Review

Short Antibacterial Peptide Surrogates Centered On A $\beta$-Turn Mimic

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Abstract

There is an urgent need for new antibiotics which are effective against drug-resistant bacteria without contributing to resistance development [1a-c]. Due to their mechanism of action, Anti-Microbial Peptides (AMPs) have shown very low bacterial, fungal and viral Resistance [2]. We designed and developed antimicrobial short peptide surrogates that include $\beta$-turn [3,4,5] mimics, with two flanking lysines[6] rests in their sequences and with cationic amphipathic structures based on the mimicry [7]of naturally occurring antimicrobial peptides at very low concentrations, less than ten μM. These short peptide surrogates exhibit this potent antimicrobial activity against a broad spectrum of bacteria including E. Coli and methicillin-resistant Staphylococcus aureus with no adverse hemolytic activity. Notably, these short peptide surrogates also did not result in any measurable resistance development in E. coli. MIC experiments indicate that D- and L-$\beta$-turn based Cationic Anti-Microbial Peptides (CAMP) surrogates are almost identical both in the eradication of both Gram+ and Gram- bacteria [91]. These results suggest similar behavior of “artificial” D- and “natural” L- peptide surrogates when binding to bacterial membranes. There is, however, sensitivity to chirality in RBC hemolysis. Our studies may contribute to further understanding of how CAMPs sense bacteria membrane as well as provide a new direction to develop novel membrane disrupting agents[8]. The peptide-mimetic design principle offers significant flexibility and diversity in the creation of new antimicrobial materials and their potential biomedical applications[9]. We would like to explore the tuning the membrane selectivity of antimicrobial peptides [10,a,b] surrogates into small molecules drug-like substances.

We have reported on the synthesis and evaluation of antibacterial compounds based on am amphipathic motif [11], observed as a moiety in many antimicrobial natural peptides, of a 5 amino-acid linear chain where the central hydrophobic 3 amino acids moiety is flanked by two lysine units and prepared some surrogates of these Penta- peptide K-K’segment 1, (2 γ 4) including compounds where privileged scaffolds are introduced, substituting a part of the hydrophobic amino acids part structure. Our studies may contribute to further understanding of how CAMPs sense bacteria membrane as well as provide a new direction to develop novel membrane disrupting agents[12].

Figure 1 : K-A1A2A3-K

The peptide surrogate 2 reveals a structure where $\beta$-turn mimic (benzodiazepine unit)[13] is conferred to A1 in 1. In this communication we report on conferring other $\beta$-turn mimics[14] as a potential center of selective attachment to bacterial cell [15]. Although drug design DE

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NUVO requires more than a structural motif [16], the identification of amino acid sequence in an antimicrobial peptide that have been linked to several pathologic disorders, may aid in the focusing of efforts as to the mimicking of that sequence (motif ) that could be referred to the particular (antibacterial for example) biological active sequence of amino acids.

Although the basic structure of biological membranes is provided by the lipid layers[17] the membrane proteins (A typical plasma membrane is somewhere in between, with protein accounting for about 50-60% of its mass[18]).

The proteins perform most of the specific functions of membranes. It is the proteins, therefore, that give each type of membrane in the cell its characteristic functional properties. Accordingly, the short linear motifs (SLiMs) are functional micro domains in proteins that play a critical role in many distinct biological processes such as cell signaling and regulation, post-translational modifications, proteolytic cleavage, and protein trafficking [19,20]. These motifs are typically found in eukaryotic disordered protein regions and vary in size from 3 to 12 aminoacids.

In general, SLiMs have less than five defined amino-acid positions, and frequently these positions have some degree of flexibility in amino acid composition. Their shortness makes them evolutionarily plastic, allowing them to evolve convergent in unrelated proteins. This can allow proteins to rapidly acquire new protein interaction function [21,22]. Their short length also present a challenge for SLiM discovery both experimentally and computationally, since there may be many false positive findings using both methods[23].

Protein-Protein recognition, very often observed in interactions of antibodies with proteins in living organisms, is one of the main phenomena’s still to be fully understood and exploited by pharmaceutical researchers. Peptides and proteins are essential to many biological processes. The interaction between the peptide ligands and their receptor targets commonly involves β-turn structures. β-Turns are a part of the receptor bound conformations of peptides with their receptors and mimics of such turn can be conferred into peptide sequences without altering the biological activity of the peptides [24]. Yet poor bioavailability and unfavorable pharmacokinetics significantly compromise the use of peptides as drugs. An example is the low-molecular-weight imaging agents target the prostate-specific membrane antigen (PSMA). PSMA is a type II integral membrane protein that has an abundant and restricted expression on the surface of Prostate Cancer (PCa), particularly in androgen-independent, advanced, and metastatic prostate cancer disease[25].

Since proteins tend to exert their biological activity through small regions of their folded surfaces, small functional units that are amenable targets for further chemical modifications could be designed to elicit the activity of the native protein [26]. This approach involves mimicking specific secondary structures of the protein molecules such as α-helix, β-sheet, β−sheet β-turn and loops that constitute the bioactive surfaces involved in the receptor-ligand interaction [27].

Protein–protein interactions (PPIs) regulate a wide array of cellular processes [28] and are attractive targets for drug design. The major outer membrane protein (MOMP), a putative poring and a multifunction surface protein of bacteria, may play an important role in the adoption of the organism to various host environments[29]. β- Turn mimics[30] can interact and bring about recognition and association of proteins. It was also noted that some of the abilities of AMPs to combine with cell walls of microbes. The possible role of a PXXP central hinge in the antibacterial activity and Membrane Interaction of PMAP-23, a member of cathelicidin family is one example. This is due to short peptide motifs PXXXP [31]. This might contribute to Cathelin[32] endocytosis. Regarding endocytosis, the structural and chemical requirement were investigated and Sequence YXRF Implicates a tight turn as the structural recognition motif for endocytosis [33]. Investigators examined the use of β-turn mimics in the protein-protein interactions involving Trans membrane receptors in nerve cells in protein interactions that involve β-turns [ ]. Such β-Turn Analogues can either mimic or disrupt Protein-Protein Interactions.

Figure 2:

Nearly half of the mass of the outer membrane of bacteria is protein. Most outer membrane proteins are thought to be located exclusively in the outer membrane, although some proteins are found in both the outer and cytoplasmic membranes. Bacteria produce cell walls except mycoplasmas, and the cell wall component common to all eubacteria is the murrain, or the peptidoglycan, which contributes mechanical rigidity. All Gram-negative bacteria contain an additional layer in the cell wall structure, i.e., the outer membrane, which is located outside the peptidoglycan layer and shows up as a trilaminar structure on the electron micrographs of thin sections of these bacteria[35,a,b].
One of the approaches is to learn about the potential application of short peptide mimics like \( \beta \)-turn mimics, on the recognition with perspective to apply this if future drug design. The appearance of \( \beta \)-turns in protein interaction is by far more common than that of other, like \( \beta \)-turns. Noncovalent \cite{36} interactions between the turn-mimics and some receptors on the cell wall of bacteria may supply enough energy differences that may allow differentiation between various bacterial transmembranal cell wall receptors due to receptor-\( \beta \)-turn mimic interactions. The interactions of some \( \beta \)-turn mimics with many classes of proteins which vary in their secondary structure (\( \beta \)-sheets, globular) have been found to rely on the interaction between \( \beta \)-turn mimics and the proteins.

Figure 3

Results and Discussion

Many variants of \( \beta \)-turn mimics have been applied so far in this area of research. One can read about benzodiazepines \cite{9}, \( \beta \)-turn mimic Hot\( =\)Tap for example \cite{37}.

Figure 4

There is a growing demand for novel antimicrobial agents \cite{38} for therapy but also for Hygiene and Agriculture, Soil Sterilization, for example. The class of compounds in the focus is the growing group of polypeptides isolate as part of the host defense systems of all organisms on earth (Antimicrobial peptides).

Strains of the bacteria that harm are becoming more resistant to drugs but also live in the vicinity, in the same organism, as other useful and needed fauna of microorganism exist in human gut, the “beneficial” various strands of Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria for example. We would like to selectively eradicate the “bad” microorganisms and leave the “useful” ones intact \cite{39}.

Figure 5: Surrogates of the conglomerate pentapeptide antibacterial motif K-A1A2A3-K

Now it is established that simple small molecules based on simple motifs \cite{40} can be prepared and eradicate Gram+ as well as Gram- bacteria. The next step is the differentiation stage (to attack only the unwanted bacteria). The selectivity mode we had in mind is to base the eradicating peptide surrogate on \( \beta \)-term mimics that are known to interact with cell wall proteins \cite{41,a,b}.

In bacteria, mainly the outer part of transmembrane receptors \cite{42,a,b} are characterized by many loops of the outer front of the receptor (Drawing 2):
The cell wall of bacteria contains proteins [43]. Mainly those that build the cell wall Toll, TLRn(n=1-13) transmembrane signaling [44] Receptors [45,46,47] for instance. This could be viewed as a suitable domain that might interact with a complementary motif or surrogates[ ] in a protein-peptide interaction [9c].

Current research reveals that short (as little as 4-5 amino acid sequences, PXXP that was mentioned above [1], for instance), polypeptide chains are the once forming a non-covalent attachment to the receptor on the outer membranes. This region of the protein in which the receptor is rich in β-turn motifs, could provide a ground for non-covalent connections. It may become feasible that antibacterial peptide surrogates, based on mimics of such short peptide sequences, may attach to the proteomic part of the outer domain that the transmembrane receptor.

K(R)-A-A-A-K part structure in transmembranal outer portion (Ref. 22) (LTR4 and LTR5 in particular). This may in this way selectively in the choice of bacteria to eradicate. Eradication will then be done by the usual membrane disassembling mechanism the bacterial cell.

**Figure 6:** K(R)-A-A-A-K part structure in transmembranal outer portion (Ref. 22)

**Concept and Idea**

We assume that the assembly of both stable and transient signaling complexes depends on a variety of highly conserved, small binding domains that are found in many intracellular signaling proteins. Each of these compact protein modules binds to a particular structural motif in the protein with which the signaling protein interacts [49]. In this communication we examined the synthesis and the preliminary testing for broad band bacteria based on β-turn mimics eradication and the hemolysis of human red blood cells (see drawing three below):

It is commonly accepted that all biological processes are underpinned by protein-protein interactions (PPI). However, we are quite far behind in our ability to understand the ‘interactome fully,’ we must know how PPI is regulated in time and space to produce biological functions [50]. These drawbacks should not prevent us from seeking solutions to the current problems. We assumed that linear peptide surrogates [51] could also function similarly [52,53] complimentary to the suitable domain.

In previous papers we have reported on the application of the β-turn mimic benzodiazepine and the Dihydro-pyridine privileged scaffolds in the synthesis of series of efficient broad-spectrum antibacterial substances (Dra 1). Some (1,2 Dra1) were comparatively activity-wise to naturally occurring AMP, (Antimicrobial peptide) [54], usually MIC values [55] of about 40, that is 5-10 times more active than the natural product (MIC values 12). The introduction of their mimic did not change otherwise the usual behavior of the AMP surrogates.
At this moment we report on the synthesis of short antibacterial peptide surrogates and the checking of their biological activities as antibacterial substances given drawing 3. The β-turn part of the conglomerate could be attached to the bends of the receptor and thereby enabling selective targeting by non covalent interaction.

Drawing 4 depicts the main idea: the Turquoise unit is the β-turn mimic conferred into the eradicating molecular structure in yellow. This is connected by supra-molecular non-covalent bound into the outer part of the transmembrane Omp receptor [56] in violet through the loops in green.

**Synthesis and Biological Testing**

Focusing on a fragment of 1 (Drawing 1), amino acids from the lysine-rich part of Dermaseptin S4 [57], Val-Leu-Lys-Ala-Ala-Ala-Lys. We have prepared the following tetra-peptide linear epitopes and found that two lysins in flanking positions are needed for enhanced biocide activity as shown below:

![Structure of peptides](image)

**Figure 9**: Tri and tetra-peptides as linear epitopes derived from Dermaseptin S4 fragment.

We further extended our peptide-mimetic approach to utilize the “snorkeling” [58] effect well Characterized in peptides to control the interaction the bioactive structure with cell membranes [59]. The “snorkeling” model is one in which peptides have long spacer “arms” in the cationic residues (lysine and arginine [60]), which can reach to the lipid-water interface.

Thus, we have focused on the 4-5 amino acid amphipathic motif where three (or two) hydrophobic amino acids (no charge) or their surrogate which are flanked by two cationic amino acids lysine (K) or arginine (R) (K-AAA-K, K-AAA-K), [26b] as examples (positive charge). They are present in frog skins, (see above) [61,62], in human lactoferrin [63]. Lactoferricin and human saliva which is among the most studied AMP derived from the milk protein.

![Lactoferricin structure](image)

**Figure 10**: Reference 33 Reference 32-34

The complete sequence of lactoferricin corresponds to lactoferrin fragment 17-41 (FKCRRWQWREMKLGLAPSITCVRRAF) and sequences from within this fragment are also antimicrobial. Svendsen and Vogel and their groups shed light on the 3D structure of Lactoferricin [64,65].

![Calculated structures of LfcinH](image)

**Figure 11**: Representations of calculated structures of LfcinH in anaqueous solvent (A and B) and membrane mimetic solvent (C and D). (A and C) Ribbon diagram representations; (B and D) charge distributions on the surface of the peptides. Positive, negative, and neutral potentials are colored blue, red and white, respectively. This figure was produced by the MOLMOL program.
Protein-Protein Interfaces [66,67] interactions can be satisfied by adding a second copy of the interface domain to the monomeric polypeptide in such a fashion to allow it to interact with the original interface. (The latter strategy was employed by Mossing and Sauer[68] when they connected via a β- turn, a partial copy of the β- ribbon interface of λ-cro - DNA protecting protein - to the end of an intact copy. This allowed the second copy to loop back and interacted with the remainder of the protein to form a stable monomer.)

Generally, The design[69]and synthesis of peptidomimetics are most important because of the dominant position peptide and protein-protein interactions play in molecular recognition and signaling, especially in living systems. The design of peptide mimetics can be viewed from several different perspectives[70,71] and peptidomimetics can be categorized in some different ways [72]. Examination of the vast literature would suggest that medicinal and organic chemists, who deal with peptide mimics, utilize these methods in many different ways.

Here we would like to suggest that the bend around Lys29-Arg31 (graphic D figure 2) might accommodate the antibacterial motif of lactoferricin. (see chart one below).

Synthesis of Surrogates Based on Tetra-Peptides

Experimental Part

Synthesis of Tetrapeptides

The following were prepared according to the Miao protocol[73]:

General Procedure for SPPS

Rink amide resin (0.4mmol) was shaken with DCM: NMP 1: 1 (3 ml) for 24 h. in a Merrifield flask. The solvent was filtrated under vacuum, and the resin was treated with 20% piperidine in DMF (v/v) to de-protect the amino groups (2×10min). Then the solid was washed five times with NMP and three times with DCM. Positive Kaiser Test indicated successful removal of the F-moc protecting group. A solution of absolute DMF (3 ml), HATU (4 eq), F-moc-AA (0.4mmol), and DIEA (3 eq) was added to the resin, and the mixture was agitated for three h. During the coupling to the secondary amine of the λ-turn mimics’ scaffold, the agitating was performed for two h at 40°C, and the next AA was added twice. At the end of the peptide synthesis, the peptide-loaded resin was dried in vacuum for 2 h. The peptide was then cleaved by addition of a solution of TFA: H2O: TIS (95:2.5: 2.5, 4ml), which turned the solid and the solution deep red. After agitating for 2 h, the solution was pressed out of the Merrifield flask, vacuum filtered, and evaporated under nitrogen. The resulting highly viscous liquid/oil was precipitated from cold diethyl ether (6ml) and lyophilized. The synthetic mimics of antimicrobial peptides (SMAMPs) were finally purified by solid-phase extraction pack (RP-18), first washed with water and then extracted with acetonitrile. Their purity was determined by HPLC (RP-18, CH3CN/0.1% TFA (aq.), 2:1).

Figure 12 : Short peptides built from S anR amino acids

Figure 13 : short peptides (All S configuration) and their ability in broad spectrum eradication of bacteria

- The peptidomimetic compounds (P1–P9) were subjected to MIC testing, identifying the lowest concentration of antimicrobial compounds that inhibit the growth of a microorganism after 24 h incubation at 37°C. Microplateautoreader E1309 Bio-tek Instrument was used to measure absorption at 690 nm. MIC values for grampositive (methicillin-resistant S. aureus) and gram negative (E. coli) bacteria were determined. The concentrations of the antimicrobial peptidomimetics in the micro plates were 400,200,100,50,25, 12.5, 6.25,3,1.5,1,0.5,0.25, 0.1, and 0.05 μg/ml. The MIC values and errors are reported as averages and standard errors of mean of three independent experiments (each experiment was performed in triplicates) respectively. The error of the experiments is less than 5%. Antimicrobial.
Activity in Plasma

Fresh human blood cells were centrifuged at 3000 rpm for 5 min. The plasma, which separated from hRBCs, was collected. P2 was dissolved in water and then diluted twofold into plasma solution so that final concentration of the compound was 800 μg/ml. This sample was pre incubated for 0, 3, or 6 h. Then the MIC values for methicillin-resistant S. aureus were determined as mentioned before min antimicrobial assay.

Hemolytic Activity The hemolytic activity of each peptidomimetic compound was tested against human red blood cells (hRBCs), obtained from healthy volunteers. Fresh heparinized hRBCs were rinsed three times with phosphate buffered saline (PBS) buffer (35mM, 150mM NaCl, pH7.4) by centrifugation (10min, 1500 rpm), followed by resuspension and dilution in PBS (10% hematocrit). Various concentrations of the peptidomimetics were then dissolved in PBS buffer and added to the hRBC solution, yielding a final erythrocyte concentration of 1% v/v. The suspensions were incubated under agitation for one h at 37 °C, followed by centrifugation (5 min, 4000 rpm). The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 450 nm. Negative controls for zero hemolysis and positive controls (100% hemolysis) consisted of hRBC suspended in PBS and Triton 1%, respectively. The degree of hemolysis is defined as the ratio of the optical density (OD) of the peptide-mimetic sample relative to the OD of the difference between the positive and negative controls for hemolysis.

References


9. a) Cell penetration cannot be excluded although Such peptides (CPPs) are larger structures (20-24 amino acids, transportan 10). (wileyonlinelibrary.com) DOI 10.1002/psc.2781.


c) Many aspects of cell signalling, trafficking, and targeting are governed by interactions between globular protein domains and short peptide segments. These domains often bind multiple peptides that share a common sequence pattern, or "linear motif" (e.g., SH3 binding to PxxP). Many domains are known, though comparatively few linear motifs have been discovered. Their short length (three to eight residues), and the fact that they often reside in disordered regions in proteins makes them difficult to detect through sequence


44. Toll-like receptors (TLRs) are a class of proteins that play a key role in the innate immune system. They are single, membrane-spanning, non-catalytic receptors usually expressed in sentinel cells such as macrophages and dendritic cells, that recognize structurally conserved molecules derived from microbes.


50. Andrea G Cochran (2001) Protein–protein interfaces: mimics and inhibitors. Current Opinion in Chemical Biology 5:654-659. New peptide scaffolds for b-turn display have been described by Cochran et al. [12••,13]. Like aPP, these structured-b-hairpins fold stably and do
not require synthetic cross-links or non-natural residues to maintain their structures. They are therefore suitable for displaying β-turn libraries on phage. Turn sequences taken from CD4 [12••] and from the B1 domain of Protein G adopt conformations indistinguishable from those in the native proteins when flanked by optimized strand sequences.


d) LACTOFERRIN N-LOBE (231-245) (HUMAN).


