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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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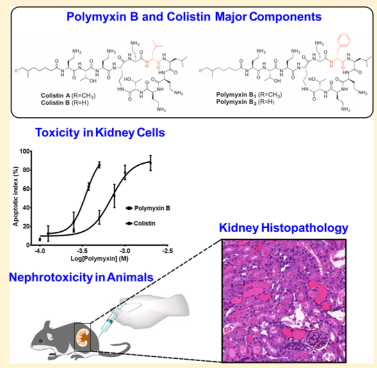
Ⓢ *Supporting Information*

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 B_1, polymyxin B_2, colistin A, and colistin B, the major cyclic lipopeptide 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 minimum inhibitory concentrations (MICs) (<0.125−4 mg/L) against a panel of clinical Gram-negative isolates. They also had comparable in vivo antimicrobial activity ($\Delta\log_{10}$ colony-forming units (CFU)/mL > −3) and nephrotoxicity (mild to moderate histological damage) in mouse models. However, polymyxin B_1 and colistin A showed significantly higher (>3-fold) in vitro apoptotic effect on human kidney proximal tubular HK-2 cells than polymyxin B_2 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 reassess pharmacopoeial standards for polymyxin 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*

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 worldwide 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 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 nonribosomal 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 nonproteogenic 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 11 of these having their chemical structures unequivocally determined (Table 1).[22−24] Differences in the structures of these individual cyclic lipopeptides center around the structure of the N-terminal fatty acyl group and the hydrophobic amino acids present at positions 6 and 7.

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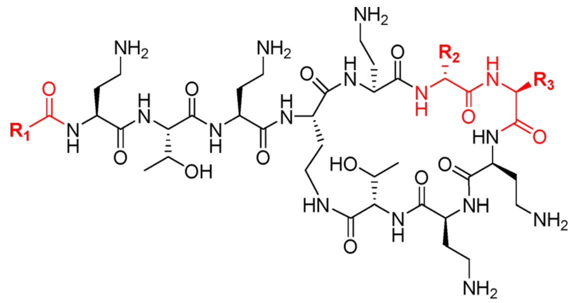
Table 1. Chemical Structures of the Individual Components Identified in Commercial Preparations of Polymyxin B and Colistin (Polymyxin E)



polymyxin	fatty-acyl group (R_1)	position 6 amino acid[a] (R_2)	position 7 amino acid[a] (R_3)
B_1	(*S*)-6-methyloctanoyl	D-Phe	L-Leu
B_2	6-methylheptanoyl	D-Phe	L-Leu
B_1-Ile	(*S*)-6-methyloctanoyl	D-Phe	L-Ile
B_2-Ile	6-methylheptanoyl	D-Phe	L-Ile
B_3	octanoyl	D-Phe	L-Leu
B_4	heptanoyl	D-Phe	L-Leu
B_5	nonanoyl	D-Phe	L-Leu
B_6	3-hydroxy-6-methyloctanoyl[b]	D-Phe	L-Leu
E_1 (colistin A)	(*S*)-6-methyloctanoyl	D-Leu	L-Leu
E_2 (colistin B)	6-methylheptanoyl	D-Leu	L-Leu
E_3	octanoyl	D-Leu	L-Leu
E_4	heptanoyl	D-Leu	L-Leu
E_7	7-methyloctanoyl	D-Leu	L-Leu
E_1-Ile	(*S*)-6-methyloctanoyl	D-Leu	L-Ile
E_1-Val	(*S*)-6-methyloctanoyl	D-Leu	L-Val
E_1-Nva	(*S*)-6-methyloctanoyl	D-Leu	L-Nva
E_2-Ile	6-methylheptanoyl	D-Leu	L-Ile
E_2-Val	6-methylheptanoyl	D-Leu	L-Val
E_8-Ile	6-methylheptanoyl	D-Leu	L-Ile

[a]D-Phe, D-phenylalanine; L-Leu, L-leucine; L-Ile, L-isoleucine; L-Val, L-valine. [b]Stereochemistry at C3 and C6 is yet to be confirmed.

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 B_1 and B_2,[22,25] whereas for colistin they are colistin A (polymyxin E_1) and colistin B (polymyxin E_2) (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 a detailed investigation examining the in vitro and in vivo activity and toxicity of the major polymyxin B and colistin lipopeptide components, polymyxin B_1 and B_2 and colistin A and B. Throughout the remainder of the paper "polymyxin B" refers to the commercial multicomponent polymyxin B product and "colistin" refers to the commercial multicomponent colistin product.

■ RESULTS AND DISCUSSION

The chemical structures of polymyxin B_1 and B_2 and colistin A and B (Table 1) are closely related. Polymyxin B_1 differs from polymyxin B_2 only by the presence of an extra methylene group, whereby the N-terminal fatty acyl group of polymyxin B_2 (6-methylheptanonyl) is extended by one carbon atom from C7 to C8 to give (*S*)-6-methyloctanoyl in polymyxin B_1. This creates a chiral center 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. By comparison of the chemical structures of polymyxins B_1 and B_2 with those of colistin A and B, differences arise only in the structure of the amino acid at position 6, with polymyxins B_1 and B_2 containing a D-phenylalanine residue at this position, whereas colistins A and 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

Table 2. MICs for Polymyxin B, Polymyxin B_1, Polymyxin B_2, Colistin, Colistin A, and Colistin B against Gram-negative Bacteria

	MIC (μg/mL)										
	P. aeruginosa			*A. baumannii*			*K. pneumoniae*		*E. cloacae*		
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

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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 B_1 represented 53% of the content (as measured by HPLC) and polymyxin B_2 23% of the content (Supporting Information, 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 (Supporting Information, Figure S2). Despite there being only a one-carbon difference in the N-terminal fatty acyl groups between polymyxin B_1 and B_2 and likewise for colistin A and B, baseline resolution of the individual components was observed under RP-HPLC conditions. As a result, polymyxin B_1, polymyxin B_2, colistin A, and colistin B were readily isolated from commercial preparations of polymyxin B and colistin with purities of >97%. The identity and purity of the final isolated cyclic lipopeptides were confirmed by LC-MS analysis (Supporting Information, Figures S3−S6).

The in vitro antimicrobial activity of polymyxin B, polymyxin B_1, polymyxin B_2, 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 2-fold dilution, and a similar observation was reported previously for polymyxin B_1 and B_2.[28] Against *P. aeruginosa* isolates, polymyxin B_1 and B_2 had MICs in the range of 1−2 μg/mL, whereas 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 B_1 and B_2 had MIC values of 0.5−1 μg/mL, whereas for colistin A and B MICs were in the range of 0.5−1 and 1−2 μg/mL, respectively. For the *K. pneumoniae* isolates, greater antimicrobial activity was observed, with polymyxin B_1 and B_2 having MICs of <0.125−0.5 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 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 and 0.25−1 μg/mL obtained for polymyxin B_1 and B_2, respectively; for colistin A and B, MIC values in the ranges of <0.125−0.25 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* MIC <0.125−0.5 μg/mL; and *E. cloacae* MIC 0.25 μg/mL) and colistin (*P. aeruginosa* MIC 1−2 μg/mL; *A. baumannii* MIC 0.5−1 μg/mL; *K. pneumoniae* MIC 0.25 μg/mL; and *E. cloacae* MIC <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 B_1, polymyxin B_2, 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 $\Delta\log_{10}$ CFU/mL > 3) was observed for the commercial products and the respective individual components (Table 3). In this model polymyxins B_1 and B_2 had comparable in vivo efficacy, and likewise for colistin A and 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.

Table 3. In Vivo Efficacy of Polymyxin B, Polymyxin B_1, Polymyxin B_2, Colistin, Colistin A, and Colistin B against *P. aeruginosa* ATCC 27853 in a Mouse Blood Infection Model (Mean ± SD; *n* = 3)

peptide	MIC (μg/mL)	mean $\Delta\log_{10}$ CFU/mL
polymyxin B	1	−3.80 ± 0.29
polymyxin B_1	1	−4.13 ± 0.47
polymyxin B_2	1	−4.24 ± 0.42
colistin	1	−3.35 ± 0.38
colistin A	2	−4.30 ± 0.39
colistin B	4	−3.99 ± 0.40

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 cell line was 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 polymyxin B, polymyxin B_1, or polymyxin B_2 or with 0.75 mM colistin, colistin A, or colistin B (Figure 1). The different concentrations used was based on the EC_{50} 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 B_2 showed significantly higher cell viability (68.5 ± 7.1%) compared to cells treated with polymyxin B_1 (11.8 ± 6.5%) and polymyxin B (19.2 ± 11.5%). Similarly, cell viability of the HK-2 cells was significantly higher following treatment with colistin B (67.8 ± 5.4%) than with colistin A (16.8 ± 9.9%) or colistin (31.2 ± 3.9%). This result is very interesting as only a 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

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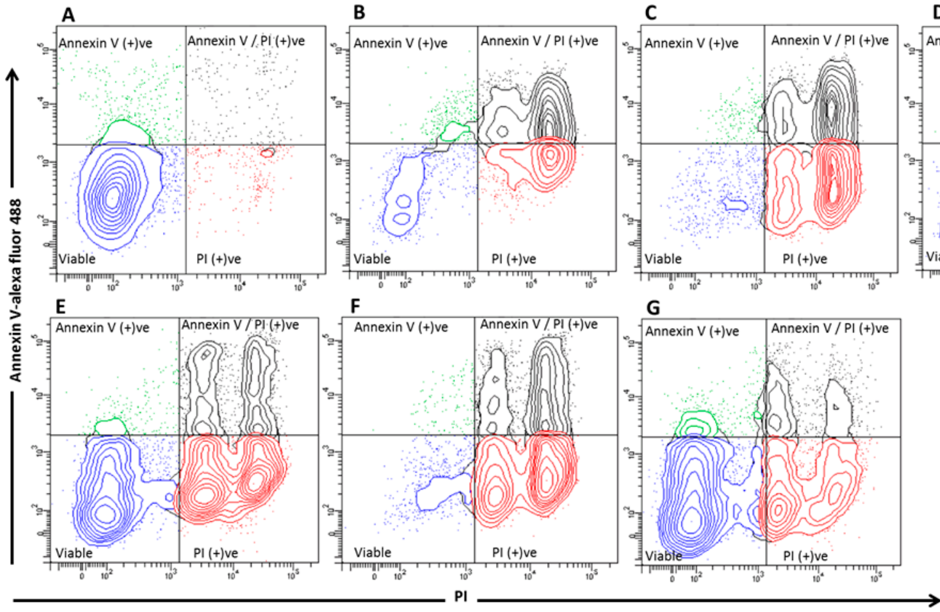

Figure 1. Staining the HK-2 cells with annexin V-alexa fluor 488 and PI: (A) control cells; (B) 0.25 mM polymyxin B; (C) 0.25 mM polymyxin B_1; (D) 0.25 mM polymyxin B_2; (E) 0.75 mM colistin; (F) 0.75 mM colistin A; (G) 0.75 mM colistin B. Upper left quadrant, cells stained by annexin V (early apoptotic cells); upper right quadrant, cells stained by both annexin V and PI (late apoptotic cells); lower right quadrant, cells stained by PI (necrotic cells); lower left quadrant, cells not stained by annexin V/PI (viable cells).

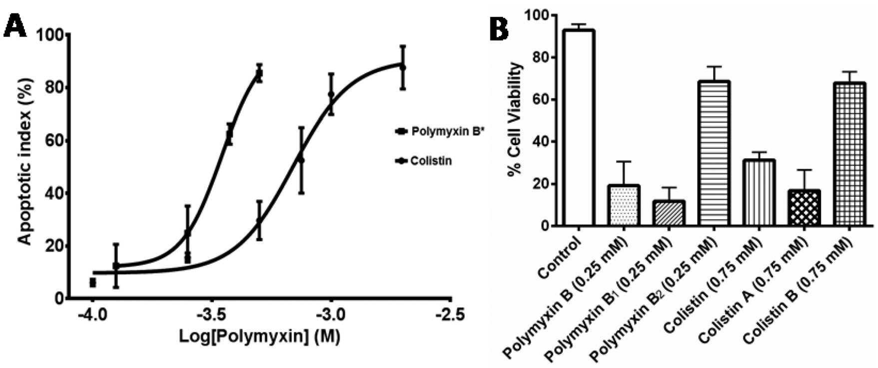

Figure 2. (A) Apoptotic effect on HK-2 cells of the commercial polymyxin B and colistin products. Note the dose response curve for polymyxin B was obtained from a previous study.[33] (B) Cell viability of HK-2 cells after treatment for 24 h with polymyxin B, polymyxin B_1, polymyxin B_2, colistin, colistin A, and colistin B (mean ± SD; $n = 3$).

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 Supporting Information supplementary table for individual kidney histology damage scores). The dosing regimen was well tolerated for each polymyxin with no acute toxicity[40] being observed. The saline control had no observable histological damage in the kidney (Figure 3A) and was given a semiquantitative score (SQS) of 0 (Table 4).[41] In comparison, histological examination of the kidneys from the mice treated with polymyxin B, polymyxin B_1, polymyxin B_2, 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

Table 4. Nephrotoxicity of Polymyxin B, Polymyxin B_1, Polymyxin B_2, Colistin, Colistin A, and Colistin B in a Mouse Model

peptide	*n*	SQS kidney histology score[a]
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
saline	3	0

[a]SQS 0 = no significant change; SQS + 1 = mild damage; SQS + 2 = mild to moderate damage.

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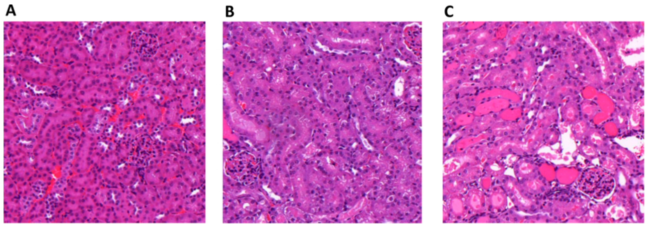

Figure 3. Representative images of kidney sections from histological examination: (A) mouse kidney from saline control group showing no histological damage; (B) mouse kidney after exposure to polymyxin B_2 with grade 1 lesions (SQS = +1), showing tubule damage with tubular dilation and degeneration; (C) mouse kidney after exposure to colistin A with grade 2 lesions (SQS = +2), showing greater tubular damage, tubular dilation, tubular casts, degeneration, and necrosis of the tubular epithelial cells.

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. These kidneys 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 B_1, polymyxin B_2, colistin, colistin A, and colistin B in terms of histological damage to the kidneys. It is interesting that although 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 the uptake of the individual polymyxins being different for isolated kidney cells.

The British (BP), European (Ph. Eur), and U.S. 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 B_1, B_2, B_3, and B_1−Ile[7] (Table 1) must be >80% of the dried polymyxin B sulfate sample. Furthermore, polymyxin B_3 must be no more than 6%, whereas polymyxin B_1-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 E_1), colistin B (polymyxin E_2), polymyxin E_3, polymyxin E_1-Ile, and polymyxin E_7 resulting from the pro-drug (Table 1) should be >77% of the dried colistin methanesulfonate sample.[43,44] Polymyxins E_3, E_1-Ile, and E_7 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 the 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 reassess pharmacopoeial standards set 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 pharmacopeial 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 B_1, polymyxin B_2, colistin A, and colistin B had comparable in vitro and in vivo antimicrobial activities and nephrotoxicities 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 B_1, Polymyxin B_2, Colistin A, and Colistin B by RP-HPLC. *Polymyxin B_1 and B_2.* Polymyxin B_1 and B_2 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 i.d., 100 Å, 10 μm], connected to a Waters 486 tunable absorbance detector (214 nm). Solvent A was 0.1% TFA/water, and solvent B was 0.1% TFA/acetonitrile. Polymyxin B (2 g) 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 i.d.] 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 m/z 200−2000. Fractions containing the desired lipopeptide components were combined and lyophilized for 2 days to give either polymyxin B_1 or polymyxin B_2, as their corresponding TFA salts. Polymyxin B_1 was isolated 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 $C_{56}H_{98}N_{16}O_{13}$: $[M + H]^+$ 1203.74, $[M + 2H]^{2+}$ 602.37, $[M + 3H]^{3+}$ 401.91. Polymyxin B_2 was isolated in a yield of 198.0 mg, t_R at 214 nm = 12.22 min (% area = 97.8%). ESI-MS

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analysis: m/z (monoisotopic) $[M + H]^+$ 1190.00, $[M + 2H]^{2+}$ 595.85, $[M + 3H]^{3+}$ 397.60. Calculated for $C_{55}H_{96}N_{16}O_{13}$: $[M + H]^+$ 1189.73, $[M + 2H]^{2+}$ 595.38, $[M + 3H]^{3+}$ 397.24.

Colistin A and B. Colistin A and colistin B were isolated and purified from a commercial colistin sulfate (Beta Pharma, China) as described above. Colistin A and 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 $C_{53}H_{100}N_{16}O_{13}$: $[M + H]^+$ 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 $C_{52}H_{98}N_{16}O_{13}$: $[M + H]^+$ 1155.75, $[M + 2H]^{2+}$ 578.37, $[M + 3H]^{3+}$ 385.92.

Measurements of Minimum Inhibitory Concentrations (MICs). MICs were determined by the broth microdilution method[46] against the following polymyxin-susceptible ATCC and clinical isolates: *P. aeruginosa* ATCC 27853, FADDI-PA022, and FADDI-PA025; *A. baumannii* ATCC 19606, FADDI-AB034, and ATCC 17978; *K. pneumoniae* ATCC 13883 and FADDI-KP032; and *E. cloacae* FADDI-EC006, FADDI-EC001, and FADDI-EC003. Experiments were performed with cation-adjusted Mueller−Hinton broth (CaMHB) in 96-well polystyrene microtiter plates. Wells were inoculated with 100 μL of bacterial suspension prepared in CaMHB (containing $\sim 10^6$ CFU/mL) and 100 μL of CaMHB containing increasing concentrations of polymyxins (0−32 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 of 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 of CaMHB and incubated overnight. On day 2, an aliquot (0.2 mL) of the overnight culture suspension was dispersed in 20 mL of CaMHB and incubated for production of early log-phase growth bacterial culture. Bacteria in the early log-phase growth suspension were concentrated by centrifugation (3220*g* for 10 min) and resuspended 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 (22−28 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 μL bolus of early log-phase bacterial suspension (4×10^8 CFU/mL). Solutions for administration of polymyxin B, polymyxin B_1, polymyxin B_2, colistin, colistin A, and colistin B were prepared at a concentration of 1 mg (free base) /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 μL/g body weight (BW) (i.e., free base 4 mg/kg BW), whereas the same volume of saline was injected into the control mice. At 0 or 4 h after the administration of antibacterial drug or saline (control), animals were euthanized by inhalation of an overdose of isoflurane. The skin on the chest and the forepaws 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 (5000 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 the blood in each mouse was calculated. For each time point, three mice were examined and the mean and standard deviation (SD) 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 [$\Delta\log = \log_{10}$ (treated) CFU/mL − $\log_{10}$ (control) CFU/mL].

Assessment of the Apoptosis and Viability of Human (HK-2) Kidney Proximal Tubular Cells Treated with Polymyxins Using Fluorescence-Activated Cell Sorting (FACS) Analysis. Methodology for the culturing of HK-2 cells, determination of the EC_{50} and percent cell viability has been 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 B_1, polymyxin B_2, 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 B_1, polymyxin B_2, 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 was placed in a preweighed 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, from 1 to <5% = 1; from 5 to <10% = 2; from 10 to <20% = 3; from 20 to <30% = 4; from 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 SQS (a scale from 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,

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from 1 to <15); SQS + 2 = mild to moderate damage (overall score, from 15 to <30); SQS + 3 = moderate damage (overall score, from 30 to <45); SQS + 4 = moderate to severe damage (overall score, from 45 to <60); and SQS + 5 = severe damage (overall score, 60).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.5b00085.

RP-HPLC profiles for polymyxin B and colistin commercial products; LC-MS analysis for polymyxin B_1, polymyxin B_2, colistin A, and colistin B (PDF)

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Notes

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■ ABBREVIATIONS

ATCC, American Type Culture Collection; CFU, colony-forming units; LC-MS, liquid chromatography−mass spectrometry; MIC, minimum inhibitory concentration; RP-HPLC, reversed-phase high-performance liquid chromatography; TFA, trifluoroacetic acid

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