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

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## Abstract

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 *E. 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 inhibition of vital respiratory enzymes in the bacterial inner membrane represents one of the secondary modes of action for polymyxins.

### Keywords

type II NADH-quinone oxidoreductase; polymyxin B; colistin; *Escherichia coli*; *Klebsiella pneumoniae*; *Acinetobacter baumannii*

## 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].

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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]. Based on 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 nicotinamide adenine dinucleotide (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 which lacks an energy-coupling site (NDH-2) and the sodium-translocating NADH-Q oxidoreductase[11–13, 15].

The inhibition of NADH oxidase enzymes family by polymyxin B was reported for Gram-positive *Bacillus spp.* and *Mycobacterium spp*.[16–19]. Moreover, a recent study in *Acinetobacter baumannii* found 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 *Acinetobacter baumannii*. To the best of our knowledge, the present study is the first to investigate the activity of this series of polymyxin analogues against the NDH-2 respiratory enzyme of Gram-negative bacteria.

## Materials and Methods

### Polymyxins

Polymyxin B sulphate (lot # 1312290; 6500 U/mg), colistin sulphate (lot # 070M1499V; 23,690 U/mg) and polymyxin B nona-peptide (lot # 088K4054) were purchased from Sigma-Aldrich ((St. Louis, MO), whereas CMS (batch # 143412, ~12500 U/mg) was purchased from Link Pharma, Auckland, New Zealand. Polymyxin $B_1$, $B_2$ and colistin nona-peptide was prepared and purified by 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) was obtained from the American Type Culture Collection (Rockville, MD, USA), while *E. coli* DH5α

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(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 colistin (Media Preparation Unit, The University of Melbourne, Parkville, Australia)[25] and further increased resistance was produced by serial subculture in cation-adjusted Mueller Hinton broth (CAMHB; containing 23.0 mg/L $Ca^{2+}$ and 11.5 mg/L $Mg^{2+}$ [Oxoid, Hampshire, England]) with increase concentration of colistin up to 100 mg/L (~70 μM)[26]. The stability of resistant variant was tested by four times subculture of stationary phase in colistin-free media. Isolates were stored in tryptone soy broth (Oxoid) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) at -80°C. Minimum inhibitory concentrations (MICs) for polymyxin B and colistin against the test strains were determined for each isolate in two replicates in CAMHB 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 sub-cultured into Mueller Hinton broth (Oxoid) and incubated aerobically for 17–24 h at 37°C to obtain approximately 1–3 g wet weight of cells. Cells were harvested from the growth medium by 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 gradual reduce of volume 100 mL, 50 mL 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, 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 (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 sulphate (AnalaR, Merck Pty. Limited, Kilsyth, Australia) were added to achieve a final concentration of 3 mg/mL and 20 mM, respectively; and the spheroplast mixture were incubated at 37°C for 30 min. The spheroplasts were disrupted by ultrasonication for 10 min, pulsation at 9 sec/9 sec 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 75000 ×*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 sulphate. The cell debris was removed by centrifugation at 800 ×*g* for 10 min. Inner membranes were isolated by 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 (Biorad Protein Assay, Hercules, CA).

## 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) were

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resuspended in the above phosphate buffer containing magnesium sulphate and dispensed into the wells in presence of 200 μM ubiquinone-1 ($Q_1$) and 5 mM potassium cyanide (KCN). A polymyxin or analogues was added as desired and the reaction mixture was incubated for 5 min at 24°C. The reaction was initiated by adding 200 μM 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 $Q_1$ concentrations were varied from 0–250μM.

### Data analysis

All kinetic data of enzyme were analysed 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 *vs.* the concentration of polymyxin in logarithm form and the concentration of polymyxin that cause 50% reduction of enzyme activity ($IC_{50}$) was estimated by concentration-response equation below:[30]

$$Y=100/[1+10^{((\mathrm{LogIC50}-X)*h)}] \quad \text{(Eq 1)}$$

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

The following model for reversible inhibition was applied to the kinetic data:[30]

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

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  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 if polymyxins can inhibit NDH-2 activity in the inner membranes of three different Gram-negative bacterial species, the electron transport chain was blocked with KCN 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

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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 (DPI) (25μM),[35, 36] inhibited the $Q_1$ dependent NADH dehydrogenase activity of the membranes (Supplementary Figure 1). Synergy between the polymyxins and DPI was not evident in a disc diffusion assay (Supplementary Figure 2). This is most likely due to the fact that polymyxins operate via a very different primary mechanism at the level of the outer membrane compared to DPI, 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 sodium (20 mM) did not impact 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 $V_{max}$ 63.4 units/mg protein for $Q_1$ (Supplementary Figure 3A). The $V_{max}$ were reduced to 39.1 and 25.6 units/mg protein and $K_m$ increased to 14.1 μM and 16.3 μM in the presence of polymyxin B 50 μM and 250 μM, respectively. The double reciprocal plot of polymyxin B inhibition kinetics in terms of $Q_1$ shows 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 $\alpha$ value from the fit of Eq. 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/mg protein for NADH. With fixed200 μM $Q_1$, the $V_{max}$ values were 43.7 and 15.4 units/mg; and the $K_m$ 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 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 $\alpha$ value from the fit of Eq. 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

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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], but 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 inner membrane of three different Gram-negative bacterial species were in most part comparable, indicating that inter-species differences in NDH-2 do not impact the inhibitory activity of the polymyxins. Polymyxin B was a better inhibitor compared to colistin, which is in line with reported results with the Gram-positive *M. 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 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 lead to 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 fall 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 catalyses the oxidation of NADH to $NAD^+$ coupled to the reduction of quinone[44–47]. Available steady-state kinetic evidence indicates 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 firstly catalyses the reduction of the prosthetic group FAD with NADH to $FADH_2$, releasing $NAD^+$; NDH-2 then binds $Q_1$ which accepts electrons from $FADH_2$[44–47]. The non-competitive inhibition with respect to NADH indicates 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 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 enzyme-substrate complex. However, as the

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binding preference for the free enzyme or 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 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 structure of the polymyxins (cyclic peptides) being distinct from those of the NDH-2 substrates NADH and $Q_1$ is 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 inhibition by gramicidin S and scopafungin were 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 inhibition of NDH-2 activity in 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 multidrug resistant in Gram-negative bacteria, NDH-2 represents an important target that can be exploited for the development of new antibiotics against these problematic pathogens.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **CMS** | colistin methanesulfonate |
| **NADH** | nicotinamide adenine dinucleotide reduced |
| **NDH-2** | type II NADH-quinone oxidoreductases |
| **$Q_1$** | ubiquinone-1 |

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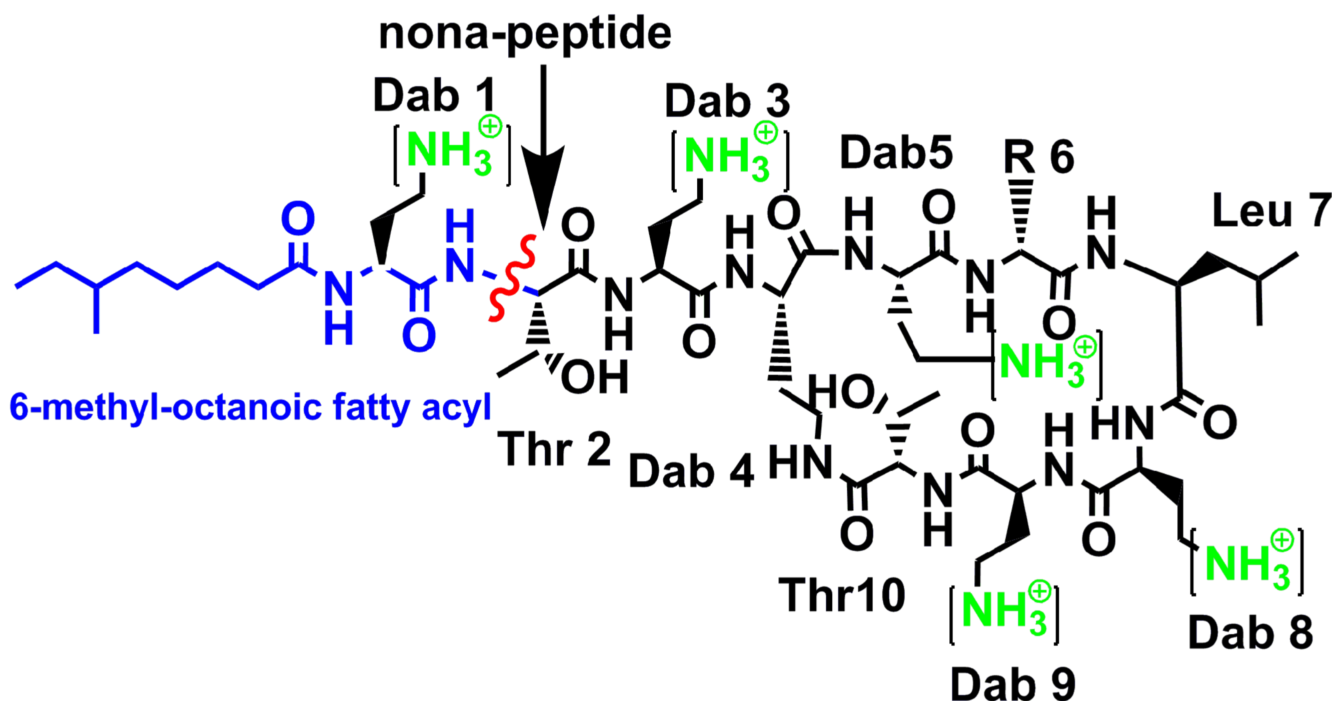


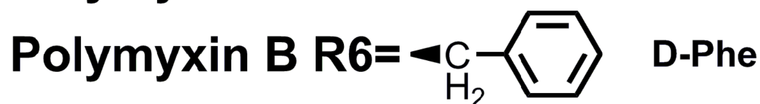


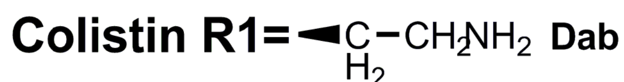


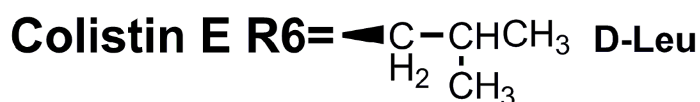


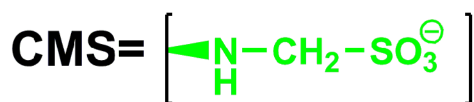


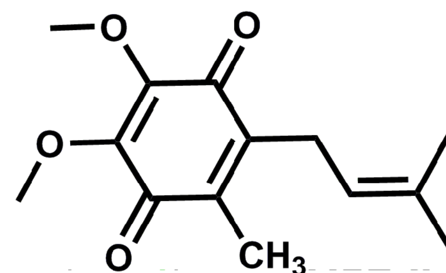


**Figure 1.**

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.

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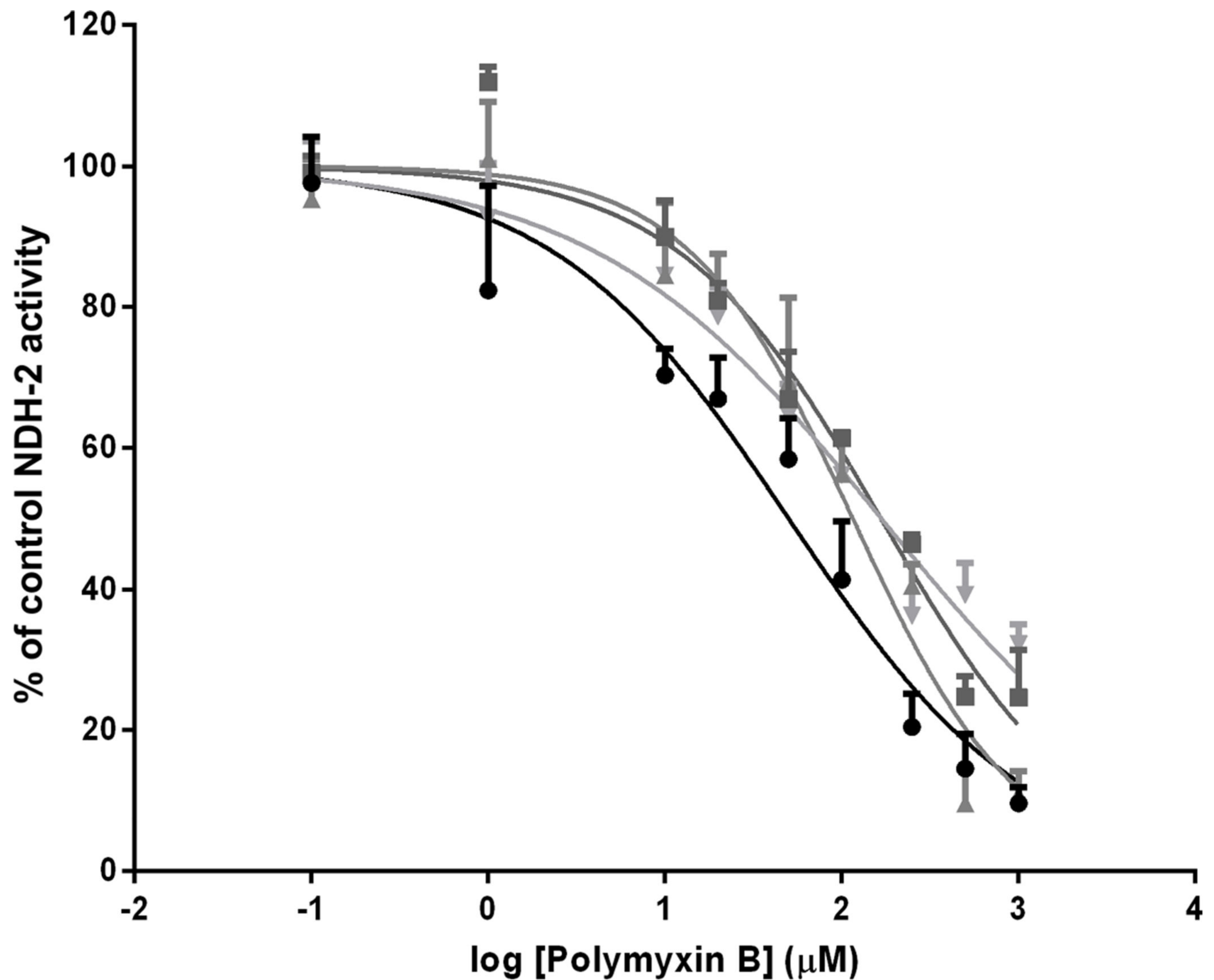


**Figure 2.**

Concentration-dependent inhibition of NDH-2 activity in Gram-negative bacterial inner membrane preparations by polymyxin B. *E. coli* DH5α (●), *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± S.D).

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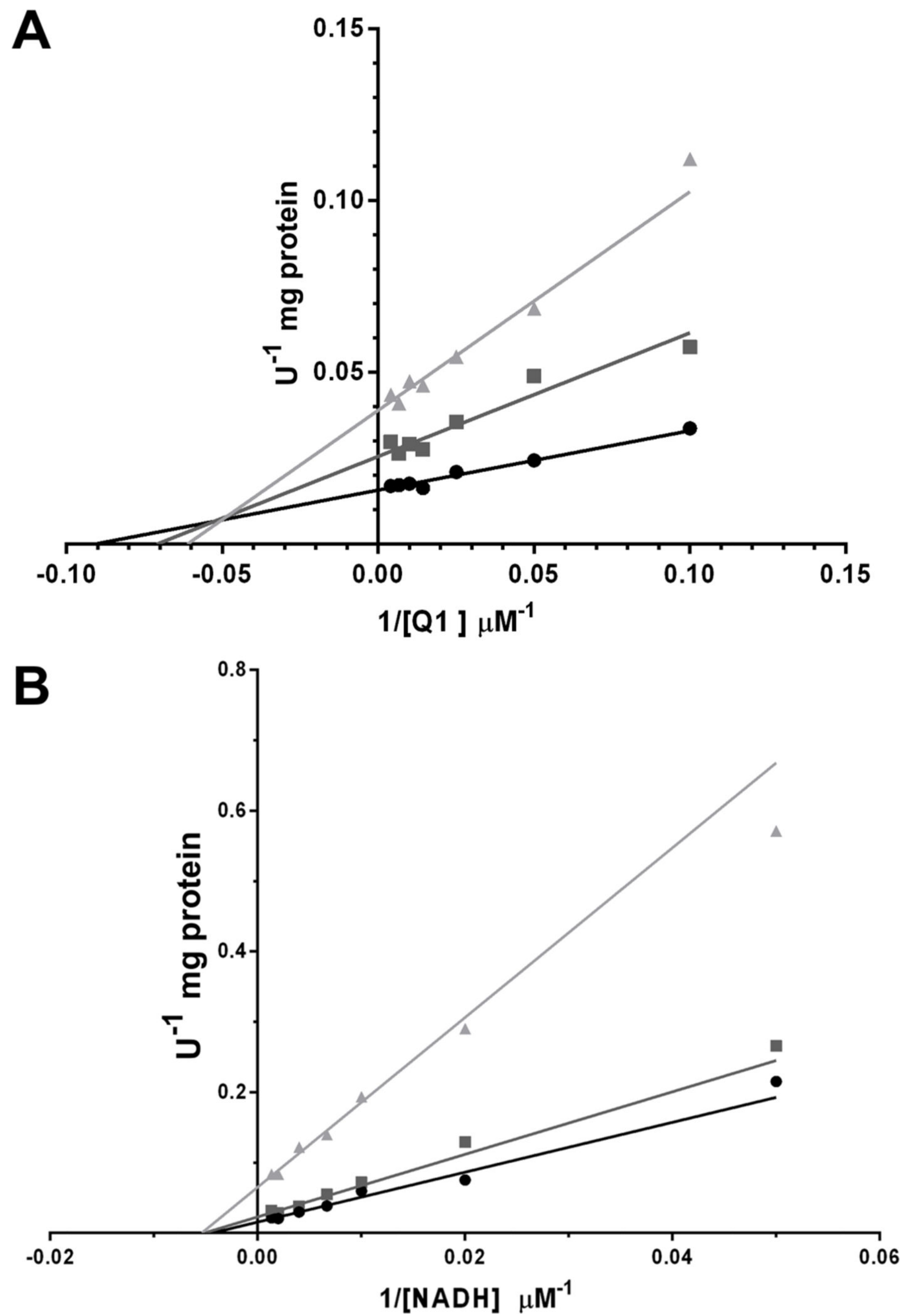


**Figure 3.**

**(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.

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## Table 1

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

| | $IC_{50}$ (μM)* | | | |
|---|---|---|---|---|
| **Strains** | ***E. coli*** **DH5α** | ***K. pneumoniae*** **ATCC 13883** | *******K. pneumoniae*** **ATCC 13883$^R$** | ***A. baumannii*** **ATCC19606** |
| 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 |

ND, Not determined.
NI, No inhibition.

*Mean ± S.D, *n*=3

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

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