A Quaternary Ammonium Warhead on Polymyxin Potentiates it Against Drug Resistant Bacteria

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Abstract

Gram-negative pathogens continue to impose a tremendous health burden across the globe. Here, we describe a novel series of polymyxin-based agents grafted with membrane active quaternary ammonium warheads to combine two important classes of Gram-negative antimicrobial scaffolds. The goal was to deliver a targeted quaternary ammonium warhead onto the surface of bacterial pathogens by using the outer membrane homing properties of polymyxin. More specifically, the fatty acid tail of polymyxin was replaced with quaternary ammonium moieties of various hydrocarbon lengths while retaining the polymyxin cyclic core. The most potent agents resulted in new scaffolds that retained the ability to target Gram-negative bacteria and had no detectable toxicity towards mammalian cells. Finally, we showed using a molecular dynamics approach that the new agents retained their ability to engage in specific interactions with lipopolysaccharide molecules. Our results serve as an example of how two membrane active scaffolds can be combined to produce a class of novel scaffolds with unique activities.

Introduction

Life expectancy has increased in every corner of the world during the past century in part due to the emergence of antibiotics that began with the discovery of penicillin. But recently, emergence of bacterial resistance to the existing antimicrobials and a sharp decrease in new antimicrobials discovery has eroded a large fraction of the gains made in the fight against bacteria.(1, 2) The World Health Organization recently labeled infections due to β-lactamase producing strains of *Acinetobacter baumannii* (*A. baumannii*), *Pseudomonas aeruginosa* (*P. aeruginosa*) and Enterobacteriaceae as being of critical concern.(3) Therefore, there is a need for continued development of agents that can lead to potential clinical candidates.

Gram-negative bacteria are difficult to eliminate because they have a complex cell envelope architecture.(4-6) In additional to the plasma membrane, they display an asymmetric outer membrane (OM) within which the outer leaflet is composed of amphipathic lipopolysaccharides (LPS) and the inner leaflet contains phospholipids.(7) LPS is composed of: O-antigen at the outer displaying portion, followed by core oligosaccharides, and lipid A at the base (**Fig. 1A**). The core oligosaccharide (inner and outer) contains various sugar units including 2-keto-3-deoxyoctulosonate (Kdo) [7]. Kdo is usually modified with phosphate groups that can add to the negative surface charge of Gram-negative bacteria. In *Escherichia coli* (*E. coli*), lipid A is made up of a β-1,6-linked glucosamine disaccharide phosphorylated at the 1 and 4 positions and acylated with six acyl groups.

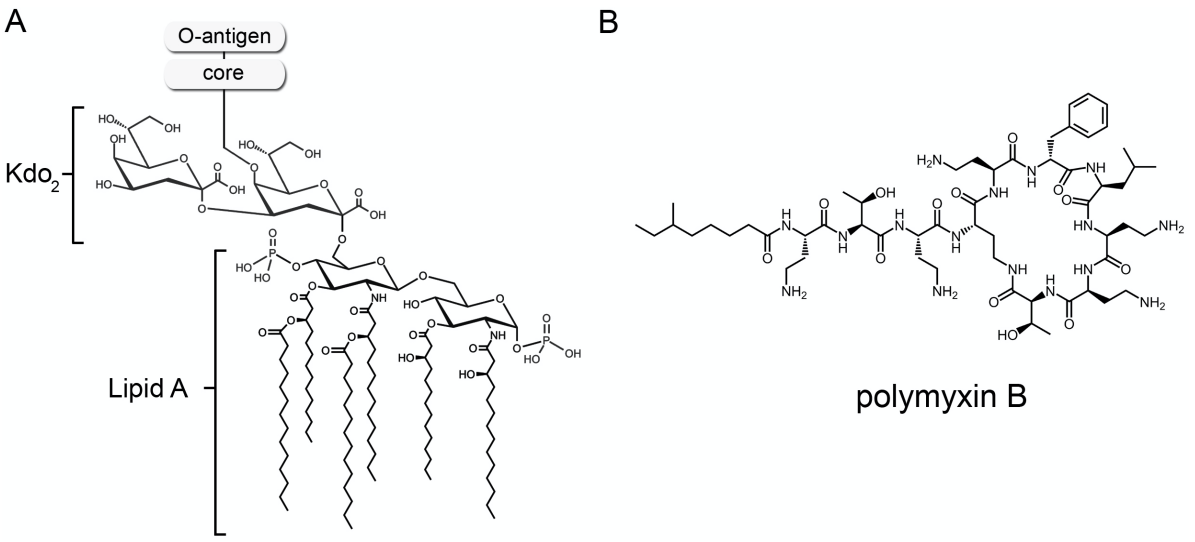

Figure 1. (A) Chemical structure of the inner core of LPS. (B) Structure of the parent compound PMB.

While LPS poses a formidable barrier for the passive permeation of large biomolecules and smaller therapeutic agents, a select number of natural product antibiotics operate by interacting directly with components of the OM accessible from the extracellular space. As a likely byproduct of bacterial warfare, polymyxins produced by soil bacteria *Paenibacillus polymyxa* bind LPS with high specificity and avidity.(8, 9) Within this group of natural products, two variants have been the focus of further clinical development for their therapeutic activity: polymyxin B (PMB) and the similarly structured polymyxin E (also clinically known as colistin) (**Fig. 1B**).(10, 11) PMB is composed of a 7-residue lactam ring with an exocyclic tripeptide and an acyl lipid tail attached to the *N*-terminus. At physiological pH, positively charged diaminobutyric acid (Dab) residues within the ring structure are proposed to form salt bridges with lipid A phosphate groups, thereby displacing Ca^{2+} and Mg^{2+} ions that crosslink LPS molecules into a stable bilayer.(12, 13) As a result, these physicochemical changes trigger OM instability, cell lysis, and death. While polymyxins have been used in clinical settings for over 50 years, polymyxin-resistant strains have been relatively rare according to a recent SENTRY

Antimicrobial Surveillance Program.(14) More recently, the clinical prescription of colistin has narrowed towards patients presenting with multidrug resistance (e.g., β-lactam resistant) as a "last resort" antibiotic in light of its reported nephrotoxicity following parenteral administration.(15) Due to its relatively low incidence of drug-resistance profile and activity against carbapenem-resistant pathogens, there has been a recent resurgence in the clinical use of PMB/colistin.(16)

The basic scaffold of polymyxins has two primary components. The cationic ring is proposed to interact with the anionic portions of lipid A while the acyl tail plays a more critical role in membrane-anchoring. To this end, the nonapeptide of PMB (7 residues composing the ring and 2 exocyclic residues) retains the ability to associate with lipid A *in vitro* and in live cells.(17, 18) Since its initial discovery, a large number of polymyxin-inspired derivatives have been developed and explored clinically.(19-22) Most recently, a chimeric peptidomimetic composed of polymyxin linked to a β-hairpin peptide macrocycle proved to be highly effective against drug-resistant Gram-negative ESKAPE pathogens.(23) Excision of the exocyclic Dab-1 residue was also found to produce a polymyxin derivative that was less toxic while retaining potent activity.(24) Our laboratory reported a polymyxin derivative conjugated to the antigenic epitope dinitrophenol to promote an immune response against Gram-negative pathogens.(25) Collectively, these efforts towards making next-generation polymyxin-inspired antibiotics highlight the unique mode of action of polymyxins and indicate the potential utility of polymyxin-based derivatives in clinical settings.

Naturally existing PMB displays a simple saturated hydrocarbon acyl tail connected to the *N*-terminus. Here, we designed hybrid molecules composed of quaternary ammonium and polymyxin (QAPs) with the goal of concentrating membrane-active moieties on the OM. More specifically, we designed a series of polymyxin-like agents grafted with quaternary ammonium

compound (QAC) on the *N*-terminus, a functional handle that has potential antimicrobial activities. A similar strategy of QAC grafting was recently demonstrated by the Boger lab to potentiate vancomycin against drug resistant Gram-positive pathogens.(26) Unlike the positive charge on an amine, the cationic charge on QAC is permanent irrespective of pH. QACs have been proposed to exert their bactericidal activity by electrostatic attraction to the negatively charged moieties on the bacterial surface followed by the interaction of the long alkyl chains with the OM, leading to solubilization of the membrane and cell lysis. QACs have an extensive history in having potent antimicrobial activity(27) and continue to inspire novel potent agents(28, 29) including some recent scaffolds we recently developed.(30-32) A number of QACs have widespread use as disinfectants and as surfactants. A prominent example is Cepacol®, a popular throat remedy that contains cetylpyridinium chloride (CPC). Our results showed that the introduction of QAC within PMB yields derivatives that retain their potency against Gram-negative pathogens while extending a therapeutic profile towards broad-spectrum activity.

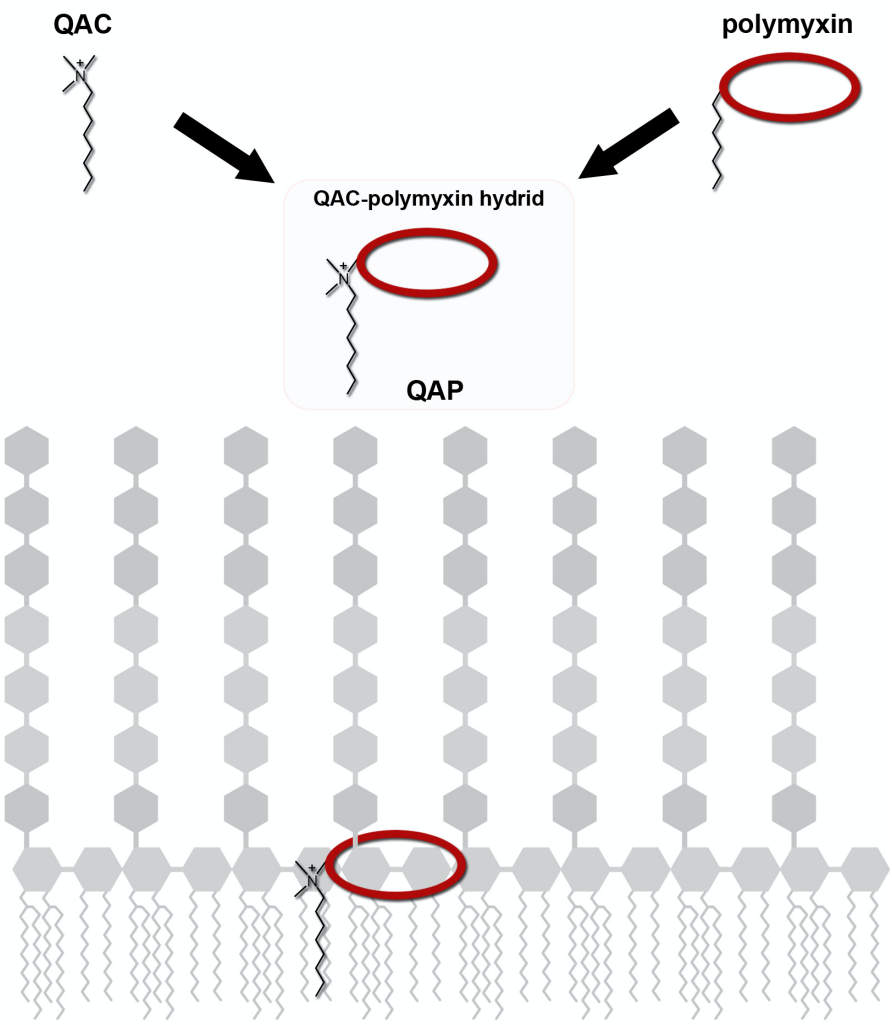

Figure 2. Schematic representation of the design of quaternary ammonium polymyxins (QAPs). Features of polymyxin responsible for lipid A binding are combined with the membrane active portion of QACs.

Results

All QAPs were synthesized by building the quaternary ammonium moiety on solid support – a procedure with little literature precedence; therefore, we had to optimize the conditions necessary to yield a high level of conversion of the final product. Briefly, all derivatives were built by solid-phase peptide synthesis starting from Wang resin preloaded with Fmoc-L-Thr(tBu)

using the following sequence: elongation to the final linear peptide using standard peptide coupling conditions, installation of the quaternary ammonium moiety on solid support, release from solid support to reveal a single free carboxylic acid and one free amino group, cyclization to generate the lactam ring, and global deprotection. Final products were purified by RP-HPLC and characterized by analytical RP-HPLC, MALDI-TOF, and NMR. In all, four main strategies were used to build the quaternary ammonium unit: (1) coupling of *N*,*N*-dimethylglycine followed by exhaustive alkylation to install the longer alkyl chain, (2) exhaustive reductive amination to install di-alkyl groups followed by alkylation with the longer alkyl chain, (3) installation of an electrophile onto the polymyxin backbone followed by displacement with an amine nucleophile, and (4) exhaustive alkylation to install identical tri-alkyl chains. We found that solvent choice for alkylation steps was important and discovered that in all cases DMF resulted in cleaner conversion to the quaternary ammonium.

In all, fourteen QAPs were built that varied in alkyl chain length, position of alkyl chain, number of alkyl modifications, and location of quaternary moiety (**Fig. 3**). These variations were chosen to cover a wide chemical space while attempting to optimize the grafting of QAC onto the polymyxin scaffold. Initially, the antimicrobial activity of the QAPs was assessed against *E. coli* to guide the re-design of the molecules. A single long alkyl chain was installed in **1** to yield a QAP that most closely resembled the combination of a traditional QAC and polymyxin. For the first set of QAPs (**1**-**4**), the exocyclic tripeptide from PMB was retained. It was found that the minimum inhibitory concentration (MIC) of **1** was elevated relative to PMB, which is consistent with previous attempts to lengthen the hydrophobic tail of polymyxins.(33) Introduction of a heptyl chain (**2**) on the quaternary ammonium derivatized from dimethyl glycine or an octyl chain (**3**) installed directly on the *N*-terminus amino group resulted in MIC values closer to the parent PMB. The introduction of a biphenyl group (**4**) was not well tolerated, contrary to a previous example showing the replacement of the fatty acid tail with biphenyl.(34)

The next series of QAPs only included the dipeptide exocylic component. The goal in removing the terminal L-Dab residue was to potentially shift the quaternary ammonium closer to the membrane-aqueous interface in register with the parent PMB. Matching the longest chain (heptyl) of PMB with a quaternary ammonium resulted in a QAP (**5**) that was equipotent to PMB. Similar to **1**, the elongation of the alkyl chain in **6** led to poor biological activity. Reduced activity was also observed upon the introduction of tri-heptyl, biphenyl, and pyridinium (**7**-**9**).(35) For the next series of derivatives, we aimed to retain the basic fatty acid tail of polymyxin and, instead, install the quaternary ammonium group on the sidechain (**10**-**13**). It was clear that additional alkyl or pyridyl chains did not lead to improvements in biological activity. Interestingly, quaternization of terminal L-Dab with methyl groups was well tolerated. On the hand other, extensive trimethylation of all L-Dab residues (**14**) was not well tolerated (MIC = 30 μM) (**Fig. S1** for the chemical structure), which likely interfered with phosphate binding.

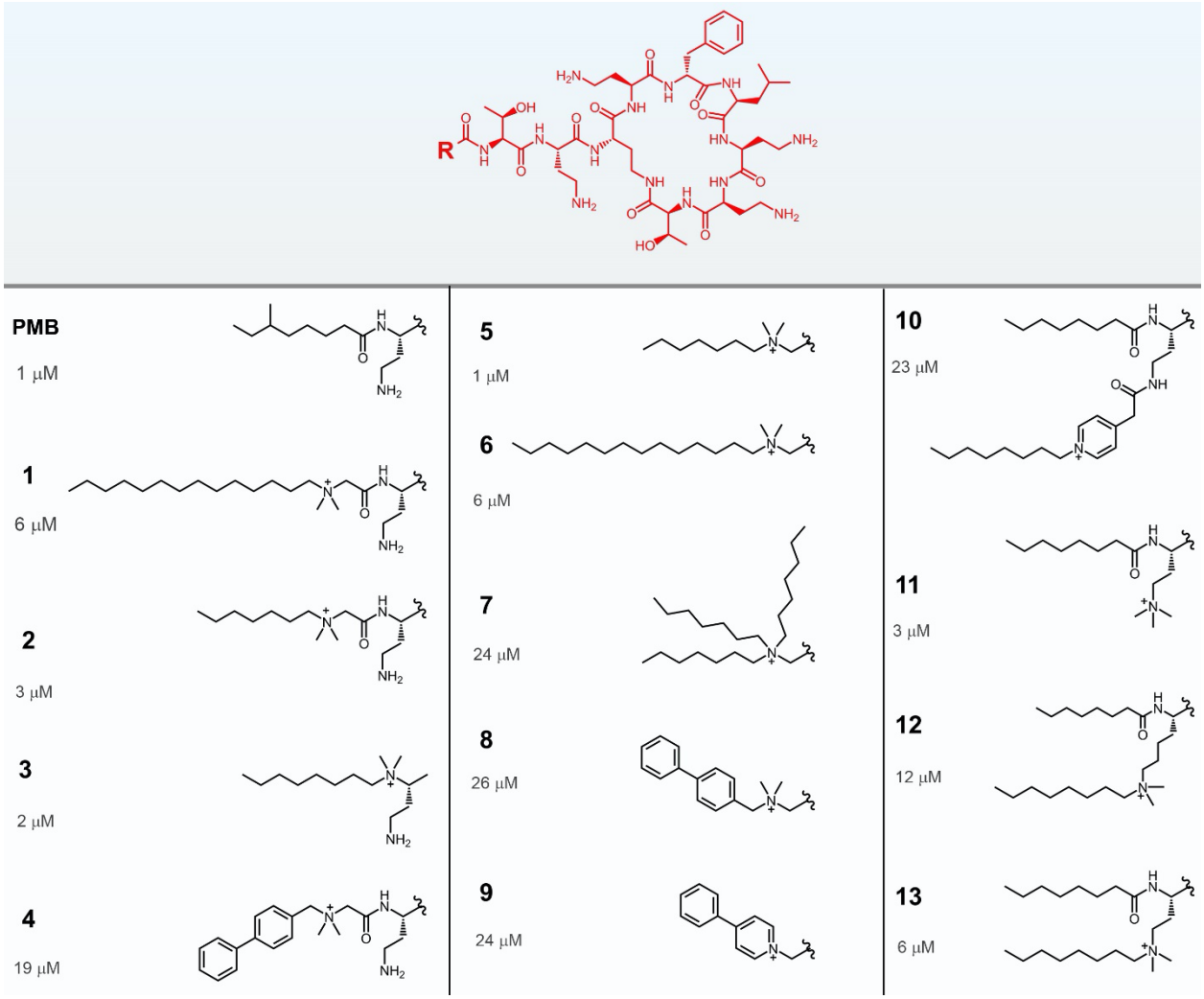

Figure 3. Chemical structure of QAPs. MIC is shown below the compound label against *E. coli* (ATCC 25922). MIC for compound **14** is 30 μM (structure in **Fig. S1**).

Next, the same panel of QAPs was tested against a number of Gram-negative pathogens and the Gram-positive pathogen *S. aureus* USA300 (**Table 1**). There is a clear difference in patterns of MIC values between *E. coli* and the rest of the Gram-negative pathogens tested. More specifically, the most potent agent (**5**) against *E. coli* had minimal effect against *A. baumannii* while retaining decent antimicrobial activity against two different strains of *P. aeruginosa*. Shorter alkyl chains in **2** and **3** resulted in potent activity against *P. aeruginosa* with little activity against *A. baumannii* and *S. aureus*. Most interesting was the finding that the increase in the

length or number of alkyl chains (QAPs **1**, **6**, and **7**) can result in activity in two Gram-negative bacteria (*P. aeruginosa* and *E. coli*) and also be potent against *S. aureus*. To the best of our knowledge, this is the first example of a polymyxin derivative with potency against *P. aeruginosa* that contains a long alkyl chain.

compound	MIC (μM)					
	A. baumannii ATCC19606	***A. baumannii*** DoD17943	***P. aeruginosa*** PAO1	***P. aeruginosa*** PA14	***S. aureus*** USA300	**Hemolysis**
PMB	2	>52	1	0.5	52	32
1	6	>47	3	6	6	32
2	13	>50	2	1	>50	32
3	7	>52	2	1	>52	32
4	>48	>48	>48	>48	>48	32
5	>55	>55	7	7	>55	32
6	>51	>51	3	3	3	32
7	>48	>48	12	6	12	32
8	>52	>52	>52	52	>52	63
9	>46	>46	>46	>46	>46	32
10	>44	>44	>44	>44	>44	63
11	13	>51	3	2	>51	32
12	>46	>46	24	46	>46	32
13	>47	>47	>47	>47	>47	32
14	>44	>44	>44	>44	>44	63
12(3)2(3)12	250	250	4	4	2	8

Table 1. Summary of MIC values for PMB, QAPs, and a positive control **12(3)2(3)12**. **12(3)2(3)12** is a cationic antimicrobial agent previously described by the Wuest lab. Hemolysis was also determined.

To explore the dynamic behaviors of PMB and **3** and their interactions with *P. aeruginosa* LPS membrane, we performed molecular dynamics (MD) simulations of PMB and **3** in the Pa.G2.O11 bilayers (see Methods for the details and **Fig. SX**). We started our simulation with inserting PMB agents in the middle of the LPS core region (17.5 Å ~ 32.5 Å from the membrane center, z = 0 Å) so as to investigate the positions and interactions of PMB and **3** in *P. aeruginosa* LPS environments. In our 1-μs simulation, the PMB agents in all systems exhibited fairly restricted mobility in the LPS core region. Especially, it is worth noting that PMB agents

prefer to localize between two layers of phosphate groups in heptose (Hep; in inner core) and lipid A (**Fig. 4A** and **B**). This implies that the two negatively charged phosphate layers provide high electron density and prevent the agent from moving to the acyl tail region of the membrane directly. As shown in **Fig. SX**, each L-Dab residue frequently interacted with both phosphate layers, which helps the PMB head to settle near lipid A head groups. The structural differences of PMB with **3** are mainly found in the tail part, i.e., the primary amine in the PMB is changed to quaternary ammonium in **3**, giving an additional +1 charge in the **3** tail. As these results showed, the interaction rate of the **3** tail to the PO_4-lipid A was higher than PMB, implying that the accessibility of the **3** tail to the lipid A head can be driven by the positively charged quaternary ammonium group. In the **3** simulation, we observed an insertion of D-phenylalanine (D-Phe) and L-Leucine (L-Leu) side chains into the membrane acyl tails region, showing a similar tendency with the previous PMB simulation results (Berglund et al, 2015).[18] When the **3** head was spanning the phosphate layer in the core region, the cyclic polypeptide ring assumed a vertical shape which made D-Phe and L-Leu side chain face outward (**Fig. 4F**, **G**, and **H**). As the **3** head approaches the lipid A head region, the cyclic polypeptide ring gradually assumed a laid down shape which facilitated the approach of D-Phe and L-Leu to the membrane acyl tail region (**Fig. 4C**, **D**, and **E**). We speculate that the quaternary ammonium group in **3** helps not only settle PMB acyl tail deeper in the membrane, but also drives the insertion of the **3** head.

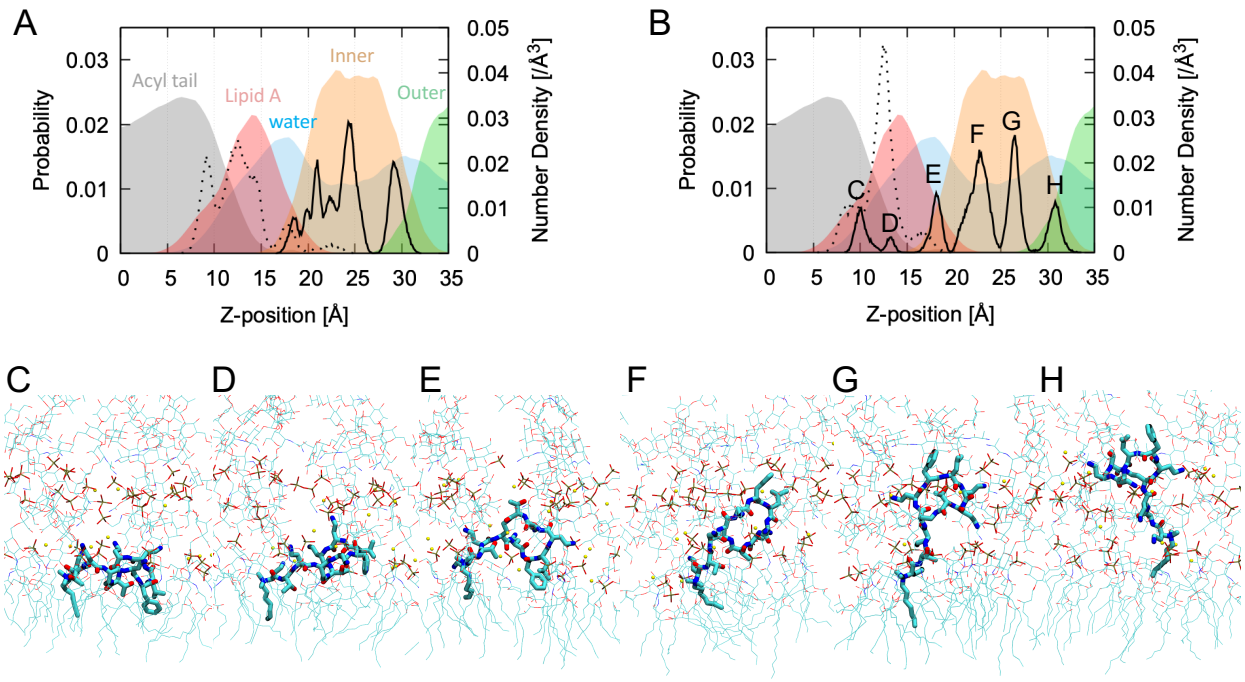

Figure 4. Probability of Z-position of the cyclic polypeptides (line) and the tail (dotted line) for (A) PMB, and (B) **3** with the heavy-atom number density profiles (**acyl tail**, gray; **lipid A head**, pink; **inner core**, ivory; **outer core**, green). The membrane center was located at Z = 0, and positions over four replicas were used to get results. Snapshots of compound **3** in the LPS membrane (C−H). Each snapshot represents a corresponding peak in panel (B).

Given that the core cyclic portion of PMB remained unmodified for the most potent QAPs, we anticipated that these agents may retain the ability to perturb the OM and thus synergize with other antibiotics that cannot typically cross the OM to reach their targets. To test this, a checkerboard assay was set up with **1** and **3** in combination with either vancomycin or rifamycin (**Fig. SX**). Synergy, additivity, and antagonism were designated based on the calculation of the FIC index. Similar to other known OM disruptors, co-treatment with vancomycin did not lead to synergistic activity. However, the combination of the QAPs tested had clear and strong synergistic effects with rifamycin. These results suggest that QAPs retain the ability to actively

facilitate the penetration of antibiotics and may be appropriate for drug cocktails in treatment regimens. Finally, we tested the potential toxicity of QAPs by measuring their hemolysis level and their effect on cultured kidney cells. Hemolysis was assessed by the red blood cell lysis assay (Lysis20). For all QAPs tested, hemolysis levels were found to be 32 μM or higher, which is considerably greater than the MIC concentration of the most potent QAPs (**Table 1**). For a select number of derivatives, additional cytotoxicity testing was performed using HEK-293 (human embryonic kidney cells) to evaluate the potential nephrotoxicity. QAPs X, Y, and Z were chosen due to their favorable MIC values and imposed no measurable loss of cellular viability at the highest concentrations tested (**Fig. SX**).

CONCLUSION

The recent discovery that dual-targeting polymyxin derivatives can target difficult Gram-negative pathogens shows that polymyxins have unique targeting properties and can form the basis of efficacious and safe antibiotic classes (23). Similarly, we envisioned that we could increase the effective concentration of QACs at the membrane level by grafting quaternary ammonium moieties onto the tail of PMB, thus yielding a hybrid series of QAPs. We showed that QAPs had strong antimicrobial activity against Gram-negative bacteria. Interestingly, some QAPs showed broader activity against both Gram-negative and -positive pathogens. As the arsenal of antibiotics that are clinically efficacious starts to dwindle, strategies are needed to assemble novel agents that combine well-established agents with orthogonal mechanisms of action.

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