Novel Antimicrobial Peptides: Targeting Wound Infections Caused by 'Superbugs' Resistant to All Current Antibiotics

Tony Velkov, Chongyu Zhu, David M. Haddleton, and Jian Li

1 Introduction

The annual costs of wound care in Australia ($2.6 billion AUD), the UK (£2.3–3.1 billion) and the USA ($50 billion USD) are staggering. The cost of chronic wound infections is often due to lengthy hospitalisations because of infections caused by multidrug-resistant (MDR) bacterial pathogens [1]. There is an urgent unmet medical need for new antibiotics for wound and burn infections caused by MDR Gram-negative 'superbugs' *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*. Resistance to the last-line therapy polymyxins (polymyxin B and colistin) has been increasingly reported, which virtually means no antibiotic will be available for treatment of wound and burn infections. Considering potential systemic toxicity and suboptimal pharmacokinetic/pharmacodynamic attainment, the topical use of antibiotics often remains a superior approach for wound infections than parenteral administration.

The present chapter covers the development of novel broad-spectrum lipopeptides that are very active against not only polymyxin-resistant Gram-negative pathogens but also MDR Gram-positive *Staphylococcus aureus* and *Enterococcus faecium* that also commonly cause serious wound infections. Furthermore, we have developed a chitosan-based colistin self-healable hydrogel that provides high localised release of colistin for the treatment of burn wound infections. The development of these novel topical lipopeptide agents could slash the billion-dollar annual cost of wound treatment and result in improved healthcare on a global scale.

2 MDR Bacterial Wound Infections

Wound and burn infections are a major medical challenge worldwide and represent a considerable healthcare burden [2–4]. They are a common risk for patients with chronic non-healing wounds which cause high morbidity and mortality. As a poignant

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example, ~75% of all deaths following major burn injuries are related to bacterial infections [3, 4]. Wound and burn infections caused by the aforementioned Gram-negative 'superbugs' are immensely concerning [5]. Burns are particularly susceptible to infections due to the disruption of the epidermal barrier, the systemic apoptotic response and immunosuppression that disrupts self-defence mechanisms to fight infection [3, 6]. Even though systemic antibiotic treatment is usually the most common therapeutic option, the significant difficulties are adverse effects and the risk of an insufficient tissue penetration due to impaired blood circulation. The use of topical chemotherapy has been fundamental and helped to improve the survival of patients with major burns and to minimise the incidence of life-threatening burn wound sepsis [7]. The topical use of antibiotics plays a significant role in the management of serious wound infections caused by Gram-negative bacteria *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* and Gram-positive *S. aureus* and *E. faecium* [3, 4]. Very worryingly, these bacteria are increasingly resistant to almost all current topical antibiotics [8, 9]. These bacterial 'superbugs' have been identified by the Infectious Diseases Society of America (IDSA) and Centre for Disease Control and Prevention (CDC) as the top-priority dangerous 'superbugs' that require urgent attention for discovery of novel antibiotics [10–13].

Polymyxins are an important last-line therapy against Gram-negative 'superbugs'.

Polymyxins consist of a linear tripeptide fragment having an N-terminal fatty acyl tail attached to a cyclic heptapeptide (Fig. 24.1). They are polycations at pH 7.4 owing to the five diaminobutyric acid (Dab) residues. Polymyxins were discovered more than 60 years ago. Because the early experience in the 1960s with parenteral polymyxins led to some cases of nephrotoxicity and neurotoxicity, their clinical use waned [14–17]. Since the mid-1990s, there has been a greatly renewed interest in polymyxins because of the increasing prevalence of MDR *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* [14–17]. Polymyxin B and colistin (polymyxin E) are the two clinically available polymyxins that are most commonly administered parenterally in patients as a last-line therapy for serious infections, when all other available antibiotics are inactive. Our in vitro studies have shown that resistance can rapidly emerge in *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* [18–20], and polymyxin resistance in hospitalised patients has been increasingly reported [10, 21, 22]. Even more worrying is the recent reports in the *Lancet Infect Dis* of the emergence of plasmid-mediated colistin resistance [23, 24], which implies resistance to these important last-line antibiotics can now rapidly spread. Resistance to polymyxins implies a total lack of antibiotics for treatment of life-threatening Gram-negative infections.

The majority of the modern pharmacological data of polymyxins are obtained by our group [18, 20, 25–43]. We were the first to characterise the modern pharmacokinetics of colistin and polymyxin B in patients [25, 30, 39, 40] and demonstrate that polymyxins exhibit rapid, concentration-dependent killing of susceptible strains of *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* [20, 34, 41]. Our studies in both in vitro and animal infection models have, for the first time, elucidated that fAUC/MIC (i.e. ratio of the area under the free plasma concentration—time curve to minimal inhibitory concentration [MIC]) is the pharmacokinetic/pharmacodynamic (PK/PD) index best correlating with colistin activity [44]. Our findings are led to the first scientifically based dosage regimens in patients. Our recent data suggest that intravenous polymyxins are not ideal for treatment of lung infections and wound infections due to suboptimal PK/PD exposure at infection sites. We first reported colistin heteroresistance (i.e. colistin-resistant subpopulations in an isolate susceptible based upon MIC) in the Gram-negative pathogens [20, 31, 36] and the potential for resistant subpopulations to rapidly amplify upon exposure to colistin in an in vitro PK/PD model that mimics clinical dosing regimens in humans [18–20]. The latter highlights the urgency to develop new antibiotics active against isolates which are resistant to polymyxins and all other current antibiotics.

3 Mechanisms of Polymyxin Activity and Resistance

The initial cellular target of polymyxins is the lipid A component of lipopolysaccharide (LPS) in the outer membrane (OM). The purported

primary mechanism of polymyxin activity involves an initial electrostatic interaction of the cationic Dab residues of the polymyxin molecule with the negatively charged phosphate groups of lipid A [45]. This initial polar interaction is followed by insertion of the fatty acyl tail of the polymyxin into the lipid A fatty acyl layer in the outer membrane. Many of the Gram-negative bacterial mechanisms of resistance to polymyxins are based on modifications to lipid A which reduce or abolish this initial electrostatic interaction. Modification of the phosphates of lipid A with positively charged moieties such as 4-amino-4-deoxy-L-arabinose or phosphoethanolamine reduces the net negative charge of lipid A, thereby increasing resistance to polymyxins [46–53].

4 Discovery of New Polymyxin-Like Lipopeptides Targeting MDR 'Superbugs'

We were invited by the *Journal of Medicinal Chemistry* to review the current state of development of polymyxin analogues [54]. Previous medicinal chemistry strategies for improving the antibacterial activity of polymyxins have been empirical and limited to modifications of the Dab residues, the heptapeptide ring and the length of the N-terminal fatty acyl chain (Fig. 1) [55–60]. Notably, numerous attempts have been made to modify the N-terminus with polar and lipophilic groups but with little success [54]. One such notable N-terminal analogue (CB-182804) came from Cubist; unfortunately this analogue failed in a Phase 1 clinical trial. Importantly, CB-182804 was not active against any polymyxin-resistant isolates [61]. None of the previous discovery programmes were specifically driven by an SAR approach nor was polymyxin resistance targeted. These major shortcomings led to the failure of the novel polymyxin discovery programmes by Cubist, AstraZeneca and Pfizer. To the best of our knowledge, we are the first to apply an SAR-based mechanistic model (Fig. 2) to discover novel lipopeptides against polymyxin-resistant Gram-negative 'superbugs' [62]. The SAR model has allowed us to identify key structural properties of polymyxins that confer antibacterial activity. In our model, the polymyxin-lipid A complex is stabilised by a combination of polar and hydrophobic interactions (Fig. 2). The positive charges on Dab1

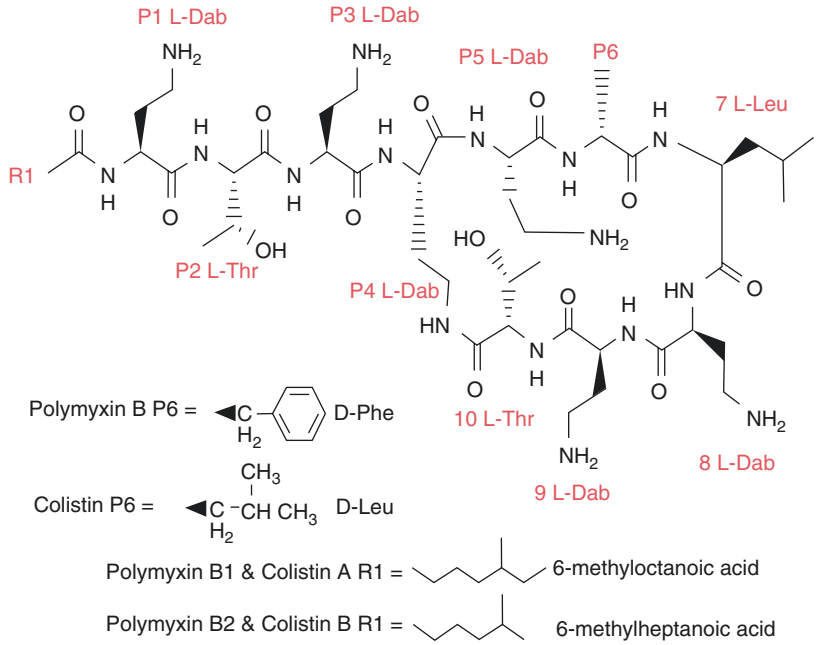

Fig. 1 Structures of colistin and polymyxin B. *Thr* threonine, *Leu* leucine, *Phe* phenylalanine, *Dab* α,γ-diaminobutyric acid

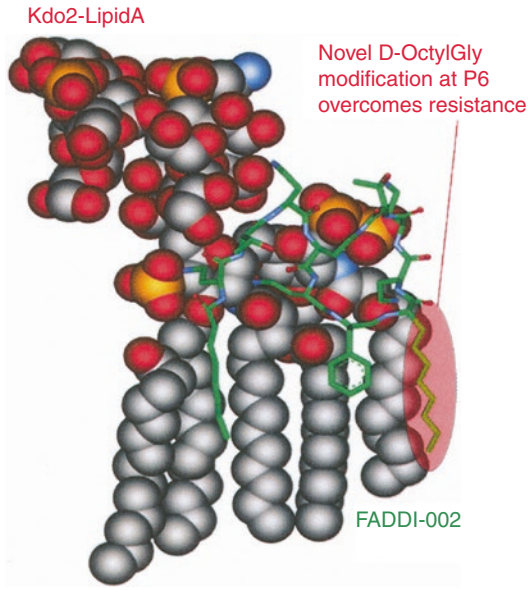

Fig. 2 SAR model of novel lipopeptide FADDI-002 in complex with *E. coli* Kdo2-lipid A

and Dab5 of polymyxin B interact with the negatively charged 4′-phosphate group of lipid A, and Dab8 and Dab9 similarly interact with the 1-phosphate of lipid A. The polymyxin-lipid A complex is further stabilised by hydrophobic contacts between the N-terminal fatty acyl chain and position 6/7 D-Phe-L-Leu segment of the polymyxin molecule, with the fatty acyl chains of lipid A. Evidently, the SAR model indicates that the polymyxin-lipid A interaction in both polymyxin-susceptible and polymyxin-resistant strains can be significantly accentuated through the introduction of additional hydrophobic contacts. To circumvent bacterial resistance mechanisms due to modifications of lipid A, in one series of our novel FADDI lipopeptides, hydrophobic modifications were introduced at position 6 or 7 to enhance penetration into the lipid A fatty acyl layer as suggested by the SAR model (Fig. 2). The SAR model was validated when our first generation lipopeptides (e.g. FADDI-002) (Table 1) with L-octylglycine substituted at position 7 displayed potent antimicrobial activity against polymyxin-resistant Gram-negative clinical isolates [62]. Subsequently, expansion of our SAR-based design strategy to include compounds incorporating lipidic groups at position 6 and the N-terminus

Table 1 Structures of representative lipopeptides

Lipopeptide	Structure
FADDI-002	Octanoyl-Dab-Thr-Dab-Dab*-Dab-D-Phe-OctGly-Dab-Dab-Thr*
FADDI-003	Biphenyl-Dab-Thr-Dab-Dab*-Dab-D-Phe-OctGly-Dab-Dab-Thr*
FADDI-019	Octanoyl-Dab-Thr-Dab-Dab*-Dab-D-OctGly-Leu-Dab-Dab-Thr*
FADDI-043	Dansylgly-OctGly-Dab-Thr-Dab-Dab*-Dab-D-Phe-Leu-Dab-Dab-Thr*
FADDI-052	Biphenyl-Dab-Thr-Dab-Dab*-Dab-D-Cys(6F3-Hex)-Leu-Dab-Dab-Thr*

*Cyclisation point of the peptide

also generated potent lipopeptides active against polymyxin-resistant Gram-negative 'superbugs' (Table 1). Notably, FADDI-019 and FADDI-020 which have the same or very similar MICs (1–4 mg/L) as colistin against colistin-susceptible *P. aeruginosa* isolates displayed significant activity (MICs 1–4 mg/L) against colistin-resistant *P. aeruginosa* (colistin MICs 32 or >128 mg/L). In static time-killing studies, FADDI-019 (MIC 1 mg/L) at 4 × MIC achieved ~6 $\log_{10}$ kill against a polymyxin-resistant MDR clinical *P. aeruginosa* isolate (colistin MIC >128 mg/L) with no viable cells detected even at 2 h; no killing was observed with colistin even at 32 mg/L. Against polymyxin-susceptible *P. aeruginosa* ATCC 27853 (colistin MIC 1 mg/L), FADDI-019 (MIC 1 mg/L) had comparable bacterial time-kill to colistin. For most of our lipopeptides, the ratios of MBCs (minimal bactericidal concentrations) to MICs were ≤4 indicating a low potential for development of resistance. Isothermal titration calorimetry studies confirmed that the hydrophobic contribution from the N-terminal fatty acyl chain is the predominant driving force for polymyxin-lipid A complexation [63, 64].

Serendipitously, a series of our lipopeptides have unexpected activity against MDR Gram-positive *S. aureus* and *E. faecium* which are intrinsically resistant to polymyxins (colistin and polymyxin B MIC >128 mg/L). Transcriptome analysis using RNA-seq revealed that virulence determinants controlled by SaeRS and the expression of enterotoxins yent2, sei, sem and seo were all significantly downregulated by FADDI-019 [65]. Clearly, our SAR-based mechanistic model

has led to unique opportunities to optimise the polymyxin structure to overcome both adaptive and intrinsic resistance to current polymyxins. There was no haemolysis in human red blood cells treated with the tested FADDI lipopeptides or polymyxins at 128 mg/L (the highest concentration examined). After administration of FADDI-002, FADDI-003, FADDI-019 or FADDI-020 to rats (intravenous, 0.75 mg/kg) and mice (subcutaneous, 40 mg/kg), no adverse effects were observed.

Preliminary in vitro studies to examine the impact of the lipopeptides on human keratinocytes and murine fibroblast cells, polymyxin B, FADDI-019 and FADDI-073 at 1.5, 5, 15 and 50 mg/L had little effect over 48 h on the morphology of fibroblasts (3 T3) and keratinocytes (HaCaT). FADDI-019, FADDI-073 and polymyxin B stimulated metabolic activity above mock-treated cultures in 3T3 cells in a dose-dependent manner at 24 and 48 h. However, similar responses were not observed in HaCaT cells; neither FADDI-019 nor FADDI-073 affected the cellular metabolic activity at any of the four concentrations at 24 or 48 h, while only 1.5 and 5 mg/L polymyxin B slightly decreased the cellular metabolic activity at both time points. It is noteworthy that for many years a topical formulation containing polymyxin B has been used for treating skin infections, with negligible toxicity [66]. Our results suggest that our lipopeptides have at least similar tolerability to colistin and polymyxin B.

5 Synthesis of a Chitosan-Colistin Hydrogel and Testing in a Mouse Burn Infection Model

We have synthesised a chitosan-colistin hydrogel and assessed its efficacy in a mouse burn infection model (Fig. 3) [67]. The chitosan-colistin hydrogel is an inexpensive, self-healable and highly biocompatible material which provides up to 95% colistin release within 24 h and showed excellent in vitro activity against *P. aeruginosa* in a disc diffusion assay. The physical properties of the hydrogel were unaffected by colistin; this allowed us to load a wide range of colistin concentrations into the hydrogel matrix without impacting its size. Serendipitously, the hydrogel formation process was accelerated in the presence of colistin. Excitingly, the chitosan-colistin hydrogel dressing (containing 0.3 mg colistin) displayed excellent in vivo activity, producing a ~4 log reduction in the bacterial load in a burn wound (1 cm^2) infection, established in mice by inoculating 100 μL 10^8 CFU/mL of *P. aeruginosa* ATCC 27853. We are currently exploring lipopeptide-hydrogel dressing systems using the superior FADDI lipopeptides, for which formulation characteristics (e.g. lipopeptide loading and mechanical properties) will be investigated and optimised.

Silk proteins serve as excellent scaffolds for wound healing and in tissue engineering [68].

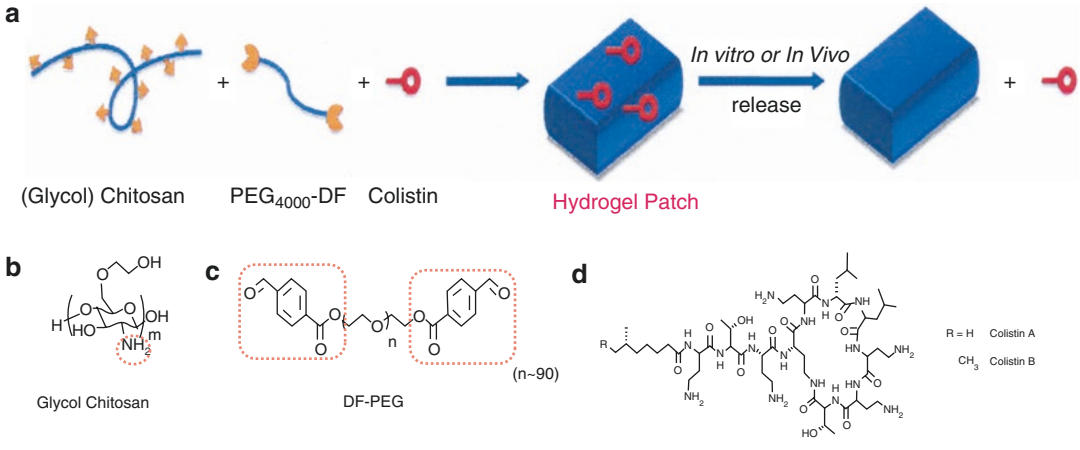

Fig. 3 (**a**) Synthesis of chitosan-colistin hydrogel. (**b**) Glycol chitosan. (**c**) DF-PEG. (**d**) Colistins A and B

Steinstraesser et al. [68] loaded ST-silk protein membranes (thickness, 100 μm; pore size, <100 nm) with colistin (0.027–270 mg/mL) and examined their efficacy against *P. aeruginosa* in animal wound infection models. The ST-silk membranes loaded with 270 mg/mL colistin demonstrated $a > 3$ log reduction in colony-forming units of *P. aeruginosa* ATCC 27853 after 4 days (~25-fold decrease from the carrier control). Similarly, in a porcine wound infection model, the ST-silk membranes loaded with 270 mg/mL colistin demonstrated an almost complete clearance of the infection after the entire follow-up of 6 days.

6 Perspective

The World Health Organization has identified antimicrobial resistance as one of the three greatest threats to human health. The last-line therapy polymyxins are losing their activity; however, no new antibiotic will be available for many years to come. The prevalence of wound infections caused by the bacterial 'superbugs' highlights the urgency of discovering novel antibiotics for topical treatment of serious wound infections. As the Infectious Diseases Society of America highlighted, 'as antibiotic discovery stagnates, a public health crisis brews', the recent emergence of plasmid-borne resistance to the last-line polymyxins highlights the urgency to develop novel antibiotics to combat these very problematic pathogens. This chapter details the development of novel polymyxin lipopeptides and hydrogel formulations as new antibiotics for topical use in wound treatment against problematic 'superbugs' that are resistant to all current antibiotics. These next-generation polymyxins hold significant potential for the treatment of chronic wound infections caused by problematic Gram-negative 'superbugs'.

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