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Identification of *N*-Arylated NH125 Analogues as Rapid Eradicating Agents against MRSA Persister Cells and Potent Biofilm Killers of Gram-Positive Pathogens

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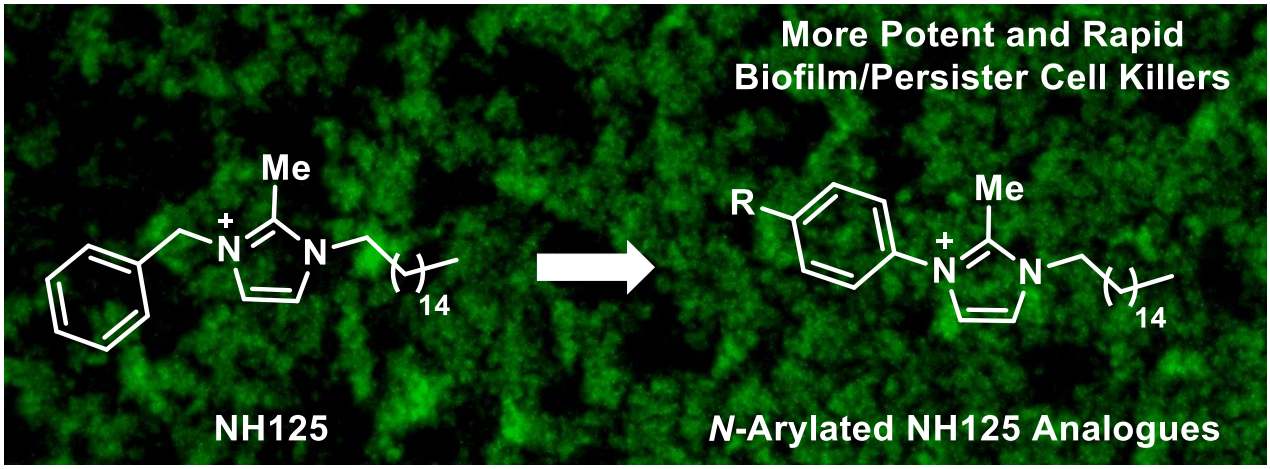
Abstract

Bacterial biofilms housing dormant persister cells are innately tolerant to antibiotics and disinfectants, yet several membrane-active agents are known to eradicate tolerant bacterial cells. NH125, a membrane-active persister-killer and starting point for development, led to the identification of *N*-arylated analogues **1** and **2** and displayed improved biofilm-killing potencies and rapid persister-killing activities in stationary cultures of methicillin-resistant *Staphylococcus aureus* (MRSA). We found **1** and **2** to be superior to other membrane-active agents in biofilm eradication assays with **1** demonstrating minimum biofilm eradication concentrations (MBEC) of 23.5, 11.7 and 2.35 μM against MRSA, methicillin-resistant *S. epidermidis* (MRSE) and vancomycin-resistant *Enterococcus faecium* (VRE) biofilms, respectively. We tested our panel of membrane-active agents against MRSA stationary cultures and found **1** to rapidly eradicate MRSA stationary cells by 4-logs (99.99%) in 30 minutes. The potent biofilm eradication and rapid persister cell killing exhibited by *N*-arylated NH125 analogues could have significant impact addressing biofilm-associated problems.



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Introduction

Bacterial biofilms are ubiquitous, surface-attached communities of slow- or non-replicating bacteria (e.g., persister cells) housed within a protective extracellular matrix of biomolecules and display high levels of tolerance to biocides, antibiotics and other chemical insults (i.e., host immune response during infection).[1-5] Although biofilm-associated infections have received much attention over the past two decades, it should be no surprise that biofilms affect multiple sectors in our society (i.e., medical[1-7], industrial[8,9] and agricultural[10,11]). Numerous applications exist for small molecule biofilm-control strategies, a few of which include: disinfectants, therapeutic agents, crop protection agents.[12,13]

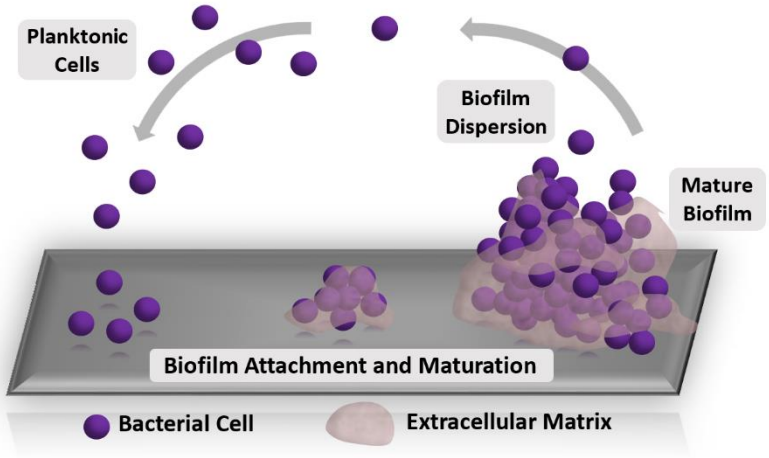

Figure 1: The process of biofilm formation, maturation and dispersion.

Individual, free-swimming planktonic bacteria use organic signaling molecules to communicate with each other in a process called quorum sensing to monitor their population density and coordinate group behavior, including the simultaneous surface attachment, colonization and development into a mature biofilm.[14] Biofilms disperse some bacterial cells back into their surrounding environment in a process called biofilm dispersion, which enables bacteria to establish biofilms in new locations (Figure 1).[2] Over the last several years, small molecules have been identified that inhibit biofilm formation and disperse established biofilms through various

mechanisms, including the modulation of quorum sensing.[12,13] Alternatively, a much smaller collection of compounds have been identified that eradicate biofilm cells, including non-dividing persister cells.[5,7,15,16]

Antimicrobial peptides (AMPs) are produced by an extensive variety of organisms to defend themselves against bacterial infection and operate through various membrane-destroying mechanisms.[17] A diverse array of AMP-inspired, membrane-active amphiphilic compounds have been reported to demonstrate potent antibacterial and anti-biofilm activities while evading the development of bacterial resistance (i.e., quaternary ammonium cations, QAC).[16-23] Membrane-targeting compounds often are challenging to develop for human therapeutic use due to membrane selectivity issues[19]; however, such agents have diverse biomedical applications and can be highly effective as disinfectants. For instance, a lack of sterilized hospital environments, including surfaces and instruments, is a major problem that leads to life-threatening hospital-acquired infections (HAI).[24,25]

Recently, NH125 (Figure 2A) was reported to eradicate methicillin-resistant *Staphylococcus aureus* (MRSA) biofilms and persister cells in stationary cultures through the depolarization of the outer membrane,[26] then later lipid bilayer destruction.[27] We found this to be interesting as NH125 also has reported anticancer[28,29] and neuromodulatory[30,31] activities *in vitro* and *in vivo*. The charged nitrogen atom of the imidazole heterocycle along with the long aliphatic tail led us to believe that NH125 elicits its antibacterial activities through the destruction and lysis of bacterial membranes, similar to other quaternary ammonium cations.[16,18,32] In addition, we felt new synthetic analogues of NH125 could be rapidly synthesized and lead to the identification of more effective persister- and biofilm-killing agents against drug-resistant and tolerant pathogenic bacteria.

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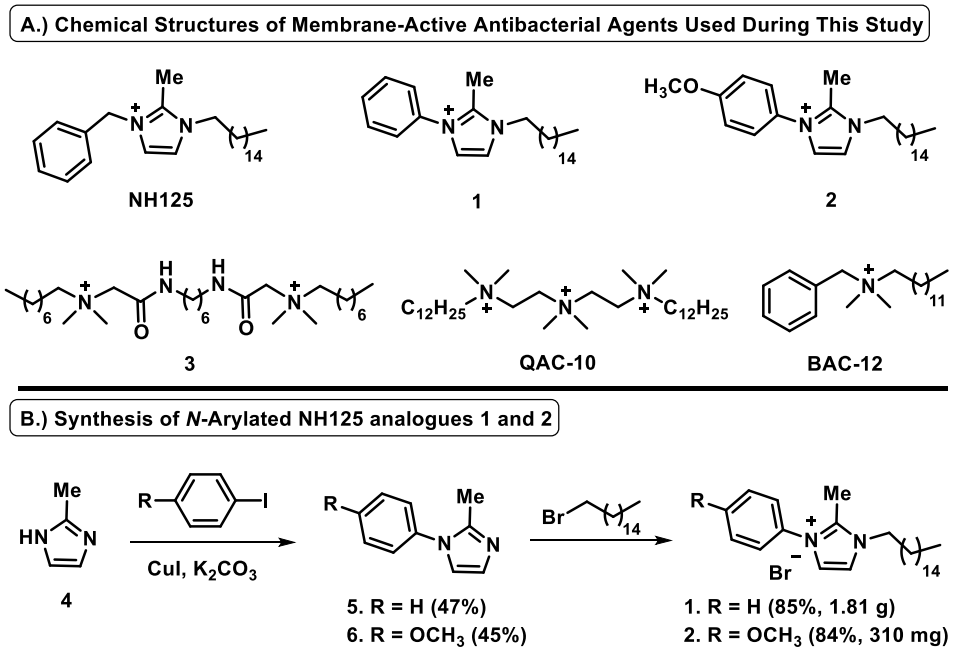

Figure 2. A) Structures of membrane-targeting agents investigated. B) Synthesis of *N*-Arylated NH125 analogues **1** and **2**.

Results and Discussion

Here, we report the identification of *N*-arylated NH125 analogues **1** and **2** that outperform NH125 and other membrane-active agents (Figure 2A) in biofilm eradication assays against MRSA, methicillin-resistant *Staphylococcus epidermidis* (MRSE) and vancomycin-resistant *Enterococcus faecium* (VRE). In kill kinetic experiments against MRSA persister cells in stationary cultures, which have enriched persister cell populations, **1** and **2** proved to be the most potent and rapid killing agents in our panel of membrane-targeting antibacterial agents. We identified **1** and **2** from a library of diverse NH125 analogues we prepared and evaluated against a panel of pathogenic bacteria (a full paper detailing these findings will be reported in due course). The chemical synthesis of **1** and **2** involves a copper-catalyzed coupling reaction between iodobenzene, or 4-iodoanisole, and 2-methylimidazole **4** to yield *N*-arylated imidazoles **5** and **6**, in 47% and 45% yields, respectively (Figure 2B). Following the copper-catalyzed C-N coupling reactions, a final

alkylation of the *N*-3-position with 1-bromohexadecane gives **1** in 85% yield (from **5**) and **2** in 84% yield (from **6**). This 2-step route was carried out to generate 1.81 grams of **1** and 310 milligrams of **2** on single runs to check the scalability of this route, which yields pure analogues without the use of chromatography.

We were interested in evaluating a panel of chemically diverse membrane-targeting compounds for antibacterial, biofilm eradication, MRSA persister killing and red blood cell lysis (hemolysis) including: NH125, **1**, **2**, **3**[33], QAC-10[16], BAC-12, and daptomycin. This small but diverse membrane-active panel was compiled for the following reasons: 1) BAC-12 is a commercial disinfectant with antibacterial properties, 2) QAC-10 and **3** are membrane-active quaternary ammonium cations (QAC)[16,33], and 3) daptomycin is a clinically used lipopeptide antibiotic known to depolarize bacterial membranes and eradicate *Staphylococcal* biofilms.[34]

We initially tested this panel of membrane-active agents against methicillin-resistant *Staphylococcus aureus* (MRSA) isolates (MRSA-2, clinical isolate from Shands Hospital, Gainesville, Florida; MRSA BAA-1707, a multidrug resistant strain purchased from ATCC), methicillin-resistant *Staphylococcus epidermidis* (MRSE), vancomycin-resistant *Enterococcus faecium* (VRE), multidrug-resistant *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Escherichia coli* (UAEC-1, clinical isolate) for antibacterial activity in minimum inhibitory concentration (MIC) assays (Table 1). We found **1** and **2** to have the most potent antibacterial activities against two MRSA strains (MIC = 1.56-3.13 μM) in this panel edging out NH125 (MIC = 2.35-4.69 μM), QAC-10 (MIC = 3.13-4.69 μM), daptomycin (MIC = 3.13-4.69 μM) and BAC-12 (MIC = 6.25 μM). We found compound **3** to have significantly reduced antibacterial activities against these MRSA strains (MIC = 12.5-25 μM) in these assays. Similar activity trends were observed against MRSE and VRE; however, daptomycin reported a significantly higher MIC against VRE (MIC = 125 μM). Analogues **1** and **2** reported the most potent antibacterial activities against *K. pneumoniae* and *E. coli* (MIC = 6.25 μM) among the panel

with moderate activities against multidrug-resistant *A. baumannii* (MIC = 12.5 μM for **1**; 18.8 μM for **2**). Only QAC-10 reported more potent antibacterial activities against *A. baumannii* in this panel (MIC = 6.25 μM) and was found to be the only membrane-active compound to demonstrate antibacterial activity against *P. aeruginosa* (MIC = 9.38 μM; all other compounds on this panel reported MIC values >100 μM). As expected, daptomycin was inactive (MIC >100 μM) against all gram-negative pathogens. Following antibacterial assays, we tested this panel in hemolysis assays to determine hemolytic activities (HC_{50} values; the concentration at which 50% of red blood cells are lysed) and found that **1**, **2**, QAC-10 and NH125 demonstrate potent hemolytic activities, correlating with their antibacterial activities. Analogue **3**, BAC-12 and daptomycin reported HC_{50} values >100 μM (highest concentration tested).

Table 1. Summary of antibacterial activity and hemolysis results. All values are reported in μM.

Compound	MRSA-2	MRSA BAA-1707	MRSE 35984	VRE 700221	*A. baumannii* 1794	PAO1	*K. pneumoniae* 13883	UAEC-1	RBC HC_{50}
NH125	4.69[a]	2.35[a]	4.69[a]	3.13	37.5[a]	>100	18.8[a]	18.8[a]	12.3
1	3.13	1.56	1.17[a]	3.13	12.5	>100	6.25	6.25	7.84
2	3.13	3.13	2.35[a]	1.56	18.8[a]	>100	6.25	6.25	13.4
3	25	12.5	9.38[a]	50	>100	>100	100	25	>100
QAC-10	3.13	4.69[a]	2.35[a]	2.35[a]	6.25	9.38[a]	25	12.5	7.92
BAC-12	6.25	6.25	3.13	25	75[a]	>100	75[a]	50	>100
Daptomycin	4.69[a]	3.13	3.13	125	>100	>100	>100	>100	>100

Notes: [a] Midpoint value for a 2-fold range in independent experiments. All MIC and HC_{50} values were obtained from a minimum of three independent experiments. MRSA BAA-1707, MRSE 35984, VRE 700221, *A. Baumannii* 1794, *K. pneumoniae* 13883 were purchased from ATCC. MRSA-2 and UAEC-1 are clinical isolates.

We then turned our attention to biofilm eradication assays using the Calgary Biofilm Device[7,15,35] and tested our panel of membrane-active compounds against MRSA BAA-1707, MRSE ATCC 35984 and VRE ATCC 700221 (Table 2). Using a Calgary Biofilm Device, bacterial biofilms are established on pegs suspended to the bottom of a 96-well plate lid. Following biofilm establishment on lid pegs, lids are then transferred to new 96-well plates containing serial dilutions of test compound and allowed to incubate. After compound treatment, 96-well plate lids with pegs are once again transferred to 96-well plates containing fresh media. During this final phase of the

assay, viable biofilms will grow and disperse planktonic cells into the media where they will grow and give a turbid well result. Conversely, wells that have no growth are a result of pegs that had eradicated bacterial biofilms. The lowest concentration at which no turbidity is observed is the minimum biofilm eradication concentration (MBEC). Using the Calgary Biofilm Device, both minimum biofilm eradication concentrations and minimum bactericidal concentrations (MBC) for planktonic bacterial can be determined using one assay from a single bacterial culture (Figure 3A). Effective biofilm-eradicating agents typically have MBEC:MBC ratios of 1-3 for biofilm:planktonic killing.[7,15]

Table 2. Summary of biofilm eradication from CBD assays. All values are reported in μM.

Compound	MRSA BAA-1707 MIC	MRSA BAA-1707 MBC / MBEC	MRSE 35984 MIC	MRSE 35984 MBC / MBEC	VRE 700221 MIC	VRE 700221 MBC / MBEC
NH125	2.35[a]	23.5[a] / 46.9[a]	4.69[a]	15.6 / 5.9[a]	3.13	5.9[a] / 11.7[a]
1	1.56	11.7[a] / 23.5[a]	1.17[a]	5.9[a] / 11.7[a]	3.13	4.69[a] / 2.35[a]
2	3.13	7.8[b] / 11.7[a]	2.35[a]	5.9[a] / 5.9[a]	1.56	3.0[a] / 5.9[a]
3	12.5	62.5 / >1000	9.38[a]	5.9[a] / 7.8	50	46.9[a] / 46.9[a]
QAC-10	4.69[a]	93.8[a] / 93.8[a]	2.35[a]	31.3 / 31.3	2.35[a]	3.0[a] / 3.0[a]
BAC-12	6.25	15.6 / >1000	3.13	11.7[a] / >2000	25	31.3 / 23.5[a]
Daptomycin	3.13	125 / >2000	3.13	31.3 / >2000	125	375[a] / 93.8[a]

Notes: [a] Midpoint value for a 2-fold range in independent experiments. All MIC, MBC, MBEC values were obtained from a minimum of three independent experiments.

We found analogues **1** and **2** to be the most potent analogues against MRSA BAA-1707 biofilms and reported minimum biofilm eradication concentration (MBEC) values of 23.5 μM and 11.7 μM, respectively (Table 2). The biofilm-killing activities of **1** and **2** were 2- to 4-fold more potent than NH125 (MBEC = 46.9 μM) and 4- to 8-fold more potent than QAC-10 (MBEC = 93.8 μM) against MRSA BAA-1707 (Figure 3A). Interestingly, **3**, BAC-12 and daptomycin all reported MBECs >1,000 μM, despite reporting MBC values of 15.6-125 μM against planktonic cells in the same assays. When viable biofilm cell counts were assessed using the Calgary Biofilm Device, the entire panel reduced MRSA BAA-1707 ~2-logs at sub-MBEC values (Figure 3B). NH-125 and **1** showed a clear dose-response in these assays while **1** reduced viable MRSA BAA-1707 biofilm cells by >4-logs at 15.6 μM (MBEC = 23.5 μM), which was a more dramatic biofilm killing effect

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than NH-125 at 62.5 (MBEC = 46.9 μM; 3 to 4-log reduction of viable biofilm cells). Live/Dead staining of MRSA BAA-1707 biofilms treated with NH-125 and **2** show that these analogues have an effective clearance of established biofilms at 31.3 μM (Figure 4).

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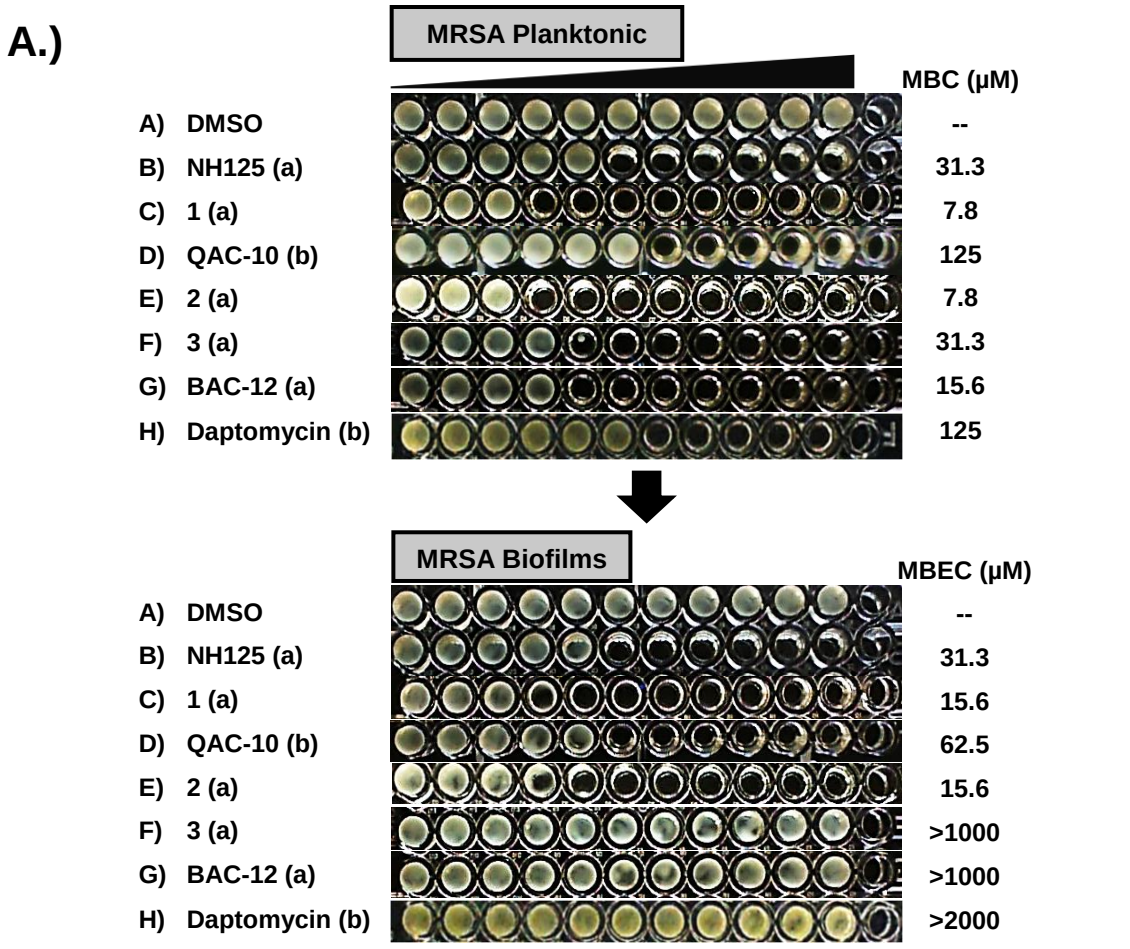

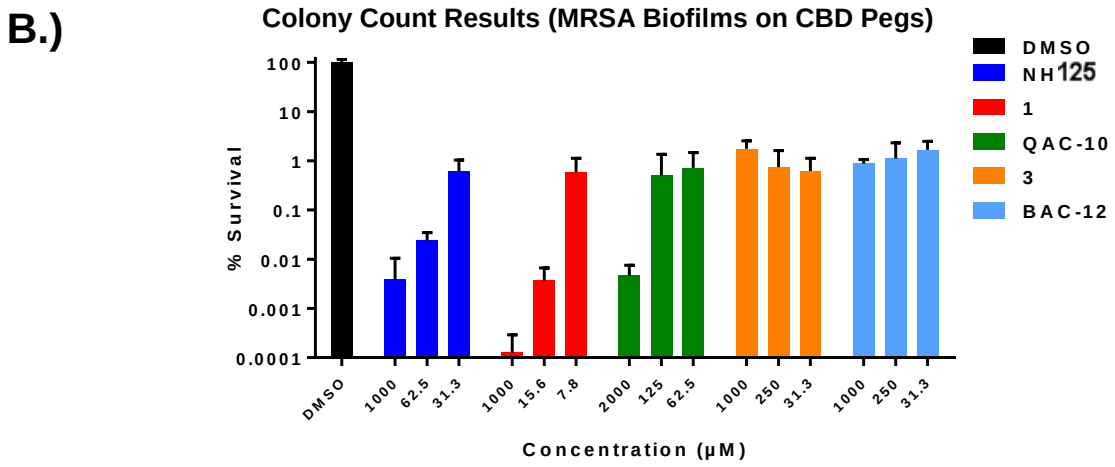

Figure 3: A.) Image of Calgary Biofilm Device (CBD) assay against MRSA BAA-1707. This assay can be used to determine the planktonic and biofilm killing effects of antibacterial agents from a single bacterial culture. B) Viable MRSA BAA-1707 biofilm cells from colony counts of CBD pegs.

10.1002/cbic.201600622

A similar trend in biofilm eradication was observed against MRSE ATCC 35984 although *N*-arylated NH125 analogues **1** and **2** did not demonstrate improvements in potencies against MRSE biofilms compared to NH125 (MBECs 5.9-11.7 μM). Interestingly, **3** and QAC-10 demonstrated potent biofilm eradication activities against MRSE biofilms with MBEC values of 7.8 μM and 31.3 μM, respectively. Against VRE biofilms, **1** and QAC-10 proved to be the most potent reporting MBEC values of 2.35 μM (>4-fold more potent than NH-125) and 3.0 μM, respectively.

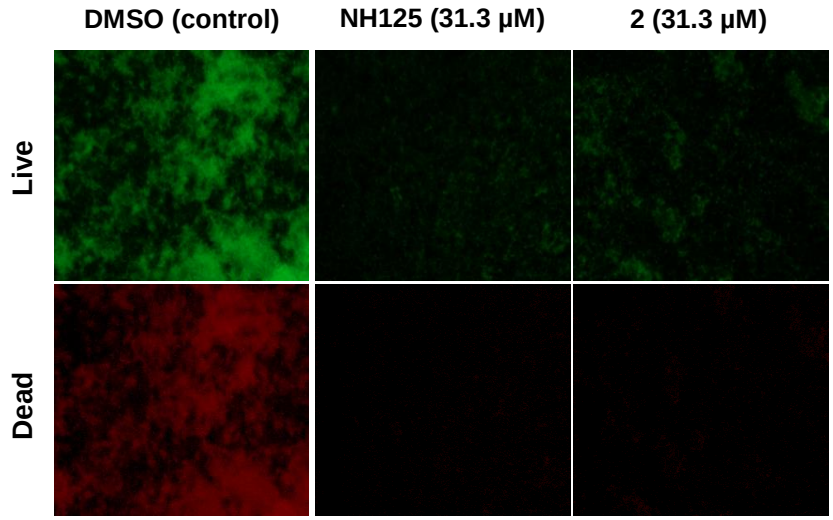

Figure 4. Live/Dead stain of MRSA BAA-1707 biofilms 24 hours after treatment with vehicle (DMSO), NH125 and **2**. Fluorescence images show potent clearance of MRSA biofilms for NH125 and **2**.

We then evaluated the panel of membrane-active compounds head-to-head at 50 μM against MRSA BAA-1707 stationary cultures in kinetic killing experiments. Stationary cultures of *S. aureus* have elevated populations of metabolically dormant persister cells[36,37] and using this experiment, we could get multiple early time points to see if **1** and **2** were rapid killers of MRSA persister cells. The entire panel reduced the number of viable stationary cultures after 24 hours; however, **1** and **2** were the only compounds to elicit a rapid killing effect of 4-logs (99.99% killing of stationary cells) in 30 minutes and >5-logs after 90 minutes. The remaining compounds on the

panel, showed very little activity at 30 minutes and only NH125 showed ~1-log reduction of stationary MRSA cells after 90 minutes. The rapid killing of stationary MRSA cultures with **1** and **2** was sustained for 6 hours and no viable MRSA cells could be found at 24 hours upon treatment with **1** whereas there was a slight recovery of MRSA cultures after 24 hours with **2**.

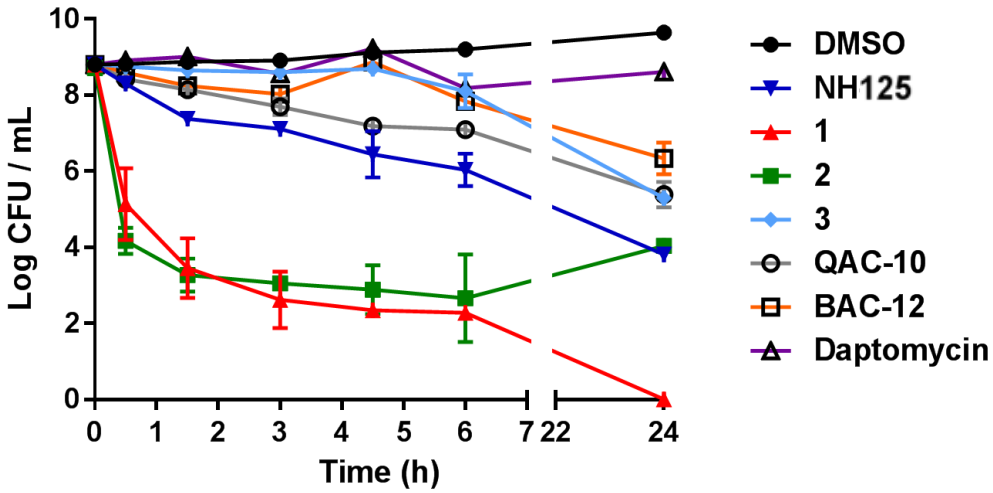

Figure 5. Experimental results for the killing kinetics of MRSA BAA-1707 stationary cultures, which are highly populated with persister cells. All compounds were tested at 50 μM from three independent experiments.

In conclusion, we have identified two new *N*-arylated NH125 analogues **1** and **2**, designed from NH125, that demonstrate broad-spectrum antibacterial activities against multiple pathogenic bacteria, including drug-resistant strains. We found **1** and **2** to demonstrate potent hemolysis activity, similar to quaternary ammonium cationic agents (i.e., QAC-10), and eradicate MRSA, MRSE and VRE biofilms with the most potent activity in a panel of diverse membrane-active agents. Interestingly, **1** and **2** were found to be rapid killers of MRSA persister cells in stationary cultures. *N*-Arylated NH125 analogues are promising agents for the development of new disinfectants and antiseptics to effectively eradicate bacteria, biofilms and persister cells.

Materials and Methods

General. Reported biological data result from a minimum of three independent experiments. Bacterial strains used during these investigations include: methicillin-resistant *Staphylococcus aureus* (Clinical Isolate from Shands Hospital in Gainesville, FL: MRSA-2; ATCC strain: BAA-1707) methicillin-resistant *Staphylococcus epidermidis* (MRSE strain ATCC 35984), vancomycin-resistant *Enterococcus faecium* (VRE strain ATCC 700221), *Acinetobacter baumannii* (ATCC 1794), *Pseudomonas aeruginosa* (PAO1), *Klebsiella pneumonia* (ATCC 13883) and *Escherichia coli* clinical isolate (UAEC-1). All compounds were stored as DMSO stocks at room temperature in the absence of light for several months at a time without observing any loss in biological activity.

Minimum Inhibitory Concentration (MIC) Microdilution Assays. The minimum inhibitory concentration (MIC) for each tested compound was determined by the broth microdilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI). In a 96-well plate, eleven two-fold serial dilutions of each compound were made in a final volume of 100 µL Luria Broth. Each well was inoculated with ~10^5 bacterial cells at the initial time of incubation, prepared from a fresh log phase culture (OD_{600} of 0.5 to 1.0 depending on bacterial strain). The MIC was defined as the lowest concentration of compound that prevented bacterial growth after incubating 16 to 18 hours at 37 °C (MIC values were supported by spectrophotometric readings at OD_{600}). DMSO served as our vehicle and negative control in each microdilution MIC assay. DMSO was serially diluted with a top concentration of 1% v/v.

Biofilm Eradication Assays (MBEC Determination; Calgary Biofilm Device Assays). Biofilm eradication experiments were performed using the Calgary Biofilm Device to determine MBC/MBEC values for various compounds of interest (Innovotech, product code: 19111). The Calgary device (96-well plate with lid containing pegs to establish biofilms on) was inoculated with 125 µL of a mid-log phase culture diluted 1,000-fold in tryptic soy broth with 0.5% glucose (TSBG) to establish bacterial biofilms after incubation at 37 °C for 24 hours. The lid of the Calgary device was then removed, washed and transferred to another 96-well plate containing 2-fold serial

dilutions of the test compounds (the “challenge plate”). The total volume of media with compound in each well in the challenge plate is 150 µL. The Calgary device was then incubated at 37 °C for 24 hours. The lid was then removed from the challenge plate and MBC/MBEC values were determined using different final assays. To determine **MBC values**, 20 µL of the challenge plate was transferred into a fresh 96-well plate containing 180 µL TSBG and incubated overnight at 37 °C. The MBC values were determined as the concentration giving a lack of visible bacterial growth (i.e., turbidity). For determination of **MBEC values**, the Calgary device lid (with attached pegs/treated biofilms) was transferred to a new 96-well plate containing 150 µL of fresh TSBG media in each well and incubated for 24 hours at 37 °C to allow viable biofilms to grow and disperse resulting in turbidity after the incubation period. MBEC values were determined as the lowest test concentration that resulted in eradicated biofilm (i.e., wells that had no turbidity after final incubation period). In select experiments, pegs from the Calgary device were removed from lead biofilm eradicators after final incubation, sonicated for 30 minutes in PBS and plated out to determine biofilm cell killing of lead biofilm-eradicating agents (i.e., colony forming unit per milliliter, CFU/mL).

MRSA Persister Cell Kill Kinetics. An overnight culture of MRSA BAA-1707 was diluted in fresh TSBG (1:13 to 1:20 fold) and allowed to grow with shaking. Once the culture reached stationary phase (4-6 hours), test compounds were added at a final test concentration of 50 µM. The cultures were incubated with shaking at 250 rpm and aliquots were removed and plated out at predetermined time points. Colony forming units (CFU) per milliliter data was recorded and plotted using Graphpad Prism 6.0.

Live/Dead Staining of MRSA Biofilms. A mid-log culture of MRSA BAA-1707 was diluted 1:1000-fold and 500 µL was transferred to each compartment of a four-compartment CELLview dish (Greiner Bio-One 627871). The dish was then incubated for 24 hours at 37 °C to establish MRSA-1707 biofilms. After this time, the cultures were removed and the plate was washed with

0.9% saline. The dish was then treated with test compounds in fresh media at various concentrations. DMSO was used as a negative control in this assay. The dish was incubated with the test compound for 24 hours at 37 °C. After this time, the cultures were removed and the dish was washed with 0.9% saline for 2 minutes. Saline was then removed and 500 µL of the stain (Live/Dead BacLight Viability Kit, Invitrogen) were added for 15 minutes and left in the dark. After this time, the stain was removed and the dish was washed twice with 0.9% saline. Then the dish was fixed with 500 µL 4% paraformaldehyde in PBS for 30 minutes. Images of remaining MRSA biofilms were then taken with a fluorescence microscope.

Hemolysis of Red Blood Cells. Freshly drawn human red blood cells (hRBC) with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant were washed with Tris-buffered saline (0.01M Tris-base, 0.155 M sodium chloride, pH 7.2) and centrifuged for 5 minutes at 3,500 rpm. The washing was repeated three times with the buffer. In a 96-well plate, test compounds were added to the buffer from DMSO stocks. Then 2% hRBCs (50 µL) in buffer were added to test compounds to give a final concentration of 200 µM. The plate was then incubated for 1 hour at 37 °C. After incubation, the plate was centrifuged for 5 minutes at 3,500 rpm. Then 80 µL of the supernatant was transferred to another 96-well plate and the optical density (OD) was read at 405 nm. DMSO served as our negative control (0% hemolysis) while Triton X served as our positive control (100% hemolysis). The percent of hemolysis was calculated as (OD_{405} of the compound- OD_{405} DMSO) / (OD_{405} Triton X- OD_{405} buffer). All reported data correspond to three independent experiments. Ten-point dose-response curves were generated in 96-well plates to determine 50% hemolysis (HC_{50} values) of red blood cells.

General C-N Coupling Procedure for the Synthesis of 5 and 6. To a stirring solution of 2-methyl-1H-imidazole **4** (800 mg, 9.74 mmol) in 6 mL anhydrous dimethyl sulfoxide under argon was added iodobenzene (1.09 mL, 9.74 mmol), then copper(I) iodide (185 mg, 0.97 mmol) and finally anhydrous potassium carbonate (2.69 g, 19.49 mmol). The reaction mixture was allowed

to stir at 130 °C for 48 hours in a sealed tube. After the completion of the reaction, the contents of the reaction mixture were transferred to a separatory funnel containing ethyl acetate (200 mL). The organic layer was washed with water (4x40 mL), then brine (2x30 mL) before the organic layers were collected, dried with anhydrous sodium sulfate, filtered, and concentrated *in vacuo* to give the crude product which was then purified via flash column chromatography using hexanes:ethyl acetate (1:1 to 1:4) to elute pure 2-methyl-1-phenyl-1H-imidazole **5** as a clear oil (730 mg, 47%).

General Alkylation Procedure for the Synthesis of 1 and 2. To a stirring solution of 2-methyl-1-phenyl-1H-imidazole **5** (700 mg, 4.46 mmol) in 5 mL anhydrous acetonitrile in a glass tube at room temperature was added 1-bromohexadecane (1.42 mL, 4.87 mmol) and the mixture was sealed and heated at 110 °C for 24 hours. The reaction was then allowed to cool to room temperature before being concentrated via rotovap. The crude product was stirred in anhydrous ether under argon for 5 hours and the resulting white precipitate was filtered and washed with anhydrous ether and dried under vacuum to obtain **1** as a pure white solid (1.81 g, 85%).

Associated Content

The Supporting Information is available free of charge on the ACS Publications website at DOI: (insert DOI here). Biological images of biofilm eradication assays, synthetic procedures for new compounds synthesized during these experiments are characterized by ^{1}H and ^{13}C NMR, HRMS and MP (solids) can be found in the supporting information document associated with this publication.

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Notes

The authors declare no competing financial interests.

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