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NH125 kills methicillin-resistant *Staphylococcus aureus* persisters by lipid bilayer disruption

Background: NH125, a known WalK inhibitor kills MRSA persisters. However, its precise mode of action is still unknown. **Methods & results:** The mode of action of NH125 was investigated by comparing its spectrum of antimicrobial activity and its effects on membrane permeability and giant unilamellar vesicles (GUVs) with walrycin B, a WalR inhibitor and benzyldimethylhexadecylammonium chloride (16-BAC), a cationic surfactant. NH125 killed persister cells of a variety of *Staphylococcus aureus* strains. Similar to 16-BAC, NH125 killed MRSA persisters by inducing rapid membrane permeabilization and caused the rupture of GUVs, whereas walrycin B did not kill MRSA persisters or induce membrane permeabilization and did not affect GUVs. **Conclusion:** NH125 kills MRSA persisters by interacting with and disrupting membranes in a detergent-like manner.

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Staphylococcus aureus is a Gram-positive human pathogen that colonizes about a third of healthy humans [1–3] and causes a wide range of infections from minor skin colonization to life-threatening infections such as toxic shock syndrome [3–6]. Antibiotic-resistant *S. aureus*, such as methicillin-resistant *S. aureus* (MRSA), is prevalent both in hospitals and in the community [4–6]. In the USA, MRSA causes approximately 19,000 deaths and accrues about \$3–4 billion of healthcare costs per year [7]. In addition to its ability to acquire antibiotic-resistance, *S. aureus* readily forms persisters, which are nongrowing dormant cells that exhibit a high level of tolerance to most conventional antibiotics [8–11]. Recent studies have shown that persisters are responsible for the recalcitrance of chronic infections to antibiotic therapy [12,13]. Vancomycin is currently used as the drug of last resort for *S. aureus*, but vancomycin-intermediate or -resistant strains are arising [14] and this agent is not effective

against MRSA persisters [11]. Therefore, new antibiotics against both antibiotic-resistant and -tolerant *S. aureus* are urgently needed.

Two component signal transduction systems (TCS) are major tools that enable bacteria to sense, respond and adapt to environmental changes [15,16]. Generally, TCS consist of a sensor histidine kinase (HK), which is auto-phosphorylated upon sensing environmental stimuli and a response regulator (RR), which is phosphorylated by its cognate sensor kinase and subsequently modulates the transcription of specific genes [15]. TCS are involved in many cellular processes in bacteria, such as sporulation, virulence, biofilm formation and antibiotic resistance [15]. Also, some TCS such as WalK/R (also known as YycF/G) and YhcS/R (also known as AirS/R) in *S. aureus* are essential for bacterial survival [17,18]. Essential TCS are considered to be promising targets for the development of antibiotics due to their indispensability for bacterial survival and their absence in mammalian cells [16]. In

Wooseong Kim¹, Nico Fricke², Annie L Conery^{3,4}, Beth Burgwyn Fuchs¹, Rajmohan Rajamuthiah^{1,3}, Elamparithi Jayamani^{1,3}, Petia M Vlahovska², Frederick M Ausubel^{3,4} & Eleftherios Mylonakis^{*,1}

¹Division of Infectious Diseases, Rhode Island Hospital, Alpert Medical School of Brown University, Providence, RI, USA

²School of Engineering, Brown University, Providence, RI, USA

³Department of Molecular Biology, Massachusetts General Hospital, Boston, MA, USA

⁴Department of Genetics, Harvard Medical School, Boston, MA, USA

*Author for correspondence:

eleftherios_mylonakis@brown.edu

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particular, the WalK/R system, which is highly conserved in low GC content Gram-positive pathogens such as *S. aureus*, *Streptococcus pneumonia* and *Enterococcus faecalis*, has been exploited as a target for developing new antibiotics [19–26]. Indeed, the WalK inhibitors NH125 [19,20], signermycin B [25], walkmycin B [22] and waldiomycin [26], as well as the WalR inhibitors walrycin A [23] and walrycin B [23], have been shown to have antimicrobial activity against *S. aureus* or MRSA.

As previously reported, we developed a fluorescence-based screening strategy for identifying antimicrobial agents against MRSA persisters and found that the WalK inhibitor NH125 killed MRSA persisters by inducing rapid membrane permeabilization [11]. In this paper, we elucidate the mode of action by which NH125 kills MRSA persisters by comparing the activity of NH125 with a functional analog, the WalR inhibitor walrycin B and a well-characterized structural analog, benzyldimethylhexadecylammonium chloride (16-BAC), a cationic surfactant. In order to determine whether the mechanism by which NH125 kills MRSA persisters is similar to walrycin B or 16-BAC, we tested the spectrum of antibiotic activity of these compounds and their effects on MRSA persister membrane permeability. We also studied the effects of these compounds on the stability of giant unilamellar vesicles (GUVs), a system for modeling the cell membrane [27,28]. Our results show NH125 kills MRSA persisters similarly to 16-BAC, by directly interacting with and disrupting membranes and not by inhibiting the activity of the WalK/R system.

Materials & methods

Bacterial strains, growth conditions & persister isolation

Methicillin-resistant *S. aureus* (MRSA) strain MW2 BAA-1707 [29], *Enterococcus faecium* E007 [30,31], *Klebsiella pneumoniae* WGLW2 (BEI Resources, Manassas, VA, USA), *Acinetobacter baumannii* ATCC 17978 [32], *Pseudomonas aeruginosa* PA14 [33], *Enterobacter aerogenes* ATCC 13048 and 11 clinical *S. aureus* isolates [34] were used to test antimicrobial activity. All bacterial strains were grown in tryptic soy broth (TSB) (BD, NJ, USA) at 37°C. Similarly to others [8–10], we previously found that essentially 100% of stationary-phase *S. aureus* MW2 cells become persisters, which are tolerant to conventional antibiotics such as gentamicin, ciprofloxacin and vancomycin [11]. Thus, *S. aureus* MW2 persisters were prepared by growing 25 ml of an *S. aureus* MW2 culture overnight to stationary phase at 37°C at 225 rpm [11].

Antimicrobial agents & chemicals

Vancomycin, ciprofloxacin, gentamicin, NH125 and benzyldimethylhexadecylammonium chloride

(16-BAC) were purchased from Sigma-Aldrich (MO, USA) and walrycin B was purchased from MedChem Express (NJ, USA), while 10 mg/ml stocks of all antibiotics were made in DMSO or ddH₂O.

Persister membrane permeability assay

Black, clear-bottom 96-well plates (Corning no. 3904) were filled with 50 µl of phosphate buffered saline (PBS)/well containing the indicated concentration of antibiotics. Stationary bacteria were then washed three times with the same volume of PBS. The washed cells were diluted to OD₆₀₀ = 0.4 (~10⁸ CFU/ml) with PBS. SYTOX Green (Molecular Probes) was added to 10 ml of the diluted persister suspension to a final concentration of 5 µM and incubated for 30 min at room temperature in the dark. A 50 µl of the persister/SYTOX Green mixture was added to each well of 96-well plates containing antibiotics and fluorescence was measured at room temperature using a spectrophotometer (SpectraMax M2, Molecular Devices) with excitation and emission wavelengths of 485 nm and 525 nm, respectively. All experiments were conducted in triplicate.

Time course assay

In order to correlate decreases in bacterial cell viability with membrane permeability, the same initial concentration as used in the membrane permeability assay was used for the time course killing assay. Overnight cultures of persisters were washed three times with PBS and diluted to OD₆₀₀ = 0.2 (5 × 10⁷–10⁸ CFU/ml) with the same buffer; 1 ml of the persister suspension containing the indicated concentrations of antibiotics was added to the wells of a 2 ml deep well assay block (Corning Costar 3960) and incubated at 37°C, with shaking at 225 rpm. At specific times, 50-µl samples were removed, serially diluted and spot-plated on tryptic soy agar (TSA, BD) plates to enumerate the number of persister cells. These experiments were conducted in triplicate.

Minimum inhibitory concentration assay

The minimum inhibitory concentration (MICs) of antibiotics were determined by the standard microdilution method [35]. Briefly, bacterial overnight cultures were diluted to approximately 1 × 10⁵ CFU/ml in cation-adjusted Mueller-Hinton (CaMH) broth; 50 µl of the diluted cultures were added to each well of a 96-well plate containing 50 µl serial twofold dilutions of antibiotic compounds in CaMH broth. The 96-well plate was incubated at 37°C for 18 h and bacterial growth was evaluated by measuring the optical density (OD) at 600 nm using a spectrophotometer (SpectraMax M2, Molecular Devices). Bac-

terial growth was defined as $OD_{600nm} \geq 0.1$. Assays were conducted in triplicate.

Preparation of GUVs & observation of effects of compounds on GUVs

Dioleoyl-glycero-phosphocholine (DOPC), Dipalmitoyl-glycero-phosphoglycerol (DOPG) and Dioleoyl-glycero-phosphoethanolamine-N-lissamine rhodamine B sulfonyl (18:1 Liss Rhod PE) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). GUVs were prepared by slightly modifying the electroformation method described in previous studies [36–38]. DOPC/DOPG/18:1 Liss Rhod PE (7:3:0.005) were dissolved in chloroform at a total lipid concentration of 4 mM. 40 μ l of the lipid solution was spread onto two indium tin oxide (ITO)-coated slides (50 $\times$ 75 $\times$ 1.1 mm, Delta Technologies, Loveland, CO, USA) and dried under low pressure for 2 h to remove the organic solvents. An electroformation chamber was assembled by inserting a 2-mm thick Teflon spacer between the two ITO slides. The chamber was then filled with 2 ml of 100 mM sucrose and sealed with binder clips. The swelling of the lipid bilayers was controlled using an electric AC-field (10 Hz) first by increasing the field-strength from 0 to 0.5 kV/m for 30 min and then keeping it constant for another 30 min. To enhance the detachment of the GUV from the glass surfaces, the frequency was reduced to 5 Hz for 20 min. The vesicle suspension was diluted (1:8) in a 110 mM glucose solution to obtain a slightly larger density inside the GUVs than outside. A 45 μ l of the diluted vesicle suspension (~500 vesicles) was transferred to a black, clear-bottom 384-well plate (Corning no. 3712). To allow all the vesicles to settle down to the bottom of the well, the plate was left in the dark at room temperature for 30 min. After adding 5 μ l of a 100X MIC compound to a well (final compound concentration: 10X MIC), the GUVs were observed and recorded by an optical microscope equipped with fluorescence contrast and a digital camera (40 $\times$ or 63 $\times$ objectives, Axio Observer. A1 & AxioCam MRm, Zeiss, Germany). Images and videos are representative of three independent experiments.

Human blood hemolysis

Hemolytic activity of compounds on human erythrocytes (Rockland Immunochemicals, PA, USA) was evaluated by slightly modifying a previously described method [34]. Briefly, 100 μ l of 4% human erythrocytes suspended in PBS was added to 100 μ l of twofold serial dilutions of compounds in PBS, 0.2% DMSO (negative control), or 2% Triton-X 100 (positive control) in a 96-well plate. The 96-well plate was incubated at 37°C for 1 h and then, centrifuged at 500 g for 5 min; 100 μ l of the supernatant was transferred to a fresh 96-well

plate and absorbance of supernatants was measured at 540 nm. Percent hemolysis was calculated using the following equation: $(A_{540nm} \text{ of compound treated sample} - A_{540nm} \text{ of 0.1\% DMSO treated sample}) / (A_{540nm} \text{ of 1\% Triton X-100 treated sample} - A_{540nm} \text{ of 0.1\% DMSO treated sample}) \times 100$. HC_{50} (concentration of a compound causing 50% hemolysis) was determined using SigmaPlot 10.0 (Systat Software Inc.).

Results

NH125 eradicates persisters of 11 clinical *S. aureus* isolates

In a previous study, we found that NH125 eradicated persisters formed by a reference MRSA strain MW2 at 5 μ g/ml, which was nontoxic to *Caenorhabditis elegans*, a model nematode for assessing toxicity [11]. To evaluate the potential of NH125 for clinical applications, we tested whether NH125 can kill growing and persister cells of 11 clinical *S. aureus* isolates [34]. The 11 isolates consisted of 9 MRSA strains, BF1, 2, 3, 4, 5, 7, 8, 10 and 2 methicillin-susceptible *S. aureus* (MSSA) strains, BF6 and 9 (Table 1). Whereas growing cells of all 11 isolates were susceptible to conventional antibiotics including gentamicin, ciprofloxacin and vancomycin (Table 1), their stationary-phase cells showed tolerance to 10X MICs of all three antibiotics (Supplementary Figure 1). This result indicates that the antibiotic tolerance of stationary phase cells appears to be a consistent feature of *S. aureus* strains, regardless of their sensitivity to methicillin. MICs of NH125 against the 11 clinical isolates were 2–4 μ g/ml (Table 1). However, in contrast to gentamicin, ciprofloxacin and vancomycin, 5 μ g/ml of NH125 eradicated approximately 5×10^7 – 10^8 CFU/ml persister cells of all of the clinical isolates to the limit of detection within 4 h (Figure 1). These results demonstrate that NH125 can not only block the growth of a broad spectrum of *S. aureus* strains under standard MIC conditions, but also kill persistent cells at a concentration that is nontoxic to human red blood cells (as described below in Figure 2).

NH125 shows antimicrobial activity against both Gram-positive & Gram-negative bacteria

The WalK/R system is specifically found in low GC content Gram-positive bacteria [17,39]. We hypothesized that if NH125 kills MRSA solely by inhibiting the WalK/R system, NH125 should not show activity against Gram-negative bacteria. We, therefore, determined the antimicrobial activity of NH125 against four Gram-negative species, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa* and *E. aerogenes* in comparison to two low GC content Gram-positive species, *E. faecium* and *S. aureus*. These six bacterial species are known as the ESKAPE pathogens, which are responsible for the

Table 1. Minimum inhibitory concentration against 11 clinical <i>staphylococcal</i> isolates.					
Strains	Oxacillin (µg/ml)	NH125 (µg/ml)	Gentamicin (µg/ml)	Ciprofloxacin (µg/ml)	Vancomycin (µg/ml)
BF1	>64	4	1	0.5	1
BF2	>64	2	1	1	1
BF3	32	2	1	1	1
BF4	16	2	1	0.5	0.5
BF5	>64	2	1	0.5	0.5
BF6	1	2	1	0.5	0.5
BF7	>64	2	1	0.5	0.5
BF8	>64	2	1	0.5	0.5
BF9	0.25	2	1	0.5	0.5
BF10	>64	2	1	0.25	0.5
BF11	>64	2	1	0.25	1

majority of nosocomial infections and have an ability to ‘escape’ antibiotic treatment [40].

Since WalR can be phosphorylated by other kinases or small molecule phosphate donors despite inhibition of WalK [41,42], WalR inhibitors are more effective than WalK inhibitors in blocking the regulatory cascades governed by the WalK/R system [23]. Thus, walrycin B, a WalR inhibitor (Figure 3B) was chosen as a functional analog of NH125 for testing its WalK/R inhibitory activity. Consistent with a previous study [23], walrycin B demonstrated antimicrobial activity against the Gram-positive bacteria *E. faecium* (MIC 2 µg/ml) and *S. aureus* (MIC 4 µg/ml) (Table 2), but not against

the four Gram-negative bacterial species tested. In contrast, NH125 showed activity against both the Gram-positive and Gram-negative bacteria, although NH125 exhibited better antimicrobial activity against the Gram-positive (MIC 1–2 µg/ml) than the Gram-negative bacteria (MIC of 8–16 µg/ml for all four species) (Table 2). These results suggest that NH125 may have other targets in addition to the WalK/R system.

To explore another potential mode of action for NH125, we also compared the antimicrobial activity of NH125 to benzyldimethylhexadecylammonium chloride (16-BAC), an NH125 structural analog consisting of a cationic head and a C16 alkyl chain (Figure 3C).

Figure 1. NH125 eradicates persisters formed by 11 clinical *staphylococcal* isolates. Persister cells of 11 clinical *staphylococcal* isolates were treated with 0.1% DMSO (control) and 5 µg/ml NH125 for 4 h. Persister viability was measured by serial dilution and plating on TSA plates. The asterisks on the x-axis are below the level of detection (2×10^2 CFU/ml). Results are shown as means $\pm$ SD; n = 3.

16-BAC is a cationic detergent and is known to kill bacteria by directly disrupting membranes [43,44]. As shown in Table 2, similarly to NH125, 16-BAC demonstrated antimicrobial activity against both Gram-positive (MIC 1–2 µg/ml) and Gram-negative bacteria (MIC 8–64 µg/ml). These results suggest that NH125 may directly disrupt bacterial membranes, similar to its structural analog 16-BAC.

The bactericidal activity of NH125 on MRSA persisters is not mediated by the inhibition of the WalK/R system.

As we previously reported [11], MRSA MW2 persisters exhibit a high level of tolerance to 10X MIC of conventional antibiotics, but are rapidly killed by NH125. Similarly to NH125, 10X MIC 16-BAC (20 µg/ml) induced rapid membrane permeabilization and completely killed approximately 10^8 CFU/ml MRSA persisters to the limit of detection (2×10^2 CFU/ml) within 15 min (Figure 4A & B). In contrast, the DMSO control or walrycin B at 10X MIC (40 µg/ml) did not kill MRSA persisters or induce membrane permeabilization (Figure 4C & D). Moreover, unlike NH125 and 16-BAC, walrycin B did not induce membrane permeabilization up to 64 µg/ml (Supplementary Figure 2). This result indicates that the inhibition of WalK may not play an important role in inducing membrane permeabilization. To further examine the effects of the WalK inhibition on membrane permeabilization, we treated growing MRSA cells with 10X MIC walrycin B, NH125 and 16-BAC. Consistent with the results of persistent MRSA cells (Figure 4), NH125 and 16-BAC caused rapid membrane permeabilization whereas walrycin B did not permeabilize the growing MRSA cell membranes (Supplementary Figure 3). These results demonstrate that both NH125 and 16-BAC kill persisters by a common mechanism involving direct disruption of persister cell membranes, which is independent of the WalK/R system. Also, antibiotic activity of NH125 against growing MRSA may be achieved by multiple mechanisms including membrane permeabilization, in addition to the inhibition of WalK phosphorylation [19,20].

NH125 disrupts giant unilamellar vesicles

To provide direct evidence that NH125 disrupts bacterial membranes, we examined the effects of NH125 on giant unilamellar vesicles (GUVs), a biomimetic model of cellular membranes, which is widely used for biophysical studies of lipid bilayer properties and interactions with biomolecules [27,28]. GUVs have been used for elucidating modes of action of membrane-active antimicrobial agents, such as daptomycin [37,45–48]. In this series of experiments, we

Figure 2. NH125 shows less hemolytic activity against human erythrocytes compared with 16-BAC. 1% human erythrocytes were treated with twofold serially diluted concentration of NH125 (solid circles) or 16-BAC (open circles) for 1 h at 37°C. A sample treated with 1% Triton-X 100 was used as the control for 100% hemolysis. Results are shown as means $\pm$ SD; n = 3.

used GUVs with a phospholipid composition that had been used previously for modeling the anionic bacterial membrane of *S. aureus* [37,49–50]. As shown in Figure 5 & Supplementary Video 1, 10X MIC (20 µg/ml) NH125 led to bursting of GUVs. Similar to NH125, rupture of GUVs was observed after treating them with 10X MIC (20 µg/ml) 16-BAC (Figure 5 & Supplementary Video 2), consistent with its mode of action described in previous studies [43,44]. For both NH125 and 16-BAC, an expansion of the GUVs was observed before bursting, which is indicative of inflow of the extravesicular solution due to a damaged lipid bilayer (Figure 5 & Supplementary Video 1 & 2). Also, the sudden decrease in the fluorescence observed before bursting of GUVs indicates loss of rhodamine-tagged lipid components, resulting from solubilization of lipid components by 16-BAC and NH125. In contrast, no such disruption of GUVs was observed when they were treated with 10X MIC (40 µg/ml) walrycin B (Figure 5 & Supplementary Video 3). These results show that the major mechanism by which NH125 kills MRSA persisters is by interacting with and disrupting membranes in a manner similar to cationic detergents.

NH125 has less hemolytic activity than 16-BAC

16-BAC is a benzalkonium chloride (BAC) analog, which is commonly used as a component of ophthalmic products, antiseptics, disinfectants and sanitizers [44]. However, it has been reported that BAC is cytotoxic and can cause lysis of human erythrocytes [51], corneal epithelial cells [52] and nasal epithelial cells [53]. Because NH125 disrupts GUVs relatively slowly compared with 16-BAC (Figure 5 & Supplementary Video 1 & 2), we hypothesized that NH125 may be less cytotoxic than

Figure 3. Chemical structure of compounds. (A) NH125, (B) Walrycin B and (C) 16-BAC.

16-BAC. To test this hypothesis, we compared hemolytic activities of NH125 with that of 16-BAC using human erythrocytes. At 8 $\mu\text{g/ml}$, 16-BAC caused approximately 7% hemolysis and its HC_{50} (concentration causing 50% hemolysis) was 25 $\mu\text{g/ml}$ (Figure 2). However, 8 $\mu\text{g/ml}$ NH125 did not cause hemolysis and its HC_{50} was 38 $\mu\text{g/ml}$, which is approximately 1.5-fold higher than the HC_{50} of 16-BAC (Figure 2). Considering that 5 $\mu\text{g/ml}$ NH125 is enough to completely eradicate persisters formed by a broad spectrum of *S. aureus* strains (Figure 1), these results suggest that NH125 may be less toxic than 16-BAC and a better alternative for 16-BAC.

Discussion

Persister cells are a nongrowing dormant subpopulation of a bacterial culture showing high tolerance to conventional antibiotics [54,55] and play an important role in the recalcitrance of chronic infections and the antibiotic tolerance of biofilms [12–13,56]. However, currently there are only a few effective ways to treat persisters. Previously, we identified NH125 and found that it kills MRSA persisters and eradicates MRSA biofilms [11]. NH125 was originally synthesized as a potential antimicrobial that inhibits phosphorylation of essential bacterial histidine kinases [19] and showed a strong correlation between antibiotic activity and WalK inhibitory activity [20]. Specifically, subtle structural modifications while maintaining its amphipathic character resulted in the decrease in both the kinase inhibitory activity and antimicrobial activity [19,20]. Further, the WalK/R system is known to regulate biofilm formation and cell wall synthesis [57]. Thiazolidine derivatives targeting WalK show bactericidal activity on *Staphylococcus epidermidis* biofilms where persisters exist [58]. Based on previously published studies of NH125, we hypothesized that the WalK/R system would be a target for killing persisters through membrane permeabilization. Here, we present evidence that the WalK inhibitory activity may not be the mode of action for the persister killing phenotype. The aim of this study was to evaluate the antimicrobial activity of NH125 against a broad spectrum of *S. aureus* strains and to elucidate the mode of action of NH125

in the killing of MRSA persisters. By comparing the activity of NH125 with its functional analog, walrycin B and its structural analog, 16-BAC, we found that NH125 appears to kill MRSA persisters by disrupting lipid bilayers in a manner apparently independent of WalK/R inhibition.

Although membrane-active agents have excellent potential as antimicrobial agents, with low MICs, fast killing rates and a low possibility of resistance selection, they often exhibit toxicity in mammalian cells [59]. However, membrane-active agents that have high selectivity for bacteria do exist and reduction of toxicity to mammals can be achieved by structural modification [59]. For instance, daptomycin, a lipopeptide antibiotic produced by *Streptomyces roseosporus*, kills Gram-positive pathogen including MRSA by disrupting the lipid bilayer [60]. Daptomycin is highly selective for bacterial membranes and is considered safe for clinical use, with few deleterious effects, such as an increase of serum creatine phosphokinase (CPK) level [61] and minor side effects on skeletal muscle [62]. Further, α -mangostin, a natural xanthone extracted from a Southeast Asian fruit, *Garcinia mangostana*, is a membrane-active agent effective in killing MRSA, with known toxicity to mammalian cells [63,64]. However, modification of the α -mangostin structure significantly decreased its toxicity to mammalian cells while maintaining its bacterial killing efficacy [64]. Considering the hemolytic activity of NH125, it appears that this agent is not as selective for bacterial membranes as daptomycin (Figure 2). However, NH125 demonstrated relatively low toxicity to red blood cells compared with 16-BAC, which is widely used as a topical antiseptic [65]. Although further studies concerning efficacy and cytotoxicity are required, we expect that NH125 could be used as part of a topical treatment for the management of chronic skin infections involving persistent bacteria and biofilms. Structure–activity relationship studies correlating chemical structure, antimicrobial activity and cytotoxicity need to be performed to explore the possibility of expanding the clinical usage of NH125.

GUVs are a versatile biomembrane-mimicking system that are widely used for studying the interaction

Strains	NH125 (μg/ml)	16-BAC (μg/ml)	Walrycin B (μg/ml)	Gentamicin (μg/ml)	Ciprofloxacin (μg/ml)	Vancomycin (μg/ml)
<i>E. faecium</i> E007	1	1	2	>64	>64	1
<i>S. aureus</i> MW2	2	2	4	1	0.5	1
<i>K. pneumonia</i> WGLW2	8	16	>64	1	0.063	>64
<i>A. baumannii</i> ATCC 17978	16	8	>64	1	0.25	>64
<i>P. aeruginosa</i> PA14	16	64	>64	2	0.063	>64
<i>E. aerogenes</i> ATCC 13048	16	16	>64	2	0.031	>64

between membrane-active compounds and lipid bilayers because their diameter (10–100 μm) is large enough to directly observe dynamic changes in morphology by optical microscopy [66,67]. We used GUVs to investigate the interaction between NH125 and lipid bilayers and observed that NH125 and 16-BAC rapidly solubilized GUVs (Figure 5 & Supplementary Video 1 & 2). The mechanism of rapid solubilization of lipid bilayers by detergents has been well-described [67,68]. First, detergents are inserted into outer monolayers of the lipid bilayers [67,68]. Then, detergents flip into the inner monolayers [67,68]. It is known that rapid solubilization occurs only when detergents can flip into the inner monolayers of the lipid bilayers [67–69]. Following the detergent flip, lipid bilayers are saturated with detergents and mixed lipid/detergent micelles are formed [67,68]. Finally, lipid bilayers are decomposed into the mixed micelles and detergent monomers [67,68]. As shown in Supplementary Video 1, when treated with 10X MIC (20 μg/ml) NH125, the size of the GUVs became increased up to the 24-s mark and then GUVs suddenly burst. This result demonstrates that NH125 is initially incorporated into the outer monolayer of the lipid bilayer by electrostatic forces between the positively charged head of NH125 and anionic GUVs. NH125 then flips into the inner leaflet of the bilayer, fully saturating the lipid bilayers with NH125, thereby causing complete solubilization of the lipid bilayers.

NH125 solubilized GUVs relatively slowly as compared with 16-BAC (Figure 5 & Supplementary Video 1 & 2). The rate of solubilization of lipid bilayers by detergents is dependent on the rate of trans-bilayer movement (known as the flip-flop) [67–69]. Also, the rate of flip-flop is influenced by the polarity and size of the detergent headgroup [70]. In fact, detergents containing a large head group have shown relatively slow flip-flop rate compared with detergents with a smaller head group [68,71–72]. The only structural difference between NH125 and 16-BAC is the relatively

large head group of NH125 (Figure 3A & C). Therefore, the relatively large head group of NH125 is likely to be related to the slower rate of solubilization (Figure 5 & Supplementary Video 1 & 2) and may be the reason why we found it to have less hemolytic activity compared with 16-BAC.

Since NH125 shares many common properties with 16-BAC, including antimicrobial activity, membrane permeability and destruction of GUVs, the mode of action of both compounds may be similar. 16-BAC is a homolog of the quaternary ammonium cationic surfactant benzalkonium chloride (BAC), which consists of *N*-alkyldimethylbenzylammonium chlorides with a C8 to C18 alkyl chain. BAC is known to have two modes of action depending on its concentration [43,44]. First, the positively charged quaternary nitrogen head binds to the acidic head of phospholipids in membranes and then, its alkyl chain embeds into the hydrophobic tails in the membrane core [43,44]. At low concentrations (~MIC), this interaction increases the surface pressure in the exposed membranes and decreases the fluidity of membranes, which leads to a loss of physiological function, such as osmoregulation, eventually causing bacterial death [43,44]. At high concentrations used in commercial products (0.004–0.02%), BAC solubilizes membrane components, such as lipid A and phospholipids, by forming micellar aggregates, which results in lysis of bacteria [43,44].

Since a decrease in SYTOX green fluorescence can be evidence of degradation of nucleic acids released from lysed bacteria [73], changes in the profiles of SYTOX Green fluorescence as a function of concentrations can be reflective of two different modes of action of 16-BAC depending on the drug concentration (Supplementary Figure 2A). Exposure of bacterial cells with up to 8 μg/ml of 16-BAC caused the SYTOX green fluorescence intensity to increase in a sigmoidal-like pattern and the maximum fluorescence intensity increases in a dose–response manner

Figure 4. Inhibition of the Walk/R system does not lead to death of MRSA persisters or rapid membrane permeabilization. MRSA persisters were treated with 10X MIC NH125 (A), 10X MIC 16-BAC (B), 10X MIC walrycin B (C) and 0.1% DMSO (D). Persister viability (solid circles) was measured by serial dilution and plating on TSA plates. Membrane permeability (open circles) was measured spectrophotometrically by monitoring the uptake of SYTOX Green (Ex = 485 nm, Em = 525 nm). The data points on the x-axis are below the level of detection (2×10^2 CFU/ml). Results are shown as means $\pm$ SD; $n = 3$

(Supplementary Figure 2A). At 16 μ g/ml of 16-BAC, although the SYTOX green fluorescence over time profile showed a sigmoidal-like pattern, its maximum intensity was lower than that of 8 μ g/ml 16-BAC. Further, at 32 μ g/ml and 64 μ g/ml 16-BAC, a rapid decrease in fluorescence was observed immediately after exposure to 16-BAC and the maximum fluorescence intensity decreased in a dose-response manner (Supplementary Figure 2A). These results suggest that at low concentrations (up to 8 μ g/ml), 16-BAC kills MRSA by damaging membranes allowing entry of SYTOX Green but not by solubilizing membrane lipids, whereas at high concentration (over 16–32 μ g/ml) 16-BAC appears to lyse persisters most likely by solubilizing membrane lipid bilayers. Similar to 16-BAC, SYTOX Green fluorescence profiles of NH125 also showed two different patterns depending on its concentration (Supplementary Figure 2B). Up to 16 μ g/ml, the fluorescence intensity showed a sigmoidal-like pattern and increased in a dose-response manner, whereas at higher concentrations (32–64 μ g/ml),

the fluorescence intensity showed a dose-dependent decrease (Supplementary Figure 2B). The similar patterns of SYTOX Green fluorescence between NH125 and 16-BAC indicates that NH125 appears to have a similar mode of action as 16-BAC. Whereas 16-BAC started showing a decrease in maximum fluorescence intensity at 16 μ g/ml, NH125 exhibited this effect at 32 μ g/ml (Supplementary Figure 2), demonstrating that 16-BAC induces membrane solubilization at lower concentrations than NH125. The stronger membrane activity of 16-BAC observed in the fluorescence profiles is consistent with relatively faster rupture of GUVs by 16-BAC (Figure 5) and relatively higher hemolytic activity (Figure 2) compared with NH125.

Conclusion

NH125 kills growing MRSA by multiple modes of action, including inhibition of Walk and membrane disruption. However, the mode of action of NH125 against MRSA persister cells is mostly likely by disrupting membranes in a detergent-like fashion, inde-

Figure 5. Time-lapse fluorescence micrograph showing effects of antimicrobial agents on giant unilamellar vesicles. Giant unilamellar vesicles consisting of DOPC/DOPG (7:3) labeled with 18:1 Liss Rhod PE (0.05%) were treated with 10X MIC NH125, 10X MIC 16-BAC, 10X MIC Walrycin B and 0.1% DMSO. After adding compounds at $t = 0$ s, changes of giant unilamellar vesicles were observed for 100 s using a fluorescent microscope (40 $\times$ objective, Ex = 460 nm, Em = 483 nm).

pendent of the inhibition of the WalK/R system. Considering its strong antimicrobial activity against a range of *S. aureus* strains and relatively low cytotoxicity compared with 16-BAC, NH125 could be a promising lead compound for treating chronic skin infection and an alternative to 16-BAC.

Future perspective

The rise of antibiotic-resistant bacteria and bacterial persisters are two big challenges in antimicrobial chemotherapy. Antimicrobial agents targeting bacterial membranes are promising candidates against both antibiotic-resistant bacteria and bacterial persisters. In the past, membrane-active agents were ignored as antimicrobial candidates due to their propensity to

show toxicity to in mammals. However, the clinical success of daptomycin has led to renewed interest in membrane-active agents and many compounds targeting bacterial membranes are currently under evaluation. Using a broad spectrum of clinical *S. aureus* strains and GUVs, we demonstrated that NH125 is a membrane-active agent with robust antimicrobial activity against both growing and persister cells. We also demonstrated the NH125 is likely to be less toxic than 16-BAC, which is currently used as a disinfectant. These results suggest that NH125 is a promising candidate for treating chronic skin infection and analogs should be studied to optimize antimicrobial activity and minimize cytotoxicity, followed by *in vivo* studies to evaluate the potential of NH125.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/full/10.4155/bio.15.189

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Executive summary

- Stationary-phase cells of 11 clinical *S. aureus* isolates showed tolerance to 10X MIC of conventional antibiotics irrespective of whether they were methicillin resistant, which indicates that the persistency of stationary phase cells appears to be a consistent feature of *S. aureus* strains.
- A nonhemolytic concentration of NH125 (5 µg/ml) completely eradicated approximately 5×10^7 CFU/ml persister cells formed by 11 clinical *S. aureus* isolates within 4 h.
- While walrycin B showed antimicrobial activity only against Gram-positive bacteria, NH125 showed antimicrobial activity against both Gram-positive and Gram-negative bacteria, similar to 16-BAC.
- Unlike walrycin B, both NH125 and 16-BAC induced rapid membrane permeabilization and completely killed MRSA persisters.
- Both 16-BAC and NH125 caused expansion of GUVs followed by their rupture, whereas walrycin B did not affect GUVs.
- Our results showed that NH125 kills MRSA persisters by disrupting membranes, in a manner most likely independent of Walk/R inhibition.
- NH125 showed lower hemolytic activity than 16-BAC.
- NH125 could be a promising topical antibiotic candidate against both antibiotic-resistant bacteria and bacterial persisters.

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