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Antimicrobial Activity and Toxicity of the Major Lipopeptide Components of Polymyxin B and Colistin: Last-line Antibiotics against Multidrug-Resistant Gram-negative Bacteria

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Abstract

Polymyxin B and colistin are currently used as a ‘last-line’ treatment for multidrug-resistant Gram-negative bacteria. However very little is known about the pharmacological differences between polymyxin B₁, polymyxin B₂, colistin A, colistin B, the major cyclic lipopeptides components present in polymyxin B and colistin products. Here, we report on the *in vitro* and *in vivo* antimicrobial activity and toxicity of these major lipopeptide components. All four lipopeptides had comparable MICs (<0.125–4 mg/L) against a panel of clinical Gram-negative isolates. They also had comparable *in vivo* antimicrobial activity ($\log_{10}$ CFU/mL >-3) and nephrotoxicity (mild to moderate histological damage) in mouse models. However, polymyxin B₁ and colistin A showed significantly higher (> 3-fold) *in vitro* apoptotic effect on human kidney proximal tubular HK-2 cells than polymyxin B₂ and colistin B, respectively. Compared to the commercial polymyxin and colistin products, the individual lipopeptide components had slightly more *in vivo* antimicrobial activity. Our results highlight the need to re-assess pharmacopoeial standards for polymyxins B and colistin and to standardize the composition of the different commercial products of polymyxin antibiotics.

Keywords

Polymyxin; colistin; nephrotoxicity; Gram-negative bacteria; multidrug-resistance

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ASSOCIATED CONTENT

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI:

RP-HPLC profiles for polymyxin B and colistin commercial products. LC-MS analysis for polymyxin B₁, polymyxin B₂, colistin A and colistin B.

The authors declare no competing financial interest.

INTRODUCTION

The polymyxins are a family of cyclic lipopeptides isolated from *Paenibacillus polymyxa* that display exceptional antimicrobial activity against a range of Gram-negative bacteria.^{1,2} First discovered in 1947 and introduced into clinical medicine in the late 1950s,^{3–5} their use in clinical practice waned in the 1970s because of the potential for adverse effects, primarily nephrotoxicity.^{6–9} However, the continuing world-wide emergence of multidrug-resistant (MDR) Gram-negative bacteria and the lack of new antibiotics to treat infections caused by these ‘superbugs’ have seen a polymyxin revival. Polymyxins are being increasingly used for last-line therapy against problematic MDR Gram-negative pathogens, namely *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae*.^{1,2,10–13} Currently, polymyxin B and colistin methanesulfonate (the inactive pro-drug of colistin,¹⁴ also known as polymyxin E) are the only polymyxins clinically available for use. The issues of toxicity with the polymyxins still remain with recent clinical studies reporting that polymyxin-associated nephrotoxicity can occur in up to 60% of patients when administered intravenously; and is the major dose-limiting factor for their optimal clinical use.^{15–18}

In terms of their chemical structures, the polymyxins are non-ribosomal cyclic lipopeptides which are represented by the general structure illustrated in Table 1. The polymyxin decapeptide core contains an intramolecular cyclic heptapeptide amide-linked loop between the amino group of the side chain of the diaminobutyric acid (Dab) residue at position 4 and the carboxyl group of the C-terminal threonine residue. They also have several other distinguishing structural features, including five non-proteogenic Dab residues which are positively charged at physiological pH, conserved hydrophobic residues at positions 6 and 7 and an N-terminal fatty acyl group.¹⁹ Commercial preparations of polymyxin B and colistin that are used in the clinic are mixtures of closely related lipopeptides obtained from fermentation. In the case of polymyxin B, up to 39 distinct lipopeptides have been identified to date in these fermentation products, with only seven of these having their chemical structures unequivocally determined (Table 1).^{20–22} For colistin, up to 36 distinct lipopeptides have been identified to date, with eleven of these having their chemical structures unequivocally determined (Table 1).^{22–24} Differences in the structures of these individual cyclic lipopeptides centre around the structure of the N-terminal fatty acyl group and the hydrophobic amino acids present at positions 6 and 7.

For commercial preparations of both polymyxin B and colistin, the majority of the lipopeptide content (>70 %) is represented by only two cyclic lipopeptides. For polymyxin B, they are polymyxin B₁ and B₂,^{22,25} whilst for colistin they are colistin A (polymyxin E₁) and colistin B (polymyxin E₂) (Table 1).^{22,26} However, it has been found that the proportion of these major cyclic lipopeptide components in commercial preparations of polymyxin B and colistin can vary between different brands and even between different batches from the same manufacturer.^{25–27} Variations in the nature of the heterogeneity among different polymyxin batches or products can contribute to variability in the pharmacokinetics of polymyxin B, colistin methanesulfonate and formed colistin *in vivo*, and subsequent pharmacodynamics and toxicodynamics. Except for a preliminary *in vitro* antimicrobial activity study on polymyxin B components,²⁸ to date no extensive studies have been undertaken to examine the antimicrobial efficacy and toxicity of the individual components

of polymyxin B and colistin *in vitro* and *in vivo*. This is a reflection of the fact that they were approved for clinical use long before the rigorous requirements for modern pharmaceutical drug approval were put into place. Considering the expanding use of polymyxins in the clinic in the face of increasing antimicrobial resistance, the question has to be asked: *what is the significance and contribution of the major lipopeptide components to the efficacy and toxicity of the polymyxin B and colistin antibiotics?* The answer can have important implications for the use of polymyxin B and colistin methanesulfonate in the clinic, as well as in the production of polymyxin B and colistin methanesulfonate products. To this end, we present here for the first time detailed investigation examining the *in vitro* and *in vivo* activity and toxicity of the major polymyxin B and colistin lipopeptide components, polymyxin B₁ and B₂ and colistin A and B. Throughout the remainder of the paper ‘polymyxin B’ refers to the commercial multi-component polymyxin B product and ‘colistin’ refers to the commercial multi-component colistin product.

RESULTS AND DISCUSSION

The chemical structures of polymyxin B₁, polymyxin B₂, colistin A and colistin B (Table 1) are closely related. Polymyxin B₁ only differs from polymyxin B₂ by the presence of an extra methylene group, whereby the *N*-terminal fatty acyl group of polymyxin B₂ (6-methylheptanonyl) is extended by one carbon atom from C7 to C8 to give (*S*)-6-methyloctanoyl in polymyxin B₁. This creates a chiral-centre at position 6 in this fatty acyl chain. This structural difference is also observed between colistin A and B. Although this difference is minor, it may still have an impact on the biological properties of these lipopeptide components. Comparing the chemical structures of polymyxin B₁ and polymyxin B₂ with colistin A and colistin B, differences arise only in the structure of the amino acid at position 6, with polymyxin B₁ and polymyxin B₂ containing a D-phenylalanine residue at this position, whilst colistin A and colistin B contain a D-leucine residue (Table 1). Although both are considered highly hydrophobic residues, they are structurally different, with D-phenylalanine having an aromatic benzyl side chain and leucine an aliphatic isobutyl side chain. Here the aromaticity and larger size of the D-phenylalanine side chain may afford different biological activity. In order to examine the antimicrobial activity and toxicity of these lipopeptide components, they were first isolated from commercial products of polymyxin B and colistin by RP-HPLC. For the commercial polymyxin B product used here, polymyxin B₁ represented 53% of the content (as measure by HPLC) and polymyxin B₂ 23% of the content (Supplementary Info, Figure S1). For the commercial colistin product used in this study, colistin A represented 58% of the content and colistin B 19% of the content (Supplementary Info, Figure S2). Despite there only being a one carbon difference in the *N*-terminal fatty acyl groups between polymyxin B₁ and B₂ and likewise for colistin A and B, base-line resolution of the individual components was observed under RP-HPLC conditions. As a result, polymyxin B₁, polymyxin B₂, colistin A and colistin B were readily isolated from commercial preparations of polymyxin B and colistin with purities of greater than 97%. The identity and purity of the final isolated cyclic lipopeptides was confirmed by LC-MS analysis (Supplementary Info, Figures S3–S6).

The *in vitro* antimicrobial activity of polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A and colistin B were measured against a panel of Gram-negative polymyxin-

susceptible ATCC type strains and MDR clinical isolates of *P. aeruginosa*, *A. baumannii*, *K. pneumoniae* and *Enterobacter cloacae* (Table 2). Overall, no substantial differences in the MICs were observed between all polymyxin materials (commercial mixtures and individual components) against all of the isolates examined; MICs were generally within a two-fold dilution and a similar observation was reported previously for polymyxin B₁ and B₂.²⁸ Against *P. aeruginosa* isolates, polymyxin B₁ and B₂ had MICs in the range of 1–2 µg/mL, whilst colistin A and B were slightly less active with MICs of 1–4 µg/mL for colistin A and 2–4 µg/mL for colistin B. A similar trend was also observed for *A. baumannii* where polymyxin B₁ and B₂ had MIC values of 0.5–1 µg/mL, whilst for colistin A and B MICs were in the range of 0.5–1 µg/mL and 1–2 µg/mL, respectively. For the *K. pneumoniae* isolates greater antimicrobial activity was observed, with polymyxin B₁ and B₂ having MICs of <0.125–0.5 µg/mL and 0.25–0.5 µg/mL, respectively; colistin A and colistin B had corresponding MIC values in the range of <0.125–0.25 µg/mL and 0.25 µg/mL. A similar trend was also observed for the *E. cloacae* isolates where MIC values were in the range of <0.125–0.5 µg/mL and 0.25–1 µg/mL obtained for polymyxin B₁ and B₂, respectively; for colistin A and B, MIC values in the range of <0.125–0.25 µg/mL and 0.25–0.5 µg/mL were obtained. Likewise, polymyxin B (*P. aeruginosa* MIC 1–2 µg/mL; *A. baumannii* MIC 0.5–1 µg/mL; *K. pneumoniae* <0.125–0.5 µg/mL and *E. cloacae* 0.25 µg/mL) and colistin (*P. aeruginosa* MIC 1–2 µg/mL; *A. baumannii* MIC 0.5–1 µg/mL; *K. pneumoniae* 0.25 µg/mL and *E. cloacae* <0.125–0.25 µg/mL) had similar *in vitro* antimicrobial activity to their respective individual components. It is possible that the minor lipopeptide components (not isolated in this work) present in the commercial polymyxin B and colistin products do not have a greater or synergistic influence on the *in vitro* antimicrobial activity. The *in vivo* efficacy of polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A and colistin B was examined against *P. aeruginosa* ATCC 27853 in a neutropenic mouse blood infection model. Significant reduction in the bacterial burden (mean log₁₀ CFU/mL >3) was observed for the commercial products and the respective individual components (Table 3). In this model polymyxin B₁ and polymyxin B₂ had comparable *in vivo* efficacy, and likewise for colistin A and colistin B. Notably, the commercial polymyxin B and colistin products were slightly less active than their respective individual components *in vivo* (Table 3). Considering that >20% of the content of the commercial products of polymyxin B and colistin used here is represented by minor lipopeptide components, the results in this study suggest that the minor lipopeptide components may be less active *in vivo*. Further evaluation is warranted to determine whether these relative activity profiles *in vivo* extend to other Gram-negative species.

Nephrotoxicity remains an important clinical issue for the polymyxins because it impacts the ability of clinicians to increase the dose for treatment of MDR Gram-negative infections.^{2,15–18,29,30} Furthermore, suboptimal dosing may promote the emergence of polymyxin resistance.^{29–32} In the present study, we first examined the *in vitro* apoptotic effect of the major lipopeptide components of polymyxin B and colistin on human kidney proximal tubular cells (HK-2).³³ The HK-2 cells were chosen in this study, as pharmacokinetic studies have demonstrated that polymyxin B and colistin are significantly reabsorbed by renal tubular cells after filtration by glomeruli.^{34,35} The significant accumulation of polymyxins in both rat and human renal tubular cells has also been

confirmed recently using a novel iodine-labeled polymyxin probe with a correlative microscopy approach.³⁶ Moreover, it has been demonstrated that polymyxins can cause apoptosis in renal tubular cells (e.g. HK-2).^{33,37} In the present study, HK-2 cells were incubated with 0.25 mM of polymyxin B, polymyxin B₁ or polymyxin B₂; or 0.75 mM of colistin, colistin A or colistin B (Figure 1). The different concentrations used was based on the EC₅₀ values for polymyxin B (0.35 [95% CI 0.29–0.42] mM),³³ and colistin (0.69 [0.60–0.81] mM), which suggested that the former was more toxic to the HK-2 cells (Figure 2a). Interestingly, significant differences in the cell viability between the individual lipopeptide components of polymyxin B and colistin were observed (Figure 2b). Cells treated with polymyxin B₂ showed significantly higher cell viability ($68.5 \pm 7.1\%$) compared to the cells treated with polymyxin B₁ ($11.8 \pm 6.5\%$) and polymyxin B ($19.2 \pm 11.5\%$). Similarly, cell viability of the HK-2 cells was significantly higher following treatment with colistin B ($67.8 \pm 5.4\%$) than with colistin A ($16.8 \pm 9.9\%$) or colistin ($31.2 \pm 3.9\%$). This result is very interesting as only one carbon difference in the *N*-terminus (Table 1) led to a significant difference in the *in vitro* apoptotic effect against HK-2 cells. Furthermore, the results demonstrate that in this *in vitro* cell assay the lipopeptide components of polymyxin B were significantly more apoptotic than colistin and its two major components; *in vitro* toxicity of commercial polymyxin B and colistin products has been observed against HK-2 cells using different metabolic and biochemical assays.^{38,39} This result would point to an apoptotic role for the D-leucine to D-phenylalanine substitution at position 6 between colistin and polymyxin B against kidney tubular cells.

We further examined the nephrotoxicity of the major components of polymyxin B and colistin in a mouse nephrotoxicity model. The model involved 2-hourly subcutaneous administration of the polymyxin until an accumulated dose of 72 mg base/kg was achieved, followed by histological examination of the kidneys (Table 4 and Supplementary Table for individual kidney histology damage scores). The dosing regimen was well tolerated for each polymyxin with no acute toxicity⁴⁰ being observed. The saline control had no observable histological damage in the kidney (Figure 3A) and was given a semi-quantitative score (SQS) of 0 (Table 4).⁴¹ In comparison, histological examination of the kidneys from the mice treated with polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A or colistin B showed comparable histological damage (Table 4). The kidneys from the animals treated with the polymyxins were identified to have either grade 1 or grade 2 lesions. Kidneys showing grade 1 lesions had tubule damage with tubular dilation and degeneration (Figure 3B). Tubular casts were identified mainly in the cortex regions. These kidney samples were given a SQS score of +1, representing mild histological damage (Table 4). For the kidneys showing grade 2 damage the tubules were severely damaged, with tubular dilation, degeneration and necrosis of the tubular epithelial cells (Figure 3C). Numerous tubular casts were identified within both medulla and cortex regions. The kidneys were identified as having grade 2 lesions and were given a SQS score of +2 (Table 4) which represents mild to moderate histological damage. Overall, the results from the *in vivo* study do not reveal any significant differences between polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A and colistin B in terms of histological damage to the kidneys. It is interesting that while no significant differences in nephrotoxicity between the individual cyclic lipopeptide components was observed *in vivo*, significant differences in apoptotic effect were observed

in our *in vitro* cell model, as discussed above. This disparity between apoptotic effects *in vitro* and *in vivo* nephrotoxicity has been observed for other polymyxin-like lipopeptides in our group and by others and requires further investigation.⁴² It may be due to regeneration of the kidney *in vivo* or that the uptake of the individual polymyxins is different for isolated kidney cells.

The British (BP), European (Ph. Eur) and USA Pharmacopoeias have recently established limits on the minimum amount of certain lipopeptide components required in polymyxin B and colistin methanesulfonate products.^{43–45} For polymyxin B products, the sum of polymyxin B₁, B₂, B₃ and B₁-Ile⁷ (Table 1) must be >80% of the dried polymyxin B sulfate sample. Furthermore, polymyxin B₃ must be no more than 6% whilst polymyxin B₁-Ile must be less than 15% of the content. For colistin methanesulfonate products only the Ph. Eur and BP have established limits where the sum of colistin A (polymyxin E₁), colistin B (polymyxin E₂), E₃, E₁-Ile and E₇ resulting from the pro-drug (Table 1) should be >77% of the dried colistin methanesulfonate sample.^{43,44} Polymyxin E₃, E₁-Ile and E₇ individually should be no more than 10% of the content of the sample. It should be pointed out that no scientific evidence is provided for the limits proposed for these major components in the pharmacopoeias. No limits have been set on the other minor cyclic lipopeptide components that have been structurally identified to date (Table 1) present in commercial polymyxin B and colistin products. Our results suggest that the commercially available products of polymyxin B and colistin examined had slightly less *in vivo* antimicrobial activity than their respective individual major components. It is possible that the minor components in the commercial products of polymyxin B and colistin, which can represent up to 30% of their lipopeptide content are less active than the major components. Further work should be undertaken to examine the antimicrobial activity as well as toxicity of these minor components. This would require the structural elucidation of all the lipopeptide components in the polymyxin B and colistin products. Nevertheless, our study further highlights the need to standardize the composition of different commercial parenteral products of polymyxin B and colistin, and the need to re-assess pharmacopoeial standards set out for polymyxins B and colistin. This was recently highlighted in the Prato polymyxin consensus as one of the key objectives for the optimization of the clinical use of polymyxin B and colistin products.¹³ As a result, the European Commission is currently undertaking a review of the pharmacopoeial limits. Ideally, polymyxin B and colistin antibiotics should be limited to a single cyclic lipopeptide component for standardization of different batches and products.

In conclusion, we have examined in detail the *in vitro* and *in vivo* antimicrobial activity and toxicity of the major lipopeptide components of the clinically utilized polymyxin B and colistin. Overall, polymyxin B₁, polymyxin B₂, colistin A and colistin B had comparable *in vitro* and *in vivo* antimicrobial activity and nephrotoxicity in mice. However, differences in their *in vitro* apoptotic effect on HK-2 cells were observed and further studies are being conducted in our laboratory to investigate this phenomenon.

METHODS

Isolation and purification of polymyxin B₁, polymyxin B₂, colistin A and colistin B by RP-HPLC

Polymyxin B₁ and polymyxin B₂—Polymyxin B₁ and Polymyxin B₂ were isolated and purified from commercial polymyxin B sulfate (Beta Pharma, China) using RP-HPLC. This was carried out on a Waters Prep LC system, employing a Phenomenex Axia column (Luna C8(2), 250 × 50.0 mm ID, 100 Å, 10 micron), connected to a Waters 486 tuneable absorbance detector (214 nm). Solvent A was 0.1% TFA/water, and Solvent B was 0.1% TFA / acetonitrile. Polymyxin B (2 gram) was dissolved in 0.1% TFA / 10% acetonitrile / water (20 mL) of which half (10 mL) was injected onto the column. Lipopeptide components were eluted with a gradient of 0–60% Solvent B over 60 min at a flow rate of 40 mL/min. This step was repeated for the remaining 10 mL of the polymyxin B solution. The fractions collected were analyzed by LC-MS. A Shimadzu 2020 LC-MS system was employed, incorporating a photodiode array detector (214 nm) coupled to an electrospray ionization source and a single quadrupole mass analyzer. Solvent A was 0.05% TFA/water, and Solvent B was 0.05% TFA/acetonitrile. A Phenomenex column (Luna C8(2), 100 × 2.0 mm ID) was used, eluting with a gradient of 0–60% solvent B over 10 min at a flow rate of 0.2 mL/min. Mass spectra were acquired in the positive ion mode with a scan range of 200 – 2,000 *m/z*. Fractions containing the desired lipopeptide components were combined and lyophilized for two days to give either polymyxin B₁ or polymyxin B₂, as their corresponding TFA salts. Polymyxin B₁ was isolated as in a yield of 307.0 mg, retention time (*t_R*) at 214 nm = 12.57 min (% Area: 97.9%). ESI-MS analysis: *m/z* (monoisotopic) [M + H]⁺ 1204.00, [M+2H]²⁺ 602.80, [M+3H]³⁺ 402.30. Calculated for C₅₆H₉₈N₁₆O₁₃ [M + H]⁺ 1203.74, [M+2H]²⁺ 602.37, [M+3H]³⁺ 401.91. Polymyxin B₂ was isolated in a yield of 198.0 mg, *t_R* at 214 nm = 12.22 min (% Area: 97.8%). ESI-MS analysis: *m/z* (monoisotopic) [M+H]⁺ 1190.00, [M+2H]²⁺ 595.85, [M+3H]³⁺ 397.60. Calculated for C₅₅H₉₆N₁₆O₁₃ [M+H]⁺ 1189.73, [M+2H]²⁺ 595.38, [M+3H]³⁺ 397.24.

Colistin A and colistin B—Colistin A and Colistin B were isolated and purified from a commercial colistin sulfate (Beta Pharma, China) as described above. Colistin A and colistin B were obtained as their corresponding TFA salts. Colistin A was isolated in a yield of 189.0 mg, *t_R* at 214 nm = 12.30 min (% Area: 97.9%). ESI-MS analysis: *m/z* (monoisotopic) [M+H]⁺ 1170.00, [M+2H]²⁺ 585.85, [M+3H]³⁺ 390.95. Calculated for C₅₃H₁₀₀N₁₆O₁₃ [M + H]⁺ 1169.77, [M+2H]²⁺ 585.38, [M+3H]³⁺ 390.59. Colistin B was isolated in a yield of 629.0 mg, *t_R* at 214 nm = 11.95 min (% Area: 99.3%). ESI-MS analysis: *m/z* (monoisotopic) [M+H]⁺ 1156.00, [M+2H]²⁺ 578.85, [M+3H]³⁺ 386.30. Calculated for C₅₂H₉₈N₁₆O₁₃ [M + H]⁺ 1155.75, [M+2H]²⁺ 578.37, [M+3H]³⁺ 385.92.

Measurements of minimum inhibitory concentrations (MICs)

MICs were determined by the broth micro-dilution method⁴⁶ against the following polymyxin-susceptible ATCC and clinical isolates: *P. aeruginosa* ATCC 27853, FADDI-PA022, FADDI-PA025; *A. baumannii* ATCC 19606, FADDI-AB034, ATCC 17978; *K. pneumoniae* ATCC13883, FADDI-KP032; and *E. cloacae* FADDI-EC006, FADDI-EC001, FADDI-EC003. Experiments were performed with cation-adjusted Mueller-Hinton broth

(CaMHB) in 96-well polystyrene microtiter plates. Wells were inoculated with 100 μ L of bacterial suspension prepared in CaMHB (containing $\sim 10^6$ colony forming units (CFU) per mL) and 100 μ L of CaMHB containing increasing concentrations of polymyxins (0 to 32 mg/L). The MIC measurements were carried out in duplicates with the MIC being defined as the lowest concentration at which visible growth was inhibited following 18–20 h incubation at 37°C.

***In vivo* efficacy study using a neutropenic mouse blood infection model**

All animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee (Monash University; Approval ID: MIPS.2010.35) and were conducted according to the Australian Code for the Care and Use of Animals for Scientific Purposes (8th edition, 2013). The polymyxin-susceptible strain *P. aeruginosa* ATCC 27853 was subcultured on nutrient agar plates. One colony was dispersed in 10-mL CaMHB and incubated overnight. On day 2, an aliquot (0.2 mL) of the overnight culture suspension was dispersed in 20-mL CaMHB and incubated for production of early log-phase growth bacterial culture. Bacteria in the early log-phase growth suspension were concentrated by centrifugation (3,220 *g* for 10 min) and re-suspended in sterile 0.9% saline for inoculation into mice. The bacterial cell concentration (CFU/mL) in saline was estimated by determining the optical density (OD) of the suspension at 600 nm, and confirmed by plating the suspension on nutrient agar plates. Swiss mice (22 to 28 g) were rendered neutropenic by injecting two doses of cyclophosphamide intraperitoneally, 4 days (150 mg/kg) and 1 day (100 mg/kg) prior to inoculation. Bloodstream infection was established by injecting intravenously 50 μ L bolus of early log-phase bacterial suspension (4×10^8 CFU/mL). Solutions for administration of polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A and colistin B were prepared at a concentration of 1 mg (free base) per mL in sterile 0.9% saline. At 2 h after inoculation, a mouse in the treatment groups was injected intravenously with one of the above solutions at 4 μ L/g body weight (BW) (i.e. free base 4 mg/kg BW), while the same volume of saline was injected into the control mice. At 0 h or at 4 h after the administration of antibacterial drug or saline (control), animals were euthanized by inhalation of overdose isoflurane. The skin on the chest and fore-paws of each animal were thoroughly cleaned with 70% ethanol and Betadine®. Blood was collected via cardiac puncture using a 1-mL syringe rinsed with heparin (5,000 IU/mL), diluted serially in sterile 0.9% saline and plated on nutrient agar plates using a spiral plater. The agar plates were incubated at 37°C overnight. Bacterial colonies on the plate were counted and the bacterial load ($\log_{10}$ CFU/mL) in blood in each mouse was calculated. For each time point, three mice were examined and the mean and standard deviation (SD) were calculated. The *in vivo* efficacy of the compounds was calculated as the difference of the $\log_{10}$ CFU/mL values between the treated mice and the control mice at 4 h ($\log = \log_{10}(\text{treated})\text{CFU/mL} - \log_{10}(\text{control})\text{CFU/mL}$).

Assessment of the apoptosis and viability of human (HK-2) kidney proximal tubular cells treated with polymyxins using FACS (Fluorescence-activated Cell Sorting) Analysis

Methodology for the culturing of HK-2 cells, determination of the EC₅₀ and % cell viability was previously described in detail.³³

Measurement of nephrotoxicity in mice

All animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. Stock solutions of polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A and colistin B in saline (5 mg base/mL) were stored at 4°C before use. Mice were subcutaneously administered either polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A or colistin B at 12 mg base/kg every 2 h until an accumulated dose of 72 mg was achieved. At 20 h after the last dose, mice were euthanized by inhalation of an overdose of isoflurane. Immediately after sampling of blood by cardiac puncture, the right kidney from each mouse was collected and placed in 10% buffered formalin pH 7.4 (Sigma, Australia) in a 5-mL plastic tube, and the left kidney placed in a pre-weighed 14-mL plastic tube, weighed again and stored at -20°C pending homogenization and analysis of polymyxin B and colistin. The frozen kidney samples were thawed, homogenized in 2 mL of Milli-Q water and stored in a -20°C freezer. The formalin-fixed kidneys were subjected to histological examination at the Australian Phenomics Network-Histopathology and Organ Pathology (University of Melbourne, Parkville, VIC, Australia). Samples were examined by a pathologist who was blind to the treatment groups. Lesions were rated as described previously.⁴¹ A brief description of the rating system follows. The nature and severity of the histological changes were initially graded: Grade 1: mild acute tubular damage with tubular dilation, prominent nuclei and a few pale tubular casts; Grade 2: severe acute tubular damage with necrosis of tubular epithelial cells and numerous tubular casts; Grade 3: acute 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. The percentages of the kidney slices affected were scored as follows: <1% = 0, 1 to <5% = 1, 5 to <10% = 2, 10 to <20% = 3, 20 to <30% = 4, 30 to <= 40% = 5, and <40% = 6. The overall kidney histology score was calculated as the product of percentage score and grade score. Finally a simplified semi-quantitative score (SQS) (a scale of 0 to +5) for renal histological changes was assigned as follows: SQS 0 = no significant change (overall score, <1); SQS +1 = mild damage (overall score, 1 to <15); SQS +2 = mild to moderate damage (overall score, 15 to <30); SQS +3 = moderate damage (overall score, 30 to <45); SQS +4 = moderate to severe damage (overall score, 45 to <60); and SQS +5 = severe damage (overall score, 60).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ATCC American Type Culture Collection

CFU	colony forming units
LC-MS	liquid chromatography-mass spectrometry
MIC	minium inhibitory concentration
RP-HPLC	Reversed Phase–High Performance Liquid Chromatography
TFA	trifluoroacetic acid

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Figure 1.

Staining the HK-2 cells with annexin V-alexa fluor 488 and PI: (A) Control cells; (B) 0.25 mM polymyxin B; (C) 0.25 mM polymyxin B₁; (D) 0.25 mM polymyxin B₂; (E) 0.75 mM colistin; (F) 0.75 mM colistin A; (G) 0.75 mM colistin B. Upper-left Quadrant: Cells stained by annexin V (early apoptotic cells), Upper-right Quadrant: Cells stained by both annexin V-PI (late apoptotic cells), Bottom-right Quadrant: Cells stained by PI (necrotic cells), and Bottom-left Quadrant: Cells not stained by annexin V/PI (viable cells).

Figure 2.
 (A) Apoptotic effect on HK-2 cells of the commercial polymyxin B and colistin products. Note the dose response curve for polymyxin B was obtained from a previous study.³³ (B) Cell viability of HK-2 cells after treatment for 24 h with polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A and colistin B (Mean $\pm$ SD; n = 3).

Figure 3.

Representative images of kidney sections from histological examination: **(A)** Mouse kidney from saline control group showing no histological damage; **(B)** Mouse kidney after exposure to polymyxin B₂ with grade 1 lesions (SQS = +1), showing tubule damage with tubular dilation and degeneration; **(C)** Mouse kidney after exposure to polymyxin B with grade 2 lesions (SQS = +2), showing greater tubular damage, tubular dilation, tubular casts, degeneration and necrosis of the tubular epithelial cells.

Table 1

The chemical structures of the individual components identified in commercial preparations of polymyxin B and colistin (polymyxin E).

Polymyxin	Fatty-acyl Group (R₁)	Pos 6. Amino acid (R₂)	Pos 7. Amino acid (R₃)
B₁	(<i>S</i>)-6-Methyloctanoyl	D-Phe	L-Leu
B₂	6-Methylheptanoyl	D-Phe	L-Leu
B₁-Ile	(<i>S</i>)-6-Methyloctanoyl	D-Phe	L-Ile
B₂-Ile	6-Methylheptanoyl	D-Phe	L-Ile
B₃	Octanoyl	D-Phe	L-Leu
B₄	Heptanoyl	D-Phe	L-Leu
B₅	Nonanoyl	D-Phe	L-Leu
B₆	3-Hydroxy-6-methyloctanoyl [#]	D-Phe	L-Leu
E₁(colistin A)	(<i>S</i>)-6-Methyloctanoyl	D-Leu	L-Leu
E₂(colistin B)	6-Methylheptanoyl	D-Leu	L-Leu
E₃	Octanoyl	D-Leu	L-Leu
E₄	Heptanoyl	D-Leu	L-Leu
E₇	7- Methyl octanoyl	D-Leu	L-Leu
E₁-Ile	(<i>S</i>)-6-Methyloctanoyl	D-Leu	L-Ile
E₁-Val	(<i>S</i>)-6-Methyloctanoyl	D-Leu	L-Val
E₁-Nva	(<i>S</i>)-6-Methyloctanoyl	D-Leu	L-Nva
E₂-Ile	6-Methylheptanoyl	D-Leu	L-Ile
E₂-Val	6-Methylheptanoyl	D-Leu	L-Val
E₈-Ile	6-Methylheptanoyl	D-Leu	L-Ile

D-Phe = D-Phenylalanine, L-Leu = L-Leucine, L-Ile = L-Isoleucine, L-Val = L-Valine

[#] Stereochemistry at C3 and C6 is yet to be confirmed

Table 2

MICs for polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A and colistin B against Gram-negative bacteria.

MIC (µg/mL)													
<i>P. aeruginosa</i>				<i>A. baumannii</i>				<i>K. pneumoniae</i>				<i>E. cloacae</i>	
Peptide	ATCC 27853	FADDI-PA022	FADDI-PA025	ATCC 19606	FADDI-AB034	ATCC 17978	ATCC 13883	FADDI-KP032	FADDI-EC006	FADDI-EC001	FADDI-EC003		
Polymyxin B	1	1	2	1	0.5	1	<0.125	0.5	0.25	0.25	0.25		
Polymyxin B ₁	1	2	2	0.5	0.5	1	<0.125	0.5	0.5	<0.125	0.25		
Polymyxin B ₂	1	1	2	1	1	0.5	0.25	0.5	0.5	1	0.25		
Colistin	1	2	2	1	1	0.5	0.25	0.25	0.25	<0.125	<0.125		
Colistin A	2	1	4	2	1	1	0.25	<0.125	0.25	>0.125	<0.125		
Colistin B	4	2	2	2	1	1	0.25	0.25	0.5	0.5	0.25		

Table 3

In vivo efficacy of polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A and colistin B against *P. aeruginosa* ATCC 27853 in a mouse blood infection model (Mean $\pm$ SD; n = 3).

Peptide	MIC (μ g/mL)	Mean log ₁₀ CFU/mL
Polymyxin B	1	-3.80 $\pm$ 0.29
Polymyxin B ₁	1	-4.13 $\pm$ 0.47
Polymyxin B ₂	1	-4.24 $\pm$ 0.42
Colistin	1	-3.35 $\pm$ 0.38
Colistin A	2	-4.30 $\pm$ 0.39
Colistin B	4	-3.99 $\pm$ 0.40

Table 4

Nephrotoxicity of polymyxin B, polymyxin B₁, polymyxin B₂, colistin, colistin A and colistin B in a mouse model.

Peptide	n	SQS Kidney histology score
Polymyxin B	3	+1
Polymyxin B ₁	3	+1
Polymyxin B ₂	3	+1→+2
Colistin	3	+1→+2
Colistin A	3	+2
Colistin B	3	+1→+2

SQS 0 = no significant change; SQS +1 = mild damage; SQS +2 = mild to moderate damage