ACS Infectious Diseases

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Article

Quaternary Phosphonium Compounds: An Examination of Non-Nitrogenous Cationic Amphiphiles That Evade Disinfectant Resistance

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Cite This: *ACS Infect. Dis.* 2022, 8, 387−397

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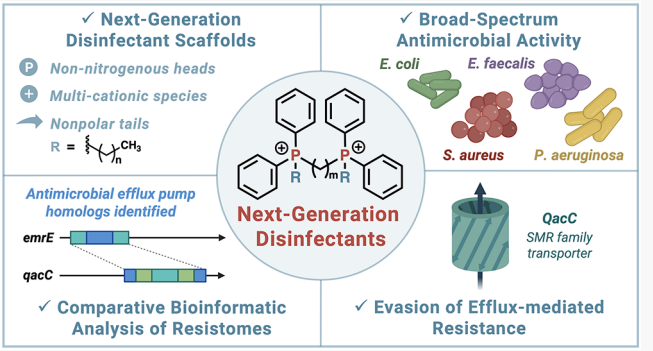
ABSTRACT: Quaternary ammonium compounds (QACs) serve as mainstays in the formulation of disinfectants and antiseptics. However, an over-reliance and misuse of our limited QAC arsenal has driven the development and spread of resistance to these compounds, as well as co-resistance to common antibiotics. Extensive use of these compounds throughout the COVID-19 pandemic thus raises concern for the accelerated proliferation of antimicrobial resistance and demands for next-generation antimicrobials with divergent architectures that may evade resistance. To this end, we endeavored to expand beyond canonical ammonium scaffolds and examine quaternary phosphonium compounds (QPCs). Accordingly, a synthetic and biological investigation into a library of novel QPCs unveiled biscationic QPCs to be effective antimicrobial scaffolds with improved broad-spectrum activities compared to commercial QACs. Notably, a subset of these compounds was found to be less effective against a known QAC-resistant strain of MRSA. Bioinformatic analysis revealed the unique presence of a family of small multiresistant transporter proteins, hypothesized to enable efflux-mediated resistance to QACs and QPCs. Further investigation of this resistance mechanism through efflux-pump inhibition and membrane depolarization assays illustrated the superior ability of P6P-10,10 to perturb the cell membrane and exert the observed broad-spectrum potency compared to its commercial counterparts. Collectively, this work highlights the promise of biscationic phosphonium compounds as next-generation disinfectant molecules with potent bioactivities, thereby laying the foundation for future studies into the synthesis and biological investigation of this nascent antimicrobial class.



KEYWORDS: *quaternary phosphonium compounds (QPCs), quaternary ammonium compounds (QACs), disinfectants, antimicrobial resistance, small multiresistant (SMR) family efflux pumps, QacC*

INTRODUCTION

Estimates predict that antimicrobial resistance (AMR) will represent the leading cause of global mortality by the year 2050,[1] underscoring the urgent need for novel antimicrobial compounds to forestall a postantibiotic era. The COVID-19 pandemic has further emphasized the centrality of antimicrobial compounds to modern public health, as disinfectant use has rapidly become critical for mitigating the spread of SARS-CoV-2, as well as opportunistic pathogens. Of the 558 disinfectant products currently listed by the EPA as effective against SARS-CoV-2, 260 (46.6%) list quaternary ammonium compounds (QACs) as an active ingredient.[2]

Many studies over the past century have investigated the antimicrobial properties of QACs, yet only modest structural innovation of QAC architectures has been explored.[3] As a result of their structural similarities, QACs exploit a common mechanism of action wherein the cationic head(s) of these amphiphiles are electrostatically attracted toward the net anionic charge of the bacterial cell membrane surface (Figure 1).[4] This interaction is followed by the insertion of the nonpolar tail(s) into the lipid bilayer of the membrane, coinciding with the cationic portion of the disinfectant, promoting the clustering of nearby phospholipids, ultimately resulting in the loss of membrane integrity and eventual cell lysis.[5,6]

As a result of their nonspecific mechanism of action, it was initially suggested that these compounds would innately evade

Received: November 19, 2021
Published: January 25, 2022

https://doi.org/10.1021/acsinfecdis.1c00611
ACS Infect. Dis. 2022, 8, 387−397

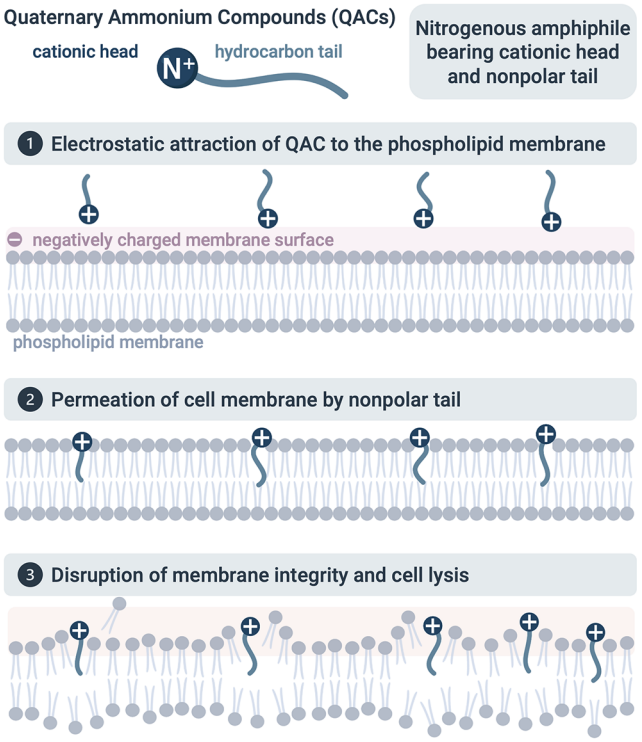


Figure 1. General mechanism of action for QACs in which the amphiphilic nature of the molecule facilitates membrane permeation, ultimately resulting in a loss of membrane integrity and subsequent cell lysis.

resistance development. However, tolerance to QACs emerged in the decades following their widespread use, culminating in the first identification of genetic QAC resistance in the 1980s.[7,8] These decreases in QAC susceptibility have been found to arise primarily through alterations to the membrane, biofilm formation, and stimulation of efflux systems, such as those encoded by the *qac* family of genes.[9]

Since the first discovery of *qac* genes in the 1980s,[10] 11 efflux pumps (QacA-J and QacZ) mediating QAC resistance have been identified across both Gram-positive and Gram-negative species. Furthermore, these genes have been found to rapidly spread through horizontal gene transfer (HGT), leading to an alarming prevalence of disinfectant resistance genes in clinical isolates globally.[11−15] Moreover, concomitant with the spread of disinfectant resistance, QAC treatments at subinhibitory concentrations have been demonstrated to promote co-resistance to common antibiotics via selective pressure for the spread of resistance plasmids encoding both *qac* and other AMR genes.[16] Thus, the ubiquity of disinfectant use spurred by the SARS-CoV-2 pandemic has led to heightened concern for the accelerated spread of QAC and antibiotic resistance.[17]

Collectively, these discoveries into the mechanisms of QAC resistance call for the development of next-generation disinfectant compounds that evade resistance while preserving broad-spectrum efficacy. Previous studies, including our own, have focused on elucidating structure−activity relationships (SARs) and structure−resistance relationships (SRRs) to enable the rational design of novel QAC compounds. These studies provided insights into the number and ratio of charges, length and types of side chains, core structural rigidity, and the inclusion of organometallic substituents have all contributed to the advancement of discovering next-generation QAC scaffolds.[5,18−30]

However, the design of cationic disinfectants is not limited to ammonium-based compounds; heteroatoms beyond nitrogen have also been a subject of interest, although not as prevalent. For example, trivalent sulfonium compounds and QPCs were recently the subject of review by our groups, in which the promise of these non-nitrogen-based cationic amphiphiles as potential solutions to QAC-related resistance and toxicity was highlighted.[31,32]

Inspired by these previous works, we envisioned further probing the underrepresented class of QPCs (Figure 2A).

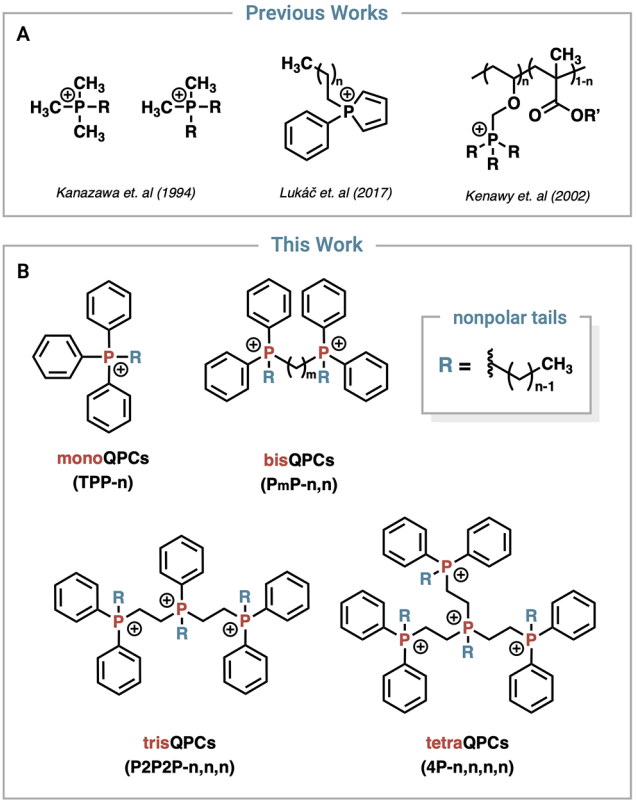


Figure 2. (A) Representative previous works on the synthesis and biological investigation of QPCs. (B) The present work expands beyond previous works to examine the structure−activity and SRRs of QPCs bearing nonpolar tails (R) of varying lengths (n) and multicationic scaffolds.

QPCs are characterized by their tetravalent phosphorous atom with a positive formal charge and have previously served as antitumor agents,[33,34] catalysts,[35] and mitochondrial antioxidants.[36,37] Polymeric QPCs have been widely utilized as surfactants,[38] water desalination agents,[39] and most importantly, antimicrobials.[40−45] Notably, QPC-based polymers have shown enhancements in activity relative to their nitrogen counterparts.[46−50] Despite this demonstrated promise of quaternary phosphoniums as effective antimicrobial scaffolds, few systematic investigations into the SAR of monomeric QPCs have been pursued. Previous reports have suggested that factors such as the saturation and number of hydrophobic tails may play an important role in the potency of these compounds, further enforcing the notion that optimizing the ratio of polar to nonpolar character is a major determinant of their antimicrobial activity.[31,51−53] Therefore, expanding upon

https://doi.org/10.1021/acsinfecdis.1c00611
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these previous works while leveraging our background in the design of potent QACs, we synthesized a library of QPC analogues and interrogated their SAR and SSR.

RESULTS AND DISCUSSION

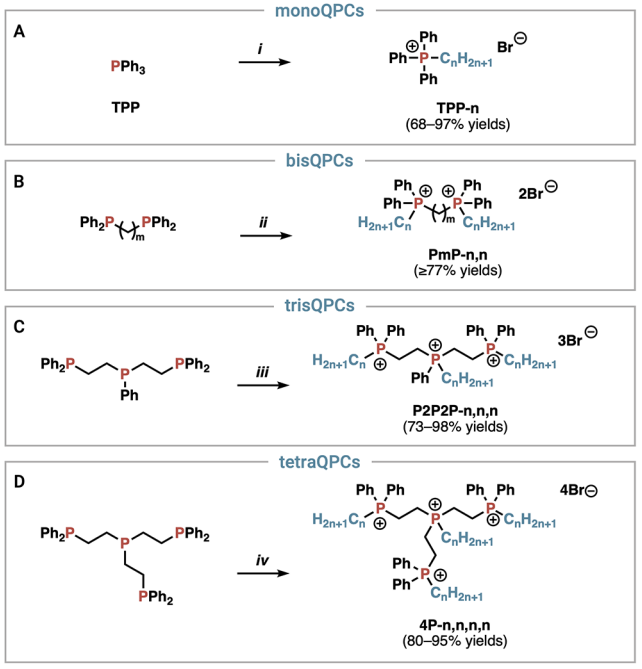
Preparation of a QPC Library through Facile Methods. We set out to identify a core phosphine scaffold that could provide access to a diverse library of QPCs through facile alkylation across a variety of chain lengths. We envisioned that this may be achieved through quaternization of phenylphosphine compounds such as 1,3-bis-(diphenylphosphino)propane (dppp) and triphenylphosphine (TPP) due to their ubiquity in organic synthesis[54,55] and relative air-stabilities.[56] Furthermore, previous studies have demonstrated promising antimicrobial activity for TPP derivatives against Gram-positive bacteria,[57−61] although a systematic investigation into TPP-based QPC architectures remains unexplored. Additionally, while several of these previously synthesized TPP derivatives have shown low micromolar antimicrobial activities, efflux pump-mediated resistance to TPP-derived QPCs has precluded their widespread utility.[62−64] Therefore, we began our investigations with the synthesis of TPP- and dppp-derived QPCs bearing hydrocarbon tails of varying lengths (Figure 2B), seeking to interrogate whether increasing the amphiphilic and cationic nature of the QPCs may increase broad-spectrum antimicrobial activity and aid in the evasion of efflux-pump-mediated resistance.

We posited that access to monocationic QPCs (monoQPCs) could be gained via alkylation of TPP. In an initial experiment, TPP was treated with excess 1-bromotetradecane in acetonitrile at reflux for 72 h, which afforded **TPP-14** in good yield. These conditions were found to be broadly effective for the synthesis of eight monoQPCs bearing hydrocarbon tails of 8−18 carbons in length, which were subsequently labeled **TPP-n** to represent the TPP core and respective tail lengths (n) (Scheme 1A).

To extend our study to the examination of phenylphosphonium compounds containing multiple sites for quaternization, we first turned our attention to the synthesis of biscationic QPCs (bisQPCs). Members from this QPC subclass were derived from alkyl-linked diphenylphosphine moieties, as inspired by the work of Long et al.[65] Beginning with dppp, an initial experiment with excess 1-bromohexadecane in acetonitrile at reflux for 48 h produced the corresponding biscationic phosphonium salt in high yield. This method was applied to the generation of seven additional dppp-derived bisQPCs. Next, we sought access to analogous structures by varying the alkyl linker from two up to six carbons in length. To our delight, application of the same technique was met with similar success, albeit reactions employing longer hydrocarbon tail lengths and/or shorter alkyl linkers required extended reaction times. Collectively, this procedure was utilized to prepare 40 bisQPCs classified as **PmP-n,n** to reflect the alkyl linker length (m) and hydrocarbon tail length (n) (Scheme 1B).

Continuing our preparation of multicationic QPCs (multiQPCs), we next turned to the synthesis of tris-cationic QPCs (trisQPCs), which we hypothesized could be derived from bis(2-diphenylphosphinoethyl)phenylphosphine in a similar approach as the bisQPCs. As predicted, tris-alkylation was possible by treating the starting material with a large excess of bromoalkane in acetonitrile at reflux for 96 h. To reduce the reaction time, transitioning to more vigorous conditions [dimethylformamide (DMF) at 110−120 °C] proved to be highly successful, generating the corresponding tris-cationic phosphonium salts in no longer than 24 h. In total, eight trisQPCs were generated and due to their structural similarities with the bisQPCs were referred to as **P2P2P-n,n,n**, indicating the ethylene-phosphorus backbone and the hydrocarbon tail length (n) (Scheme 1C).

Scheme 1. Overview of the General Syntheses for the Four Quaternary Phosphonium Compound (QPC) Subclasses; Detailed Synthetic Procedures are Available in the Supporting Information; (i) $C_nH_{2n+1}Br$ (1.5 equiv), MeCN, 80 °C, 72 h; (ii) $C_nH_{2n+1}Br$ (2.5 equiv), MeCN, 80 °C, 24−96 h; (iii) $C_nH_{2n+1}Br$ (3.5−4.0 equiv), MeCN (80 °C, 96 h) or DMF (110−120 °C, 21−24 h); and (iv) $C_nH_{2n+1}Br$ (4.5 equiv), DMF, 105 °C, 24 h



Owing to the facile alkylation of the phenylphosphines thus far, we speculated whether access to tetra-cationic QPCs may also be possible. While tetra-cationic quaternary ammonium species have been reported by our group in the past, their syntheses have presented challenges.[26] However, we were pleasantly surprised to find that the tetra-alkylation of tris[2-(diphenylphosphino)ethyl]phosphine, containing three diphenylphosphine moieties as well as a trialkylphosphine, was not only successful but also attained through a one-step synthesis under conditions nearly identical to those of the trisQPC series. Employing this approach, three tetraQPCs were isolated in high yields and designated as **4P-n,n,n,n** to represent the tetraphosphonium core and respective hydrocarbon tail lengths (n) (Scheme 1D). Synthetic procedures and full characterization of all synthetic compounds are presented in the Supporting Information.

Evaluation of Bioactivity of the QPC Library. Of the 59 QPCs that were synthesized, we focused on assessing the bioactivities of 8 monoQPCs, 29 bisQPCs, 6 trisQPCs, and 3 tetraQPCs, noting that QPCs bearing long hydrocarbon tails displayed poor water solubility. To mitigate solubility issues, a 2.5% dimethyl sulfoxide (DMSO) carrier concentration was

https://doi.org/10.1021/acsinfecdis.1c00611
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used to solubilize the compounds at the highest test concentration (250 μM). The antimicrobial activity, as well as hemolytic activity, was assessed using red blood cell (RBC) lysis as a proxy for the latter. For comparison to commercially employed QACs, benzalkonium chloride (BAC; 70% benzyldimethyldodecylammonium chloride and 30% benzyldimethyltetradecylammonium chloride) and cetylpyridinium chloride (CPC) were included in the assays. Additionally, **2Pyr-11,11**, a pyridinium-based biscationic QAC displaying best-in-class potency[5] out of roughly 650 compounds synthesized to-date by our group, was also included for comparison. The complete set of minimum inhibitory concentration (MIC) values against a panel of six bacterial strains [community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA; USA 300−0114), hospital-acquired methicillin-resistant *S. aureus* (HA-MRSA; ATCC 33591), methicillin-susceptible *S. aureus* (MSSA; SH1000), *Enterococcus faecalis* (OG1RF), *Escherichia coli* (MC4100), and *Pseudomonas aeruginosa* (PAO1)] along with the RBC lysis (represented by $Lysis_{20}$) are presented in Table 1.

Elucidation of SARs. First, inspection of the bioactivity profiles of the monoQPCs indicated trends that were unique from the multicationic species examined. Optimal activity against MSSA, CA-MRSA, and HA-MRSA was observed for compounds bearing 11−13-carbon tail lengths (MIC = 0.5, 1, and 2 μM, respectively). For example, **TPP-13** displayed the enhanced potency among the monoQPCs against *E. coli*, with a ∼63-fold improvement in activity (MIC = 4 μM) compared to its 12-carbon analogue. This trend between bioactivity and tail length closely aligns with previous reports from our group,[66] as hydrophobic tails between 12 and 14 carbons have been established as ideal for disruption of the intermolecular forces of the lipid bilayer in Gram-positive bacteria.[4] At longer chain lengths, activity against *E. faecalis* and *P. aeruginosa* continued to increase, with **TPP-16** resulting in ∼63-fold and ∼16-fold improvement in activity (MIC = 4 and 16 μM) compared to CPC.

In comparison to the monoQPCs, the bisQPCs followed a slightly different pattern in bioactivity, with compounds bearing shorter tail lengths displaying optimal activities across all strains. Against MSSA and CA-MRSA, compounds with 8-carbon tail lengths displayed the best activities, with **P2P-8,8**, **P5P-8,8**, and **P6P-8,8** each reporting sub-micromolar activity (MIC = 0.5 μM) for at least one of the two strains. In contrast, against *E. faecalis*, *E. coli*, and *P. aeruginosa*, alkyl tail lengths of 10 and 11 carbons resulted in >fourfold enhancements in activity compared to their 8-carbon counterparts. However, as the hydrocarbon tail length increased beyond 11 carbons, activity across all strains began to decline, with this trend being most evident against *E. faecalis*, *E. coli*, and *P. aeruginosa*. Interestingly, these findings contrast our previous investigations of multiQACs, in which growing chain lengths displayed improved activity,[67] suggesting that the interaction between bisQPCs and the cellular membrane may deviate from that of QACs. Furthermore, the effect of varying the alkyl linker separating the two quaternized phosphines was also a subject of interest. While this modification led to slight improvement in activity for five of the strains, increasing the alkyl linker length did lead to a noteworthy increase in activity against *E. faecalis*.

Notably, **P6P-10,10**, bearing a six-carbon linker, displayed the greatest broad-spectrum activity of all the prepared QPCs. Moreover, **P6P-10,10** displayed improved activity compared to both commercial QACs, as well as our best-in-class biscationic QAC, **2Pyr-11,11**. To further examine the broad-spectrum efficacy of **P6P-10,10** compared to the leading commercial QAC (BAC), transmission electron microscopy (TEM) imaging was performed. As depicted in Figure 3, devastating amounts of cell lysis are observed in the presence of QPCs but are absent after treatment with BAC. The results

Table 1. Antimicrobial Activity and Cytotoxicity Hemolysis, Measured as MIC and $Lysis_{20}$ (Lys_{20}), Respectively

		Minimum Inhibitory Concentration (μM)						
	Compound	MSSA	CA-MRSA	HA-MRSA	*E. faecalis*	*E. coli*	*Pa*[b]	Lys_{20} (μM)[a]
QACs	BAC	4	4	8	250	63	250	16
	CPC	1	1	2	250	32	250	4
	2Pyr-11,11	1	2	2	8	2	16	4
monoQPCs	TPP-8	4	4	125	>250	>250	>250	63
	TPP-10	1	2	8	250	250	250	16
	TPP-11	1	1	4	125	125	250	16
	TPP-12	0.5	1	2	63	250	63	8
	TPP-13	1	1	2	16	4	32	4
	TPP-14	2	2	2	8	16	16	4
	TPP-16	2	2	4	4	16	16	4
	TPP-18	4	8	8	16	125	125	4
bisQPCs	P2P-8,8	1	0.5	8	250	>250	250	63
	P2P-10,10	1	1	2	8	16	8	4
	P2P-11,11	1	1	2	8	32	16	4
	P2P-12,12	1	2	8	32	63	63	4
	P3P-8,8	1	1	4	250	>250	250	63
	P3P-10,10	2	1	2	4	63	4	2
	P3P-11,11	1	1	16	4	8	16	4
	P3P-12,12	1	1	4	32	125	125	4
	P3P-13,13	2	2	4	63	63	63	4
	P3P-14,14	2	2	8	125	63	>250	2
	P4P-8,8	1	1	4	125	250	125	63
	P4P-10,10	1	2	2	4	16	8	2
	P4P-11,11	1	2	2	4	16	16	2
	P4P-12,12	2	2	4	16	63	>250	1
	P4P-13,13	2	2	8	125	125	>250	2
	P4P-14,14	8	4	4	63	125	>250	1
	P5P-8,8	0.5	1	2	125	250	125	32
	P5P-10,10	1	1	4	2	16	4	2
	P5P-11,11	2	2	4	2	8	16	2
	P5P-12,12	2	2	4	16	32	63	2
	P5P-13,13	4	4	4	63	125	250	2
	P5P-14,14	4	8	8	63	125	>250	1
	P5P-16,16	8	16	16	125	125	250	1
	P6P-8,8	0.5	0.5	2	63	125	63	16
	P6P-10,10	1	1	2	2	8	4	2
	P6P-11,11	2	2	4	2	16	16	2
	P6P-12,12	2	2	2	16	32	32	2
	P6P-13,13	4	2	4	32	63	>250	2
	P6P-14,14	4	4	8	63	63	250	2
trisQPCs	P2P2P-8,8,8	2	1	2	250	250	>250	16
	P2P2P-10,10,10	2	2	8	250	32	>250	2
	P2P2P-11,11,11	2	4	4	63	63	>250	1
	P2P2P-12,12,12	2	4	8	125	125	>250	1
	P2P2P-13,13,13	2	4	16	125	125	>250	2
	P2P2P-14,14,14	16	16	32	250	250	>250	1
tetraQPCs	4P-10,10,10,10	16	32	32	250	250	>250	8
	4P-11,11,11,11	16	32	125	125	125	>250	4
	4P-12,12,12,12	16	16	63	250	250	>250	4

[a]Due to the low solubility of compounds bearing long tail lengths, 2.5% DMSO was used as a carrier, resulting in cell lysis above 63 μM. [b]Pa represents *P. aeruginosa*.

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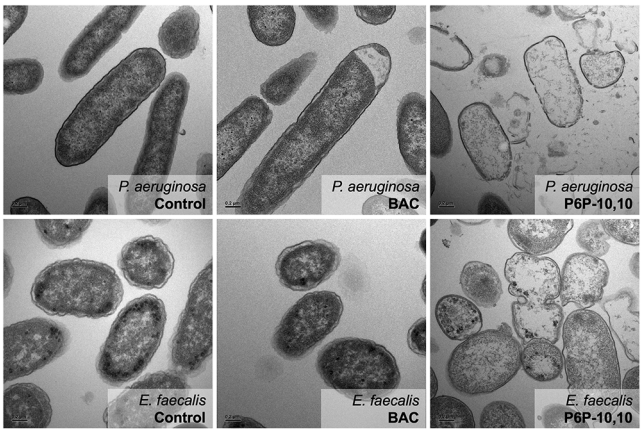

Figure 3. TEM images of *P. aeruginosa* and *E. faecalis* treated with one-half of their respective MICs of the leading QAC (BAC) and best-in-class QPC, **P6P-10,10**. A 2.5% DMSO solution was used as a control.

of the TEM analysis underscore the distinct efficacy of **P6P-10,10** against both Gram-positive (*E. faecalis*) and Gram-negative (*P. aeruginosa*) pathogens over BAC. Together, these results highlight the promise of bisQPCs, namely, **P6P-10,10**, as next-generation disinfectant compounds that warrant further investigation.

Lastly, analysis of the multiQPCs revealed that additional phosphonium residues did not enhance bioactivity. Rather, tris-cationic multiQPCs unveiled a similar trend to that of the bisQPCs, with shorter alkyl tail lengths (8−11 carbons) exhibiting greater potencies. In particular, **P2P2P-8,8,8** illustrated the best activity against the MSSA, CA-MRSA, and HA-MRSA strains (MIC = 2, 1, and 2 μM, respectively). However, in general, increasing the number of phosphonium atoms led to an overall decrease in antimicrobial activity for both tris- and tetra-cationic QPCs compared to their biscationic counterparts.

Determination of QPC Hemolytic Activity. RBC lysis (measured as $Lysis_{20}$) appeared to increase with alkyl chain length for mono- and bisQPCs. For mono-, bis-, and trisQPCs, eight-carbon species consistently displayed the lowest hemolytic activity, with no compound reporting toxicity greater than BAC ($Lysis_{20} \geq 16$ μM). However, increasing the chain length from 8 to 10 carbons had a profound impact on toxicity, leading to at least an eightfold increase in hemolytic activity for bis- and trisQPCs. For compounds with alkyl chains above 10 carbons, hemolytic activity continued to gradually increase. Additionally, the length of the alkyl linker had a minor impact on the toxicity of bisQPCs, with longer spacers leading to increased hemolytic activity. These results parallel previous studies on antimicrobial and anticancer amphiphiles in which increasing hydrocarbon tail length correlates with the increase in hemolytic activity due to membrane disruption.[68,69]

Elucidation of QPC Resistance Mechanisms. Upon comparison of the QPC bioactivities between strains, several trends emerged. First, in agreement with previous literature on QACs, Gram-negative species were less susceptible than Gram-positive species to compound treatment, presumably owing to the presence of the outer-membrane.[4] Next, between the Gram-positive *S. aureus* and *E. faecalis* strains examined, the latter generally displayed significantly decreased susceptibility. This result contrasts our previous QAC investigations, in which MRSA and *E. faecalis* display similar susceptibilities.[67,70] However, this result does align with the pattern of increased AMR in enterococci species, especially toward cell−wall targeting molecules,[71] and further suggests a degree of divergence in the mechanisms between QAC and QPC activities.

Intriguingly, among the *S. aureus* strains, a pattern of resistance for HA-MRSA emerged. Specifically, mono- and bisQPCs possessing short to moderate hydrocarbon tail lengths (8−12 carbons) displayed significantly higher MICs against the HA-MRSA strain compared to the MSSA and CA-MRSA strains. This pattern of resistance directly contrasts our previous findings with QACs, in which CA-MRSA typically displays a degree of resistance while MSSA and HA-MRSA display higher susceptibilities.[66,67,70] Because the efflux of cationic TPP derivatives, such as methyltriphenylphosphonium (methylTPP) (Figure 4A), has been previously documented in *S. aureus*,[62−64] we postulated that the observed resistance may be the result of the distinct presence of an efflux pump in HA-MRSA.

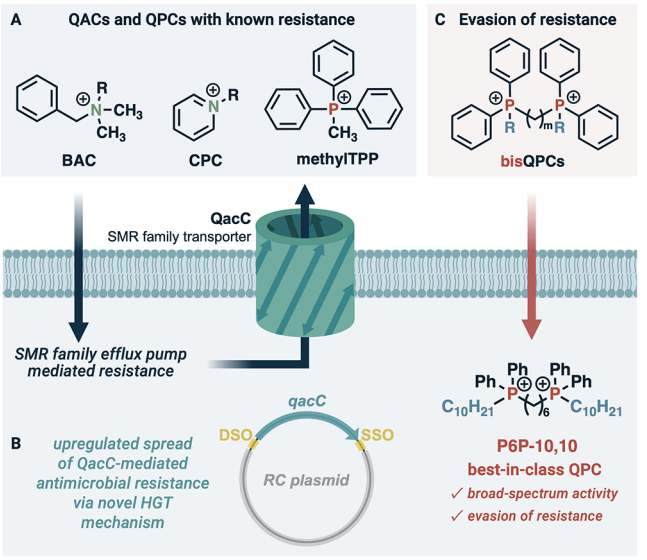

Figure 4. Proposed mechanism for QacC-mediated resistance to QACs and QPCs in HA-MRSA. (A) The SMR family transporter, QacC, is known to efflux commercial QACs such as BAC and CPC in addition to QPCs, such as methyltriphenylphosphonium (methylTPP). (B) The spread of QacC-mediated resistance is purported to be facilitated by the novel "DSO-gene-SSO" HGT mechanism, characterized by the presence of the gene on RC plasmids between the DSO and SSO.[71] (C) Notably, the bisQPC, **P6P-10,10**, evades this resistance mechanism, conferring broad-spectrum activity.

Implication of SMR Family Transporters in QPC Resistance. To further investigate this hypothesis, bioinformatic analysis was used to identify potential efflux pump-encoding genes in the bacterial genomes of the strains tested in our study. Comparative analysis of the resistomes of our bacterial panel using the Comprehensive Antibiotic Resistance Database and the National Center for Biotechnology Information Basic Local Alignment Search Tool unveiled the unique presence of the QAC resistance gene, *qacC*, in HA-MRSA (Table 2).

The *qacC* gene encodes the small multidrug resistance (SMR) family transporter protein, QacC, first identified by Lyon and Skurray in 1987 and implicated in the efflux of

https://doi.org/10.1021/acsinfecdis.1c00611
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Table 2. Comparative Bioinformatic Analysis of the Bacterial Panel[a]

	Transporter Family	MFS					RND		ABC		MATE		SMR		
	gene	*qacA*	*qacB*	*norA*	*norB*	*tetA*	*mexB*	*mtrC*	*macB*	*tolC*	*mepA*	*mdtG*	*qacC*	*qacE*	*emrE*
Strain	MSSA			●	●	●					●	●			
	CA-MRSA			●	●	●					●	●			
	HA-MRSA			●	●	●					●		●		
	E. faecalis					●			●					●	
	E. coli						●	●	●	●	●	●			●
	P. aeruginosa					●	●	●	●						●

[a]Representative resistance-mediating efflux pumps from the five multidrug efflux pump families are shown, highlighting the unique presence of SMR family genes *qacC*, *qacE*, and *emrE* in strains refractory to QAC and QPC treatments. (MFS = major facilitator superfamily; RND = resistance/nodulation/cell division family; ABC = ATP-binding cassette family; MATE = multidrug and toxic compound extrusion family; and SMR = small multidrug resistance family).

QACs, in addition to methylTPP and tetraphenylphosphonium (Figure 4A).[10,62] Notably, the Gram-negative QacC homologue, EmrE, was also identified in both the *E. coli* and *P. aeruginosa* strain genomes. Furthermore, QacE, a closely related SMR family efflux pump was identified in the *E. faecalis* strain genome (Table 2).[64,72] Taken together, the unique presence of these small multidrug pump class of SMR-family transporters may be responsible for the observed refractory nature of these strains to treatment with QAC and QPC disinfectants.

In the context of AMR, the association between QacC and resistance to disinfectant compounds in HA-MRSA raises a particular alarm for AMR, as a novel HGT mechanism for *qacC* was purported and correlated with its recent spread by Wassenaar and coworkers in 2016.[73] Specifically, the unusually high degree of sequence conservation for *qacC* coupled with its aberrant location between the double-strand replication origin (DSO) and single-strand replication origin (SSO) on rolling-circle (RC) plasmids in the absence of mobilizing genetic elements suggest that *Staphylococcus* populations may have recently evolved a new mechanism for the efficient horizontal transfer of the *qacC* gene (Figure 4B).

Moreover, the only other RC-plasmid gene currently suspected to involve a similar transfer mechanism is *lnuA*, a gene conferring resistance to lincosamides in *Staphylococcus* species, leading Wassenarr and coworkers to posit that this novel "DSO-gene-SSO" HGT mechanism was selected for through the use of disinfectants and antibiotics.[73] This hypothesis coincides with a multitude of studies investigating evolutionary selection for the spread of QAC resistance genes, as well as QAC-mediated co-resistance to antibiotics.[16,74−76] Hence, the rapidly growing prevalence of AMR demands for renewed interest in the development of next-generation antibiotics and disinfectants, such as those disclosed herein (Figure 4C).

Investigations into efflux-mediated resistance mechanisms. To probe the putative SMR-mediated mechanism of resistance, we hypothesized that an efflux pump inhibitor would potentiate the activities of the QAC and QPC molecules. Therefore, we re-evaluated the MICs of QAC and QPC molecules in the presence and absence of carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), a protonophore that inhibits the proton-motive force necessary for transport in SMR family efflux pumps.[77] As a result of its mechanism of action, CCCP affects cellular metabolism and thus exhibits an MIC. Therefore, the MIC of CCCP was determined for each of the six strains, and half of this inhibitory concentration was utilized. In combination with CCCP, we assessed one commercial QAC (BAC), our best-in-class biscationic QAC (**2Pyr-11,11**), a derivative of the efflux-susceptible methylTPP QPC (**TPP-8**), and our best-in-class biscationic QPC (**P6P-10,10**) against the bacterial panel. To our surprise, the presence of CCCP decreased microbial susceptibility when combined with the biscationic QAC (**2Pyr-11,11**) and QPC (**P6P-10,10**) molecules against several strains (Figure 5A). Notably, **P6P-10,10** displayed the greatest attenuation in combination with CCCP with greater than fourfold increases in MIC against all strains. This unexpected result, namely, the dramatic decrease in **P6P-10,10** efficacy in the presence of CCCP, made us speculate that membrane depolarization may

A

Minimum Inhibitory Concentration (μM)	BAC		2Pyr-11,11		P6P-10,10		TPP-8	
	– CCCP	+ CCCP	– CCCP	+ CCCP	– CCCP	+ CCCP	– CCCP	+ CCCP
MSSA	4	4	1	2	1	4	4	4
CA-MRSA	4	4	2	2	1	4	4	4
HA-MRSA	8	4	2	2	2	16	125	125
E. faecalis	250	>250	8	>250	2	>250	>250	>250
E. coli	63	63	2	4	8	125	>250	>250
P. aeruginosa	250	250	16	>250	4	250	>250	>250

B

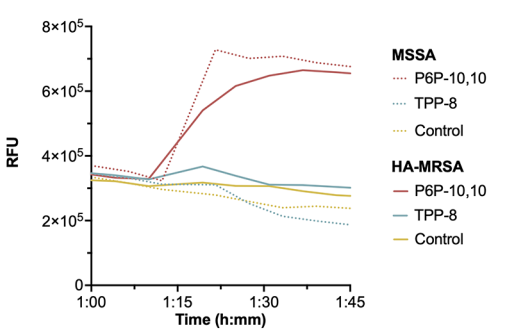

Figure 5. (A) MIC values of QACs (**BAC** and **2Pyr-11,11**) and QPCs (**P6P-10,10** and **TPP-8**) in combination with CCCP. (B) After 70 min of preincubation with $DiBAC_4(3)$, MSSA and HA-MRSA cells were treated with **P6P-10,10** (2.0 μM), **TPP-8** (4.0μM), or 2.5% DMSO (control), and the fluorescence intensity was measured. Membrane depolarization results in increased $DiBAC_4(3)$ fluorescence.

https://doi.org/10.1021/acsinfecdis.1c00611
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be the central in the mechanism by which this QPC exerts its broad-spectrum potency and evades apparent mechanisms of resistance.

To further investigate this role of membrane depolarization in the mechanism of **P6P-10,10**, we postulated that the fluorescent probe $DiBAC_4(3)$ [bis-(1,3-dibutylbarbituric acid)-trimethine oxonol], which emits fluorescence as a result of membrane depolarization, could be utilized to examine the differential effects of **P6P-10,10** and **TPP-8** on membrane depolarization in MSSA and HA-MRSA. Specifically, while **P6P-10,10** displayed low micromolar potency against both MSSA and HA-MRSA, **TPP-8** was significantly less effective against HA-MRSA, purportedly as a result of efflux-mediated resistance. Therefore, we hypothesized that **P6P-10,10** would have a greater effect on membrane depolarization against HA-MRSA compared to **TPP-8**, as the presence of efflux pumps in this strain may abate membrane depolarization for TPP-derived molecules that are susceptible to efflux. The results of the fluorescence assay (Figure 5B) suggest that **P6P-10,10** deviates significantly from **TPP-8** in its ability to depolarize the membrane, as **P6P-10,10** treatment led to a twofold increase in depolarization compared to **TPP-8** treatment. Collectively, we posit that the enhanced ability of **P6P-10,10** to perturb the membrane through depolarization underlies the mechanism by which this compound displays improved broad-spectrum potency.

CONCLUSIONS

An overreliance on select quaternary ammonium-based disinfectants and antiseptics has driven AMR, urgently necessitating the development of diverse antimicrobials for sanitization.[3,78] Herein, we have disclosed the synthesis and biological investigation of 59 QPCs, unveiling the promise of these underexplored cationic amphiphiles.

The results of this study elucidated the relationship between alkyl chain length and number of cationic residues with antimicrobial efficacy and hemolytic activity for QPCs. Both monoQPCs with moderate hydrocarbon tail lengths (11−13 carbons) and bisQPCs with shorter tail lengths (8−11 carbons) demonstrated promising antimicrobial activities against a panel of six Gram-positive and Gram-negative bacteria. Notably, **P6P-10,10** emerged as an effective broad-spectrum antimicrobial, exhibiting comparable hemolytic activity and ≥fourfold increases in activity against Gram-negative species compared to commercial QACs, BAC, and CPC. The broad-spectrum potency of this novel bisQPC was further highlighted through TEM imaging, wherein the improved lytic activity of **P6P-10,10** over the leading commercial QAC (BAC) was illustrated. Furthermore, this bisQPC was able to evade QPC resistance posited to be conferred by the presence of the SMR family of efflux pumps, such as QacC in HA-MRSA. This potential relationship between disinfectant resistance and QacC is noteworthy, as a study reporting the recent spread of the *qacC* gene through a novel HGT mechanism[73] underscores the need for next-generation disinfectants, such as **P6P-10,10**, that overcome mounting resistance. To further probe this mechanism of resistance, efflux pump inhibition and membrane depolarization assays were performed, together elucidating the central role of membrane depolarization in the mechanism by which **P6P-10,10** exhibits broad-spectrum potency and evades resistance. Taken together, the results of our investigation have provided the impetus for further exploration of QPC architectures, as well as further investigation into the mechanisms of resistance observed with these compounds, both of which are subjects of ongoing collaborative research in our laboratories.

METHODS

Synthesis of the QPC Library. Reagents and solvents were used from Sigma-Aldrich, Accela, Strem Chemicals Inc., and Fisher Chemical without further purification. All reactions were carried out under an ambient atmosphere unless otherwise noted, with reagent grade solvents and magnetic stirring. Detailed procedures for the preparation of each of the synthesized compounds are provided in the Supporting Information. ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded with a 400 or 500 MHz JEOL spectrophotometer, and chemical shifts were reported on a δ-scale (ppm) downfield from TMS or 85% H_3PO_4. Coupling constants were calculated in hertz. The solvents used were chloroform-*d* ($CDCl_3$), with an internal reference of 7.26 ppm for ^{1}H NMR and 77.16 ppm for ^{13}C NMR, and methanol-d_4, with internal references of 4.78 and 3.31 ppm for ^{1}H NMR and 49.15 ppm for ^{13}C NMR. Accurate mass spectrometry data were acquired on an AB Sciex 5600 TripleTOF using electrospray ionization in the positive mode. Melting ranges were taken on an SRS DigiMelt MPA160.

Biological Assays. For all biological assays, laboratory strains of methicillin-susceptible *S. aureus* MSSA (SH1000), *E. faecalis* (OG1RF), *E. coli* (MC4100), *P. aeruginosa* (PAO1), community-acquired methicillin-resistant *S. aureus* CA-MRSA (USA300-0114), and hospital-acquired methicillin-resistant *S. aureus* HA-MRSA (ATCC 33591) were grown with shaking at 37 °C overnight from freezer stocks in 5 mL of the indicated media: SH1000, OG1RF, MC4100, USA300-0114, and PAO1 were grown in a BD Mueller−Hinton broth (MHB), whereas ATCC 33591 was grown in a BD tryptic soy broth (TSB). Optical density (OD) measurements were obtained using a SpectraMax iD3 plate reader (Molecular Devices, United States).

MIC Assays. Compounds were serially diluted twofold from stock solutions (1.0 mM) to yield twelve 100 μL test concentrations, wherein the starting concentration of DMSO was 2.5%. Overnight *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, USA300-0114 (CA-MRSA), and ATCC 33591 (HA-MRSA) cultures were diluted to ca. 106 CFU/mL in MHB or TSB and regrown to the mid-exponential phase, as determined by OD recorded at 600 nm (OD600). All cultures were then diluted again to ca. 106 CFU/mL and 100 μL were inoculated into each well of a *U*-bottom 96-well plate (corning, 351,177) containing 100 μL of the compound solution. Plates were incubated statically at 37 °C for 72 h upon which wells were evaluated visually for bacterial growth. The MIC was determined as the lowest concentration of the compound resulting in no bacterial growth visible to the naked eye based on the highest value in three independent experiments. Aqueous DMSO controls were conducted as appropriate for each compound.

RBC Lysis Assay ($Lysis_{20}$). RBC lysis assays were performed on mechanically defibrinated sheep blood (Hemostat Labs: DSB030). An aliquot of 1.5 mL of blood was placed into a microcentrifuge tube and centrifuged at 3800 rpm for 10 min. The supernatant was removed, and the cells were resuspended with 1 mL of phosphate-buffered saline (PBS). The suspension was centrifuged as described above, the

https://doi.org/10.1021/acsinfecdis.1c00611
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supernatant was removed, and cells were resuspended four additional times in 1 mL of PBS. The final cell suspension was diluted 20-fold with PBS. Compounds were serially diluted with PBS twofold from stock solutions (1.0 mM) to yield 100 μL of 12 test concentrations on a flat-bottom 96-well plate (corning, 351,172), wherein the starting concentration of DMSO was 2.5%. To each of the wells, 100 μL of the 20-fold suspension dilution was then innoculated. The concentration of DMSO in the first well was 2.5%, resulting in DMSO-induced lysis at all concentrations >63 μM. TritonX (1% by volume) served as a positive control (100% lysis marker) and sterile PBS served as a negative control (0% lysis marker). Samples were then placed in an incubator at 37 °C and shaken at 200 rpm. After 1 h, the samples were centrifuged at 3800 rpm for 10 min. The absorbance of the supernatant was measured with a UV spectrometer at a 540 nm wavelength. The concentration inducing 20% RBC lysis was then calculated for each compound based upon the absorbances of the TritonX and PBS controls. Aqueous DMSO controls were conducted as appropriate for each compound.

Efflux Inhibition Assay. Using the above procedure, the MIC for carbonyl cyanide 3-chlorophenylhydrazone 97% (CCCP) (Sigma-Aldrich) was determined against *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, USA300-0114 (CA-MRSA), and ATCC 33591 (HA-MRSA). For the efflux inhibition assay, half the determined MIC of CCCP against each strain was used (Table S1). Respective QPC and QAC compounds were serially diluted twofold from stock solutions (1.0 mM) to yield 12 test concentrations of 50 μL each, wherein the starting concentration of DMSO was 2.5%. To each well containing 50 μL of the QAC or QPC solution, 50 μL of CCCP (in respective media, 10% DMSO carrier) at the designated test concentration against each strain (Table S1) was added. Overnight *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, USA300-0114 (CA-MRSA), and ATCC 33591 (HA-MRSA) cultures were diluted to ca. 106 CFU/mL in MHB or TSB and regrown to the mid-exponential phase as determined by OD recorded at 600 nm (OD600). All cultures were then diluted again to ca. 106 CFU/mL and 100 μL were inoculated into each well of a *U*-bottom 96-well plate (corning, 351,177) containing 100 μL of the compound solution. Plates were incubated statically at 37 °C for 72 h upon which wells were evaluated visually for bacterial growth. The MIC was determined as the lowest concentration of compound resulting in no bacterial growth visible to the naked eye based on the highest value in three independent experiments. Aqueous DMSO controls were conducted as appropriate for each compound.

DiBAC$_4$(3) Fluorescence Assay. Overnight *S. aureus* (SH1000) and ATCC 33591 (HA-MRSA) cultures were diluted to ca. 10^6 CFU/mL in MHB and TSB, respectively, and regrown to the mid-exponential phase, as determined by OD recorded at 600 nm (OD_{600}). The cultures were then centrifuged at 3,800 rpm for 10 min, the supernatant was removed, and the cells were resuspended and washed three times with PBS. After washing, the cells were resuspended in 5 mL of PBS. To this cell suspension, 125 μL of 1.0 M sterile-filtered glucose solution was added (25 mM final concentration). The cells were then incubated for 15 min at 37 °C. After incubation, 25 μL of a 50 μM solution of DiBAC$_4$(3) (Biotium) was added to the suspensions (244 nM final concentration) and the suspensions were mixed thoroughly. To a black 96-well plate with clear flat-bottom wells (Greiner Bio-One, 655,097), 150 μL of the suspension was then transferred. The plate was then placed in the prewarmed (37 °C) microplate reader, and the cells were acclimated for 40 min or until the fluorescence was stabilized (fluorescence at 490 nm excitation and 516 nm emission measured once per minute). Next, 50 μL of three test concentrations (2× MIC, 1× MIC, and 0.5× MIC) of compound was transferred quickly to the wells using a multichannel pipette and the plate was returned to the microplate reader. The fluorescence was again monitored for 100 min. Biological triplicates were completed with PBS and DMSO controls.

TEM Imaging. *E. faecalis* and *P. aeruginosa* cultures were diluted to ca. 10^6 CFU/mL in MHB media and regrown to the mid-exponential phase as determined by OD recorded at 600 nm (OD_{600}). Aliquots of the cultures (0.5 mL) were then treated with 0.5 mL of compound solutions (half their MIC of either BAC or **P6P-10,10**). The treated cultures were then incubated under shaking at 37 °C for 48 h. After 48 h, the samples were centrifuged for 10 min at 13,000 rpm. The supernatant was removed and the samples were washed three times with PBS. The pellets were then fixed in Karnovsky's fixative at 4 °C for 24 h. The pellets were then washed twice with 0.1 M cacodylate buffer and then fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h at room temperature. The samples were next washed with double-distilled water twice, followed by a series of dehydrations with ascending pure ethanol concentrations (25, 50, 70, 95, 100, and 100%). After dehydration, the samples were treated for 10 min twice with pure propylene oxide (PPO). Following these washes, samples were infiltrated with a series of ascending concentrations of Eponate 12 resin in PPO with agitation overnight (1:2, 1:1, 2:1, and 1:0 ratios of Eponate 12 resin to PPO, respectively). Lastly, the samples were polymerized with Eponate 12 resin at 60 °C for 48 h. The samples were then sectioned, placed on grids, and visualized using a JEOL JEM-4100 transmission electron microscope (JEOL, Ltd., Japan) at 120 kV.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsinfecdis.1c00611.

MIC; RBC lysis assay (lysis20); efflux inhibition assay; DiBAC4(3) fluorescence assay; TEM imaging; synthesis of the QPC library; and ^{1}H NMR, ^{13}C NMR, ^{31}P NMR, and HRMS images (PDF)

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https://doi.org/10.1021/acsinfecdis.1c00611
ACS Infect. Dis. 2022, 8, 387−397

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Author Contributions

#K.J.S. and M.E.M. contributed equally to this work and each have the right to list their name first on their CV. K.J.S., C.E.H., L.E.A., A.A.P., and R.G.C. performed the synthesis of all the compounds. M.E.M. and A.M.S. performed the inhibitory assays. M.E.M. carried out the bioinformatic analysis and TEM imaging. K.P.C.M. and W.M.W. conceived the project and experimental design, advised the implementation, and outlined the paper. All authors contributed to the writing and editing of the manuscript and have given approval to the final version of the manuscript.

Funding

This work was funded by the National Institute of General Medical Sciences (R35 GM119426 to W.M.W.) and Major Research Instrumentation grants from the National Science Foundation (CHE-1827930 and CHE-2018399 to K.P.C.M.).

Notes

The authors declare the following competing financial interest(s): The authors have intellectual property covering the novel quaternary phosphonium compounds disclosed.

■ ACKNOWLEDGMENTS

The TEM imaging in this work was supported by the Robert P. Apkarian Integrated Electron Microscopy Core (IEMC) at Emory University, which is subsidized by the School of Medicine and Emory College of Arts and Sciences. Additional support was provided by the Georgia Clinical & Translational Science Alliance of the National Institutes of Health under award number UL1TR000454. Figure illustrations were created with BioRender.com.

■ REFERENCES

(1) O'Neill, J. *Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations*; Review on Antimicrobial Resistance, 2016.
(2) United States Environmental Protection Agency. List N: Disinfectants for Coronavirus (COVID-19) https://www.epa.gov/coronavirus/about-list-n-disinfectants-coronavirus-covid-19-0 (accessed Jun 19, 2021).
(3) Jennings, M. C.; Minbiole, K. P. C.; Wuest, W. M. Quaternary Ammonium Compounds: An Antimicrobial Mainstay and Platform for Innovation to Address Bacterial Resistance. *ACS Infect. Dis.* **2015**, *1*, 288−303.
(4) Gilbert, P.; Moore, L. E. Cationic Antiseptics: Diversity of Action under a Common Epithet. *J. Appl. Microbiol.* **2005**, *99*, 703−715.
(5) Al-Khalifa, S. E.; Jennings, M. C.; Wuest, W. M.; Minbiole, K. P. C. The Development of Next-Generation Pyridinium-Based Multi-QAC Antiseptics. *ChemMedChem* **2017**, *12*, 280−283.
(6) Alkhalifa, S.; Jennings, M. C.; Granata, D.; Klein, M.; Wuest, W. M.; Minbiole, K. P. C.; Carnevale, V. Analysis of the Destabilization of Bacterial Membranes by Quaternary Ammonium Compounds: A Combined Experimental and Computational Study. *ChemBioChem* **2020**, *21*, 1510−1516.
(7) Soprey, P. R.; Maxcy, R. B. Tolerance of Bacteria for Quaternary Ammonium Compounds. *J. Food Sci.* **1968**, *33*, 536−540.
(8) Tennent, J. M.; Lyon, B. R.; Gillespie, M. T.; May, J. W.; Skurray, R. A. Cloning and Expression of Staphylococcus Aureus Plasmid-Mediated Quaternary Ammonium Resistance in Escherichia Coli. *Antimicrob. Agents Chemother.* **1985**, *27*, 79−83.
(9) Minbiole, K. P. C.; Jennings, M. C.; Ator, L. E.; Black, J. W.; Grenier, M. C.; LaDow, J. E.; Caran, K. L.; Seifert, K.; Wuest, W. M. From Antimicrobial Activity to Mechanism of Resistance: The Multifaceted Role of Simple Quaternary Ammonium Compounds in Bacterial Eradication. *Tetrahedron* **2016**, *72*, 3559−3566.
(10) Lyon, B. R.; Skurray, R. Antimicrobial Resistance of Staphylococcus Aureus: Genetic Basis. *Microbiol. Rev.* **1987**, *51*, 88−134.
(11) Liu, W. J.; Fu, L.; Huang, M.; Zhang, J. P.; Wu, Y.; Zhou, Y. S.; Zeng, J.; Wang, G. X. Frequency of Antiseptic Resistance Genes and Reduced Susceptibility to Biocides in Carbapenem-Resistant Acinetobacter Baumannii. *J. Med. Microbiol.* **2017**, *66*, 13−17.
(12) Abuzaid, A.; Hamouda, A.; Amyes, S. G. B. Klebsiella Pneumoniae Susceptibility to Biocides and Its Association with CepA, QacΔE and QacE Efflux Pump Genes and Antibiotic Resistance. *J. Hosp. Infect.* **2012**, *81*, 87−91.
(13) Liu, Q.; Liu, M.; Wu, Q.; Li, C.; Zhou, T.; Ni, Y. Sensitivities to Biocides and Distribution of Biocide Resistance Genes in Quaternary Ammonium Compound Tolerant Staphylococcus Aureus Isolated in a Teaching Hospital. *Scand. J. Infect. Dis.* **2009**, *41*, 403.
(14) Mayer, S.; Boos, M.; Beyer, A.; Fluit, A. C.; Schmitz, F. J. Distribution of the Antiseptic Resistance Genes QacA, QacB and QacC in 497 Methicillin-Resistant and -Susceptibile European Isolates of Staphylococcus Aureus [4]. *J. Antimicrob. Chemother.* **2001**, *47*, 896−897.
(15) Ignak, S.; Nakipoglu, Y.; Gurler, B. Frequency of Antiseptic Resistance Genes in Clinical Staphycocci and Enterococci Isolates in Turkey. *Antimicrob. Resist. Infect. Control* **2017**, *6*, 1−7.
(16) Han, Y.; Zhou, Z.-C.; Zhu, L.; Wei, Y.-Y.; Feng, W.-Q.; Xu, L.; Liu, Y.; Lin, Z.-J.; Shuai, X.-Y.; Zhang, Z.-J.; Chen, H. The Impact and Mechanism of Quaternary Ammonium Compounds on the Transmission of Antibiotic Resistance Genes. *Environ. Sci. Pollut. Res.* **2019**, *26*, 28352−28360.
(17) Chen, B.; Han, J.; Dai, H.; Jia, P. Biocide-Tolerance and Antibiotic-Resistance in Community Environments and Risk of Direct Transfers to Humans: Unintended Consequences of Community-Wide Surface Disinfecting during COVID-19? *Environ. Pollut.* **2021**, *283*, 117074.
(18) Allen, R. A.; Jennings, M. C.; Mitchell, M. A.; Al-Khalifa, S. E.; Wuest, W. M.; Minbiole, K. P. C. Ester- and Amide-Containing MultiQACs: Exploring Multicationic Soft Antimicrobial Agents. *Bioorg. Med. Chem. Lett* **2017**, *27*, 2107−2112.
(19) Kontos, R. C.; Schallenhammer, S. A.; Bentley, B. S.; Morrison, K. R.; Feliciano, J. A.; Tasca, J. A.; Kaplan, A. R.; Bezpalko, M. W.; Kassel, W. S.; Wuest, W. M.; Minbiole, K. P. C. An Investigation into Rigidity−Activity Relationships in BisQAC Amphiphilic Antiseptics. *ChemMedChem* **2019**, *14*, 83−87.
(20) Leitgeb, A. J.; Feliciano, J. A.; Sanchez, H. A.; Allen, R. A.; Morrison, K. R.; Sommers, K. J.; Carden, R. G.; Wuest, W. M.; Minbiole, K. P. C. Further Investigations into Rigidity-Activity Relationships in BisQAC Amphiphilic Antiseptics. *ChemMedChem* **2020**, *15*, 667−670.
(21) Sommers, K. J.; Bentley, B. S.; Carden, R. G.; Post, S. J.; Allen, R. A.; Kontos, R. C.; Black, J. W.; Wuest, W. M.; Minbiole, K. P. C. Metallocene QACs: The Incorporation of Ferrocene Moieties into MonoQAC and BisQAC Structures. *ChemMedChem* **2021**, *16*, 467−471.
(22) Schallenhammer, S. A.; Duggan, S. M.; Morrison, K. R.; Bentley, B. S.; Wuest, W. M.; Minbiole, K. P. C. Hybrid BisQACs: Potent Biscationic Quaternary Ammonium Compounds Merging the

Structures of Two Commercial Antiseptics. *ChemMedChem* **2017**, *12*, 1931−1934.
(23) Laverty, G.; Gorman, S. P.; Gilmore, B. F. The Potential of Antimicrobial Peptides as Biocides. *Int. J. Mol. Sci.* **2011**, *12*, 6566−6596.
(24) Palermo, E. F.; Vemparala, S.; Kuroda, K. Cationic Spacer Arm Design Strategy for Control of Antimicrobial Activity and Conformation of Amphiphilic Methacrylate Random Copolymers. *Biomacromolecules* **2012**, *13*, 1632−1641.
(25) Faig, A.; Arthur, T. D.; Fitzgerald, P. O.; Chikindas, M.; Mintzer, E.; Uhrich, K. E. Biscationic Tartaric Acid-Based Amphiphiles: Charge Location Impacts Antimicrobial Activity. *Langmuir* **2015**, *31*, 11875−11885.
(26) Mitchell, M. A.; Iannetta, A. A.; Jennings, M. C.; Fletcher, M. H.; Wuest, W. M.; Minbiole, K. P. C. Scaffold-Hopping of Multicationic Amphiphiles Yields Three New Classes of Antimicrobials. *ChemBioChem* **2015**, *16*, 2299−2303.
(27) Xie, X.; Cong, W.; Zhao, F.; Li, H.; Xin, W.; Hou, G.; Wang, C. Synthesis, Physiochemical Property and Antimicrobial Activity of Novel Quaternary Ammonium Salts. *J. Enzyme Inhib. Med. Chem.* **2018**, *33*, 98−105.
(28) Starodubtseva, R. R.; Gibadullina, E. M.; Pazilova, N. B.; Sapunova, A. S.; Voloshina, A. D.; Sudakov, I. A.; Vyshtakalyuk, A. B.; Pudovik, M. A.; Burilov, A. R.; Bukharov, S. V. Design, Synthesis, and Biological Activity of Novel Ammonium Salts Containing Sterically Hindered Phenolic Fragment and Phosphoryl Group. *MedChemComm* **2018**, *9*, 2106−2120.
(29) Bazina, L.; Maravić, A.; Krce, L.; Soldo, B.; Odžak, R.; Popović, V. B.; Aviani, I.; Primožič, I.; Šprung, M. Discovery of Novel Quaternary Ammonium Compounds Based on Quinuclidine-3-Ol as New Potential Antimicrobial Candidates. *Eur. J. Med. Chem.* **2019**, *163*, 626−635.
(30) Fedorowicz, J.; Sączewski, J.; Konopacka, A.; Waleron, K.; Lejnowski, D.; Ciura, K.; Tomašič, T.; Skok, Ž.; Savijoki, K.; Morawska, M.; Gilbert-Girard, S.; Fallarero, A. Synthesis and Biological Evaluation of Hybrid Quinolone-Based Quaternary Ammonium Antibacterial Agents. *Eur. J. Med. Chem.* **2019**, *179*, 576−590.
(31) Carden, R. G.; Sommers, K. J.; Schrank, C. L.; Leitgeb, A. J.; Feliciano, J. A.; Wuest, W. M.; Minbiole, K. P. C. Advancements in the Development of Non-Nitrogen-Based Amphiphilic Antiseptics to Overcome Pathogenic Bacterial Resistance. *ChemMedChem* **2020**, *15*, 1974−1984.
(32) Feliciano, J. A.; Leitgeb, A. J.; Schrank, C. L.; Allen, R. A.; Minbiole, K. P. C.; Wuest, W. M.; Carden, R. G. Trivalent Sulfonium Compounds (TSCs): Tetrahydrothiophene-Based Amphiphiles Exhibit Similar Antimicrobial Activity to Analogous Ammonium-Based Amphiphiles. *Bioorg. Med. Chem. Lett* **2021**, *37*, 127809.
(33) Kumar, V.; Malhotra, S. V. Study on the Potential Anti-Cancer Activity of Phosphonium and Ammonium-Based Ionic Liquids. *Bioorg. Med. Chem. Lett* **2009**, *19*, 4643−4646.
(34) Iksanova, A. G.; Gabbasova, R. R.; Kupriyanova, T. V.; Akhunzyanov, A. A.; Pugachev, M. V.; Vafiva, R. M.; Shtyrlin, N. V.; Balakin, K. V.; Shtyrlin, Y. G. In-Vitro Antitumor Activity of New Quaternary Phosphonium Salts, Derivatives of 3-Hydroxypyridine. *Anticancer Drugs* **2018**, *29*, 682−690.
(35) Macarie, L.; Simulescu, V.; Ilia, G. Phosphonium-Based Ionic Liquids Used as Reagents or Catalysts. *ChemistrySelect* **2019**, *4*, 9285−9299.
(36) Skulachev, V. P. How to Clean the Dirtiest Place in the Cell: Cationic Antioxidants as Intramitochondrial ROS Scavengers. *IUBMB Life* **2005**, *57*, 305.
(37) Skulachev, V. P.; Anisimov, V. N.; Antonenko, Y. N.; Bakeeva, L. E.; Chernyak, B. V.; Erichev, V. P.; Filenko, O. F.; Kalinina, N. I.; Kapelko, V. I.; Kolosova, N. G.; Kopnin, B. P.; Korshunova, G. A.; Lichinitser, M. R.; Obukhova, L. A.; Pasyukova, E. G.; Pisarenko, O. I.; Roginsky, V. A.; Ruuge, E. K.; Senin, I. I.; Severina, I. I.; Skulachev, M. V.; Spivak, I. M.; Tashlitsky, V. N.; Tkachuk, V. A.; Vyssokikh, M. Y.; Yaguzhinsky, L. S.; Zorov, D. B. An Attempt to Prevent Senescence: A Mitochondrial Approach. *Biochim. Biophys. Acta Bioenerg.* **2009**, *1787*, 437−461.
(38) Pindzola, B. A.; Jin, J.; Gin, D. L. Cross-Linked Normal Hexagonal and Bicontinuous Cubic Assemblies via Polymerizable Gemini Amphiphiles. *J. Am. Chem. Soc.* **2003**, *125*, 2940−2949.
(39) Zhou, M.; Nemade, P. R.; Lu, X.; Zeng, X.; Hatakeyama, E. S.; Noble, R. D.; Gin, D. L. New Type of Membrane Material for Water Desalination Based on a Cross-Linked Bicontinuous Cubic Lyotropic Liquid Crystal Assembly. *J. Am. Chem. Soc.* **2007**, *129*, 9574−9575.
(40) Muñoz-Bonilla, A.; Fernández-García, M. Polymeric Materials with Antimicrobial Activity. *Prog. Polym. Sci.* **2012**, *37*, 281−339.
(41) Xue, Y.; Xiao, H. Characterization and Antipathogenic Evaluation of a Novel Quaternary Phosphonium Tripolyacrylamide and Elucidation of the Inactivation Mechanisms. *J. Biomed. Mater. Res.* **2016**, *104*, 747−757.
(42) Xue, Y.; Pan, Y.; Xiao, H.; Zhao, Y. Novel Quaternary Phosphonium-Type Cationic Polyacrylamide and Elucidation of Dual-Functional Antibacterial/Antiviral Activity. *RSC Adv.* **2014**, *4*, 46887−46895.
(43) Xue, Y.; Xiao, H.; Zhang, Y. Antimicrobial Polymeric Materials with Quaternary Ammonium and Phosphonium Salts. *Int. J. Mol. Sci.* **2015**, *16*, 3626−3655.
(44) Kenawy, E.-R.; Abdel-Hay, F. I.; El-Shanshoury, A. E.-R. R.; El-Newehy, M. H. Biologically Active Polymers. V. Synthesis and Antimicrobial Activity of Modified Poly(Glycidyl Methacrylate-Co-2-Hydroxyethyl Methacrylate) Derivatives with Quaternary Ammonium and Phosphonium Salts. *J. Polym. Sci., Part A: Polym. Chem.* **2002**, *40*, 2384−2393.
(45) Kenawy, E.; Abdel-Hay, F. I.; El-Raheem, A.; El-Shanshoury, R.; El-Newehy, M. H. Biologically Active Polymers: Synthesis and Antimicrobial Activity of Modified Glycidyl Methacrylate Polymers Having a Quaternary Ammonium and Phosphonium Groups. *J. Controlled Release* **1998**, *50*, 145−152.
(46) Kanazawa, A.; Ikeda, T.; Endo, T. Polymeric Phosphonium Salts as a Novel Class of Cationic Biocides. IX. Effect of Side-Chain Length between Main Chain and Active Group on Antibacterial Activity. *J. Polym. Sci., Part A: Polym. Chem.* **1994**, *32*, 1997−2001.
(47) Kanazawa, A.; Ikeda, T.; Endo, T. Polymeric Phosphonium Salts as a Novel Class of Cationic Biocides. III. Immobilization of Phosphonium Salts by Surface Photografting and Antibacterial Activity of the Surface-treated Polymer Films. *J. Polym. Sci., Part A: Polym. Chem.* **1993**, *31*, 1467−1472.
(48) Kanazawa, A.; Ikeda, T.; Endo, T. Polymeric Phosphonium Salts as a Novel Class of Cationic Biocides. V. Synthesis and Antibacterial Activity of Polyesters Releasing Phosphonium Biocides. *J. Polym. Sci., Part A: Polym. Chem.* **1993**, *31*, 3003−3011.
(49) Kanazawa, A.; Ikeda, T.; Endo, T. Polymeric Phosphonium Salts as a Novel Class of Cationic Biocides. VII. Synthesis and Antibacterial Activity of Polymeric Phosphonium Salts and Their Model Compounds Containing Long Alkyl Chains. *J. Appl. Polym. Sci.* **1994**, *53*, 1237−1244.
(50) Kanazawa, A.; Ikeda, T.; Endo, T. Synthesis and Antimicrobial Activity of Dimethyl- and Trimethyl- Substituted Phosphonium Salts with Alkyl Chains of Various Lengths. *Antimicrob. Agents Chemother.* **1994**, *38*, 945−952.
(51) Kanazawa, A.; Ikeda, T.; Endo, T. Polymeric Phosphonium Salts as a Novel Class of Cationic Biocides. IX. Effect of Side-chain Length between Main Chain and Active Group on Antibacterial Activity. *J. Polym. Sci., Part A: Polym. Chem.* **1994**, *32*, 1997−2001.
(52) Lukáč, M.; Devínsky, F.; Pisárčik, M.; Papapetropoulou, A.; Bukovský, M.; Horváth, B. Novel Phospholium-Type Cationic Surfactants: Synthesis, Aggregation Properties and Antimicrobial Activity. *J. Surfactants Deterg.* **2017**, *20*, 159−171.
(53) Terekhova, N. V.; Tatarinov, D. A.; Shaihutdinova, Z. M.; Pashirova, T. N.; Lyubina, A. P.; Voloshina, A. D.; Sapunova, A. S.; Zakharova, L. Y.; Mironov, V. F. Design and Synthesis of Amphiphilic 2-Hydroxybenzylphosphonium Salts with Antimicrobial and Antitumor Dual Action. *Bioorg. Med. Chem. Lett* **2020**, *30*, 127234.

https://doi.org/10.1021/acsinfecdis.1c00611
ACS Infect. Dis. 2022, 8, 387−397

(54) Lao, Z.; Toy, P. H. Catalytic Wittig and Aza-Wittig Reactions. *Beilstein J. Org. Chem.* **2016**, *12*, 2577−2587.
(55) Zhao, Y.-L.; Li, Y.; Li, Y.; Gao, L.-X.; Han, F.-S. Aryl Phosphoramides: Useful Electrophiles for Suzuki-Miyaura Coupling Catalyzed by a NiCl2/Dppp System (Dppp=1,3-Bis-(Diphenylphosphino) Propane). *Chem.—Eur. J.* **2010**, *16*, 4991−4994.
(56) Stewart, B.; Harriman, A.; Higham, L. J. Predicting the Air Stability of Phosphines. *Organometallics* **2011**, *30*, 5338−5343.
(57) Pugachev, M. V.; Shtyrlin, N. V.; Sapozhnikov, S. V.; Sysoeva, L. P.; Iksanova, A. G.; Nikitina, E. V.; Musin, R. Z.; Lodochnikova, O. A.; Berdnikov, E. A.; Shtyrlin, Y. G. Bis-Phosphonium Salts of Pyridoxine: The Relationship between Structure and Antibacterial Activity. *Bioorg. Med. Chem.* **2013**, *21*, 7330−7342.
(58) Pugachev, M. V.; Shtyrlin, N. V.; Sysoeva, L. P.; Nikitina, E. V.; Abdullin, T. I.; Iksanova, A. G.; Ilaeva, A. A.; Musin, R. Z.; Berdnikov, E. A.; Shtyrlin, Y. G. Synthesis and Antibacterial Activity of Novel Phosphonium Salts on the Basis of Pyridoxine. *Bioorg. Med. Chem.* **2013**, *21*, 4388−4395.
(59) Kayumov, A. R.; Nureeva, A. A.; Trizna, E. Y.; Gazizova, G. R.; Bogachev, M. I.; Shtyrlin, N. V.; Pugachev, M. V.; Sapozhnikov, S. V.; Shtyrlin, Y. G. New Derivatives of Pyridoxine Exhibit High Antibacterial Activity against Biofilm-Embedded Staphylococcus Cells. *BioMed Res. Int.* **2015**, *15*, 1−10.
(60) Shtyrlin, N. V.; Vafina, R. M.; Pugachev, M. V.; Khaziev, R. M.; Nikitina, E. V.; Zeldi, M. I.; Iksanova, A. G.; Shtyrlin, Y. G. Synthesis and Biological Activity of Quaternary Phosphonium Salts Based on 3-Hydroxypyridine and 4-Deoxypyridoxine. *Russ. Chem. Bull.* **2016**, *65*, 537−545.
(61) Tsepaeva, O. V.; Nemtarev, A. V.; Grigor'eva, L. R.; Mironov, V. F. Synthesis of C(28)-Linker Derivatives of Betulinic Acid Bearing Phosphonate Group. *Russ. Chem. Bull.* **2021**, *70*, 179−182.
(62) Grinius, L. L.; Goldberg, E. B. Bacterial Multidrug Resistance Is Due to a Single Membrane Protein Which Functions as a Drug Pump. *J. Biol. Chem.* **1994**, *269*, 29998−30004.
(63) Costa, S.; Junqueira, E.; Palma, C.; Viveiros, M.; Melo-Cristino, J.; Amaral, L.; Couto, I. Resistance to Antimicrobials Mediated by Efflux Pumps in Staphylococcus Aureus. *Antibiotics* **2013**, *2*, 83−99.
(64) Ovchinnikov, V.; Stone, T. A.; Deber, C. M.; Karplus, M. Structure of the Emre Multidrug Transporter and Its Use for Inhibitor Peptide Design. *Proc. Natl. Acad. Sci. U.S.A.* **2018**, *115*, E7932−E7941.
(65) Hemp, S. T.; Hudson, A. G.; Allen, M. H.; Pole, S. S.; Moore, R. B.; Long, T. E. Solution Properties and Electrospinning of Phosphonium Gemini Surfactants. *Soft Matter* **2014**, *10*, 3970−3977.
(66) Forman, M. E.; Fletcher, M. H.; Jennings, M. C.; Duggan, S. M.; Minbiole, K. P. C.; Wuest, W. M. Structure-Resistance Relationships: Interrogating Antiseptic Resistance in Bacteria with Multicationic Quaternary Ammonium Dyes. *ChemMedChem* **2016**, *11*, 958−962.
(67) Paniak, T. J.; Jennings, M. C.; Shanahan, P. C.; Joyce, M. D.; Santiago, C. N.; Wuest, W. M.; Minbiole, K. P. C. The Antimicrobial Activity of Mono-, Bis-, Tris-, and Tetracationic Amphiphiles Derived from Simple Polyamine Platforms. *Bioorg. Med. Chem. Lett* **2014**, *24*, 5824−5828.
(68) Groothuis, F. A.; Timmer, N.; Opsahl, E.; Nicol, B.; Droge, S. T. J.; Blaauboer, B. J.; Kramer, N. I. Influence of in Vitro Assay Setup on the Apparent Cytotoxic Potency of Benzalkonium Chlorides. *Chem. Res. Toxicol.* **2019**, *32*, 1103−1114.
(69) Yousif, L. F.; Stewart, K. M.; Kelley, S. O. Targeting Mitochondria with Organelle-Specific Compounds: Strategies and Applications. *ChemBioChem* **2009**, *10*, 1939−1950.
(70) Joyce, M. D.; Jennings, M. C.; Santiago, C. N.; Fletcher, M. H.; Wuest, W. M.; Minbiole, K. P. Natural Product-Derived Quaternary Ammonium Compounds with Potent Antimicrobial Activity. *J. Antibiot.* **2016**, *69*, 344−347.
(71) Kristich, C. J.; Rice, L. B.; Arias, C. A. *Enterococcal Infection—Treatment and Antibiotic Resistance*; Massachusetts Eye and Ear Infirmary, 2014.
(72) Paulsen, F. T.; Skurray, R. A.; Tam, R.; Saier, M. H.; Weiter, J. H.; Goldberg, E. B.; Grinius, L. L. The SMR Family: A Novel Family of Multidrug Efflux Proteins Involved with the Efflux of Lipophilic Drugs. *Mol. Microbiol.* **1996**, *19*, 1167.
(73) Wassenaar, T. M.; Ussery, D. W.; Ingmer, H. The QacC Gene Has Recently Spread between Rolling Circle Plasmids of Staphylococcus, Indicative of a Novel Gene Transfer Mechanism. *Front. Microbiol.* **2016**, *7*, 1528.
(74) Chapman, J. S. Disinfectant Resistance Mechanisms, Cross-Resistance, and Co-Resistance. *Int. Biodeterior. Biodegrad.* **2003**, *51*, 271−276.
(75) Paul, D.; Chakraborty, R.; Mandal, S. M. Biocides and Health-Care Agents Are More than Just Antibiotics: Inducing Cross to Co-Resistance in Microbes. *Ecotoxicol. Environ. Saf.* **2019**, *174*, 601−610.
(76) Pal, C.; Bengtsson-Palme, J.; Kristiansson, E.; Larsson, D. G. J. Co-Occurrence of Resistance Genes to Antibiotics, Biocides and Metals Reveals Novel Insights into Their Co-Selection Potential. *BMC Genom.* **2015**, *16*, 964.
(77) Mombeshora, M.; Mukanganyama, S. Development of an Accumulation Assay and Evaluation of the Effects of Efflux Pump Inhibitors on the Retention of Chlorhexidine Digluconate in Pseudomonas Aeruginosa and Staphylococcus Aureus. *BMC Res. Notes* **2017**, *10*, 1.
(78) Mahoney, A. R.; Safaee, M. M.; Wuest, W. M.; Furst, A. L. The Silent Pandemic: Emergent Antibiotic Resistances Following the Global Response to SARS-CoV-2. *iScience* **2021**, *24*, 102304.

https://doi.org/10.1021/acsinfecdis.1c00611
ACS Infect. Dis. 2022, 8, 387−397