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Topological effects on the designability and bactericidal potency of antimicrobial peptides

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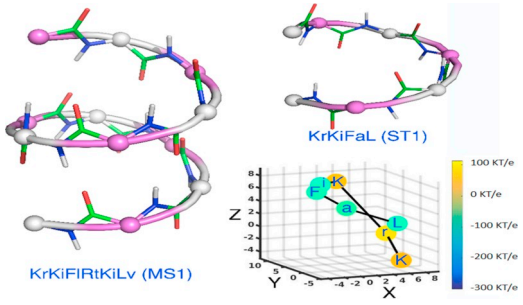
HIGHLIGHTS

- Peptide length affects bactericidal potency of AMPs.
- Conformationally constrained AMPs retain bactericidal potency.
- Disordered conformations tend to lose bactericidal potency of AMPs.
- Optimal amphipathicity reduces cytotoxic potential of AMPs.

GRAPHICAL ABSTRACT



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ABSTRACT

New ideas and methods are being developed to generate highly designable small functional protein folds beyond the confines of natural structures, from secondary to quaternary level. Highly designable folds can have multiple sequence solutions, which are thermodynamically and kinetically stable. We have previously described how short syndiotactic helices can be exceptionally stable energetically, and how they can be used as a template for designing antibacterial agents. In this work, we have designed four syndiotactic, single turn, amphipathic; cationic 7-mer peptides which are the sequence and structural subset of earlier published 12-mer sequences. We examined the stability of the designed structures and its effects on the biological activity of such short peptide sequences. This was achieved by making objective comparisons between 12-mer and 7-mer sequences in terms of their antibacterial activity. Further, we investigated the mechanistic origins of clearly different bactericidal potency of single (7-mer) and double (12-mer) turn syndiotactic helices using molecular dynamics simulations. Our results suggest that conformationally constrained stable short double turn peptide scaffolds are highly designable, whereas single turn structures are more likely to be disordered. The stability of the designed peptide structure provides a platform for inclusion of multiple sequence variables and defined electrostatic fingerprints. Therefore, a stable peptide scaffold along with pre-defined electrostatic signatures can together be utilized for the design of novel antimicrobial peptides.

1. Introduction

Traditionally, efforts to design protein structures *de novo,* have focused on modifying naturally occurring proteins. Of late, design efforts have moved on to totally new functional folds beyond natural alphabets, folding into their global free energy minimum, optimized by molecular force fields at the design phase. Conventional peptide design only guarantees functional group constitution by opting specific amino

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acid sequence, and not their topology, because peptide molecules are very fluxional, and its conformation therefore is subjected to the external flux [1]. This is principally due to the isotactic (poly L) stereochemistry of naturally occurring peptide and protein backbone, where adoption of a conformation is a consequence of its amino-acid sequence, and this sequence-structure combination will eventually modulate its prescribed function [1]. Attempts of protein or peptide *de novo* design so far, have also been conservative, being mimics of natural protein folds, with few exceptions such as TOP7 and CC-Hept [2]. Stereo-chemical engineering of the peptide main chain offers expansion of the design space, possibly offering greater functional possibilities as future materials for therapeutic [3,4] and non-therapeutic applications [5]. Depending on the stereo-chemical sequence, a polymeric chain like protein can be isotactic (poly L or Poly D), syndiotactic (LDLD) and hetero-tactic (random distribution of L and D) [6]. With few exceptions, all proteins in the existing 'protein universe' are exclusively isotactic, with sequence of L-chiral amino acid polymers [5].

Antimicrobial peptides (AMPs) are natural molecules of innate immunity that counter microbial invasion. Peptides like defensins, cathelicidins, cecropins, dermaseptins, magainins *etc.* are some of the most widely studied classes of antimicrobial peptides (AMPs) [7–9]. Gramicidin, a syndiotactic peptide antibiotic from *Bacillus brevis* was the first ever clinically tested AMP [10,11]. Despite different sequence, structure and amino acid composition, these molecules share common characteristic features like cationicity and amphipathicity in their primary molecular construct [12], which enables them to permeabilize bacterial membranes, resulting in broad spectrum antimicrobial and anti-biofilm activity [13–15]. In *de novo* design attempts, side-chain charge distribution ensuring amphipathicity was primarily achieved by choosing amino-acid sequences by rational design, with little emphasis on the possible structure it adopts in (i) extracellular matrix, (ii) upon interaction with the membrane and (iii) after penetration or membrane de-formation. The structure of short peptides is primarily directed by short and long range electrostatic interactions of the backbone and side-chain [16]. In an earlier work, we have reported how in a folded short isotactic poly peptide like alpha helix, local electrostatic interactions are in conflict, with their dipoles pointing in the same direction, but harmonious in extended structures [1]. It is, however, difficult to design well-defined electrostatic zones in extended or coiled structures. Syndiotactic helices (Gramicidin or beta helix) on the other hand, possess harmonious local and non-local interactions, complementing each other, adding to the overall stability of the structure. Short peptides with stable structures can have relatively smooth and deep energy minimum in their potential energy landscape, ensuring better thermodynamic stability. Stable conformation of Gramicidin in different solvent systems provides the basic template for designing novel sequences with well-defined electrostatic signatures [17,18].

In another paper, we reported our attempt to experiment with this conceptual possibility by designing amphipathic peptide systems of diversified stereochemistry, which can potentially act as bactericidal agents. This was achieved by systematically varying the main-chain stereochemistry or tacticity of isotactic poly-L 12-mer cationic amphipathic peptides to four variants of LDLD stereo-chemical sequence with the same amino acid composition [8] (Fig. 1). *De novo* designed stereo-engineered LDLD or DLDL 12-mer peptides have retained their potency while their poly L variants rapidly lost their activity, when the experiment was repeated in human serum [4]. Stability against enzymatic degradation was one of the main reasons for the under-utilization of natural peptides, which could otherwise be a good therapeutic option with minimal side effects [19]. Our assessment with alternating LDLD (or DLDL) stereo-chemical sequence of antimicrobial peptides suggest that stereo-chemical engineering of the designed peptides can address this most significant, associated limitation of peptide-based treatment regimen against bacterial infections. The earlier reported peptides were having gramicidin helical conformations with two turns (Fig. 1). All the designed syndiotactic peptides have shown very impressive values of

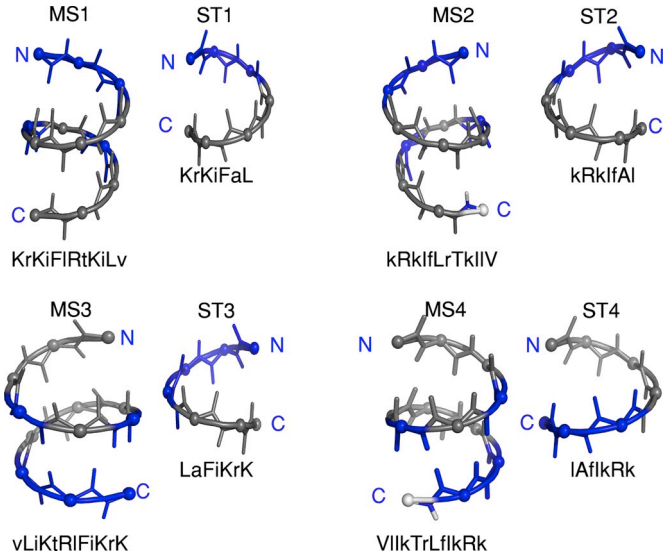

Fig. 1. Comparative backbone structure of *7 mer* peptides (ST1 – ST4) with reference to their *12 mer* mother sequence (MS1 – MS4). All the peptide sequences are syndiotactic, having alternating L- and D-amino acids in its back bone. The N- and C– terminus of the peptides are shown in each case along with their sequence. Small letter in the sequence indicates D-amino acids.

antibacterial potency, both 'in-media' and 'in-serum' conditions [4]. In order to explore the possibility of bringing down the sequence length and understand how topology of the designed sequence play crucial role in their antimicrobial activity, we designed and synthesized an approximate sequence subset of these peptides with 7 amino acids (Fig. 1). Shorter sequences are relatively easy to synthesize in laboratory and they are hence more economical [20]. We have retained the basic design protocol and to a great extent sequence, so that we can make an objective assessment of the effects of size reduction and topological variation, by conserving the sequence. We observed a significant reduction in antimicrobial potency of peptides (ST1 – ST4) upon chain length reduction, with varied bactericidal activity profiles. To investigate further the possible reasons for reduced bactericidal potency, we performed molecular dynamics simulations of both double and single turn candidate structures under identical conditions. Our results provide some important design directives while generating functionally active peptides for therapeutic applications.

2. Materials and methods

2.1.1. Peptide design and electrostatic profiling

All the peptides (PDB files) used in the study was designed with software PDBMake [7], and a modified version of Ribosome [7]. Delphi software was used to calculate the electrostatic profiles of PDB files, by solving Finite difference Poisson-Boltzmann eq. (FD-PBE) [21,22]. Electrostatic potential was calculated for each residue and summed up at their respective chromophoric centres as described elsewhere [7]. The electrostatic potential maps were plotted using MATLAB, showing distinct zones of cationic and hydrophobic zones within each peptide.

2.1.2. Molecular dynamics simulations

Molecular Dynamics simulations have been performed with single turn (7 mer) and double turn (12 mer) peptide to study their respective membrane interactions under identical conditions. The lipid bilayer was prepared and equilibrated using MemBuilder II server [23], comprised of POPC:POPG [24] in the ratio3:1with uniform distribution in each leaflet of the bilayer [25]. The bilayer was oriented perpendicularly to the Z axis within the simulation box. Four peptide molecules

were placed near one side of the bilayer surface at 1 nm distance from each other. All atom molecular dynamics simulations were performed using the GROMACS 5.0.4 package [26]. United atom Berger lipid parameters and GROMOS 53a6 force field were used for MD simulations. The peptides, along with the lipid bilayer were solvated in a triclinic box and neutralized to a total charge of zero by randomly replacing few water molecules with counter ions (Na^+ and Cl^-). The entire system was subjected to steepest descent energy minimization until a tolerance of 1000 KJ mol^{-1} nm^{-1} was achieved. The energy minimized system was then equilibrated followed by the production run for 160 ns at 303 K, with periodic boundary at an integration time step of 2 fs. The entire system comprising of the peptides, lipids and solvent along with the counter ions were maintained at a constant temperature of 303 K using the Nosé-Hoover thermostat with a time constant of 0.5 ps. A pressure of 1 bar was maintained using semi isotropic pressure coupling with the Parrinello-Rahman barostat at a time constant of 2 ps. Electrostatics (Particle-mesh Ewald) and van der Waal cut-off was fixed at 1.2 nm. Bond lengths were constrained by LINCS algorithm.

2.1.3. Circular dichorism

Circular dichroism (CD) experiment was performed to check secondary structure of the peptide sequence. The spectral readings were recorded in a 1 mm quartz cuvette using Jasco J-1500 instrument and the slit width was maintained at 1 nm. Fresh peptides samples were dissolved in deionized (18.2 MΩ-cm) water to prepare stock concentration of 100 μM and the spectral data point was recorded 260 nm to 190 nm at room temperature. The spectral data points are shown as Mean Residue Ellipticity (MRE -θ). MRE was calculated using the formula: [θ]MRE × 10^3 (deg.cm^2. $dmol^{-1}$) = (Mr* θ mdeg)/(10 * l * c) [Mr(mean residue weight, *i.e.* peptide molecular weight/number of residues), θ mdeg (ellipticity in millidegrees),l(length in centimetres), c (peptide concentration in mg/mL] and plotted using Origin 8 software [27].

2.1.4. Peptide synthesis and characterization

Standard Solid Phase Peptide Synthesis protocol was employed for the synthesis of peptide molecules using Fmoc chemistry. Post synthesis, the peptides were cleaved in a chemical cocktail (m-Cresol: Thioanisole: EDT: TFA) for 12 h duration. Subsequently, the peptides were precipitated in ice-cold ether, followed by repeated washing. The peptides were further purified by reverse phased liquid chromatographic run (10 to 100% acetonitrile), at a flow rate of 0.5 mL/ min. The purified peptide molecules were further verified using mass spectrometry (MALDI) [7,8].

2.1.5. Antimicrobial assay and microscopic analysis (field emission scanning Electron microscopy)

Overnight grown bacterial cultures (*Staphylococcus aureus*, *Escherichia coli* and Gentamicin resistant MRSA) were washed and resuspended (bacterial count of 2 × 10^6 CFU/mL) in 10 mM phosphate buffer (pH 7.4). 50 μL of bacterial suspension was mixed with the required concentrations of peptide in buffer and incubated for 2 h at 37 °C. Post incubation, 20 μL of 10-fold diluted peptide-bacterial broth was plated in Nutrient Broth (NB) agar plates. The petri plates were incubated for 12 to 16 h at 37 °C. Percent killing efficiency of test molecules were calculated by comparative analysis with untreated ones [8,28]. For FE-SEM analysis, glutaraldehyde was added up to 4% post incubation, followed by subsequent incubation of 30 min at room temperature. Bacterial suspension was then washed using an increasing concentration of alcohol (30 to 100%), followed by gold coating [8].

2.1.6. Hemolytic activity

Fresh sample of blood was collected from a healthy individual and mixed with anticoagulant, ethylene diamine tetra acetic acid (EDTA) salt at a concentration of 2 mg/mL. A 10% hematocrit suspension was prepared in 5 mM HEPES buffer saline (pH 7.4) and 50 μL of the hematocrit was treated with peptides at their respective minimum inhibitory concentration (MIC). The peptide-blood mixture was incubated at 37 °C for 2-h duration. Percent hemolysis was calculated spectroscopically, by comparative analysis with control (untreated) samples at 540 nm [7,29].

Table 1
Designed peptide codes, sequences and their respective molecular mass. The letters in uppercase represent L chiral amino acid, and in lower case, 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

3. Results

3.1.1. Peptide design and electrostatic profiling

We have designed four 7-mer syndiotactic peptide sequences (Table 1) as single turn Gramicidin helix, a subset of the earlier design in sequence and structure (Fig. 1) to make a comparative assessment of their designability and bactericidal potency.

The earlier reported 12-mersyndiotactic beta helical structure [8] was found to be highly designable, and the four peptides were showing very impressive bactericidal potency in the range of 1–10 μM (MIC value).The 7-mer sequences were designed to have a Gramicidin-like helical conformation with distinct cationic and hydrophobic side-chains (Fig. 1).

Specifically, peptide ST2 is the stereo-chemically reversed model of ST1. ST3 is the sequence and stereochemistry reversed ST1, and ST4 is sequence reversed model of ST1 (Table 1). The designed peptide molecules are amphipathic in nature, owing to the presence of both cationic (hydrophilic) and hydrophobic side-chains in the sequence. These characteristics are known to play a significant role in the selective membrane interaction, which is primarily electrostatic in nature, followed by lysis causing bacterial cell death. The amphipathic nature of the *de-novo* designed peptides was verified by electrostatic potential mapping of the designed peptides (Supplementary Fig. 1). The electrostatic potential maps were generated by solving Finite Difference Poisson Boltzmann (FD-PB) equation using Delphi software [22]. The variable electrostatics within a peptide sequence can be attributed to its side chain sequence, which was apparently visible in the designed peptide sequences.

In general, optimum amphipathicity is one of the design mainstay of ideal AMPs with significant bactericidal potency as well as minimal mammalian cytotoxicity [13,14,30]. Hence, designed peptides with ideal electrostatic fingerprint can be expected to have the desired physiological activity. The designed peptide sequences are made of similar amino acid composition. However, inter-peptide variants were created by altering the stereochemistry and sequence reversal. No two peptides within the set have the same sequence or stereochemistry (Table 1).

3.1.2. Assessment of secondary structure using CD spectra

The CD experiment was performed to check the conformations of 7-mer syndiotactic peptides. The CD spectra of the designed 7-mer peptides lack the characteristic peaks associated with a defined secondary structure (Fig. 2). The CD spectra of Gramicidin in primary alcohols

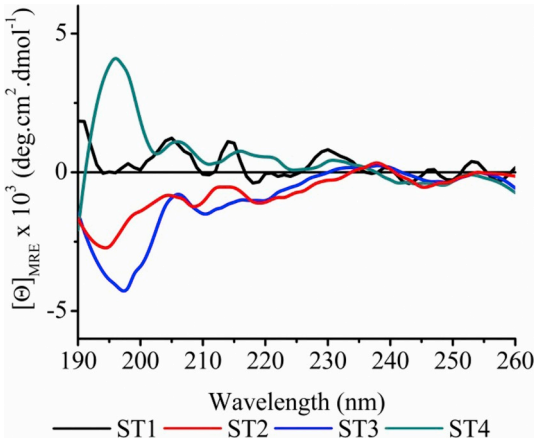

Fig. 2. Circular dichroism (CD) spectra of the peptides ST1- ST4 (7-mer) showing presence of unordered secondary structure.

Table 2
Minimal Inhibitory Concentration (MIC) values of peptides in MS and ST series against *S.aureus, E.coli and* Gentamicin resistant MRSA at micro-molar (μM) concentration.

Peptide code	MS1	ST1	MS2	ST2	MS3	ST3	MS4	ST4
S.aureus	8	> 100	8	> 100	6	> 100	8	> 100
E.coli	0.5	> 100	4	> 100	1	> 100	4	> 100
Gentamicin resistant MRSA	4	> 100	6	100	8	> 100	8	> 100

show a positive maxima near 200 nm and negative minima near 215 nm and 230 nm [31], but our experiments were performed in water. Understandably, the CD spectra for Gramicidin is different from traditional α-helices and β-sheets [32,33]. Further, the CD spectra of the designed ST1-ST4 peptides does not confer with the earlier reports of CD spectra, of either Gramicidin A or 12-mer syndiotactic peptides in water [34]. Together, these observations suggest a disordered conformation for the designed peptides (ST1-ST4).

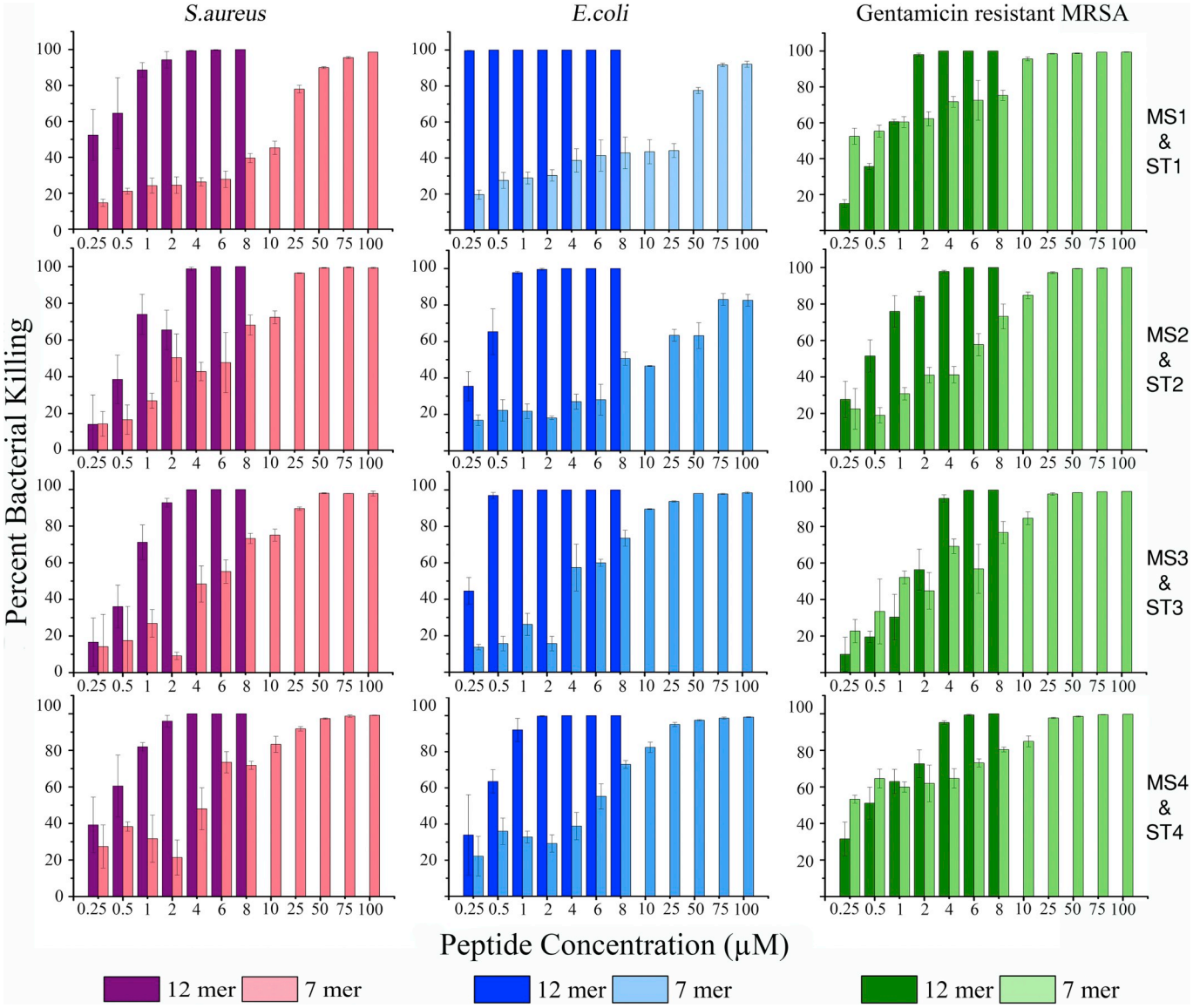

Fig. 3. Comparative antimicrobial activity of peptides (MS1 to MS4 and ST1 to ST4) against *Staphylococcus aureus*, *Escherichia coli* and Gentamicin resistant MRSA. The standard deviation has been shown as error bars in the graph. Single turn ST-1 peptides have significantly lesser antibacterial activity compared to MS-1 peptide. ST-1 to ST-4 is a structural and sequential subset of MS-1 to MS-4.

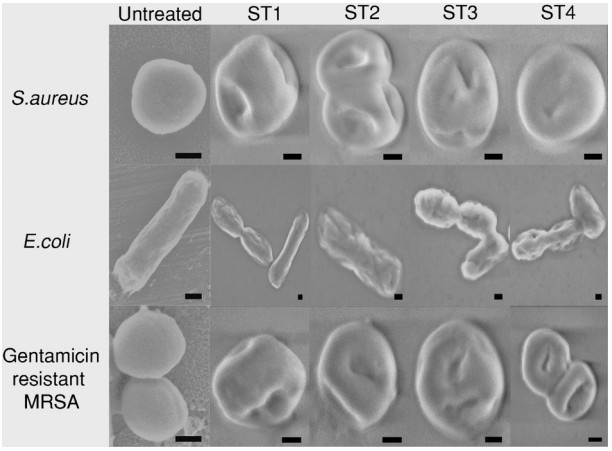

Fig. 4. Field Emission Scanning Electron Microscopy (FE-SEM) of bacterial cells treated with peptides (ST1 – ST4) at 100 μM concentration and untreated cells as control. Bacterial cells of *Staphylococcus aureus*, *Escherichia coli* and Gentamicin resistant MRSA were used in this study and all the peptide treated cell were seen to develop deformed membrane texture as compared to untreated control cells. The scale bars correspond to 200 nm.

3.1.3. Antimicrobial assay

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 observed to have markedly reduced compared to the earlier reported mother sequence MS1 – MS4 (Fig. 3, Table 2).

In the earlier reported study, the 12-mer peptides were designed to form a Gramicidin helix with two turns, unlike the 7-mer peptide sequences in this work, which can only form a single turn, if at all it adopts a similar conformation.

The microscopic analysis (FE-SEM) provides a qualitative picture of the deformed bacterial membrane architecture when treated with the designed peptides. The bacterial cells (*S.aureus*, *E.coli* and Gentamicin resistant MRSA) were treated with peptides at their minimum inhibitory concentration, followed by fixation and imaging (Fig. 4). The treatment of Gram-positive, Gram-negative and multi-drug resistant bacterial cells with 7-mer peptides (ST1-ST4) showed significant membrane deformation. However, the respective MIC values for 7-mer peptides is significantly higher than those of the 12-mer peptides (MS1-MS4) against the same bacterial cells [8].

Therefore, the need of optimal amphipathicity on a stable structure of AMPs is very important. Ideal amphipathicity also dictates the selective lysis of bacterial membrane, with minimal or no notable damage to the mammalian cells [13,14].

3.1.4. Hemolytic assay

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 a hemolytic activity of peptides against mammalian (RBC). Hemolytic activity of the peptide was found to be well within the permitted levels of toxicity (Supplementary Fig. 2).However, it has little significance under the present circumstances, that the designed 7-mer sequence is not likely to be a potential drug candidate owing to its relatively poor antibacterial activity.

3.1.5. Molecular dynamics simulations

To study the differential effects in the designability and resulting antibacterial potency, we performed Molecular Dynamics simulations of 12 mer gramicidin helical peptide (MS-1) and its sequential and structural subset ST-1, with the model lipid bilayers. The membrane

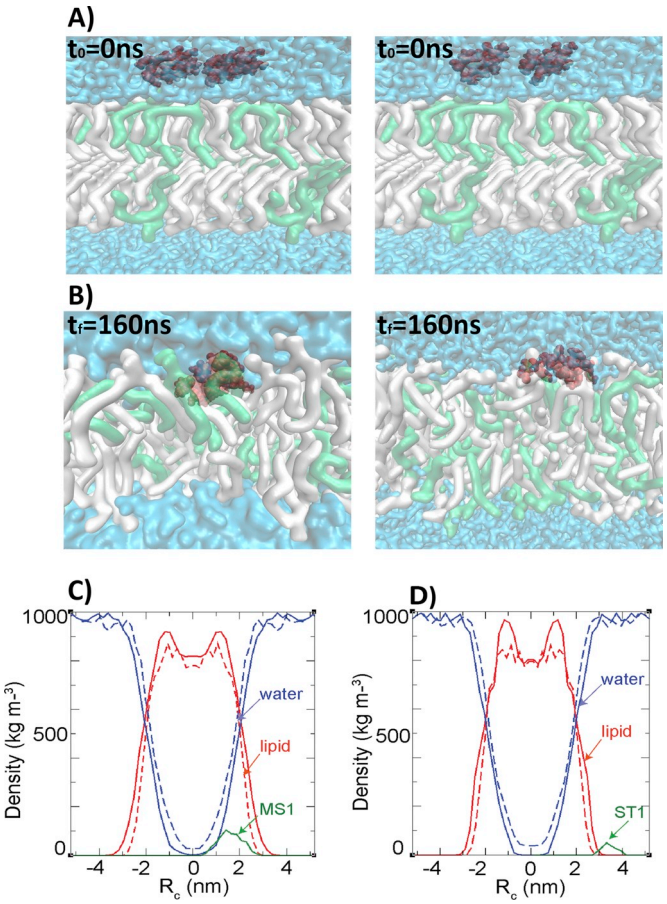

Fig. 5. Comparative summary of MS1 (left panel) and ST1 (right panel) peptides with mixed lipid bilayer. The peptides are shown at the beginning and end of simulations in 5A and 5B, with the mixed lipid bilayer (POPC: white, POPG: green, Peptides: red, Water: cyan) in 3A and Dynamic bonds format in 5B. 5C and 5D show mass density of the system showing difference between peptide free system (dotted lines) and the system after being exposed to the peptides for 160 ns (solid lines). R_c denotes the distance from the centre of the box in nm. The different peak maxima (green line) of 1.3 for MS-1 and 3.4 for ST-1 (5C) are indicative of their respective peptide-membrane interaction and possibly the extent of membrane deformation centre at 0 nm) with the lipid head-group maxima at −1.1 and 1.1 nm. The peptide density was seen to be ranging from 0.5 to 3 nm and 2.7 to 4.1 nm for MS1 and ST-1 respectively with their peak maxima at 1.3 nm and 3.4 nm. The different peak maxima of 1.3 and 3.4 are indicative of the membrane deforming potential of MS-1 and ST-1 peptides on typical bacterial membranes upon identical conditions. This differential behaviour in peptide membrane interaction has actually been translated to their respective MIC values (Table 2) indicating bactericidal potency.

model used was 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG) bilayer in the ratio of 3:1. A POPC: POPG mixed bilayer is reportedly used as a representative of the Gram-positive bacteria such as *S.aureus* [35–38]. Standard MD protocols were followed, using GROMOS96 force field under NPT conditions and thermally equilibrated coordinates in earlier reported studies using POPC and POPG. Two independent simulations were setup for the comparative study. Four peptides were placed randomly above POPC: POPG bilayer. During the first 10 ns of the simulation, the MS1 peptide units assembled to form a multimeric complex, followed by subsequent close interaction of the aggregated peptide complex with the upper leaflet of the membrane. In the case of ST-1 peptide, two dimers of ST-1 peptides was formed, one of them getting adsorbed to the upper membrane, while the other remained distant from the bilayer (Fig. 5). The positively charged amino acids of Lysine and arginine facilitated the strong attraction of the

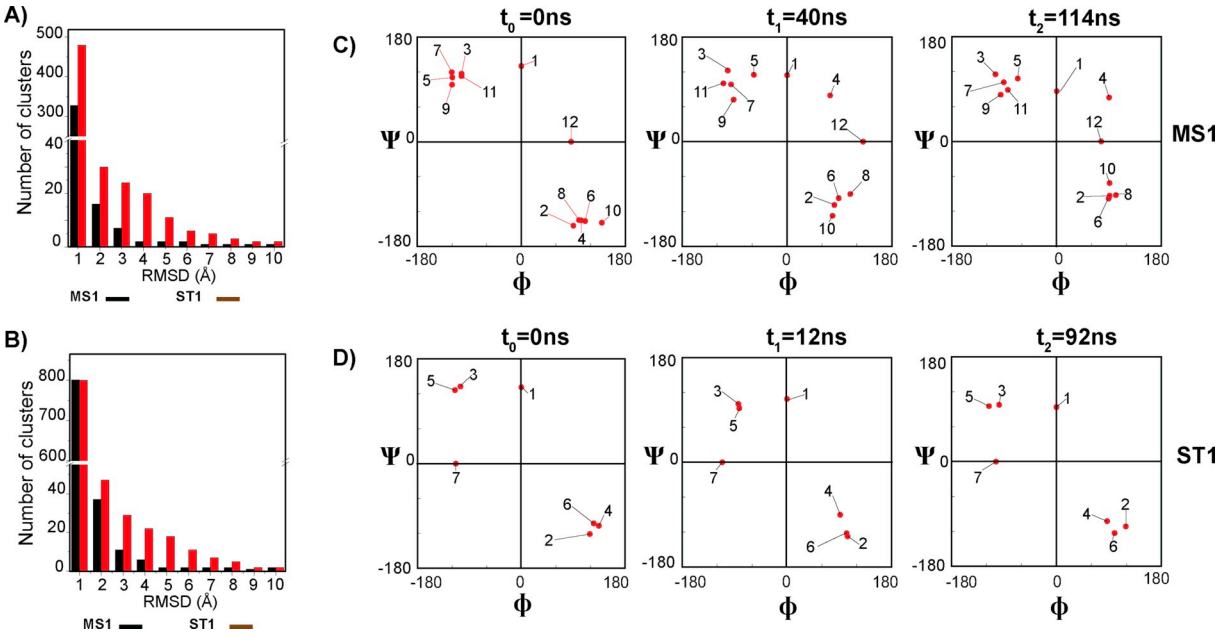

Fig. 6. A & B) Cluster analysis of MS-1 and ST-1 peptide backbone and peptide complexes. C & D) Ramachandran plot for MS1 and ST1 peptides respectively at various time frames. Three time points t_0, t_1 and t_2 represents the mean structure of the largest clusters formed at three different time points; i) while the peptide is in water medium, ii) while the peptide interacts with the lipid bilayer and iii) time of penetration of the peptide into the upper membrane. (Time frames were obtained through clustering analysis). Red dots represent Ramachandran φ, ψ combinations of individual amino acids of the peptide at respective times t_0, t_1 and t_2. MS-1 (syndiotactic double turn peptide) could approximately retain its phi and psi angle combinations determining the structure of the backbone (6C), while ST-1 peptide has moved away from the designed gramicidin beta helical conformation (6D).

peptide complex to the negatively charged POPC:POPC bilayer. On the other hand, the aggregated peptide complex of MS-1 established contacts with the membrane surface within 40 ns, but insertion of the tetramer peptide complex into the upper membrane was evident only after 110 ns (Supplementary video file 1). In the case of ST-1 peptide, one of the dimer complexes got adsorbed to the upper bilayer, but insertion of the dimer was not evident during the entire course of the simulation (Supplementary video file 2). The initial aggregation of the peptide units in the case of MS-1 might have facilitated the uptake of the peptide, which was not apparent in the case of ST-1. MS-1 peptide conserved its gramicidin helical conformation throughout the simulation, while ST-1 peptides deviated from the designed phi and psi angles during the course of simulation (Fig. 6C and 6D). The instability of ST-1 structure was more evident from the account of number of clusters formed at different RMSD cut-off (Fig. 6A, B). The partial density profiles were calculated along the membrane normal for the POPC:POPG bilayer, water and the peptide. During the entire simulation, the POPC:POPG membrane displayed similar density profiles ranging from −3 nm to 3 nm (bilayer.

Dynamics of peptide molecule on a rugged landscape generally yields a myriad of local minima, identified as clusters that are nearby in their free energy, but can well be conformationally non-identical. Cluster analysis of MS-1 and ST-1 resulted in very different landscapes, with ST-1 peptide has total number of clusters almost two times while compared to MS-1. ST-1 shows little preference for the ground state, whereas MS-1 has clearly differentiated global minima (Supplementary Fig. 3). MS-1 energy landscape has the character of a peptide or protein molecular system with a stable structure, while ST-1 peptide clusters appear to be energetically near but topologically distant, with relatively small energy barrier.

4. Discussion

Antibiotic resistance reduces the therapeutic activity of antibiotics. Reduced number of novel antibiotics and recurrent rise in antibiotic resistant superbugs has caused a situation demanding new chemical entities [39]. With the drug discovery pipeline showing signs of drying up, AMPs can be a useful alternative, due to its broad spectrum activity [40], selectivity towards microbial cells and negligible mammalian cytotoxicity [41]. However, there are notable limitations of peptide-based molecules, which has withheld their clinical applications as potent antibiotics. Premature cleavage by proteolytic enzymes and reduced activity in presence of salts are among the major drawbacks of peptide-based antibiotics [19]. Strategies like the incorporation of D-amino acid within the peptide sequences have reportedly addressed these limitations, resulting in improved bactericidal activity [19,42,43]. We have previously described the antimicrobial potency of 12-mer syndiotactic peptides [4]. In the present study, we have designed a new series of peptides with similar characteristics in terms of cationicity and amphipathicity and varying sequence length (7-mer) (Fig. 1). A double turn Gramicidin helical structure was designed in the case of syndiotactic 12-mer peptides. The syndiotactic helix formed by the 12-mer peptides were thermodynamically and kinetically stable [4]. However, the 7-mer peptides do not retain their designed structure in solution as is evident from the CD spectra of ST1-ST4 (Fig. 2). The disordered structure of 7-mer peptides, therefore, destabilizes the electrostatic signatures imbibed during their design.

We investigated the effects of the disordered structure due to the reduced length of the designed peptides on their antimicrobial potency. The peptides were tested against *Staphylococcus aureus*, *Escherichia coli* and Gentamicin resistant MRSA bacterial species. The antimicrobial potency of the designed 7-mer peptides was compared to the potency of the previously reported 12-mer peptides [8] in terms of minimum inhibitory concentrations (MIC) against the three bacterial species. The bactericidal activity of 7-mer peptides was lower than that of 12-mer peptides by a huge margin (Table 2, Fig. 3). Despite reduced antimicrobial potency, these peptides were designed to be membrane active in nature. The surface of bacterial cells was deformed when treated by 7-mer peptides at 100 μM concentration as seen from FESEM analysis (Fig. 4), indicating their membranolytic potential. Further, both 7-mer peptides were evaluated for their possible hemolytic potential. The

designed peptides showed minimal levels of toxicity towards mammalian RBCs. However, due to decreased antibacterial potency in comparison to 12-mer peptides, the designed 7-mer peptides do not present themselves as potential AMPs.

Furthermore, we evaluated the interactions of 7-mer and 12-mer peptides with model POPC, POPG bilayers using molecular dynamics simulation. The peptides were simulated in a system comprising of a POPC:POPG::3:1 bilayer and water molecules for 160 ns. Our comparative molecular dynamics simulation suggests that the aggregation of individual peptide into an assembly is the first event before membrane interaction for syndiotactic amphipathic peptides. This is primarily a result of structural stability of beta helix conformation and conservation of hydrophobic and cationic zones. Recent study by Phambu et al. [44] with RW peptides of different lengths also supports the theory that formation of assemblies of individual peptides is a prerequisite for antimicrobial activity [44]. The 12-mer peptides (MS1-MS4) were able to penetrate the outer leaflet of the lipid bilayer, whereas the 7-mer peptides were present on the membrane surface (Fig. 5). The number of clusters for the peptide backbone structures involving 7-mer peptides was higher those observed with the 12-mer peptides (Fig. 6). This indicates that the 12-mer peptides have a more stable backbone architecture than the 7-mer peptides. Therefore, molecular dynamics simulation analysis provides ample proof that the 7-mer peptides do not retain the single turn Gramicidin helical structure.

5. Conclusion

In this study, we report the bactericidal activity of syndiotactic sequences in relation to their chain length and conformational rigidity. We observed that chain length and conformational locking are among the most important criteria while designing potential antimicrobial peptides. Further, we infer that a smaller chain length of ST1-ST4 peptides in comparison to MS1-MS4 peptides, lead to a reduction in their antimicrobial potency. The smaller chain length relates to the inability of ST1-ST4 peptides to maintain their designed electrostatic features, thereby affecting their bactericidal activity. Therefore, the antimicrobial potency of the designed peptides is affected by a reduced chain-length, which results in structural instability which results in a reduced bactericidal activity.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bpc.2019.02.005.

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