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Synthetically Tuning the 2-Position of Halogenated Quinolines: Optimizing Antibacterial and Biofilm Eradication Activities via Alkylation and Reductive Amination Pathways

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We dedicate this work to the memory of the late Professor Alan Katritzky

Abstract: Agents capable of eradicating bacterial biofilms are of great importance to human health as biofilm-associated infections are tolerant to our current antibiotic therapies. We have recently discovered that halogenated quinoline (HQ) small molecules are: 1) capable of eradicating methicillin-resistant *Staphylococcus aureus* (MRSA), methicillin-resistant *Staphylococcus epidermidis* (MRSE) and vancomycin-resistant *Enterococcus faecium* (VRE) biofilms, and 2) synthetic tuning of the 2-position of the HQ scaffold has a significant impact on antibacterial and antibiofilm activities. Here, we report the chemical synthesis and biological evaluation of 39 HQ analogues that have a high degree of structural diversity at the 2-position. We identified diverse analogues that are alkylated and aminated at the 2-position of the HQ scaffold and demonstrate potent antibacterial (MIC $\leq$ 0.39 μM) and biofilm eradication (MBEC 1.0–93.8 μM) activities against drug-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecium* strains while demonstrating $<$5% haemolysis activity against human red blood cells (RBCs) at 200 μM. In addition, these HQs demonstrated low cytotoxicity against HeLa cells. Halogenated quinolines are a promising class of antibiofilm agents against Gram-positive pathogens that could lead to useful treatments against persistent bacterial infections.

Introduction

Small molecules capable of eradicating free-floating planktonic bacteria and surface-attached bacterial biofilms serve as promising therapeutic leads as persistent, biofilm-associated bacterial infections are innately tolerant to our current arsenal of antibiotics.[1–3] Bacteria exist in two distinct lifestyles, which include: 1) rapidly-dividing, free-floating (planktonic) bacteria, and 2) surface-attached communities of specialized, non-replicating persister cells encased within an extracellular matrix of biomolecules (i.e., biofilm; Figure 1).[1,2,4] Bacterial biofilms play a significant role in human health as it is estimated that 17 million new biofilm infections will result in $>$500 000 deaths in the United States each year.[2,5]

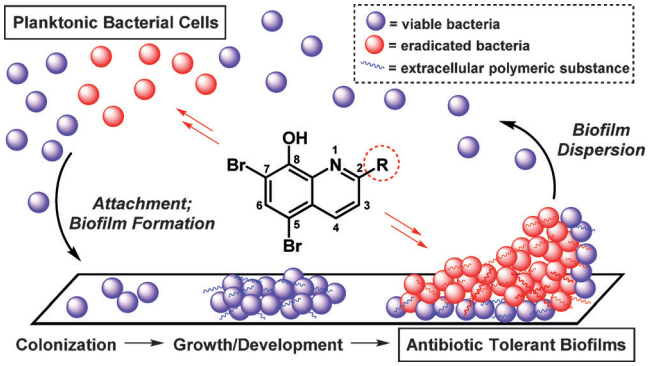

Figure 1. Illustration of the dynamic "biofilm cycle" including free-floating planktonic and surface-attached bacteria. Halogenated quinolines eradicate both planktonic and persistent biofilm-associated bacteria.

Since penicillin's discovery in 1928, each new class of antibiotic has been discovered as bacterial growth inhibitors that either kill or inhibit the proliferation of planktonic cells. As a consequence, our antibiotics operate through growth-dependent mechanisms and are rendered ineffective against non-replicating, persistent biofilms.[1,2,6] Several classes of biofilm inhibitors and dispersal agents have been discovered and although these are promising agents, they typically operate through the perturbation of bacterial signaling processes (i.e., quorum sensing)[3,7,8] and do not kill persister cells housed within biofilms.

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In order to eradicate persister cells that live within biofilms, new small molecules are needed that operate through growth-independent mechanisms. The most prominent class of biofilm-eradicating agents are antimicrobial peptide mimics (i.e., quaternary ammonium cations[9,10]); however, other small molecules have demonstrated biofilm eradication, including: dicationic porphyrins (i.e., XF-73)[11], carbonyl cyanide *m*-chlorophenylhydrazone (CCCP),[12] *N*-acetyl cysteine (NAC),[13] mitomycin C[14] and halogenated phenazines.[15,16] In addition, the co-treatment of ADEP4 (acyldepsipeptide antibiotic and ClpP activator) and rifampin successfully eradicated a methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm infection using a mouse model.[17]

Recently, we reported a series of five halogenated quinolines with potent biofilm-eradicating activities against MRSA, MRSE (methicillin-resistant *Staphylococcus epidermidis*) and VRE (vancomycin-resistant *Enterococcus faecium*).[18] These HQs were discovered through a reductive amination reaction at the 2-position of the HQ scaffold, which plays a critical role in antibacterial activities. Here, we describe a full account of our investigations of HQ analogues that have been diversified at the 2-position of the HQ scaffold, including: analogue design, chemical synthesis, biological evaluation and structure-activity relationship analysis.

In our previous studies, we observed that the 2-position of HQ scaffold plays a significant role on the corresponding antibacterial activities on HQ analogues.[19] Broxyquinoline (Brox-Q; Figure 2) has a hydrogen atom in the 2-position compared to **HQ-1**, which has a methyl group in the 2-position of the HQ scaffold. This single methyl group difference enhances the antibacterial activity of **HQ-1** 16-fold against staphylococcal pathogens while eliminating antibacterial activity against the Gram-negative pathogen *Acinetobacter baumannii* 128-fold compared to Brox-Q. In addition, we found that **HQ-1** is capable of eradicating MRSA biofilms.[18,20] These observations motivated us to design multiple synthetic routes to achieve rapid and highly diverse analogues at the 2-position of the HQ scaffold for evaluation in antibacterial and biofilm eradication assays against drug-resistant strains of major human pathogens.

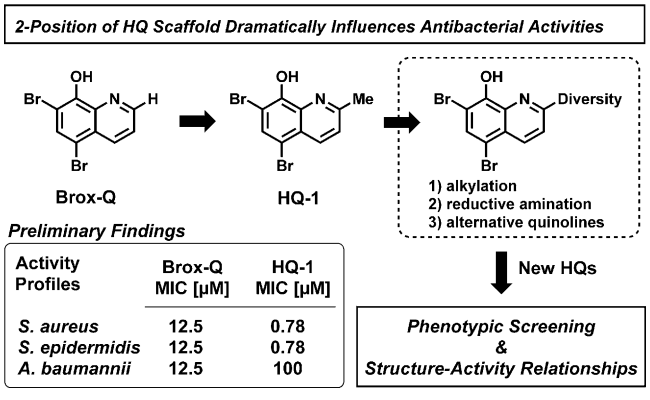


Figure 2. Preliminary findings that the 2-position of the HQ scaffold controls antibacterial properties led to the discovery of HQ biofilm-eradicating agents.

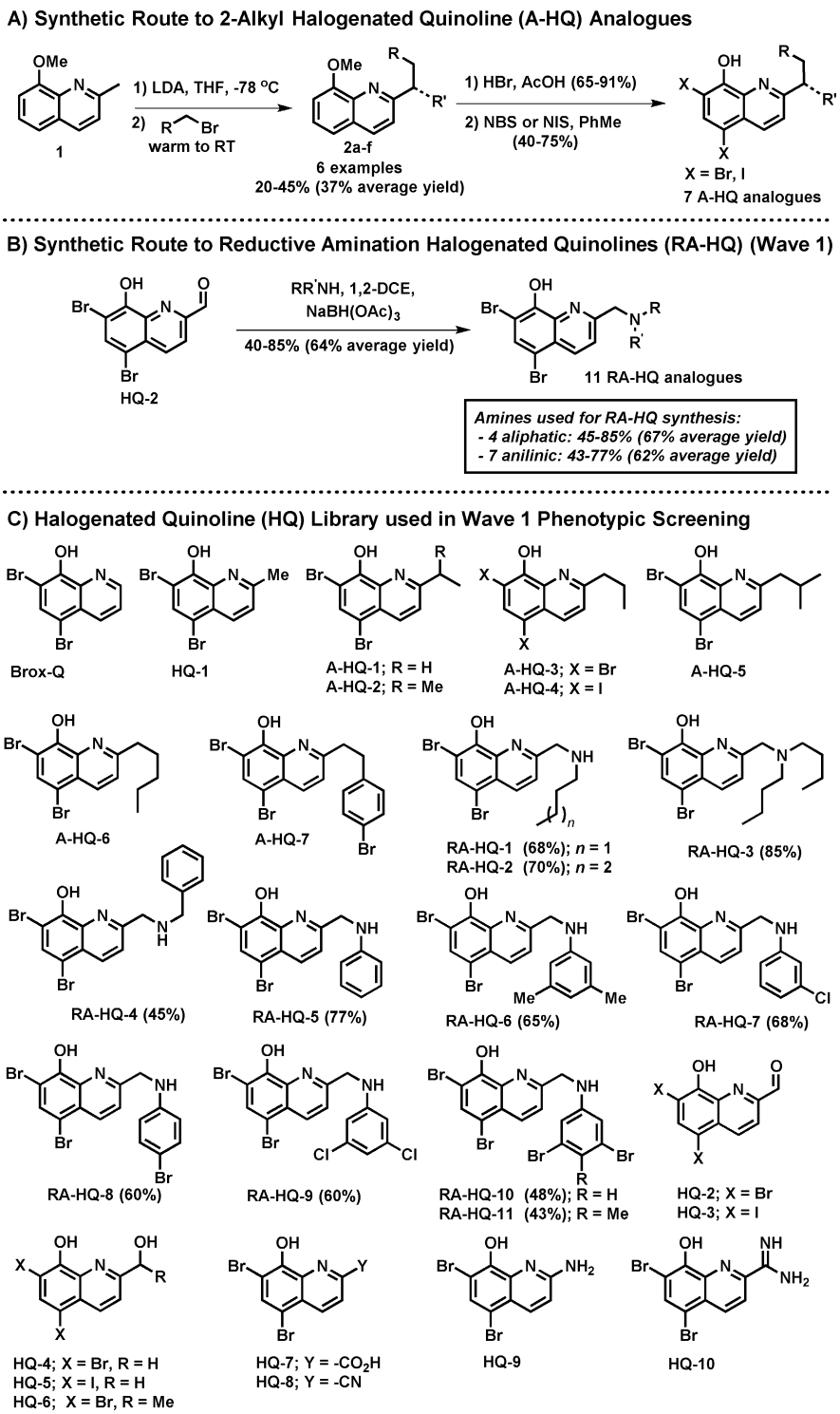
Results and Discussion

Chemical synthesis of wave 1 HQ analogues

With **HQ-1** demonstrating potent antibacterial (minimum inhibitory concentration or MIC 0.78 μM) and biofilm eradication (minimum biofilm eradication concentration or MBEC 250 μM) activities against MRSA,[20] our initial goal was to synthesize a series of HQ analogues that possessed diverse alkylated products at the 2-position of the HQ scaffold (**A-HQs**). To this end, we treated **1** with 1.1 equivalents of lithium diisoproplyamine (LDA) to generate the corresponding carbanion at the methyl group of the 2-position, followed by subsequent addition of an alkyl halide to serve as an electrophile in an S_N2 reaction (Scheme 1 A). This alkylation reaction proceeded in 20–45% yield to generate 6 diverse alkylated products **2a**–**f**. Methylation with methyl iodide gave a 20% yield of the bismethyl product **2b**, which was unanticipated; however, we carried this analogue forward to 2-isopropyl **A-HQ-2** (Scheme 1). Following the alkylation step, 8-methoxyquinolines **2a**–**f** were demethylated using hydrobromic acid in acetic acid to afford the corresponding 8-hydroxyquinolines in 65–91% yield. Final halogenation with *N*-bromosuccinimide (NBS) or *N*-iodosuccinimide (NIS) yielded **A-HQ-1** through **A-HQ-7** in 40–75% yield.

In addition to 2-alkylated HQ analogues, we designed an alternative route to incorporate a diverse series of amines and anilines at the 2-position of the HQ scaffold through reductive amination. **HQ-2** (Scheme 1 B), which was synthesized by the NBS bromination of 8-hydroxyquinoline-2-carbaldehyde, was used as a key building block for analogue synthesis through a divergent reductive amination reaction. Initial attempts to carry out reductive amination on **HQ-2** were unsuccessful despite extensive scouting of solvents (i.e., toluene, acetonitrile, methanol), temperatures (i.e., room temperature, reflux), reaction times (i.e., hours to multiple days) and catalysts (i.e., acid, no acid). Upon close examination of these reaction conditions, we encountered problems with initial imine formation which led to no or unacceptable yields of reductive amination. However, we found that changing the solvent to 1,2-dichloroethane (1,2-DCE) allowed the desired reductive amination to proceed smoothly at room temperature. We condensed **HQ-2** with a diverse panel of amines and anilines for 15 min to 1 h before adding sodium triacetoxyborohydride ($NaBH(OAc)_3$) to afford eleven reductive amination HQ analogues (**RA-HQ-1** through **RA-HQ-11**) in 40–85% yield. Aliphatic amines (4 examples; 45–85% yield) and anilines (7 examples; 43–77% yield) demonstrated near identical efficiencies using this reductive amination route (Scheme 1 B).

Commercially available or easily synthesized 8-hydroxyquinolines with different substitution at the 2-position were brominated or iodinated to yield a diverse collection of HQs (see Scheme 1 C for structures; see the Supporting Information for synthesis details). Several of these analogues were used to probe diverse electronic properties at the 2-position of the HQ scaffold, including: **HQ-2/HQ-3** (aldehyde), **HQ-7** (carboxylic acid), **HQ-8** (nitrile), **HQ-9** (amine) and **HQ-10** (amidine). **HQ-4,**



Scheme 1. Synthetic routes involving: A) alkylation, and B) reductive amination pathways for the diversification of the 2-position of the HQ scaffold. C) Initial HQ library synthesized for biological evaluation in antibacterial and biofilm eradication assays.

HQ-5 and **HQ-6** possessed alcohol functional groups, complementing analogues the alkylated series.

Biological investigations of wave 1 HQ analogues

With this panel of 29 HQ analogues (including **Brox-Q** and **HQ-1**; Scheme 1 C), our strategy was to screen this library against *Staphylococcus aureus* (methicillin-sensitive; ATCC 29213), *Staphylococcus epidermidis* (methicillin-sensitive; ATCC 12228) and *Enterococcus faecium* (ATCC 700221; vancomycin-resistant, VRE) to identify potent antibacterial agents in microdilution MIC assays (MIC $\leq$ 3.13 μM; full summary in the Supporting Information Table S1) before advancing the most active antibacterial agents to biofilm eradication studies using Calgary Bio-

film Device (CBD) assays[21] (Table 1). We began with an MIC screen since these assays are operationally simple and HQs that eradicate biofilms typically demonstrate potent antibacterial activities. In addition, all HQ analogues were screened against human red blood cells for haemolytic activity at 200 μM (Table 1) while select analogues were evaluated for mammalian cytotoxicity against HeLa cells (see the Supporting Information).

Comparing the 27 new HQs (Wave 1; Scheme 1C) to the potent antibacterial activity of **HQ-1** (MIC = 0.59 μM; Supporting Information Table S1), we identified nine HQs that demonstrated equipotent antibacterial activities (i.e., MIC values 0.39–1.17 μM) against *S. aureus* 29213. Fifteen new HQs lost antibacterial activity (MIC values between 1.56 and 100 μM) and three HQs were inactive (MIC > 100 μM) against *S. aureus* 29213 in our assays. Five of the seven **A-HQs** and three **RA-HQs** proved to be the most potent antibacterial HQs against *S. aureus* 29213. When evaluated against MRSA-2 and MRSA BAA-44, several HQs demonstrated potent antibacterial activities (MIC ≤ 1.56 μM), including: **A-HQ-1**, **A-HQ-3**, **RA-HQ-5** (Supporting Information Table S1; Supporting Information Table S2 has additional MRSA clinical isolate MIC data).

Interestingly, against *S. epidermidis* 12228, four new HQs (**A-HQ-2**, **RA-HQ-7**, **RA-HQ-8**, **RA-HQ-9**) demonstrated two- to threefold more potent antibacterial activities (MIC = 0.39 to 0.59 μM) compared to **HQ-1** (MIC = 1.17 μM). Eight new HQs demonstrated equipotent antibacterial activities (MIC = 0.78–2.35 μM) while 15 new HQs reported partial or complete loss in antibacterial activity against *S. epidermidis* (MIC = 3.13 to > 100 μM). Select HQs were evaluated against methicillin-resistant *S. epidermidis* (ATCC 35984), which demonstrated an increased sensitivity towards HQ analogues as **HQ-1** (MIC = 0.30 μM), **A-HQ-1** (MIC = 0.39 μM), **A-HQ-3** (MIC = 0.59 μM), **RA-HQ-5** (MIC = 0.15 μM) and **RA-HQ-9** (MIC = 0.30 μM) demonstrated the most potent antibacterial activities against MRSE (Table 1; Supporting Information Table S1).

Halogenated quinolines displayed a range of antibacterial activities against VRE 700221. **HQ-1** demonstrated good antibacterial activity (MIC = 2.35 μM) against VRE while eight new HQs demonstrating increased antibacterial potencies (MIC = 0.39–0.78 μM) with **A-HQ-4** and **RA-HQ-9** proving to be the most potent (MIC = 0.39 μM; eightfold more potent than **HQ-1**). From this series of wave 1 HQs, seven new HQs demonstrated equipotent activity (MIC = 1.17–3.13 μM) while 12 HQs lost antibacterial activities (MIC = 6.25–100 μM) against VRE when compared to **HQ-1**.

Similar antibacterial trends were observed between staphylococcal pathogens (*S. aureus* and *S. epidermidis*) and *E. faecium*

Table 1. Summary of antibacterial, biofilm eradication and haemolysis activities for select halogenated quinoline (HQ) analogues, relevant conventional antibiotics and controls. All concentrations are reported in μM.

Compound	MRSA-2 MIC	MRSA-2 MBC/MBEC	MRSA BAA-1707 MBC/MBEC	MRSE MIC	MRSE MBC/MBEC	VRE MIC	VRE MBC/MBEC	% Haemolysis at 200 μM
HQ-1	0.78	23.5[a]/188[a]	62.5/46.9[a]	0.30[a]	31.3[b]/93.8[a]	2.35[a]	2.0/1.5[a]	≤1
A-HQ-1	0.78	93.8[a]/1000	–	0.39	31.3/>1000	0.78	1.5[a]/2.0	≤1
A-HQ-3	0.78	125/93.8[a]	31.3/46.9[a]	0.59[a]	9.38[a]/62.5	0.78	2.0[b]/2.0[b]	≤1
A-HQ-4	1.56	188[a]/188[a]	–	1.17[a]	125/125	0.39	7.8/7.8	≤1
A-HQ-5	0.78	–	31.3/23.5[a]	0.59[a]	–	100	–	12.3
A-HQ-7	9.38[a]	375[a]/>1000	–	1.17[a]	250/>1000	2.35[a]	9.38[a]/1.5[a]	≤1
RA-HQ-1	4.69[a]	46.9[a]/>1000	–	2.35[a]	9.38[a]/62.5	3.13	15.6/3.9	21.3
RA-HQ-2	2.35[a]	31.3[a]/93.8	–	1.56	23.5[a]/62.5	3.13	15.6[b]/7.8	87.9
RA-HQ-5	0.78	125/188[a]	62.5[b]/93.8[a]	0.15[a]	7.8/3.0[a]	0.78	7.8[b]/1.5	3.1
RA-HQ-7	1.17[a]	62.5[b]/188[a]	–	0.30[a]	9.38[a]/5.9[a]	0.78	1.5[a]/1.0[c]	18.8
RA-HQ-8	3.13	125/125	–	0.15[a]	5.9[a]/31.3	0.78	3.9[b]/1.0[c]	3.7
RA-HQ-9	1.56	62.5/750[a]	–	0.30[a]	1.5[a]/23.5[a]	0.39	2.0[b]/1.0[c]	10.6
RA-HQ-11	18.8[a]	500/>1000	–	9.38[a]	250/>1000	75[a]	125/9.38[a]	≤1
RA-HQ-12	0.39	31.3[b]/93.8[a]	15.6[b]/7.8	0.39	3.9/5.9[a]	0.78	1.0[c]/1.0[c]	≤1
RA-HQ-13	0.78	31.3/93.8[a]	–	0.30[a]	31.3/46.9[a]	0.78	1.5[a]/1.5[a]	17.6
RA-HQ-14	4.69[a]	250/750[a]	–	0.78	46.9[a]/188[a]	4.69[a]	46.9[a]/1.5[a]	11.0
RA-HQ-15	1.56	188[a]/750[a]	–	0.20	31.3[b]/93.8[a]	0.78	7.8[b]/23.5[a]	37.3
RA-HQ-16	1.56	188[a]/500	93.8[a]/375[a]	0.20	46.9[a]/125	0.78	7.8[b]/1.5[a]	≤1
RA-HQ-17	3.13	750[a]/>1000	–	0.59[a]	188[a]/>1000	1.56	31.3[b]/3.0[a]	7.4
RA-HQ-22	>100	>1000/>1000	–	4.69[a]	250/375[a]	9.38[a]	31.3[b]/7.8	3.0
RA-HQ-23	25	>1000/>1000	–	12.5	125/375[a]	25	31.3[b]/15.6	3.3
vancomycin	0.59[a]	3.0/>2000	5.9[a]/>2000	0.78	3.0[a]/>2000	>100	>200/150[a]	≤1
daptomycin	4.69[a]	62.5[b]/>2000	125/>2000	12.5	–	–	–	1.7
linezolid	3.13	15.6/>2000	31.3/>2000	3.13	–	3.13	4.69[a]/1.56	≤1
QAC-10	3.13	31.3[b]/125	–	2.35[a]	31.3/31.3	2.35[a]	3.0[a]/3.0[a]	>99
CCCP	3.13	31.3/1000	–	6.25	31.3/93.8[a]	–	–	3.5
NAC	–	>2000/>2000	–	–	>2000/>2000	–	>2000/>2000	–
pyrazinamide	–	>2000/>2000	–	–	–	–	–	≤1
EDTA	–	2000/>2000	–	–	1000/>2000	–	–	3.0

[a] Midpoint value for a 2-fold range in independent experiments. [b] Midpoint value for a 4-fold range in independent experiments. [c] Lowest concentration tested. All MIC, MBC, MBEC values and haemolysis data were obtained from at least three independent experiments. MRSA = methicillin-resistant *Staphylococcus aureus*, MRSE = methicillin-resistant *Staphylococcus epidermidis*, VRE = vancomycin-resistant *Enterococcus faecium*.

for wave 1 HQs with several alkylated and reductive aminated analogues demonstrating the most potent antibacterial activities. This observation guided our wave 2 synthesis efforts. Interestingly, a subpanel of 12 new HQs was found to be inactive (MIC > 100 μM; Supporting Information Table S3) against a panel of Gram-negative pathogens, including: *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Acinetobacter baumannii*. In addition, all HQs were screened against RBCs at 200 μM and demonstrated a range of haemolytic activities (≤1 to 88% haemolysis; Table 1; Supporting Information Table S1). Select HQs were evaluated against HeLa cells (lactate dehydrogenase release assays) and demonstrated good to excellent mammalian cytotoxicity (IC_{50} > 50 to > 100 μM; Supporting Information Table S1).

Chemical synthesis of wave 2 HQ analogues

Following initial antibacterial studies with wave 1 HQs, we set out to synthesize a second series (wave 2) of reductive aminated HQ analogues that contained diverse aniline moieties due to the impressive biological activities of **RA-HQ-5** (aniline moiety) and **RA-HQ-8** (4-bromoaniline moiety), which were found to possess highly potent antibacterial activities against *S. aureus*, *S. epidermidis* and *E. faecium* (MIC = 0.59–0.78 μM) while demonstrating < 4% haemolytic activity against RBCs at 200 μM and low cytotoxicities against HeLa cells (Figure 3). In addition to aniline moieties, diiodo-versions of **RA-HQ-5** and **RA-HQ-8** were also synthesized (i.e., **RA-HQ-22** and **RA-HQ-23**).

For the synthesis of wave 2 **RA-HQs**, we used our reductive amination conditions that provided **RA-HQs** during wave 1 analogue synthesis. Using these conditions, we were able to rapidly generate ten new aniline-based RA analogues in 42 to 73% yield (58% average yield; Scheme 2). These anilines were selected to probe several structural features that would enable a greater understanding of the structure–activity relationships (SAR) of HQ analogues, including: 1) overall diversity of the aniline moiety by using various substitutions of halogens, alkyl and methoxy groups, 2) the role of the 4-bromine atom on the aniline moiety by substituting this position with other halogen atoms, alkyl and methoxy groups, and 3) alternative bromination patterns on the aniline moiety to compare to active **RA-HQ-8**. In addition to new RA analogues that possess the 5,7-dibromo-8-hydroxyquinoline moiety, **HQ-3** was used as a synthetic template to yield diiodinated analogues **RA-HQ-22** (55% yield from **HQ-3**) and **RA-HQ-23** (35% yield from **HQ-3**) for our SAR investigations.

Antibacterial activities of wave 2 HQ analogues

Following the chemical synthesis of wave 2 RA-HQ analogues, antibacterial assays revealed five new analogues (**RA-HQ-12**, **RA-HQ-13**, **RA-HQ-15**, **RA-HQ-16**, **RA-HQ-17**) that possess potent antibacterial activities (MIC ≤ 1.17 μM) against *S. aureus*, *S. epidermidis* and *E. faecium*, including methicillin-resistant staphylococcal isolates (Supporting Information Table S1). Impressively, **RA-HQ-12** and **RA-HQ-16** demonstrate the highest level of potency in antibacterial activities (MIC 0.20–1.56 μM) against these pathogens, including drug-resistant isolates while demonstrating no haemolysis against RBCs at 200 μM. In general, the halogenated aniline moieties of the RA-HQ analogues possessed good-to-highly potent antibacterial activities while alkyl and methoxy aniline moieties generally gave decreased antibacterial activities. Diiodinated HQ analogues **RA-HQ-22** and **RA-HQ-23** demonstrated a significant reduction of antibacterial activities when compared to their corresponding potent brominated analogues **RA-HQ-5** and **RA-HQ-8**, respectively.

Biofilm eradication investigations of wave 1 and 2 HQs

Following antibacterial studies, 21 HQ analogues were evaluated for biofilm eradication activities using the Calgary Biofilm Device (CBD), which has proven to be a useful tool for evaluating small molecules for antibiofilm activities.[21–23] CBD (biofilm eradication) protocols involve three phases, including: Phase 1) establishment of bacterial biofilms in the absence of test compound, Phase 2) challenging the biofilm with a test compound (i.e., HQ), and Phase 3) recovery of viable biofilms in the absence of test compounds (viable biofilms grow and disperse planktonic bacteria into fresh media, resulting in turbidity). Using the CBD, biofilms are established/treated/recovered on pegs that are attached to a 96-well plate lid and submerged in media. Eradicated biofilms following compound treatment are

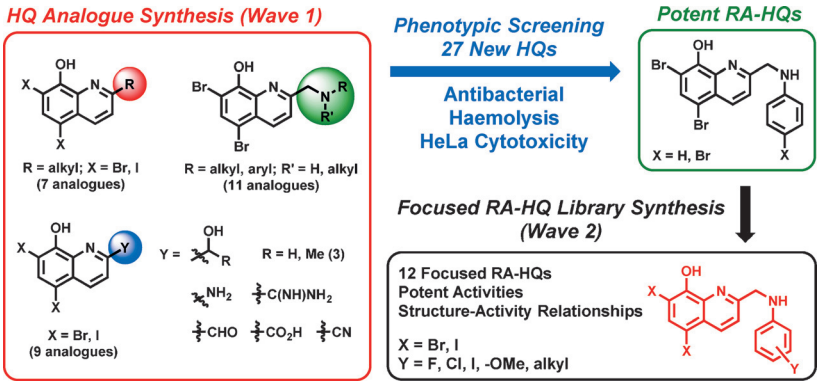
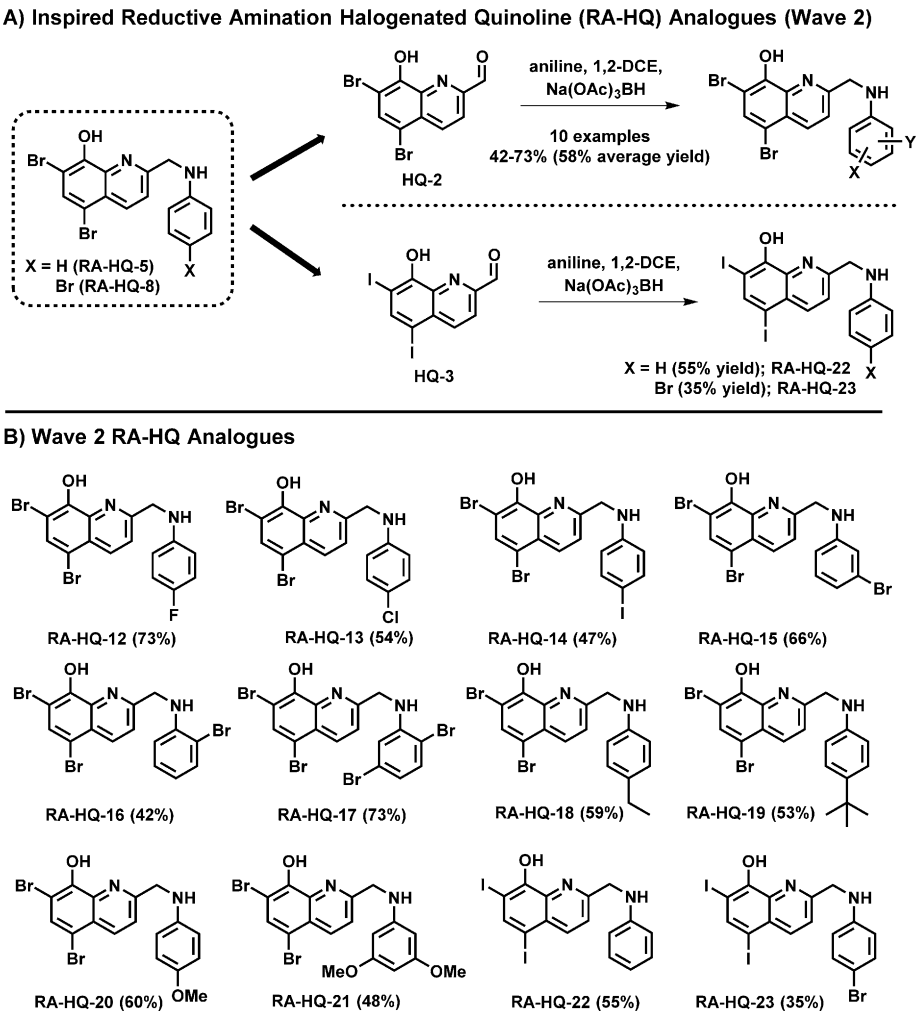


Figure 3. Progression from wave 1 HQ analogue synthesis and biological evaluation to the design of wave 2 HQs focused primarily on diverse reductive amination analogues with aniline moieties.



Scheme 2. Chemical synthesis of wave 2 **RA-HQ** analogues based on lead **RA-HQ-5** and **RA-HQ-8**.

unable to recover, thus resulting in non-turbid microtiter wells following the final incubation (i.e., recovery) period. The lowest test concentration that results in eradicated biofilms (non-turbid microtiter wells) corresponds to the MBEC value (Figure 4).

In addition to determining biofilm-eradication activities, the Calgary Biofilm Device can be used to quantify planktonic killing through the determination of minimum bactericidal concentrations (MBC) of compounds. This enables the assessment of biofilm and planktonic eradication activities from the same experiment through the evaluation of MBEC/MBC ratios. Biofilm-eradicating agents should demonstrate MBEC/MBC $\approx$ 1, which is preferred to comparing MBEC and MIC values obtained under different assay parameters.

Staphylococcal pathogens, in particular *S. aureus* and *S. epidermidis*, are the leading cause of biofilm-associated, hospital-acquired bacterial infections and are often life-threatening.[24,25] Staphylococcal biofilm infections are highly prevalent in indwelling medical device-related infections (i.e., heart valve, joint replacement).[25] *S. epidermidis* biofilms are a major clinical problem as this pathogen is frequently encountered during cerebral shunt[26] and catheter related treatments.[27] *E. feacium* biofilms are involved in endocarditis, catheter-associated urinary tract infections and peridontitis.[28] Due to the significant role these pathogens play in biofilm-related infections, we evaluated our most promising HQs in biofilm eradication assays against these bacteria.

HQ analogues displayed a range of biofilm eradication activities against MRSA-2 (a clinical isolate) biofilms (Table 1). **HQ-1** demonstrated good biofilm eradication activities against MRSA-2 reporting an MBEC value of 188 μM using CBD assays. **A-HQ-3** (MBEC = 93.8 μM), **RA-HQ-8** (MBEC = 125 μM), **RA-HQ-12** (MBEC = 93.8 μM) and **RA-HQ-13** (MBEC = 93.8 μM) reported improved biofilm eradication activities against MRSA-2 compared to **HQ-1**. Using CBD assays, we found that lead biofilm-eradicating HQs against MRSA-2 reported MBEC/MBC ratios between 1 and 3 demonstrating near equipotent biofilm and planktonic killing efficiencies for these HQ analogues. Three new HQs (**A-HQ-4**, **RA-HQ-5**, **RA-HQ-7**) demonstrated equipotent biofilm eradication activities against MRSA-2 compared to **HQ-1** while 12 new HQs were found to be less active (MBEC = 500 to $>$1000 μM). Surprisingly, the 2-ethyl analogue **A-HQ-1** proved to have less potent biofilm eradication activity against MRSA-2, despite the 2-methyl (**HQ-1**) and 2-propyl (**A-**

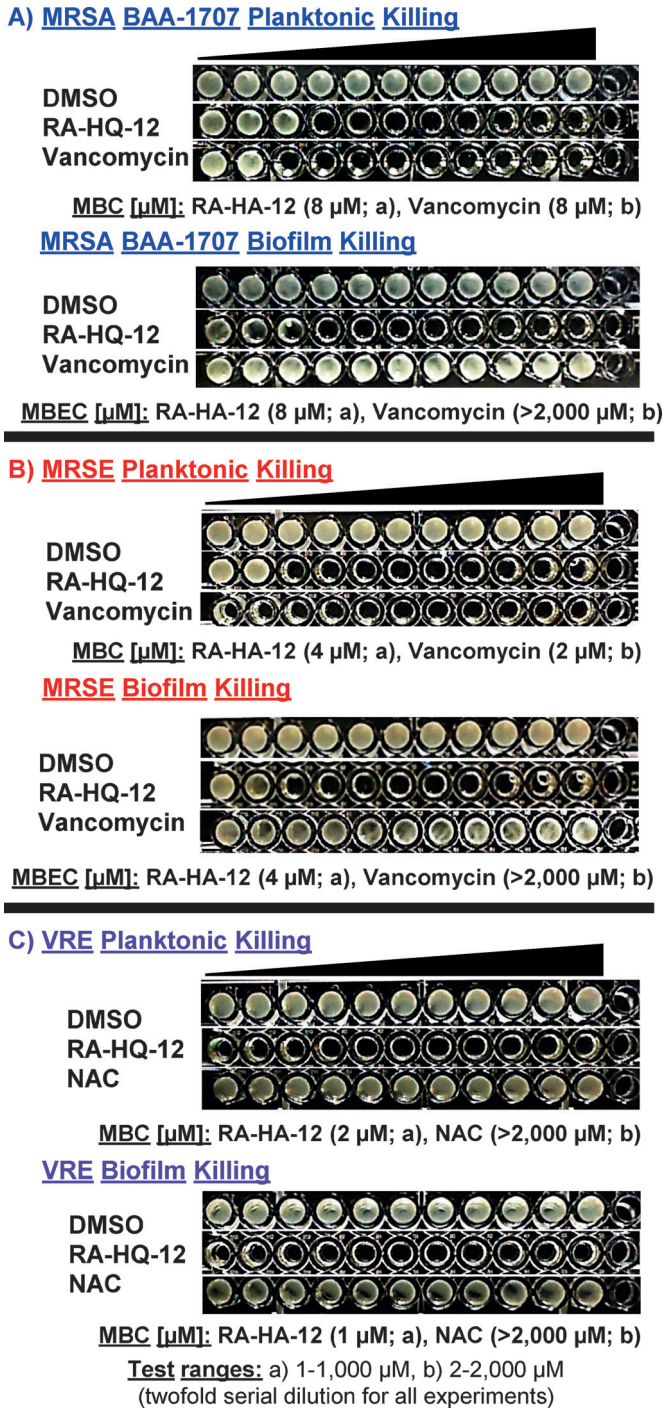


Figure 4. Calgary Biofilm Device (CBD) assays for **RA-HQ-12**. CBD is used to determine planktonic and biofilm cell eradication in a single assay for evaluating small molecules of interest.

HQ-3) analogues demonstrating potent activities. We were puzzled by this result and repeated antibacterial and biofilm eradication assay numerous times, further inspected multiple samples of **A-HQ-1** for stability and purity only to find this compound to be both stable and pure. **A-HQ-1** possesses potent antibacterial activities against MRSA (and MRSE; Table 1) without similar biofilm eradication activities, which is a phenotype rarely encountered with HQ small molecules against staphylococcal pathogens. In addition to **A-HQ-1**, we observed a similar activity trend with **A-HQ-7** which possess a large 2-ethyl-4-bromophenyl moiety; however, this compound is not as potent as the other more structurally simple 2-alkyl HQ analogues.

In addition to new HQ analogues, we evaluated front-running MRSA treatments (i.e., vancomycin) in CBD assays against MRSA-2 alongside new HQ analogues. From these experiments, we found vancomycin, daptomycin and linezolid were unable to eradicate MRSA-2 biofilms at the highest test concentration (MBEC $>2000\ \mu M$) despite reporting moderate-to-excellent planktonic killing (MBC $=3.0$–$62.5\ \mu M$) in the same experiment. These large planktonic versus biofilm killing activities (MBEC/MBC ratio for vancomycin is >667 against MRSA-2) is illustrative of the high levels of tolerance that biofilms display towards conventional antibiotics. Against MRSA-2, lead HQ analogues are >20-fold more potent as biofilm-eradicating agents when compared to current anti-MRSA therapeutic agents (Table 1). Other reported biofilm-eradicating agents and persister cell killers (i.e., QAC-10,[9] CCCP,[12,15] NAC,[13] pyrazinamide;[1,29] Table 1) were also evaluated as positive controls during our investigations. QAC-10, an antimicrobial peptide mimic and membrane-lysing agent, proved to be the most potent control in this panel; however, lead HQs identified during these investigations were found to be more potent than QAC-10 against MRSA, MRSE and VRE biofilms (Table 1).

We tested a small panel of active HQ analogues against a second MRSA strain, ATCC BAA-1707, in CBD assays and found this multi-drug resistant MRSA strain to be more sensitive to HQ compounds than MRSA-2, which is a clinical isolate from a patient at Shands Hospital (Gainesville, FL). Impressively, **RA-HQ-12** reported an MBEC value of $7.8\ \mu M$ (Figure 4A), which proved to be sixfold more potent than **HQ-1** against MRSA BAA-1707. Similar to MRSA-2, vancomycin, daptomycin and linezolid all proved to be unable to eradicate MRSA BAA-1707 biofilms despite demonstrating moderate to potent planktonic killing activity in CBD assays (Table 1).

We found that MRSE biofilms demonstrated an increased sensitivity to our HQs (compared to MRSA-2 results) as nine new HQs showed improved biofilm eradication activities compared to **HQ-1** (MBEC $=93.8\ \mu M$; Table 1) with **RA-HQ-5** (MBEC $=3.0\ \mu M$), **RA-HQ-7** (MBEC $=5.9\ \mu M$) and **RA-HQ-12** (MBEC $=5.9\ \mu M$; Figure 4) proving to be the most potent analogues (MBEC/MBC ratios ≈ 1 for these analogues). In addition, **RA-HQ-9** (MBEC $=23.5\ \mu M$), **RA-HQ-8** (MBEC $=31.3\ \mu M$) and **RA-HQ-13** (MBEC $=46.9\ \mu M$) demonstrated excellent eradication activities against MRSE biofilms. Ten HQs demonstrated equipotent or reduced biofilm eradication activities against MRSE (MBEC $=125$ to $>1000\ \mu M$) compared to **HQ-1**. Vancomycin was unable to eradicate MRSE biofilms (MBEC $>2000\ \mu M$), despite effectively killing planktonic cells (MBC $=3.0\ \mu M$) in CBD assays (Figure 4A and B).

Against VRE biofilms, 13 new HQ analogues were found to be highly potent biofilm-eradicating agents (MBEC $=1.0$–$3.9\ \mu M$, Table 1) while demonstrating equipotent planktonic and biofilm cell killing by comparing MBC/MBEC ratios. From

our HQ panel, four analogues eradicated VRE biofilms at 1 μM (i.e., **RA-HQ-7, RA-HQ-8, RA-HQ-9, RA-HQ-12**), which is the lowest concentration tested during these investigations. Interestingly, *N*-acetyl cystine (NAC) was unable to eradicate VRE biofilms at the highest test concentration (MBEC >2000 μM) when tested alongside these HQ analogues (Figure 4) despite a previous report that NAC eradicates *E. faecium* biofilms.[13] During these studies, linezolid reported an MBEC value of 1.56 μM against VRE 700221 biofilms while QAC-10 (positive-control) registered an MBEC value of 3.0 μM.

Biological activity profiles of HQ analogues

From these extensive biological investigations, critical insights into the structure–activity relationships and biological activity profiles integrating antibacterial, biofilm eradication and haemolysis activities of alkylated and reductive aminated analogues at the 2-position of the HQ scaffold were gained. Alkylated HQ analogues proved to be promising as several analogues in this subclass maintained potent antibacterial activities against *S. aureus* (including drug-resistant MRSA isolates), *S. epidermidis* (including MRSE) and VRE while select analogues demonstrated potent biofilm eradication activities without any detectable haemolytic activity against RBCs with the exception of **A-HQ-5** which showed moderate levels of haemolysis at high concentrations (12% RBC lysis at 200 μM).

Reductive amination HQ analogues demonstrated a broad range of antibacterial activities, from highly potent to completely inactive against the Gram-positive pathogens, including a panel of MRSA isolates. Unlike the 2-alkylated HQ series, RA-HQ analogues registered drastically different haemolysis activities (≤1 to 88% lysis of RBCs at 200 μM; Table 1 and Supporting Information Table S1). The aniline or 4-halogenated aniline moieties on lead HQ scaffolds (i.e., **RA-HQ-5**, **RA-HQ-8**, **RA-HQ-12**) proved to demonstrate potent biofilm eradication activities. This activity was sensitive to moving the halogen atoms on the aniline ring (i.e., **RA-HQ-15**) and further modification (i.e., **RA-HQ-11**). Biological profiles regarding the antibacterial, biofilm eradication and haemolysis activities change dramatically among related analogues as illustrated in Figure 5.

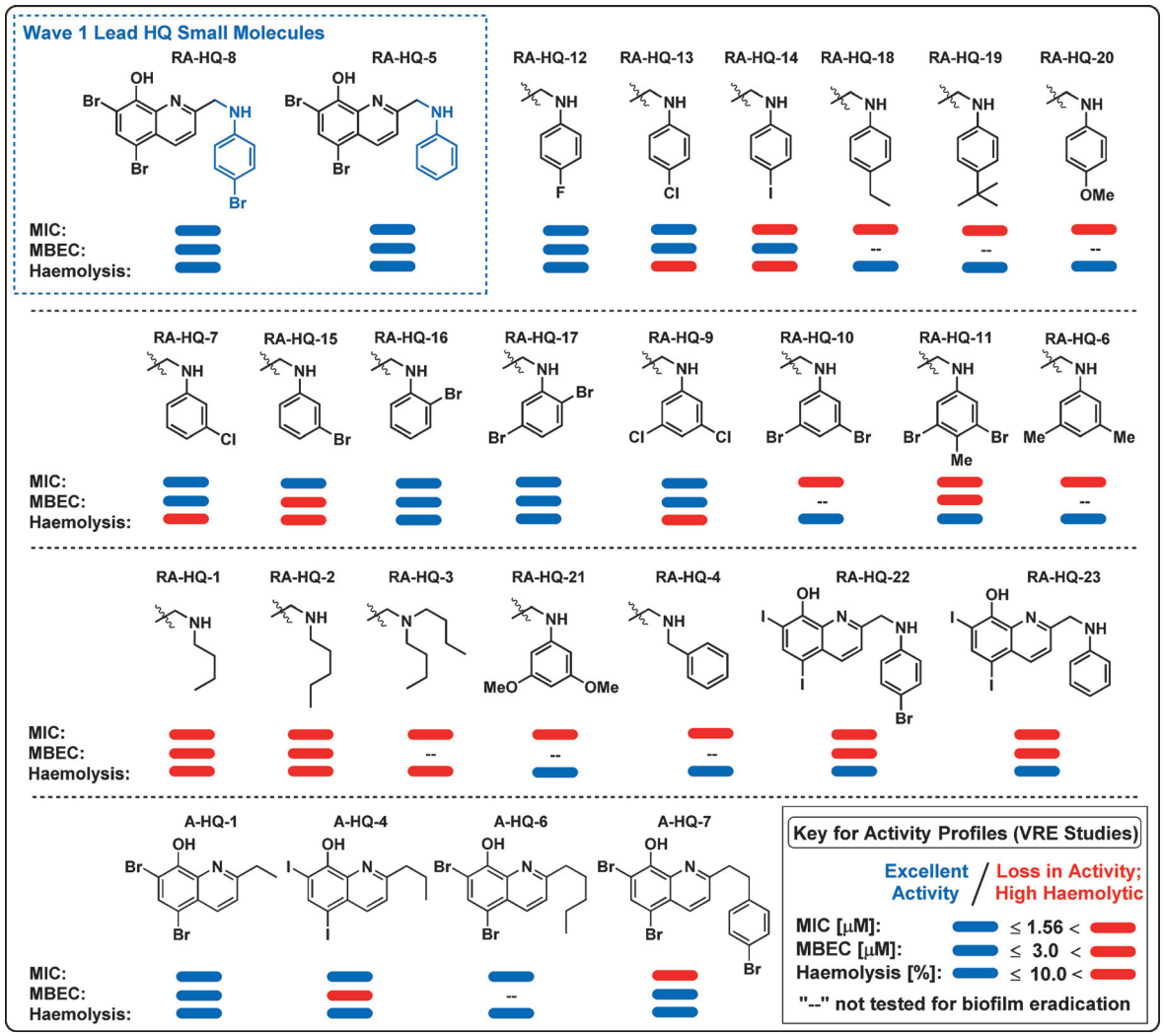


Figure 5. Activity profiles (antibacterial, biofilm eradication, haemolysis) of select HQ analogues from wave 1 and wave 2 against VRE. These activity profiles will be useful in directing the development of future HQ biofilm-eradicating agents.

Straight-chain aliphatic- along with select aniline-based reductive aminated analogues demonstrated moderate to high levels of hemolytic activities against RBCs (>10% at 200 μM, Figure 5). Overall, we are very encouraged by these findings and feel that the ability to synthetically tune the 2-position of the HQ scaffold will be critical in developing HQ biofilm-eradicating agents as both small molecule probes and therapeutic agents.

Halogenated quinolines bear an 8-hydroxyquionline substructure, which is an established metal-chelating moiety.[30,31] Although detailed mechanistic studies are required, we believe that HQs operate through a metal(II) cation-dependent mechanism. Interestingly, when co-treated with various metal(II) cations, we have found that the antibacterial activities of HQs can be enhanced (i.e., Zn^{2+}), suppressed (i.e., Cu^{2+}, Fe^{2+}) or unaffected (i.e., Mg^{2+}) depending on the metal(II) ions (Supporting Information Tables S4 and S5). Mechanistic studies are underway in our labs and will be reported in due course. We believe these HQ small molecules will lead to important discoveries concerning biofilm viability and new avenues for small molecule biofilm control and therapeutic strategies.

Conclusions

In conclusion, we have demonstrated the ability to synthetically tune the 2-position of the halogenated quinoline scaffold through the use of alkylation and reductive amination pathways. Promising analogues from this study demonstrate impressive antibacterial and biofilm-eradication activities against several Gram-positive human pathogens, including MRSA isolates. **RA-HQ-12** proved to be a highly potent eradicating agent against MRSA, MRSE and VRE biofilms. Lead HQ small molecules also demonstrated low haemolytic and HeLa cell cytotoxicity. Halogenated quinolines represent a promising class of antibacterial agents that are highly effective in eradicating persistent bacterial biofilms and could lead to novel therapeutic agents against chronic and recurring infections.

Experimental Section

Full experimental details concerning the chemical synthesis and biological evaluation of these HQ compounds can be found in the Supporting Information associated with this manuscript. All novel compounds synthesized during these studies have ^{1}H and ^{13}C NMR spectra, HRMS and melting point (for solids) are reported in the Supporting Information. Images of select MIC and Calgary Biofilm Device experiments are included in the Supporting Information document.

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Keywords: antibiotic tolerance · bacterial biofilms · halogenated quinolone · medicinal chemistry · structure–activity relationships

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