

ORIGINAL ARTICLE

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.

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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 led 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,⁴ 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.^{11–15} The exact organization of enzymes varies among different bacteria.^{11–13} In complex 1, 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 nona-peptide (lot no. 088K4054) were purchased from Sigma-Aldrich ((St. Louis, MO, USA), whereas CMS (batch no. 143412, $\sim 12\,500$ U mg⁻¹) was purchased from Link

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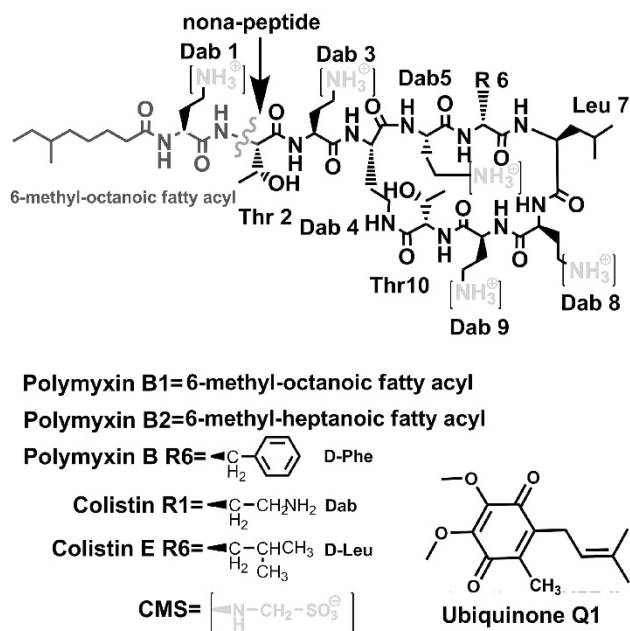


Figure 1 Chemical structures of the compounds used in this study. Polymyxin residues: Thr: threonine; Leu: leucine; Phe: phenylalanine; Dab: α,γ -diaminobutyric acid. CMS = colistin methanesulfonate. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Pharma, Auckland, New Zealand. Polymyxin B₁, B₂ and colistin nona-peptide were prepared and purified using HPLC as previously described.^{21,22} All polymyxins were diluted in deionised water and all except CMS were kept in 4 °C up to a month as they are stable in this condition.²³ CMS was prepared immediately before experiment as CMS is a pro-drug.²⁴

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* DH5 α (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)²⁵ 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 μ M).²⁶ 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 1.²⁷

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 3220 g 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 mM Tris-HCl (Trizma base, Sigma-Aldrich), pH 8.0, containing 20% sucrose at 21 °C.²⁸ EDTA iron (III) salt (Sigma-Aldrich), pH 7.5, and lysozyme (Sigma-Aldrich) were added to achieve final concentrations of 10 mM and 1 mg ml⁻¹, respectively, and the suspensions were retained for 30 min at 21 °C. The spheroplast suspensions were centrifuged at 16 000 g for 30 min at 4 °C (Beckmann 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, Kilsyth, Victoria, Australia) were added to achieve a final concentration of 3 mg ml⁻¹ and 20 mM, 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/9 s on-off, on ice using a VCX 500 sonicator 19-mm probe (Sonics Vibracell, Sonics & Materials, Inc, Newtown, CT, USA). The lysate was centrifuged at 75 000 g for 30 min at 4 °C (Beckmann Avanti) to obtain crude inner membrane. Membranes were resuspended at 10 mg wet weight per ml into 50 mM phosphate buffer (pH 7.5), which contained 5 mM magnesium sulfate. The cell debris was removed using centrifugation at 800 g for 10 min. Inner membranes were isolated using centrifugation at 75 000 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-quinone oxidoreductase activity assay

Enzymatic activity measurements were performed at 37 °C in 96-well plates (Greiner Bio-one, Frickenhausen, Germany). Membrane-bound NADH-quinone oxidoreductase activity was measured as previously reported.^{11,29} Briefly, bacterial membranes (3 mg ml⁻¹) were resuspended in the above phosphate buffer containing magnesium sulfate and dispensed into the wells in presence of 200 μ M ubiquinone-1 (Q₁) and 5 mM 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 μ M NADH that was prepared immediately before each experiment. The NADH oxidase activity was measured by following the decrease in absorbance at 340 nm (NADH ϵ = 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 μ M.

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:³⁰

$$Y = 100 / \left[1 + 10^{((\text{LogIC}_{50} - X) * h)} \right] \quad (1)$$

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:³⁰

$$v = [V_{\max} * S / (1 + [I] / (\alpha K_i))] / ([K_m * (1 + [I] / K_i) / (1 + [I] / (\alpha K_i))] + S) \quad (2)$$

where, v is the enzyme velocity, V_{max} is the maximum enzyme velocity without inhibitor, K_m is the Michaelis-Menten constant, K_i is the inhibition constant, [I] is the concentration of inhibitor, [S] is the concentration of substrate and α is mechanism determinant, which is diagnostic of the mode of inhibition. The non-competitive mode of inhibition is indicated when α = 1 (inhibitor displays equal affinity for both free enzyme and enzyme substrate complex) and mixed inhibition when α \neq 1, in which if α > 1 the inhibitor preferentially binds to the free enzyme and if α < 1 the inhibitor has a greater affinity to enzyme-substrate complex.³⁰

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 Q_1 was monitored spectrophotometrically. Polymyxin B, B1, B2 and colistin inhibited NDH-2 activity in a concentration-dependent manner (Figure 2); the calculated IC_{50} values are documented in Table 1. The IC_{50} 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 1 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).³¹ Rotenone (20 μM), a specific inhibitor of the NDH-1 and the sodium-dependant quinone oxidoreductase (note that NDH-2 is insensitive to rotenone),^{12–14,32–34} 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),^{35,36} inhibited the Q_1 -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.^{35,36} Moreover, deamino-NADH, a NADH cofactor analog that can only be utilized by NDH-1 and the sodium-dependant quinone oxidoreductase, and

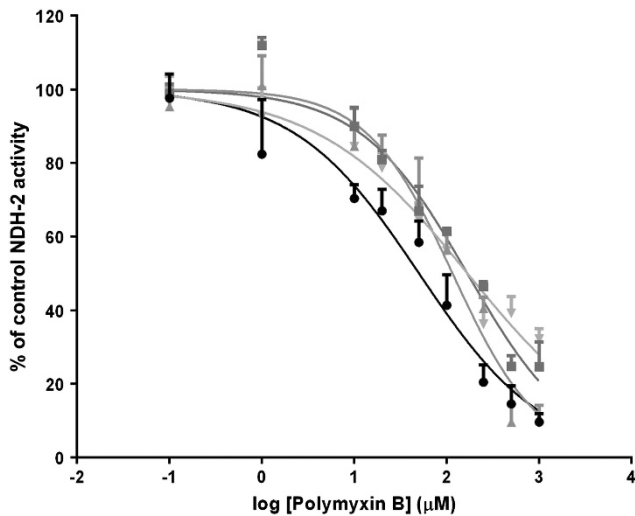


Figure 2 Concentration-dependent inhibition of NDH-2 activity in Gram-negative bacterial inner membrane preparations by polymyxin B. *E. coli* DH5 α (●), *K. pneumoniae* ATCC 13883 (■), *K. pneumoniae* ATCC 13883^R (▲) and *A. baumannii* ATCC 19606 (▼). Data are presented as the percentage of control activity in the absence of inhibitor. The experiments were performed in triplicate (shown as the mean \pm s.d.).

Table 1 IC_{50} values for the inhibition of NDH-2 oxidase activity in the inner membranes of Gram-negative bacteria by polymyxins

Strains	IC_{50} (μM) ^a			
	<i>E. coli</i> DH5 α	<i>K. pneumoniae</i> ATCC 13883	^b <i>K. pneumoniae</i> ATCC 13883 ^R	<i>A. baumannii</i> ATCC19606
Polymyxin B	49.8 \pm 19.6	168 \pm 18.6	117 \pm 18.7	167 \pm 9.4
Polymyxin B1	44.6 \pm 16.9	ND	ND	ND
Polymyxin B2	56.9 \pm 25.7	ND	ND	ND
Colistin	251 \pm 66.1	376 \pm 50.0	359 \pm 81.8	346 \pm 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.

^aMean \pm s.d., $n = 3$.

^b*K. pneumoniae* ATCC 13883^R is a colistin-resistant variant of *K. pneumoniae* ATCC 13883 after serial exposure to colistin-containing media (see Methodology).

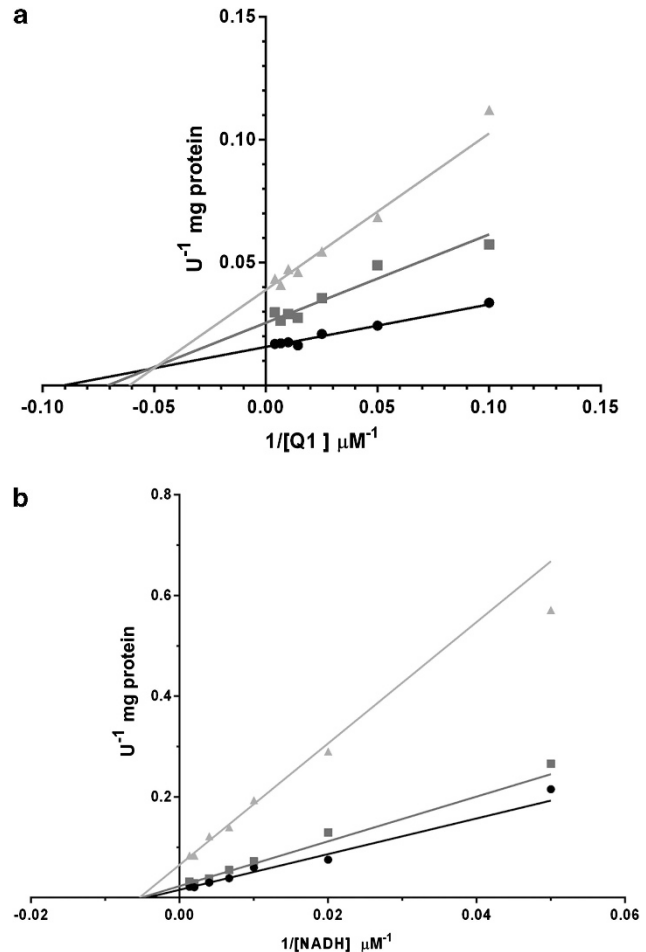


Figure 3 (a) Lineweaver-Burk plot showing mixed inhibition by polymyxin B of Q_1 -dependent NDH-2 activity. (b) Lineweaver-Burk plot showing non-competitive inhibition by polymyxin B of NADH-dependent NDH-2 activity.

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 Q_1 using *E. coli* inner membrane preparations. The Q_1 -dependent NDH-2 activity displayed Michaelis–Menten kinetics with an apparent K_m of 11.0 μM and V_{\max} of 63.4 units per mg protein for Q_1 (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 μM in the presence of polymyxin B 50 and 250 μM , respectively. The double reciprocal plot of polymyxin B inhibition kinetics in terms of Q_1 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 Q_1 (Figure 3a). Moreover, the α value from the fit of Equation 2 was 2.32 ± 1.28 , which is diagnostic of a mixed mode of inhibition for Q_1 . The NADH-dependent NDH-2 activity displayed Michaelis–Menten kinetics with an apparent K_m of 228 μM and V_{\max} 64.3 units per mg protein for NADH. With fixed 200 μM Q_1 , the V_{\max} values were 43.7 and 15.4 units mg^{-1} and the K_m values were 195 and 185 μM NADH in the presence of polymyxin B 50 and 250 μM , 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 α 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

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. Coincidentally, 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 Q_1 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 Q_1 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 Q_1 , together with the α value > 1 , which indicates that polymyxin B favours binding to the free enzyme, which lowers the apparent affinity of NDH-2 for Q_1 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 Q_1 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 NADH 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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- Li, J. & Nation, R. L. Old polymyxins are back: is resistance close? *Clin. Infect. Dis.* **43**, 663–664 (2006).
- Li, J. *et al.* Colistin: the re-emerging antibiotic for multidrug-resistant Gram-negative bacterial infections. *Lancet. Infect. Dis.* **6**, 589–601 (2006).
- Lim, L. M. *et al.* Resurgence of colistin: a review of resistance, toxicity, pharmacodynamics, and dosing. *Pharmacotherapy* **30**, 1279–1291 (2010).
- Hancock, R. E. W. Peptide antibiotics. *Lancet* **349**, 418–422 (1997).
- Hancock, R. E. W. & Scott, M. G. The role of antimicrobial peptides in animal defenses. *Proc. Natl Acad. Sci. USA* **97**, 8856–8861 (2000).
- Cho, J. & Kim, S. Non-membrane targets of antimicrobial peptides: novel therapeutic opportunities? In *Advances in Molecular and Cellular Microbiology* 18pp. 128–140 (CABI, Wallingford, 2010).
- Otvos, L. Antibacterial peptides and proteins with multiple cellular targets. *J. Pep. Sci.* **11**, 697–706 (2005).
- Velkov, T., Thompson, P. E., Nation, R. L. & Li, J. Structure–activity relationships of polymyxin antibiotics. *J. Med. Chem.* **53**, 1898–1916 (2010).
- Storm, D. R., Rosenthal, K. S. & Swanson, P. E. Polymyxin and related peptide antibiotics. *Annu. Rev. Biochem.* **46**, 723–763 (1977).
- Teuber, M. Action of polymyxin B on bacterial membranes. *Arch. Microbiol.* **100**, 131–144 (1974).
- Kim, M. S. & Kim, Y. J. Enzymatic properties of the membrane bound NADH oxidase system in the aerobic respiratory chain of *Bacillus cereus*. *J. Biochem. Mol. Biol.* **37**, 753–756 (2004).
- Kerscher, S., Dröse, S., Zickermann, V. & Brandt, U. In *Bioenergetics. Results and Problems in Cell Differentiation* (Eds Schäfer, G. & Penefsky, H.) pp. 185–222 (Springer, Berlin/Heidelberg, 2008).
- Yagi, T., Yano, T., Di Bernardo, S. & Matsuno-Yagi, A. Procarboxyl complex I (NDH-1), an overview. *Biochim. Biophys. Acta* **1364**, 125–133 (1998).
- Yagi, T. Bacterial NADH-quinone oxidoreductases. *J. Bioenerg. Biomembr.* **23**, 211–225 (1991).
- Yagi, T. & Matsuno-Yagi, A. The proton-translocating NADH-quinone oxidoreductase in the respiratory chain: the secret unlocked. *Biochemistry* **42**, 2266–2274 (2003).
- Mogi, T. & Kita, K. Gramicidin S and polymyxins: the revival of cationic cyclic peptide antibiotics. *Cell Mol. Life Sci.* **66**, 3821–3826 (2009).
- Mogi, T. *et al.* Identification of new inhibitors for alternative NADH dehydrogenase (NDH-II). *FEMS. Microbiol. Lett.* **291**, 157–161 (2009).
- Mogi, T. *et al.* Polymyxin B identified as an inhibitor of alternative NADH dehydrogenase and malate: quinone oxidoreductase from the gram-positive bacterium *Mycobacterium smegmatis*. *J. Biochem.* **146**, 491–499 (2009).
- Tochikubo, K., Yasuda, Y. & Kozuka, S. Decreased particulate NADH oxidase activity in *Bacillus subtilis* spores after polymyxin B treatment. *J. Gen. Microbiol.* **132**, 277–287 (1986).
- Sampson, T. R. *et al.* Rapid killing of *Acinetobacter baumannii* by polymyxins is mediated by a hydroxyl radical death pathway. *Antimicrob. Agents Chemother.* **56**, 5642–5649 (2012).
- Azad, M. A. *et al.* Structure-activity relationships for the binding of polymyxins with human alpha-1-acid glycoprotein. *Biochem. Pharmacol.* **84**, 278–291 (2012).
- Cao, G. *et al.* Development and validation of a reversed-phase high-performance liquid chromatography assay for polymyxin B in human plasma. *J. Antimicrob. Chemother.* **62**, 1009–1014 (2008).
- Li, J., Milne, R. W., Nation, R. L., Turnidge, J. D. & Coulthard, K. Stability of colistin and colistin methanesulfonate in aqueous media and plasma as determined by high-performance liquid chromatography. *Antimicrob. Agents Chemother.* **47**, 1364–1370 (2003).
- Bergen, P. J., Li, J., Rayner, C. R. & Nation, R. L. Colistin methanesulfonate is an inactive prodrug of colistin against *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **50**, 1953–1958 (2006).
- Velkov, T. *et al.* Surface changes and polymyxin interactions with a resistant strain of *Klebsiella pneumoniae*. *Innate Immun.* (e-pub ahead of print 25 July 2013; doi:10.1177/1753425913493337).
- Li, J. *et al.* Heteroresistance to colistin in multidrug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* **50**, 2946–2950 (2006).
- EUCAST ECoAST. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin. Microbiol. Infect.* **9**, ix–xv (2003).
- Dancey, G. F., Levine, A. E. & Shapiro, B. M. The NADH dehydrogenase of the respiratory chain of *Escherichia coli*. I. Properties of the membrane-bound enzyme, its solubilization, and purification to near homogeneity. *J. Biol. Chem.* **251**, 5911–5920 (1976).
- Matsushita, K., Ohnishi, T. & Kaback, H. R. NADH-ubiquinone oxidoreductases of the *Escherichia coli* aerobic respiratory chain. *Biochemistry* **26**, 7732–7737 (1987).
- Copelan, R. A. *Evaluation of Enzyme Inhibition in Drug Discovery: A Guide for Medicinal Chemist and Pharmacologist*. 2nd edn (ed A John Wiley & Sons) 57–201 (Inc. Publication, 2013).
- Dimroth, P. & Thomer, A. A primary respiratory Na⁺ pump of an anaerobic bacterium: the Na⁺-dependent NADH:quinone oxidoreductase of *Klebsiella pneumoniae*. *Arch. Microbiol.* **151**, 439–444 (1989).
- Melo, A. M. P., Bandeira, T. M. & Teixeira, M. New insights into type II NAD(P)H:quinone oxidoreductases. *Microbiol. Mol. Biol. Rev.* **68**, 603–616 (2004).
- Krebs, W., Steuber, J., Gemperli, A. C. & Dimroth, P. Na⁺ translocation by the NADH:ubiquinone oxidoreductase (complex I) from *Klebsiella pneumoniae*. *Mol. Microbiol.* **33**, 590–598 (1999).
- Steuber, J., Schmid, C., Rufibach, M. & Dimroth, P. Na⁺ translocation by complex I (NADH:quinone oxidoreductase) of *Escherichia coli*. *Mol. Microbiol.* **35**, 428–434 (2000).
- Shiemke, A. K., Arp, D. J. & Sayavedra-Soto, L. A. Inhibition of membrane-bound methane monooxygenase and ammonia monooxygenase by diphenyliodonium: implications for electron transfer. *J. Bacteriol.* **186**, 928–937 (2004).
- Biagini, G. A. *et al.* Generation of quinolone antimalarials targeting the *Plasmodium falciparum* mitochondrial respiratory chain for the treatment and prophylaxis of malaria. *Proc. Natl Acad. Sci. USA* **109**, 8298–8303 (2012).
- Bjorklöv, K., Zickermann, V. & Finel, M. Purification of the 45 kDa, membrane bound NADH dehydrogenase of *Escherichia coli* (NDH-2) and analysis of its interaction with ubiquinone analogs. *FEBS Lett.* **467**, 105–110 (2000).
- Dong, C. K. *et al.* Type II NADH dehydrogenase of the respiratory chain of *Plasmodium falciparum* and its inhibitors. *Bioorg. Med. Chem. Lett.* **19**, 972–975 (2009).
- Weinstein, E. A. *et al.* Inhibitors of type II NADH:menaquinone oxidoreductase represent a class of antitubercular drugs. *Proc. Natl Acad. Sci. USA* **102**, 4548–4553 (2005).
- Koul, A., Arnould, E., Lounis, N., Guillemont, J. & Andries, K. The challenge of new drug discovery for tuberculosis. *Nature* **469**, 483–490 (2011).
- Fisher, N., Bray, P. G., Ward, S. A. & Biagini, G. A. Malaria-parasite mitochondrial dehydrogenases as drug targets: too early to write the obituary. *Trends Parasitol.* **24**, 9–10 (2008).
- Fisher, N., Bray, P. G., Ward, S. A. & Biagini, G. A. The malaria parasite type II NADH:quinone oxidoreductase: an alternative enzyme for an alternative lifestyle. *Trends Parasitol.* **23**, 305–310 (2007).
- Biagini, G. A. *et al.* Generation of quinolone antimalarials targeting the *Plasmodium falciparum* mitochondrial respiratory chain for the treatment and prophylaxis of malaria. *Proc. Natl Acad. Sci. USA* **109**, 8298–8303 (2012).
- Velazquez, I. & Pardo, J. P. Kinetic characterization of the rotenone-insensitive internal NADH: ubiquinone oxidoreductase of mitochondria from *Saccharomyces cerevisiae*. *Arch. Biochem. Biophys.* **389**, 7–14 (2001).
- Yano, T., Li, J. S., Weinstein, E., Teh, J. S. & Rubin, H. Steady-state kinetics and inhibitory action of antitubercular phenothiazines on mycobacterium tuberculosis type-II NADH:menaquinone oxidoreductase (NDH-2). *J. Biol. Chem.* **281**, 11456–11463 (2006).
- Teh, J. S., Yano, T. & Rubin, H. Type II NADH: menaquinone oxidoreductase of *Mycobacterium tuberculosis*. *Infect. Disord. Drug Targets* **7**, 169–181 (2007).
- Eschemann, A., Galkin, A., Oettmeier, W., Brandt, U. & Kerscher, S. HDQ (1-Hydroxy-2-dodecyl-4(1H)quinolone), a high affinity inhibitor for mitochondrial alternative NADH dehydrogenase: evidence for a ping-pong mechanism. *J. Biol. Chem.* **280**, 3138–3142 (2005).

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