ChemPubSoc Europe

DOI: 10.1002/cmdc.201600095

CHEMMEDCHEM Communications

Structure–Resistance Relationships: Interrogating Antiseptic Resistance in Bacteria with Multicationic Quaternary Ammonium Dyes

Megan E. Forman,[a] Madison H. Fletcher,[b] Megan C. Jennings,[b] Stephanie M. Duggan,[a] Kevin P. C. Minbiole,*[a] and William M. Wuest*[b]

Bacterial resistance toward commonly used biocides is a widespread yet underappreciated problem, one which needs not only a deeper understanding of the mechanisms by which resistance proliferates, but also means for mitigation. To advance our understanding of this issue, we recognized a polyaromatic structural core analogous to activators of QacR, a negative transcriptional regulator of the efflux pump QacA, and envisioned a series of quaternary ammonium compounds (QACs) based on this motif. Using commercially available dye scaffolds, we synthesized and evaluated the antimicrobial activity of 52 novel QACs bearing 1–3 quaternary ammonium centers. Striking differences in antimicrobial activity against bacteria bearing QAC resistance genes have been observed, with up to a 125-fold increase in minimum inhibitory concentration (MIC) for select structures against bacteria known to bear efflux pumps. Based on these findings, general trends in structure–resistance relationships have been identified, laying the groundwork for future mechanistic studies.

The proliferation of antibiotic-resistant bacteria is a significant threat to human health, and accordingly, the scientific and medical communities are making a concerted effort to stem the tide of resistance. Less alarm has been sounded, however, about a directly related problem: the decrease in efficacy of antiseptics often used by the general public to decontaminate surfaces and by hospitals to sterilize equipment. Over the past thirty years the identification of bacterial isolates with quaternary ammonium compound (QAC) resistance genes has risen dramatically,[1] and as a result, there have been efforts to better understand the mechanisms by which antiseptics can lose efficacy.[2,3] Resistance to traditional disinfectants such as benzalkonium chloride (BAC) and didecyldimethylammonium chloride (DDAC) has been identified in both Gram-positive and Gram-negative bacteria[4,5] and has presumably arisen through overuse and prolonged sub-lethal exposure. These compounds can, in fact, activate numerous resistance mechanisms, including physiological changes to bacterial cell membranes, as well as the production of transporter proteins, which efflux antibacterial agents.[6] More specifically, the *qacAB/R* system is one of the primary methods by which Gram-positive bacteria, specifically *S. aureus*, minimizes exposure to QACs. An overview of the resistance machinery is depicted in Figure 1 A. Although QACs are lytic to cell membranes, they are capable of entering the cell at sub-MIC values by passive diffusion. The compounds can then either be exported by the basal level of QacA (a transmembrane efflux pump) activity that is present, or bind with QacR, a negative transcriptional regulator of *qacA*. Following the binding of QACs to the recognition site, QacR disassociates, allowing the transcription of the *qacA* gene. This leads to the increased production of QacA and the rapid efflux of antimicrobial compounds from the cell. Other efflux proteins in Gram-positive bacteria include NorA;[1,7] an analogous system has also been observed in Gram-negative bacteria through the efflux pumps AcrAB-TolC in *E. coli* (EC) and MexAB-OprM in *P. aeruginosa* (PA).[4,5,8,9]

It has been posited that efflux pumps are, in fact, multidrug transporters with alternate primary functions, having evolved to recognize and export a wide range of antibacterial and biocidal scaffolds.[2,6] The evolutionary origins of some of these resistance mechanisms have been attributed to the recognition of natural product QACs such as berberine, sanguinarine, and chelerythrine produced by plants.[2,3] This is evidenced by the crystal structure of berberine bound to QacR, which highlights the key electrostatic (acidic amino acid residues) and π–π (aromatic residues) interactions as shown in Figure 1 B.[2] Brennan et al. demonstrated that commercially available dyes—crystal violet and malachite green—fit neatly into the binding site for berberine; they noted, however, that this recognition motif was limited to mono- and biscationic QACs.[2]

Previous research from our group[3] and others confirm this common QacR recognition motif, with ample evidence that mono- and biscationic QACs and those with aryl moieties display significant increases in MIC values (up to 60-fold) for *qacA/B*-bearing bacteria. In contrast, some of the most potent antiseptics developed in our lab are multicationic; these often exhibit low-micromolar concentrations against both Gram-positive and Gram-negative bacteria.[10–15] We thus sought to expand on these earlier findings, exploring series of compounds with varied cationic character as well as aromatic

[a] M. E. Forman, S. M. Duggan, Prof. K. P. C. Minbiole
Department of Chemistry, Villanova University,
800 East Lancaster Avenue, Villanova, PA 19085 (USA)
E-mail: kevin.minbiole@villanova.edu

[b] M. H. Fletcher, M. C. Jennings, Prof. W. M. Wuest
Department of Chemistry, Temple University,
1901 North 13th Street, Philadelphia, PA 19122 (USA)
E-mail: wwuest@temple.edu

Supporting information for this article can be found under http://dx.doi.org/10.1002/cmdc.201600095. Details provided include chemical synthesis, characterization and spectral data, biological protocols, and complete biological data.

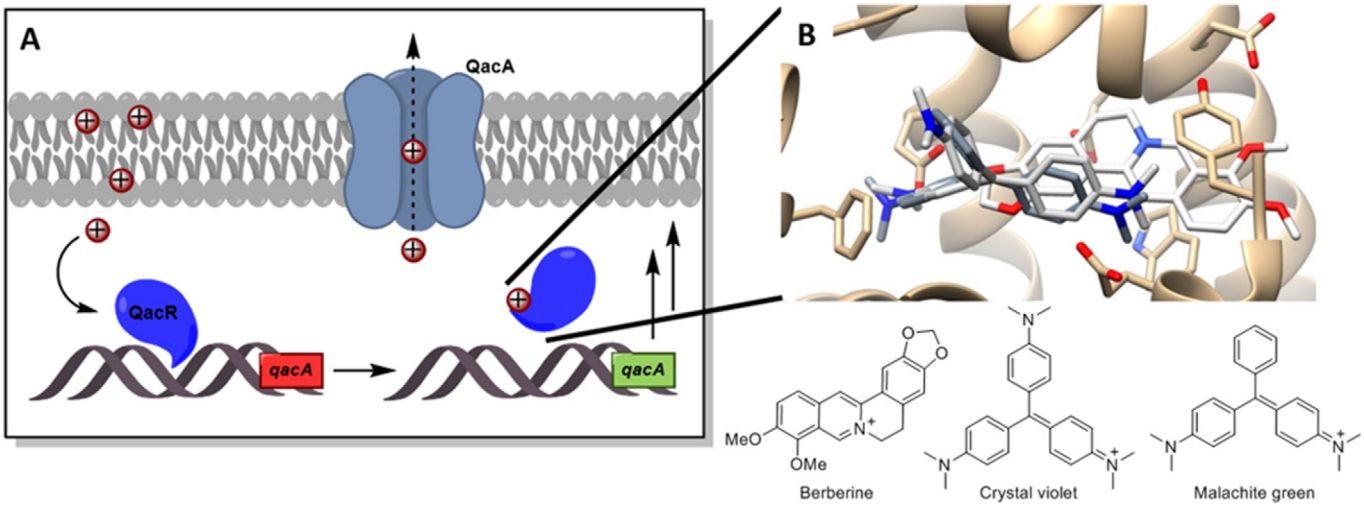


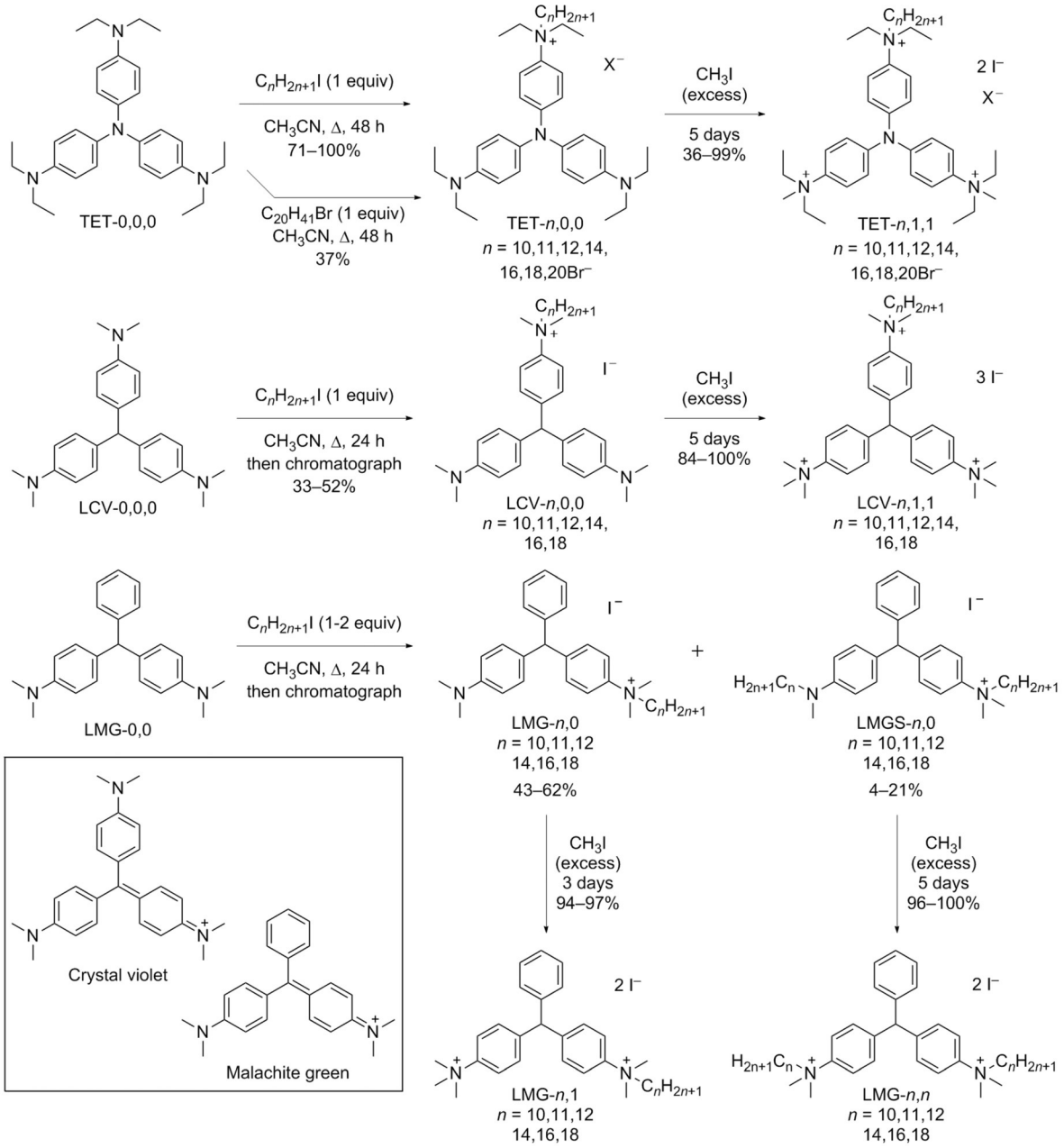
Figure 1. A) QAC resistance mechanism: QACs (red circles) penetrate the membrane and associate with QacR (dark blue), causing dissociation from DNA and allowing transcription of *qacA*. The resulting QacA proteins in the membrane (light blue) facilitate efflux of QACs. B) Top: Overlay of QAC structures (bottom) bound to QacR: berberine (light gray), crystal violet (medium gray), malachite green (dark gray). Residues with proposed importance are structurally highlighted in beige (E57, E58, W61, E90, Y93, Y103, E170, F162).

groups, to investigate whether aryl substrates are indeed particularly prone to QacR recognition. We rationally designed a library of QACs featuring dye scaffolds known to permeate cellular membranes, taking advantage of the reported QacR binding of crystal violet and malachite green.[16] Akin to structure–activity relationship (SAR) assays ubiquitous in the pharmaceutical sector, we designed a series of QAC structures to investigate what structural features would trigger resistance in bacterial strains with known efflux pumps, which we will refer to as a "structure–resistance relationship". The library focused on interrogating the role that permanent charge (+1 to +3) and alkyl length play on efficacy and susceptibility to resistance. Due to the presence of many conjugated ring systems in the dyes, we postulated that there would be an increased amount of recognition and efflux based on previous work demonstrating the binding of aromatic substrates.

Synthesis of the dye-based QACs began with two commercially available dye scaffolds in their reduced (and thus more nucleophilic) form: the bisamine leukomalachite green (LMG) and the trisamine leukocrystal violet (LCV), as shown in Scheme 1. Also readily available was the tetraamine analogue shown (here abbreviated as TET), the aromatic rings of which are more electron rich due to a central nitrogen atom that was expected to be unreactive to alkylation. Each polycyclic aromatic core system was subjected to an analogous synthetic sequence to generate a series of dye-based amphiphiles bearing varied ratios of tertiary amines, quaternary amines, and long alkyl chains. Thus each dye was first exposed to one equivalent of an alkyl iodide ($C_nH_{2n+1}I$) at reflux to furnish compounds abbreviated as TET-*n*,0,0, LCV-*n*,0,0, and LMG-*n*,0, bearing varying alkyl chain lengths, in moderate yields, as shown in Scheme 1. The inaccessibility of 1-iodoeicosane led us to employ the 20-carbon bromide analogue, which resulted in diminished yields in the formation of TET-20,0,0 as the bromide salt. These singly quaternized dyes were then exposed to neat methyl iodide over three to five days to provide fully quaternized tris-QACs (TET-*n*,1,1 and LCV-*n*,1,1) or bis-QACs (LMG-*n*,1) in moderate to high yields. Full experimental details and characterization for all novel compounds are presented in the Supporting Information.

Initial exposure of each dye structure to an excess of the long-chained alkyl halide led to complex mixtures, although in the case of the simpler LMG series, we were able to purify a by-product. Through NMR characterization, we uncovered an unexpected reaction—it was inferred that some bis-alkylation of LMG was occurring, but under these conditions an iodide counter-ion reacted with the product, displacing one of the methyl groups to furnish a monocationic compound bearing two long-chain alkyl substituents. We termed this monoQAC subset a "swapped" series (i.e., LMGS) due to the net swap of one methyl group for a long-chain alkyl group. Recognizing that we had accessed both an unexpected monoQAC series, as well as opened a route to prepare symmetrical bisQAC dye derivatives, we exposed the LMGS compounds to neat methyl iodide to fully quaternize the dye compound in high yields. This resulted in the LMG-*n*,*n* series bearing extended carbon chains on both nitrogen atoms in the molecule.

The complete set of MIC values against six bacteria [*Staphylococcus aureus* (SA), hospital-acquired methicillin-resistant SA (HA-MRSA), community-acquired methicillin-resistant SA (CA-MRSA), *Enterococcus faecalis* (EF), *Escherichia coli* (EC), and *Pseudomonas aeruginosa* (PA)] is presented in Table 1, wherein multiQACs are grouped with their monoQAC counterparts. The dye-based QACs with greatest inhibition of the complete range of Gram-positive and Gram-negative bacteria featured bis- or tris-quaternization, with the optimal alkyl total ranging from 17–24 carbons. For example, comparison of two bis-cationic series—LMG-*n*,1 and LMG-*n*,*n*—evaluates the effect of total alkyl chain length in retention of efficacy against the more challenging CA-MRSA and the Gram-negative strains EC



Scheme 1. Synthesis of mono-, bis-, and tris-quaternary ammonium compounds by alkylation of dye-based scaffolds.

and PA. LMG compounds bearing a single long-chain alkyl group exhibited some elevated MIC values against CA-MRSA and PA, while LMG-10,10, LMG-11,11, and LMG-12,12 showed virtual equipotency across the board (≤ 2 μM). We have previously demonstrated that multiQACs can eradicate established biofilms with minimum biofilm eradication concentrations < 100 μM corresponding to bactericidal activity; therefore, we posit that the compounds reported herein act in an analogous fashion.[12]

When tested against non-resistant SA, most of the 52 QAC structures displayed MIC values in the single-digit micromolar range. Very similar results were observed against two additional strains lacking the *qacAB/R* machinery, known to be susceptible to QACs, namely HA-MRSA and EF. However, when testing against CA-MRSA, a strain known to contain efflux pump genes, increases in MIC were observed, albeit to greatly varying degrees. Marked differences in MIC values against the SA strains were observed for all monoQACs tested, which are bolded in Table 1. In fact, up to 125-fold increases in MIC values were noted (e.g., TET-14,0,0 and TET-16,0,0), even though the most active compounds (e.g., TET-16,1,1) showed no difference in activity. In comparison with other published results, multi-amine QAC scaffolds developed in our labs (N-, Q-, P-, C-, T-series) exhibited significantly less dramatic MIC changes (usually under 16-fold) when comparing SA and CA-MRSA, even when monocationic,[10–14] suggesting that the mul-

Table 1. Minimum inhibitory concentration (MIC) of QAC dye analogues, based on three independent trials.[a]

Compd	MIC [μM]						Compd	MIC [μM]					
	SA	CA-MRSA	HA-MRSA	EF	EC	PA		SA	CA-MRSA	HA-MRSA	EF	EC	PA
TET-10,0,0	**N.T.**	**N.T.**	**N.T.**	**N.T.**	**N.T.**	**N.T.**	**TET-1,1,0**	**4**	**125**	**32**	**32**	**250**	**>500**
TET-10,1,1	2	32	8	8	8	125	TET-1,1,1	500	>500	>500	250	>500	>500
TET-11,0,0	**4**	**32**	**2**	**2**	**63**	**125**	LMG-1,1	>500	>500	>500	>500	>500	>500
TET-11,1,1	1	4	2	4	4	63	**LMG-10,0**	**1**	**8**	**0.5**	**0.5**	**4**	**8**
TET-12,0,0	**2**	**125**	**1**	**1**	**125**	**125**	LMG-10,1	2	63	8	8	8	63
TET-12,1,1	1	4	2	2	4	16	LMG-10,10	0.5	1	≤0.25	0.25	1	2
TET-14,0,0	**1**	**125**	**1**	**2**	**63**	**250**	**LMG-11,0**	**≤0.25**	**4**	**≤0.25**	**≤0.25**	**4**	**8**
TET-14,1,1	0.5	2	1	1	4	16	LMG-11,1	1	32	4	4	2	32
TET-16,0,0	**2**	**250**	**4**	**4**	**63**	**500**	LMG-11,11	0.5	1	0.5	0.5	1	2
TET-16,1,1	1	0.5	1	1	1	8	**LMG-12,0**	**2**	**32**	**0.5**	**≤0.25**	**32**	**32**
TET-18,0,0	**8**	**125**	**8**	**16**	**63**	**500**	LMG-12,1	0.5	16	1	1	1	16
TET-18,1,1	1	0.5	0.5	1	1	8	LMG-12,12	1	1	0.5	0.5	2	1
TET-20,0,0	**N.T.**	**N.T.**	**N.T.**	**N.T.**	**N.T.**	**N.T.**	**LMG-14,0**	**2**	**32**	**4**	**2**	**32**	**63**
TET-20,1,1	1	2	1	1	4	16	LMG-14,1	0.5	2	0.5	0.5	1	8
LCV-1,1,1	>500	>500	>500	>500	>500	>500	LMG-14,14	2	8	1	2	8	63
LCV-10,0,0	**1**	**16**	**1**	**1**	**8**	**32**	**LMG-16,0**	**4**	**32**	**4**	**4**	**32**	**125**
LCV-10,1,1	4	125	16	32	16	125	LMG-16,1	0.5	1	≤0.25	0.5	1	2
LCV-11,0,0	**0.5**	**16**	**0.5**	**0.5**	**16**	**32**	LMG-16,16	1	4	1	2	4	16
LCV-11,1,1	4	125	16	32	16	125	**LMG-18,0**	**8**	**32**	**16**	**8**	**32**	**250**
LCV-12,0,0	**0.25**	**16**	**0.5**	**0.5**	**8**	**32**	LMG-18,1	0.5	2	0.5	0.5	2	8
LCV-12,1,1	4	125	16	32	16	125	LMG-18,18	8	32	4	8	16	250
LCV-14,0,0	**2**	**32**	**2**	**2**	**63**	**125**	**LMGS-10,0**	**8**	**125**	**2**	**2**	**125**	**500**
LCV-14,1,1	4	1	2	4	4	32	**LMGS-11,0**	**8**	**125**	**2**	**8**	**63**	**500**
LCV-16,0,0	**4**	**125**	**2**	**2**	**63**	**250**	**LMGS-12,0**	**16**	**125**	**16**	**16**	**63**	**500**
LCV-16,1,1	1	1	2	2	1	16	**LMGS-14,0**	**125**	**125**	**32**	**32**	**125**	**500**
LCV-18,0,0	**16**	**250**	**8**	**8**	**500**	**500**	**LMGS-16,0**	**8**	**125**	**32**	**32**	**63**	**500**
LCV-18,1,1	1	0.5	0.5	1	0.5	4	**LMGS-18,0**	**63**	**125**	**250**	**250**	**250**	**500**

[a] N.T.: not tested due to low solubility. MonoQACs (bold) are grouped with their corresponding multiQACs. Error is within one dilution from the reported value.

tiaromatic nature of dye scaffolds are unique in their ability to trigger bacterial resistance.

Additionally, antiseptics that display increased MIC values for CA-MRSA (as compared to SA) also show increased MIC values for PA; often the MIC values against PA were greatly elevated (500 μM). In the past we have postulated that the second membrane present in Gram-negative bacteria may be a significant factor in the decreased activity of monoQACs; however, our results are consistent with previous reports that a family of MDR efflux pumps (MexAB-OprM in PA and AcrAB-TolC in EC) is responsible.[5,6] Therefore we propose that this QAC resistance may not be solely attributed to the additional cell membrane but instead to multidrug efflux transporters present within the PAO1 strain. Such proteins as those found in the Mex family (MexA-F; MexAB-OmpR) have been shown to efflux BAC as well as a number of dye compounds.[5]

Direct comparisons of dye-based monoQACs with their multiQAC analogues led to some of our most surprising observations. Superior activity of the multiQACs was expected, but the magnitude of the differences was not; for example, when comparing the MIC values of monocationic TET-16,0,0 with that of triscationic TET-16,1,1 against CA-MRSA (a species known to have QacR), a 500× difference (250 μM vs. 0.5 μM) was observed! While the same differential was observed in a second case (LCV-18,0,0 vs. LCV-18,1,1), the smallest monoQACs prepared (i.e., LMG-10,0 and LMG-11,0) were quite potent against CA-MRSA; one can only speculate that they were too small or too hydrophilic to evoke significant resistance. Additionally, analysis of the MIC values for the prepared trisQACs leads to another unexpected observation: in the fully quaternized LCV series, we identified the first triscationic QAC (LCV-10,1,1) that demonstrates a 32-fold decrease in antibacterial effectiveness against CA-MRSA. In fact, dye-based multiQACs with alkyl chain substitution of less than 14 total carbons repeatedly displayed elevated MICs, suggesting bacterial resistance. This stands in complete contrast to dozens of previously reported *non-aromatic* tris-QAC structures prepared in our laboratories,[4,11–14] which showed no notable difference in MIC against SA versus CA-MRSA. However, similar trends can be identified with those compounds that feature aryl moieties, such as the natural product scaffolds of quinine and nicotine.[10] Indeed, previous studies in our lab have demonstrated the ability for SA and more recently MRSA to develop resistance against monoQACs versus their multiQAC counterparts within 170-700 generations.[3] Future experiments will look into the rate of resistance development within the Gram-negative species.

The drastic increase in bacterial tolerance of most dye-based monoQACs and other aromatic multiQACs can arise from one of three proposed modes of action (Figure 1). The first possibility is that, in contrast to monoQACs (as well as other QACs with shorter alkyl lengths), multiQACs are less likely to traverse the cell membrane due to their increased cationic charge. This would prevent the intracellular buildup of QACs, and accordingly, the overexpression of QacA. A second mechanism in-

volves recognition of the compounds by QacR, but an inability of multiQAC efflux by QacA resulting in the accumulation of QAC inside the cell ultimately leading to membrane disassembly and eventual cell death. The inability of QacA to efflux multiQACs could be attributed to a number of causes, for example, the protein may not be able to accommodate the extra positive charges thereby limiting its ability to efflux the compounds. The final proposal is based on the inability of QacR to recognize more complex substrates such as the multiQACs. As with QacA, QacR may not have the necessary residues to stabilize and therefore recognize the multivalent nature of the multiQACs resulting in decreased binding efficiency. This would result in a lack of overexpression of the QacA efflux pump, again resulting in the detrimental buildup of QAC substrate. With these proposals in mind, we sought to use our QAC dyes as tool compounds to determine if QacR was responsible for the limited development of resistance.

We postulated that by using a reactivation assay (essentially the inverse of a potentiation assay), we could better understand the role QacR plays in monoQAC resistance. Specifically, we dosed the CA-MRSA strain with sub-MIC concentrations of a compound known to be tolerated (i.e., TET-14,0,0), which we reasoned might activate efflux pumps, causing an increase in MIC for a different QAC, such as trisQAC TET-14,1,1. However, dosing of the CA-MRSA strain with sub-MIC concentrations of TET-14,0,0 and varying concentrations of the triscationic variant did not result in any significant modification of observed MIC values. This result fails to confirm the role of QacR in the resistance experienced by our monoQAC dye analogues, although it may hint at the inability of QacR to recognize these structural entities. Other possible explanations include that QacR may be activated by our monoQACs but that QacA cannot efflux the trisQACs or that the resistance mechanism implicated for TET-14,0,0 may be orthogonal to the Qac system. Further studies are currently being undertaken in our laboratory to pinpoint the difference in recognition of the mono- versus trisQACs.

In summary, we have taken inspiration from a subset of structures (berberine and two polyaromatic dyes) that are known to trigger the QacR resistance mechanism, and prepared a series of compounds that showed the promise to bind similarly. The resulting set of 52 QACs, all derived from dyes, included structures with excellent antimicrobial activity, as well as quite similar structures evoking markedly different MIC values in bacteria with or without efflux pumps (up to 125-fold change). This variation in bacterial susceptibility suggests that the presence of efflux pumps is a greater protective factor than the second bacterial membrane of Gram-negative strains. These findings provide credence to the synthetic approach implemented to characterize the "structure–resistance relationship" of QAC resistance in bacteria. We believe that this method nicely complements the current microbiological arsenal of genetic mutants, enzymology, and crystallography to characterize resistance mechanisms. Future work in our laboratory will use these tool compounds in chemical genetic approaches to better understand how trisQACs are able to evade resistance.

Conflict of interest

W.M.W. and K.P.C.M. are equity shareholders in NovaLyse BioSolutions.

Acknowledgements

This work was funded by a University City Science Center QED Proof-of-Concept Grant (S1403 to W.M.W.), as well as by Temple University and Villanova University. M.C.J. acknowledges a National Science Foundation Pre-Doctoral Fellowship (DGE1144462). The authors thank Dr. Charles W. Ross (Temple Mass Spectrometry Facility) for obtaining mass spectrometry data.

Keywords: antibiotic resistance · biocides · MRSA · quaternary ammonium compounds

[1] M. C. Jennings, K. P. C. Minbiole, W. M. Wuest, *ACS Infect. Dis.* **2015**, *1*, 288 – 303.
[2] M. A. Schumacher, M. C. Miller, S. Grkovic, M. H. Brown, R. A. Skurray, R. G. Brennan, *Science* **2001**, *294*, 2158 – 2163.
[3] M. C. Jennings, B. A. Buttaro, K. P. C. Minbiole, W. M. Wuest, *ACS Infect. Dis.* **2015**, *1*, 304 – 309.
[4] S. S. Costa, M. Viveiros, L. Amaral, I. Couto, *Open Microbiol. J.* **2013**, *7*, 59 – 71.
[5] K. Poole, *Clin. Microbiol. Infect.* **2004**, *10*, 12 – 26.
[6] K. Poole, *J. Antimicrob. Chemother.* **2005**, *56*, 20 – 51.
[7] E. Marchi, L. Fuir, S. Arioli, I. Morrissey, V. Di Lorenzo, D. Mora, L. Giovannetti, M. R. Oggioni, C. Viti, *Microbiol. Res.* **2015**, *170*, 184 – 194.
[8] S. R. Holdsworth, C. J. Law, *J. Antimicrob. Chemother.* **2013**, *68*, 831 – 839.
[9] X.-Z. Li, H. Nikaido, K. Poole, *Antimicrob. Agents Chemother.* **1995**, *39*, 1948 – 1953.
[10] M. D. Joyce, M. C. Jennings, C. N. Santiago, M. H. Fletcher, W. M. Wuest, K. P. C. Minbiole, *J. Antibiot.* **2016**, DOI: 10.1038/ja.2015.107.
[11] M. A. Mitchell, A. A. Iannetta, M. C. Jennings, M. H. Fletcher, W. M. Wuest, K. P. C. Minbiole, *ChemBioChem* **2015**, *16*, 2299 – 2303.
[12] M. C. Jennings, L. E. Ator, T. J. Paniak, K. P. C. Minbiole, W. M. Wuest, *ChemBioChem* **2014**, *15*, 2211 – 2215.
[13] J. W. Black, M. C. Jennings, J. Azarewicz, T. J. Paniak, M. C. Grenier, W. M. Wuest, K. P. C. Minbiole, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 99 – 102.
[14] T. J. Paniak, M. C. Jennings, P. C. Shanahan, M. D. Joyce, C. N. Santiago, W. M. Wuest, K. P. C. Minbiole, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 5824 – 5828.
[15] L. E. Ator, M. C. Jennings, A. R. McGettigan, J. J. Paul, W. M. Wuest, K. P. C. Minbiole, *Bioorg. Med. Chem. Lett.* **2014**, *24*, 3706 – 3709.
[16] M. J. Wilhelm, J. B. Sheffield, M. Sharifian, Y. Qu, C. Spahr, G. Gonella, B. Xu, H.-L. Dai, *ACS Chem. Biol.* **2015**, *10*, 1711 – 1717.

Received: February 15, 2016
Revised: March 9, 2016
Published online on March 30, 2016