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Metallocene QACs: The Incorporation of Ferrocene Moieties into monoQAC and bisQAC Structures

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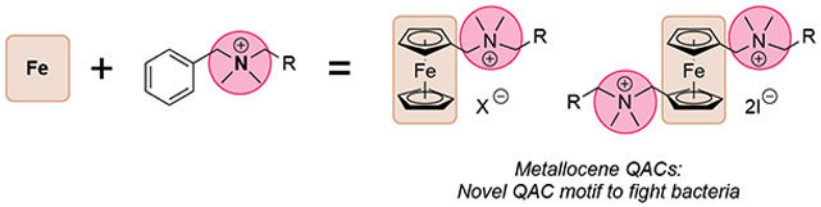
Abstract

Inspired by the incorporation of metallocene functionalities into a variety of bioactive structures, particularly antimicrobial peptides, we endeavored to broaden the structural variety of quaternary ammonium compounds (QACs) by the incorporation of the ferrocene moiety. Accordingly, 23 ferrocene-containing mono- and bisQACs were prepared in high yields and tested for activity against a variety of bacteria, including Gram-negative strains and a panel of clinically-isolated MRSA strains. Ferrocene QACs were shown to be effective antiseptics with some displaying single-digit micromolar activity against all bacteria tested, demonstrating yet another step in the expansion of structural variety of antiseptic QACs.

Graphical Abstract



Given the utility of siderophore-based antimicrobials as well as the established efficacy of quaternary ammonium compounds (QACs), the examination of ferrocene-based QACs was explored for the development of next-generation antiseptics. For both mono and bisQACs, alkyl chains ~11-12 carbons in length displayed the best antibacterial activity, with bisQACs generally displaying superior activities, particularly against Gram-negative strains.

Keywords

antiseptics; bisQAC; ferrocene; methicillin-resistant *Staphylococcus aureus* (MRSA); quaternary ammonium compounds; benzalkonium chloride

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The effective use of antiseptic compounds functions as a frontline defense against infections caused by pathogenic bacteria; thorough hygiene procedures that include antiseptics promise to lessen the burden of continuously developing novel antibiotics.[1] Fortunately, antiseptics are available in a wide variety of classes, including bleach, alcohols, peroxides, and particularly relevant to this work, amphiphiles.[2,3] Modern amphiphilic antiseptics draw inspiration from nature, for nearly every eukaryotic organism utilizes antimicrobial peptides (AMPs) as defense mechanisms, produced by plant and animal species alike in response to the threat of infection.[4] Such compounds exploit a fundamental strategy – permeabilization of the bacterial cell envelope through amphiphile-driven formation of open pores – and their inherent structural variability serves to slow the development of resistance. Representing a class of synthetic analogs to AMPs, amphiphilic quaternary ammonium compounds (QACs) bear at least one cationic nitrogen atom that is drawn towards the negatively charged bacterial cell surface.[5] This electrostatic attraction is followed by intercalation of the nonpolar tail(s) of the QAC into the hydrophobic membrane core of the bacterial cell, perturbing the lipid bilayer and resulting in a loss of membrane integrity and eventual cell lysis.[5,6] It is lamentable, though, that QACs have shown only modest structural innovation in the past century, and accordingly have displayed levels of resistance.[7]

One unorthodox and creative technique in the development of novel antibiotics is the attachment of metal-binding moieties to known structures. One specific example is the “Trojan Horse” approach to pathogen eradication, wherein a siderophore-held iron atom can be actively taken up by bacteria, only to simultaneously deliver a tethered antibiotic structure, as pioneered by Marvin Miller and others.[8,9] Alternatively, many AMPs have been embellished with organometallic substituents including metallocenes, leading to improved activity.[10-12] Ferrocene in particular is inexpensive and known to display rather low toxicity (rat, oral LD50 = 1320 mg/kg);[13] accordingly, many ferrocene-based antimicrobials have been reported and show improved efficacy as antimicrobial agents compared to their more traditional analogs.[14-17] In one example, synthetically derived ferrocene-containing penems had superior anti-MRSA activity, exhibiting as much as a ~4-fold elevation, while also displaying a modest 2-fold enhancement in activity against Gram-negative strains compared to their non-organometallic antibiotic counterparts (Figure 1, left).[14,15] For other small molecule antibiotic mimics, the incorporation of ferrocene with dithiothione and dithioketone moieties resulted in good activity against both *E. coli* and *S. pyogenes* (MIC = 10 μg/mL) (Figure 1, center).[16] In natural product antibiotics such as platensimycin, the administration of ferrocene moieties led to the formation of derivatized compounds that demonstrated modest activity against *S. aureus* (MIC = 128μg/mL) (Figure 1, right).[17] Due to the versatility of these organometallic species, this sort of creative structural modification might represent a means to take back the upper hand in the fight against bacterial resistance.

Previous studies by many groups, including our own, have examined structure-activity and structure-resistance relationships for a multitude of QAC scaffolds, from which insights regarding the number and ratio of charges, lengths and types of side chains, and core structural rigidity have been developed.[7,18] This has led to the preparation of next-generation QACs exhibiting enhanced antimicrobial activity against many different Gram-positive and Gram-negative bacterial strains, including methicillin-resistant *Staphylococcus*

aureus (MRSA) strains.[19] With this in mind, our group has been particularly interested in developing more distinct QAC scaffolds that are effective against a wide range of pathogenic bacterial strains. Transition metal-containing QAC antiseptics, to our knowledge, represent an unexplored avenue for development, and may provide structures for which bacteria have no established resistance mechanisms. We therefore explored the synthesis and antimicrobial evaluation of mono- and bisQACs bearing ferrocene cores, exploiting commercially available starting materials and rapid synthetic routes.

To this end, we set out to identify ferrocene core structures that could swiftly lead to mono- and bisQACs that varied in their alkyl chain length. For monoQACs, we envisioned the quaternization of the nitrogen atom on the relatively inexpensive (dimethylaminomethyl)ferrocene, available from multiple suppliers. In an initial experiment, the starting material was treated with 2 equivalents of 1-bromooctane in acetonitrile at reflux for 24 h, which afforded **Fe-8 Br** in 65% yield (Scheme 1; see supporting information for experimental details). Minor adjustments to these conditions (1.2-1.25 equiv RX, CH_3CN, 72 h, Ar, rt) were found to be broadly effective for the synthesis of ferrocene monoQACs bearing 8-18 carbon chains (termed **Fe-n X** to reflect chain length and counterion) as either bromide or iodide salts (52-83%).

We also extended our study towards the examination of ferrocene-based bisQACs derived from 1,1'-bis(dimethylaminomethyl)ferrocene,[20] whereby each ferrocene unit is now equipped with two tertiary nitrogens capable of quaternization. Similar to above, the preparation of these compounds was achieved by treatment of the starting material with excess 1-iodoalkane (2.5 equivalents) in either acetonitrile or acetone at reflux for 24 h (Scheme 2), furnishing ferrocene bisQACs bearing two 8-18 carbon chains as iodide salts.

With 15 monoQACs and 8 bisQACs in hand, we turned our attention towards assessing the biological activity of these novel compounds. Antimicrobial activity as well as toxicity were assessed, using red blood cell (RBC) lysis as a proxy for the latter. Two antimicrobial standards, benzalkonium chloride (BAC; 70% benzyldimethyldodecylammonium chloride and 30% benzyldimethyltetradecylammonium chloride) and cetylpyridinium chloride (CPC), were also included for comparison (Figure 2). The complete set of MIC values against six bacteria [community-acquired methicillin-resistant *S. aureus* (USA 300-0114), hospital-acquired methicillin-resistant *S. aureus* (ATCC 33591), methicillin-susceptible *S. aureus* (SH1000), *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa*] along with the RBC lysis (presented as $Lysis_{20}$, the lowest concentration at which >20% of all RBCs are lysed), is presented in Table 1. Further, select compounds were tested against a battery of nine invasive methicillin-resistant clinically-isolated *S. aureus* strains, as illustrated in Table 2.

Inspection of the bioactivity profiles of the 23 prepared novel antiseptics indicated some clear trends. First and foremost, we again observe that bisQACs generally demonstrate antimicrobial activities superior to their monocationic counterparts. MonoQACs exhibited surprisingly consistent activity against methicillin-susceptible *S. aureus* and HA-MRSA, repeatedly displaying single digit micromolar MIC values; however, they expressed weak

activity (MIC usually ≥125 μM) versus *E. faecalis* and *P. aeruginosa*, echoing the efficacy of commercially available QAC antiseptics BAC and CPC.

While the role of counterion did not appear to play a significant role in antimicrobial activity for monoQACs, though the diminished solubility of iodide salts might have suppressed some bioactivity, the length of the nonpolar alkyl chains of these QACs was crucial for antimicrobial potency. The incorporation of ~11-12 carbons in the alkyl side chain led to optimal activity, particularly against Gram-negative strains, which is due in part to a lack of solubility in aqueous media for QACs with longer chain lengths.[18,20] MonoQACs **Fe-12 Br** and **Fe-12 I** displayed the best activity against both *E. faecalis* and *P. aeruginosa* (MIC = 63 μM), with at least a 2-fold increase in activity compared to all other monoQACs including BAC and CPC. This alone may suggest some advantage of the ferrocene moiety as compared to more traditional QACs. **Fe-11,11** was the most effective bisQAC in this entire set of antiseptics, showing single digit micromolar activity across all Gram-positive and Gram-negative strains. While quite effective, this does not show a distinct advantage over a comparable bisQAC lacking the ferrocene moiety (e.g., TMEDA-derived QACs such as 12(2)12),[22] although resistance properties might be distinct.

As highlighted above, QACs exhibiting alkyl chain length(s) of ~11-12 carbons displayed optimal activity in this investigation. This trend has been continuously reinforced from plethora of previous studies by our group.[19] Activity peaking for compounds with these chain lengths trends with the length necessary to sufficiently penetrate and disrupt the intermolecular forces of the hydrophobic tails of phospholipids that constitute the cell membrane.[5,18]

Additionally, when comparing our HA-MRSA and CA-MRSA strains, MICs for CA-MRSA tended to be 2- to 4-fold higher than those for HA-MRSA, particularly for monoQACs. This may indicate that the QacA efflux pump found in our lab strain of CA-MRSA is more flexible at accepting variable substrates, such as ferrocene QACs, than the HA-MRSA lab strain that lacks this specific multidrug efflux pump.[23,24] Such an event is supported by reports on the QacA efflux pump, where aromatic QAC substrates were found to bind in QacA via π-π stacking, which could be the case for these ferrocene derivatives.[25] Of course, other factors such as membrane composition may be important determinants in conferring resistance to QACs, due to its ability in affecting the fluidity, flexibility, and phase behavior of the targeted bacterial cell membrane.[26]

When the antimicrobial activity of four top-performing compounds was assessed against a broader panel of methicillin-resistant *S. aureus* (Table 2), a surprisingly consistent picture emerged. Select ferrocene bisQACs were 2-4-fold more effective against MRSA strains than BAC, varying little from strain to strain. Fe-10,10 was repeatedly shown to be four times more effective than BAC, irrespective of the clinical MRSA strain used.

Red blood cell lysis (measured as $Lysis_{20}$), serving as an approximation for human toxicity, appeared to roughly parallel antimicrobial activity. Regardless, these QACs did not display elevated hemolytic properties, with no compound reporting a $Lysis_{20}$ concentration lower than that for the commercially available monoQAC CPC ($Lysis_{20}$ = 8 μM). Generally,

bisQACs were more hemolytic compared to monoQACs. Interestingly, the most active compound for all ferrocene-based QACs, **Fe-11,11**, actually exhibited a two-fold reduction in hemolytic activity ($Lysis_{20}$ = 16 μM) compared to **Fe-12,12**, **Fe-13,13** and **Fe-14,14**, as well as CPC.

We have herein demonstrated that metallocene moieties can be incorporated into QAC structures, and that ferrocene-containing bisQACs represent highly effective QAC antiseptics. While the antimicrobial activity of the prepared compounds still suggest that the number of cationic residues outweigh the importance of the presence of a metal in this line of antiseptics, this hopefully serves to significantly broaden the architecture for QACs. Our group will continue to pursue the installation of other metal centers in QACs, including non-iron structures, as well as more redox active moieties that might be able to eradicate bacteria via more than one mechanism of action.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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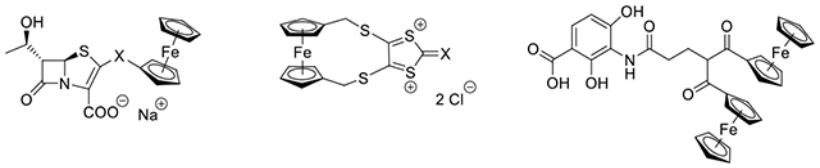


Figure 1:
Past examples of ferrocene-enhanced antimicrobial compounds[13-16]

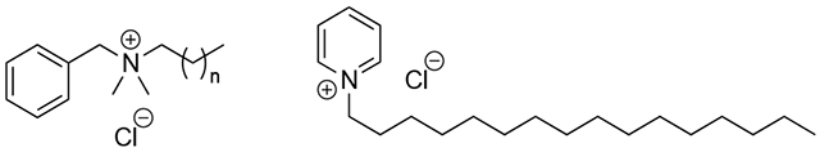
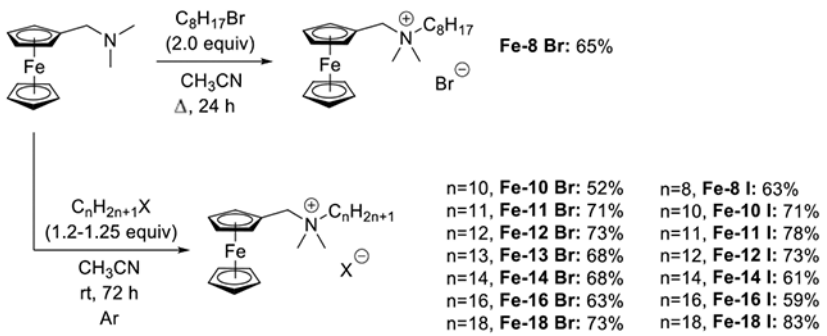
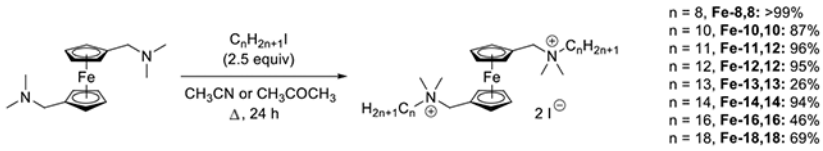


Figure 2:
Commercially available disinfectants BAC (left) and CPC (right).



Scheme 1:
Synthesis of ferrocene monoQACs



Scheme 2:
Synthesis of ferrocene bisQACs

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Table 1.

Antimicrobial activity and red blood cell lysis data for amphiphiles, presented in μM

Compound	Minimum Inhibitory Concentration (μM)						$Lysis_{20}$ (μM)
	S. aureus	***E. faecalis***	***E. coli***	***P. aeruginosa***	**CA-MRSA**	**HA-MRSA**	
BAC	2	250	32	125	2	8	32
CPC	1	125	32	125	1	1	8
Fe-8 Br	32	>250	>250	>250	32	125	250
Fe-8 I	32	>250	>250	>250	32	125	250
Fe-10 Br	4	>250	125	>250	63	16	250
Fe-10 I	4	>250	63	>250	63	16	125
Fe-11 Br	2	125	32	125	32	4	125
Fe-11 I	4	250	63	250	32	8	63
Fe-12 Br	1	63	16	63	8	4	32
Fe-12 I	2	63	16	63	1	4	32
Fe-13 Br	1	>250	8	>250	8	2	16
Fe-14 Br	2	250	8	250	8	2	16
Fe-14 I	1	250	8	250	8	2	16
Fe-16 Br	2	125	63	250	2	2	16
Fe-16 I	2	>250	63	>250	32	2	16
Fe-18 Br	2	125	125	250	63	4	32
Fe-18 I	4	250	125	>250	2	4	32
Fe-8,8	8	>250	63	>250	32	32	250
Fe-10,10	1	32	4	32	2	2	63
Fe-11,11	1	8	2	8	2	1	16
Fe-12,12	2	32	4	32	2	2	8
Fe-13,13	2	63	32	63	4	4	8
Fe-14,14	2	125	32	125	2	2	8
Fe-16,16	4	125	63	250	32	4	8
Fe-18,18	8	125	125	125	63	8	16

Gram negative bacteria (*E. coli* and *P. aeruginosa*) are shaded in grey. All MIC data was acquired through compilation of the highest value of three independent trials (lowest for $Lysis_{20}$). All trials were consistent within one dilution.

Table 2.

Antimicrobial activity of select amphiphiles against clinical-isolated MRSA strains.

	Minimum Inhibitory Concentration (μM)									
Compound	**SH1000***	**1289**	**G-1891**	**G-382**	**123**	**120336**	**CA11**	**EUH 13**	**115**	**221**
BAC	2–4	8	4	8	8	8	8	8	8	4
Fe-10,10	1–2	2	2	2	4	2	2	2	2	2
Fe-11,11	1–2	4	4	4	4	4	4	4	8	4
Fe-12,12	2	4	4	4	4	4	4	4	4	4
Fe-16,16	4	4	4	8	4	4	4	4	4	8

*SH1000 is a methicillin-susceptible strain