Proline Rich Antimicrobial Peptides-----A tool to Counter Antimicrobial Resistance

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Abstract

Bacterial resistant to antibiotics is a global health problem that necessitates the production of new effective therapeutics. Antimicrobial peptides (AMPs) are commonly accepted as viable alternatives to currently used antibiotics. Some AMPs have the ability to work against not only planktonic bacteria but microbial biofilms as well. Proline rich antimicrobial peptides (PR-AMPs) are a distinctive group of AMPs, isolated in various animal sources, exhibiting an exceptionally high proline content. PR-AMPs function through non-lytic mechanisms and are generally regarded as non-toxic to mammalian cells. These peptides block bacterial protein translation by binding on and inactivating the ribosome. Proline, the only cyclic proteinogenic amino acid has the ability to undergo cis-trans isomerisation. When two different pyrrolidine ring conformations (exo and endo pucker) are adopted, these rotational isomers along the peptidyl-proline bond have different stabilities. Protein structure has ability to regulate itself either by preferring unique conformations and by improving hydrophobic interactions. Pseudoprolines (ΨPro) are mimetics of proline and function as molecular hinges. These WPro can be used for tailoring cis / trans isomerization around the Xaa- ΨPro amide bond (Xaa can be any amino acid) and hydrophobicity modulation.

1. INTRODUCTION

Antibiotics is a significant clinical success story in which, for nearly 70 years, pharmacy and chemistry have revolutionized bacterial treatment and thereby stopped bacterial epidemics. Despite this overwhelming success, the excessive global use of antibiotics has increased the evolutionary pressure on bacteria, promoting the generation of pathogens that are increasingly immune to antibiotics 1. In addition, most human infections are characterized by the presence of microorganisms in a biofilm state (microbial sessile group of cells that are irreversibly attached to a substratum and embedded in a matrix of extracellular polymeric substances formed by them) 2. Because of the physiological properties of the biofilm phenotype, bacteria in the populations become highly resistant to many standard antibiotics, displaying significantly higher levels of antibiotic resistance (up to 1000fold) than those usually found during planktonic (freefloating) development 2. Among various mechanisms for antibiotic resistance, the most common one is that of drugs based on β -lactam. β -lactam antibiotic resistance is due to β lactamases 3, i.e. enzymes which inactivate this class of antibiotics by opening the β -lactam ring. Extended-spectrum β -lactamases (ESBL) in *E. coli* and broad-range β -lactamases such as Klebsiella pneumoniae carbapenemase (KPC) 4-6 are significant causes of Gram-negative resistance. This health hazard has recently been partially overcome for Gram-positive pathogens such as MRSA 1, but there are growing concerns related to Gram-negative pathogens multi- or pan-resistance 7. Novel treatment options are therefore needed, using novel antimicrobials, preferably with new modes of action and/or belonging to new classes of drugs 8.

Keywords: Antimicrobial resistance, Antimicrobial peptides, Proline-rich antimicrobial peptides, Proline, *cis-trans* isomerization, pseudoproline

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Antimicrobial peptides (AMPs) are a promising class of compounds and an effective weapon against the ability of microorganisms that have developed resistance 9, because of their potency and different modes of action. Due to this reason, they possess weak cross reactivity towards human analogues 10. In addition, recent studies have also shown the ability of some AMPs to act not only against planktonic bacteria, but also against microbial biofilms, in particular during early phases of biofilm development 11, 12.

1. Antimicrobial Peptides (AMPs)

Antimicrobial peptides are a diverse group of molecules that are formed as a part of all multicellular organisms' innate immune response 13. AMPs have been reported that are potent against a wide range of micro-organisms, including Gram-positive and Gram-negative bacteria and fungi 14. Most AMPs are small peptides of 15-70 amino acid residues forme by post-translation processing of larger precursors, the synthesis of which is induced by the Toll and Toll-like receptors 15, 16. AMPs typically have a cationic character (net charge from +2 to +9) and an amphipathic structure containing around 50 percent hydrophobic residues 17. In addition to the importance of this cationic character in mediating initial interactions with the target membranes of gram-negative bacteria, the net positive charge is also essential for the so-called self-promoted uptake of antimicrobial peptides. Hydrophobicity is undoubtedly an unavoidable structural trait, influencing the possible interaction between antimicrobial peptides and different membrane compositions, and dictating the degree of peptide penetration into the lipid bilayer. Based on their structures or sequences, AMPs can be classified into many categories as α -helical, cysteine-rich, glycine-rich and proline-rich peptides 18.

2.1. Proline-Rich Antimicrobial Peptides (PR-AMPs)

In recent years, PR-AMPs have gained significant attention as a potential way of counteracting the rapid growth in bacterial resistance to traditional antibiotics 19, 20. Wide and heterogeneous group of small, medium-sized peptides are composed of proline-rich peptides, distinguished by proline residues, which often consist of unique sequences. The characteristic structure of the various biological functions of these molecules is determined by this feature.

PR-AMPs are distinguished by an uncommon proline residue content which is found to be very high in some cases (up to 70 percent of the whole sequence). PR-AMPs have been demonstrated to possess antimicrobial properties and are also known as modulators of *trans*duction signal and intermolecular interactions. Owing to their wide distribution and unusual mechanism of destroying bacteria without cell membrane disruption 21, the PR-AMPs expressed in mammals and insects have attracted special interest.

Proline-rich peptides typically share similar structural arrangements from a structural point of view; the presence of proline residues can induce a higher or lower tendency to assume a left-handed PPII helix conformation "all-trans." The formation of PPII helix is a folding process strongly affected by the steric interaction between proline and the residues immediately preceding it; arginine, glutamine and serine which can also function together to stabilize the helix of PPII 22. In backbone of PPII, all residues take dihedral angles of / or close to -75° (Φ) and $+146^{\circ}$ (Ψ) with all prolyl bonds in the form of trans isomerization, with three residues per direction. In comparison, a PPI helix occurs when the proline residues are in the all-cis conformation. The transition is most often energetically desirable in free peptides than in the *cis* form. The arrangement of the trans PPII tends to be balanced by the presence of water molecules and the sequences flanking proline residues 23.

PR-AMPs show low sequence homology, but are structurally homologous (proline content, one of many motives for Pro-Arg-Pro, disordered solution structure, relatively high proportion of specific amino acid residues) and tend to destroy bacteria by similar mechanisms 24.

The Pro-Arg-Pro or related motif-repeats help the entry into the host and ultimately into bacterial cells without the ability to destabilize the cells, and thus without showing eukaryote toxicity. Specialized *trans*porters *trans*port PR-AMPs into the cytoplasm, such as SbmA in Gram – ve bacteria 25, where they inhibit unique intracellular targets. In view of the absence of such *trans*port pathways in mammalian cells and the discovery of only small interactions with intracellular eukaryotic proteins, PR-AMPs are commonly considered to be non-toxic 25 and thus a desirable alternative to current antimicrobials.

Interestingly, some PR-AMPs may pass the blood brain barrier (BBB) to selectively target brain cells, further emphasizing their capacity for cerebral infection treatment or for the delivery of brain-specific drugs 26. High antimicrobial and minimal hemolytic activity of these peptides do not include just one factor but need a good balance between (i) peptide helicity, (ii) optimal core segment hydrophobicity, (iii) positive charges and their distribution, (iv) membrane dimerization and/or oligomerization, and (v) minimization of aggregation in aqueous solution 27.

2.1.2. Mode of Action

Initial attempts to identify bacterial targets for PR-AMPs led to the discovery of the prime candidate for inhibition of the heat shock protein DnaK 28. Short PRPs (18-20 aa) such as oncoin, drosocin, pyrrocoricin or apidaecin have previously been shown to interact stereo-specifically with the substratum-binding domain of chaperone DnaK, with dissociation constants in the micromolar range resulting in protein misfolding and aggregation, and subsequent bacterial death 29. However, the view has been questioned by two recent reports. Krizsan et al. 30 showed that apidaecin derivatives (Api88 and Api137) and oncocin derivatives (Onc72 and Onc112) inhibit bacterial protein translation by binding to (with Kd value in the nanomolar range) and inactivating the 70S ribosome, a property that has been shown to rely not only on cationic residues inside peptides, but also on a few conserved hydrophobic side chains. This result reflects a novel mode of action of antimicrobial peptides encoded by genes. Mardirossian, Grzela 31 also noted that a PR-AMP, Bac7 (1-35), by binding to the ribosome, inhibits protein synthesis.

Significantly Onc112(19 residue PRP) binds the 70S ribosome to about 50 times more than Dnak 30, making it the favored destination. Seefeldt, Nguyen 32, Roy, Lomakin 33 have recently reported Onc112 crystal structure linked to ribosome *Thermus thermophilus* 70S. Strikingly Onc112 peptide binds within the ribosomal exit tunnel and extends to the peptidyl *trans*ferase core where it overlaps with the aminoacyl-tRNA binding site. Biochemically it is shown that Onc112 binding blocks and destabilizes the initiation complex, thus preventing entry into the elongation phase 32.

The mechanism by which oncocin inhibit protein synthesis has been shown by these high-resolution structures. For other PR-AMPs including drosocin, pyrrhocoricin and apidaecin, which share many of the residues of Onc112, are essential for their ribosome binding and antimicrobial activity, this mechanism is likely to be the same. Several strategies for improved or even new antimicrobial products have been developed for the exit of the ribosome tunnel 32. The ability to generate peptide-based new scaffolds, such as Onc112, with powerful activity against various Gram-negative bacteria, provides an exciting way to grow potential antimicrobials.

2.2. AM-PRPs derived from Insects and Mammals

Much of the information about the structure-activity relationship for PR-AMPs comes from insect-derived peptides such as apidaecin, a small 18 amino acid residue peptide (GNNRPVYIPQPRPPHPRL) 35 isolated from honeybees, with a 33% 36 proline content, drosocin, a 19-mer glycopeptide (GKPRPYSPRPTSHPRPIRV) 37 isolated from Drosophila and pyrrrhocorycin, а glycopeptide (VDKGSYLPRPTPPRPIYYY) 38. Similarly, arasin-1 is also bactericidal in antimicrobial activity, as isolated in 37 amino acid long PR-AMP (SRWPSPGRPPGRPKPIFRPC1NC2YAPPC2PC1DRW) 39. Oncocin: is a novel 19 AM-PRP residue, active against Gram



Figure 1. (a) Model for Onc112(b) A relatively binding location for Onc112 on the ribosome in comparison with well-known chloramphenicol, clindamycin, tiamulin and erythromycin *trans*lation inhibitors, and the site-link A Phe-tRNAPhe 34.

-ve bacteria, showing "canonical" -PRP-antimicrobial motive (VDKPPYLPRPRPRRIYNR-NH2). It has the ability to traverse the BBB in very successful fashion. Penaeidins: penaeidins are expressed as precursor molecules composed of a signal peptide (19-21 residues) that precedes the mature bioactive peptide. They exert antimicrobial effects on Gram +ve, and -ve bacteria and fungicidal activity on filamentous fungi 40, 41. PR-bombesin: A member of this family isolated from the skin of the amphibians. Its sequence, H2N-EKKPPRPPQWAVGHFM, is characterized by the -PRPpattern 42, 43.

2.2.1. AM-PRPs in Mammals

Cathelicidins: AM-PRPs are also known as part of the cathelicidin family in mammals: a group of N-terminal and C-terminal peptides that have an antimicrobial activity, which is distinguished by different sequences and lengths 44. PR-39, belonging to this class, is a multifunctional peptide that exerts a heavy antimicrobial activity and participates in many other cellular processes such as chemo attraction, angiogenesis, apoptosis and inflammation 45. Other members of this family are the prophenins-1 and -2, two 79 residue-rich proline peptides, which differ for one residue and are active against Gram-negative bacteria 46.

2.2.2. PRPs as Cell Penetrating peptides

The natural cell penetrating peptides (CPPs) are also antimicrobial PRPs. These peptides can penetrate cells and target intracellular molecular partners without disrupting the external membrane and manipulate their biologic activities in are typically inactivated by extracellular efflux, as the cytosol. CPPs also may be used as cargoes for drugs that overexpressed p-glycoprotein is used to extract doxorubicin from the tumor cells. A good example of the promising supply of anticancer drugs is the acid amphipathic proline-rich peptide (Ac-CGGWVELPPPPPVELPPP-NH2) related to doxorubicin 10.

Modules of proline-rich cell penetration for antibacterial peptides in nature may be common. For example, the hydrophobic cathelicidin sequences show clear similarities with pyrrhocoricin, drosocin or apidecin C-terminal tails 47, 48. Hansen *et al.* stated that by ligation with the penetrating cell peptide (CPP), the activity range of insect derived PR-AMPs could be extended to Gram-positive bacteria. These constructions are able to enter mammalian cells and have only a marginal toxicity in comparison to native PR-AMPs 1.

2. UNIQUE FEATURES OF PROLINE

Because of the conformational restriction of backbone cyclization and the presence of a tertiary amide bond 49-54, proline residues are unique among canonical amino acids. On the other hand, an imide bond formed with the preceding residue (Xaa-Pro) is readily subject to *cis-trans* isomerisation, characterized by a higher population of *cis* (lower free energy difference Δ Gtc) as compared to other Xaa-Xaa bonds, and low activation energy (Δ G‡ct) of *cis* to *trans* bonds. Proline ring serves to intrinsically restrict its φ dihedral angle around -60 °C ± 15 55, 56.

The conformational limit and absence of a hydrogen bond donor contribute to preference in some structural contexts, including the termination of secondary structures, loops, turns and polyproline helices (PPII). Proline residues are also preferentially used in biomolecular identification on the basis of their differentiation from other canonical amino acids; the pyrrolidine ring's hydrophobicity and the potential for favorable aromatic-proline interactions 57 are important. Proline is central to protein folding 58-61, regulatory switches 62-64, antigens of peptides 65 and hormones of peptides 50. Proline has two key conformational equilibria, endo versus exo ring pucker in the side chain, and trans versus cis amide bond in the peptide backbone 66. Proline ring pucker correlates with protein Φ and Ψ main chain conformation, with an exo ring pucker favoring more compact conformations (PPII) and endo ring pucker favoring more extended conformations 67. Trans versus cis amide bond conformation defines the ω torsion angle, with the *cis* conformation preferring the endo ring pucker. Thus, control of proline ring pucker permits control of all protein backbone torsion angles (Φ, Ψ, ω) 68. Using substituted proline units is a simple way of manipulating the stability of peptides or proteins. A substituent bias the ring puckers and thus induces changes in the propensities of the trans / cis population 69



Figure 2. The side-chain conformation of the pyrrolidine ring of Pro is considered to be governed by the Cγ-envelope exo-endo pucker equilibrium. This conformational *trans*ition affects the *trans-cis* isomerization states of the Xaa-Pro amide bond as well as other dihedral angles in the peptide backbone.

3.1. Cis-trans Isomerism

As the energy difference of the ground-state between *trans* and *cis* forms is less than 4 kJ / mol where the *trans* shape remains somewhat preferred, it is often observed that Xaa-pro bonds have around 80% *trans* and 20% *cis* in an unfolded peptide chain. In Xaa-Pro bonds the *trans* conformer is favoured by an interaction between the neighboring carbonyl groups over the *cis* conformer. This $n \rightarrow \pi^*$ interaction involves the donation of electron density from the oxygen Oi-1 lone pair (n) into the π^* orbital of the adjacent carbonyl group (Ci=Oi) 70, 71. In addition, steric effects can further favor or disfavor the *trans* over the *cis* conformer 72-74.

3.1.1. Means to influence cis-trans Isomerism

Although, most peptide bonds occur in the *trans* geometry, those that include proline (Xaa-Pro) can occur in *cis* or *trans*. The conformation of proline residue with *cis* limits the conformation space of the peptide chain, which during folding and final native protein structure significantly alters the local protein chain path. Extraordinarily high *cis* amide content provides an easy and attractive way to restrict the selective bond of peptides, in particular for temporary incorporation of β -turns within the polypeptide chain. Isomerization of prolines is believed to restrict the rate of folding of protein 75. In order to produce ordered structures of the biologically active peptides, tertiary amide bond *cis / trans* isomerism is essential to control 76-78.





Nature has identified a family of prolyl-peptidylcis-transisomersases (PPIases), such as cyclophilins 49 and FKBPs 79, which are commonly used to catalyze in vivo 80 and in vitro peptidyl-prolyl bond cis-trans-isomerasis. PPIases 49 catalyzing peptidyl-Pro cis-trans isomerisation have also emerged as an important regulatory mechanism for cell growth and signaling and have been involved in cancer pathology, Alzheimer's disease, aging, asthma and microbial infection. Pro-cis-trans interconversion is normally a molecular change between protein-conformations and is used to control timing of protein-protein interactions in an enzyme-controlled way. The PPIases catalytic conversion of peptide bonds between phosphorylated residue (Ser or Thr), modulates the folding processes of a protein, has been shown to be able to control Phosphorylation 81. Environmental polarity affects trans: cis isomer ratio of Xaa-Pro bonds significantly. There is an intricate balance between polarity effects and $n \rightarrow \pi^*$ interactions of adjacent carbonyl groups. Polar solvents prefer the less polar trans conformer to the cis conformer. This is an example of how the trans: cis conformer ratio is affected by the environment and influences the conformational properties of peptides and proteins.

To change the conformational and functional properties of peptides and proteins, various mimetics, proline derivatives have been formed with electron-withdrawing, sterically demanding or H-bond donating substituents that benefit or disadvantage the trans over the cis conformer compared to unsubstituted proline residues 82-87, e.g. 3- and 4fluoroprolins are among the most widely used proline derivatives. The electron-withdrawing fluoro-substituent is known for a stereoelectronic gauche effect 88 that regulates the ring puckering of these derivatives. Incorporating a CF3group not only induces substantial steric demand and potentially modulates the pyrrolidine ring's electronic configuration, but it is also known to make template structures more lipophilic 72, which directly affects the role of the membrane-active peptides. Pseudo-prolines are mimetics of proline, which can be used to convert amide bond into cis conformation. While cis-Pro bonds are commonly found in unfolded proteins, a number of native globular protein structures 89, 90 have also reported cis-Xaa-Pro peptide bonds. A single cis peptide bond that occurs in the center of an otherwise all-trans backbone can dramatically alter the orientation of the conformation and the protein chain.

Pseudo-prolines

Pseudoprolines (Ψ Pro) serve as molecular hinges to induce *cis* amide bonds in the backbones of peptides. (Ψ Pro) mechanistically act by disrupting the secondary structure and increasing the solubilization of covered peptides 91, 92. (Ψ Pro) is a powerful instrument for investigating the effect of

ring substitution on imide bond isomerization. They offer a wide variety of applications, such as in drug and prodrug design based on peptides, molecular recognition studies, or processes of protein folding and self-aggregation. In the case of lactam-based systems which integrate a pseudoproline, the yield of cyclized peptide is recorded to increase and the cyclization reaction 91 is accelerated.

4.1. Oxazolidines And Thiazolidines Pseudoprolines

Serine, threonine derived oxazolidine-carboxylic acid and the cysteine-derived thiazolidine carboxylic acid, denoted as pseudo-proline ((Xaa[\PR1, R2Pro], Figure 4) 93. Variation of the C2-substituents within the heterocyclic system results in different physicochemical and conformational properties. C2-unsubstituted systems show a preference similar to that of the proline residue for the *trans* form, whereas 2,2-dimethylated derivatives adopt the *cis* amide conformation in high content. Ring opening under acidic conditions allows to reconstitute the regular all-*trans* peptide. For 2-monosubstituted \Pro, the *cis*-trans distribution depends on the C2-relative stereochemistry.



trans amide bond

cis amide bond

Figure 4. Amide rotamer equilibrium in N-acyl pseudoprolines (ΨPro)

The introduction of trifluoromethyl-group (CF3) at C2carbon of the oxazolidine ring of pseudoprolines strongly increases its stability in acidic medium and favor the *cis* amide conformation. This is mainly due to the strong electronwithdrawing effect of the CF3-group and its steric hindrance 93. Despite the deactivation of the nitrogen atom, these oxazolidines can be efficiently N-acylated to give stable pseudoproline-type structures as *cis/trans* conformers. CF3substituted pseudoprolines are reported to be hydrolytically stable proline surrogates 94.



4.2 δ-azaproline

 δ -azaproline, a five membered cyclic α -hydrazino acid, closely resembles proline in structure and ring size, the only difference being a nitrogen atom at the δ position instead of carbon 95-97.

δ-Azaproline, a new *bis*-nitrogen proline surrogate has been developed in order to control the conformation of Xaa-ΨPro bond. This bond adopts a *trans* conformation when Nδ-atom is protected with a carbamate protecting group (e.g. Boc, Cbz) and adopts *cis* conformation when Nδ-position is not protected. Thus δ-azaproline serves as a conformational switch of the protected or non-protected forms, which is a unique property of this proline 98.



trans amide bond

cis amide bond

Where PG = Boc, Cbz

Figure 6. Trans- or cis-amide conformational preference in δ -azaprolines can be switched by the use of a carbamate protection group.

4.3 δ-Oxaproline

These pseudoprolines has oxygen atom at the ω -position of the ring. This amino acid has been reported for use in chemoselective α -ketoacid hydroxylamine (KAHA) ligations. Karvonen *et al.* have reported that peptides containing 5-oxaproline act as specific syncatalytic inactivators of isolated prolyl 4-hydroxylase, the enzyme that catalyzes the formation of 4-hydroxyproline in collagens 99.

Shireman, Miller 100 has reported the conformations of representative acylnitroso-derived amino acid derivatives that exhibit elevated populations of the *trans* conformer.



3. CONCLUSION

The spread of multi-resistant bacterial pathogens presents a significant challenge to the global population in light of widely occurring drug-resistant infections in hospitals and in the environment. Therefore, there is an urgent need to look for new potent antimicrobial agents to cope with the evolving pathogen invasion while reducing the risk of induction of resistance in bacteria. Antimicrobial peptides (AMPs) are commonly accepted as viable alternatives to the antibiotics currently used. The ability to generate new peptide-based scaffolds, which show powerful activity against a variety of Gram-negative bacteria, is an exciting avenue for future antimicrobial growth. A new technique for increasing protein stability and/or behavior is the ability to regulate protein structure, either by preferring unique conformations and by enhanced hydrophobic interactions. Because of their

particular method of destroying bacteria, PR-AMPs are among the best alternatives to antibiotics currently available.

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