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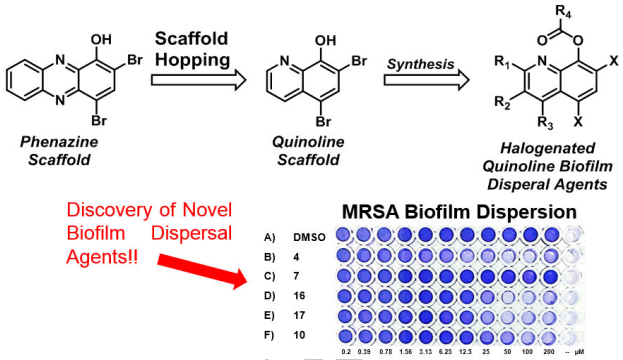

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ABSTRACT

Staphylococcus aureus and *Staphylococcus epidermidis* are recognized as the most frequent cause of biofilm-associated nosocomial and indwelling medical device infections. Biofilm-associated infections are known to be highly resistant to our current arsenal of clinically used antibiotics and antibacterial agents. To exacerbate this problem, no therapeutic option exists that targets biofilm-dependent machinery critical to *Staphylococcal* biofilm formation and maintenance. Here, we describe the discovery of a series of quinoline small molecules that demonstrate potent biofilm dispersal activity against methicillin-resistant *S. aureus* and *S. epidermidis* using a scaffold hopping strategy. This interesting class of quinolines also has select synthetic analogues that demonstrate potent antibacterial activity and biofilm inhibition against *S. aureus* and *S. epidermidis*.

Bacterial biofilms are surface-attached bacterial communities that form when free-floating (planktonic) bacteria use endogenous signaling molecules in a process called quorum sensing to coordinate the simultaneous attachment to a surface.[1-4] Biofilms are notorious for their resistance to conventional antibiotics, microbicidal agents and host immune responses.[1,2] These bacterial communities are highly prevalent in medicine as bacterial biofilms are associated with ~80% of all bacterial infections.[2,5] To make matters worse, we currently have no approved therapeutic treatment options that target machinery critical to biofilm formation or biofilm maintenance.

Staphylococci are frequently encountered by humans as commensal bacteria found on skin and mucous surfaces, which contributes significantly to their status of being involved in more biofilm-associated infections than other pathogens.[6] Drug-resistant *S. aureus* and *S. epidermidis* infections are a major clinical problem in biofilm-related diseases, such as: osteomyelitis, indwelling medical device infection (in particular, central venous catheter *S. epidermidis* infections), chronic wound infection, endocarditis, periodontitis, peri-implantitis, mixed biofilm infections and ocular infection.[6-9] Despite the alarming need, very few small molecule chemotypes or scaffolds exist that target *Staphylococcal* biofilms.[10,11]

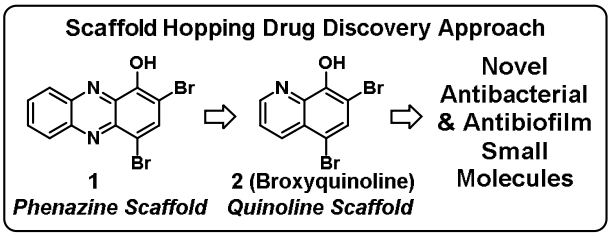


Figure 1. Scaffold hopping strategy to discover novel antibacterial and antibiofilm small molecules.

Our group recently discovered that bromophenazine **1** possesses potent antibacterial activity against *S. aureus* and *S. epidermidis* (MICs between 0.78-1.56 μM or 0.28-0.55 μg/mL).[12] Using a scaffold hopping approach[13,14] (Figure 1), we were curious to know how halogenated quinolines structurally similar to **1**, such as broxyquinoline **2**, would perform in antibacterial and antibiofilm assays against *S. aureus* and *S. epidermidis* as a major goal for our group is to identify novel antibacterial and/or antibiofilm compounds.

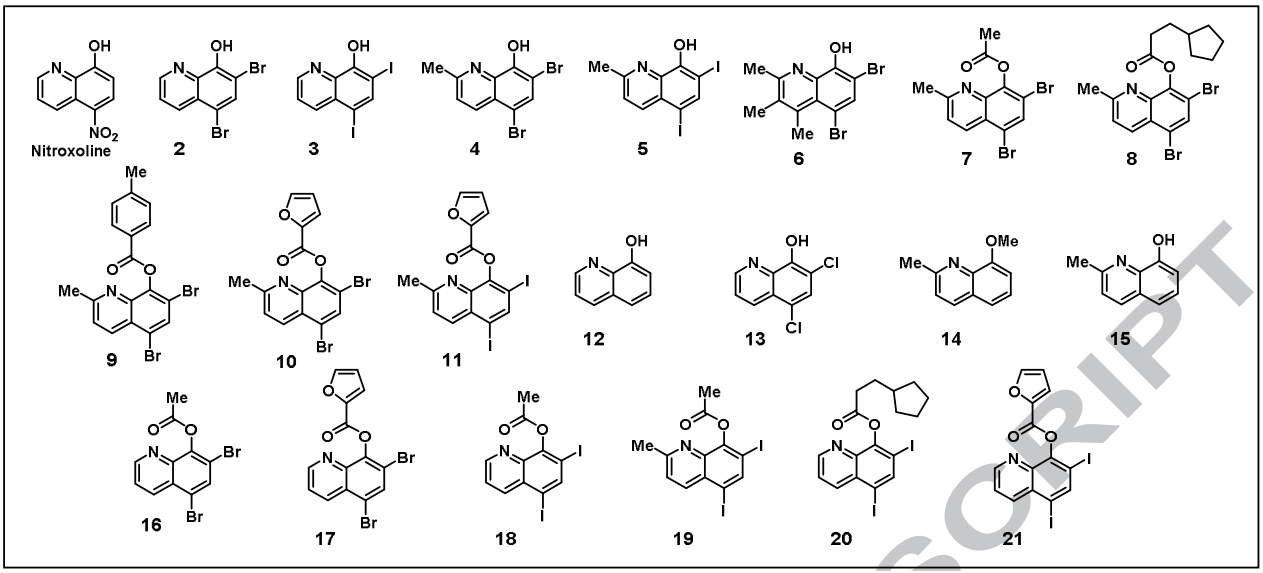
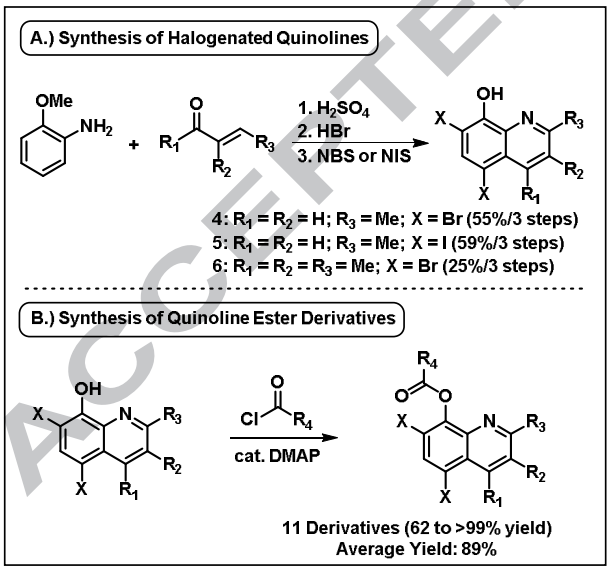


Figure 2. Structures of the quinolines evaluated against *Staphylococcal* pathogens.

Using this scaffold hopping approach, we evaluated a focused library of 21 quinoline small molecules (Figure 2). We initiated this study with the chemical synthesis of 16 quinolines using standard synthetic approaches to construct appropriate halogenated quinoline scaffolds or esters of halogenated 8-hydroxyquinoline derivatives (Scheme 1). Five quinolines, including broxyquinoline **2** and nitroxoline, were obtained commercially for these investigations. Nitroxoline is an antibiotic used to treat urinary tract infections due to its bacteriostatic activity against *Escherichia coli*[15,16] and antibiofilm activity against *Pseudomonas aeruginosa*.[17] Nitroxoline elicits these activities through the chelation of various metals, including zinc.[15-17]



Scheme 1. Rapid synthesis of (A) halogenated quinolines and (B) ester derivatives using standard synthetic approaches.

With our library of 21 quinolines in hand, we first evaluated the ability of these quinolines to inhibit bacterial growth in microdilution MIC experiments head-to-head against **1** (positive control) using a panel of four clinically relevant pathogens, which included: *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *A. baumannii* ATCC 19606 and *P. aeruginosa* PAO1/BAA-47. This panel of pathogens included both gram-positive (*S. aureus* and *S. epidermidis*) and gram-negative (*A. baumannii* and *P. aeruginosa*) bacteria to determine the spectrum of activity of our quinolines. In addition to ATCC strains, we evaluated these quinolines against several *S. aureus* clinical isolates, including several methicillin-resistant *S. aureus* isolates and a *S. epidermidis* clinical isolate (Supplementary Table 2; Supporting Information) from patients treated at UF Health Shands Hospital (Gainesville, FL).

Interestingly, in head-to-head MIC experiments, quinoline **2** was 8- to 16-fold less potent than bromophenazine **1** against *S. aureus* and *S. epidermidis* strains (Table 1.) despite being 4- to 8-fold more potent against *A. baumannii* (Supplementary Table 1; Supporting Information). Neither bromophenazine **1** nor quinoline **2** displayed any detectable growth inhibition activity against *P. aeruginosa* at the highest concentration tested (i.e., 100 μM). Despite the reduction in antibacterial activity from **1** to **2**, quinolines **4**, **7** and **10** demonstrated a 2- to 4-fold increase in potency in antibacterial activity (MIC 0.39-0.78 μM) against *S. aureus*[18] ATCC 29213 compared to bromophenazine **1** (MIC 1.56 μM). Quinolines **7** and **10** also demonstrated similar potency increases in antibacterial activity against *S. epidermidis* when compared to **1**. In addition, we found quinolines **4**, **7**, **9** and **10** to demonstrate potent antibacterial activity (MIC values between 0.2 and 1.56 μM) against a panel of six clinical isolates of *S. aureus* (Supplementary Table 2; Supporting Information).

We then evaluated our quinoline library in biofilm dispersion assays against methicillin-resistant *S. aureus* clinical isolate MRSA-2 and *S. epidermidis* ATCC 12228. The dispersion, or clearance, of established *S. aureus* and *S. epidermidis* biofilms has continued to remain a critical biomedical challenge and we were curious to know if our library of quinolines was capable of dispersing established *Staphylococcal* biofilms. We selected MRSA-2 and *S. epidermidis* ATCC12228 as model strains for this biofilm study due to their ability to form robust biofilms as determined by crystal violet staining in 96-well polystyrene plates.

Table 1. Antibacterial and biofilm dispersion activities of select quinolines against *S. aureus* and *S. epidermidis*.

Compound	*S. aureus* [a] antibacterial activity MIC (μM)	*S. aureus* [b] biofilm dispersion EC_{50} (μM)	*S. epi.* [c] antibacterial activity MIC (μM)	*S. epi.* [c] biofilm dispersion EC_{50} (μM)
2	12.5	9.49	12.5	14.0
3	12.5	14.9	12.5	>200
4	0.39	2.60	0.78-1.56	9.17
5	3.13-6.25	6.55	3.13	11.8
6	6.25	13.6	3.13	5.56*
7	0.39-0.78	2.55	0.39-0.78	12.8
8	0.78-1.56	2.09	1.56	3.26
9	1.56	2.06	>100	>200
10	0.39	2.74	0.39	19.8
11	6.25	3.30	3.13	12.3
nitroxoline	25	10.5	12.5-25	14.2

[a] *S. aureus* strain ATCC 29213 used for MIC. [b] *S. aureus* strain MRSA-2 used for biofilm dispersion. [c] *S. epidermidis* strain ATCC 12228 used for MIC and biofilm dispersion. *EC_{50} value obtained for a biofilm dispersal agent that demonstrates 51±1% biofilm dispersion at 200 μM compared to DMSO controls. All other biofilm dispersal agents were ≥80% effective in biofilm dispersion assays against *S. epidermidis* ATCC 12228.

Several quinolines demonstrated potent biofilm dispersal activity against MRSA-2 (Table 1 & Figure 3). Four quinoline derivatives (i.e., quinolines **4**, **7**, **9**, **10**) demonstrated very potent biofilm dispersion against MRSA-2 with EC_{50} values <2.80 μM. Quinoline **9** (EC_{50} = 2.06 μM) demonstrated the most potent biofilm dispersion activity during the course of our investigations. As a collection, this focused library of 21 quinolines was highly effective in the dispersion, or clearance, of established MRSA-2 biofilms. Results from our biofilm dispersion experiments with MRSA-2 show that 17 of the 21 quinolines (81%) evaluated were found to be "active" biofilm dispersal agents by reporting EC_{50} values of ≤15 μM. Further analysis of our MRSA-2 biofilm dispersion results reveal that 14 of the 21 quinolines (67%) demonstrated "good" potency with EC_{50} values ≤10 μM while 7 of the 21 quinolines (33%) demonstrated "outstanding" biofilm dispersion potency with EC_{50} values ≤5 μM (complete biofilm dispersion data in Supporting Information).

With the goal of identifying promising quinolines capable of completely clearing established MRSA biofilms, we determined EC_{90} values for each of our quinolines (Supplementary Table 3; Supporting Information). Quinolines **8** and **9** were found to be the most potent quinolines in our library with EC_{90} values of 16.6 μM and 17.4 μM, respectively. Six of the 21 quinolines in this study reported EC_{90} values <30 μM against established MRSA-2 biofilms.

In addition to *S. aureus*, we also evaluated our library of quinolines against *S. epidermidis* ATCC 12228 in biofilm dispersion assays (Table 1 & Figure 3). As a collection, these quinoline small molecules were slightly less potent against *S. epidermidis* compared to the MRSA-2 biofilm dispersion assay results; however, quinolines **8** and **4** demonstrated outstanding potency against established *S. epidermidis* biofilms with EC_{50} values of 3.26 μM and 5.56 μM, respectively (see Table 1). As a library, 16 of the 21 (76%) quinolines were found to be "active" dispersal agents and reported EC_{50} values of ≤30 μM. From our biofilm dispersion studies, we found 13 of the 21 (62%) quinolines demonstrated "good" potency as biofilm dispersal agents (EC_{50} values of ≤15 μM) while 4 of the 21 (19%) quinolines demonstrated "outstanding" potency as dispersal agents against *S. epidermidis* biofilms (EC_{50} values of ≤10 μM; complete biofilm dispersion data in Supporting Information).

We also determined EC_{90} values for the biofilm dispersal activity of our quinoline library against *S. epidermidis* (Supplementary Table 4; Supporting Information). Quinoline **8** reported an EC_{90} value of 28.8 μM, which was the most potent biofilm dispersal activity in our quinoline library. The large majority of the biofilm dispersion active quinolines reported EC_{90} values between 110 and 200 μM.

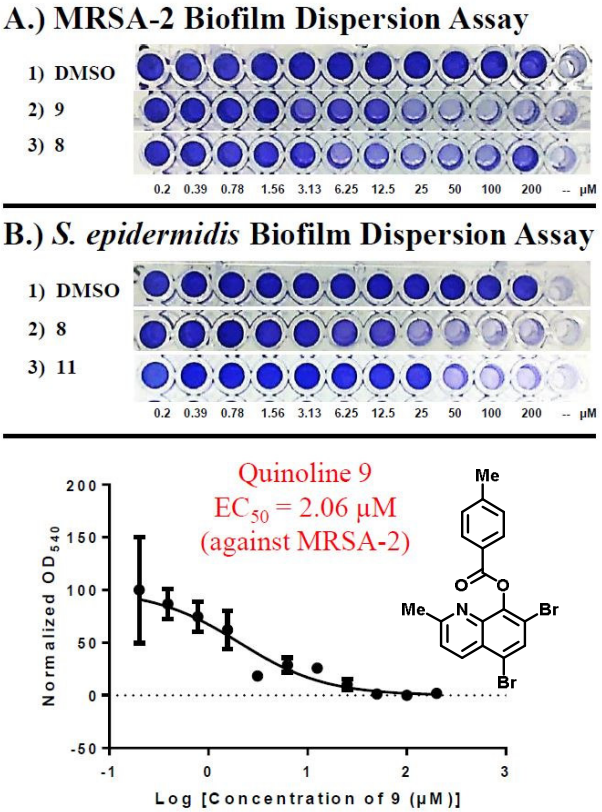


Figure 3. Biofilm dispersion assays of potent biofilm dispersal agents against: A.) MRSA-2 (quinolines **8** and **9**) and B.) *S. epidermidis* (quinolines **8** and **11**). A dose-response curve is presented for quinoline **9** against MRSA-2 biofilms.

In addition to biofilm dispersion, a second major goal of our research program is to identify small molecule biofilm inhibitors that operate via non-growth inhibiting mechanisms. Biofilm inhibitors that operate via mechanisms independent of growth inhibition mimic quorum sensing antagonists and place little, if any, stress on bacteria to develop resistance. Although we determined that our quinoline small molecules had moderate to good antibacterial (i.e., growth inhibiting) activities in our initial MIC experiments, we were curious to

see if any of our quinoline derivatives possessed biofilm inhibition activity that was independent of growth inhibition against *Staphylococcal* strains.

Biofilm inhibition assay parameters were optimized for *S. aureus* and *S. epidermidis* biofilm formation (i.e., glucose supplementation, higher bacterial load and longer incubation periods) which was necessary to achieve a dense and robust *Staphylococcal* biofilm in microtiter plates. As a result of these growth promoting conditions, a new set of MIC values for "biofilm inhibition conditions" was generated for our quinoline library (Table 2). MIC values in these biofilm inhibition assays were typically higher (up to >16-fold higher) than standard MIC assay protocols across the quinoline library. The advantage of using this approach to evaluate potential biofilm inhibitors is that the evaluation of planktonic "toxicity" and biofilm inhibition can be carried out using a single assay. We compared the "biofilm inhibition" assay MIC values to the biofilm inhibition activity (IC_{50} values obtained from crystal violet staining of biofilms in 96-well plates) generated in these biofilm inhibition assays and obtained a MIC:IC_{50} ratio to characterize the biological activity of our quinolines as either "biofilm inhibitors" or "antibacterial agents" (growth inhibitors).

From our biofilm inhibition experiments, we determined that quinolines **3** and **6** possess "biofilm inhibition" activity against *S. aureus* (Table 2) and *S. epidermidis* (see Supplementary Table 3; Supporting Information). Quinoline **3** is commercially available; however, quinoline **6** is a novel small molecule that was found to be essentially non-toxic to planktonic (MIC >100 µM) while inhibiting biofilm formation with good potency (IC_{50} = 4.45 µM) by reporting an MIC:IC_{50} ratio of >22.5 against *S. aureus* ATCC 29213. Quinoline **6** demonstrated antibacterial activity (MIC = 6.25 µM) under standard MIC conditions, but it is clear from our biofilm inhibition assays that **6** possesses the ability to inhibit *S. aureus* biofilm formation at concentrations that do not inhibit planktonic growth (Table 2; Supporting Information for images of this experiment). Other quinolines from this library were determined to have antibacterial activity and reported MIC:IC_{50} ratios between 2.3 and 13.1 in head-to-head biofilm inhibition assays against *S. aureus* (see Supporting Information for details on all quinoline library members). MIC:IC_{50} ratios between 4 and >20 have been previously reported for 2-aminobenzimidazole biofilm inhibitors; however, that study reported MIC values and biofilm inhibition activity using separate assay conditions.[19]

In addition to our quinolines, we determined MIC:IC_{50} ratios for two antibiotics (vancomycin and erythromycin) to serve as a reference for "antibacterial activity" in head-to-head biofilm inhibition experiments (Table 2). In our biofilm inhibition experiments against *S. aureus*, vancomycin gave a MIC:IC_{50} ratio of 3.1 while erythromycin reported a MIC:IC_{50} ratio of 7.8. From this series of biofilm inhibition experiments against *S. aureus*, we concluded that MIC:IC_{50} ratios of compounds obtained from the same biofilm inhibition experiment that are ≤10 most likely inhibit biofilm formation via the same mechanism used to inhibit bacterial growth, thus we have characterized such compounds as "antibacterial" and not "biofilm inhibitor" agents.

In biofilm inhibition experiments against *S. aureus* ATCC 29213, nitroxoline reported an MIC:IC_{50} ratio of 3.8. The large differences in MIC:IC_{50} ratios between quinoline **6** and nitroxoline (known metal chelator and clinically used antibiotic) suggests that these two quinoline small molecules possibly operate via alternative primary mechanisms.

TPEN is an established zinc-chelator which was used as a control in our investigations in *Staphylococcal* biofilm inhibition experiments (Table 2). TPEN has previously reported to demonstrate planktonic toxicity against *S. aureus*.[20] In our biofilm inhibition assays against *S. aureus* ATCC 29213, TPEN reported an MIC:IC_{50} ratio of 1.8 which corresponds to high antibacterial (growth inhibiting) phenotype being the primary mechanism for biofilm inhibition.

Table 2. Biofilm inhibition assay results for quinolines and antibiotic controls against *S. aureus* (ATCC 29213).

Compound	*S. aureus* [a] antibacterial activity MIC (µM)	*S. aureus* [a] biofilm inhibition IC_{50} (µM)	MIC:IC_{50} Ratio	Phenotype
2	25	2.55	9.8	Antibacterial
3	>100	3.49	>28.7	Biofilm Inh.
4	6.25	1.14	5.5	Antibacterial
5	6.25	0.89	7.0	Antibacterial
6	>100	4.45	>22.5	Biofilm Inh.
7	6.25	0.76	8.2	Antibacterial
8	6.25-12.5	2.66	7.0	Antibacterial
9	>100	>100	--	Inactive
10	6.25	1.03	6.1	Antibacterial
11	6.25	1.06	5.9	Antibacterial
nitroxoline	25	6.50	3.8	Antibacterial
TPEN	62.5	34.8	1.8	Antibacterial
erythro.	1.56-3.13	0.30	7.8	Antibacterial
vanco.	1.56	0.51	3.1	Antibacterial

[a] *S. aureus* strain ATCC 29213 was used to obtain MIC and IC_{50} values from same biofilm inhibition experiment (assay optimized for biofilm formation). NOTE: Antibiotic abbreviations: erythro.= erythromycin; vanco. = vancomycin.

It is interesting to note activity profile differences between nitroxoline and several active brominated quinolines identified during these investigations. Nitroxoline is known to have bacteriostatic activity against *E. coli* and antibiofilm activity against *P. aeruginosa*, which is why this agent is used to treat reccurring urinary tract infections. In these studies, we found nitroxoline to demonstrate good antibacterial activity against *A. baumannii* (MIC 6.25 µM; Supplementary Table 1.) and only moderate to weak antibacterial activity against *S. aureus* and *S. epidermidis* (MICs 12.5-50 µM). Nitroxoline's activity profile does not highly correlate with the *Staphylococcal* active bromoquinoline small molecules that were identified during the course of these investigations. These differences could point to alternative mechanisms as the primary mode-of-action for the halogenated quinolines described here. Future investigations will aim to interrogate mechanistic differences between nitroxoline and the brominated quinoline small molecules; however, a metal-chelating mechanism for these quinolines cannot be eliminated with our current findings. Metal-chelating

compounds have previously been reported to have biofilm dispersion[21-23] and biofilm inhibition activity.[24,25]

In conclusion, we have identified several quinolines with potent biofilm dispersal and antibacterial activity against methicillin-resistant *S. aureus* and *S. epidermidis* using a scaffold hopping strategy. Our team is currently developing this class biofilm dispersal agents and biofilm inhibitors against *S. aureus* and *S. epidermidis*. Several of the brominated quinolines that were identified during the course of these investigations have the potential to target *Staphylococcal* biofilms and provide clinically effective treatment options for patients suffering from multidrug-resistant, biofilm-associated *Staphylococcal* infections.

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Supplementary Material

Supplemental material for the following items can be found in the Supporting Information document associated with this manuscript, which include: (1) MIC, biofilm inhibition, biofilm dispersion assay protocols; (2) supporting data tables with MIC/biofilm assay results; (3) supporting images of MIC/biofilm assays; (4) synthetic procedures; (5) NMR spectra for all new compounds/synthesized quinolines.