Strategies against methicillin-resistant *Staphylococcus aureus* persisters

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Chronic *Staphylococcus aureus* infections are complicated by frequent relapses not only from the development of drug resistance to conventional antibiotics, but also through the formation of persister bacterial cells. Bacterial persisters are in a transient, metabolically inactive state, making conventional antibiotics that target essential cellular growth processes ineffective, resulting in high clinical failure rates of antibiotic chemotherapy. The development of new antibiotics against persistent *S. aureus* is an urgent issue. Over the last decade, new strategies to identify *S. aureus* persister-active compounds have been proposed. This review summarizes the proposed targets, antipersister compounds and innovative methods that may augment conventional antibiotics against *S. aureus* persisters. The reviewed antipersister strategies can be summarized as two broad categories; directly targeting growth-independent targets and potentiating existing, ineffective antibiotics by aiding uptake or accessibility.

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Staphylococcus aureus is a Gram-positive opportunistic pathogen carried by approximately a third of the human population [1]. It is a leading cause of both hospital-acquired and community-acquired infections, which can range from minor skin infections to life-threatening disease, such as endocarditis and osteomyelitis [2]. Even if patients are treated with a regimen of antibiotics active against *S. aureus*, they require prolonged therapy and many experience complications due to antibiotic-resistance [3,4], or due to shift to a nongrowing, dormant state that confers a high level of tolerance to most conventional antibiotics [5–11].

Methicillin-resistant *S. aureus* (MRSA), is resistant to β -lactams, a widely-used class of antibiotics. MRSA is widespread in hospital and community settings and is responsible for approximately 60% of all *S. aureus* infections in the USA [12,13]. The Centers for Disease Control and Prevention estimates that MRSA causes 80,461 severe infections, 11,285 deaths, and US\$3–4 billion in healthcare costs per year in the USA alone [14,15]. Currently, vancomycin is used as an antibiotic of last-resort against MRSA. However, the high selective pressure from increased usage of vancomycin has promoted the emergence of vancomycin-intermediate or -resistant *S. aureus* [3,16].

In addition to acquiring antibiotic-resistance, as noted above, *S. aureus* can evade antibiotics by readily entering into nongrowing, dormant states [5,6]. Most antibiotics target biosynthetic processes, such as DNA, protein, and cell wall synthesis during bacterial growth [5,17]. However, these biosynthetic processes are inactive, or significantly minimized in the nondividing, dormant bacteria, known as persisters; therefore current antibiotics are largely ineffective against these cells [5]. *S. aureus* persisters were first identified in the 1940s, a couple of years after the initial discovery of penicillin-resistant bacteria [18]. In 1944, Bigger found that nongrowing *S. aureus* was not susceptible to penicillin [19]. He induced *S. aureus* to enter a nondividing state by culturing at a lower temperature, using 1:800 diluted media, or adding a bacteriostatic agent, boric acid. He discovered that the nondividing cells prepared under each condition were highly tolerant to penicillin, however, unlike resistant mutants, descendants of penicillin-tolerant cells remained susceptible to penicillin. He concluded that the activity of penicillin was bacterial growth-dependent and the tolerance resulted from dormant and nondividing states [19].

Recent studies demonstrate that almost all stationary phase *S. aureus* cells are persisters, and that they are tolerant to multiple antibiotics, each with a different target, such as gentamicin (protein synthesis inhibitor), ciprofloxacin (DNA replication inhibitor), and vancomycin (cell wall synthesis inhibitor) [7–11]. *S. aureus* persisters are also found in biofilms, a bacterial complex in which bacteria adhere to surfaces and are encapsulated in a matrix of extracellular polymeric substances [20] and these persisters are, at least partially, responsible for the high antibiotic-tolerance of biofilms [5,21]. Importantly, *S. aureus* is able to form persisters inside host tissues, and thus avoids host immune systems [9,22]. Several studies have shown that *S. aureus* persisters are responsible for the recalcitrance of chronic infections [5,23]. For instance, antibiotic treatments have reportedly failed in up to 38% of MRSA bacteremia cases even though the infecting MRSA were susceptible to the same antibiotics *in vitro* [24,25]. *S. aureus* persisters in biofilms are associated with high rates of antibiotic treatment failures and recurrence in endocarditis and osteomyelitis [26–28].

Since *S. aureus* infections often involve both multi-drug resistant and persistent *S. aureus*, these cases are hard to treat and ultimately cure with conventional antibiotics alone. The development of new therapeutics is urgently needed. In this review, we focus on targets and compounds effective against MRSA persisters and discuss their limitations. We also review new methodologies to kill *S. aureus* persisters using conventional antibiotics.

Bacterial protease ClpP activators, ADEPs

Bacterial proteases enzymatically cleave peptide bonds to break down proteins and polypeptides, playing important roles in stress responses, pathogenicity, and viability [29]. Bacterial proteases are a promising target for antimicrobial development because deregulation of key proteases results in reduced bacterial survival and virulence [29,30]. A well-known example is the caseinolytic protease system, which consists of a tetradecameric cytoplasmic serine protease (ClpP) and its cognate ATPase chaperones including ClpX, ClpA and ClpC [31,32]. The caseinolytic protease system plays a pivotal role in maintaining protein homeostasis by degrading misfolded and damaged proteins [31,32]. It is also involved in cell division, virulence and the breakdown of short-lived regulatory proteins [31,32]. Because ClpP nonspecifically degrades proteins, its proteolytic activity is strictly regulated by its corresponding cytoplasmic serine protease and its cognate ATPase chaperones in an ATP-dependent manner [29,30]. Briefly, ClpP-ATPases recognize, unfold, and translocate specific proteins into the proteolytic core of ClpP in an energy-dependent manner [32].

In 1985, the first antimicrobials targeting ClpP were identified by scientists from Eli Lilly and Company [33]. They isolated eight acyldepsipeptides (ADEPs), known as the A54556 complex, with antimicrobial activity against Gram-positive bacteria, including staphylococci and streptococci from *Streptomyces hawaiiensis* NRRL 15010 [33]. The representative ADEPs in the A54556 complex are A54556 factor A (later designated as ADEP 1) and factor B (Figure 1A). A few years later, Osada *et al.* isolated other ADEPs, enopeptin A and B (Figure 1A) having antimicrobial activity from *Streptomyces* sp. RK-1051 [34]. However, their mode of actions remained unknown for two decades. In 2005, Brötz-Oesterhelt *et al.* elucidated that ADEPs target ClpP by sequencing ADEP 1-resistant *Escherichia coli* mutants and then confirming the binding between ClpP and ADEP 1 analogs using affinity chromatography and chemical crosslinking [35]. Crystallography studies demonstrated that ADEPs bind at the interfaces between two adjacent ClpP subunits in the ClpP tetradecamer, which also function as the main docking site for the ClpP-ATPases [36,37]. The insertion of ADEPs leads to a change in the quaternary structure of the ClpP tetradecamer complex including the expansion of its axial pores, which subsequently confers the ability to degrade cellular protein without aid of ClpP-ATPases (Figure 1B) [36,37]. The overactivated ClpP protease indiscriminately breaks down oligopeptides and nascent proteins including proteins essential for bacterial survival [35,38].

Despite their unique mode of action, natural ADEPs have many drawbacks as drug candidates, including poor solubility and rapid systemic clearance [39]. Further, they demonstrated inefficacy in a mouse model of bacterial infection [39]. To overcome these constraints, structure–activity relationship (SAR) studies and structural optimization were conducted based on ADEP 1. The rigidification of the macrocyclic scaffold by replacing *N*-methylalanine with pipecolate reduced the entropic energy barrier for binding to ClpP [39]. To improve biological activity and stability, the polyunsaturated side chain was replaced with a heptenoyl moiety; two fluorines were added to the positions 3 and 5 of the phenyl moiety, yielding an improved ADEP 1 analog, termed ADEP 4 (Figure 1A) [39]. Indeed, ADEP 4 showed at least tenfold improved antimicrobial activity against MRSA (MIC 0.05 µg/ml) as compared with ADEP 1 (MIC 6.3 µg/ml). Moreover, it was more efficacious in the mouse model of *S. aureus* infection than the last resort antibiotic linezolid [35,39].

Recently, further optimization of ADEP 4 has been carried out. Carney *et al.*, hypothesized that an increase in the conformational rigidity of ADEP 4 would improve ClpP binding to and permeability of bacterial membranes [40].

Figure 1. Acyldepsipeptides kill MRSA persisters by deregulating the ClpP protease. Structures of natural acyldepsipeptides and their synthetic analogs **(A)** and mode of action **(B)**.

To create more conformationally constrained analogs, they replaced the serine residue of the ADEP 4 macrocycle with allo-threonine and substituted 4-methylpipecoate for the pipercolated residue of ADEP 4. The modified ADEP 4 analog, termed ADEP B315 (Figure 1A) exhibited significantly improved antimicrobial activity against *S. aureus* with an MIC of 0.024 µg/ml [40]. Arvanitis *et al.* showed that ADEP B315 was effective in mouse models of both methicillin-sensitive *S. aureus* (MSSA) and MRSA infections, performing comparable to vancomycin treatment [41]. Further ADEP B315 did not have significant toxicity on murine kidney or liver cells at a dose of 50 mg/kg, while vancomycin caused nephrotoxicity at the same dose [41].

In contrast to conventional antibiotics that mediate antimicrobial activity through inhibiting targets, ADEPs activate their target, resulting in nonspecific protein degradation. Since the ADEP killing mechanism is growth-independent, Conlon *et al.* reasoned ADEPs could be effective against both growing and dormant persistent bacteria [9]. Indeed, ADEP 4 induced considerable protein degradation in MRSA persister cells, as assessed through proteome analysis [9]. Further, the treatment of 10⁹ CFU/ml stationary-phase MRSA persisters with 5 µg/ml ADEP 4 decreased bacterial viability up to 4 logs in 2 days [9]. Although MRSA developed ADEP4 resistance after 3-days of exposure, 5 µg/ml ADEP 4 combined with 0.4 µg/ml rifampicin completely eradicated 10⁹ CFU/ml MRSA persisters within this time frame [9]. This combination of ADEP 4 and rifampicin also entirely eliminated MRSA persisters in biofilms within 3 days [9]. To test *in vivo* efficacy of ADEP 4 combined with rifampicin, Conlon *et al.* used the deep-seated mouse thigh infections model to mimic a chronic MRSA infection [9]. Combined treatment with ADEP 4 and rifampicin completely cleared the MRSA-infected thigh tissues, whereas vancomycin alone, rifampicin alone, or vancomycin and rifampicin could not clear the infection [9]. These results indicate that compounds targeting ClpP are promising antibiotic candidates to treat relapsing chronic infections.

Identification of candidate compounds through interrogation of membrane permeability

The bacterial cell envelope is composed of the lipid bilayer membrane and the cell wall. Since the bacterial cell envelope functions as a protective barrier and includes many proteins essential for cell survival, it is a promising target for novel antibiotics [42]. In fact, evolution tells us the bacterial cell envelope is an excellent target for killing bacteria. For instance, bacteria and bacteriophages produce hydrolytic enzymes, such as lysostaphin [43] and endolysin [44,45], which kills *S. aureus* by cleaving a major cell wall component peptidoglycan [43,44]. Mammalian immune systems secrete antimicrobial peptides, such as defensin, which inhibit *S. aureus* by disrupting membrane lipid bilayers [46]. Despite the differences in the specific modes of action, these antimicrobials rapidly permeabilize a part of the bacterial cell envelope [47,48]. Indeed, several antimicrobial peptides (AMPs) and enzymes show potency against bacterial persisters, which has been discussed in several recent reviews [49–53]. Here, we will focus on two membrane-active small molecules that effectively kill MRSA persisters, a recently proposed screen method, and its hit compound.

In 2009, Ooi *et al.* found that a porphyrin antibacterial agent, XF-73 (Figure 2A) exhibited strong bactericidal activity against *S. aureus* [54]. The MIC of XF-73 against *S. aureus* was 1 µg/ml, and it killed exponential-phase *S. aureus* cells faster than daptomycin [54]. Further, Ooi *et al.* found that XF-73 induced rapid membrane perturbation, which correlated with its bactericidal activity [54]. Based on these results, the authors hypothesized that XF-73 would be able to kill slow-growing and nondividing *S. aureus* cells by membrane disruption. Indeed, 4X MIC of XF-73 demonstrated bactericidal activity against both nongrowing *S. aureus* induced by incubation at 4°C and stationary-phase *S. aureus* cells, which are tolerant to the equivalent concentration of daptomycin [55]. These results provided the important insight that membrane-active small molecules could effectively kill nondividing bacterial persisters. Currently, XF-73 is in Phase II clinical trials for nasal decolonization of *S. aureus* [56].

Hu *et al.* conducted a screen assay to discover antimicrobials capable of targeting MRSA persisters [57]. They isolated nonmultiplying *S. aureus* from long-term stationary phase cultures and screened compounds that killed these nonmultiplying *S. aureus* cells [57]. The small quinolone-derived compound HT61 (Figure 2A) was identified from this screen. Hu *et al.* found that HT61 induced rapid membrane permeabilization and cell lysis at over 20 µg/ml. Therefore, they concluded that the membrane disruption is HT61's major mode of action to kill MRSA persisters. A recent report described the molecular details of this membrane-active mechanisms [58]. Combining cell-based membrane leakage and artificial lipid bilayers assays, Hubbard *et al.* suggested that HT61 directly interacted with lipid bilayers and subsequently caused unrecoverable membrane damage [58]. Interestingly, although the MIC of HT61 was 8 µg/ml, which is higher than the measured MICs of linezolid (0.8 µg/ml) or daptomycin (1 µg/ml) against *S. aureus*, HT61 eradicated 10⁷ CFU/ml of persisters formed by both MSSA and MRSA at 10 µg/ml within 6 h [57]. It also exhibited excellent bactericidal activity against persisters formed by a collection of clinical

Compound	MRSA MW2	BAA-1707	Human erythrocytes	Ref.
	MIC (μM)	MBEC (μM)	HC50 (μM)	
NH125	2.35	46.9	12.3	[77, 78]
Analog 1	1.56	23.5	7.8	[77, 78]
Analog 2	3.13	11.7	13.4	[77]
Analog 8	0.39	31.3	13.0	[78]
Analog 11	0.39	46.9	6.8	[78]
Analog 30	3.13	23.5	12.6	[78]

Figure 2. Membrane-active antimicrobials effective against MRSA persisters. Structures of XF-13 and HT61 **(A)**, and structures and bioactivities of NH125 and its analogs **(B)**.

MBEC: Minimum biofilm eradication concentration; MIC: Minimum inhibitory concentration; HC₅₀: The concentration at which 50% of erythrocytes are lysed.

MRSA strains, consisting of 103 isolates [57]. *In vivo* evaluation of the compound revealed that 1% HT61 gel was significantly efficacious in MRSA mouse skin infection model. Within the effective concentration, a toxicity assessment found that administration of 1–10% HT61 gels for 14 days did not cause adverse effects on mini pig skin [57]. Due to its high efficacy and low skin toxicity, HT61 has progressed to Phase III clinical trials for nasal decolonization of *S. aureus* [59].

Although XF-73 and HT61 have advanced into clinical trials for topical use, each has limitations when used to treat invasive systemic infections, due to their highly hydrophobic nature. High hydrophobicity often results in high serum binding and low tissue penetration rates. Therefore, XF-73 and HT61 may show lower bioavailability and significantly reduced *in vivo* efficacy, which is a major obstacle to the development of new membrane-active agents for systemic use [42]. In spite of their limitations, however, these two membrane-active agents can be further optimized as our understanding of the relationship among membrane activity, hydrophobicity, and bioavailability is further developed. Moreover, new drug delivery systems might help address the high serum binding and low tissue penetration issues [60–62]. For example, current drug delivery technologies using nanoparticles and liposomes enable the systemic administration of small molecules having issues of bioavailability, solubility and toxicity [62,63].

A systematic strategy to identify membrane-active antipersister agents

The successful identification of the membrane-active small molecules, XF-73 and HT61 inspired our group to seek out additional small molecules that could induce rapid membrane permeability. To identify compounds that permeabilize MRSA persisters, we developed a high-throughput screening method using the membrane-impermeable DNA binding dye, SYTOX Green, as a marker [10]. Membrane-active agents are often nonselective, or indiscriminate between mammalian and bacterial membranes [42]. To identify antimicrobial agents that specifically target bacterial membranes, we used a whole animal *Caenorhabditis elegans*-based screen of approximately 80,000 small molecules to identify all anti-infectives that prolong the survival of *C. elegans* infected with a lethal dose of MRSA [10,64]. The major benefit of this screening step is to identify antimicrobial compounds while simultaneously excluding compounds that are toxic to the worm [65,66].

In the subsequent phase, we narrowed our interrogation to 101 compounds identified as hits from the *C. elegans*-MRSA screen [10]. During this part of the screen, we exposed MRSA persister cells to compounds in the presence of SYTOX Green and used a spectrophotometer to determine if any compounds induced rapid membrane permeabilization. Among the 101 compounds, we identified NH125 (1-Hexadecyl-2-methyl-3-(phenylmethyl)-1H-imidazolium iodide, Figure 2) as a compound that altered membrane permeability [10]. Previously, NH125 was known to kill *S. aureus* by inhibiting WalK, a histidine kinase highly-conserved in Gram-positive bacteria and essential for survival [67,68]. NH125 is also known to inhibit eukaryotic elongation factor 2 (eEF2) kinase, which contributes to its anticancer and neuromodulatory activities [69–74].

Our findings suggest that NH125 also kills MRSA persisters by inducing rapid membrane permeabilization [10]. It is effective at killing persisters formed by a range of *S. aureus* strains, including MRSA clinical isolates, at 5 µg/ml [10,11]. When used at 10 µg/ml, NH125 killed over 99% of MRSA persisters formed inside biofilms, and it eliminated 50% of the biomass from mature biofilms. At 80 µg/ml, it completely destroyed established MRSA biofilms [10].

The structure of NH125 is similar to a benzalkonium chloride (BAC), an amphipathic disinfectant that acts on membrane lipid bilayers. Using giant unilamellar vesicles, a widely-used biomembrane-mimicking lipid bilayer system to examine interaction between membrane-active antimicrobials and bacterial lipid bilayers [75,76], we found that like BAC, NH125 exposure led to expansion and bursting of giant unilamellar vesicles [11]. This result demonstrates that the positively charged head of NH125 attaches to the head of the lipid bilayers and enters using hydrophobic interaction between its alkyl chain and the lipid bilayer's hydrophobic core. The interjection of NH125 into a bilayer results in the full saturation of lipid bilayers. Thus, evidence suggests that the major mechanism of action of NH125 against MRSA persisters is the disruption of lipid bilayers rather than the inhibition of WalK.

Recently, the Huigens group synthesized various NH125 analogs (Figure 2B) to improve its antimicrobial activity and to better understand its SAR [77,78]. The optimal alkyl chain length of NH125 for antimicrobial activity was known to be C₁₆, and analogs with chain lengths of <C₈ or >C₁₈ showed no remarkable antibiotic activity [67,68]. Therefore, they focused on head group modifications [77,78]. First, they found that *N*-arylated NH125 analogs, such as Analogs 1, 2 (Figure 2B) showed significantly enhanced antimicrobial activity against both stationary-phase MRSA persisters and biofilm MRSA persisters, as compared with its parental NH125. Both Analogs 1 and 2 had faster killing kinetics against MRSA persisters than NH125 and also exhibited minimum biofilm eradication

concentrations (MBEC) of 23.5 and 11.7 μM against MRSA biofilms, respectively, which is two- to fourfold lower than the MBEC of NH125 (Figure 2B) [77]. Although, alkylated NH125 (Analog 8 and 11, Figure 2) exhibited four- to sixfold lower MICs against MRSA than NH125, their MBECs against MRSA biofilms were equal or only slightly improved (Figure 2B). Analog 11 alone led to a 4-log reduction in stationary-phase MRSA persisters within 6 h at 50 μM , while NH125 provided only a 2-log reduction at the same concentration [78]. The substitution of triazole for the imidazole in NH125 (Analog 30, Figure 2) exhibited a slightly improved MBEC (23.5–31.3 μM) against MRSA biofilms, compared with NH125 (46.9 μM) (Figure 2B) [78].

In sum, a positively charged head group and an optimized alkyl tail (C_{16}) are essential for the antimicrobial activity of NH125 and its derivatives. This SAR is similar to BAC [79,80]. Therefore, NH125 most likely attaches to a negatively charged bacterial membrane using its positively charged imidazole head, and penetrates into the lipid bilayers through interaction between its alkyl tail and the hydrophobic tails of lipid bilayers.

Although NH125 has excellent antimicrobial activity against both stationary-phase persisters and biofilm persisters, it causes significant hemolysis at high concentration. Its HC_{50} (the concentration which causes 50% hemolysis) is 38 $\mu\text{g}/\text{ml}$ [11]. Despite their improved antimicrobial activity, the NH125 analogs do not exhibit reduced hemolytic activity compared with NH125 (Figure 2B) [77,78]. Thus, due to its narrow therapeutic window, further investigations and improvements in toxicity profiles are required for broader applications to MRSA persistent infections. However, NH125 exhibits higher HC_{50} than BAC ($\text{HC}_{50} = 25 \mu\text{g}/\text{ml}$), which is currently used as a topical antiseptic and a preservative in eye drops [81]. Therefore, NH125 has the potential to be used for treating skin infections that incorporate *S. aureus* persisters.

Repurposing of FDA-approved anticancer drugs

New antimicrobial agents need not always be synthesized from scratch or identified among collections of natural materials. Indeed, they can come from drugs that are already approved to treat disease clinically, albeit for different diseases. The search and identification of new uses for existing drugs has become known as drug repurposing [82]. The clinically approved drugs have passed extensive toxicity tests and clinical safety procedures, and their pharmacological properties are well understood. Most have track records in clinical trials with known outcomes and evaluated effects to humans. Thus, drug repositioning allows us to significantly reduce times and costs of drug discovery and development, as compared with *de novo* drug development [82,83].

Many US FDA-approved cancer drugs were originally identified as antimicrobial agents from soil-bacteria [84]. For instance, one of the most effective classes of antineoplastic drugs, anthracyclines (for example doxorubicin and daunorubicin), were isolated as antimicrobials from *Streptomyces peucetius* [85]. These antimicrobial-anticancer agents induce the alkylation or intercalation of DNA, resulting in permanent DNA damage [85,86]. Kwan *et al.* reasoned that the DNA alkylating or crosslinking antineoplastic agents could be effective against bacterial persisters since this killing mechanism is not growth-dependent [87]. They chose an FDA-approved anticancer drug, mitomycin C (MMC, Figure 3), as a potential antipersister drug because it can passively diffuse into cells and spontaneously causes DNA crosslinking [87].

MMC was originally isolated from *Streptomyces caespitosus* as an antimicrobial agent in the 1950s [88]. Since, MMC FDA approval in 1974 for treating gastric and pancreatic cancer, it has been used for treating various cancers, such as breast and bladder cancers [89]. MMC showed excellent bactericidal activity against persisters formed by both Gram-positive and Gram-negative pathogens [87]. MMC completely eradicated 10^8 CFU/ml *S. aureus* stationary-phase persisters at 10 $\mu\text{g}/\text{ml}$ within 3 h [87]. It eliminated 10^8 CFU/ml *S. aureus* persisters formed inside biofilms in 24 h [87]. MMC killed stationary-phase and biofilm persisters of *E. coli* O157:H7 and *P. aeruginosa* at 10 $\mu\text{g}/\text{ml}$ [87]. Further, MMC was effective in the *in vitro* wound biofilm model mimicking *P. aeruginosa*–*S. aureus* polymicrobial infections and the *C. elegans*–*E. coli* O157:H7 infection model [87]. In addition, recent studies demonstrate that MMC also effectively kills *Borrelia burgdorferi* [90] and *Acinetobacter baumannii* persister cells [91].

Based on this finding, Chowdhury *et al.* evaluated the antipersister activity of seven additional DNA crosslinking compounds [92]. Among the seven crosslinking agents, they found that cisplatin (Figure 3) was effective against persister cells of *E. coli* and *S. aureus* [92]. Like MMC, cisplatin is an FDA-approved antineoplastic drug used for treating various cancers, including testicular, ovarian, cervical, bladder and head/neck tumors [93]. Cisplatin crosslinks DNA by binding between two neighboring guanine bases on the same DNA strand [93]. Cisplatin showed antimicrobial activities against both growing and persistent bacteria at high concentrations. For instance, the MIC of cisplatin against *S. aureus* was 300 $\mu\text{g}/\text{ml}$. Its MICs against Gram-negative bacteria, such as *E. coli* and *P. aeruginosa* were 50–400 $\mu\text{g}/\text{ml}$ [92]. Interestingly, cisplatin eradicated the stationary-phase persisters of *S.*

Figure 3. Structures and antimicrobial activities of DNA-crosslinking antimicrobial-anticancer drugs.
ECK99: Effective concentration for killing over 99% of persister cells; MIC: Minimum inhibitory concentration.

aureus, *E. coli* O157, and *P. aeruginosa* at 250–500 µg/ml [92]. In addition to its antimicrobial activity, low-doses of cisplatin (0.1 and 0.5 mg/kg) were found to reduce bacterial burden by enhancing the phagocytosis activity of macrophages in a murine sepsis model [94]. Considering that cisplatin is known to cause nephrotoxicity [95] and high concentrations are required for killing bacterial persisters, most likely clinical development of cisplatin for antimicrobial use will be limited to topical treatment, such as chronic wound infections.

Metabolite-mediated uptake of gentamicin into persisters

In addition to discovering novel antipersister compounds, scientists have explored novel strategies that use existing antibiotics to kill persisters. Although, persisters are in nondividing dormant states, a reduced rate of protein translation does occur [96,97]. Thus, antibiotics targeting translation processes, such as aminoglycosides may have some effect against persisters [8]. However, one such antibiotic, gentamicin does not affect *S. aureus* persister viability even when provided at 100X MIC [8,10]. Cellular uptake of aminoglycosides requires the proton motive force (PMF), which is generated during bacterial growth [98,99]. Because the PMF is significantly reduced in persisters' dormant state, aminoglycosides do not enter persisters, resulting in persisters' high tolerance to aminoglycosides [8]. Allison *et al.* reasoned that metabolites could stimulate persisters to be susceptible to aminoglycosides [8]. To verify this hypothesis, they screened carbon sources that stimulated persisters to become susceptible to gentamicin. Only particular carbon sources (such as glucose, mannitol, and fructose) combined with gentamicin led to a 3-log decrease in *S. aureus* persister viability [8]. These carbon sources specifically potentiated gentamicin, but not ampicillin or ofloxacin, suggesting that the carbon sources were not simply converting persisters to growing cells [8]. Since the effect was specific to gentamicin, they hypothesized that these carbon sources were only increasing the persister PMF, which then facilitated the uptake of gentamicin. They proved their hypothesis by the following experimental results.

First, they found that the proton ionophore, carbonyl cyanide *m*-chlorophenyl hydrazone, which inhibits the PMF, inhibited the uptake of gentamicin and subsequently nullified the carbon source potentiation of gentamicin [8]. Second, they confirmed that the metabolites increased PMF in persisters. This pioneering strategy was remarkably effective to kill both Gram-positive and Gram-negative bacterial persisters [8]. For instance, gentamicin combined with fructose led to a 2-log decrease in *S. aureus* stationary-phase persisters and a 1.5-log decrease in *S. aureus* biofilm persisters within 4 h [8]. In addition, gentamicin combined with mannitol reduced the viability of *E. coli* stationary-phase persisters up to 3-log within 2 h, and more than 4-log within 4 h in *E. coli* biofilm persisters [8].

Last, the combinatorial treatment of 1 mg/kg gentamicin and 1.5 g/kg mannitol reduced *E. coli* biofilm persisters in a mouse chronic urinary tract infection model by 1.5-log [8].

Persister membrane permeable tobramycin

Another approach to augment the uptake of aminoglycoside into persisters was investigated by Schmidt *et al.* [100]. They focused on the membrane activity of AMPs and cell-penetrating peptides. Although, AMPs and cell-penetrating peptides generally have lower antimicrobial activity than aminoglycosides, they can selectively interact with bacterial membranes, which is independent of bacterial metabolic states [42]. A recent series of studies demonstrated that AMPs and CCPs share a common key functionality of generating saddle-splay (also known as negative Gaussian) curvature in target membranes, which is the topologically required process for membrane destabilization, such as pore formation [101–104]. Arginine, lysine and hydrophobic content in AMPs have been found to play a key role in generating saddle-splay membrane curvature [101,102,104]. Based on these findings, Schmidt *et al.* hypothesized that a hybrid aminoglycoside conjugated with the specific amino acid sequences that induce saddle-splay membrane curvature could be effective to kill bacterial persisters [100].

To interrogate their hypothesis, Schmidt *et al.* linked a peptide consisting of 12 amino acid residues, (RQIKI-WFQNRRW, originally from penetratin), to the drug tobramycin, and designated the new compound as pentobra (Figure 4A) [100]. Indeed, unlike tobramycin, pentobra permeated and killed *S. aureus* persisters. Pentobra led to 4-log and 6-log decreases in *S. aureus* persister viability at 6.4 and 25.7 μ M, respectively [100]. Pentobra was also effective at killing *E. coli* persisters, with 12.8 and 25.7 μ M resulting in 1.5 and 1.8-log reductions, respectively. Further, pentobra had high bacterial membrane selectivity, causing no damage to eukaryotic cell membranes or no eukaryotic cytotoxicity up to 100 μ M [100].

Antibody- or peptide-antibiotic conjugates targeting intracellular MRSA persisters

Although, *S. aureus* is generally regarded as an extracellular pathogen, it does invade and survive inside mammalian cells, including nonprofessional phagocytes [105,106]. The intracellular environment not only provides *S. aureus* with a protective barrier to extracellular host defenses, but also stimulates the transition from normal dividing-cells to small colony variants and persister cells, both of which have higher tolerances to conventional antibiotics [107,108]. For instance, MRSA sequestered in murine macrophages is known to have high tolerance to last resort antibiotics, such as vancomycin, daptomycin and linezolid [22].

To kill intracellular MRSA, Lehar *et al.* designed an antibody-antibiotic conjugate (AAC). After evaluating the binding affinity of over 40 anti-*S. aureus* antibodies, they chose an antibody targeting *S. aureus* wall-teichoic acids, anionic glycopolymers that connect cell wall peptidoglycan layers [109]. For the antibiotic component, they selected a member of the rifamycin class of antibiotics (RNA polymerase inhibitors), rifalogue due to its high potency, stability in the intracellular environment and feasibility to conjugate the linker [22]. The antibody and rifalogue were conjugated by a cathepsin-cleavable linker (Figure 4B) [22]. Thus, the AAC itself does not have antimicrobial activity and is not toxic to host cells due to its large size. But, once host cells engulf AAC- opsonized bacteria, intracellular proteases break down the linker, and the rifalogue is subsequently released to attack the bacteria inside the host cells (Figure 4B) [22]. Remarkably, AACs were effective to kill MRSA located in various types of host cells including human macrophages, endothelial, and epithelial cell lines [22]. Further, AACs were efficacious at a daily dose of 50 mg/kg in a murine model of bacteremia, while vancomycin failed to cure at twice daily doses of 110 mg/kg [22].

In addition to an antibody-mediated therapy, the Seleem group employed a short antimicrobial peptide that penetrates into mammalian cells to facilitate the uptake of aminoglycosides [110,111]. Previously, this group synthesized a proline-rich short antimicrobial peptide, P14LRR, that effectively penetrated into mammalian membranes without causing cell lysis [112]. They conjugated P14LRR to kanamycin using a reducible disulfide linker and designated this kanamycin–peptide conjugate as P14KanS (Figure 4C) [110,111]. Since, the mammalian cell cytosol is a reducing environment, the disulfide linker is broken down and the components are released [110,111]. Interestingly, P14KanS exhibited superior antimicrobial activity to either P14LRR or kanamycin alone [111]. The MIC of P14KanS against MRSA was 1 μ M, which was a 4 and a 64-fold lower than MICs of kanamycin (4 μ g/ml) and P14LRR (64 μ g/ml), respectively [111]. This improved antimicrobial activity most likely results from enhanced kanamycin uptake due to the membrane-active antimicrobial peptide. P14KanS was also effective at eradicating *S. aureus* biofilms and destroyed over 65 and 82% of *S. aureus* biofilms at 1 and 8 μ M, respectively [111]. Importantly, P14KanS killed intracellular *S. aureus*. The treatment of infected human keratinocytes with 15 μ M P14KanS for 24 h led to

Figure 4. Engineered existing antibiotics effectively kill bacterial persisters. Structures and functional mechanisms of pentobra (A), antibody-antibiotic conjugate (B), and kanamycin-peptide conjugate (C).

approximately 2.5-log reduction of MSSA viability and approximately 1.24-log reduction of MRSA viability [111]. Further, P14KanS showed efficacy in the *C. elegans*–*S. aureus* infection model. Treatment of *C. elegans* infected with *S. aureus* with 10 μ M P14KanS resulted in 100% *C. elegans* survival for 72 h [111].

Conclusion & future perspective

Compared to the study of antibiotic-resistant mechanisms and the development of new drugs effective against drug-resistant bacteria, the study of persisters has a relatively short history. Even though persisters were first identified in 1944, intensive investigation on bacterial persisters did not mature until the 2000s, and strategies to treat them were not formulated until the 2010s. In this report, we take the opportunity to review the current targets, their corresponding antimicrobials, and novel strategies effective against *S. aureus* persisters that have been reported since 2010. To sum up, *S. aureus* persister eradication strategies can be classified into two categories: directly targeting persisters by disrupting growth-independent targets, and, facilitating the uptake or accessibility of existing antibiotics. Although, each compound discussed here has its own drawbacks, such as cytotoxicity or the potential for developed resistance, the bacterial cell envelope and proteases remain promising targets for inhibiting persisters. Further, the disadvantages of each compound can be lessened, and knowledge from these pioneering approaches can and will be employed as a starting point to develop next generation therapeutics to treat *S. aureus* persisters infections.

Due to an increasing immunocompromised patient population and medical devices usage, chronic and relapsing infections caused by persisters will continue and most likely become more prevalent. Our current arsenal of antibiotics and means of treating active infections are not sufficient for long term patient care. Thus, anti-infectives that cure chronic infections are urgently needed. As discussed above, membrane-active agents and protease activators are promising candidates to combat persisters. We are provided with insights to discover or rationally design membrane-active antibiotics with high bacterial membrane selectivity through the knowledge gleaned on the currently identified compounds. Further, natural methods employed by AMPs and how they distinguish between

Executive summary

There is urgent need to develop antimicrobials effective against *Staphylococcus aureus* persisters

- In addition to resistance development, *S. aureus* evade antibiotics by shifting into nondividing, dormant states.
- *S. aureus* persisters are found in stationary-phase planktonic culture, biofilms and host cells and have high tolerance to most of conventional antibiotics.
- *S. aureus* persisters are responsible for relapsing and chronic infections, which are difficult to cure by conventional antibiotics chemotherapy.

Overactivation of ClpP is effective to kill *S. aureus* persisters

- Acyldepsipeptides bind to cytoplasmic serine protease (ClpP) and induces its conformational changes, which subsequently leads to ATP-independent activation of a ClpP protease.
- ADEP4-dependent ClpP activation indiscriminately breaks down cellular proteins, resulting in *S. aureus* persisters death.
- Combination of ADEP4 with rifampicin is effective to kill *S. aureus* persisters and is efficacious in a deep-seated mouse thigh infection model.

Bacterial cell envelope is a promising target for killing *S. aureus* persisters

- The bacterial cell envelope is an evolutionary verified and growth-independent target.
- The SYTOX Green-persister membrane permeability screen identifies bacterial envelop targeting antimicrobial agents.
- Membrane-active compounds with low membrane selectivity can be excluded by an initial *Caenorhabditis elegans*-based screen.
- XF-73, HT61 and NH125 kills Methicillin-resistant *S. aureus* (MRSA) persisters by disrupting membrane lipid bilayers.

Novel strategies to augment the potentiation of conventional antibiotics

- Metabolites increase proton motive force, facilitating aminoglycoside uptake of into *S. aureus* persisters.
- Gentamicin combined with fructose effectively kills *S. aureus* persisters.
- Tobramycin conjugated with a small peptide inducing saddle-splay membrane curvature (Pentobra) is potent against *S. aureus* persisters.
- An anti-*S. aureus* antibody conjugated to rifallog effectively kills MRSA persisters formed inside host cells and highly efficacious in a murine model of bacteremia.
- A kanamycin-antimicrobial peptide conjugate significantly clears intracellular MRSA.

bacterial and host membranes have been better elucidated. With a better understanding about the ways to inhibit persister cells and better reduce biofilm accumulation, improved compounds can be generated that will inhibit active infections while offering protection against the development of chronic infections.

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