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Development of novel membrane active lipidated peptidomimetics active against drug resistant clinical isolates

Sandeep Lohan [a], Arneesh Kalanta [a], Praveen Sonkusre [b], Swaranjit Singh Cameotra [b], Gopal Singh Bisht [a,*]

[a] Department of Pharmacy, Jaypee University of Information Technology, Solan 173234, India
[b] Environmental Biotechnology & Microbial Biochemistry, Institute of Microbial Technology, Chandigarh 160036, India

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ABSTRACT

A new series of small cationic lipidated peptidomimetics have been synthesized and found to be highly active against several susceptible as well as drug resistant clinical isolates of bacteria and fungi. All lipidated peptidomimetics do not cause significant lysis of human erythrocytes (HC_{50} > 200 μg/mL). Calcein dye leakage experiment revealed membranolytic effect of LPEP08 which was further confirmed by scanning electron microscopy (SEM). The involvement of intracellular targets as an alternate mode of action was precluded by DNA retardation assay. Additionally, LPEP08 exhibit high proteolytic stability and dose not elicit resistance against drug resistant clinical isolate of *Staphylococcus aureus*, even after 16 rounds of passaging. These results demonstrate the potential of lipidated peptidomimetics as biocompatible anti-infective therapeutics.

1. Introduction

The dramatically increased frequency of infections caused by multi-drug resistant bacterial and opportunistic fungal strains has driven research to expand the arsenal of anti-infective agents. Recently, WHO has recognized the infection caused by multidrug-resistant pathogens viz. methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant *Staphylococcus aureus* (VRSA), drug resistant *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* as major causes of morbidity and mortality.[1,2] In addition, majority of the invasive fungal infections caused by *Aspergillus* and *Candida* species emerged as major threat to public health.[3] These trends have accentuate to develop new class of antibiotics possessing novel mode of action as well as different cellular targets compared to conventional antibiotics in order to decrease the possibility of resistance development.

Antimicrobial peptides (AMPs) are found in virtually all multicellular organisms and functionally act as weapons to ward off pathogenic microbes in order to survive and thrive on this planet.[4,5] In general, AMPs are typically composed of 20–50 amino acid residues and carrying a net positive charge (provided by Arg and Lys residues) with ≈50% hydrophobic residues. The mode of action of AMPs is of particular interest, as it is thought to be non-specific unlike conventional antibiotic drugs (usually directed against a specific cellular receptor).[6,7] Mechanically majority of AMPs are bind and permeate cell membranes and others have found to modulate the immune response or have targets within the cell. Taking these findings together, AMPs display unique mode of action that could not deriving the development of resistance.[8,9]

Lipopeptides constitute another class of native AMPs, which are produced non-ribosomally in bacteria and fungi during cultivation on various carbon sources.[10–12] Structurally, native lipopeptides are complex molecules composed of aliphatic acid attached to the N-terminus of cationic or anionic peptidic moiety.[13] The mode of lytic action of lipopeptides is via perturbation of the cell membrane by unknown mechanisms which is similar to most of the AMPs.[14,15] Mechanistically electrostatic interaction between cationic lipopeptides and negative membrane surface charge of bacteria is the initial step of their bactericidal activity. On the other hand in the fungi lipopeptides bind to the negatively charged membrane phosphatidylinositol (PI) and to the negatively charged terminal sialic acid moieties.[16,17] Clinically used members of this novel class of antimicrobials includes daptomycin (active only toward Gram-positive bacteria), polymyxin B (active only toward Gram-negative bacteria), and echinocandins (β-1,3-D-glucan synthase inhibitors; active only toward fungi).[18] The major drawback associated with this class of antimicrobials is that the toxic dose is close to the therapeutic dose.[18]

It is well documented that conjugation of linear fatty acids to small cationic AMPs resulted into enhancement of antimicrobial potential against Gram-negative and Gram-positive bacteria.[19–21]

* Corresponding author. Tel.: +91 1792 239336; fax: +91 1792 245362.
E-mail address: bishtchem2005@gmail.com (G.S. Bisht).

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In addition, Chongsiriwatana et al. demonstrated that lipidation of small peptoid sequences renders them more selective towards microbes without losing antimicrobial activity.[22] The aliphatic tail region of lipopeptides was found to be essential for their antimicrobial action as it may improves the hydrophobic interaction with cytoplasmic membrane.[23] Thus, the development of small synthetic congeners which mimics integral structural features may overcome some of the drawbacks associated with current lipopeptide antibiotics.

Herein, we reported a new series of small cationic lipidated peptidomimetics synthesized by incorporating 3-amino benzoic acid (3-ABA) as a turn motif between peptidic and aliphatic tail region of the molecules. We confirmed the broad-spectrum antimicrobial potential and low hemolytic action of lipidated peptidomimetics. For an initial investigation of bactericidal mechanism of the lead molecules, calcein leakage experiments on model membranes were performed and which was complemented by observing their effect on intact cells. Finally, the proteolytic stability even in human blood plasma and inability of drug resistant *S. aureus* to develop resistance aid in the future development of small lipidated peptidomimetics to treat fatal infections.

2. Results and discussion

2.1. Design and synthesis

In a drug development perspective, proteolytic enzyme susceptibility is one of the biggest issues associated with the clinical applicability of peptide based therapeutics. Thus, with aim to develop novel potent biocompatible anti-infective agents, we carried out the synthesis of lipidated peptidomimetics using non-natural amino acid ornithine (Orn) as cationic charge residue. To synthesize lipidated molecules, small peptide scaffold composed of 3 and 4 Orn residues were acylated with fatty acid tail varying from 10 to 18 carbon atoms. It is well known fact about AMPs that the antimicrobial activity is somewhat depends on their secondary structure. For that reason, incorporation of any suitable moiety in the molecular framework which provides a specific turn might boost up the antimicrobial action. The incorporation of constrained aromatic amino acid (3-ABA) in peptide sequence resulted into the improvement of stability of folded conformation[24,25] as well as antimicrobial potential.[26] On the basis of these findings, we incorporated 3-ABA as a linker between peptide and hydrophobic tail region (Scheme 1).

2.2. Antimicrobial activity

All synthesized lipidated peptidomimetics were screened against representative Gram-positive and Gram-negative bacteria and fungi, including antibiotic resistant clinical isolates. Lipidated peptidomimetics composed of 3 Orn residues and 10 carbon atoms long aliphatic tail (LPEP01) was found to be almost inactive as antimicrobial. LPEP01 exhibits MIC > 50 μg/mL against all tested pathogens with an exception of having MIC = 31.5 μg/mL against *S. aureus* MTCC 3160 (Table 1). A closer examination of the activity results revealed that lipopeptides with aliphatic chain length ranging from 12 to 18 carbon atoms showed improved antimicrobial activity. Among the lipidated peptidomimetics composed of 3 Orn residues (LPEP01-LPEP05), highest activity was observed in case of lipopeptide having N-terminus myristic acid (LPEP03) with MIC values of 2.5 μg/mL for *Escherichia coli* and *P. aeruginosa* and 3.1 μg/mL for *S. aureus*. In addition, LPEP03 showed good antibacterial activity against antibiotic resistant clinical isolates of *E. coli* (MIC = 4 μg/mL) and *S. aureus* (MIC = 4.5 μg/mL). LPEP03 display moderate activity against *Bacillus subtilis* with MIC = 15.5 μg/mL. Further increase in the length of aliphatic tail resulted into decrease in antibacterial potential (LPEP04 & LPEP05; Table 1). LPEP06, composed of 4 Orn residues and 10 carbon atoms long aliphatic tail, displayed moderate activity (MIC values in the range of 17.5–35 μg/mL) against all tested bacterial strains with an exception of having minimum killing effect against *B. subtilis* (MIC = 100 μg/mL; Table 1). Similar impact of aliphatic chain length on antibacterial potential was observed in case of lipidated peptidomimetics comprised of 4 Orn residues as significant improvement in antibacterial activity was observed for lipidated peptidomimetics bearing bulky aliphatic tail. LPEP08 exhibits maximum antibacterial potential with MIC values of 1.5 μg/mL for *E. coli* and *S. aureus* and 2 μg/mL for *P. aeruginosa* (Table 1). Further increment in aliphatic tail was not fruitful as somewhat decrease in activity was observed in case of lipopeptides (LPEP09 & LPEP10) having 16 and 18 carbon atoms long aliphatic tail. It was interesting to note that all synthesized lipidated peptidomimetics exhibit broad antibacterial activity spectrum with insignificant difference between MIC values against susceptible pathogens and drug resistant clinical isolates.

Lipidated peptidomimetics with comparatively bulky aliphatic tail were found to be more active towards fungal strains. LPEP10 has highest antifungal activity with MIC values of 1.5 μg/mL against *Candida albican* and *Aspergillus fumigatus*, 2.5 μg/mL for *A. niger*, and 5.5 μg/mL for *Cryptococcus neoformans*. It was encouraging to observe similar antifungal potential of LPEP10 against drug resistant clinical isolates of *C. albican* (MIC = 1.5 μg/mL) and *A. fumigatus* (MIC = 2 μg/mL; Table 2).

Analyzing the antimicrobial activity results of lipidated peptidomimetics, we conclude that activity is depends on the content of both cationic charge and hydrophobic bulk. Importantly, in contrast to the most AMPs or natural lipopeptides that are active either against bacteria or fungi alone,[27] lipidated peptidomimetics reported here are highly potent against both bacteria and fungi. The broad-spectrum antimicrobial potential of this library of compounds reflected their candidature to develop as novel anti-infective agents.

2.3. Hemolytic activity

All synthesized lipidated peptidomimetics displayed batter selectivity towards microbial cells (HC_{50} > 200 μg/mL) as summarized in Table 1. Lipidated peptidomimetics with bulky aliphatic tail (16 & 18 carbon atoms long aliphatic tail) showed higher affinity towards hRBC as compared to the molecules bearing small fatty acid chain (10, 12, and 14 carbon atoms long aliphatic tail). Thus, it seems that, as the length of aliphatic tail increases, the ability of lipidated peptidomimetics to discriminate between anionic bacterial surface and zwitterionic mammalian membrane decreases. These outcomes were in accordance with our earlier findings.[28] Noticeably, most potent antibacterial lipidated peptidomimetic (LPEP08) has significantly wider therapeutic index (SR = 333), which we defined as $HC_{50}/MIC_{E.c}$ and $HC_{50}/MIC_{S.a}$ (Table 1).

2.4. Bactericidal kinetic assay

In contrast to the majority of the conventional antibiotics, AMPs are usually bactericidal, rather than bacteriostatic.[29] To determine whether this ability is inherent to newly synthesized lipidated peptidomimetics we performed time-kill assay by exposing *E. coli* and *S. aureus* to various concentrations of LPEP08.The results clearly showed that LPEP08 was bactericidal at 4 × MIC and 8 × MIC against both *E. coli* (Fig. 1A) and *S. aureus* (Fig. 1B). At lower concentrations (MIC and 2 × MIC) LPEP08 was able to inhibit the growth. The results also demonstrated the rapid killing effect of LPEP08 at higher concentration levels (4 × MIC and 8 × MIC), as nearly 5-log reduction in the growth of *E. coli* and *S. aureus* was observed within 30 min of incubation (Fig. 1A & B).