

Antimicrobial Peptide Surrogates Based on Freidger Lactam

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Abstract

The efforts to eradicate microbes, in particular those that have developed resistance toward antibiotic drugs demands the exploration of novel ideas that will bring about the eradication of those microbes. Recently, we have published on the identification and synthesis of short peptide surrogates based on the amphipathic K-A-A-A-K (lys-Ala-Ala-Ala-Lys) sequence.

One approach is to confer β -turn mimics in the central region of the motif. In this way defined structural moieties like benzodiazepine were introduced. The bioactive result showed very potent ability to eradicate broad band spectrum of bacteria without harming hRBCs.

At this research work we sought to examine the two opposite spatial arrangements of such β -turn mimics based surrogates. We have focused on the two enantiomer Friedinger lactam as basis to examine the chiral situations on biological activity:

1. Eradication of bacteria and
2. Toxicity to human Red Blood Cells (hRBCs)

Introduction

Short antimicrobial peptides [1,2,3] are targeted for many years as potential antimicrobial agents urgently needed for the worldwide combat against bacteria, in particular sleepers, persister and resistant [4] microbes [5].

β -turn mimics and privileged scaffolds are a powerful in drug designs in center actions with outer membrane proteins can result in biological response of the protein acquiring new features from the non-covalent supra-molecular construct achieved. We have prepared short antimicrobial peptide surrogate and showed that conferring β -turn mimics and privileged scaffolds into the K-A-A-A-K motif Y (Figure 1).

That was extended to mimics Y a,b,c [2,3,4] (Figure 2).

Results in bioactive molecules that eradicate microbes were encouraging since very high potency was revealed at the sub nano-molar [6] concentrations. The choice of the lysine [7] as basic unit, the Friedinger lactam [8,9,10] fell due to the comfortable access to both S and R forms) 3a and 3b in respect) based on the starting material R and S methionine. The two sorts of cells tested for biological targets for the antimicrobial agent are bacteria (prokaryotes) an human red blood cells (eukaryotes). There are major differences between the two sorts of targets that might be depending on the structure of the peptide mimics structure [11]. Infact, no good correlation was found by testing different antimicrobial peptides but the analysis of relationship between peptide structure and its hemolytic activity confirmed the effect of charge and hydrophobicity on the biological activity studied.

Results and Discussion

In our work we have focused on the penta-peptide P12 that showed superb activity as a broad-spectrum antimicrobial peptide and did not cause hRBC hemolysis [12,4]. We designed similar mimics to p12 as depicted below (Figure 3,4).

Conferring the two amino acids into p12 will afford 5:

The general scheme aims at surrogates where the mimic unit is conferred to the hydrophobic region of 1.

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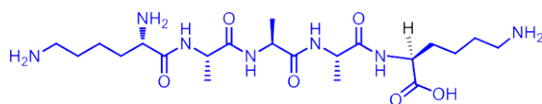


Figure 1: Structure of lys-Ala-Ala-Lys

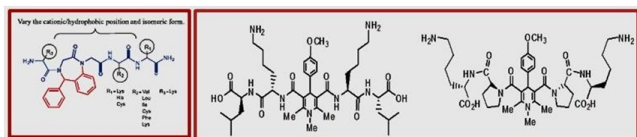


Figure 2: The cationic/hydrophobic position and isomeric form

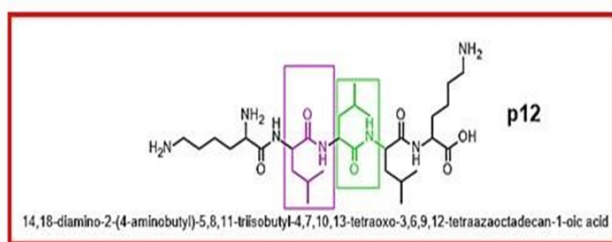


Figure 3: The sequence of P12

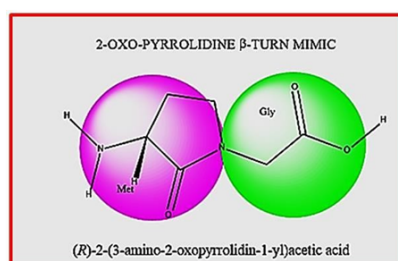


Figure 4: Designed Surrogate 5 of P12

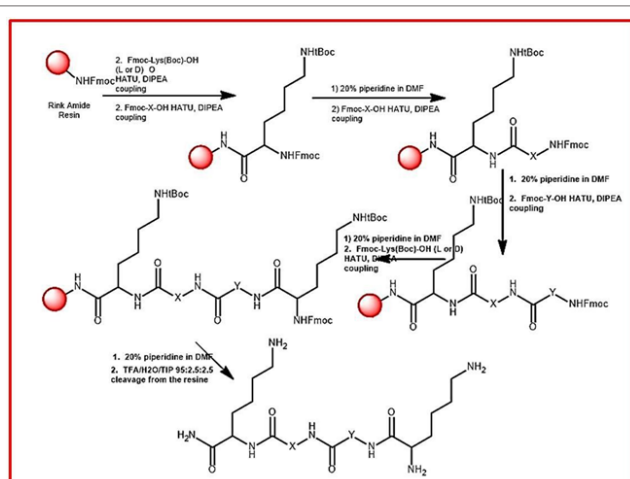


Figure 5: General synthesis of precursors to peptide surrogates on solid support

In Scheme 2 the solid supported synthesis of precursors is depicted (Figure 5,6).

We focused on the leucine (X) and the central leucine as conferring regions in P12 (see structure above): The magenta leucine will be replaced by Friedinger's lactam where as the central (green) leucine unit

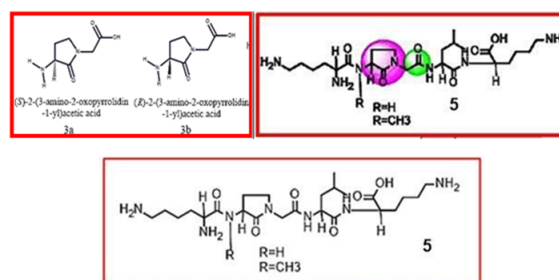
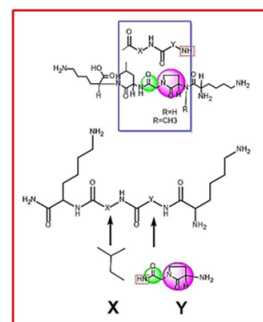


Figure 6: Focusing on the design of the Friedinger based surrogate of short peptide motif K-A-A-A-K

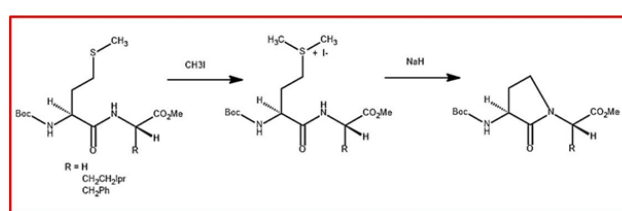


Figure 7: Synthesis of Friedinger Lactam and related compounds from methionine [12]

will be replaced by glycine. In this way we aim at both enantiomers of 5 (its two mirror images). The surrogate of p12.

The synthesis of Friedinger lactams [11]. The general scheme is depicted in this way it was possible to prepare the two amino acids mimic (magenta and green. See P12 previously) by the dipeptide surrogate 4. In the course of this synthesis, we observed some methylation of the amino group of the Friedinger lactam. On use of NaH in the methylation stage of the methionine unit, we could increase the yield of cyclization and isolate the byproduct. However, the design and the synthesis of the surrogates has been carried out by preparation of peptide precursors prior to the cyclization as follows (Figure 7,8).

Having the two enantiomers of N-methyl Friedinger lactams, and having in mind the publication of Gilon and Kessler and their co-workers [13] on the increased biological activity of N-methyl peptides, we embarked on the synthesis and evaluation of 32 and 33 as compared to the N-methylated 26 and 27 respectively. This N-methylation increased the biological activity in eradication of both Gram- and Gram+ by a factor of up to 10 (Table 1).

The R and S lactams were prepared according to literature procedure [5]. We have approached the synthesis of our target surrogates.

The Goals and importance of research were as follows to the study is a synthesis of hybrid peptide-mimetic new anti-bacterial peptide surrogates. These are based on Friedinger lactams and derivatives. The Multi-stage analog of cyclic structure based on the targets is based on the analogy to the solid supported preparation of benzodiazepine containing peptidomimetics [2]. We were aiming at compounds 4 and

5. This will provide a possibility to examine chirality on the two important biological features of 4 and 5 namely:

1. eradication of bacteria
2. Hemolysis of hRBSs.

Controls for Hemolysis

Hemolysis of Human Red Blood Cells (hRBC) reveals that the all (S) tetra peptide is very different from the others. It affects hemolysis to the extent of 14% where as in all other cases throughout this paper only very poor, up to 3% hRBC hemolysis, was obtained, although the eradication was very efficient (MIC) in the concentration range of 50-0.5 μM /ml.

Early studies using all D-enantiomers of native and model peptides demonstrated equivalent antimicrobial activities of D- and L- is forms. Thus, the prevailing dogma supported a non-receptor type interaction for antimicrobial peptides with most pathogen membranes [14,15]. Since then, several studies suggest there may be important exceptions to this generalization [17,18]. However, a number of studies have now shown non-equivalent activities for native all-L peptides, versus their all- D enantiomers [19] For example, in intriguing studies using PR-39, a proline-and arginine-rich peptide of porcine origin, the all-D enantiomer showed 1000- fold differences in species- specific activity against bacterial organisms [20]. These studies suggest receptor- type interactions may be important for some peptides in targeting specific epitopes on the microbial surface. However, AMPs

are practically cell-selective, and a great number of studies focused on the improvement of cell selectivity of AMPs were in the right direction. The results suggest that strong antimicrobial activity and less cytotoxicity can be achieved by increasing then positive charge of the peptide with minimal hydrophobicity above a threshold. This is consistent with the hypothesis that the lipid composition of cell surfaces primarily determines cell selectivity. The hydrophobicity effective lyresponsible for cytotoxicity is that on the hydrophobic face of the amphipathic secondary structure formed upon binding to the membrane. Residues close to the ends of a helix do not fully contribute to the effective hydrophobicity [21].

With this in mind, we have prepared a series of tetra-peptides in which two lysine rests flank 2 hydrophobic units. The MIC [20] results show that all are eradicating bacteria in 10^{-8} M concentration (Table 2).

We have synthesized and tested (MIC) tri-, tetra- and the above mentioned penta-peptides in which the central part of the amphipathic compounds consists of hydrophobic amino acids and this is flanked by two lysine units as mimics of the natural situation. In our hands, tri-peptides did show poor biocide activity. However, the tetra-peptides shown below were very potential eradication of both Gram+ (Staph. Auer.) as well as Gram- (*E.Coli*) bacteria in 10^{-7} - 10^{-9} M/ml concentrations. Similar to the natural CAMPs, only little (>5-3%) hRB Chemolysis is obtained on incubation of hRBCs (from healthy human blood).

Above table tetra-peptides are of “unnatural” configuration. The results indicate the following main features:

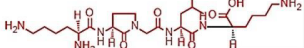
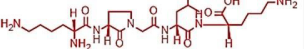
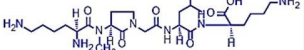
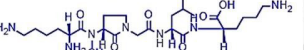
	STRUCTURE	MS	MIC RESULTS 10 ⁶ M		NRBC human Red Blood Cells Hemolysis
			COLI	STAF	
26			120	90	3%
27			100	100	12%
32			10	15	3%
33			15	20	17%

Table 1: Biological activity of surrogates based on Freidinger's lactams, N-methylated as compared to the N-methylated analogs, the R and S short peptide surrogates based on Freidinger's lactams

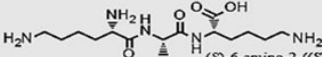
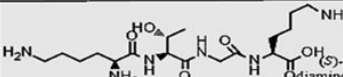
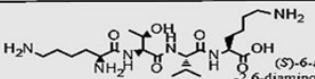
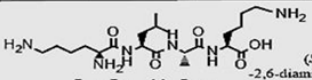
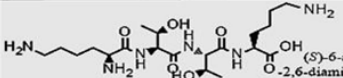
cpd	structure	MIC		hRBC %
		Coli	Aur	
1	 (S)-6-amino-2-((S)-2-(S)-2,6-diaminohexanamido)propanamido)hexanoic acid Lys-Ala-Lys	not active	14	
2	 (S)-6-amino-2-((2-(2S,3R)-2-((S)-2,6-diaminohexanamido)-3-hydroxybutanamido)acetamidohexanoic acid Lys-Thr-Gly-Lys	0.5µg/ml 10 ⁻⁷ - 10 ⁻⁸ M	<3	
3	 (S)-6-amino-2-((S)-2-((2S,3R)-2-((S)-2,6-diaminohexanamido)-3-hydroxybutanamido)-3-methylbutanamido)hexanoic acid Lys-Thr-Val-Lys	0.5µg/ml 10 ⁻⁷ - 10 ⁻⁸ M	<3	
4	 (S)-6-amino-2-((S)-2-((S)-2-((S)-2,6-diaminohexanamido)-4-methylpentanamido)propanamido)hexanoic acid Lys-Leu-Ala-Lys	0.5µg/ml 10 ⁻⁷ - 10 ⁻⁸ M	>8	
5	 (S)-6-amino-2-((2S,3R)-2-((2S,3R)-2-((S)-2,6-diaminohexanamido)-3-hydroxybutanamido)hexanoic acid Lys-Thr-Thr-Lys	0.5µg/ml 10 ⁻⁷ - 10 ⁻⁸ M	<3	

Table 2: structures and bactericidal activity (MIC) of retro-enantiomeric tetra peptides

1. Cell mortality is caused by the 2 flanking Lysine units that are connected to hydrophilic dipeptides constructed of 2 “unnatural” (R)-configured hydrophobic amino acids in the amphipathic short peptide.
2. The special arrangements of the structures of these units are not relevant as for the bacteria eradication process. Presumably, the special arrangement of bacterial receptors does not dictate any event in the course of action of the eradicating peptide. The mortality of these bacteria strands most likely takes its course by disintegration of the cell membrane.
3. It seems that the cationic ends of the biocide peptides are causing the membrane disruption. Katchalski, Volcani and collaborators reported the biocide activity of poly- flank. Unfortunately also causing blood cells agglutination [22]. Biocidalco-polymers have cationic amino acids likely sine to affect eradication of microbes [23].
4. We have recently showed that the distance between the flanking cationic moieties can vary from 50 nm to 110 nm without effecting considerable the bactericidal activity [24,25,26].

Al though bacteria eradication is the same with all enantiomers, hRBC hemolysis depending on the chirality. The general results with the tetra peptides were: The L enantiomer gave up to 13% hemolysis where as the DL and DD were as active as the L in bacterial eradication but gave only up to 3% hemolysis (figure 9).

Similarly, literature [8] report by German scientists focuses on the biological activity of short peptides, eradicates bacteria in all chiral forms. However, Compared to the all-LC8-lipidated lead sequence, diastereomeric peptides had very similar antibacterial properties, but were over 30 times less hemolytic (Table 2). They show that the observed hemolysis and antibacterial activity is affected by both differences in lipophilicity of the different peptides and specific combinations of L- and D- amino acid residues [24,25,26]. In our hands, the surrogates that we prepared and tested showed practically no hemolysis (>3%) of hRBC in unnatural diastereomeric chiral arrangements. It seems that the killing of bacteria and hemolysis of hRBC are following different routes, where as the hemolysis process involves at least one chiral sensitive event, the eradication is not involving any event sensitive to the special arrangement of the components involved. The fact that natural (all S) arrangement of the short peptides facilitates hemolysis may indicate

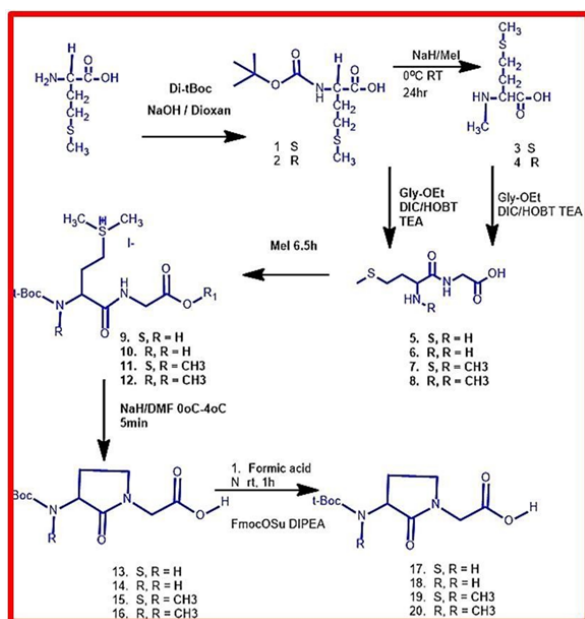


Figure 8: Synthesis of -NH₂ and NHMe Friediger lactams

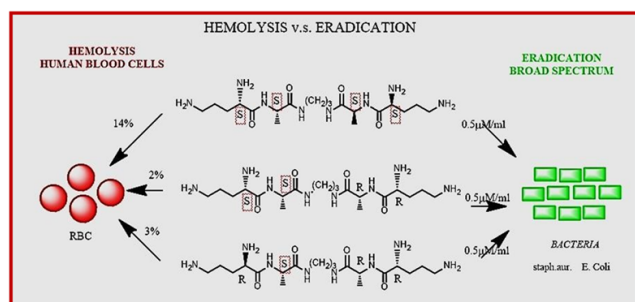


Figure 9: Some enantiomers of tetra-peptide (KR1R2K) in various special arrangement and their biological features in broad-spectrum eradication of bacteria and RB Chemolysis.

an arrangement with proteins on the cell membrane in the course of events bringing about disruption of the hRBC.

It is well known that eukaryotic cell membranes, in contrast to prokaryotic membranes, are generally characterized by zwitterionic phospholipids, a relatively large amount of cholesterol and sphingomyelin, and the absence of the high, inside negative trans membrane potential that is present in prokaryotic membranes [27,28]. Hence, if the peptides form pores/channels in the hydrophobic core of the eukaryotic bilayer, they would cause the hemolysis of erythrocytes; in contrast, for prokaryotic cells, the peptides lyse cells in a detergent-like mechanism [28], as described by the carpet mechanism [30]. Our "membrane discrimination" mechanism is consistent with the results of previous studies of model membranes, which demonstrated that the pore formation mechanism ("barrel-stave" mechanism) was used by antimicrobial peptides in zwitter ionic membranes, while a detergent-like mechanism ("carpet" mechanism) for the peptides was shown when the peptides interacted with negatively charged membranes [28,29,30]. Of course, model membrane studies do not address the complexity of the cell envelope and its contributions to the biological activity of these peptides.

Experimental Section

The peptides were prepared employing standard SPPS F-moc-strategy [31-36]. For the first coupling, the Rink Amide resin was preswelled in DCM/NMP 1:1, and treated with piperidine (20% in

DMF) at room temperature to remove the N- α -protecting group. The resin was further washed with DCM and NMP three times in each solvent. All peptide coupling reactions were performed with 3 equiv of amino acid, 3.5 equiv HATU in enough DMF to dissolve the reagents. The coupling was verified by Kaiser test on a small washed (NMP $\times$ 3, DCM $\times$ 3) and filtered sample of resin after each step. After final coupling, there in was washed (NMP $\times$ 5, DCM $\times$ 5, ether $\times$ 2) and dried.

Cleavage of the peptide from the resin was performed employing a mixture of TFA/TIS/H₂O (95:2.5:2.5) v/v/v which yielded 20-40 mg of 26-40 (90-95% by HPLC). α -protecting group. The resin was further washed with DCM and NMP three times in each solvent. All peptide coupling reactions were performed with 3 equiv of amino acid, 3.5 equiv HATU in enough DMF to dissolve the reagents. The coupling was verified by Kaiser test on a small washed (NMP $\times$ 3, DCM $\times$ 3) and filtered sample of resin after each step. After final coupling, the resin was washed (NMP $\times$ 5, DCM $\times$ 5, ether $\times$ 2) and dried. Cleavage of the peptide from the resin was performed employing a mixture of TFA/TIS/H₂O (95:2.5:2.5) v/v/v which yielded 20-40 mg of 26-30 (90-95% by HPLC).

Conclusions

Microbial infections still cause around one quarter of all the deaths worldwide [28], compounded by the increasing incidence of antibiotic-resistance, which is responsible for the majority of surgical site infections.

Infections are typically treated with oral or intravenous antibiotics. Treatment success relies on efficient penetration of drug from the blood into the tissue. Inhaled delivery of antibiotics directly into the infected airways could represent a more efficient means of achieving therapeutic concentrations at the infection site. Antimicrobial peptides (AMPs) are promising alternative to conventional antibiotics that can avoid problems such as antimicrobial resistance. However, their peptidic nature and subsequent enzymatic lability, high molecular weight and tissue binding represent challenges to their clinical development. In our research we developed low (MW ca, 600) molecular weight AMP surrogates that are efficient broad-spectrum antibiotics. Better understanding of the barriers to efficient AMP penetration into the target tissue, lung mucus for example, will expedite their development into the next-generation of antibiotic therapeutics.

This project involves the development of a triggered antibiotic delivery system that efficiently penetrates the mucus layer and attach membrane proteins turn mimics. Then delivers its antibiotic cargo selective to the site of infection. The project involves: 1) synthesis of peptide AMPs surrogates and b-turn mimics as linkers, 2) UV and fluorescence spectroscopic studies of peptide-membrane interactions and 3) microbiological testing against pathogens.

Therefore, new strategies for effective prevention and control are urgently required. In this regard, cationic antimicrobial peptide surrogates present numerous advantages as compared to antibiotics or antimicrobial peptides, e.g. synthetic peptides exert similar or even higher antimicrobial efficacy than natural host defense peptides. Although they present high efficacy and resistance to proteolysis, their preparation is still cumbersome and remains an obstacle to their wide spread application. However, mimics of antibacterial short peptide motifs containing quaternary ammonium cations in flanking position and relay on a β -turn mimic which are probably on the way to copy the biological series of events causing mortality of the microbes by the antimicrobial systems. They can be cost-effective if synthesized in large-scale production and with a broad diversity of targeted [14] antimicrobial ability.

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