Dual-Action Antibiotic Peptides

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Targeting Intracellular Pathogenic Bacteria with Unnatural Proline-Rich Peptides: Coupling Antibacterial Activity with Macrophage Penetration**

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The emergence of drug-resistant bacteria is a growing challenge to anti-infective therapy. Pathogens such as methicillin- and vancomycin-resistant *Staphylococcus aureus* (MRSA and VRSA) have undermined most, and sometimes all, clinically usable antibacterial drugs.[1] Another challenge in the development of effective antibacterial agents arises from bacterial pathogens that have evolved to inhabit mammalian cells, such as phagocytic macrophages.[2] Bacteria, such as *Mycobacterium tuberculosis, Salmonella, Listeria*, and *Brucella*, reproduce within these host mammalian cells and form a repository that a significant subset of antibiotics, such as β-lactams and aminoglycosides, are unable to access.[3] These difficulties have spurred efforts to target intracellular pathogens using delivery vehicles containing antibiotics.[4]

Antimicrobial peptides (AMPs) are a class of antibiotics that generally act by targeting the microbial cell membrane, resulting in cell lysis.[5] This mechanism of action is also one of the major drawbacks associated with clinical use of AMPs owing to host cell toxicity. However, a small class of non-membrane lytic AMPs have been identified.[6] Unifying features of these non-membrane lytic AMPs, including bactenectin, PR-39, and drosocin, are a high proline content and an overall cationic charge owing to high levels of arginine. While proline-rich AMPs (P-AMPs) are less toxic than membrane-lytic AMPs, as seen in their lack of hemolytic activity and safety in animal models,[6] the majority do not enter mammalian cells,[7] with a few exceptions.[8]

AMP-inspired analogues have been widely explored.[9–12] We sought to interrogate the interplay of structure and function for P-AMPs with idealized, de novo designed sequences. At the same time, we wished to enhance the antibiotic activity of these proline-rich structures with an innate mammalian cell penetrating ability. Ultimately we sought to generate non-lytic antibacterial peptides with the ability to localize within mammalian cells, thereby providing agents that target elusive intracellular pathogenic bacteria.

P-AMPs have an abundance of proline residues (25–50%), and as a result have a propensity to adopt a polyproline type II helical (PPII) conformation.[6a,c] P-AMPs, such as PR-39 and bactenicin, contain triad repeats of PRP and RPP, respectively, often with hydrophobic amino acids.[6c] With these key features in mind, we designed a series of peptides starting with a sequence containing four copies of the PRP triad repeat found in PR-39, namely Fl-PRP-4 (Figure 1a), and a sequence with more cationic PRR triad (Fl-PRR-4) in an effort to improve mammalian cell uptake (Figure 1a). This latter sequence was further modified to contain all proline-based residues, including an unnatural amino acid containing a guanidinium group (P_R, Fl-PP_RP_R-4) to investigate possible links between activity and the ability to adopt a PPII conformation (Figure 1b,c). Finally, using PR-39 and bactenicin as models, we introduced additional hydrophobicity into the PP_RP_R design by replacing proline with an isobutyl-modified proline analogue (P_L) in two different length peptides Fl-$P_LP_RP_R$-3 and Fl-$P_LP_RP_R$-4 (Figure 1b). These latter three peptides were designed to possess a PPII scaffold such that every third amino acid along the peptide backbone would be aligned along the same face of the helix, resulting in amphiphilic structures (Figure 1c). Additionally, fluorescein (Fl) was incorporated at the N-terminus of each peptide, after a glycine spacer, to image cellular localization.

The peptides were synthesized using an Fmoc-based, solid-phase strategy on the Rink amide resin. The unnatural proline-based amino acids with the appropriate protecting groups were synthesized as previously described.[13] All of the peptides were purified to homogeneity by reverse-phase HPLC and characterized by MALDI mass spectrometry. Circular dichroism (CD) was used to evaluate the conformation of the designed peptides, as a PPII conformation displays a characteristic maximum in the CD spectrum at 225 nm.[14] The CD spectrum of Fl-PRP-4 exhibited a very weak maximum at 225 nm, while Fl-PRR-4 lacked the characteristic 225 nm maximum entirely (see the Supporting Information). Alternatively, Fl-PP_RP_R-4, Fl-$P_LP_RP_R$-3, and Fl-$P_LP_RP_R$-4 each displayed a strong peak at 225 nm, indicating that an all-proline backbone facilitated the formation of a PPII helix.

The antibacterial activity of the five peptides was explored with *E. coli* and *S. aureus*. The minimalistic PRP-based

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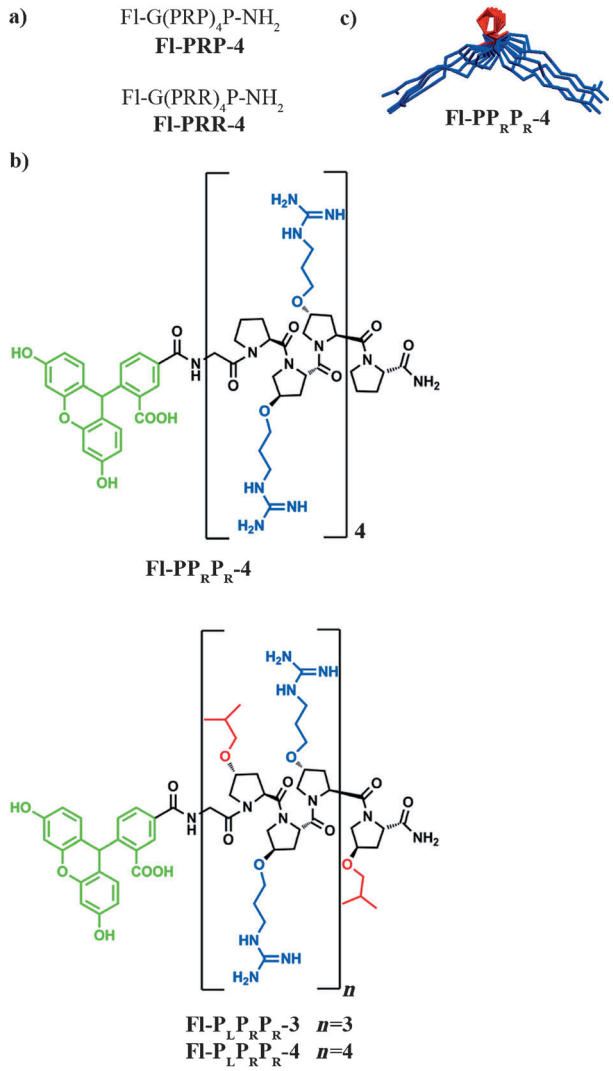

Figure 1. Structures of the idealized P-AMP sequences containing a) natural amino acids and b) unnatural proline-based residues P_R (blue modification) and P_L (red modification). c) Model of Fl-PP_RP_R-4 looking down the PPII helix (Pro in red, P_R in blue).

peptide (Fl-PRP-4) was not active against both bacterial strains up to a concentration of 100 μM. However, increasing the cationic character with the PRR-based peptide (Fl-PRR-4) led to modest but observable activity in both Gram-positive and -negative bacteria (Table 1). Electrostatic interactions between cationic AMPs and the negatively charged bacterial membrane are believed to be the first step in antibacterial action,[5] thereby explaining the improved antibacterial activity when going from Fl-PRP-4 (+4 charge) to Fl-PRR-4 (+8 charge). A similar trend was also observed for Fl-$P_LP_RP_R$-3 and Fl-$P_LP_RP_R$-4. Interestingly, restricting Fl-PRR-4 to a rigid PPII conformation had a detrimental effect on activity, as seen with Fl-PP_RP_R-4. However, the addition of hydrophobic isobutyl groups to the proline backbone of Fl-PP_RP_R-4 (Fl-$P_LP_RP_R$-4) resulted in a dramatic improvement in activity against both bacteria (Table 1). For *E. coli*, this data is comparable to the MIC for melittin (4.6 μM) and buforin II (1.6 μM), but significantly better than magainin II

Table 1: Antibacterial and hemolysis activity of designed P-AMPs.

	E. coli MIC[a] [μM]	*S. aureus* MIC [μM]	Hemolysis [μM]
Fl-PRP-4	>100	>100	>100
Fl-PRR-4	40	20	>100
Fl-PP_RP_R-4	60	>100	>100
Fl-$P_LP_RP_R$-3	60	60	>100
Fl-$P_LP_RP_R$-4	4	12	>100
Melittin	4.6[15]	2.1[15]	5[b]

[a] The minimum inhibitory concentration (MIC). [b] Greater than 80% hemolysis was observed at this concentration.

(40.5 μM).[15] Replacing the fluorescein moiety in this peptide with an acetyl group led to no discernible change in the antibacterial activity. The de novo designed proline-rich peptides also caused only low levels of damage to human red blood cells (hRBCs) (<5%) after 1 h of incubation up to a concentration of 100 μM (see Table 1 and the Supporting Information), whereas melittin was highly hemolytic at 5 μM. This lack of observed hemolysis with our designed P-AMPs is a crucial feature for potential in vivo applications. Fl-$P_LP_RP_R$-4 was therefore identified as the most potent, non-hemolytic antibacterial among our designed proline-rich peptides.

To rescue mammalian cells infected with intracellular bacteria, antimicrobial agents need to effectively penetrate within these host cells. We evaluated the cell penetrating ability of all five peptides by flow cytometry with J774A.1 macrophage cells. Macrophages are commonly invaded by intracellular bacteria and were thus selected for uptake studies. The three peptides (Fl-PRR-4, Fl-PP_RP_R-4, Fl-$P_LP_RP_R$-4) with the highest cationic charge (+8) showed significantly higher cellular fluorescence as compared to Fl-$P_LP_RP_R$-3 (+6) and Fl-PRP-4 (+4) (Figure 2).[16] The fluorescence associated with Fl-PP_RP_R-4 uptake, however, was significantly reduced with trypan blue, a dye used to quench external fluorescence that may be due to cell surface binding.[17] Among the most cationic peptides, there was approximately a four-fold increase in uptake in going from Fl-PRR-4 to Fl-$P_LP_RP_R$-4. The higher uptake of this structurally restricted peptide is in agreement with reports where rigidity

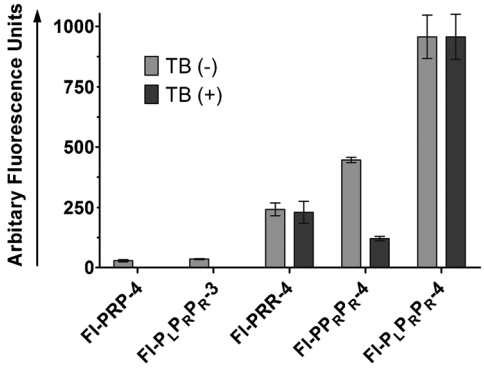

Figure 2. Cellular internalization of the designed P-AMPs. J774A.1 cells were incubated with the peptides (15 μM) for 1 h and analyzed by flow cytometry. Trypan blue (TB) was used to quench fluorescence of membrane-bound peptide.[17]

and an enforced PPII conformation, enhanced cellular internalization of arginine rich peptides.[13a,18] Additionally, the designed peptides were found to not compromise the integrity of the cellular membranes using propidium iodide (see the Supporting Information). Thus among the designed P-AMPs, Fl-$P_LP_RP_R$-4 was found to be most potently internalized within J774A.1 macrophage cells. Fl-$P_LP_RP_R$-4 was also found to satisfy two vital properties for application against intracellular bacteria: limited toxicity towards J774A.1 macrophage cells (at 15 μM) and resistance to proteolytic degradation by trypsin (see the Supporting Information).

Confocal microscopy was used to investigate the subcellular localization of Fl-$P_LP_RP_R$-4 within J774A.1 cells (Figure 3). We observed co-localization with each of the

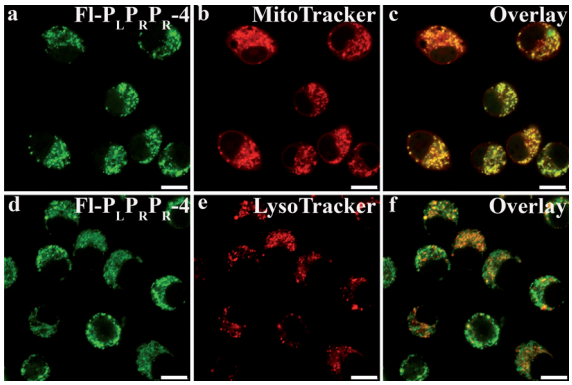

Figure 3. Confocal microscopy images of co-localization studies using Fl-$P_LP_RP_R$-4 (15 μM) and MitoTracker (a–c) or LysoTracker (d–f) within J774A.1 cells after 1 h. Scale bars: 10 μm.

tracking dyes and Fl-$P_LP_RP_R$-4 across a range of time points (1 h: Figure 3; 10 and 30 min: see the Supporting Information) pointing to association with both mitochondria and endosomes. Under these conditions, Fl-$P_LP_RP_R$-4 was found to have a limited influence on mitochndrial membrane depolarization (ca. 10%), whereas the known mitochondrial depolarizer FCCP showed strong depolarization (ca. 30%; see the Supporting Information). Arginine-rich cell-penetrating peptides and synthetic vectors are believed to internalize by endocytosis or direct translocation or a combination of both pathways depending on concentration, cell type, and cargo.[19] For instance, Fl-$P_LP_RP_R$-4 has previously been shown to preferentially localize with mitochondria or lysosomes in HeLa cells depending upon the concentration used.[20] The presence of Fl-$P_LP_RP_R$-4 in both endosomes and at the mitochondria of J774A.1 cells suggests that both mechanisms are at play. Crucially these data suggest that Fl-$P_LP_RP_R$-4 could access bacteria residing within infected J774A.1 cells while having a limited impact on mitochondrial function.

As Fl-$P_LP_RP_R$-4 displayed potent antibacterial activity and macrophage penetration, we proceeded to test this peptide against a range of bacterial pathogens. Fl-$P_LP_RP_R$-4 has potent broad-spectrum antibacterial activity (Table 2), including MRSA (8 μM) as well as the biological warfare agents *Bacillus anthra*cis (8 μM) and *Brucella* (16 μM). Also of interest was the activity against the biofilm-forming clinical-

Table 2: Antibacterial activity of Fl-$P_LP_RP_R$-4 against pathogenic bacteria.

Pathogen	MIC[a]/MBC[b] [μM]
Gram positive	
*Bacillus anthra*cis	8/8
S. aureus	8/32
methicillin-resistant S. aureus (MRSA)	8/16
Listeria monocytogenes	16/32
Gram negative	
Salmonella typhimurium	8/8
Brucella abortus	16/32
Pseudomonas aeruginosa	32/32

[a] The minimum inhibitor concentration (MIC) was the lowest concentration of the peptide where no growth was observed. [b] The minimum bactericidal concentration (MBC) was reported as the lowest concentration of the peptide producing more than 99.9% reduction of the initial inoculum.

isolates of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Fl-$P_LP_RP_R$-4 was further shown to inhibit the growth of *Salmonella, Listeria*, and *Brucella* at 8, 16, and 16 μM respectively, which are all classified as intracellular pathogens.

AMPs have been shown to often exert their antimicrobial activity by lysing the bacterial membrane. For instance, *E. coli* treated with melittin has been reported to rapidly release β-galactosidase as a consequence of membrane lysis. To test the effect of Fl-$P_LP_RP_R$-4 on the membrane integrity of *E. coli*, we performed the β-galactosidase leakage assay, with melittin as a positive control.[9c,21] In contrast to melittin, Fl-$P_LP_RP_R$-4 showed no β-galactosidase leakage at both its MIC (4 μM) as well as 5 times its MIC (20 μM). These data support a mechanism of antibacterial action for Fl-$P_LP_RP_R$-4 that does not involve lysing the bacterial membrane. Fl-$P_LP_RP_R$-4 was found to associate with *E. coli*, as visualized by confocal microscopy.[22] Strong fluorescence was visible within 10 min of treatment with Fl-$P_LP_RP_R$-4, and after 6 hours the bacteria displayed a fragmented morphology, pointing to a possible mode of antibacterial action (see the Supporting Information).

With a knowledge of the separate cell penetrating and antibacterial activities of Fl-$P_LP_RP_R$-4, we investigated the ability of this P-AMP to clear intracellular bacterial pathogens, *Salmonella* and *Brucella*, within J774A.1 cells using an in vitro bacterial protection assay (Figure 4).[23] While intra-

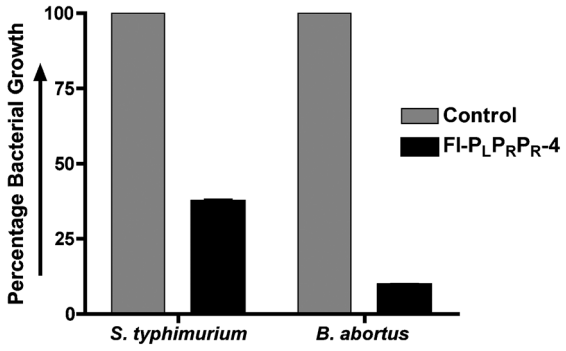

Figure 4. Intracellular antibacterial activity of Fl-$P_LP_RP_R$-4 (15 μM) in J774A.1 cells infected with *Salmonella typhimurium* and *Brucella abortus* after 9 h of treatment. The data without error bars indicate that the standard deviation is too small to be seen.

cellular S*almonella* was reduced by approximately 62% with the addition of Fl-$P_LP_RP_R$-4, intracellular *Brucella* was found to be significantly reduced by 90%. The difference in inhibition between *Salmonella* and *Brucella* could be the result of many factors, such as the sub-cellular localization of *Salmonella* and compartmentalization within phagosomes.

Overcoming bacterial resistance will necessitate the continuous development of therapeutic agents that also target intracellular bacterial reservoirs, so as to avoid recurrence and emergence of resistance. The substantial reduction in intracellular pathogen levels observed upon treatment with Fl-$P_LP_RP_R$-4 provides strong evidence for its potential application for treatment of stubborn intracellular bacterial pathogens.

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