Bioorganic & Medicinal Chemistry Letters 24 (2014) 556–559

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Dimeric unnatural polyproline-rich peptides with enhanced antibacterial activity

Victor Hernandez-Gordillo, Iris Geisler, Jean Chmielewski *

Department of Chemistry, Purdue University, 560 Oval Drive, West Lafayette, IN 47901, USA

ARTICLE INFO

Article history:
Received 23 October 2013
Revised 4 December 2013
Accepted 4 December 2013
Available online 11 December 2013

Keywords:
Antimicrobial peptide
Proline-rich
Dimerization
Non-lytic

ABSTRACT

We report a dimerization strategy to enhance the antibacterial potency of an otherwise weak cationic amphiphilic polyproline helical (CAPH) peptide. Overall, the dimeric CAPHs were more active against *Escherichia coli* and *Staphylococcus aureus* than the monomeric counterpart, reaching up to a 60-fold increase in potency. At their minimum inhibitory concentration (MIC), the dimeric peptides demonstrated no hemolytic activity or bacterial membrane disruption as monitored by β-galactosidase release in *E. coli*. At higher concentrations the dimeric agents were found to induce β-galactosidase release, but maintained negligible hemolytic activity, pointing to a potential shift in the mechanism of action at higher concentrations. Thus, discontinuous dimerization of an unnatural proline-rich peptide was a successful strategy to create potent de novo antibacterial peptides without membrane lysis.

The difficulty in treating multidrug resistant and extensively drug resistant bacteria is a profound problem worldwide.[1–6] Pathogens resistant to the 'drugs of last resort' such as carbapenem-resistant *Enterobacteriaceae* (CRE), and methicillin- and vancomycin-resistant *Staphylococcus aureus* (MRSA, VRSA) have undermined almost all usable antibiotics.[7–9] Particularly worrisome is the fact that no new antibacterial drugs have been approved in recent years,[10] and newly designed antibacterials, such as the boron-based class of aminomethyl benzoxaboroles intended to target CRE, have failed in clinical trials due to the appearance of resistant bacteria.[11] Therefore, the need for new drugs for the treatment of pathogenic bacteria is of a very high priority.

Antimicrobial peptides (AMPs) are a diverse group of molecules found in virtually all living organisms.[12–15] The majority of AMPs target the bacterial membrane, which leads to rapid bacterial cell death.[16–18] A subset of AMPs with a high content of proline residues acts through a non-membrane lytic mechanism.[13,17,19,20] These proline-rich AMPs (P-AMPs) are less toxic to mammalian cells, have reduced hemolytic activity, and have shown limited toxicity in animal models[19–21] when compared to membrane lytic AMPs.[18,22] Thus, P-AMPs are preferred for drug development as anti-infective agents.[23,24]

P-AMPs such as PR-39, pyrrhocoricin, drosocin, and the synthetic A3-APO peptides contain consensus amino acid triad repeats of PRP or RPP. This high content of proline residues is responsible for the tendency of P-AMPs to adopt a polyproline type II (PPII) helix.[25–27] Arginine (R) residues in the motif provide the peptides with an overall cationic character.[19,28] Inspired by these motifs, we recently reported the design of an unnatural polyproline-rich peptide (**FL-$P_LP_RP_R$-4**), with broad-spectrum activity against Gram-positive and Gram-negative bacteria.[29] **FL-$P_LP_RP_R$-4** is composed of repeating units of modified proline residues containing either hydrophobic isobutyl groups (P_L) or positively charged guanidinium groups (P_R). The peptide forms a cationic amphiphilic PPII helix (CAPH) with a hydrophobic face composed of five isobutyl groups, and a hydrophilic face, composed of eight guanidinium groups (Fig. 1a).[29] Interestingly, a shorter CAPH (**FL-$P_LP_RP_R$-3**), lacking one $P_LP_RP_R$ repeat (Fig. 1a), demonstrated significantly diminished antimicrobial activity as compared to **FL-$P_LP_RP_R$-4** against *Escherichia coli* and *S. aureus* bacteria (Table 1). The difference in antibacterial potency was hypothesized to be due to the lower number of positively charged residues in the **FL-$P_LP_RP_R$-3** peptide, a feature that limited interactions with bacteria.

Dimerization has proven to be a successful strategy to enhance the antibacterial activity of naturally occurring AMPs and AMP-inspired analogues.[30–33] For instance, pyrrhocoricin dimers are more active against Gram-positive bacteria than the monomeric counterpart.[34] Likewise the P-AMP-inspired A3-APO dimeric peptide is highly active against clinically isolated pathogenic bacteria and has shown the potential to clear bacterial infection in various animal models.[21,35–37] The increased positive charge within the dimers is believed to contribute to the improved potency against bacteria.[30] However, linear continuous dimers are less active than branched dimers sharing the same number of positive charges, suggesting that branched dimerization strategies may be more beneficial to increase the antimicrobial activity of a given AMP.[38] Thus, we wished to create a potent unnatural antimicrobial

* Corresponding author.
E-mail address: chml@purdue.edu (J. Chmielewski).

0960-894X/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmcl.2013.12.023

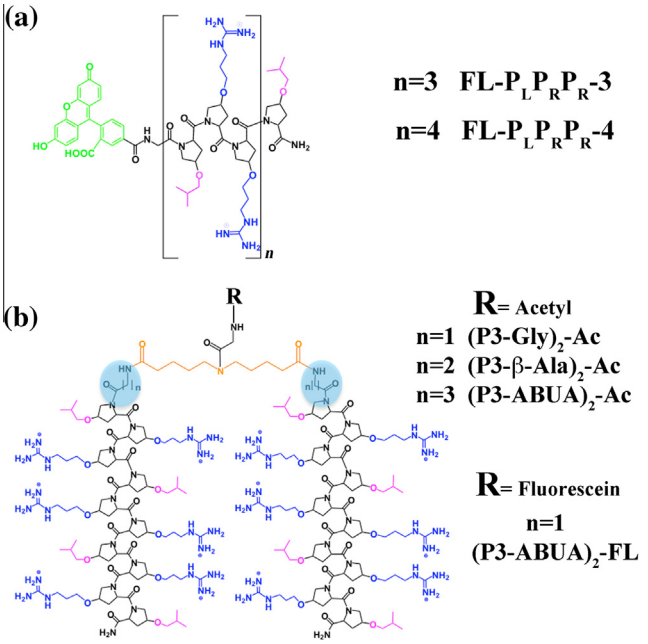

Figure 1. Structure of the cationic amphiphilic polyproline helices (CAPHs) composed of unnatural proline-based amino acids in (a) monomeric and (b) dimeric forms. Hydrophilic residues are shown in blue and hydrophobic residues are shown in pink. The fluorescein moiety is depicted in green, the spacers are highlighted with a blue circle and the linker is shown in orange.

Table 1
Antibacterial activity of designed CAPHs

Peptide	*E. coli* (MIC[a]) [μM]	*S. aureus* MIC [μM]
FL-P$_L$P$_R$P$_R$-3	60[29]	60[29]
(P3-Gly)$_2$-Ac	1	2
(P3-β-Ala)$_2$-Ac	4	8
(P3-ABUA)$_2$-Ac	1	4
FL-P$_L$P$_R$P$_R$-4	4[29]	12[29]
Melittin	4.6[42]	2.1[42]

[a] The minimum inhibitory concentration (MIC).

agent by covalently linking the amino-termini of two monomeric AMPs in a branched fashion (Fig. 1b). Herein, we describe such a dimerization of **P$_L$P$_R$P$_R$-3** to probe whether this modification increases the antibacterial activity of an otherwise weakly active AMP.

The **P$_L$P$_R$P$_R$-3** peptide was dimerized at the N-terminus with a dicarboxylic acid linking moiety and an intervening amino acid (glycine, β-alanine and aminobutyric acid—ABUA) to adjust the length of the spacer (Fig. 1b). The crosslinker was designed so as to allow association between the hydrophobic faces of the two **P$_L$P$_R$P$_R$-3** peptides. The linker also included an internal secondary amine to allow for a point of attachment for fluorescein (Fig. 1b).[39] The synthesis of monomeric and dimeric **P$_L$P$_R$P$_R$-3** based peptides was carried out with unnatural proline-based, Fmoc-protected amino acids using a solid-phase strategy as previously described.[39,40] The peptides were purified to homogeneity by reverse-phase HPLC and characterized by MALDI mass spectrometry.[39] In our initial studies the secondary amine in the linking moiety was acetylated, resulting in dimeric CAPHs (**P3-Gly**)$_2$-**Ac**, (**P3-β-Ala**)$_2$-**Ac** and (**P3-ABUA**)$_2$-**Ac** (Fig. 1b).

The antibacterial activity of the dimeric and monomeric CAPHs was explored against *E. coli* and *S. aureus* bacteria, using a broth micro-dilution assay.[41] The antibacterial activity of **FL-P$_L$P$_R$P$_R$-3** against both bacteria was low, with a MIC of 60 μM, as reported previously.[29] In contrast, the dimeric peptides were highly active against both bacteria, reaching a 15- to 60-fold increase in potency with *E. coli* and a 7.5- to 30-fold increase in potency with *S. aureus* as compared to their monomeric counterpart (Table 1). When compared to the membrane lytic peptide, melittin, the (**P3-Gly**)$_2$-**Ac** and (**P3-ABUA**)$_2$-**Ac** dimers were 4-fold more potent against *E. coli*, while (**P3-β-Ala**)$_2$-**Ac** was equipotent with melittin. Overall, the dimers were more potent against *E. coli* than *S. aureus*, a trend that has been previously observed with P-AMPs—greater activity against Gram-negative than Gram-positive bacteria.[19] Although there was no clear trend in potency versus the length of the dimer spacer—shorter (Gly) and longer (ABUA) spacers were equipotent—there was a significant improvement in activity of the dimers (+12) when compared to the monomeric **FL-P$_L$P$_R$P$_R$-3** (+6) and **FL-P$_L$P$_R$P$_R$-4** (+8) peptides. Thus increasing the cationic character by means of dimerization led to an increase in potency. Most likely the increase in the positive charge increases the attraction between the negatively charged bacteria and the dimeric CAPHs, which is the first step in the mechanism of AMP-mediated bacteria toxicity.[17]

A critical feature in the design of AMPs is the ability to preferentially target bacteria without affecting the viability of mammalian cells. To test the safety of the designed dimeric peptides, we performed a hemolysis assay with human red blood cells (hRBCs) and measured the release of hemoglobin upon incubation with the peptides. hRBCs were incubated with the dimeric peptides at varying concentrations. Melittin and 1% Triton X-100 were used as positive controls.[43] As expected, melittin and Triton X-100 caused hRBCs lysis and hemoglobin release (Fig. 2), with melittin inducing hemolysis at the lowest concentration tested (1 μM). In contrast, negligible hemolytic activity was observed with the designed dimeric peptides at concentrations up to 32 μM. Thus, the dimeric peptides had an increase in potency in antimicrobial activity without causing damage to hRBCs. These dimeric peptides have also been reported to have minimal toxicity against MCF7 cells.[39]

AMPs that kill bacteria through membrane disruption have been shown to cause leakage of cytosolic β-galactosidase in *E. coli*. For instance melittin, a well-know membrane lytic AMP, promotes leakage of β-galactosidase as a consequence of membrane lysis.[44,45] To gain further insights into the possible mechanism of antibacterial activity of the dimeric CAPHs, we treated *E. coli* with the dimers and evaluated the leakage of intracellular β-galactosidase (Fig. 3).[46] As expected, melittin caused an 8-fold in-

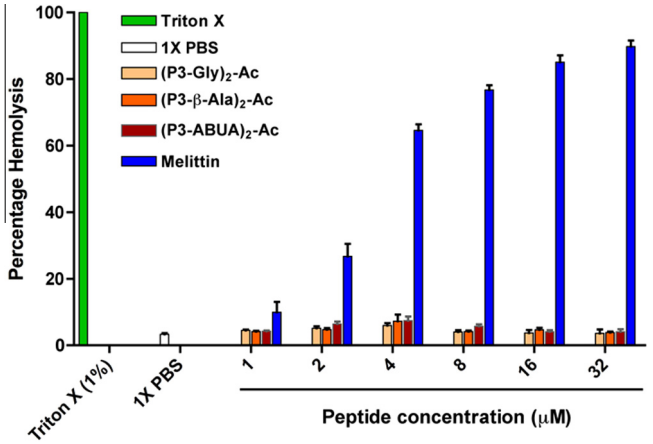

Figure 2. Hemolytic activity of the designed dimeric CAPHs and controls. hRBC were incubated with the peptides or control for 1 h and analyzed for the hemoglobin release at OD_{405}. The data represent the average of two independent experiments.