A secondary mode of action of polymyxins against Gram-negative bacteria involves the inhibition of NADH-quinone oxidoreductase activity

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Polymyxin B and colistin were examined for their ability to inhibit the type II NADH-quinone oxidoreductases (NDH-2) of three species of Gram-negative bacteria. Polymyxin B and colistin inhibited the NDH-2 activity in preparations from all of the isolates in a concentration-dependent manner. The mechanism of NDH-2 inhibition by polymyxin B was investigated in detail with Escherichia coli inner membrane preparations and conformed to a mixed inhibition model with respect to ubiquinone-1 and a non-competitive inhibition model with respect to NADH. These suggest that the inhibition of vital respiratory enzymes in the bacterial inner membrane represents one of the secondary modes of action for polymyxins.

INTRODUCTION

The absence of novel antibiotics in the drug discovery pipeline and the increasing incidence of infection caused by multi-drug-resistant (MDR) Gram-negative bacteria has lead us to re-evaluate ‘old’ antibiotics, such as polymyxin B and colistin, which retain activity against these MDR pathogens.1–3

Although cationic peptides such as the polymyxins are traditionally thought of as outer membrane-active agents,5 the bacterial outer membrane is not necessarily the sole target for their mode of action.5–7 Secondary targets involved in the bactericidal activity of polymyxins remain poorly characterized.8 On the basis of the available evidence, one possible secondary mode of action of polymyxin B and colistin in Gram-negative bacteria involves the inhibition of bacterial respiration.9,10

In general, the bacterial respiratory chain consists of three complexes with quinones and reduced NADH acting as the carriers that shuttle electrons and protons between large protein complexes.11–13 The exact organization of enzymes varies among different bacteria.11–13 In complex I, three inner membrane respiratory enzymes of the NADH oxidase family have been identified: proton-translocating NADH-quinone (Q) oxidoreductase (NDH-1), NADH-Q oxidoreductase that lacks an energy-coupling site (NDH-2) and the sodium-translocating NADH-Q oxidoreductase.11–13,15

The inhibition of the NADH oxidase enzyme family by polymyxin B was reported for Gram-positive Bacillus spp. and Mycobacterium spp.16–19 Moreover, a recent study in Acinetobacter baumannii found that the mechanism of bacterial killing by polymyxins is mediated by release of hydroxyl radicals that might be related to aberrant bacterial respiration.20 Taken together, these findings open up the possibility that a secondary mode of action of polymyxin B and colistin against Gram-negative bacteria may involve inhibition of vital respiratory enzymes in the bacterial inner membrane.

The aim of this study was to investigate the ability of polymyxin B, colistin, colistin methanesulfonate (CMS) and the nona-peptides of polymyxin B and colistin (Figure 1) to inhibit NDH-2 oxidoreductase activity in the inner membrane of the Gram-negative bacteria E. coli, Klebsiella pneumoniae and A. baumannii. To the best of our knowledge, the present study is the first to investigate the activity of this series of polymyxin analogs against the NDH-2 respiratory enzyme of Gram-negative bacteria.

MATERIALS AND METHODS

Polymyxins

Polymyxin B sulfate (lot no. 1312290; ≥6500 U mg⁻¹), colistin sulfate (lot no. 070M1499V; ≥23 690 U mg⁻¹) and polymyxin B nonapeptide (lot no. 088K0544) were purchased from Sigma-Aldrich (St. Louis, MO, USA), whereas CMS (batch no. 143412, ~12 500 U mg⁻¹) was purchased from Link

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The colonies were successively subcultured into Mueller Hinton broth (Oxoid) and incubated aerobically for 17–24 h at 37°C. The following model for reversible inhibition was applied to the kinetic data:

\[ Y = \frac{100}{1 + 10^{(LogIC50 - X)/h}} \]  

where, \( Y \) is the percentage of NDH-2 activity inhibition, \( X \) is logarithm of polymyxin concentration and \( h \) is the Hill coefficient.

The following model for reversible inhibition was applied to the kinetic data:

\[ v = \frac{V_{\text{max}} \times S}{(1 + [I]/(aK_i))} / ([K_i] * (1 + [I]/[K_i])/(1 + [I]/(aK_i)) + S) \]  

where, \( v \) is the enzyme velocity, \( V_{\text{max}} \) is the maximum enzyme velocity without inhibitor, \( K_i \) is the Michaelis–Menten constant, \( K_i \) is the inhibition constant, \([I]\) is the concentration of inhibitor, \([S]\) is the concentration of substrate and \( a \) is mechanism determinant, which is diagnostic of the mode of inhibition. The non-competitive mode of inhibition is indicated when \( a = 1 \) (inhibitor displays equal affinity for both free enzyme and enzyme substrate complex) and mixed inhibition when \( a \neq 1 \), in which if \( a > 1 \) the inhibitor preferentially binds to the free enzyme and if \( a < 1 \) the inhibitor has a greater affinity to enzyme–substrate complex.

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Bacterial isolates

*K. pneumoniae* ATCC 13883 (KpS) and *A. baumannii* ATCC 19606 (Abs) were obtained from the American Type Culture Collection (Rockville, MD, USA), whereas *E. coli* DH5α (Ec) strain was employed in this study. Colistin-resistant variant of *K. pneumoniae* ATCC 13883 (designated 13883R; KpR) was selected by direct plating of parent strain onto Mueller Hinton agar containing 10 mg l⁻¹ colistin (Media Preparation Unit, The University of Melbourne, Parkville, Victoria, Australia) and further increased resistance was produced by serial subculture in cation-adjusted Mueller Hinton broth (containing 23.0 mg l⁻¹ Ca²⁺ and 11.5 mg l⁻¹ Mg²⁺ (Oxoid, Hampshire, England)) with increased concentration of colistin up to 100 mg l⁻¹ (≈70 μm). The stability of resistant variant was tested by four times subculture of the stationary phase in colistin-free media. Isolates were stored in tryptone soy broth (Oxoid) with 20% glycerol (Ajax Finechem, Seven Hills, New South Wales, Australia) at −80°C. MICs for polymyxin B and colistin against the test strains were determined for each isolate in two replicates in cation-adjusted Mueller Hinton broth via broth microdilution, and the MIC of working isolates are documented in Supplementary Table 1.  

**Inner membrane preparation**

Bacterial strains from frozen stock cultures were inoculated onto nutrient agar plates (Media Preparation Unit) and incubated for 18 h aerobically at 37°C. The colonies were successively subcultured into Mueller Hinton broth (Oxoid) and incubated aerobically for 17–24 h at 37°C to obtain ~1–3 g wet weight of cells. Cells were harvested from the growth medium using centrifugation in sterile centrifuge bottles at 3220g for 30 min at 4°C (Eppendorf 5810R, Eppendorf AG, Hamburg, Germany). Cells were washed at least three times in gradually reduced volumes (100, 50 and 20 ml) of sterile saline. To prepare spheroplasts, the cells were resuspended at a ratio of 1 g wet weight per 10 ml of 30 molar Tris-HCl (Trizma base, Sigma-Aldrich), pH 8.0, containing 20% sucrose at 21°C. EDTA iron (III) salt (Sigma-Aldrich), pH 7.5, and lysozyme (Sigma-Aldrich) were added to achieve final concentrations of 10 μmol and 1 μg ml⁻¹, respectively, and the suspensions were retained for 30 min at 21°C. The spheroplast suspensions were centrifuged at 16000 g for 30 min at 4°C (Beckman Avanti J-25, Rotor RA25.50, Beckman Coulter, Brea, CA, USA). The spheroplast pellet was resuspended in 20 ml of 0.1 M phosphate buffer, pH 7.5, containing 20% sucrose. DNase (Sigma-Aldrich) and magnesium sulfate (AnalaR, Merck Pty. Limited, Kellyville, Victoria, Australia) were added to achieve a final concentration of 3 mM and 20 μmol, respectively, and the spheroplast mixture was incubated at 37°C for 30 min. The spheroplasts were disrupted using ultrasonication for 10 min, pulsation at 9/s on-off, on ice using a VCX 550 sonicator 19-mm probe (Sonics VibraCell, Sonics & Materials, Inc, Newtown, CT, USA). The lysate was centrifuged at 75000 g for 30 min at 4°C (Beckman Avanti) to obtain crude inner membrane. Membranes were resuspended at 10 mg wet weight per ml into 30 molar phosphate buffer (pH 7.5), which contained 5 μmol magnesium sulfate. The cell debris was removed using centrifugation at 8000 g for 10 min. Inner membranes were isolated using centrifugation at 75000 g for 1 h at 4°C, and the membrane preparation was stored at −80°C until required for experiments. Protein was quantified via Bradford assay (Bio-Rad Protein Assay, Hercules, CA, USA).

**NADH-quione oxidoreductase activity assay**

Enzymatic activity measurements were performed at 37°C in 96-well plates (Greiner Bio-one, Frickenhausen, Germany). Membrane-bound NADH-quione oxidoreductase activity was measured as previously reported. Briefly, bacterial membranes (3 mg ml⁻¹) were resuspended in the above phosphate buffer containing magnesium sulfate and dispersed into the wells in presence of 200 μmol ubiquinone-1 (Q₁) and 5 μmol potassium cyanide. A polymyxin or its analogs were added as desired and the reaction mixture was incubated for 5 min at 24°C. The reaction was initiated by adding 200 μmol NADH that was prepared immediately before each experiment. The NADH oxidase activity was measured by following the decrease in absorbance at 340 nm (NADH e = 6.22 mm⁻¹ cm⁻¹) using a Versamax Microplate Reader with SoftMax Pro Microplate Data Acquisition Software (Molecular Devices, Sunnyvale, CA, USA). For inhibition studies with polymyxins, the NADH or Q₁ concentrations were varied from 0 to 250 μmol.

**Data analysis**

All kinetic data of enzyme were analyzed using Graphpad Prism 6 (GraphPad Software Inc, San Diego, CA, USA). For the NADH oxidase inhibition activity, we plotted the percentage of NDH-2 activity versus the concentration of polymyxin in logarithm form, and the concentration of polymyxin that cause 50% reduction in enzyme activity (IC₅₀) was estimated by concentration-response equation below:

\[ Y = 100 / \left[ 1 + 10^{(LogIC50 - X)/h} \right] \]  

**Chemical structures of the compounds used in this study.** Polymyxins residues: Thr: threonine; Leu: leucine; Phe: phenylalanine; Dab: α,γ-diaminobutyric acid. CMS = colistin methanesulfonate. A full color version of this figure is available at The Journal of Antibiotics journal online.
RESULTS

Inhibition of NDH-2 activity by polymyxins

To test whether polymyxins can inhibit NDH-2 activity in the inner membranes of three different Gram-negative bacterial species, the electron transport chain was blocked with potassium cyanide and NADH oxidation in the presence of 200 μM Q₁ was monitored spectrophotometrically. Polymyxin B, B₁, B₂ and colistin inhibited NDH-2 activity in a concentration-dependent manner (Figure 2); the calculated IC₅₀ values are documented in Table 1. The IC₅₀ values for the inner membrane preparations from the paired polymyxin-susceptible and -resistant K. pneumoniae strains were comparable for each of polymyxin B and colistin (Table 1; Figure 2). We also examined the effect of polymyxin B and colistin nonapeptides and CMS on NDH-2 activity. The NDH-2 activity was not inhibited by CMS, polymyxin B nona-peptide and colistin nona-peptide (Table 1).

Control data with specific inhibitors and selective co-factors for each of the complex I NDH enzymes demonstrated that our assay system is monitoring NDH-2 activity and that the polymyxin inhibition we report is specific for NDH-2 and not NDH-1 or the sodium-dependant quinone oxidoreductase activity (Supplementary Figure 1). Rotenone (20 μM), a specific inhibitor of the NDH-1 and the sodium-dependant quinone oxidoreductase (note that NDH-2 is insensitive to rotenone), did not inhibit the NADH dehydrogenase activity of the membranes (Supplementary Figure 1).

As secondary controls, we showed that the NDH-2-selective inhibitor, diphenyliodonium iodide (25 μM), inhibited the Q₁-dependent NADH dehydrogenase activity of the membranes (Supplementary Figure 1). Synergy between the polymyxins and diphenyliodonium iodide was not evident in a disc diffusion assay (Supplementary Figure 2). This is most likely because of the fact that polymyxins operate via a very different primary mechanism at the level of the outer membrane compared with diphenyliodonium iodide, whose primary mode of action involves NDH-2 inhibition. Moreover, deamino-NADH, a NADH cofactor analog that can only be utilized by NDH-1 and the sodium-dependant quinone oxidoreductase, and

Table 1 IC₅₀ values for the inhibition of NDH-2 oxidase activity in the inner membranes of Gram-negative bacteria by polymyxins

| Strains          | E. coli DH5α | K. pneumoniae ATCC 13883 | K. pneumoniae ATCC 13883R | A. baumannii ATCC 19606 |
|------------------|--------------|--------------------------|---------------------------|--------------------------|
| Polymyxin B      | 49.8 ± 19.6  | 168 ± 18.6               | 117 ± 18.7                | 167 ± 9.4                |
| Polymyxin B₁     | 44.6 ± 16.9  | ND                       | ND                        | ND                       |
| Polymyxin B₂     | 56.9 ± 25.7  | ND                       | ND                        | ND                       |
| Colistin         | 251 ± 66.1   | 376 ± 50.0               | 359 ± 81.8                | 346 ± 62.6               |
| Polymyxin B nona-peptide | NI          | NI                       | NI                        | NI                       |
| Colistin nona-peptide | NI          | NI                       | NI                        | NI                       |
| Colistin methanesulfonate | NI         | NI                       | NI                        | NI                       |

Abbreviations: ND, not determined; NI, no inhibition.

*Mean ± s.d., n = 3.

K. pneumoniae ATCC 13883R is a colistin-resistant variant of K. pneumoniae ATCC 13883 after serial exposure to colistin-containing media (see Methodology).
sodium (20 mM) did not have an impact on the NDH-2 activity of the membranes (Supplementary Figure 1). 31,33,34

To define the mode of inhibition by polymyxin B, we performed steady-state inhibition kinetic analysis for both NADH and Q1 using E. coli inner membrane preparations. The Q1-dependent NDH-2 activity displayed Michaelis–Menten kinetics with an apparent $K_m$ of 11.0 $\mu$M and $V_{max}$ of 63.4 units per mg protein for Q1 (Supplementary Figure 3A). The $V_{max}$ values were reduced to 39.1 and 25.6 units per mg protein and $K_m$ increased to 14.1 and 16.3 $\mu$M in the presence of polymyxin B 50 and 250 $\mu$M, respectively. The double reciprocal plot of polymyxin B inhibition kinetics in terms of Q1 shows that the lines converge to the left of the y axis, above the x axis, which is diagnostic of a mixed mode of inhibition in terms of Q1 (Figure 3a). Moreover, the $z$ value from the fit of Equation 2 was 2.32 ± 1.28, which is diagnostic of a mixed mode of inhibition for Q1. The NADH-dependent NDH-2 activity displayed Michaelis–Menten kinetics with an apparent $K_m$ of 228 $\mu$M and $V_{max}$ 64.3 units per mg protein for NADH. With fixed 200 $\mu$M Q1, the $V_{max}$ values were 43.7 and 15.4 units mg$^{-1}$ and the $K_m$ values were 195 and 185 $\mu$M NADH in the presence of polymyxin B 50 and 250 $\mu$M, respectively (Supplementary Figure 3B). Our $K_m$ and $V_{max}$ values for E. coli NDH-2 are in agreement with values previously reported for E. coli NDH-2 from native membranes and purified E. coli NDH-2 enzyme. 29,37 The double reciprocal plot of polymyxin B inhibition kinetics in terms of NADH showed that the slopes of the lines converged to the left of the y axis and on the x axis, which is consistent with a non-competitive mode of inhibition (Figure 3b). The $z$ value from the fit of Equation 2 was 0.70 ± 0.38, which is diagnostic of a non-competitive mode of inhibition for NADH.

**DISCUSSION**

It is well established that the initial site of action for the polymyxins is the outer membrane. 8 Nevertheless, the antibacterial action of polymyxins on Gram-negative bacteria is believed to involve multiple sites of action. 8 Our data suggest that one of the secondary target sites of polymyxins is the type II NADH-quinone oxidoreductase respiratory enzyme that forms an integral part of the bacterial electron transport pathway; Type II NADH-quinone oxidoreductases are flavoenzymes that are found in the respiratory chain of a variety of organisms. 29 NDH-2 is often referred to as the ‘alternative’ NADH quinone reductase that does not pump protons across the inner membrane. 29 It is acknowledged that the similar NDH-2 enzyme is an important target for antimicrobial development, particularly against malaria and tuberculosis. 38-43 however, the study of this enzyme in Gram-negative bacilli is very limited.

The IC$_{50}$ values for the inhibition by polymyxin B and colistin of NDH-2 activity in the inner membrane of three different Gram-negative bacterial species were in most part comparable, indicating that interspecies differences in NDH-2 do not have an impact on the inhibitory activity of the polymyxins. Polymyxin B was a better inhibitor compared with colistin, which is in line with reported results with the Gram-positive Mycobacterium smegmatis NDH-2.18 Notably, also colistin inhibited NADH-quinone oxidoreductase activity in the polymyxin-susceptible strain of K. pneumoniae with a comparable IC$_{50}$ to that of the polymyxin-resistant strain, suggesting that polymyxin resistance in these strains is not at the level of the inner membrane respiratory enzymes. Our previous study had indicated that the resistant derivative of K. pneumoniae exhibited less negative charge than the wild type that leads to the failure of polymyxin interaction at the outer membrane. 25-27

The loss of inhibitory activity seen with the polymyxin nonapeptide and CMS suggests that the N-terminal fatty acyl chain and the positive charges of the polymyxin molecule are critical for NDH-2 inhibitory activity. 8 Although polymyxin B and colistin display high IC$_{50}$ values for NDH-2 inhibition, under in vivo conditions there remains the possibility that very high local concentrations of the antibiotic can accumulate at the site of infection that falls within these IC$_{50}$ value ranges. Coincidently, we have garnered in vitro evidence that suggests that polymyxins can accumulate in the inner membrane of Gram-negative bacteria (manuscript submitted for publication). Therefore, the high IC$_{50}$ values do not dismiss the possibility that NDH-2 represents one of the secondary pathways that is targeted once the polymyxin penetrates the outer membrane.

NDH-2 contains a non-covalently bound flavin adenine dinucleotide prosthetic group (FAD) and it catalyzes the oxidation of NADH to NAD$^+$ coupled to the reduction of quinone. 44-47 Available steady-state kinetic evidence indicates that the reaction kinetics of NDH-2 follows a ‘ping-pong’ (double displacement) reaction mechanism where the enzyme interacts with NADH and quinone separately and does not form a ternary complex with both substrates. 44-47 This mechanism predicts that NDH-2 first catalyzes the reduction of the prosthetic group FAD with NADH to FADH$_2$ releasing NAD$^+$; NDH-2 then binds Q1 that accepts electrons from FADH$_2$. 44-47 The non-competitive inhibition with respect to NADH indicates that polymyxins bind to a site on the enzyme away from the NADH-binding site, which slows the reaction rate. In line with a non-competitive mode of inhibition, our results showed that polymyxin inhibition decreases the $V_{max}$ but does not markedly change the $K_m$. 30 Furthermore, a non-competitive inhibition mode of action indicates that the polymyxin likely binds with equal affinity to either the free enzyme or the enzyme–substrate complex. Similarly, the mixed inhibition mode observed with respect to Q1 involves binding to an allosteric site on either the free enzyme or the enzyme–substrate complex. However, as the binding preference for the free enzyme or the enzyme–substrate complex is disproportional, this inhibition mode usually affects both the $K_m$ and $V_{max}$ as per our results. 30 Polymyxin B inhibition increased the $K_m$ for Q1, together with the $z$ value > 1, which indicates that polymyxin B favours binding to the free enzyme, which lowers the apparent affinity of NDH-2 for Q1 and thereby decreases the apparent maximum enzyme reaction rate ($V_{max}$).

The structures of the polymyxins (cyclic peptides) being distinct from those of the NDH-2 substrates, NADH and Q1, are supportive of the inhibition kinetic data, in that they are unlikely to compete for the same sites on the enzyme. Our kinetic data are in line with the reported data for Gluconobacter oxydans, which showed that inhibition by gramicidin S and scopafungin was non-competitive with respect to NADH. 17 Scopafungin, which like polymyxin B and colistin possesses a cyclic ring and a long acyl chain in its structure, displayed a mixed inhibition mode with respect to ubiquinone, whereas gramicidin S was a competitive inhibitor. 17

We have shown for the first time that the secondary mechanism of polymyxins involves the inhibition of NDH-2 activity in the inner membrane of Gram-negative bacteria. Further studies are underway to elucidate the effect of polymyxins on NDH oxidoreductases downstream of NDH-2 and polymyxin response networks, which will shed further light on the role of inner membrane respiratory enzymes in polymyxin-mediated bacterial cell death. In view of the dry antibiotic pipe-line, together with the increasing incidence of multi-drug resistance in Gram-negative bacteria, NDH-2 represents an important target that can be exploited for the development of new antibiotics against these problematic pathogens.
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