Antibiofilm Compounds

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Halogenated Phenazines that Potently Eradicate Biofilms, MRSA Persister Cells in Non-Biofilm Cultures, and *Mycobacterium tuberculosis*

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Abstract: *Conventional antibiotics are ineffective against non-replicating bacteria (for example, bacteria within biofilms). We report a series of halogenated phenazines (HP), inspired by marine antibiotic* ***1****, that targets persistent bacteria. HP* ***14*** *demonstrated the most potent biofilm eradication activities to date against MRSA, MRSE, and VRE biofilms (MBEC = 0.2–12.5 μM), as well as the effective killing of MRSA persister cells in non-biofilm cultures. Frontline MRSA treatments, vancomycin and daptomycin, were unable to eradicate MRSA biofilms or non-biofilm persisters alongside* ***14****. HP* ***13*** *displayed potent antibacterial activity against slow-growing M. tuberculosis (MIC = 3.13 μM), the leading cause of death by bacterial infection around the world. HP analogues effectively target persistent bacteria through a mechanism that is non-toxic to mammalian cells and could have a significant impact on treatments for chronic bacterial infections.*

Current antibiotics operate primarily through growth-dependent mechanisms and effectively target rapidly-dividing bacteria; however, non-replicative bacteria (for example, dormant persister cells or bacterial biofilms) display high levels of antibiotic tolerance that contribute to persistent and recurring bacterial infections.[1–3] In recent years, our knowledge of bacterial biofilms (surface-attached bacterial communities with altered physiologies, gene expression profiles, and growth-rates)[4,5] and persister cells[6] has grown considerably, yet our ability to target persistent bacterial phenotypes remains a challenge. To target and eradicate non-replicating bacteria, innovative strategies to identify antibacterial agents that operate through growth-independent mechanisms must therefore be employed.

Although there has been much interest in identifying non-growth altering biofilm inhibitors and dispersal agents over the past two decades,[7] few classes of biofilm-eradicating agents are known. Biofilm-eradicating agents typically operate through the disruption of bacterial membranes (for example, antimicrobial peptides,[8,9] quaternary ammonium cations/QACs).[10] Although these compounds are indeed valuable, new biofilm-eradicating agents with complementary modes of action are of great importance and have multiple therapeutic applications to address persistent bacterial infections.

Considering the marine environment as an extensive source of microbial diversity and new antibacterial agents,[11] it stands to reason that such sources are fertile grounds for the discovery of biofilm-eradicating agents. Despite marine sources being largely unexplored, several classes of chemically diverse quorum sensing (the bacterial signaling process that controls biofilm formation and maintenance) modulators[12–15] and biofilm inhibitors/dispersal agents have been identified from marine organisms.[7b,16] Our group recently discovered that halogenated phenazine (HP) analogue **2** (Figure 1), a synthetic analogue of marine phenazine antibiotic **1**, displays biofilm eradication activities against MRSA with a minimum biofilm eradication concentration (MBEC) of 150 ± 50 μM,[17] which is on pace with the most potent eradicating agents previously reported.[10]

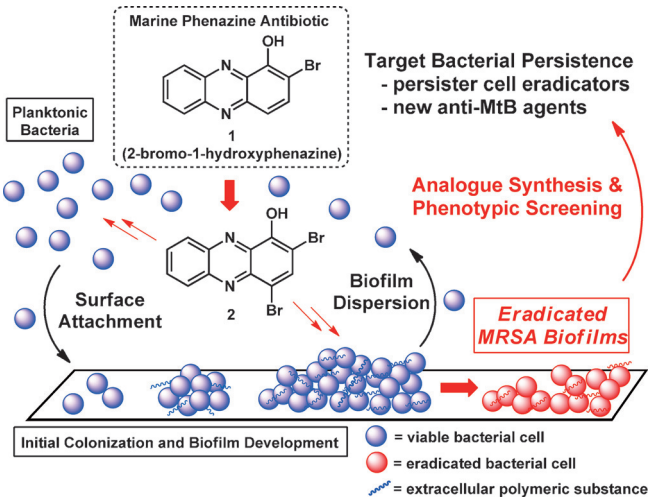

Figure 1. Marine phenazine antibiotic **1** and MRSA biofilm-eradicating agent, halogenated phenazine **2**, as unique starting points to target persistent bacteria.

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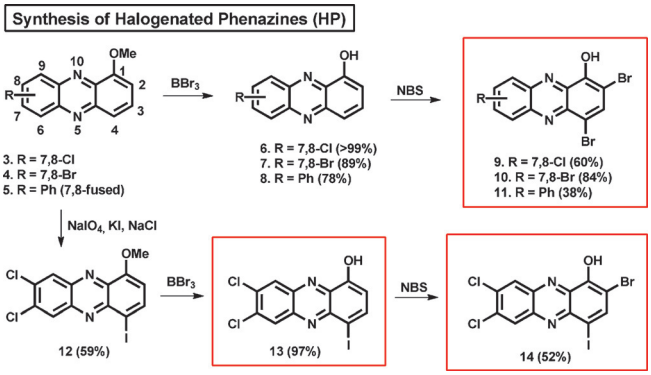
We were curious to probe the mechanism of **2**, since 2-bromo-1-hydroxyphenazine **1** belongs to the family of redox-active phenazine antibiotics produced by *Pseudomonas* and *Streptomyces* bacteria.[18] Furthermore, our goals were to synthesize more efficient biofilm-eradicating agents, target persister cells in non-biofilm cultures, and evaluate HP analogues against the slow-growing pathogen *Mycobacterium tuberculosis* (MtB). Compounds that can effectively eradicate biofilms, persister cells, and MtB are promising agents to address problems associated with bacterial persistence.

We synthesized a focused library of five HP analogues that contained various substitutions in the 7- and 8-position of the phenazine related to previous work by Cushman and co-workers (Scheme 1).[19] Demethylation of 1-methoxyphenazines **3–5** proceeded smoothly using boron tribromide (BBr_3) to afford 1-hydroxyphenazines **6–8** (78-99%), which were



Scheme 1. Chemical synthesis of halogenated phenazine compounds to target bacterial persistence.

then brominated using *N*-bromosuccinimide (NBS) to yield **9–11** (38-84% yield). 1-Methoxyphenazine **3** was selectively iodinated at the 4-position using sodium periodate ($NaIO_4$)/potassium iodide (KI)/sodium chloride (NaCl) to afford **12** (59% yield), followed by demethylation using BBr_3 to give **13** (97% yield). A final bromination reaction at the 2-position of phenazine **13** afforded mixed HP **14** (52% yield).

HP analogues, frontline MRSA treatments (vancomycin, daptomycin, linezolid), and control compounds were evaluated for bacterial biofilm eradication activity against MRSA-2 using the Calgary Biofilm Device (CBD),[20] which allows biofilms to be established on pegs that are submerged in inoculated media in 96-well plates. Pegs with established biofilms are then transferred to a second 96-well plate containing serial dilutions of test compounds for biofilm eradication. Following compound treatment, pegs are transferred to fresh media to allow viable biofilms to recover (grow and disperse), resulting in turbid wells (Figure 2A). During these investigations, we found CBD assays to be superior to biofilm eradication assays that regrow biofilms on the inside of microtiter wells.[17] The CBD allows for the determination of the minimum biofilm eradication concentration (MBEC) and planktonic-killing (minimum bactericidal concentration, MBC) dynamics from a single experiment. Typically, MBEC

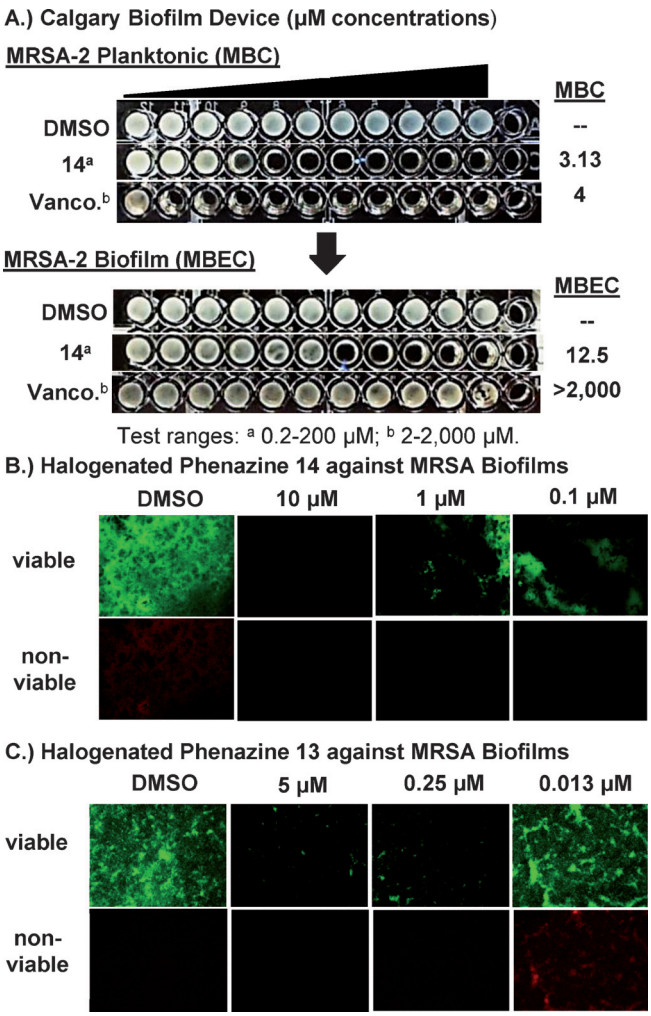


Figure 2. A) Calgary Biofilm Device assay of HP **14** and vancomycin against MRSA-2. B) LIVE/DEAD staining of MRSA-2 biofilms following treatment with **14**. C) LIVE/DEAD staining of MRSA-2 biofilms following treatment with **13**.

and MIC values are compared using different assays[10,20] (bacterial density, media, incubation times), which can have a significant impact on these values.

In CBD assays against MRSA-2 (clinical isolate, Shands Hospital; Gainesville, FL), HP **2** exhibited MBC values of 31.3 μM and MBEC values of 93.8 μM (Table 1). Several new HP analogues showed improved biofilm eradication activities against MRSA-2 biofilms with HP **14** being the most potent analogue reporting an MBEC of 12.5 μM (MBC = 3.13 μM; Figure 2A). Despite the improved biofilm eradication potencies for HP **9**, **10**, **13**, and **14**, phenazine **11** was inactive as a biofilm eradicator at the highest concentration tested (MBEC > 200 μM). Active HP biofilm eradicators demonstrated near equipotent killing of MRSA-2 biofilm and planktonic cells, which is a desirable characteristic of a biofilm-eradicating agent, with MBEC:MBC ratios between 1.0 and 4.0.

To support our CBD assay findings, we performed LIVE/DEAD assays with potent biofilm eradicators **13** and **14** (Figure 2B,C). Interestingly, LIVE/DEAD staining of MRSA-2 biofilms treated with **14** shows a significant

Table 1: Summary of biological investigations of HP analogues, conventional antibiotics and other control agents used in this study.[a]

Compound	MRSA-2 MBC/MBEC	MRSA-2 stationary killing	MRSE MBC/MBEC	VRE MBC/MBEC	% Hemolysis at 200 μM	Mtb MIC	HeLa Cytotox. IC_{50}
2	31.3[c]/93.8[b]	–	23.5[b]/250[c]	23.5[b]/9.38[b]	<1	25	>100
9	18.8[b]/50	–	1.56/4.69[b]	0.39/0.39	<1	6.25	>100
10	25/25	–	1.56/6.25	0.39/0.39	<1	>50	>100
11	200/>200	–	–	–	<1	–	–
13	25/37.5[b]	–	9.38[b]/75	–	1.4	3.13	>100
14	3.13/12.5	>3 log (12.5 μM)	1.56/1.56	0.20[d]/0.20[d]	2.7	12.5	>50
Vancomycin	3.0[b]/>2000	none (100 μM)	3.0[b]/>2000	>200/150[c]	<1	–	–
Daptomycin	62.5[c]/>2000	none (100 μM)	–	–	1.7	–	–
Linezolid	15.6/>2000	~1 log (100 μM)	–	–	<1	–	–
QAC-10	31.3[c]/125	~2 log (100 μM)	–	3.0[b]/3.0[b]	>99	–	–
CCCP	31.3/1000	–	–	–	3.5	–	–

[a] All concentrations are reported as μM; [b] midpoint value for a 2-fold range in independent experiments; [c] midpoint value for a 4-fold range in independent experiments; [d] lowest concentration tested; All MBC/MBEC values were obtained from 2 to 6 independent experiments. Percent hemolysis (red blood cell lysis) and HeLa cytotoxicity (24 hour; LDH assay) were determined using a spectrophotometer (96-well plates).

amount of biofilm clearance at 0.1 μM against MRSA-2 (Figure 2B). Similar MRSA-2 biofilm clearance was observed at 0.25 μM, and biofilm killing was also observed at 0.013 μM with **13** (red signal; Figure 2C). Thus, HP agents are able to effectively clear and kill biofilms.

HPs demonstrated enhanced biofilm eradication activities against methicillin-resistant *Staphylococcus epidermidis* (MRSE; ATCC 35984) and vancomycin-resistant *Enterococcus faecium* (ATCC 700221) compared to MRSA-2 (Table 1). HPs **9**, **10**, **13**, and **14** showed improved eradication activities against MRSE/VRE biofilms compared to **2**. HP **14** demonstrated the most potent eradication activities against MRSE (MBEC = 1.56 μM; 160-fold more potent than HP **2**) and VRE (MBEC = 0.20 μM; 47-fold more potent than HP **2**) and displays the most potent biofilm-eradicating activities reported to date.

The frontline MRSA treatments vancomycin, daptomycin, and linezolid, were evaluated alongside our HP analogues in CBD assays and, despite these antibiotics demonstrating moderate to excellent potency against MRSA-2 planktonic cells, all of the conventional antibiotics were unable to eradicate biofilms, even at the highest concentration tested (MBEC > 2000 μM). As with MRSA-2, vancomycin exhibited potent bactericidal activity (MBC = 3.0 μM; Table 1) against MRSE planktonic cells in CBD assays, yet was unable to eradicate biofilms against both pathogens (MBEC > 2000 μM; Table 1). The corresponding MBEC:MBC ratios of this small panel of anti-MRSA antibiotics against MRSA-2 were between >32 and >667, reflecting the high level of antibiotic tolerance in these biofilms.

Our HP analogues are derived from a larger class of redox-active phenazine antibiotics, which are believed to demonstrate antimicrobial activities through the generation of superoxide radicals.[18] When HP analogues were co-treated with tiron, a superoxide radical quenching agent,[21] the antibacterial activities of HP analogues were not reduced against MRSA-2, MRSE, or VRE (Supporting Information). 8-Hydroxyquinoline was used as a positive control in tiron-quenching experiments, and showed a complete loss of antibacterial activity against MRSA-2.

Two known biofilm eradicators were evaluated as positive controls, including the membrane disruptor QAC-10[10] and the proton ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP; Table 1).[22] Both controls demonstrated biofilm eradication against MRSA-2 with QAC-10 being significantly more potent than CCCP. HP analogues demonstrated enhanced biofilm eradication potencies up to 10-fold against MRSA-2, and up to 15-fold against VRE biofilms compared to QAC-10 (Table 1).

All of the compounds were screened for hemolytic activity against red blood cells at 200 μM (single concentration). QAC-10 reported >99% hemolysis at 200 μM, a feature associated with membrane-lysing agents. HP analogues did not demonstrate hemolytic activity at 200 μM (<3% hemolysis, Table 1). Owing to the drastic differences in hemolytic activities between HP analogues and QAC-10, we conclude that HP analogues do not eradicate biofilms through disruption of bacterial membranes.

HP analogues and antibiotics were also evaluated against persistent bacteria (that is, MRSA-2 persister cells and MtB) in non-biofilm cultures. Stationary cultures of *S. aureus* are known to consist of high populations of metabolically dormant persister cells.[23,24] When stationary cultures of MRSA-2 were treated with HP **14** and the frontline MRSA antibiotics, only **14** demonstrated a dramatic killing effect (>99.9%/>3-log reduction of viable cells) that continues to increase over the 24 hour experiment against MRSA-2 persisters at 12.5 μM (Figure 3; Table 1). Vancomycin and daptomycin were unable to kill MRSA-2 persister cells at 100 μM (>100-fold the MIC value for vancomycin), while linezolid showed initial killing of MRSA-2 persisters at 100 μM (2-log reduction after 3 h) despite MRSA-2 recovering to an overall 1 log reduction of viable stationary cells after 24 h. We also evaluated QAC-10 as a positive control at 100 μM and observed rapid and sustained killing of 2–3 logs against stationary MRSA-2 cells (Figure 3; Table 1).

Since the HP analogues proved to be effective at eradicating non- or slow-growing bacterial biofilms and stationary cultures, we evaluated HP analogues against the slow-growing human pathogen *M. tuberculosis* (MtB). Tuber-

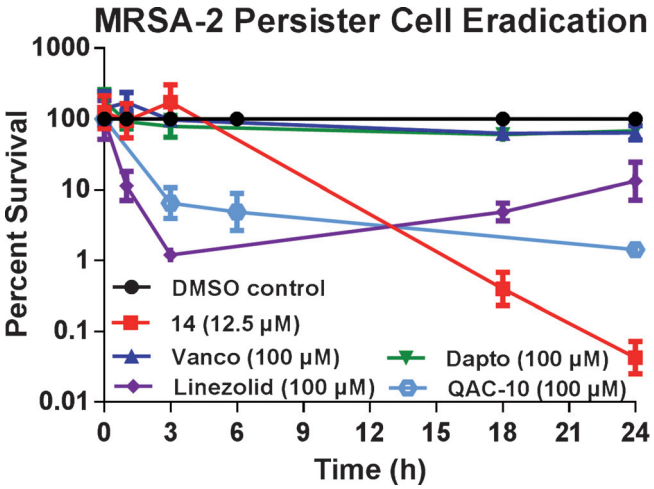


Figure 3. Eradication of MRSA-2 persister cells in a non-biofilm (stationary) culture. HP **14** demonstrated effective persister cell killing compared to lead MRSA antibiotics and QAC-10.

culosis continues to be the leading cause of death by bacterial infection worldwide,[25] largely owing to its persistent nature. Moreover, phenazine small molecules have been reported with potent antibacterial activities against MtB.[26] A small panel of HP analogues were tested against the *M. tuberculosis* H37Ra strain. Parent HP **2** displayed a moderate MIC value of 25 μM against *M. tuberculosis*, while **13** demonstrated the most potent anti-tuberculosis activity in our HP panel with an MIC of 3.13 μM. Streptomycin was used as a positive control in these assays with an MIC of 1.32 μM against *M. tuberculosis* H37Ra.

Biofilm-eradicating HP analogues were evaluated for mammalian cytotoxicity in 24 hour lactate dehydrogenase (LDH) release assays against HeLa cells at 25, 50, and 100 μM. Four of the five biofilm-eradicating agents (HPs **2**, **9**, **10**, **13**) had IC_{50} values >100 μM, while HP **14** had an IC_{50} value >50 μM (Table 1). The HeLa cytotoxicity for HPs, taken together with the lack of toxicity against human red blood cells at 200 μM, indicates that the mechanism for HP analogues is indeed selective for bacterial cells over mammalian cells. This general lack of toxicity corroborates our in vivo studies (Supporting Information), which supports previous work showing that HP **2** is safely administered to mice at 200 mg kg^{-1} per day for four days.[27]

In conclusion, we have discovered a series of highly potent marine phenazine antibiotic-inspired small molecules that target persistent bacteria, including: bacterial biofilms, persister cells in stationary culture, and MtB. HP **14** demonstrated the most potent biofilm-eradicating activities to date (MRSA MBEC = 12.5 μM; MRSE MBEC = 1.56 μM; VRE MBEC = 0.20 μM), while effectively killing MRSA persister cells. Multiple HPs demonstrated antibacterial activity against the slow-growing pathogen *M. tuberculosis*, with **13** being the most potent (MIC = 3.13 μM). HP analogues displayed excellent initial toxicity profiles against RBCs, HeLa cells, and mice. Marine antibiotics are a promising source of compounds to combat persistent bacterial phenotypes. HP analogues could lead to promising new treatment options to effectively treat persistent and recurring bacterial infections.

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