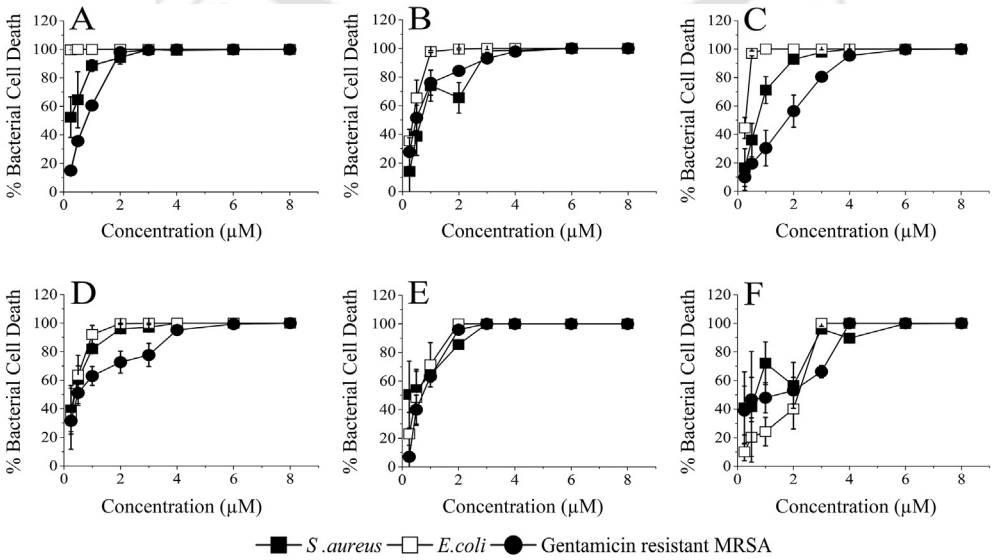


**Fig. 3.** Antibacterial activity of the designed peptide systems (MS1 to MS6), against *Staphylococcus aureus*, *Escherichia coli* and Gentamicin resistant MRSA. The percent bacterial cell death has been plotted for the designed peptides treated with various peptide concentrations ranging from 0.25 μM to 8 μM. All the peptides show complete killing at 8 μM concentration against the three bacterial cell types. Standard deviation in each case is shown as error bars.

**Table 2**
Minimal inhibitory concentration (MIC) of designed peptides. All MIC values are in micromolar concentration.

| Bacteria | Peptides | | | | | |
|---|---|---|---|---|---|---|
| | MS1 | MS2 | MS3 | MS4 | MS5 | MS6 |
| *S. aureus* | 8 | 8 | 6 | 8 | 3 | 8 |
| *E. coli* | 0.5 | 4 | 1 | 4 | 2 | 3 |
| Gentamicin-resistant MRSA | 4 | 6 | 8 | 8 | 3 | 4 |

tried to examine the potential to expand the peptide design space manifold, by adopting the simplest designed system, the LDLD chiral polypeptide to verify its possible structural and resultant physiological effects.

Examination of peptide backbone at an atomistic scale for folded and extended conformations of poly-L and alternating-L, D structure reveals that short range and long range electrostatics are either conflicting or harmonious depending upon the chain stereochemistry. The stereochemical transformation of inter peptide electrostatics from a condition of conflict to one of harmony respectively for poly-L and alternating-L, D explains why peptides differ in properties such as "stiffness" and solvent sensitivity. Our molecular dynamics simulation results further underline this earlier observation. The big difference in the number of clusters among syndiotactic and isotactic variants points to the rugged energy landscape of poly-L sequences compared to a more facile one in LDLD (Table 1, Supplementary Fig. 4). Since the sequences are identical, this extra stability may have been realized through favorable short range and long range electrostatics as explained by Ramakrishnan et al., in an earlier published work [15]. As a consequence of this, gramicidin helical conformation of LDLD peptides is more or less insensitive to external stimulus, whereas multiple conformational possibilities of relatively similar energy in their energy landscape allows poly L variants to switch from one conformation to another, an observation similar to earlier reported studies from Ghadiri and Gellman's group, with unnatural amino acids [19,26–28,38–40,47]. Therefore, amphipathicity in LDLD is clearly defined (Fig. 1), well-crafted and conformationally retained, independent of external stimulus.

Side-chains with appreciable cationicity such as arginine and lysine cannot be designed to be co-localized on an isotactic poly L peptide chain, due to short range electrostatics, because the resultant repulsive forces are not compensated by favorable backbone interactions. In LDLD diastereomers, short range and long range electrostatics are favorable in a gramicidin helical conformation, so that side-chain interactions between positive charges of and between lysine and arginine can be compensated. The conflicting interactions at the side-chain may be balanced by LDLD backbone electrostatics while they assume gramicidin-helical conformation. Poly-L backbone, on the other hand, remains extended thus generating sequence dependent structures of relatively similar energy profiles. The relatively large number of clusters in the poly-L

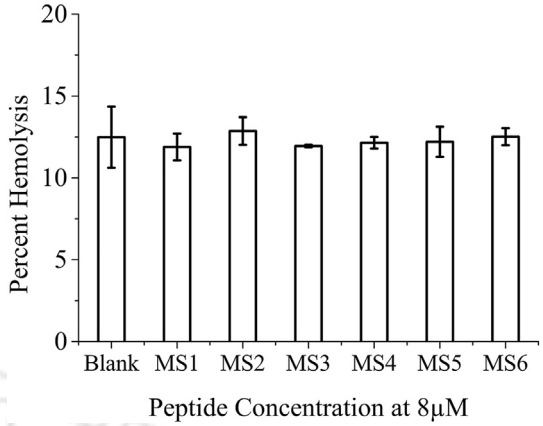


**Fig. 5.** Hemolytic assay of peptides against human RBC. Human RBC was treated with buffer and 8 μM concentration of the designed peptides. The experiment was performed in triplicates and the error bars represent standard deviation.

ensemble in our MD simulations supports this assumption. Bacterial cell systems comprise a negatively charged plasma membrane, where the complexity of the membrane varies from a Gram-positive to Gram-negative bacteria. The Gram-positive cell wall consists of a 20–80 nm thick layer of peptidoglycans and is rich in lipoteichoic acid and its derivatives, with teichoic acid endowing majority of the negative charge to the cell surface. The cell wall for Gram-negative bacteria is however, more complex than others, consisting of a 2–7 nm thick layer of peptidoglycans surrounded by a 7–8 nm thick outer membrane. In contrast to the lipoteichoic acid of Gram-positive bacteria, the negative charge on the Gram-negative bacterial surface is due to the presence of lipopolysaccharide.

As per the prediction of Sir Alexander Fleming, the rise of resistant species can be attributed to the overuse of antibiotics. Persistent use of antibiotics caused by drug abuse, spared resistant species which triggered their number due to bacterial multiplication and mutation through gene transfer [74–79]. Such a biochemical adaptation provides a shielding effect against traditional antibiotics. The designed peptides show better activity against antibiotic resistant Gram-positive species as compared to the native Gram-positive one.

The stiffness of an LDLD diastereomeric backbone, thus facilitates the maintenance of the designed amphiphilicity, while the poly-L backbone undergoes major changes in conformations owing to the same charge repulsions and dipole moment. Despite having the same amino acid content and only two sequence variations, the six designed peptide systems are distinct by themselves and have unique structure dependent electrostatic fingerprints (Supplementary Fig. 2), and therefore have differential membranolytic potential. Significantly, all designed systems are showing comparable or better efficiency compared to typical

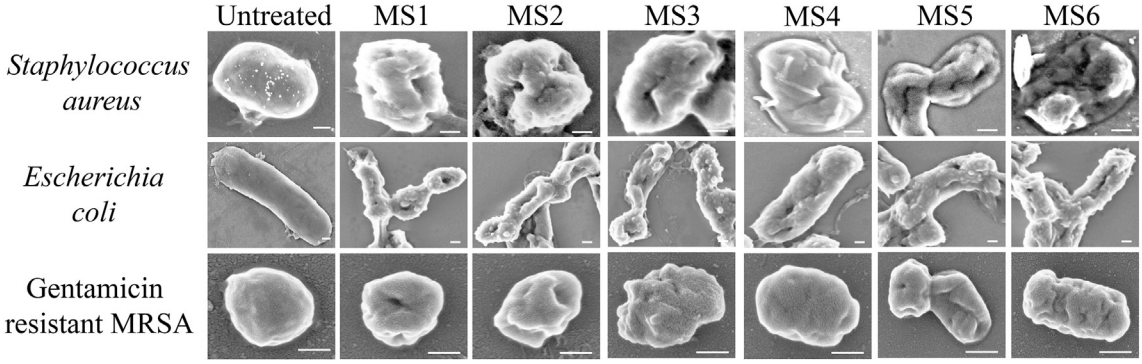


**Fig. 4.** FE-SEM images indicating membranolytic activity of designed peptides. Membrane deformation as a result of bactericidal action of the designed peptides against (A) *Staphylococcus aureus*, (B) *Escherichia coli* and (C) Gentamicin resistant MRSA at their respective minimum inhibitory concentrations (Table 2) is shown. The scale bars correspond to 200 nm.

molecules reported as broad spectrum peptide antibiotics (Supplementary Table 1). For Gram-positive bacteria, the potency is comparable across designed systems, but all LDLD peptides are showing better activity compared to their poly L variants in Gram-negative bacteria with an MIC value as low as 0.5 μM, possibly owing to the unique charge disposition of negative species on Gram-negative bacteria. The poly-L backbone in our model systems may be adopting a complementary structure to the membrane surface resulting in much better MIC values against antibiotic resistant species. This may not be the case always as there could be situations wherein, the poly-L version may not adapt a suitable amphiphilic profile, crucial for bacterial membrane lysis. The hetero-chiral versions (LDLD and DLDL) on the other hand are expected to retain their designed amphiphilicity independent of any modulation in the membrane surface charge. At 4 μM, both poly-L peptides have 100% killing efficiency, as against 6–8 μM for 3 out of 4 LDLD sequences. However, more elaborate study is necessary to fully verify this hypothesis.

The MIC values for peptides against Gram-negative bacteria are, lower against other species, indicating better performance in lysing bacterial cells. The designed peptide MS1 (Table 2) is showing excellent activity against Gram-negative bacteria, emerging as an important molecule for further verification as a potential drug molecule. This can again be attributed to the retention of designed amphiphilicity of the syndiotactic variants and relatively fluxional nature of homochiral sequences. Adaptation of diastereomeric variants by changing the stereochemical sequence thus offers a unique possibility to lock the topology of the designed peptide, thus modulating its specificity and activity.

Receptor based design of peptide drugs has traditionally been facing issues of the lack of specificity, principally due to the degenerate conformational variants offered by poly L diastereomeric sequences. Conformational locking by stereochemical engineering offers the possibility of fixing the structure and therefore imparting a good degree of specificity to the designed constructs. Differential activity profiles of six designed systems against three different bacterial membranes tested and constituent cells in human blood underlines this distinct possibility.

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## Transparency document

The Transparency document associated with this article can be found, in the online version.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamem.2017.05.002.

## References

[1] A. Mucherino, S. Costantini, D. di Serafino, M. D'Apuzzo, A. Facchiano, G. Colonna, Understanding the role of the topology in protein folding by computational inverse folding experiments, Comput. Biol. Chem. 32 (2008) 233–239.
[2] Y.J.M. Bollen, C.P.M. van Mierlo, Protein topology affects the appearance of intermediates during the folding of proteins with a flavodoxin-like fold, Biophys. Chem. 114 (2005) 181–189.
[3] G. Jerath, P.K. Hazam, S. Shekhar, V. Ramakrishnan, Mapping the geometric evolution of protein folding motor, PLoS One 11 (2016), e0163993.
[4] V. Ramakrishnan, S.P. Srinivasan, S.M. Salem, S.J. Matthews, W. Colón, M. Zaki, B. Christopher, Geofold: topology-based protein unfolding pathways capture the effects of engineered disulfides on kinetic stability, Proteins Struct. Funct. Bioinf. 80 (2012) 920–934.
[5] A. Kumar, V. Ramakrishnan, Creating novel protein scripts beyond natural alphabets, Syst. Synth. Biol. 4 (2010) 247–256.
[6] S. Durani, Protein design with L- and D-alpha-amino acid structures as the alphabet, Acc. Chem. Res. 41 (2008) 1301–1308.
[7] F.W. Billmeyer, Textbook of Polymer Science, 1971.
[8] K. Suat, S.D. Jois, Design of β-turn based therapeutic agents, Curr. Pharm. Des. 9 (2003) 1209–1224.
[9] N.W. Schmidt, A. Mishra, G.H. Lai, M. Davis, L.K. Sanders, D. Tran, A. Garcia, K.P. Tai, P.B. McCray, A.J. Ouellette, M.E. Selsted, G.C. Wong, Criterion for amino acid composition of defensins and antimicrobial peptides based on geometry of membrane destabilization, J. Am. Chem. Soc. 133 (2011) 6720–6727.
[10] C.K. Wang, G.J. King, A.C. Conibear, M.C. Ramos, S. Chaousis, S.T. Henriques, D.J. Craik, Mirror images of antimicrobial peptides provide reflections on their functions and amyloidogenic properties, J. Am. Chem. Soc. 138 (2016) 5706–5713.
[11] L. Otvos Jr., Peptide-based drug design: here and now, Methods Mol. Biol. 494 (2008) 1–8.
[12] K. Saikia, Y.D. Sravani, V. Ramakrishnan, N. Chaudhary, Highly potent antimicrobial peptides from N-terminal membrane-binding region of *E. coli* MreB, Sci. Rep. 7 (2017) 42994.
[13] L. Huo, W.A. van der Donk, Discovery and characterization of Bicereucin, an unusual D-amino acid-containing mixed two-component Lantibiotic, J. Am. Chem. Soc. 138 (2016) 5254–5257.
[14] V. Ramakrishnan, R. Ranbhor, S. Durani, Simulated folding in polypeptides of diversified molecular tacticity: implications for protein folding and de novo design, Biopolymers 78 (2005) 96–105.
[15] V. Ramakrishnan, R. Ranbhor, A. Kumar, S. Durani, The link between sequence and conformation in protein structures appears to be stereochemically established, J. Phys. Chem. B 110 (2006) 9314–9323.
[16] D.A. Brant, P.J. Flory, The configuration of random polypeptide chains. I. Experimental results, J. Am. Chem. Soc. 87 (1965) 2788–2791.
[17] D.A. Brant, P.J. Flory, The configuration of random polypeptide chains. II. Theory, J. Am. Chem. Soc. 87 (1965) 2791–2800.
[18] P.J. Flory, Principles of Polymer Chemistry, Cornell University Press, 1953.
[19] M.R. Ghadiri, J.R. Granja, R.A. Milligan, D.E. McRee, N. Khazanovich, Self-assembling organic nanotubes based on a cyclic peptide architecture, Nature 366 (1993) 324–327.
[20] D. Liu, W.F. DeGrado, De novo design, synthesis, and characterization of antimicrobial beta-peptides, J. Am. Chem. Soc. 123 (2001) 7553–7559.
[21] H. Myrberg, L. Zhang, M. Mae, U. Langel, Design of a tumor-homing cell-penetrating peptide, Bioconjug. Chem. 19 (2008) 70–75.
[22] R. Pasqualini, E. Koivunen, R. Kain, J. Lahdenranta, M. Sakamoto, A. Stryhn, R.A. Ashmun, L.H. Shapiro, W. Arap, E. Ruoslahti, Aminopeptidase N is a receptor for tumor-homing peptides and a target for inhibiting angiogenesis, Cancer Res. 60 (2000) 722–727.
[23] G. Wang, X. Li, Z. Wang, APD2: the updated antimicrobial peptide database and its application in peptide design, Nucleic Acids Res. 37 (2009) D933–D937.
[24] P. Kapoor, H. Singh, A. Gautam, K. Chaudhary, R. Kumar, G.P.S. Raghava, TumorHoPe: a database of tumor homing peptides, PLoS One 7 (2012), e35187.
[25] P. Agrawal, S. Bhalla, S.S. Usmani, S. Singh, K. Chaudhary, G.P. Raghava, A. Gautam, CPPsite 2.0: a repository of experimentally validated cell-penetrating peptides, Nucleic Acids Res. 44 (2016) D1098–D1103.
[26] S. Fernandez-Lopez, H.-S. Kim, E.C. Choi, M. Delgado, J.R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D.A. Weinberger, K.M. Wilcoxen, M.R. Ghadiri, Antibacterial agents based on the cyclic D,L-[alpha]-peptide architecture, Nature 412 (2001) 452–455.
[27] W.S. Horne, C.M. Wiethoff, C. Cui, K.M. Wilcoxen, M. Amorin, M.R. Ghadiri, G.R. Nemerow, Antiviral cyclic D,L-alpha-peptides: targeting a general biochemical pathway in virus infections, Bioorg. Med. Chem. 13 (2005) 5145–5153.
[28] H.B. Albada, P. Prochnow, S. Bobersky, J.E. Bandow, N. Metzler-Nolte, Highly active antibacterial ferrocenoylated or ruthenocenoylated Arg-Trp peptides can be discovered by an L-to-D substitution scan, Chem. Sci. 5 (2014) 4453–4459.
[29] A.S. Ravipati, S.T. Henriques, A.G. Poth, Q. Kaas, C.K. Wang, M.L. Colgrave, D.J. Craik, Lysine-rich cyclotides: a new subclass of circular knotted proteins from Violaceae, ACS Chem. Biol. 10 (2015) 2491–2500.
[30] S. Gautam, T. Kim, E. Lester, D. Deep, D.A. Spiegel, Wall teichoic acids prevent antibody binding to epitopes within the cell wall of *Staphylococcus aureus*, ACS Chem. Biol. 11 (2016) 25–30.
[31] B. Mishra, G. Wang, Ab initio design of potent anti-MRSA peptides based on database filtering technology, J. Am. Chem. Soc. 134 (2012) 12426–12429.
[32] M. Stach, T.N. Siriwardena, T. Kohler, C. van Delden, T. Darbre, J.L. Reymond, Combining topology and sequence design for the discovery of potent antimicrobial peptide dendrimers against multidrug-resistant *Pseudomonas aeruginosa*, Angew. Chem. Int. Ed. Eng. 53 (2014) 12827–12831.
[33] G. Michaud, R. Visini, M. Bergmann, G. Salerno, R. Bosco, E. Gillon, B. Richichi, C. Nativi, A. Imberty, A. Stocker, T. Darbre, J.-L. Reymond, Overcoming antibiotic resistance in *Pseudomonas aeruginosa* biofilms using glycopeptide dendrimers, Chem. Sci. 7 (2016) 166–182.

[34] H. Wu, Y. Niu, S. Padhee, R.E. Wang, Y. Li, Q. Qiao, G. Bai, C. Cao, J. Cai, Design and synthesis of unprecedented cyclic [gamma]-AA peptides for antimicrobial development, Chem. Sci. 3 (2012) 2570–2575.
[35] H. Chen, C. Liu, D. Chen, K. Madrid, S. Peng, X. Dong, M. Zhang, Y. Gu, Bacteria-targeting conjugates based on antimicrobial peptide for bacteria diagnosis and therapy, Mol. Pharm. 12 (2015) 2505–2516.
[36] V. Dartois, J. Sanchez-Quesada, E. Cabezas, E. Chi, C. Dubbelde, C. Dunn, J. Granja, C. Gritzen, D. Weinberger, M.R. Ghadiri, T.R. Parr Jr., Systemic antibacterial activity of novel synthetic cyclic peptides, Antimicrob. Agents Chemother. 49 (2005) 3302–3310.
[37] W.S. Horne, C.M. Wiethoff, C. Cui, K.M. Wilcoxen, M. Amorin, M.R. Ghadiri, G.R. Nemerow, Antiviral cyclic D,L-α-peptides: targeting a general biochemical pathway in virus infections, Bioorg. Med. Chem. 13 (2005) 5145–5153.
[38] S. Fernandez-Lopez, H.-S. Kim, E.C. Choi, M. Delgado, J.R. Granja, A. Khasanov, K. Kraehenbuehl, G. Long, D.A. Weinberger, K.M. Wilcoxen, M.R. Ghadiri, Antibacterial agents based on the cyclic D,L-[alpha]-peptide architecture, Nature 412 (2001) 452–455.
[39] M.R. Ghadiri, J.R. Granja, L.K. Buehler, Artificial transmembrane ion channels from self-assembling peptide nanotubes, Nature 369 (1994) 301–304.
[40] H.S. Kim, J.D. Hartgerink, M.R. Ghadiri, Oriented self-assembly of cyclic peptide nanotubes in lipid membranes, J. Am. Chem. Soc. 120 (1998) 4417–4424.
[41] Y. Shai, Z. Oren, Diastereomers of cytolysins, a novel class of potent antibacterial peptides, J. Biol. Chem. 271 (1996) 7305–7308.
[42] N. Papo, Y. Shai, Effect of drastic sequence alteration and D-amino acid incorporation on the membrane binding behavior of lytic peptides, Biochemistry 43 (2004) 6393–6403.
[43] N. Benkirane, M. Friede, G. Guichard, J.P. Briand, M.H. Van Regenmortel, S. Muller, Antigenicity and immunogenicity of modified synthetic peptides containing D-amino acid residues. Antibodies to a D-enantiomer do recognize the parent L-hexapeptide and reciprocally, J. Biol. Chem. 268 (1993) 26279–26285.
[44] P.J. Fisher, F.G. Prendergast, M.R. Ehrhardt, J.L. Urbauer, A.J. Wand, S.S. Sedarous, D.J. McCormick, P.J. Buckley, Calmodulin interacts with amphiphilic peptides composed of all D-amino acids, Nature 368 (1994) 651–653.
[45] D. Wade, A. Boman, B. Wahlin, C.M. Drain, D. Andreu, H.G. Boman, R.B. Merrifield, All-D amino acid-containing channel-forming antibiotic peptides, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 4761–4765.
[46] L. Zhang, T.J. Falla, Cationic antimicrobial peptides — an update, Expert Opin. Investig. Drugs 13 (2004) 97–106.
[47] T.L. Raguse, E.A. Porter, B. Weisblum, S.H. Gellman, Structure-activity studies of 14-helical antimicrobial beta-peptides: probing the relationship between conformational stability and antimicrobial potency, J. Am. Chem. Soc. 124 (2002) 12774–12785.
[48] J.L. Reymond, T. Darbre, Expanding the topological space of bioactive peptides, Chimia 67 (2013) 864–867.
[49] B. Findlay, G.G. Zhanel, F. Schweizer, Cationic amphiphiles, a new generation of antimicrobials inspired by the natural antimicrobial peptide scaffold, Antimicrob. Agents Chemother. 54 (2010) 4049–4058.
[50] A. Holtzel, R.W. Jack, G.J. Nicholson, G. Jung, K. Gebhardt, H.P. Fiedler, R.D. Sussmuth, Streptocidins A–D, novel cyclic decapeptide antibiotics produced by *Streptomyces* sp. Tu 6071. II. Structure elucidation, J. Antibiot. 54 (2001) 434–440.
[51] B.I. Dahiyat, S.L. Mayo, De novo protein design: fully automated sequence selection, Science 278 (1997) 82–87.
[52] A. Kumar, R. Ranbhor, K. Patel, V. Ramakrishnan, S. Durani, Automated protein design: landmarks and operational principles, Prog. Biophys. Mol. Biol. 125 (2017) 24–35.
[53] S. Pronk, S. Pall, R. Schulz, P. Larsson, P. Bjelkmar, R. Apostolov, M.R. Shirts, J.C. Smith, P.M. Kasson, D. van der Spoel, B. Hess, E. Lindahl, GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit, Bioinformatics 29 (2013) 845–854.
[54] M. Christen, P.H. Hunenberger, D. Bakowies, R. Baron, R. Burgi, D.P. Geerke, T.N. Heinz, M.A. Kastenholz, V. Krautler, C. Oostenbrink, C. Peter, D. Trzesniak, W.F. van Gunsteren, The GROMOS software for biomolecular simulation: GROMOS05, J. Comput. Chem. 26 (2005) 1719–1751.
[55] L. Li, C. Li, S. Sarkar, J. Zhang, S. Witham, Z. Zhang, L. Wang, N. Smith, M. Petukh, E. Alexov, DelPhi: a comprehensive suite for DelPhi software and associated resources, BMC Biophys. 5 (2012) 9.
[56] C. Li, M. Petukh, L. Li, E. Alexov, Continuous development of schemes for parallel computing of the electrostatics in biological systems: implementation in DelPhi, J. Comput. Chem. 34 (2013) 1949–1960.
[57] MATLAB and Statistics Toolbox Release, The MathWorks, Inc., Natick, Massachusetts, United States, 2013.
[58] S. Wang, Y. Ishii, Revealing protein structures in solid-phase peptide synthesis by (13)C sold-state NMR: evidence of excessive misfolding for Alzheimer's β, J. Am. Chem. Soc. 134 (2012) 2848–2851.
[59] B. Dang, B. Dhayalan, S.B.H. Kent, Enhanced solvation of peptides attached to "solid-phase" resins: straightforward syntheses of the elastin sequence Pro-Gly-Val-Gly-Val-Pro-Gly-Val-Gly-Val, Org. Lett. 17 (2015) 3521–3523.
[60] N. Chaudhary, R. Nagaraj, Impact on the replacement of Phe by Trp in a short fragment of Abeta amyloid peptide on the formation of fibrils, J. Pept. Sci. 17 (2011) 115–123.
[61] S.R. Dennison, R.D. Baker, I.D. Nicholl, D.A. Phoenix, Interactions of cell penetrating peptide Tat with model membranes: a biophysical study, Biochem. Biophys. Res. Commun. 363 (2007) 178–182.
[62] S.R. Dennison, Y.S. Kim, H.J. Cha, D.A. Phoenix, Investigations into the ability of the peptide, HAL18, to interact with bacterial membranes, Eur. Biophys. J. 38 (2008) 37–43.
[63] J. Paczesny, I. Binkiewicz, M. Janczuk, K. Wybrańska, Ł. Richter, R. Hołyst, Langmuir and Langmuir–Blodgett films of unsymmetrical and fully condensed polyhedral oligomeric silsesquioxanes (POSS), J. Phys. Chem. C 119 (2015) 27007–27017.
[64] J. Varkey, R. Nagaraj, Antibacterial activity of human neutrophil defensin HNP-1 analogs without cysteines, Antimicrob. Agents Chemother. 49 (2005) 4561–4566.
[65] W.A. Velema, J.P. van der Berg, W. Szymanski, A.J.M. Driessen, B.L. Feringa, Orthogonal control of antibacterial activity with light, ACS Chem. Biol. 9 (2014) 1969–1974.
[66] K. Rasool, M. Helal, A. Ali, C.E. Ren, Y. Gogotsi, K.A. Mahmoud, Antibacterial activity of Ti3C2Tx MXene, ACS Nano 10 (2016) 3674–3684.
[67] A.S. Abd-El-Aziz, C. Agatemor, N. Etkin, D.P. Overy, M. Lanteigne, K. McQuillan, R.G. Kerr, Antimicrobial organometallic dendrimers with tunable activity against multi-drug-resistant bacteria, Biomacromolecules 16 (2015) 3694–3703.
[68] S.N. Balaji, V. Trivedi, Extracellular methemoglobin mediated early ROS spike triggers osmotic fragility and RBC destruction: an insight into the enhanced hemolysis during malaria, Indian J. Clin. Biochem. 27 (2012) 178–185.
[69] M.-R. Lee, N. Raman, S.H. Gellman, D.M. Lynn, S.P. Palecek, Hydrophobicity and helicity regulate the antifungal activity of 14-helical β-peptides, ACS Chem. Biol. 9 (2014) 1613–1621.
[70] J. Comer, F. Dehez, W. Cai, C. Chipot, Water conduction through a peptide nanotube, J. Phys. Chem. C 117 (2013) 26797–26803.
[71] P.D. Tamma, S.E. Cosgrove, L.L. Maragakisb, Combination therapy for treatment of infections with Gram-negative bacteria, Clin. Microbiol. Rev. 25 (2012) 450–470.
[72] M.M. Rigano, A. Romanelli, A. Fulgione, N. Nocerino, N. D'Agostino, C. Avitabile, L. Frusciante, A. Barone, F. Capuano, R. Capparelli, A novel synthetic peptide from a tomato defensin exhibits antibacterial activities against *Helicobacter pylori*, J. Pept. Sci. 18 (2012) 755–762.
[73] K.L. Scott, J. Lecak, J.P. Acker, Biopreservation of red blood cells: past, present, and future, Transfus. Med. Rev. 19 (2005) 127–142.
[74] The Antibiotic Resistance Crisis Part 1: Causes and Threats, PT 40, 2015 277–283.
[75] A.F. Read, R.J. Woods, Population biological principles of drug-resistance evolution in infectious diseases, Lancet Infect. Dis. 11 (2011) 236–247.
[76] H. Roy, M. Ibba, RNA-dependent lipid remodeling by bacterial multiple peptide resistance factors, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 4667–4672.
[77] R. Kumariya, S.K. Sood, Y.S. Rajput, N. Saini, A.K. Garsa, Increased membrane surface positive charge and altered membrane fluidity leads to cationic antimicrobial peptide resistance in *Enterococcus faecalis*, Biochim. Biophys. Acta Biomembr. 1848 (2015) 1367–1375.
[78] S. Maria-Neto, K.C. de Almeida, M.L.R. Macedo, O.L. Franco, Understanding bacterial resistance to antimicrobial peptides: from the surface to deep inside, Biochim. Biophys. Acta Biomembr. 1848 (2015) 3078–3088.
[79] H.G. Sahl, Y. Shai, Bacterial resistance to antimicrobial peptides, Biochim. Biophys. Acta Biomembr. 1848 (2015) 3019–3020.

# *Peptido-mimetic Approach in the Design of Syndiotactic Antimicrobial Peptides*

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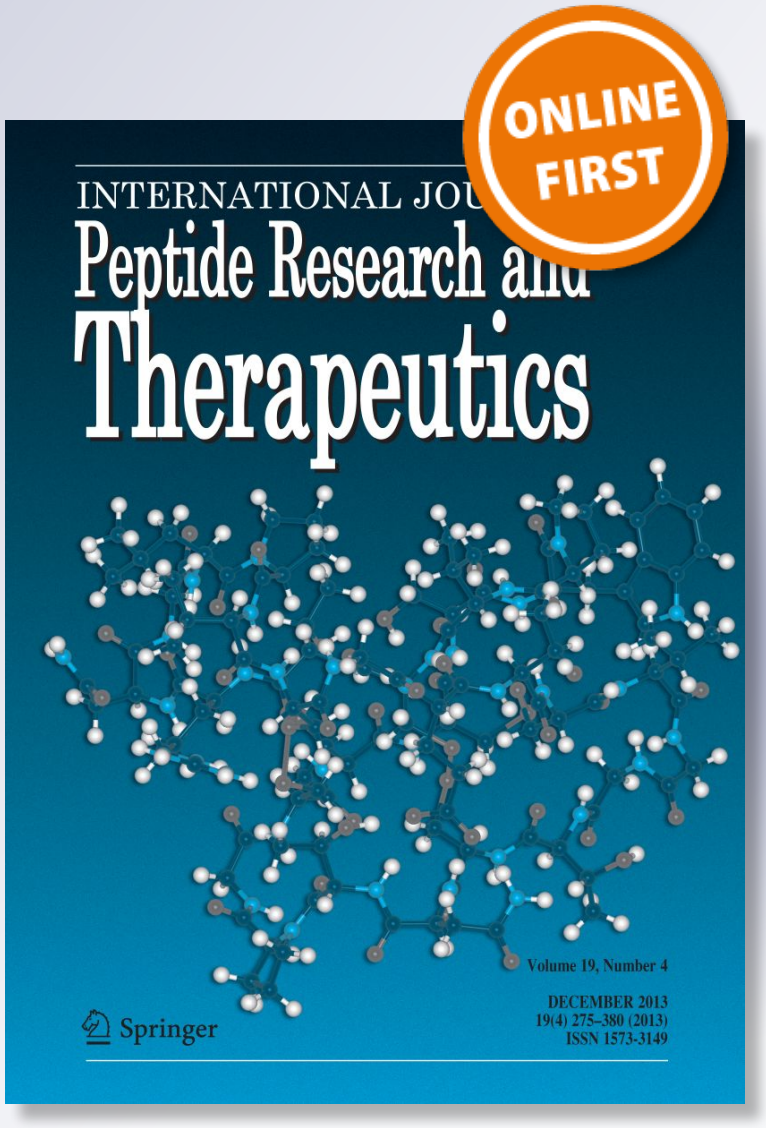


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# Peptido-mimetic Approach in the Design of Syndiotactic Antimicrobial Peptides

**Prakash Kishore Hazam[1] · Gaurav Jerath[1] · Nitin Chaudhary[1] · Vibin Ramakrishnan[1]**

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**Abstract** Biocompatibility, low toxicity and high selectivity towards bacterial cells has been the hallmark of peptide-based antibiotics. The innate immune system has been employing such molecular systems against invading pathogens as a successful defense strategy. In this study, we attempt to develop topologically constrained antimicrobial peptides with syndiotactic stereochemical arrangement, by incorporating L and D amino acids successively in its amino acid sequence. Acetylated versions of the designed peptides were also examined for its influence on bactericidal potency, against Gram-positive and Gram-negative bacteria. Syndiotactic stereochemical arrangement of the polypeptide main chain mimics stereochemistry of Gramicidin, a naturally occurring antimicrobial peptides. Gramicidin is a class of penta-deca-peptides isolated from soil bacteria *Bacillus brevis*, but their utility as antibiotic was limited to topical use due to high levels of hemotoxicity. Activity profiles of the four de novo designed peptide variants show higher specificity towards Gram-positive bacteria than Gram-negative variants, matching earlier reports on the therapeutic potential of gramicidin as a broad spectrum antibiotic. Significantly, our hemolytic assay confirms very low (<1%) levels of toxicity for the designed peptides unlike gramicidin. Earlier reports confirm that incorporation of D amino acids effectively negates the possibility of proteolytic degradation, thus pointing to the potential utility of de novo designed peptides with diversified stereochemistry as a promising new approach in the generation of novel antibiotic peptides.

**Keywords** Peptidomimetics · Polymer tacticity · Antimicrobial peptides · Peptide design · Gramicidin · Stereochemistry

**Abbreviations**

| | |
|---|---|
| MD | Molecular dynamics |
| RMSD | Root mean square deviation |
| Rg | Radius of gyration |
| FE-SEM | Field emission scanning electron microscopy |
| MS | Mass spectrometry |
| CFU | Colony forming unit |

## Introduction

Higher organisms use peptides as a defense mechanism against invading pathogens (Dosler and Karaaslan 2014; Ravichandran et al. 2017; Czihal and Hoffmann 2009; Saikia et al. 2017), Defensins (Lehrer 2004), cecropins (Moore et al. 1996), magainins (Berkowitz et al. 1990) and cathelicidins (Zanetti 2005) constitute major classes of "host defense peptide". There is a remarkable increase in peptide based antibiotics, with nearly two dozen molecules are either completed or under clinical trials as antimicrobial or immunomodulatory agents (Felicio et al. 2017). However, cost, uncertain toxicology profiles and lability to proteolytic susceptibility hampers the otherwise promising approach, especially in the light of the phenomenal increase in antibiotic resistance against conventional therapeutics (Li et al. 2015; Koh et al. 2015). Decreasing the size of peptides and machine learning approach have more

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✉ Vibin Ramakrishnan
vibin@iitg.ernet.in

[1] Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam 781039, India

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or less addressed the first two issues, while the problem of proteolytic degradation may be sorted out by the use of D-stereoisomers and unnatural amino acids in the sequence (Hamamoto et al. 2002). Designed antimicrobial peptides, in general, are cationic amphipathic peptide sequences with distinct membrane permeabilizing ability (Hazam et al. 2017; Wimley 2010). Though various mechanisms have been proposed like carpet (Tene et al. 2016), detergent (Bechinger and Lohner 2006), barrel stave (Chang et al. 2008), toroidal pore (Brogden 2005) etc., a consensus picture is yet to emerge.

We designed two amphipathic peptide sequences and their N-terminal acetylated variants mimicking the stereochemical sequence of Gramicidin, a penta-deca-peptide isolated from soil bacterial species *Bacillus brevis* (Urry 1971). Antibiotic profile of Gramicidin reports that they are particularly effective against Gram-positive bacteria. However, their utility is rather limited to topical use as a lotion or ointment in the treatment of infected surface wounds and throat infections (Wang and Ishii 2012; Wishart et al. 2008). Reported mechanism of action for Gramicidin suggests that it inserts itself into bacterial membranes resulting in membrane disruption and permeabilization, leading to a sequence of events such as the loss of intracellular solutes, dissipation of trans-membrane potential, inhibition of respiration and reduction in ATP pools, culminating in cell death (Burkhart et al. 1998). Naturally-occurring Gramicidin or Gramicidin D exists in isoforms with a variation of valine or isoleucine at first position. Gramicidin molecule is a mixture of Gramicidin A, B and C with a variation at the 11th position containing tryptophan, phenylalanine and tyrosine respectively (Burkhart et al. 1999). In this paper we present a design philosophy to synthesize cationic, amphipathic peptide molecules incorporating D-amino acids in the design alphabet. To promote the membrane permeabilizing ability, we design our sequence around Gramicidin with gramicidin helix (6.3 helix) as the target structure. Gramicidins form cation selective trans-membrane ion channel in typical model membranes (Hladky and Haydon 1972). Even though, Gramicidin acquires various folding states, two of the major conformations have been predominant in different environments; first is the channel forming single stranded helical dimer and the second type is a non-channel forming intertwined helix (Urry 1971; LoGrasso et al. 1988). Even though conformations of gramicidin are solvent dependent, single stranded β (6.3) dimer is the thermodynamically most stable structure (LoGrasso et al. 1988; Killian et al. 1988; Kelkar and Chattopadhyay 2007).

Like all natural proteins, most of the therapeutic peptides are sequences of asymmetric amino acids having L-chiral stereo-chemical structure. In other words, they are stereo-regular "isotactic" hetero-polymers. The isotactic stereochemistry of the backbone, coupled with planarity of peptide bond and steric effects limits the conformational space of a polypeptide. Even then, the predictive ability of a given amino acid sequence to fix the structure, folding intermediates and pathways are still weak, despite making enormous advancement in the basic understanding of this unsolved problem (Ramakrishnan et al. 2012; Khoury et al. 2014). This limits the success of a protein de novo design experiment in realizing its translational objectives.

Polymer sequences with alternating L and D-chiral monomers (amino acids) are referred to as syndiotactic polymers and with random distribution of L and D are heterotactic or atactic polymer (Billmeyer 1957; Durani 2008). Gramicidin is a 15 amino acid long trans membrane helical molecule with alternating L and D-chiral amino acids in its sequence and can hence be termed as a syndiotactic peptide (Nanda et al. 2007). In this study we tried to develop topologically constrained antimicrobial peptide molecules with syndiotactic stereo-chemical arrangements after making an objective assessment about their relative stability by molecular dynamics simulations. Acetylated variants of both the designed peptides, were also examined to test their possible differential efficacy, against Gram-positive and Gram-negative bacterial species. The activity profiles were considerably similar, indicating limited significance of acetylation in syndiotactic sequences. The bactericidal potency of the designed peptides was higher against Gram-positive than in Gram-negative bacteria, though the design approach may be further modified in future designs in the generation of broad spectrum antibiotics.

## Materials and Methods

### Design and Simulation

The peptides molecules used in this study were designed by using an in-house software PDB make and a modified version of Ribosome Software from G.D. Rose laboratory (Srinivasan 2013). Molecular dynamics (MD) simulations were performed using GROMACS (version: 4.6.5) (Pronk et al. 2013) with GROMOS 96 43a1 force field (Scott et al. 1999). The peptide structures were energy minimized in vacuum over 2000 steps using the steepest descent algorithm in a cubic box, with each face separated at 1.5 nm from the protein molecule. Subsequently, water molecules (SPC) were added to the simulation box and the system was further energy minimized. A 10 ns production run under NVT conditions was performed with an integration step of 2 fs. Initial velocities were taken from Maxwell distribution at 300 K, using Berendsen thermostat for temperature coupling with coupling relaxation step setting at 0.1 ps. The cut-off limit for non-bonded interactions was set at

0.8 nm. All through the simulation run, bond length was constrained with geometric accuracy of $10^{-4}$.

### Electrostatic Profiling

Electrostatic potential for the designed peptides was calculated by solving Finite Difference Poisson–Boltzmann equation using Delphi software (Li et al. 2012). These electrostatic potential maps of candidate structures were further compared to identify distinct electrostatic potential signatures for each structure. The electrostatic potentials were represented in a 3D graph using MATLAB.

### Reagents

Rink Amide resin, Fmoc amino acids, *N, N, N, N*-tetramethyl-*O*-(1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU), *N, N*-dimethylformamide (DMF), m-Cresol, Glutaraldehyde, Dimethylsulphoxide (DMSO), and Ethanol were purchased from Merck. 1-Hydroxybenzotriazole (HOBt), diethyl ether, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium phosphate monobasic anhydrous, acetonitrile (ACN), ethylene diamine tetra acetic acid (EDTA) and Piperidine were obtained from SRL laboratories. *N, N*-diisopropylethylamine (DIPEA), thioanisole, 1, 2 ethane dithiol (EDT), Trifluoroactic acid (TFA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), was purchased from Sigma-Aldrich. Nutrient Broth and Agar was purchased from Himedia laboratories. All other reagents used were of the highest purity (≥99%).

### Peptide Synthesis and Characterization

The peptides were synthesized employing standard Solid phase peptide synthesis protocol (Fmoc chemistry). Post synthesis, the peptides were cleaved from the resin using a cleavage cocktail (m-Cresol: Thioanisole: EDT: TFA :: 2:2:1:20) for 12 h in dark. The peptides were purified by means of semi-preparative reversed-phase liquid chromatography. A chromatographic run of 10% ACN to 100% ACN with 0.1% TFA, was used for the gradient elution of peptides at a flow rate of 1 mL/min. The eluents were monitored at 210 nm and verified by electrospray ionization mass spectrometry (Wang et al. 2012; Falcao et al. 2016; Chaudhary and Nagaraj 2011).

### Antibacterial Assay

*Staphylococcus aureus* (NCTC 8530) and *Pseudomonas aeruginosa* (NCTC6750) bacterial strains were used for the assay. Mid logarithmic phase bacterial cells were washed and resuspended in sodium phosphate buffer (10 mM, pH 7.4). The resulting suspension was diluted to a net absorbance value of 0.2 at 600 nm and 50 µL of the inoculum was treated with required concentrations of peptide (Net incubation broth is 100 µL), incubated for 2 h. Post incubation, 100 µL of MTT solution (0.5 mg/mL) was added to the broth and incubated for 4 h at 37 °C. Subsequently, the reaction broth was centrifuged and the bacterial pellets were mixed with DMSO. The difference in the absorbance at 570 and 660 nm was calculated and percent bacterial cell lysis was reported, relative to untreated bacterial cells (Rufian-Henares and Morales 2011; Piaru et al. 2012; Eloff 1998). A relative inhibition of 80% growth with reference to growth control was considered to be as Minimal Inhibitory Concentration, as per relative reported standard procedure (Wu et al. 2014).

### Field Emission-Scanning Electron Microscopy (FE-SEM)

Post incubation, glutaraldehyde (4%) was added to peptide-bacteria mixture, followed by an incubation of 30 min. The entire broth was centrifuged (1500×*g*, 5 min) and the bacterial pellet was placed over a glass slide. The dried samples were washed (gradient, 30–100% ethanol), coated with gold and images were recorded using scanning electron microscopy (Abd-El-Aziz et al. 2015).

### Hemolytic Assay

Fresh human blood was collected and mixed with 1.5 mg EDTA per milliliter and mixed thoroughly. These red blood cells were re-suspended and repeatedly washed with buffer (5 mM HEPES buffer saline, pH 7.4) by centrifugation at 800×*g* for 5 min, until the supernatant was colorless. A 10% cell suspension in buffer was prepared and 50 µL of the suspension was mixed with peptide solution, incubated for 2 h at 37 ± 2 °C. After incubation, the suspension was centrifuged at 800×*g* for 5 min and the absorbance of supernatant was recorded at 540 nm (Balaji and Trivedi 2012; Lee et al. 2014). Percent cell lysis was calculated in comparison to the extent of hemolysis recorded with 100% lysis of blood cells in water.

## Results and Discussion

### Design Philosophy

We designed two pairs of syndiotactic sequences mimicking Gramicidin 6.3 helix as model systems, with a starting (φ, ψ) dihedral angle pair shown in Table 1. Gramicidin has a right handed helical structure with (i to i + 7) and (i to i − 5) (Ramakrishnan et al. 2005) hydrogen bonding

pattern, and a pitch 4.7 Å (Katsaras et al. 1992), with 6.3 residues per turn (Urry 1971). Theoretically such helical structures can assume two structural variants depending on their 'handedness'; either right or left handed (Fig. 1). Therefore, we have designed two variants right handed (RH) and left handed (LH) for each sequence resulting in eight possible variants, though only four variants are synthetically possible.

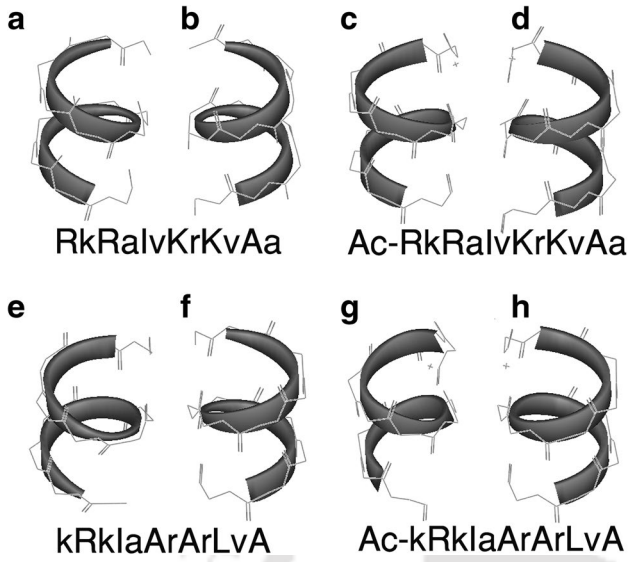


**Fig. 1** Backbone structures of the designed hetero-chiral peptides having syndiotactic stereochemistry. All the peptides can either be in left or right handed orientation, resulting in a maximum of eight theoretical backbone combinations possible for four peptides. **a** (01LH) and **b** (01RH) are the left handed and right handed back bone of sequence 01, whereas **c** (01Ac-LH) and **d** (01Ac-RH) are the left and right handed backbone of 01Ac. Likewise, **e** (02LH) and **f** (02RH) are left handed and right handed back bone of sequence 02 along with **g** (02Ac-LH) and **h** (02Ac-RH) are left and right handed version of 02Ac

The designed molecules are both cationic and amphipathic, which may play an important role in membrane lysis mediated by electrostatic interactions. Electrostatic potential maps of the designed peptides were generated by solving Finite Difference Poisson Boltzmann equation using Delphi software. The topology resulting from the side-chain orientation and the electrostatic potential environment it creates, were visibly distinct in all four models (Fig. 2). Precisely, the designed molecules display distinct topological and electrostatic fingerprint, which may get translated to their antibacterial potency.

The stereochemistry and sequences of 01 and 01-Ac pair and 02 and 02-Ac pair are the same, except that 01-Ac and 02-Ac being N-terminal acetylated (Table 1). To verify the stability of the designed peptides in a 6.3 helical conformation, similar to gramicidin helix, a 10 ns MD run at 298 K under NVT condition using GROMOS 96 force field, was performed for each structural variant. The cluster analysis of the theoretical models suggest that the structures are quite stable with the percent sampling of most populous structure being more than 95% in all eight models (Supplementary Fig. 1; Supplementary Table 1).

Further, the structural integrity of the molecules was verified by measuring Root Mean Square Deviation (RMSD) from the starting structure (Fig. 3). Radius of Gyration has been measured to verify whether the designed

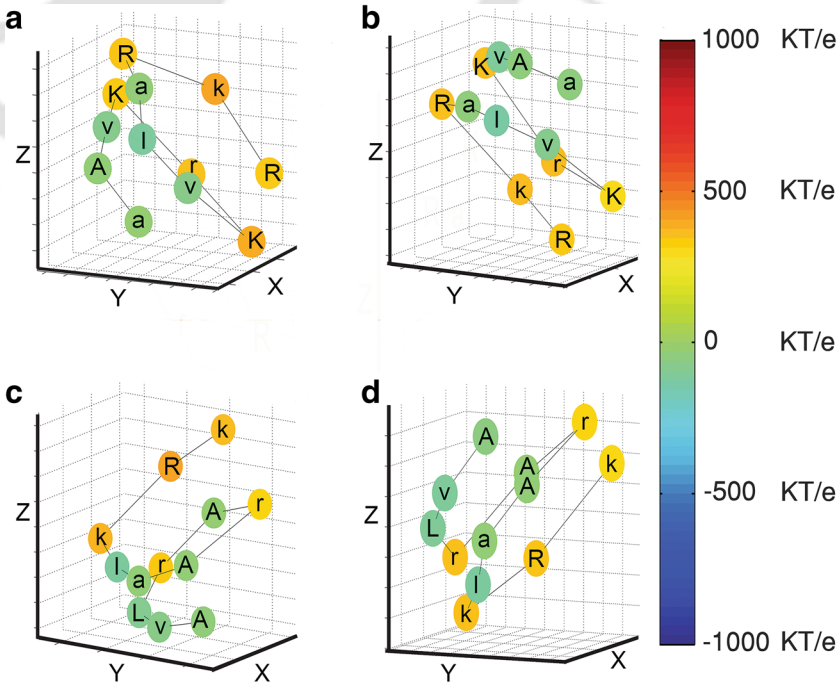


**Fig. 2** Electrostatic potential map of the designed peptides, using Delphi Software solving Finite Difference Poisson Boltzmann equation. **a, b** are the potential maps of left and right handed conformations of the peptide 01. Similarly, **c, d** are the potential maps of the left handed and right handed peptide 02 respectively. Though two conformations are theoretically possible, only one sequence can be experimentally verified. Differential *color coding* of amino acid residues, points to their electrostatic potential with reference to the color *bar*. The *color* map shows the potential distribution in KT/e units. (Color figure online)

**Table 1** Designed peptides and their sequences. The letter in the upper case denotes L-chiral amino acid whereas the letter in the lower case represents D. The sequences with N-terminal acetylation have been denoted with a prefix Ac. The dihedral angles used for the peptide sequences has been shown in details. Minimum Inhibitory Concentration of the designed peptides against *S. aureus* and *P. aeruginosa* shows preferential activity of syndiotactic sequences against Gram positive bacteria

| Code | Sequence | Handedness | Dihedral angles (L-amino acid) | Dihedral angles (D-amino acid) | Molecular mass (Dalton) | MIC (μM) | |
|---|---|---|---|---|---|---|---|
| | | | | | | *S. aureus* | *P. aeruginosa* |
| 01 | RkRaIvKrKvAa | Right | −120, 140 (φ, ψ) | 120, −110 (φ, ψ) | 1394.76 | 25 | >100 |
| | | Left | −110, 120 (φ, ψ) | 110, −150 (φ, ψ) | | | |
| 01 Ac | Ac-RkRaIvKrKvAa | Right | −120, 140 (φ, ψ) | 120, −110 (φ, ψ) | 1436.8 | 25 | >100 |
| | | Left | −110, 120 (φ, ψ) | 110, −150 (φ, ψ) | | | |
| 02 | kRkIaArArLvA | Right | −120, 140 (φ, ψ) | 120, −110 (φ, ψ) | 1351.69 | 12.5 | >100 |
| | | Left | −110, 120 (φ, ψ) | 110, −150 (φ, ψ) | | | |
| 02 Ac | Ac-kRkIaArArLvA | Right | −120, 140 (φ, ψ) | 120, −110 (φ, ψ) | 1393.73 | 12.5 | >100 |
| | | Left | −110, 120 (φ, ψ) | 110, −150 (φ, ψ) | | | |

structures remain folded throughout the simulation (Fig. 3). Detailed examination of average structure forming largest cluster, however shows that hydrogen bonding pattern has been changed during the course of MD simulations (Supplementary Figs. 2 and 3; Supplementary Table 2). In our earlier studies, we have shown that gramicidin 6.3 helical structures with alternate L and D stereo-chemical sequence have their short-range and long range backbone electrostatic interactions complementing each other, while in poly L sequences they are in conflict (Ramakrishnan et al. 2006; Kumar et al. 2009; Ranbhor et al. 2006).

The resultant modification of energy landscape leading to extra stability due to complimentary local electrostatics is the key for stereo-chemical engineering of peptide sequences. We have further shown that countervailing short range–long range interaction holds the key for side-chain sequences dictating the overall conformation of a given polypeptide sequence. Since local electrostatics of gramicidin helical backbone is complimenting and stable, the fold is much more adaptable to varied sequence solutions (Ramakrishnan et al. 2005). Our MD results on all eight variants support earlier observations and this design strategy. Since amphipathicity and cationicity has been the hallmark of antibacterial activity of natural and designed peptides, we have designed all peptides retaining amphipathicity, at the same time presenting differential electrostatic potential arising from cationic side-chains.

## Antibacterial Assay and Toxicity Studies

The in vitro antimicrobial activity of the synthesised peptides was tested against Gram-positive (*S. aureus*) and Gram-negative (*P. aeruginosa*) bacteria. All the peptides were showing better activity against Gram-positive bacteria, in comparison with Gram-negative bacteria (Fig. 4; Table 1).

This observation is consistent with the earlier reports on gramicidin. Gramicidin has a reported MIC value of 2.2 μM (Xiong et al. 2005) against *S. aureus* and is inactive against Gram-negative bacteria. Our peptides show similar trend, though the sequence similarity between gramicidin and the designed peptides are minimal. Our designed peptides primarily rely on cationicity to induce membranolytic activity. Acetylation of synthesized peptides is aimed at lowering the net positive charge by blocking the free N-terminus. We, however also could not find any notable difference in antimicrobial potency between acetylated and non-acetylated peptide variants.

## Hemolytic Activity

Hemolytic assay against mammalian RBC was performed to estimate the extent of toxicity. All four peptides exhibited negligible cytotoxicity against mammalian cells at 100 μM concentration following standard protocols. Gramicidin has a reported toxicity estimate of 50% hemolysis at 5 μM concentration (Wang et al. 2012). Unlike gramicidin, toxicity levels of all four reported peptides are well within the accepted safe threshold of 2% lysis at a very high concentration of 100 μM; and three out of the four peptide variants are showing a value less than 1% (Table 2).

## Microscopic Analysis

A qualitative evaluation of membrane rupturing potential of designed antimicrobial peptide against bacteria was performed using FE-SEM analysis. The cationic peptides caused significant membrane damage which was inferred after a comparative analysis between peptide treated (ruptured) and untreated (intact) bacterial cells. The bacterial cells were treated with 100 μM peptide resulting in deformed bacterial membrane architecture. (Fig. 5).

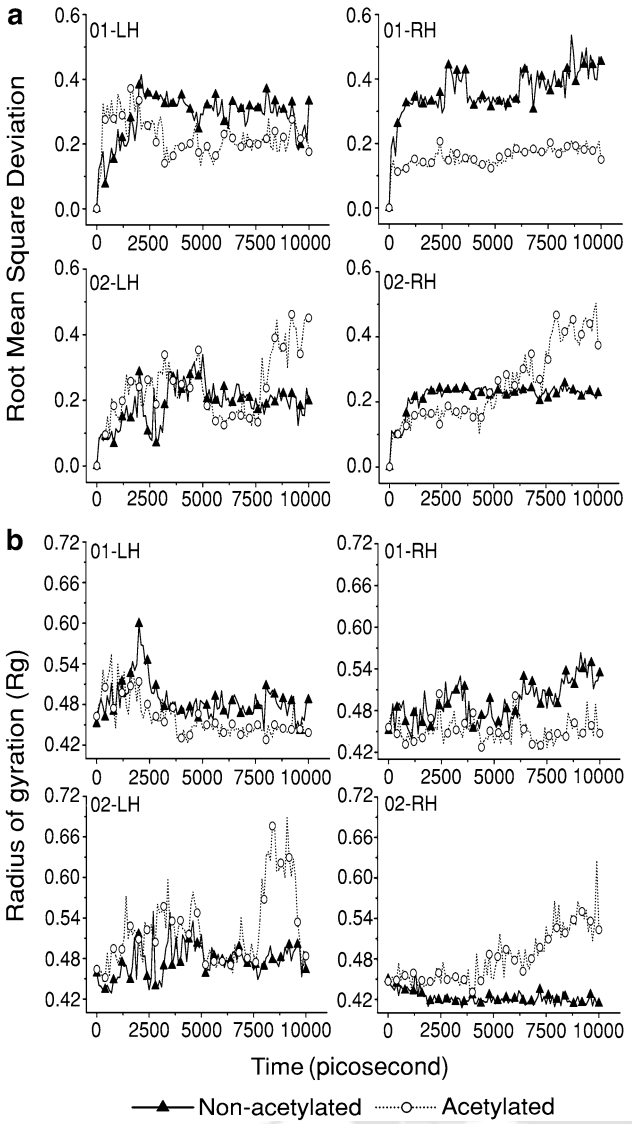


**Fig. 3** **a** Root Mean Square Deviation (RMSD) as a function of time of all the designed peptides from MD simulation in a 10 nanosecond (ns) production run, **b** Radius of Gyration (Rg) as a function of time indicating the overall folded nature of the designed peptides

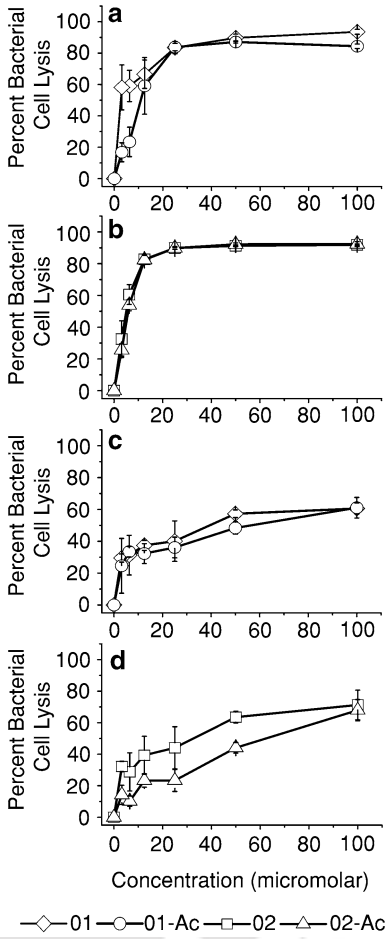


**Fig. 4** Bactericidal potency of the peptides 01 and 02 along with their acetylated variants against *S. aureus* (Gram-positive) and *P. aeruginosa* (Gram-negative) bacteria. **a** and **b** show bactericidal activity against Gram-positive bacteria, whereas **c** and **d** show activity against Gram-negative bacterial species

Qualitative pictures indicate that the membrane rupturing ability of the amphipathic cationic peptides are responsible for the desired activity profile of the designed peptides, though detailed mechanism investigation is beyond the scope of this paper.

**Table 2** Hemolytic assay of designed peptides against mammalian Red Blood Cells (RBC) at 100 μM peptide concentration

| S. no | Peptide code | % RBC lysis | Standard deviation |
|---|---|---|---|
| 1 | 01 | 0.5 | 0.02 |
| 2 | 01 Ac | 2 | 0.05 |
| 3 | 02 | 0.7 | 0.03 |
| 4 | 02 Ac | 0.09 | 0.02 |

## Conclusions

Almost all natural proteins are isotactic with chain stereochemistry (poly L). An isotactic peptide molecule roughly acquires 21% allowed conformational space in Ramachandran plot. If the stereochemical sequence is a variable, it increases the designable space manifold,

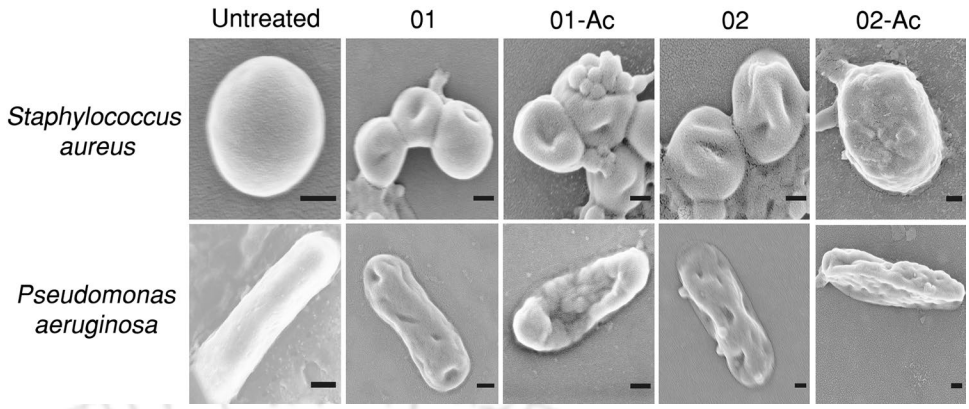


**Fig. 5** FE-SEM images of Gram-positive and Gram-negative bacteria, treated with individual peptides after 2 h of incubation. Comparison of untreated *S. aureus* (Gram-positive) and *P. aeruginosa* (Gram-negative) cells against ruptured bacterial membrane treated with 01, 01 Ac, 02 and 02 Ac at 100 μM concentration. The *scale bars* correspond to 200 nm

resulting in an informed walk across Ramachandran φ, ψ space. We employed a peptido-mimetic approach, modeling around Gramicidin adopting the simplest stereochemical sequence with syndiotactic stereochemistry. Gramicidin is a natural antibiotic peptide with Syndiotactic backbone (alternating L and D) having good bactericidal activity against Gram-positive bacteria, but could only be used topically because of very high levels of toxicity. We attempt to re-design the amino acid sequence (side-chain sequence) space retaining its stereo-chemical (main-chain) sequence, with an objective to minimize its toxicity, while retaining antimicrobial activity. The two designed cationic amphipathic peptides with alternating L and D chiral amino acid sequences, and their acetylated variants exhibit negligible levels of toxicity, well within the acceptable limits. Our best peptide sequence is showing an MIC value of 12.5 μM against a reported value of 2.2 μM (Xiong et al. 2005) for gramicidin D. Importantly, this design strategy offers an effective means to develop possible sequence variation in future designs. Incorporation of stereochemistry as an additional design variable significantly expands the design space of a peptide sequence. In a previous study (Hazam et al. 2017), we have systematically varied stereo-chemistry of the designed peptides resulting in different structure and electrostatic interaction profiles while they approach a target. However, our efforts to directly relate the structure and electrostatic profiles in defining activity and toxicity of a given peptide, is not yet conclusive.

Role of acetylation in naturally occurring proteins has not been precisely deduced but studies indicate that it improves the potency of peptides, as it mimics the native protein itself (Drazic et al. 2016). As indicated in literature, it enhances the overall potency, metabolic stability and resistance against enzymatic degradation (Di 2015; Strömstedt et al. 2009). In our study, we could not find any significant difference in activity or toxicity profiles of our designed peptides, which was also an objective of this investigation.

Apart from increasing the overall design space considerably, incorporation of D amino acids effectively negates the possibility of proteolytic degradation. This point to the potential utility of de novo designed peptides with diversified stereochemistry as a promising new approach in the generation of antibiotic peptides. Such attempts are important to effectively manage the broad antibiotic spectrum and pharmacological profile with increased instances of antibiotic resistance reported in recent times.

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## References

Abd-El-Aziz AS, Agatemor C, Etkin N, Overy DP, Lanteigne M, McQuillan K (2015) Antimicrobial organometallic dendrimers with tunable activity against multidrug-resistant bacteria. Biomacromolecules 16(11):3694–3703

Balaji S, Trivedi V (2012) Extracellular Methemoglobin mediated early ROS spike triggers osmotic fragility and RBC destruction: an Insight into the enhanced hemolysis during malaria. Indian J Clin Biochem 27(2):178–185

Bechinger B, Lohner K (2006) Detergent-like actions of linear amphipathic cationic antimicrobial peptides. Biochim Biophys Acta 1758(9):1529–1539

Berkowitz BA, Bevins CL, Zasloff MA (1990) Magainins: a new family of membrane-active host defense peptides. Biochem Pharmacol 39(4):625–629

Billmeyer FW (1957) Textbook of polymer science, 3rd edn. Interscience Publishers, New york

Brogden KA (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat Rev Microbiol 3(3):238–250

Burkhart BM, Li N, Langs DA, Pangborn WA, Duax WL (1998) The conducting form of gramicidin A is a right-handed double-stranded double helix. Proc Natl Acad Sci U S A 95(22):12950–12955

Burkhart BM, Gassman RM, Langs DA, Pangborn WA, Duax WL, Pletnev V (1999) Gramicidin D conformation, dynamics and membrane ion transport. Biopolymers 51(2):129–144

Chang WK, Wimley WC, Searson PC, Hristova K, Merzlyakov M (2008) Characterization of antimicrobial peptide activity by electrochemical impedance spectroscopy. Biochim Biophys Acta 1778(10):2430–2436

Chaudhary N, Nagaraj R (2011) Impact on the replacement of Phe by Trp in a short fragment of Abeta amyloid peptide on the formation of fibrils. J Pept Sci 17(2):115–123

Czihal P, Hoffmann R (2009) Mapping of apidaecin regions relevant for antimicrobial activity and bacterial internalization. Int J Pept Res Ther 15:157–164

Di L (2015) Strategic approaches to optimizing peptide ADME properties. AAPS J 17(1):134–143

Dosler S, Karaaslan E (2014) Inhibition and destruction of Pseudomonas aeruginosa biofilms by antibiotics and antimicrobial peptides. Peptides 62:32–37

Drazic A, Myklebust LM, Ree R, Arnesen T (2016) The world of protein acetylation. Biochim Biophys Acta 1864(10):1372–1401

Durani S (2008) Protein design with L- and D-alpha-amino acid structures as the alphabet. Acc Chem Res 41(10):1301–1308

Eloff J (1998) A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Med 64(08):711–713

Falcao LL, Silva-Werneck JO, Ramos AdR, Martins NF, Bresso E, Rodrigues MA (2016) Antimicrobial properties of two novel peptides derived from Theobroma cacao osmotin. Peptides 79:75–82

Felicio MR, Silva ON, Gonçalves S, Santos NC, Franco OL (2017) Peptides with dual antimicrobial and anticancer activities. Front Chem 5(5):1–9

Hamamoto K, Kida Y, Zhang Y, Shimizu T, Kuwano K (2002) Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions. Microbiol Immunol 46(11):741–749

Hazam PK, Jerath G, Kumar A, Chaudhary N, Ramakrishnan V (2017) Effect of tacticity-derived topological constraints in bactericidal peptides. Biochim Biophys Acta 1859(2017):1388–1395

Hladky SB, Haydon DA (1972) Ion transfer across lipid membranes in the presence of gramicidin A. I. Studies of the unit conductance channel. Biochim Biophys Acta 274(2):294–312

Katsaras J, Prosser RS, Stinson RH, Davis JH (1992) Constant helical pitch of the gramicidin channel in phospholipid bilayers. Biophys J 61(3):827–830

Kelkar DA, Chattopadhyay A (2007) The gramicidin ion channel: a model membrane protein. Biochim Biophys Acta 1768(9):2011–2025

Khoury GA, Smadbeck J, Kieslich CA, Floudas CA (2014) Protein folding and de novo protein design for biotechnological applications. Trends Biotechnol 32(2):99–109

Killian JA, Prasad KU, Hains D, Urry DW (1988) The membrane as an environment of minimal interconversion. A circular dichroism study on the solvent dependence of the conformational behavior of gramicidin in diacylphosphatidylcholine model membranes. BioChemistry 27(13):4848–4855

Koh J-J, Lin S, Aung TT, Lim F, Zou H, Bai Y (2015) Amino acid modified xanthone derivatives: novel, highly promising membrane-active antimicrobials for multidrug-resistant gram-positive bacterial infections. J Med Chem 58(2):739–752

Kumar A, Ramakrishnan V, Ranbhor R, Patel K, Durani S (2009) Homochiral stereochemistry: the missing link of structure to energetics in protein folding. J Phys Chem B 113(51):16435–16442

Lee M-R, Raman N, Gellman SH, Lynn DM, Palecek SP (2014) Hydrophobicity and helicity regulate the antifungal activity of 14-helical β-peptides. ACS Chem Biol 9(7):1613–1621

Lehrer RI (2004) Primate defensins. Nat Rev Microbiol 2(9):727–738

Li L, Li C, Sarkar S, Zhang J, Witham S, Zhang Z (2012) DelPhi: a comprehensive suite for DelPhi software and associated resources. BMC Biophys 5:9

Li J, Liu S, Koh J-J, Zou H, Lakshminarayanan R, Bai Y (2015) A novel fragment based strategy for membrane active antimicrobials against MRSA. Biochim Biophys Acta 1848(4):1023–1031

LoGrasso PV, Moll F, Cross TA (1988) Solvent history dependence of gramicidin A conformations in hydrated lipid bilayers. Biophys J 54(2):259–267

MATLAB version 7.10.0. (2010) Natick Massachusetts: The MathWorks Inc

Moore AJ, Beazley WD, Bibby MC, Devine DA (1996) Antimicrobial activity of cecropins. J Antimicrob Chemother 37:1077–1089

Nanda V, Andrianarijaona A, Narayanan C (2007) The role of protein homochirality in shaping the energy landscape of folding. Protein Sci 16(8):1667–1675

Piaru SP, Mahmud R, Perumal S (2012) Determination of antibacterial activity of essential oil of *Myristica fragrans* Houtt. Using tetrazolium microplate assay and its cytotoxic activity against vero cell line. Int J Pharmacol 8(6):572–576

Pronk S, Pall S, Schulz R, Larsson P, Bjelkmar P, Apostolov R (2013) GROMACS 4.5: a high-throughput and highly parallel open source molecular simulation toolkit. Bioinformatics 29(7):845–854

Ramakrishnan V, Ranbhor R, Durani S (2005) Simulated folding in polypeptides of diversified molecular tacticity: implications for protein folding and de novo design. Biopolymers 78(2):96–105

Ramakrishnan V, Ranbhor R, Kumar A, Durani S (2006) The link between sequence and conformation in protein structures appears to be stereochemically established. J Phys Chem B 110(18):9314–9323

Ramakrishnan V, Srinivasan SP, Salem SM, Matthews SJ, Colón W, Zaki M (2012) GeoFold: topology-based protein unfolding pathways capture the effects of engineered disulfides on kinetic stability. Proteins 80(3):920–934

Ranbhor R, Ramakrishnan V, Kumar A, Durani S (2006) The interplay of sequence and stereochemistry in defining conformation in proteins and polypeptides. Biopolymers 83(5):537–545

Ravichandran G, Kumaresan V, Bhatt P, Arasu MV, Al-Dhabi NA, Arockiaraj J (2017) A cumulative strategy to predict and characterize antimicrobial peptides (AMPs) from protein database. Int J Pept Res Ther 23:281–290

Rufian-Henares JA, Morales FJ (2011) Microtiter plate-based assay for screening antimicrobial activity of melanoidins against *E. coli* and *S. aureus*. Food Chem 111(4):1069–1074

Saikia K, Sravani YD, Ramakrishnan V, Chaudhary N (2017) Highly potent antimicrobial peptides from N-terminal membrane-binding region of *E. coli* MreB. Sci Rep 7:42994

Scott WRP, Hünenberger PH, Tironi IG, Mark AE, Billeter SR, Fennen J (1999) The GROMOS biomolecular simulation program package. J Phys Chem A 103(19):3596–3607

Srinivasan R (2013) Ribosome–program to build coordinates for peptides from sequence. http://folding.chemistry.msstate.edu/~raj/Manuals/ribosome.html

Strömstedt AA, Pasupuleti M, Schmidtchen A, Malmsten M (2009) Evaluation of Strategies for improving proteolytic resistance of antimicrobial peptides by using variants of EFK17, an internal segment of LL-37. Antimicrob Agents Chemother 53(2):593–602

Tene N, Bonnafe E, Berger F, Rifflet A, Guilhaudis L, Ségalas-Milazzo I (2016) Biochemical and biophysical combined study

of bicarinalin, an ant venom antimicrobial peptide. Peptides 79(:):103–113

Urry DW (1971) The gramicidin A transmembrane channel: a proposed pi(L, D) helix. Proc Natl Acad Sci U S A 68(3):672–676

Wang S, Ishii Y (2012) Revealing protein structures in solid-phase peptide synthesis by 13 C solid-state NMR: evidence of excessive misfolding for alzheimer's β. J Am Chem Soc 134(6):2848–2851

Wang F, Qin L, Pace CJ, Wong P, Malonis R, Gao J (2012) Solubilized gramicidin A as potential systemic antibiotics. Chembiochem 13(1):51–55

Wimley WC (2010) Describing the mechanism of antimicrobial peptide action with the interfacial activity model. ACS Chem Biol 5(10):905–917

Wishart DS, Knox C, Guo AC, Cheng D, Shrivastava S, Tzur D (2008) Drug Bank: a knowledgebase for drugs, drug actions and drug targets. Nucleic Acids Res. 36(Database issue):D901–D906

Wu X, Wang Z, Li X, Fan Y, He G, Wan Y (2014) In vitro and in vivo activities of antimicrobial peptides developed using an amino acid-based activity prediction method. Antimicrob Agents Chemother 58(9):5342–5349

Xiong YQ, Mukhopadhyay K, Yeaman MR, Adler-Moore J, Bayer AS (2005) Functional interrelationships between cell membrane and cell wall in antimicrobial peptide-mediated killing of *Staphylococcus aureus*. Antimicrob Agents Chemother 49(8):3114–3121

Zanetti M (2005) The role of cathelicidins in the innate host defenses of mammals. Curr Issue Mol Biol 7(2):179–196

THE JOURNAL OF PHYSICAL CHEMISTRY B

Article

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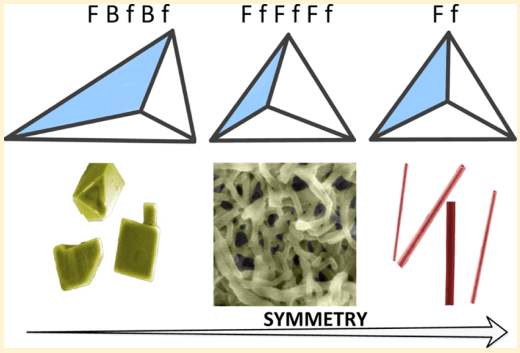
# Symmetry-Directed Self-Organization in Peptide Nanoassemblies through Aromatic $\pi-\pi$ Interactions

Sajitha Sasidharan, Prakash Kishore Hazam, and Vibin Ramakrishnan*

Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati, Guwahati 781039, India

Supporting Information

**ABSTRACT:** Almost all biological systems are assemblies of one or more biomolecules from nano- to macrodimensions. Unlike inorganic molecules, peptide systems attune with the conceptual framework of aggregation models when forming nanoassemblies. Three significant recent theoretical models have indicated that nucleation, end-to-end association, and geometry of growth are determined primarily by the size and electrostatics of the individual basic building blocks. In this study, we tested six model systems, differentially modulating the prominence of three design variables, namely, aromatic $\pi-\pi$ interactions, local electrostatics, and overall symmetry of the basic building unit. Our results indicate that the crucial design elements in a peptide-based nanoassembly are (a) a stable extended $\pi-\pi$ interaction network, (b) size, and (c) overall symmetry of the basic building blocks. The six model systems represent all of the design variables in the best manner possible, considering the complexity of a biomolecule. The results provide important directives in deciding the morphology and crystallinity of peptide nanoassemblies.




## INTRODUCTION

Almost all biological systems are functional assemblies of one or more fundamental biomolecular units from nano- to macrodimensions, mediated through nonbonding interactions. The robustness and efficient functioning of these assembled architectures are already well established. Understanding the algorithm that connects the physicochemical characteristics of the basic building blocks and stable structure formation at nano- and microlevel dimensions of these assemblies are central problems while exploring the possibility of making use of such biomolecules as fundamental units. Although bioinspired design is a topic of modern-day curriculum, pioneering work on this topic is the book "*On Growth and Form*" by D'Arcy Wentworth Thompson, written almost 100 years ago, which attempted to quantitatively assess structural patterns and formations in biological systems.[1]

Self-organization is envisaged as a spontaneous process that involves development of order or a recurring pattern as a result of local interactions between smaller subunits of an initially disordered system. A classic case of self-organization is folding and aggregation of protein chains to functional and dysfunctional forms, respectively.[2] Helmholtz in one of his recorded lectures makes an observation that "the forces so far as they cause chemical and mechanical influence in a living system, must be quite the same character as inorganic forces".[1] Uncertainty and lack of understanding in ascertaining the long-term physiological effects of nanomaterials of inorganic origin[3] are prompting researchers to consider materials of biological origin, such as nucleic acids and proteins, as basic building units. Earlier reports have suggested that the interplay between electrostatic and hydrophobic interactions, modulated by solvent effects, results in ordered aggregation and assembly over a nano- to microdimensional range.[4−8]

Protein or peptide molecules are heteropolymers made of 20 amino acids of L stereochemistry. Because of the planarity of the peptide bond and the L stereochemistry, the conformational possibilities of individual amino acids forming a protein chain are sterically restricted. This results in a peculiar situation, in which only 21% of the total $\phi$, $\psi$ space of the Ramachandran map can be accessed for the protein main chain.[9] Even then, natural proteins have an enormous number of variations in their functional space and about 1500 variations in fold space.[10] Protein molecules are long polymeric chains, sometimes even extending beyond 1000 amino acids. Smaller units or peptides are generally nonfunctional. The smallest polymer unit is a dipeptide, with two amino acids joined together through a peptide bond. However, assembly of individual peptides to form potentially functional units was a distinct possibility, although unrealized until Ghadiri first reported nanotubes formed from self-assembling units of short peptides stacked one over the other.[11,12] Usage of locking conformational basins by incorporating D amino acids was first adopted by Ghadiri. Hierarchical assembly of such conformationally rigid monomeric units forming robust nano- and microscale networks was

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PLOS ONE

RESEARCH ARTICLE

# Mapping the Geometric Evolution of Protein Folding Motor

**Gaurav Jerath, Prakash Kishore Hazam, Shashi Shekhar, Vibin Ramakrishnan***

Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, Assam, India

* vibin@iitg.ernet.in



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**Competing Interests:** The authors have declared that no competing interests exist.

## Abstract

Polypeptide chain has an invariant main-chain and a variant side-chain sequence. How the side-chain sequence determines fold in terms of its chemical constitution has been scrutinized extensively and verified periodically. However, a focussed investigation on the directive effect of side-chain geometry may provide important insights supplementing existing algorithms in mapping the geometrical evolution of protein chains and its structural preferences. Geometrically, folding of protein structure may be envisaged as the evolution of its geometric variables: $\phi$, and $\psi$ dihedral angles of polypeptide main-chain directed by $\chi_1$, and $\chi_2$ of side chain. In this work, protein molecule is metaphorically modelled as a machine with 4 rotors $\phi$, $\psi$, $\chi_1$ and $\chi_2$, with its evolution to the functional fold is directed by combinations of its rotor directions. We observe that differential rotor motions lead to different secondary structure formations and the combinatorial pattern is unique and consistent for particular secondary structure type. Further, we found that combination of rotor geometries of each amino acid is unique which partly explains how different amino acid sequence combinations have unique structural evolution and functional adaptation. Quantification of these amino acid rotor preferences, resulted in the generation of 3 substitution matrices, which later on plugged in the BLAST tool, for evaluating their efficiency in aligning sequences. We have employed BLOSUM62 and PAM30 as standard for primary evaluation. Generation of substitution matrices is a logical extension of the conceptual framework we attempted to build during the development of this work. Optimization of matrices following the conventional routines and possible application with biologically relevant data sets are beyond the scope of this manuscript, though it is a part of the larger project design.

## Introduction

The three dimensional native structure of a protein defies all concepts of aesthetics that nature generally tend to practice in its creation. However, these folded structures and the guiding principles of its construction is an important scientific problem, yet to be completely solved. The physical folding code, folding mechanism and a perfect algorithm that predicts the fold from sequence has been a matter of intense scrutiny for more than five decades now [1]. The scientific community has invested significant amount of time and resources in deciphering this

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METHODOLOGY ARTICLE

# *bPE toolkit*: toolkit for computational protein engineering

**Gaurav Jerath · Prakash Kishore Hazam · Vibin Ramakrishnan**

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**Abstract** We present a computational toolkit consisting of five utility tools, for performing basic operations on a protein structure file in PDB format. The toolkit consists of five different programs which can be integrated as part of a pipeline for computational protein structure characterization or as a standalone analysis package. The programs include tools for chirality check for amino acids (ProChiral), contact map generation (CoMa), data redundancy (DaRe), hydrogen bond potential energy (HyPE) and electrostatic interaction energy (EsInE). All programs in the toolkit can be accessed and downloaded through the following link: http://www.iitg.ac.in/bpetoolkit/.

**Keywords** Toolkit · Protein engineering · Protein design · Protein structure

## Introduction

Recently, experimentalists have started using computational methods extensively in formulating and fine tuning their research plans. Most of these groups employed in protein engineering research have shared their interest in using certain basic tools supporting their research, yet not part of a fairly large program suite which would be difficult for them to use independently without the help of a computational biologist. Additionally, we would like to have few independent tools that can be incorporated in a pipeline of various operations with protein structure files, such that they can create their own computational work flow. To accomplish these objectives, we present a protein engineering toolkit, consisting of five programs that can perform basic operations in a protein structure file.

G. Jerath · P. K. Hazam · V. Ramakrishnan (✉)
Department of Biotechnology, Indian Institute of Technology, Guwahati 781039, India
e-mail: vibin@iitg.ernet.in; vibin@iitg.ac.in

## Computational methods and their validation

The theoretical background of computational programs and their validation is described as follows. The science and logic behind their design has also been briefly illustrated.

### ProChiral: chirality check program

Chirality is a property of a molecule which doesn't have a super-imposable mirror image of itself. All amino acids, except glycine are chiral with the CA atom being the chiral center. The chirality of an amino acid can be determined by checking the orientation of the side chain with respect to the protein/peptide backbone, i.e. the orientation of the CA atom with respect to the backbone. This can be done by calculating the improper dihedral angle formed by the normals from the planes involving N-CA-C and C-CA-CB. A positive value for the angle signifies the Levorotatory form of the amino acid whereas, a negative value depicts the Dextrorotatory form. The chirality program gives the orientation of the amino acid residue side chain as Dextro or Levo-rotatory.

ProChiral was tested using the existing protein data bank (PDB) structure 1GRM.pdb, which has a mixture of L and D-amino acids. The program was also applied to other PDB structure files which are homo-chiral in nature. Furthermore, few in-house generated hetero-chiral PDB files were also tested with the program. The program successfully identified the chiral orientation of the CA atom with 100 % accuracy for all the structures tested. Additionally, the structures were

# Appendix

| Peptide | Staphylococus aureus (MIC) | Escherichia coli (MIC) | Reference |
|---|---|---|---|
| **Cecropin B** | *S. aureus* NCTC8532 (66.7 µM) | *E. coli* BUE55 (1.7 µM), *E. coli* NCTC 11954 (3.3 µM) | A.J. Moore, W.D. Beazley, M.C. Bibby, D.A. Devine, Antimicrobial activity of cecropins, J. Antimicrob. Chemother. 37 (1996) 1077-1089. |
| **Gramicidin S** | *S. aureus* FDA209P (6.25 µg/mL or 5.48 µM) | *E. coli* NIHJJC-2 (>100 µg/mL or 87.67 µM | S. Ando, H. Aoyagi, S. Shinagawa, N. Nishino, M. Waki, T. Kato, N. Izumiya, [4,4'-D-diaminopropionic acid]gramicidin S: a synthetic gramicidin S analog with antimicrobial activity against Gram-negative bacteria, FEBS Lett. 161 (1983) 89-92. |
| **Polymixin B** | *S. aureus* RN4220 (64 µg/mL or 33.58 µM) | *E. coli* UB1005 (0.5 µg/mL or 0.262 µM), *E. coli* DC2 (0.1 µg/mL or 0.0524 µM) | T.J. Falla, D.N. Karunaratne, R.E. Hancock, Mode of action of the antimicrobial peptide indolicidin, J. Biol. Chem. 271 (1996) 19298-19303. |
| **Buforin 2** | *S. aureus* (1.65 µM)[2] | *E. coli* DH5α (6.3 µM)[1] | 1) M. Bagheri, M. Beyermann, M. Dathe, Mode of Action of Cationic Antimicrobial Peptides Defines the Tethering Position and the Efficacy of Biocidal Surfaces, Bioconjugate Chem. 23 (2012) 66-74. 2) C.B. Park, K.-S. Yi, K. Matsuzaki, M.S. Kim, S.C. Kim, 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, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 8245-8250. |
| **Tritrpticin** | *S. aureus* KCTC1261 (4 µM) | *E. coli* KCTC1682 (8 µM) | W.L. Zhu, K.S. Hahm, S.Y. Shin, Cathelicidin-derived Trp/Pro-rich antimicrobial peptides with lysine peptoid residue (Nlys): therapeutic index and plausible mode of action, J. Pept. Sci. 13 (2007) 529-535. |
| **Ocellatin 4** | *S. aureus* ATCC25923 (64 µM) | *E. coli* ATCC25922 (64 µM) | A. Nascimento, A. Chapeaurouge, J. Perales, A. Sebben, M.V. Sousa, W. Fontes, M.S. Castro, Purification, characterization and |

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| | | | homology analysis of ocellatin 4, a cytolytic peptide from the skin secretion of the frog Leptodactylus ocellatus, Toxicon 50 (2007) 1095-1104. |
| **Fallaxin** | *S. aureus* ATCC5923 (>160 µM) | *E. coli* ATCC25922 (40 µM) | A. Nascimento, A. Chapeaurouge, J. Perales, A. Sebben, M.V. Sousa, W. Fontes, M.S. Castro, Purification, characterization and homology analysis of ocellatin 4, a cytolytic peptide from the skin secretion of the frog Leptodactylus ocellatus, Toxicon 50 (2007) 1095-1104. |
| **Laticeptin** | *S. aureus* ATCC 25923 (>200 µM) | *E. coli* ATCC25922 (50 µM) | A. Nascimento, A. Chapeaurouge, J. Perales, A. Sebben, M.V. Sousa, W. Fontes, M.S. Castro, Purification, characterization and homology analysis of ocellatin 4, a cytolytic peptide from the skin secretion of the frog Leptodactylus ocellatus, Toxicon 50 (2007) 1095-1104. |
| **Pentadactylin** | *S. aureus* ATCC 25923 (200 µM) | *E. coli* ATCC25922 (25 µM) | A. Nascimento, A. Chapeaurouge, J. Perales, A. Sebben, M.V. Sousa, W. Fontes, M.S. Castro, Purification, characterization and homology analysis of ocellatin 4, a cytolytic peptide from the skin secretion of the frog Leptodactylus ocellatus, Toxicon 50 (2007) 1095-1104. |
| **Cecropin A** | *S. aureus* ATCC6538 (32 µM) | *E. coli* ATCC8739 (1 µM) | C. Zhou, X. Qi, P. Li, W.N. Chen, L. Mouad, M.W. Chang, S.S.J. Leong, M.B. Chan-Park, High Potency and Broad-Spectrum Antimicrobial Peptides Synthesized via Ring-Opening Polymerization of α-Aminoacid-N-carboxyanhydrides, Biomacromolecules 11 (2010) 60-67. |

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| **Melittin** | *S. aureus* ATCC6539 (2.8 µM) | *E. coli* ATCC8740 (22.4 µM) | C. Zhou, X. Qi, P. Li, W.N. Chen, L. Mouad, M.W. Chang, S.S.J. Leong, M.B. Chan-Park, High Potency and Broad-Spectrum Antimicrobial Peptides Synthesized via Ring-Opening Polymerization of α-Aminoacid-N-carboxyanhydrides, Biomacromolecules 11 (2010) 60-67. |
| **LL-37** | *S. aureus* ATCC6540 (>57 µM) | *E. coli*ATCC8741 (>57 µM) | C. Zhou, X. Qi, P. Li, W.N. Chen, L. Mouad, M.W. Chang, S.S.J. Leong, M.B. Chan-Park, High Potency and Broad-Spectrum Antimicrobial Peptides Synthesized via Ring-Opening Polymerization of α-Aminoacid-N-carboxyanhydrides, Biomacromolecules 11 (2010) 60-67. |
| **Indolicidin** | *S. aureus* ATCC6541 (67 µM) | *E. coli* ATCC8742 (>134 µM | C. Zhou, X. Qi, P. Li, W.N. Chen, L. Mouad, M.W. Chang, S.S.J. Leong, M.B. Chan-Park, High Potency and Broad-Spectrum Antimicrobial Peptides Synthesized via Ring-Opening Polymerization of α-Aminoacid-N-carboxyanhydrides, Biomacromolecules 11 (2010) 60-67. |
| **Magainin I** | *S. aureus* ATCC6542 (53 µM) | *E. coli* ATCC8743 (53 µM) | C. Zhou, X. Qi, P. Li, W.N. Chen, L. Mouad, M.W. Chang, S.S.J. Leong, M.B. Chan-Park, High Potency and Broad-Spectrum Antimicrobial Peptides Synthesized via Ring-Opening Polymerization of α-Aminoacid-N-carboxyanhydrides, Biomacromolecules 11 (2010) 60-67. |
| **Defensin (HNP-1)** | *S. aureus* ATCC6543 (37 µM) | *E. coli* ATCC8744 (37 µM) | C. Zhou, X. Qi, P. Li, W.N. Chen, L. Mouad, M.W. Chang, S.S.J. Leong, M.B. Chan-Park, High Potency and Broad-Spectrum Antimicrobial Peptides Synthesized via Ring-Opening Polymerization of α-Aminoacid-N-carboxyanhydrides, |

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| | | | Biomacromolecules 11 (2010) 60-67. |
| **Magainin II** | *S. aureus* ( 20.58 μM) | *E. coli* (41.15 μM) | C.B. Park, K.S. Yi, K. Matsuzaki, M.S. Kim, S.C. Kim, 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, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 8245-8250. |
| **LL-37** | *S. aureus* 930918-3 (12.5 μg/mL or 2.78 μM), *S. aureus* 67395 (2.9 μg/mL or 0.645 μM), *S. aureus* 68721 (3.2 μg/mL or 0.712 μM), *S. aureus* 502A (3.6 μg/mL or 0.801 μM) | *E. coli* ML-35p (0.6 μg/mL or 0.1335 μM), *E. coli* ATCC 9637 (0.1 μg/mL or 0.0222 μM), *E. coli* ATCC 11775 (1.9 μg/mL or 0.423 μM), *E. coli* MCR106 (1.6 μg/mL or 0.356 μM) | J. Turner, Y. Cho, N.-N. Dinh, A.J. Waring, R.I. Lehrer, Activities of LL-37, a Cathelin-Associated Antimicrobial Peptide of Human Neutrophils, Antimicrob. Agents Chemother. 42 (1998) 2206-2214. |
| **Ceratotoxin A** | *S. aureus* ATCC 25923 (175 μM) | *E. coli* ATCC23739 (7 μM) | L. Marri, R. Dallai, D. Marchini, The Novel Antibacterial Peptide Ceratotoxin A Alters Permeability of the Inner and Outer Membrane of Escherichia coli K-12, Curr. Microbiol. 33 (1996) 40-43. |
| **Moricin** | *S. aureus* (6.25 μg/mL or 1.38 μM) | *E. coli* O157:H7 (6.25 μg/mL or 1.38 μM) | H. Dai, S. Rayaprolu, Y. Gong, R. Huang, O. Prakash, H. Jiang, Solution structure, antibacterial activity, and expression profile of Manduca sexta moricin, J. Pept. Sci. 14 (2008) 855-863. |
| **Protegrin-1 (PG-1)** | *S. aureus* 29213 (1.7 μg/mL or 0.79 μM) | *E. coli* 25922 (0.75 μg/mL or 0.35 μM) | D.A. Steinberg, M.A. Hurst, C.A. Fujii, A.H. Kung, J.F. Ho, F.C. Cheng, D.J. Loury, J.C. Fiddes, Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with in vivo activity, Antimicrob. Agents Chemother. 41 (1997) 1738-1742. |
| **Ranalexin** | *S. aureus* (4 μg/mL or 1.902 μM) | *E. coli* ( 32 μg/mL or 15.213 μM) | D.P. Clark, S. Durell, W.L. Maloy, M. Zasloff, Ranalexin. A novel antimicrobial peptide from bullfrog (Rana catesbeiana) skin, structurally related to the bacterial antibiotic, polymyxin, J. Biol. Chem. 269 (1994) 10849-10855. |
| **Citropin 1.1** | *S. aureus* (25 μg/mL or 15.5 μM) | *E. coli* (> 100 μg/mL or > 62 μM) | J. Doyle, C.S. Brinkworth, K.L. Wegener, J.A. Carver, |

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| | | | L.E. Llewellyn, I.N. Olver, J.H. Bowie, P.A. Wabnitz, M.J. Tyler, nNOS inhibition, antimicrobial and anticancer activity of the amphibian skin peptide, citropin 1.1 and synthetic modifications. The solution structure of a modified citropin 1.1, Eur. J. Biochem. 270 (2003) 1141-1153. |
| **Citropin 1.1.2** | *S. aureus* (25 µg/mL or 15.5 µM) | *E. coli* (> 100 µg/mL or > 62 µM) | J. Doyle, C.S. Brinkworth, K.L. Wegener, J.A. Carver, L.E. Llewellyn, I.N. Olver, J.H. Bowie, P.A. Wabnitz, M.J. Tyler, nNOS inhibition, antimicrobial and anticancer activity of the amphibian skin peptide, citropin 1.1 and synthetic modifications. The solution structure of a modified citropin 1.1, Eur. J. Biochem. 270 (2003) 1141-1153. |
| **Androctonin** | *S. aureus* (15–30 µM) | *E. coli* D31 (3–6 µM)<br>*E. coli* D22 (>30 µM)<br>*E. coli* 1106 (6–15 µM) | Ehret-Sabatier, L.; Loew, D.; Goyffon, M.; Fehlbaum, P.; Hoffmann, J. A.; van Dorsselaer, A.; Bulet, P. Characterization of novel cysteine-rich antimicrobial peptides from scorpion blood, *J.Biol.Chem*. **1996,** *271*, 29537-29544. |
| **MSI-94** | *S. aureus* (0.9-1.8 µM) | *E. coli* D31 (0.45–0.9 µM)<br>*E. coli* D22 (0.9-1.8 µM)<br>*E. coli* 1106 (0.9-1.8 µM) | Ehret-Sabatier, L.; Loew, D.; Goyffon, M.; Fehlbaum, P.; Hoffmann, J. A.; van Dorsselaer, A.; Bulet, P. Characterization of novel cysteine-rich antimicrobial peptides from scorpion blood, *J.Biol.Chem*. **1996,** *271*, 29537-29544. |
| **PGLa** | *S. aureus* (1.15-2.3 µM) | *E. coli* D31 (1.15-2.3 µM)<br>*E. coli* D22 (1.15-2.3 µM)<br>*E. coli* 1106 (0.6–1.15 µM) | L. Ehret-Sabatier, D. Loew, M. Goyffon, P. Fehlbaum, J.A. Hoffmann, A. van Dorsselaer, P. Bulet, Characterization of Novel Cysteine-rich Antimicrobial Peptides from Scorpion Blood, J. Biol. Chem. 271 (1996) 29537-29544. |
| **Ascaphin-1** | *S. aureus* (>50 µM) | *E.coli* (6 µM) | J.M. Conlon, A. Sonnevend, C. Davidson, D.D. Smith, P.F. Nielsen, The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, |

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| | | | Ascaphus truei, Biochem. Biophys. Res. Commun. 320 (2004) 170-175. |
| **Ascaphin-3** | *S. aureus* (>50 µM) | *E.coli* (6 µM) | J.M. Conlon, A. Sonnevend, C. Davidson, D.D. Smith, P.F. Nielsen, The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, Ascaphus truei, Biochem. Biophys. Res. Commun. 320 (2004) 170-175. |
| **Ascaphin-7** | *S. aureus* (25 µM) | *E.coli* (3 µM) | J.M. Conlon, A. Sonnevend, C. Davidson, D.D. Smith, P.F. Nielsen, The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, Ascaphus truei, Biochem. Biophys. Res. Commun. 320 (2004) 170-175. |
| **Ascaphin-8** | *S. aureus* (6 µM) | *E.coli* (6 µM) | J.M. Conlon, A. Sonnevend, C. Davidson, D.D. Smith, P.F. Nielsen, The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, Ascaphus truei, Biochem. Biophys. Res. Commun. 320 (2004) 170-175. |
| **Brevinin-1BYa** | *S. aureus* (2 µM) | *E. coli* (17 µM) | J.M. Conlon, A. Sonnevend, C. Davidson, D.D. Smith, P.F. Nielsen, The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, Ascaphus truei, Biochem. Biophys. Res. Commun. 320 (2004) 170-175. |
| **Brevinin-1BYb** | *S. aureus* (4 µM) | *E. coli* (16 µM) | J.M. Conlon, A. Sonnevend, C. Davidson, D.D. Smith, P.F. Nielsen, The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, Ascaphus truei, Biochem. Biophys. Res. Commun. 320 (2004) 170-175. |
| **Brevinin-1BYc** | *S. aureus* (8 µM) | *E. coli* (Not active at 50 µM) | J.M. Conlon, A. Sonnevend, C. Davidson, D.D. Smith, P.F. Nielsen, The ascaphins: a family of antimicrobial peptides from the skin |

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| | | | secretions of the most primitive extant frog, Ascaphus truei, Biochem. Biophys. Res. Commun. 320 (2004) 170-175. |
| **Ranatuerin-2BYa** | *S. aureus* (27 µM) | *E. coli* (7 µM) | J.M. Conlon, A. Sonnevend, C. Davidson, D.D. Smith, P.F. Nielsen, The ascaphins: a family of antimicrobial peptides from the skin secretions of the most primitive extant frog, Ascaphus truei, Biochem. Biophys. Res. Commun. 320 (2004) 170-175. |
| **Ranatuerin-2BYb** | *S. aureus* (Not active at 50 µM) | *E. coli* (17 µM) | J.M. Conlon, A. Sonnevend, M. Patel, C. Davidson, P.F. Nielsen, T. Pal, L.A. Rollins-Smith, Isolation of peptides of the brevinin-1 family with potent candidacidal activity from the skin secretions of the frog Rana boylii, J. Pept. Res. 62 (2003) 207-213. |
| **Temporin-1BYa** | *S. aureus* (15 µM) | *E. coli* (Not active at 50 µM) | J.M. Conlon, A. Sonnevend, M. Patel, C. Davidson, P.F. Nielsen, T. Pal, L.A. Rollins-Smith, Isolation of peptides of the brevinin-1 family with potent candidacidal activity from the skin secretions of the frog Rana boylii, J. Pept. Res. 62 (2003) 207-213. |
| **Clavanin A** | *S. aureus* (45 µM) | *E. coli* (24 µM) | O.N. Silva, I.C.M. Fensterseifer, E.A. Rodrigues, H.H.S. Holanda, N.R.F. Novaes, J.P.A. Cunha, T.M.B. Rezende, K.G. Magalhães, S.E. Moreno, M.S. Jerônimo, A.L. Bocca, O.L. Franco, Clavanin A Improves Outcome of Complications from Different Bacterial Infections, Antimicrob. Agents Chemother. 59 (2015) 1620-1626. |
| **$V_{681}$** | *S. aureus* 25923 (16 µg/ml or 5.5 µM), *S. aureus* SAP0017 (9 µg/ml or 3.09 µM) | *E.coli* UB1005 (7.1 µg/ml or 2.434 µM), *E. coli* DC2 (4.5 µg/ml or 1.54 µM) | Y. Chen, C.T. Mant, S.W. Farmer, R.E. Hancock, M.L. Vasil, R.S. Hodges, Rational design of alpha-helical antimicrobial peptides with enhanced activities and specificity/therapeutic index, J. Biol. Chem. 280 (2005) 12316-12329. |