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Polymyxin derivatives as broad-spectrum antibiotic agents†

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We designed a few polymyxin derivatives which exhibit broad-spectrum antimicrobial activity. Lead compound P1 could disrupt bacterial membranes rapidly without developing resistance, inhibit biofilms formed by *E. coli*, and exhibit excellent *in vivo* activity in an MRSA-infected thigh burden mouse model.

Polymyxins are secondary metabolite nonribosomal peptides produced by a Gram-positive bacterium *Paenibacillus polymyxa* and first recognized as antibiotic agents in the 1940s.[1] Among the five polymyxins (polymyxins A to E), two of them have been used in clinics: polymyxins B and E (also known as colistin).[2] Both polymyxin B and colistin exhibit antibacterial activities against a narrow spectrum of Gram-negative pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*.[3]

Colistin is a complex, multicomponent antibiotic mixture.[4] Two major constituents are colistin A and colistin B, with identical head groups but fatty acyl tails of different lengths: colistin A contains a 6-methyloctanoic acid residue, whereas colistin B bears a 6-methylheptanoic acyl tail (Fig. 1).[5] In the clinical setting, colistin is administered in the form of colistin methanesulfonate (CMS), a less toxic and nonactive prodrug.[6] However, the early clinical experience before the 1970s, the parenteral administration of PMB and colistin (or its nonactive prodrug colistin methanesulfonate), led to concern over their potential nephrotoxicity and neurotoxicity, and their clinical use waned for a long period.[7–9]

The mechanism of colistin has been extensively investigated and proposed. The outer membrane of Gram-negative bacteria constitutes a permeable barrier.[10] Polymyxins can directly interact with the lipid A component of the lipopolysaccharide (LPS) of the outer membrane.[11] The current understanding of the structure–activity relationship (SAR) is that the amphipathic nature of polymyxins is crucial:[12] cationic residues and hydrophobic groups. There are several key domains crucial for interaction with lipid A: a Dab side chain with positive charge, the heptapeptide backbone, a hydrophobic fatty acyl tail at the N-terminal and hydrophobic motifs at positions 6 and 7 (Fig. 1). Indeed, polymyxins exhibit weak antimicrobial activity toward Gram-positive bacteria due to the lack of LPS in their membranes.

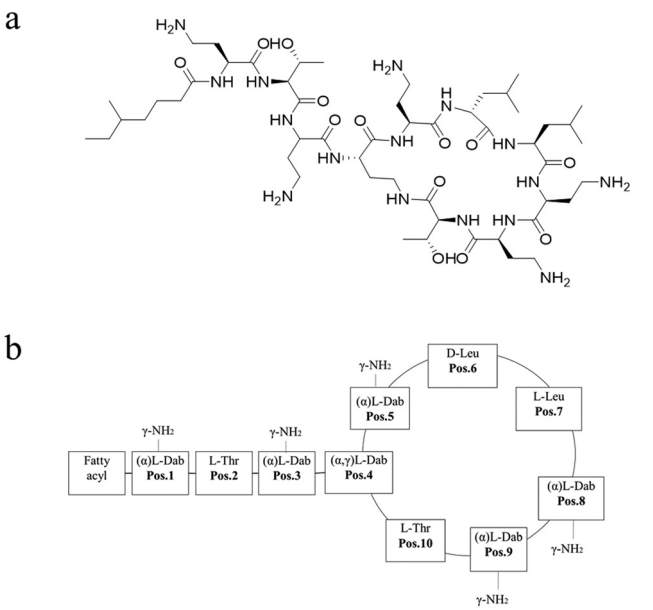

Fig. 1 (a) Structure of colistin (polymyxin E) and (b) the numbering of positions in colistin.

The WHO has identified antibiotic resistance as one of the three greatest threats to human health.[13] The world is now facing an enormous threat from the emergence of bacteria that are resistant to almost all available antibiotics.[14] In recent years, virtually no novel drugs targeting multidrug-resistant

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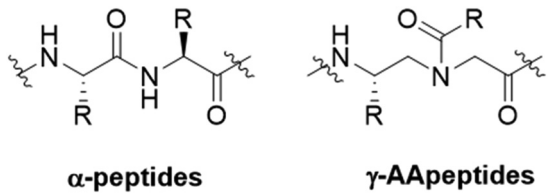


Fig. 2 The structures of α-peptides and γ-AApeptides.

(MDR) Gram-negative bacteria (especially *P. aeruginosa* and *K. pneumoniae*) have been developed.[12] Polymyxins are increasingly being used as last-line therapy to treat otherwise untreatable serious infections caused by Gram-negative bacteria that are resistant to essentially all other currently available antibiotics.[2,15–17] However, the emergence of polymyxin resistance in Gram-negative bacteria has been reported.[18,19] The most common way that Gram-negative bacteria survive from polymyxins is by remodeling LPS.[20]

Our previous studies have suggested that γ-AApeptides may be an alternative class of peptidomimetics combating antibiotic resistance (Fig. 2),[21,22] and lipidation could further enhance their antibacterial activity.[23–25] Herein, we report the design and investigation of a few polymyxin derivatives modified with γ-AApeptide building blocks and lipid tails. Intriguingly, we show that certain polymyxin derivatives exhibit broader spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria.

As shown in Table 1, we designed a few sequences with modifications on collision structural domains. First, the length of the fatty acyl group was changed from branched 8 carbons to linear 16 carbons for all the colistin derivatives. We speculated that the longer tail could help colistin to penetrate bacterial membranes. Additionally, in compound **P2**, L-Dab residues at positions 8 and 9 were replaced with a positively charged γ-AA building block, which contains two positively charged groups and presumably mimics L-Dab residues (Table 1). On the other hand, in **P3**, the L-Dab and L-Thr residues at positions 1 and 2 were substituted with another γ-AApeptide building block, which contains one positively charged and polar group. At last, we designed compound **P4**, with a change from D-Leu and L-Leu residues to a hydrophobic γ-AApeptide building block bearing two hydrophobic groups. We speculated that inclusion of hydrophobic groups, cationic groups and longer tails would enhance the interaction between these compounds with bacterial membranes, and thus increase their antibacterial activity.

Table 1 Structures of polymyxin mimic cyclic peptides

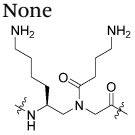
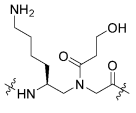
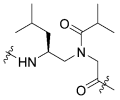
Compound	Modified positions	AApeptide building block	Fatty acyl group
P1	None	None	$C_{16}H_{31}O$
P2	8, 9		$C_{16}H_{31}O$
P3	1, 2		$C_{16}H_{31}O$
P4	6, 7		$C_{16}H_{31}O$

Table 2 Activity and selectivity of polymyxin mimic peptides

Compound	MIC (μg mL^{-1})				HC_{50} (μg mL^{-1})	SI (HC_{50}/MIC $_{MRSA}$)
	Gram positive		Gram negative			
	MRSA	MRSE	*E. coli*	*P. aeruginosa*		
P1	6.25	3.12	6.25	6.25	125	20
P2	12.5	>50	6.25	12.5	62.5	5
P3	25	>50	3.12	25	>250	>10
P4	12.5	>50	25	>50	>250	>20
Colistin	>50	>50	0.5	0.5	>250	>5

The antimicrobial activity of these compounds was tested by MIC using 4 different bacterial strains including both multi-drug resistant Gram-positive and Gram-negative bacteria, and the results are shown in Table 2. Interestingly, **P1**, **P2**, **P3** and **P4** show good activity against Gram-positive bacterial MRSA with MICs of 6.25–25 μg mL^{-1}, and as expected, colistin is not active against MRSA, suggesting that changing the lipid tail could enhance its broad-spectrum activity. It is known that colistin is only active against Gram-negative bacterial strains and does not show activity against Gram-positive bacterial strains. Interestingly, all four cyclic peptides are active against both MRSA and *E. coli*, suggesting that increasing the length of the lipid tail was sufficient to enhance the broad-spectrum activity of colistin. We believed longer tails could penetrate both Gram-positive and Gram-negative bacterial membranes, and such findings could be used to guide the future design of antibiotic agents. Intriguingly, compound **P1**, which just has a longer lipid tail compared to colistin, has shown the best activity among the 4 compounds, indicating that inclusion of γ-AApeptide building blocks did not improve the antibacterial activity. We reasoned that, due to the complexity of LPS, change of residues on the cyclic backbone may alter the conformation of the ring structure, leading to weaker interaction with bacterial cell membranes. Moreover, **P1** also exhibited good selectivity, as it shows a hemolytic activity of 125 μg mL^{-1}, which is 20-fold its activity toward MRSA.

Since compound **P1** exhibited the most potent and broad-spectrum activity, it was investigated further for its ability to disrupt the membranes of MRSA and *E. coli*. Two dyes, 4′,6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) (Fig. 3), were used to differentiate among cells with either

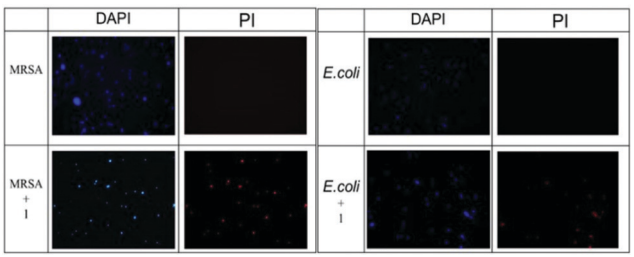


Fig. 3 Fluorescence micrographs of MRSA and *E. coli* treated or not treated with 2× MIC of compound **P1** for 2 h.

an intact or a damaged membrane. DAPI can permeate the membranes of intact cells and therefore shows blue fluorescence regardless of cell viability. In contrast, PI is a DNA intercalator but lacks cell permeability. It fluoresces in red only when cell membranes are disrupted. As shown in Fig. 3, in the DAPI channel, both MRSA and *E. coli* exhibited blue fluorescence in the absence of compound **P1**. In the PI channel, neither strain showed red fluorescence before treatment, indicating that the membranes of these bacteria were intact. However, after treatment with compound **P1** for 2 h at 2× MIC, both MRSA and *E. coli* exhibited red fluorescence, suggesting that the membranes of both MRSA and *E. coli* were compromised.

To understand its bacterial membrane-disruptive kinetics, time kill assay of compound **P1** was also carried out. MRSA and *E. coli* were treated with different concentrations of compound **P1**: 2× MIC, 4× MIC and 8× MIC. In Fig. 4, MRSA could be eliminated within 60 min at all three concentrations. The growth of *E. coli* was also effectively prevented at all three concentrations. The results indicate that compound **P1** could rapidly kill MRSA and arrest the growth of *E. coli* bacterial strains.

Inhibition of biofilms was also studied since biofilms have strong tolerance to antibiotics and can cause contamination by adhering to solid surfaces.[26] As shown in Fig. 5, 10% of a biofilm of *E. coli* bacteria was inhibited by compound **P1** at as low as 0.03 μg mL^{-1} and 50% was inhibited at 3 μg mL^{-1}. At a concentration of 6 μg mL^{-1}, more than 80% of bacteria were eradicated by compound **P1**. Biofilm data showed that **P1** could effectively inhibit the formation of a bacterial biofilm, which makes it a promising antibiotic agent.

Since compound **P1** was designed to be membrane active and disrupt bacterial membranes rather than acting on specific targets, we hypothesized that **P1** could also prevent the development of resistance in bacteria. Therefore, we carried out drug resistance studies for **P1** against *E. coli*. To do so, **P1** was incubated with *E. coli* at half of its MIC overnight, and the new MIC was measured subsequently. After 12 passages, the MICs of **P1** remained relatively stable (Fig. 5), which strongly suggests that PI does not readily induce resistance in bacteria, thereby augmenting their therapeutic potential.

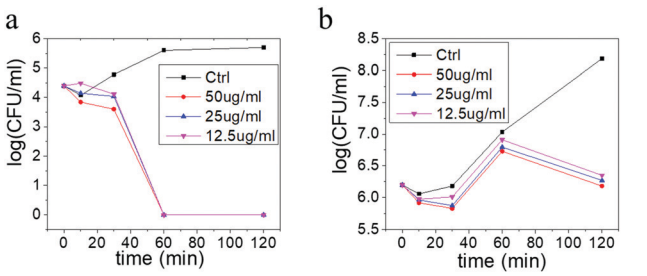


Fig. 4 Time-kill plots of compound **P1** against MRSA (a) and *E. coli* (b).

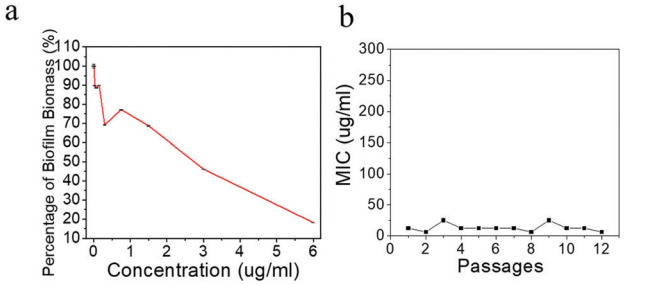


Fig. 5 Biological activity of **P1** in the inhibition of a biofilm by *E. coli* (a). Drug resistance study for compound **P1** against *E. coli* (b).

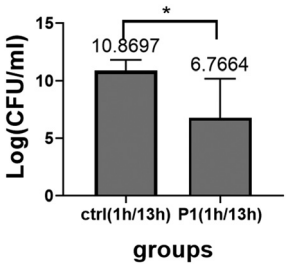


Fig. 6 *In vivo* efficacy of compound **P1** in the thigh-infection mouse model (*: 0.02 < *P* value < 0.033).

The development of membrane-active antibacterial peptides has been hindered by difficulties with systemic toxicity and tissue distribution; thus only a few compounds have been reported with *in vivo* activity and advanced into clinical trials. Polymyxins were investigated for their activity toward Gram-negative bacteria; however, their *in vivo* activity toward Gram-positive bacteria is rare. We envisioned that **P1** may show *in vivo* activity against MRSA and thus could have better therapeutic potential than colistin toward Gram-positive bacterial strains. As such, we employed the thigh burden model, which is a widely used animal model for evaluating the preclinical antimicrobial activity of compounds, to evaluate the *in vivo* anti-infective activity of compound **P1**.[27] The thigh muscles of neutropenic mice were inoculated with MRSA, followed by intravenous (i.v.) injections of compound **P1**. As shown in Fig. 6, significant activity was observed at a dose of 5 mg kg^{-1} when administered twice with a 12 h interval between each injection. A 4 log 10 decrease in colony-forming units (CFUs) was observed. The *in vivo* results suggested that compound **P1** exhibited significant antibiotic activity against infection with MRSA.

In summary, we have made a few polymyxin derivatives. Unlike colistin, these compounds exhibit potent and broad-spectrum antimicrobial activity against a panel of multidrug-resistant Gram-positive and Gram-negative bacteria. Our studies suggest that the lead compound could kill bacteria rapidly and the susceptibility of MRSA remained stable even after 12 passages. Furthermore, the results of an MRSA-infected thigh burden mouse model suggested the great antibiotic therapeutic potential of the lead compound. Therefore, the polymyxin compounds could be potential broad-spectrum antibiotic agents to combat drug resistance. Further studies on the optimization of their activity and selectivity are currently underway.

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Conflicts of interest

There are no conflicts to declare.

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