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Research paper

Identification of novel cyclic lipopeptides from a positional scanning combinatorial library with enhanced antibacterial and antibiofilm activities

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ABSTRACT

Treating bacterial infections can be difficult due to innate or acquired resistance mechanisms, and the formation of biofilms. Cyclic lipopeptides derived from fusaricidin/LI-F natural products represent particularly attractive candidates for the development of new antibacterial and antibiofilm agents, with the potential to meet the challenge of bacterial resistance to antibiotics. A positional-scanning combinatorial approach was used to identify the amino acid residues responsible for driving antibacterial activity, and increase the potency of these cyclic lipopeptides. Screening against the antibiotic resistant ESKAPE pathogens revealed the importance of hydrophobic as well as positively charged amino acid residues for activity of this class of peptides. The improvement in potency was especially evident against bacterial biofilms, since the lead cyclic lipopeptide showed promising *in vitro* and *in vivo* anti-biofilm activity at the concentration far below its respective MICs. Importantly, structural changes resulting in a more hydrophobic and positively charged analog did not lead to an increase in toxicity toward human cells.

1. Introduction

Pathogens such as ***E**nterococcus faecium*, ***S**taphylococcus aureus*, ***K**lebsiella pneumoniae*, ***A**cinetobacter baumannii*, ***P**seudomonas aeruginosa*, *and* ***E**nterobacter sp* [1,2]. (collectively called the ESKAPE pathogens) are the leading causes of hospital-acquired infections, and are resistant to the most commonly used antibiotics due to their acquisition of resistance genes, and their ability to form biofilms; leaving very few therapeutic options [3,4]. The discovery of novel antibiotics has historically focused on bacteria growing in a planktonic state, however it has recently been indicated that novel therapies addressing bacterial infections must account for the presence of biofilms [5,6]. It has been estimated that bacterial biofilms are responsible for approximately 60–80% of all chronic infections [7]. The important characteristic of chronic biofilm-associated infections is an insensitivity to both the host immune response and antimicrobial intervention [8–11]. For example, bacteria within a biofilm are up to 1000 times more resistant to the effect of antibacterial agents than the same organism circulating in a planktonic (free swimming) state [12–14]. Poor penetration of the drug through the biofilm, slow growth of the biofilm due to nutrient limitation, the activation of general stress response pathways, the emergence of a biofilm-specific phenotype and the presence of persister cells have been suggested as mechanisms of

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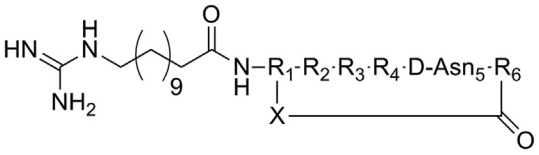
biofilm resistance [15–19]. Chronic wounds, such as diabetic ulcers, pressure ulcers and venous leg ulcers, are particularly susceptible to the development of biofilm-associated infections due to impaired healing properties of patients [20]. These infections are typically polymicrobial, with *S. aureus* and *P. aeruginosa* most frequently isolated from such wounds [21–23]. In addition, *A. baumannii* has emerged as a significant nosocomial pathogen, and is responsible for an increasing number of infections among immunocompromised and trauma patients [24–26]. A number of antibiotics have been evaluated for the prevention and disruption of biofilms, however few have proven to be effective [27,28].

Although limited therapeutic options are presently available, in order to provide effective treatments, new and innovative anti-infectives are needed, preferably with novel modes of action and/or belonging to novel classes of drugs. To this end, the structurally unique LI-F family of natural products, including fusaricidins, represent attractive candidates for the discovery and development of new antibacterial agents, capable of treating complicated infections caused by multidrug-resistant bacteria, including those found within biofilms [29–33]. Fusaricidins/LI-Fs are cyclic lipodepsipeptide antifungal antibiotics isolated from *Paenibacillus* sp. Amongst the isolated fusaricidin/LI-F antibiotics, fusaricidin A or LI-F04a has been shown to possess the most potent *in vitro* antimicrobial activity, targeting a variety of fungi and Gram-positive bacteria (MICs ranging from 0.78 to 3.12 μg/mL) [31,32].

Structural modifications of fusaricidin A/LI-F04a that include incorporation of a simpler lipidic tail, and substitution of an ester bond with an amide bond, resulted in comparably potent analogs, with improved proteolytic stability under physiologically relevant conditions, and greatly decreased nonspecific cytotoxicity [34]. Structures of the most potent fusaricidins/LI-Fs synthetic analogs, **1** and **2**, are shown in Fig. 1. In addition to being active against planktonic Gram-positive bacteria, cyclic lipopeptides **1** and **2** showed promising activity against bacterial biofilms as well. We have previously demonstrated that **1** and **2** very efficiently inhibit the growth of *S. aureus* biofilm *in vitro*, at concentrations corresponding to their MICs, and that depsipeptide **1** reduced the proliferation of community-associated MRSA (USA300) in an *in vivo* porcine wound model [35]. However, depsipeptide **1** exhibited significant *in vitro* toxicity toward human liver and red blood cells [34]. Although the mode of action of this class of antibacterial peptides is not yet fully understood, the bacterial membrane has been suggested as a potential target based on analysis of the *Bacillus subtilis* transcriptome after treatment with the fusaricidin/LI-F natural product mixtures [36]. Our initial mechanistic studies showed that **1** and **2** are able to depolarize the cytoplasmic membranes of Gram-positive bacteria in a concentration-dependent manner. However, a lack of correlation between membrane depolarization and cell lethality suggested that membrane-targeting activity is not the primary mode of action for this class of antibacterial peptides [37]. Due to its improved stability and lower cytotoxicity, cyclic lipopeptide **2** may have significant advantages over naturally occurring fusaricidin A/LI-F04a and its depsipeptide analogs as a lead structure for the development of new antibacterial agents. In addition, amide analogs are synthetically more accessible than the parent depsipeptides, allowing for further structural optimization using a combinatorial chemistry approach. Indeed, given the 20 amino acid building blocks, even small cyclic peptides such as **2** offer enormous diversity, and the potential for discovery of more potent analogs. Among combinatorial chemistry strategies, positional scanning synthetic combinatorial libraries (PS-SCL) offer a unique and rapid approach for peptide sequence optimization [38–40].

In the pilot study reported herein, we have generated a positional scanning combinatorial library of cyclic lipopeptide **2**, containing 130,321 cyclic lipopeptides to screen for enhanced antibacterial activity. A lead cyclic lipopeptide was identified and assessed for its antibacterial/antibiofilm activities and nonspecific toxicity.



	X	R_1	R_2	R_3	R_4	R_6
1	O	Thr	D-Val	Val	D-*a*Thr	D-Ala
2	NH	Dap	D-Val	Val	D-*a*Thr	D-Ala
3	NH	Dap	D-Leu	Leu	D-Ile	D-Lys
4	NH	Dap	D-Leu	Leu	D-Lys	D-Arg
5	NH	Dap	D-Leu	Phe	D-Ile	D-Arg
6	NH	Dap	D-Leu	Phe	D-Ile	D-Lys
7	NH	Dap	D-Lys	Leu	D-Ile	D-Arg
8	NH	Dap	D-Leu	Leu	D-Phe	D-Arg
9	NH	Dap	D-Leu	Phe	D-Ile	D-Arg
10	NH	Dap	D-Trp	Trp	D-Arg	D-Thr
11	NH	Dap	D-Trp	Leu	D-Trp	D-Arg
12	NH	Dap	D-Leu	Leu	D-Phe	D-Arg

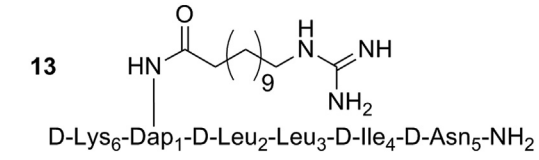


Fig. 1. Sequences of cyclic lipopeptides **1**–**12** and control lipopeptide **13**.

2. Results and discussion

To gain further insight into the amino acid requirements for antibacterial activity and toxicity of the fusaricidin/LI-F class of cyclic lipopeptides, and to identify analog(s) with improved activities, we prepared a combinatorial library of cyclic lipopeptide **2**. Cyclic lipopeptide **2** was used as a model compound due to its low nonspecific toxicity, promising antibacterial/antibiofilm activities, and ease of synthesis.

2.1. Library design and synthesis

The general strategy for cyclic lipopeptide combinatorial library preparation and library deconvolution is outlined in Scheme 1. The cyclic lipopeptide synthetic combinatorial library was generated by the process of divide, couple and recombine ("tea bag" method) using a previously developed Fmoc SPPS chemistry [34,41,42]. In brief, our synthetic strategy included attachment of the C-terminal amino acid Fmoc-D-Asp^5-OAllyl to a PEG-based amide resin *via* the side chain, use of a combination of orthogonal protecting groups, stepwise solid-phase assembly of a linear precursor, attachment of a lipidic tail followed by coupling of the N-terminal Fmoc-Dap_1(Mtt)-OH and on-resin cyclization. To maintain the same order of D- and L-amino acids as they appear in the sequences of the fusaricidin/LI-F natural products in the synthesized cyclic lipopeptides, D-Val_2, D-Thr_4, and D-Ala_6 were replaced with D-amino acid mixtures, whereas L-Val_3 was similarly replaced with an L-amino acid mixture. Fmoc deprotection, amino acid coupling reactions and final cyclization steps were monitored by a ninhydrin