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Polymyxins and analogs bind to ribosomal RNA and interfere with eukaryotic translation in vitro

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Keywords

Antibiotics; Polymyxins; Colistin; RNA; Translation

Bacterial resistance to current antibiotics is a significant threat to human health, and the lack of new antibiotics to treat multi-drug resistant infections, especially by Gram-negative bacteria, amplifies this dire situation.[1] This has renewed interest in the clinical use of polymyxins, in particular polymyxin B (PMB) and colistin (also known as polymyxin E).[2] Indeed, although abandoned as antibiotics in the 1970s due to toxicity concerns, polymyxins have been recently resurrected as last-line treatments for otherwise untreatable multi-drug resistant infections.[2b, 3]

Polymyxins are penta-cationic cyclic lipodecapeptides. Their amphiphillic nature is the presumed basis for the proposed mechanism of action against Gram-negative bacteria, which involves outer membrane (OM) and inner membrane (IM) permeation.[4] It has been suggested that polymyxins first bind to the lipid A core of the lipopolysaccharide component of the OM through electrostatic attractions between the positively charged γ-L-diaminobutyric acid (Dab) residues with the negatively charged lipid A phosphate groups (Scheme 1).[4] This then allows the hydrophobic motifs to insert into the OM, destabilizing its packing and, subsequently, through a self-promoted uptake mechanism, polymyxins pass through.[4] The exact mechanism relating IM permeation to bactericidal activity remains unclear. The amphiphillic character alone likely does not account for this activity, as common cationic detergents function as antibacterial agents at much higher concentrations than the polymyxin's MICs.[4b,4c,5] These observations have led to the hypotheses that polymyxins act on the IM by permeating the phospholipids bilayer or by pore formation.[4b,4d,6] An alternative hypothesis suggests that polymyxins may facilitate phospholipid exchange, which could create osmotic imbalance, leading to bacteriolysis.[7]

The diminished clinical use of polymyxins was due to their neurotoxicity and nephrotoxicity and, less commonly, ototoxicity and pruritus,[8] although the underlying mechanisms responsible for these adverse effects are not known. We noted that aminoglycoside antibiotics,[9] a large family of potent bactericidal agents, exhibit similar side effects,

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particularly nephrotoxicity and ototoxicity.[10] Like polymyxins, aminoglycosides are highly cationic, with typically four to six protonated amines at physiological pH. Another similarity is that aminoglycosides are proposed to have a self-promoted uptake mechanism across the OM in *P. Aeruginosa*.[11] We hypothesized that the similar toxicity profiles along with the overall chemical characteristics of aminoglycosides and polymyxins could be due to shared targets and molecular mechanisms. Here we probe the binding of polymyxins to the bacterial 16S ribosomal RNA decoding site (or A-site),[12] the cognate target of aminoglycosides, as well to the corresponding eukaryotic 18S RNA A-site, and examine their effect on prokaryotic and eukaryotic translation in vitro. We demonstrate, for the first time, that PMB, colistin and their analogs bind to the 16S and 18S A-site RNA constructs, and interfere with eukaryotic translation in vitro, but not with bacterial translation.

To examine the affinity of PMB, colistin and analogs to the 16S and 18S A-sites, we utilized a modified version of a FRET assay originally developed in our laboratory;[13] this assay has recently been used for exploring the affinity of modified aminoglycosides to the 16S RNA construct.[14] It consists of an aminoglycoside-coumarin conjugate, kanamycin-coumarin (FRET donor), which binds to a Dy-547 labeled 16S or 18S A-site RNA hairpin construct (FRET acceptor) (Scheme 2). Displacement of kanamycin-coumarin by a competing unlabeled binder results in a diminished FRET signal. Plotting the fractional fluorescent saturation versus compound concentration generates titration curves. Representative titration curves are shown in Figure 1, including kanamycin's, an established antibiotic.[15]

Polymyxins and analogs display significant affinity to the 16S A-site (Figure 1 and Table 1). PMB and colistin are the weakest binders with IC_{50} values of 52±2 μM and 77±5 μM, respectively. The analogs **1** and **2** exhibit higher affinities than PMB and colistin. Notably, **2** with an IC_{50} value of 4.5±0.2 μM is more potent than kanamycin, which is considered a mediocre A-site binder. Polymyxin B nonapeptide (PMBN), where the hydrophobic tail-Dab[1] has been proteolytically removed,[16] shows a 1.5-fold increase in affinity compared to PMB. Colistin methanesulfonate (CMS), a derivative where the primary amino groups are sulfomethylated, exhibits no displacement up to 500 μM. CMS also acts a useful negative control, as it does not interfere with the FRET signal, helping to verify that the polymyxins do not disrupt the A-site construct. Repeating the experiments using the 18S eukaryotic A-site RNA construct yields a similar trend of IC_{50} values across the polymyxins and analogs to those obtained for the titrations using the bacterial A-site construct (Figure 1 and Table 1).

All compounds were examined in vitro for their ability to hamper bacterial and eukaryotic translation. This coupled transcription/translation assay monitors the generation of a functional luciferase following the addition of luciferin and measuring the resulting luminescence. Plotting the relative luminescence versus compound concentration generates interference curves. Note that this assay does not discriminate between inhibition of translation (lower protein yield) and mistranslation (generation of defective proteins), as either results in lower relative luminescence. We therefore refer to the observed results as translation interference.

As shown in Figure 1, kanamycin, used as a positive control, preferentially hinders bacterial translation over eukaryotic translation with IC_{50} values differing by 250-fold, consistent with previously published results.[17] No activity against bacterial translation occurs up to 220 μM for PMB and colistin, and 110 μM for **1** and **2** (Figure 1 and Table 1). Importantly, PMB, colistin and the novel analogs **1** and **2** show dose-dependent interference of eukaryotic translation. All disrupt eukaryotic translation more potently than kanamycin, with **2** being the most effective with an IC_{50} value of 8.8±0.8 μM (Table 1).

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As illustrated by the results outlined above, the polymyxins show considerable binding to the bacterial and eukaryotic A-site RNA constructs. PMB and colistin are weaker binders, as compared to analogs **1** and **2**, which incorporate a hydrophobic D-octylglycine (D-OctGly) substitution at the R^6 position (Scheme 1). Notably, **2**, having a biphenyl in the R^1 position, binds to the 16S and 18S A-sites with greater and equal potency to kanamycin, respectively (Table 1). Bacterial translation is not affected by any of the polymyxins or analogs.[18] Intriguingly, all polymyxins potently prevent eukaryotic translation with the general trend corresponding to their 18S A-site binding, with **2** being the most potent. Notably, the very hydrophobic substitutions at positions R^1 and R^6 make **2** the most hydrophobic of the analog series (Table 1).

The bacterial A-site has been established to be a very selective binding site for aminoglycoside antibiotics.[19] It is intriguing to discover that polymyxins could disrupt the kanamycin–A-site complex. For comparison to the aminoglycosides, under the same experimental conditions for the 16S A-site, the IC_{50} value previously observed for neomycin (strong binder) was 1.6±0.2 μM, neamine (moderate binder) was 4.5±0.4 μM, and kanamycin (weak binder) was 7.0±0.7 μM.[14] PMB, colistin, and **1** show lower affinities compared to aminoglycosides. Derivative **2**, however, has an affinity comparable to neamine for the 16S A-site and to kanamycin for the 18S A-site under the same conditions. Polymyxins contain five primary ammonium groups that could participate in hydrogen bonding and can electrostatically interact with the RNA scaffold. It is therefore interesting that these unrelated antibiotics can be accommodated by this compact site, although allosteric effects, previously proposed for other RNA–ligand binding events, where polymyxins displace kanamycin-coumarin by inducing RNA conformation changes, cannot be excluded.[20]

Polymyxins show little selectivity between the 16S and 18S RNA A-sites. Among the parent compounds, colistin has a higher IC_{50} than PMB. The possible difference in affinity could be due to the D-Phe^6 residue in PMB compared to the D-Leu^6 of colistin, as phenylalanine, could, in principle, intercalate.[21] The compound that shows the most significant increase in A-site binding, **2**, has a D-OctGly in place of the D-Phe^6 and also a biphenyl group substituting the *N*-terminal fatty acyl chain. The modifications in **2** improve the relative affinity for the A-site dramatically. Biphenyl is known to base-stack in other systems;[22] therefore, this dramatic increase could be due to its ability to intercalate. Indeed, monitoring the fluorescence of the biphenyl residue shows dose-dependent quenching upon titration of 16S A-site RNA (See SI, Figure S5).

A substantial difference between aminoglycosides and polymyxins is the presence of a non-polar fatty acyl group in the latter. The likely bound location of the methyl octanoyl/heptanoyl chain of PMB is closer to the groove wall, in the most hydrophobic region.[23] The apparent increase in affinity for PMBN compared to PMB could indicate that the hydrophobic tail of PMB may be hindering certain attractive interactions. In **1**, a synthetic derivative, the hydrophobic D-Phe^6 is replaced by a D-OctGly^6. Compared to PMB, the IC_{50} of **1** decreases by almost four-fold. Perhaps the presence of the two hydrophobic chains causes a reorientation of the molecule to accommodate both octanoyl chains closer to the RNA skeleton.

Polymyxins and analogs show no effect on bacterial translation. A second antibacterial mode of action of polymyxins by interference of bacterial translation is therefore unlikely, despite their affinity to the 16S RNA A-site, although it should be noted that A-site binding does not necessarily correlate to in vitro translation (IVT) activities, as previously observed for the aminoglycosides.[12a,17a,24] Additionally, although the A-site is considered the cognate target of aminoglycosides and is thought to contribute to their ability to interfere

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with translation, they have been shown to bind to multiple sites on the ribosome.[25] The polymyxins and analogs do effectively interfere with eukaryotic translation and the general trend observed correlates with their relative affinity to the 18S A-site. Other targets or modes of action contributing to this outcome cannot be discounted. Whether or not the ability of polymyxins to interfere with eukaryotic translation is responsible for the adverse effects seen in the clinic remains to be explored.

Experimental Section

See Supporting Information for all experimental methods and additional data regarding the 16S and 18S FRET-based assay and the eukaryotic and prokaryotic IVT assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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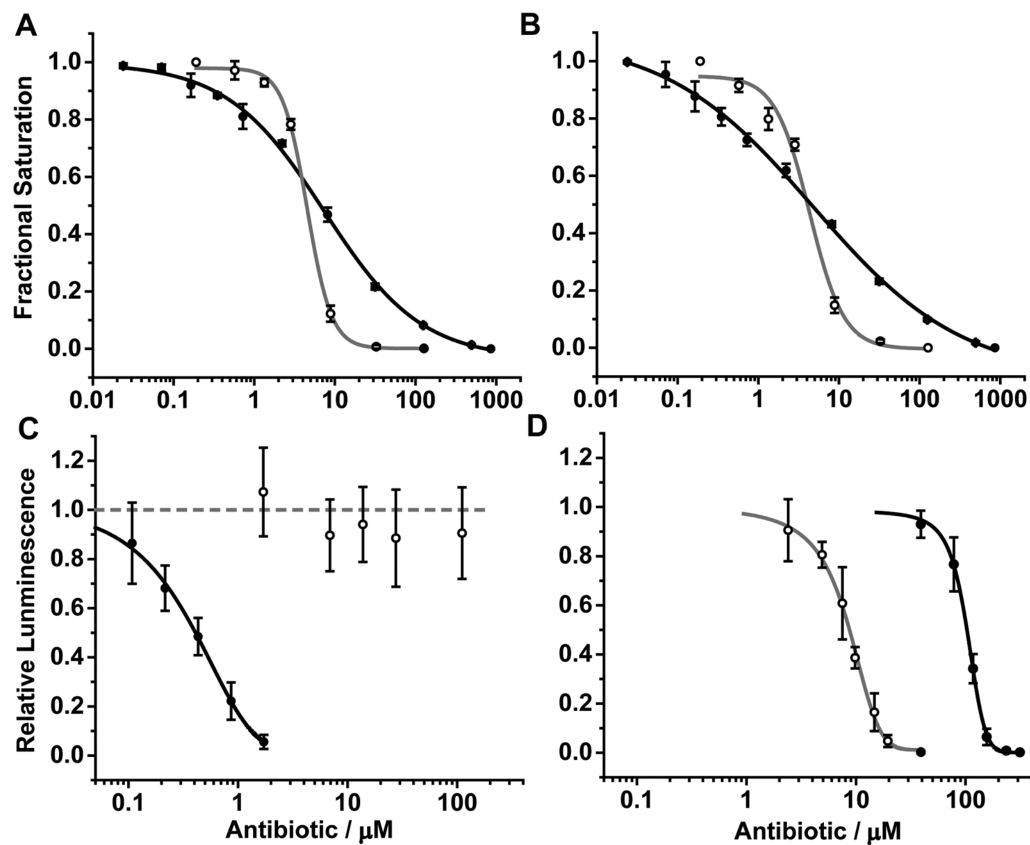

Figure 1.
A-site RNA binding of kanamycin (●) and **2** (○) overlaid for A) 16S and B) 18S A-site constructs, and C) bacterial and D) eukaryotic IVT assays. [15]

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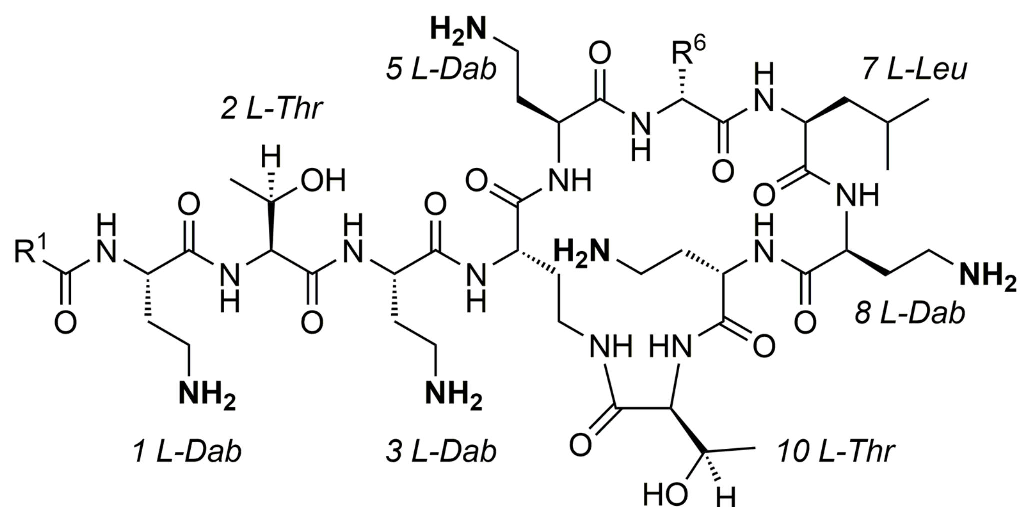

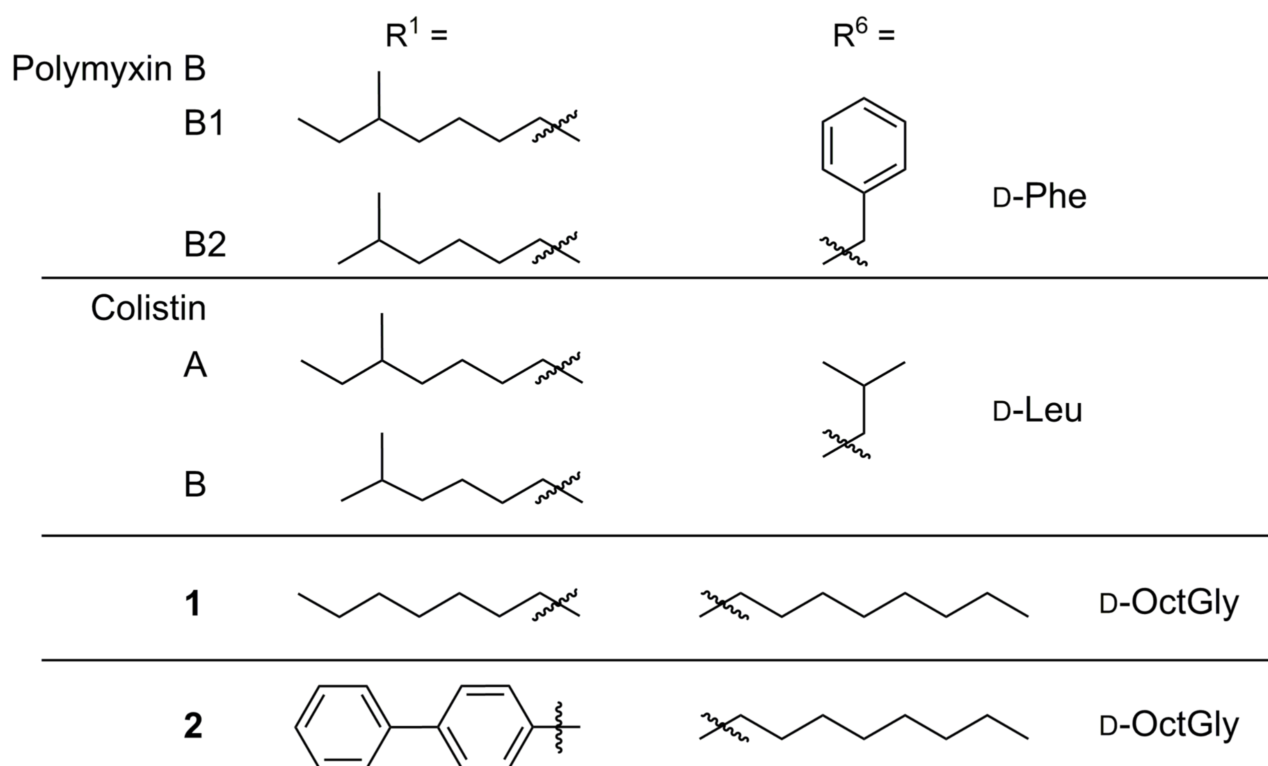

Scheme 1.
Structures of polymyxin B, colistin, and two analogs. Primary amines, likely to be protonated at physiological pHs, are in bold.

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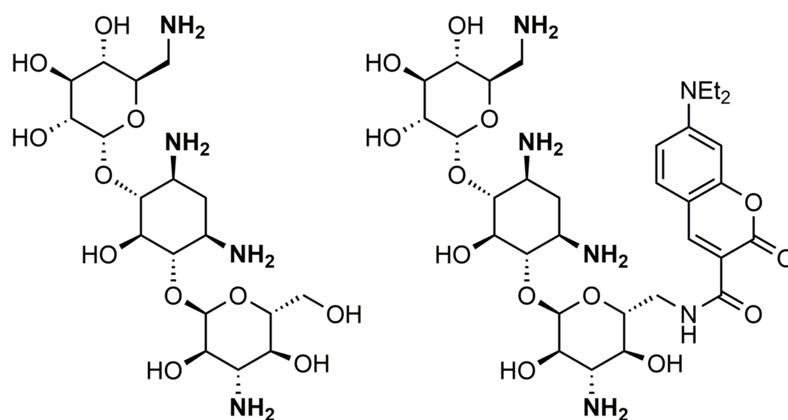

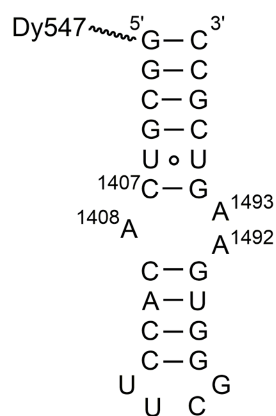

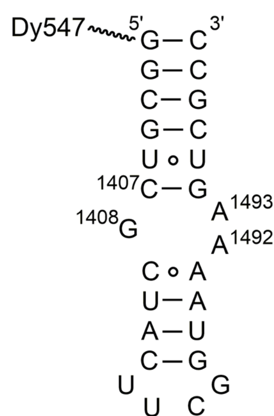

Scheme 2.
Top: Kanamycin and kanamycin-coumarin conjugate. *Bottom:* Fluorescently labeled 16S and 18S A-site RNA constructs.

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Table 1

Binding to the 16S and 18S A-sites and interference with in vitro bacterial and eukaryotic translation.

Compounds	16S A-site IC_{50} (μM)	18S A-site IC_{50} (μM)	Bacterial IVT IC_{50} (μM)	Eukaryotic IVT IC_{50} (μM)
PMB	52±2	54±5	>220	46±1
Colistin	77±5	83±5	>220	32±2
1	20±1	19±3	>110	13±1
2	4.5±0.2	4.1±0.1	>110	8.8±0.8
Kanamycin	6.6±0.2	4.5±0.5	0.41±0.02	100
PMBN	32±2	-	-	-
CMS	>500	-	-	-