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Teaching 'Old' Polymyxins New Tricks: New-Generation Lipopeptides Targeting Gram-Negative 'Superbugs'

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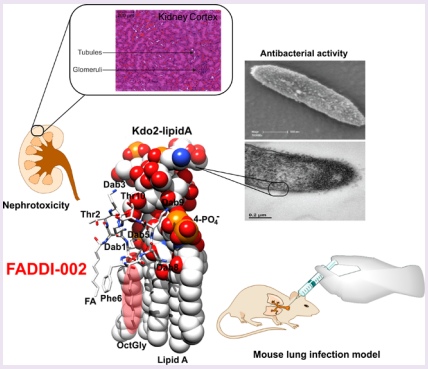
Supporting Information

ABSTRACT: The antimicrobial lipopeptides polymyxin B and E (colistin) are being used as a 'last-line' therapy for infections caused by multidrug-resistant Gram-negative pathogens. Polymyxin resistance implies a total lack of antibiotics for the treatment of life-threatening infections caused by the Gram-negative 'superbugs'. This report details the structure–activity relationships (SAR) based design, *in toto* synthesis, and preclinical evaluation of a series of novel polymyxin lipopeptides with better antibacterial activity against polymyxin-resistant Gram-negative bacteria.



Polymyxins are natural products that were discovered more than 60 years ago.[1] They have a narrow antibacterial spectrum which is mainly against Gram-negative pathogens.[2] The dry antibiotic pipeline, together with the increasing incidence of bacterial resistance in the clinic, has been dubbed 'the perfect storm'. Polymyxin B (PMB) and colistin are now being used as the last therapeutic option for infections caused by multidrug-resistant (MDR) 'superbugs' *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*.[3,4] Recent studies have shown that resistance can rapidly emerge *in vitro* in *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*.[5−7] Most worryingly, polymyxin resistance in hospitalized patients has been increasingly reported.[8] Resistance to polymyxins often implies a total lack of antibiotics for treatment of life-threatening infections caused by MDR Gram-negative 'superbugs'. There is a clear unmet medical need for the development of new antibiotics to target Gram-negative pathogens that are resistant to all current antibiotics including polymyxins.

Understanding of the structure–activity relationships (SARs) is an essential precursor for modern antibiotic discovery and development. The recent novel polymyxin discovery programs were not specifically driven by solid SAR data nor did they target polymyxin resistance.[9−12] The polymyxin molecule consists of five key structural features (Figure 1A): (i) the hydrophobic *N*-terminal fatty acyl chain; (ii) the positive charge of the five L-α,γ-diaminobutyric acid (Dab) residues (at physiological pH); (iii) the linear tripeptide segment; (iv) the hydrophobic motif at positions R6 and R7 in the cyclic heptapeptide ring; and (v) the heptapeptide backbone.[13] After an extensive analysis of all reported polymyxin analogues in the literature and our pharmacophore development studies, we proposed that polymyxin SAR data need to be interpreted

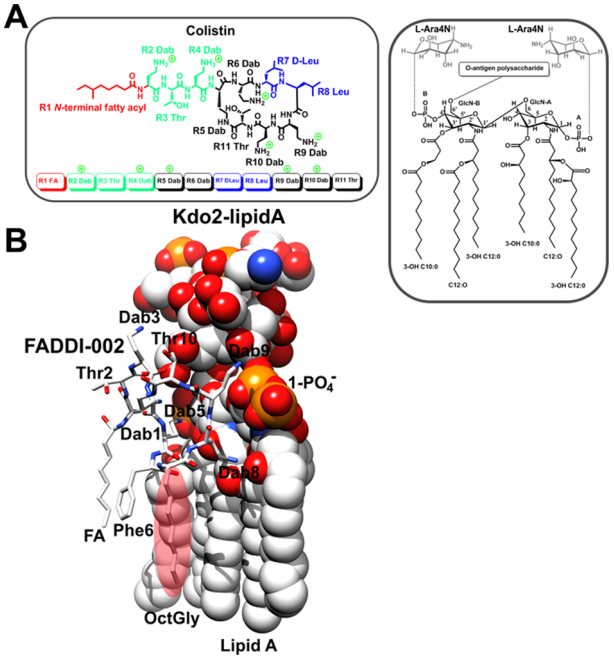


Figure 1. (A) Five key features of the polymyxin molecule. Inset: Structure of Lipid A isolated from polymyxin-resistant *P. aeruginosa* showing the aminoarabinose (Ara4N) modifications.[16] (B) An SAR model of lipopeptide FADDI-002 complexed to Kdo2lipid A.

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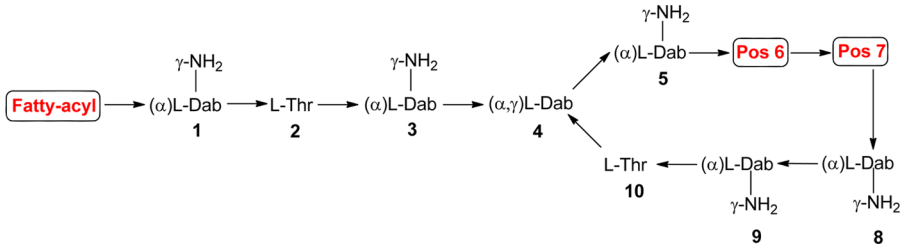
Table 1. Chemical Structures for Polymyxin Lipopeptides Synthesized



peptide	fatty acyl	Pos 6	Pos 7
polymyxin B_1	6-S-methyloctanoyl	D-Phe	L-Leu
polymyxin B_2	6-methylheptanoyl	D-Phe	L-Leu
colistin A	6-S-methyloctanoyl	D-Leu	L-Leu
colistin B	6-methylheptanoyl	D-Leu	L-Leu
FADDI-002	octanoyl	D-Phe	L-OctGly
FADDI-003	biphenylacyl	D-Phe	L-OctGly
FADDI-016	biphenylacyl	D-Phe	L-BipAla
FADDI-017	octanoyl	D-Phe	L-BipAla
FADDI-018	octanoyl	D-Phe	L-Cys(S-Hex)
FADDI-019	octanoyl	D-OctGly	L-Leu
FADDI-020	biphenylacyl	D-OctGly	L-Leu
FADDI-021	octanoyl	D-OctGly	L-OctGly
FADDI-031	octanoyl	D-OctGly	L-Ala
FADDI-036	phenacyl	D-OctGly	L-Leu
FADDI-039	octanoyl	D-BipAla	L-Leu
FADDI-040	biphenylacyl	D-BipAla	L-Leu
FADDI-047	octanoyl	D-Tyr(Bzl)	L-Leu
FADDI-063	octanoyl	D-Phe	L-Cys(S-Bzl)
FADDI-064	octanoyl	D-Phe	L-Phe

Table abbreviations: L-Cys(S-Hex) = S-Hexyl-L-Cysteine; L-Cys(S-Bzl) = S-Benzyl-L-Cysteine; D-Tyr(Bzl) = O-Benzyl-D-Tyrosine; D-BipAla = Bipheyl-D-Alanine; D-OctGly = D-OctylGlycine.

based on a mechanistic model of the polymyxin-lipid A target complex.[13,14] Unlike the empirical studies of polymyxin analogs in the literature (reviewed in ref 13), our lipopeptide discovery program is the first to use a new polymyxin SAR base-dmechanistic model to design novel polymyxin-like lipopeptides that specifically target polymyxin resistance in Gram-negative bacteria. This report details the *in toto* design, synthesis, and preclinical evaluation of a new generation of polymyxin lipopeptides that specifically target polymyxin-resistant Gram-negative 'superbugs'.

RESULTS AND DISCUSSION

***In Vitro* Antibacterial Activity.** Based on our pharmacophore modeling from all reported polymyxin analogues,[13] we generated a structural model of Kdo_2lipid A in complex with a hypothetical polymyxin-like lipopeptide (FADDI-002) specifically targeting polymyxin resistance (Figure 1B). The most common mechanism of polymyxin resistance is through the modifications of the lipid A phosphates with the positively charged sugar 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine (Figure 1A).[14−17] However, it is noted that, in many instances, Gram-negative bacteria employ a combination of both the charge−charge repulsion mechanism and addition of fatty acyl groups to lipid A to attain a high level of polymyxin resistance.[14] Hence, we hypothesized that polymyxin resistance can be overcome through the introduction of hydrophobic modifications at positions R6 or R7 (Figure 1A). In our model, the (FADDI-002)−Kdo_2lipid A complex is stabilized by a combination of polar and hydrophobic interactions that would form the basis of the ability of polymyxins to insert into the outer membrane.[18] The positive charges on Dab^1 and Dab^5 allow bonding with the negatively charged 4′-phosphate group of lipid A, and Dab^8 and Dab^9 similarly bond with the 1-phosphate of lipid A. The complex is further stabilized by the hydrophobic contacts of the polymyxin *N*-terminal fatty acyl tail and the position R6 and R7 hydrophobic motif with the fatty acyl chains of lipid A. Our SAR-based hypothesis for the design of our first-generation lipopeptide (FADDI-002) was that the modification of the core polymyxin scaffold with L-octylglycine at position R7 would overcome polymyxin resistance (due to L-Ara4N modification of the lipid A phosphates) by accentuating the hydrophobic interactions with lipid A. The model was validated when lipopeptide (FADDI-002) was synthesized and shown potent antimicrobial activity against a panel of polymyxin-resistant Gram-negative clinical isolates (Supporting Information Table S1). Subsequently, we expanded our SAR-based designs across a structural series of lipopeptides with the incorporation of lipidic groups at positions R6, R7, and the *N*-terminus (Table 1).

Antimicrobial activity was screened against a panel of ATCC and clinical isolates of polymyxin-susceptible and -resistant strains *P. aeruginosa, A. baumannii* and *K. pneumoniae, Enterobacter cloacae* and Gram-positive *Enterococcus faecium* and *Staphylococcus aureus* (Supporting Information Tables S1 and S2). One pair of isogenic strains with genetically defined polymyxin resistance mechanisms was employed for each of *P. aeruginosa* and *K. pneumoniae*.[15−17] The lipopeptides showed

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very promising activity against polymyxin-resistant strains while also maintaining their activity against polymyxin-susceptible strains. Notably, against polymyxin-resistant clinical isolates of *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, these lipopeptides had MICs of 2−8 mg/L, whereas PMB or colistin was not inhibitory even at 128 mg/L. MICs of the lipopeptides obtained with the two paired *P. aeruginosa* and *K. pneumoniae* strains with genetically defined polymyxin resistance mechanisms involving lipid A modification support our SAR model that the interaction with modified lipid A can be enhanced through additional contacts via hydrophobic modifications at positions R6 and R7 of the polymyxin scaffold. Gram-positive bacteria are usually intrinsically resistant to existing polymyxins.[2] However, several lipopeptides showed, in addition to their activities against Gram-negative 'superbugs', unexpected activity against the problematic Gram-positive vancomycin-resistant *E. faecium* and methicillin- or vancomycin-resistant *S. aureus* (Supporting Information Table S2). For most of our lipopeptides, the ratios of minimal bactericidal concentrations (MBCs) to MICs were ≤4. While the *in vitro* activity of PMB and colistin decreased in the presence of Mg^{2+}, most promisingly, addition of 16 mM of Mg^{2+} led to a maximum of only 2-fold increase in MICs for (FADDI-002) and (FADDI-003), versus 16-fold increase in PMB MICs (data not shown).

In the time-killing kinetic studies, lipopeptide FADDI-003 (MIC 4 mg/L) at 4× MIC achieved ∼6 $\log_{10}$ kill against a polymyxin-resistant 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 (Supporting Information Figure S1A). Against polymyxin-susceptible *P. aeruginosa* ATCC 27853 (colistin MIC 1 mg/L), lipopeptide FADDI-003 (MIC 4 mg/L) had comparable bacterial killing to colistin (Supporting Information Figure S1B).

In summary, the presented SAR data indicates that incorporation of longer aliphatic chains (greater than 5 carbons) at position 6 or 7 significantly increases antibacterial activity. In most cases, this increase in activity is greater when the modification is at position 6. The structure of the aliphatic side chain at position 6 and 7 does have a small effect on activity with a straight chain aliphatic groups giving the best result. For modifications to positions 6 and 7, the structure of the *N*-terminal fatty acyl group is not important for activity; for example, replacement of the octanoyl group with the biphenyl group does not have any significant effect on activity. It appears that the 'hydrophobic reach' of the substituent at position 6 and 7 that is important. The stereochemistry of the residue at positions 6 and 7 is important, D- gives better activity than L- at position 6 while at position 7, L- gives better activity than D-. Incorporation of longer aliphatic side chains (greater than 5 carbons) at position 6 or 7 significantly increases protein binding in most cases to >90%.

Impact of Lipopeptide Treatment on Outer Membrane of Gram-negative Bacteria. Scanning and transmission electron microscopy (SEM and TEM) images show that treatment with 4 mg/L of lipopeptide FADDI-003 led to the formation of blebs and protrusions (evidence of cell lysis) on the bacterial cell envelope of a polymyxin-resistant clinical *P. aeruginosa* isolate (colistin MIC >128 mg/L; FADDI-003 MIC 4 mg/L) (Figure 2A). Notably, a similar blebbing effect was observed with polymyxin-susceptible Gram-negative bacterial cells treated with polymyxin B and colistin.[19]

N-phenyl-L-naphthylamine (NPN) is a hydrophobic probe widely used to measure bacterial cell permeabilization.[20,21] The

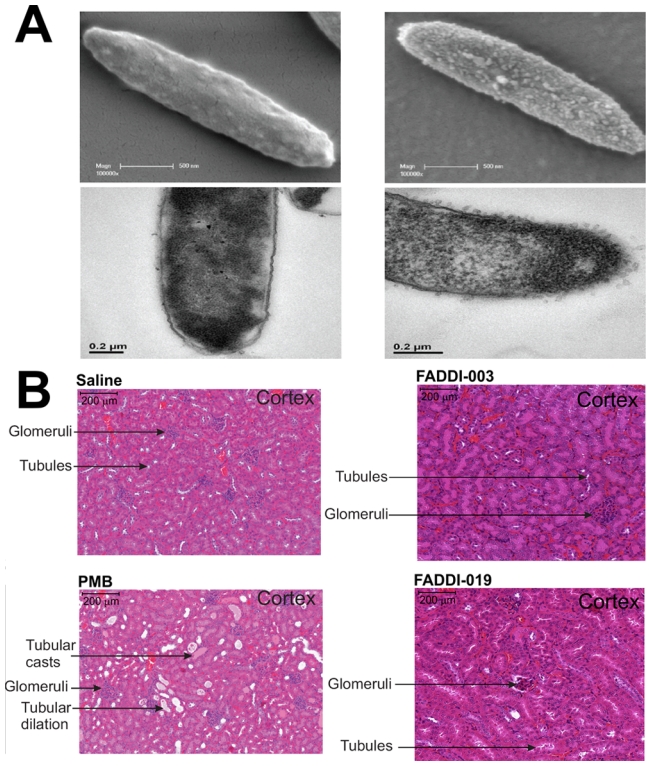

Figure 2. SEM and TEM images for the polymyxin-resistant *P. aeruginosa* FADDI-PA070 (A) untreated and treated with 4 mg/L of lipopeptide FADDI-002. (B) Microscopic image of the cortex section of the kidneys of mice treated with saline control, PMB, and lipopeptides FADDI-003 and FADDI-019 (accumulated dose 105 mg/kg).

tight packing of the fatty acyl chains of lipid A in the outer membrane leaflet limits the free diffusion of hydrophobic solutes, such as NPN. However, once permeated, intercalation of NPN into the underlying phospholipid inner leaflet and the cytoplasmic membranes produces a resultant increase in fluorescence. Exposure to lipopeptide FADDI-20 permeabilized log-phase *K. pneumoniae* cells, which was seen as an increase in NPN uptake upon addition of fixed doses of the lipopeptide (Supporting Information Figure S3). This would suggest the lipopeptide activity involves permeabilization of the Gram-negative bacterial membrane.

Interactions with LPS. Our fluorescent dansyl-polymyxin displacement assay[22] revealed significantly higher binding affinities (up to 27.1-fold) for our superior lipopeptides FADDI-002 and FADDI-003 compared to PMB and colistin (Supporting Information Table S3). The lipopeptides displayed the highest binding affinity for LPS from *S. enteric*, *E. coli*, and the polymyxin-susceptible strain of *K. pneumoniae*. Whereas, the binding affinity of the lipopeptides was lower for LPS from *P. aeruginosa* and the polymyxin-resistant *K. pneumoniae* strain.

***In Vivo* Efficacy in a Mouse Lung Infection Model.** A proof-of-concept study using a neutropenic mouse lung infection model demonstrated significantly ($p < 0.045$) better *in vivo* efficacy of lipopeptide FADDI-002 against a polymyxin-resistant clinical isolate of *P. aeruginosa* compared with colistin. With an inoculum of 5.42 ± 0.54 log CFU/lung, at 24 h after a single-dose treatment (40 mg/kg), the bacterial burden in the lungs from the mice treated with FADDI-002 was 4.75 ± 0.80 log CFU/lung, which was significantly lower than 6.71 ± 0.46

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log CFU/lung for colistin and 7.39 ± 0.17 log CFU/lung for the control.

Pharmacokinetics. In rats, lipopeptides FADDI-002 and FADDI-003 had substantially lower total clearances (0.66−1.30 mL/min/kg) and volumes of distribution (195−313 mL/kg), and longer half-lives (166−204 min), compared to colistin (5.2 mL/min/kg, 496 mL/kg, and 74.6 min, respectively).[23] Similar to colistin,[23] urinary recoveries of our lipopeptides were negligible (<1%). The plasma protein binding of the lipopeptides was measured via equilibrium dialysis with pooled healthy human plasma samples from the Australian Red Cross. PMB was ∼51% bound to human plasma proteins. In comparison the lipopeptides were highly bound to plasma proteins (>90%) (Supporting Information Table S4).

Safety and Tolerability. There was no detectable hemolysis of human red blood cells after exposure to the examined lipopeptides, PMB, and colistin at concentrations up to 32 mg/L (Supporting Information Figure S2). Despite the increase in hydrophobicity, our lipopeptides showed no significant increase in hemolytic activity against red blood cells. Following a bolus dose of four lipopeptides (FADDI-002, -019, -021, and -040) in rats (intravenous, 0.75 mg/kg) and mice (subcutaneous, 40 mg/kg), no adverse effects were observed. The results of these preliminary animal studies suggest that our lipopeptides have at least similar tolerability to PMB and colistin in rodents.

Nephrotoxicity is the major dose-limiting factor for PMB and colistin therapy.[24] The kidneys of three groups of mice (n = 3 per group) subcutaneously treated with lipopeptides FADDI-003 or FADDI-019 (accumulated dose 105 mg/kg) were subjected to histopathological examination and compared to the kidneys of mice treated with an identical concentration of PMB or a saline control (Figure 2B). Micro- and macromorphological examination of kidney sections from the lipopeptide FADDI-003 treated mice revealed no significant lesions in the cortex, medulla, and papilla regions. The kidneys of the lipopeptide FADDI-003 treated mice essentially resembled the kidneys of mice treated with the saline control and no histological grade was given. Micro-examination of the kidneys of mice treated with FADDI-019 showed mild tubular dilation and degeneration, and no tubular casts were identified. No macromorphological changes were evident, and the micromorphological changes observed in the kidneys was too mild to be graded. In comparison, the kidneys from the PMB treated mice displayed damaged tubules, with marked tubular dilation and degeneration. Tubular casts were identified in both the cortex and medulla. The kidneys appeared encapsulated by fibrotic tissue. The animals were identified to have grade 1 lesions. The lower nephrotoxicity of the lipopeptide may be due to their high plasma protein binding, which would in turn reduce the exposure of the kidneys.

In summary, our superior lipopeptides are significantly more active against polymyxin-resistant MDR Gram-negatives and have improved pharmacokinetic profiles compared to the currently used PMB and colistin. To the best of our knowledge, we are the first research group to rationally develop novel polymyxin lipopeptides using an SAR guided *in toto* synthetic strategy to introduce modifications that modulate activity against polymyxin-resistant Gram-negative 'superbugs'. The results obtained support the further development of these polymyxin-like lipopeptides as therapeutics for targeting polymyxin-resistant Gram-negative 'superbugs'.

METHODS

Example Synthesis of Lipopeptide FADDI-002. Synthesis of the protected linear peptide (coupling of residues 1−10 and N-terminal octanoyl) was carried out using out on a CEM Liberty Microwave automated peptide synthesizer using standard Fmoc solid phase peptide chemistry (Supporting Information Figure S4). Specifically, synthesis was undertaken using TCP-Resin, preloaded with Fmoc-Thr(tBu)−OH (loading 0.78 mmol/g), 0.1 mmol scale (128 mg of resin). Coupling of the Fmoc-amino acids was performed using the default instrument protocol: 5 mol equiv (relative to resin loading) of Fmoc amino acid and HCTU in dimethylformamide (DMF) with activation *in situ*, using 10 mol equiv of diisopropylethylamine (DIPEA). This was carried out for 2 min at room temperature (RT), then for 4 min at 50 °C (25W microwave power). The resin was exposed to the deprotection solution 20% piperidine in DMF for 30 s under microwave irradiation at 75 °C (35 W microwave power), the solution is removed, a fresh batch of deprotection solution and the step repeated for 3 min. The resin was then treated with DMF (4 × 2 min) then treated with 3% hydrazine in DMF (4 × 15 min) to remove the ivDde group. The resin was washed with DMF (4 × 2 min), MeOH (2 × 2 min) and diethylether (1 × 2 min) and air-dried under vacuum suction. The protected linear peptide was then cleaved from the resin by washing the resin with 1% trifluoroacetic acid (TFA) in dichloromethane (DCM) (1 × 5 min, 3 × 10 min). The resulting residue was dissolved in 50% acetonitrile/water and freeze-dried overnight. The crude protected linear peptide obtained was used in the next step without further work up. The protected linear peptide was dissolved in DMF (10 mL) to which diphenylphosphorylazide (DPPA), 0.3 mmol, 0.65 μL (3 mol equiv relative to the loading of the resin), and DIPEA 0.6 mmol, 104 μL (6 mol equiv relative to the loading of the resin) were added. This solution was stirred at RT overnight. The reaction solution was then concentrated under vacuum overnight. The resulting residue was taken up in a solution of 5% TIPS in TFA and stirred at RT for 1.5 h. The TFA was removed under a stream of nitrogen and the crude cyclic peptide precipitated with cold diethyl ether. The resulting precipitate was collected by centrifugation and air-dried in a fume food to give the crude cyclic peptide as a residue. The resulting residue was taken up in milli-Q water and desalted using a Vari-Pure IPE SAX column. The resulting solution containing the crude cyclic peptide was subjected to RP-HPLC purification. Fractions collected were analyzed by LC-MS as described in the Supporting Information online. The combined fractions were freeze-dried for two days to give the FADDI-002 TFA salt as a white solid in a yield of 11.2 mg. The purity was >95% as estimated by reversed-phase HPLC carried out employing a Phenomenex column (Luna C8(2), 100 × 2.0 mm) eluting with a gradient of 80% acetonitrile in 0.05% aqueous TFA, over 10 min at a flow rate of 0.2 mL/min, detection was at 214 nm. The compound was confirmed as having the correct molecular weight by ESI-MS analysis: m/z (monoisotopic) calculated; $C_{59}H_{104}N_{16}O_{13}S$ $[M+H]^+$1245.80, $[M+2H]^{2+}$ 623.40, $[M+3H]^{3+}$ 415.93; observed: $[M+H]^+$ 1245.90, $[M+2H]^{2+}$ 623.80, $[M+3H]^{3+}$ 416.25. The synthesis of the other lipopeptides is detailed in the online Supporting Information.

Determination of Minimum Inhibitory Concentrations (MICs). MICs were determined by the broth microdilution method.[25] Experiments were performed with Cation-Adjusted Mueller−Hinton Broth (CaMHB) in 96-well polypropylene microtiter plates. Wells were inoculated with 100 μL of bacterial suspension prepared in CaMHB (containing ∼10^6 colony forming units (cfu) per mL) and 100 μL of CaMHB containing increasing concentrations of polymyxins (0 to 128 mg/L). The MIC was defined as the lowest concentration at which visible growth was inhibited following 18−20 h incubation at 37 °C. Cell viability was determined by sampling wells at polymyxin concentrations greater than the MIC. These samples were diluted in normal saline and plated onto nutrient agar. After incubation at 37 °C for 20 h, viable colonies on the plates were counted. The limit of detection was 10 cfu/mL. Static time-kill experiments were conducted against two paired polymyxin-susceptible and -resistant strains of each species as previously described in detail.[26]

Dansyl-polymyxin LPS Binding Assay. The binding affinity of the lipopeptides for purified LPS was examined using our synthetic dansyl-polymyxin binding assay as previously reported.[22] The molar concentration of LPS was determined using the purpald Kdo assay.[27]

NPN Uptake Assay. NPN uptake was measured as described previously in detail.[20,21]

Pharmacokinetics. The pharmacokinetics (PK) of the lipopeptides were examined after single-dose intravenous administration in rats as previously described in detail.[28] Plasma and urine samples were collected over 24 h and concentrations of the lipopeptides or polymyxin B in the samples were measured by LC/MS/MS. Plasma protein binding was determined using equilibrium dialysis as previously described.[29]

***In Vivo* Efficacy in a Mouse Lung Infection Model.** *In vivo* studies were conducted using a neutropenic mouse lung infection model as we have previously reported.[30] One polymyxin-susceptible and one polymyxin-resistant *P. aeruginosa* strain were employed. Bacterial burden in the lungs was determined at 0 and 24 h after the treatment.

Nephrotoxicity. All animal experiments were approved by the Monash University Animal Experimentation Ethics Committee, which also monitored the welfare of the animals. Potential *in vivo* nephrotoxic effect of the polymyxins was evaluated in a mouse model established in our group.[24] Three groups of mice (n = 3 per group) were subcutaneous injected over 3−4 days with PMB sulfate, lipopeptides FADDI-003 or FADDI-019 (accumulated dose 105 mg/kg). At the end of the experiments, kidneys were removed and fixed in 10% formalin immediately for histopathological examination. Various levels were cut through the kidneys, capturing the cortex, medulla and papilla, and sections were stained with hematoxylin and rosin. A graded scoring matrix was employed to illustrate the degrees of change: grade 1, mild tubular damage with tubular dilation, prominent nuclei and a few pale tubular casts; grade 2, severe tubular damage with necrosis of tubular epithelial cells and numerous tubular casts; grade 3: cortical necrosis/infarction of tubules and glomeruli with or without papillary necrosis. The grades were given the following scores: grade 1 = 1; grade 2 = 4; and grade 3 = 10.[24]

■ ASSOCIATED CONTENT

Supporting Information

Tables S1 and S2: MIC values for the lipopeptides for Gram-negative and -positive bacteria strains. Table S3: binding affinity of the lipopeptides for isolated bacterial lipopolysaccharide (LPS) determined by a dansyl-polymyxin displacement assay. Table S4: plasma protein binding of lipopetides determined by equilibrium dialysis using pooled human plasma. Detailed description of the synthesis of the lipopeptides. Figure S1: static time-kill kinetics of lipopeptide FADDI-019 and colistin against a colistin-resistant *P. aeruginosa* isolate. Figure S2: hemolytic effect of lipopeptides after 1 h exposure to human red blood cells. Figure S3: NPN uptake kinetics of *K. pneumoniae* cells treated with FADDI-020. Figure S4: lipopeptide synthetic scheme. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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