Recalcitrant *Staphylococcus aureus* Infections: Obstacles and Solutions

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ABSTRACT Antibiotic treatment failure of *Staphylococcus aureus* infections is very common. In addition to genetically encoded mechanisms of antibiotic resistance, numerous additional factors limit the efficacy of antibiotics *in vivo*. Identifying and removing the barriers to antibiotic efficacy are of major importance, as even if new antibiotics become available, they will likely face the same barriers to efficacy as their predecessors. One major obstacle to antibiotic efficacy is the proficiency of *S. aureus* to enter a physiological state that is incompatible with antibiotic killing. Multiple pathways leading to antibiotic tolerance and the formation of tolerant subpopulations called persister cells have been described for *S. aureus*. Additionally, *S. aureus* is a versatile pathogen that can infect numerous tissues and invade a variety of cell types, of which some are poorly penetrable to antibiotics. It is therefore unlikely that there will be a single solution to the problem of recalcitrant *S. aureus* infection. Instead, specific approaches may be required for targeting tolerant cells within different niches, be it through direct targeting of persister cells, sensitization of persisters to conventional antibiotics, improved penetration of antibiotics to particular niches, or any combination thereof. Here, we examine two well-described reservoirs of antibiotic-tolerant *S. aureus*, the biofilm and the macrophage, the barriers these environments present to antibiotic efficacy, and potential solutions to the problem.

KEYWORDS *Staphylococcus aureus*, antibiotic tolerance, persisters

Antibiotics are arguably the most important class of medicine in modern health care. Despite their importance, their efficacy in the context of the infection environment remains poorly understood. Treatment failure is common and is becoming more widespread which imposes a heavy burden on global public health. Despite this threat, the FDA has not approved a new class of antibiotics since the lipopeptides in 2003 (1). The reasons for this dearth in drug discovery and development are complex, with economic factors playing a major role. New antibiotics are less profitable than drugs for chronic illnesses, such as diabetes or heart disease, which are frequently prescribed for a lifetime. Resistance to antibiotics, which render the drugs useless, spreads through societies and is fueled by use (2). This resistance has led to policies of antibiotic stewardship to limit resistance development to new agents, further limiting their use and the associated profits. Numerous efforts to solve this problem are ongoing, and there is reason for optimism. Nonetheless, antibiotic discovery and development remain a major challenge and emphasize the need to maximize the efficacy of our existing drugs to yield better patient outcomes and allow for shorter antibiotic-dosing regimens, which would also slow resistance development.

Staphylococcus aureus is the most common cause of nosocomial infection, is among the most common causes of community-associated bacterial infection, and is extremely difficult to treat with antibiotics (3). Antibiotic resistance (reviewed extensively elsewhere [4–6]), is a major problem in *S. aureus*; however, other factors also contribute significantly

Citation Rowe SE, Beam JE, Conlon BP. 2021. Recalcitrant *Staphylococcus aureus* infections: obstacles and solutions. Infect Immun 89:e00694-20. <https://doi.org/10.1128/IAI.00694-20>.

Editor Anthony R. Richardson, University of Pittsburgh

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Accepted manuscript posted online 1 February 2021

Published 17 March 2021

to poor antibiotic efficacy. Here, we will discuss treatment failure, focusing on the barriers to antibiotic penetration, the formation of antibiotic-tolerant *S. aureus* populations, and ways to overcome those barriers.

OBSTACLES

Biofilms. A biofilm is a community of bacteria surrounded by an extracellular matrix that protects the bacteria from an array of assaults, including uptake by host phagocytic cells. Biofilms are also extremely difficult to kill with antibiotics. Up to 80% of all bacterial infections are estimated to be biofilm-associated (7), including many chronic infections frequently caused by *S. aureus* (endocarditis, osteomyelitis, chronic wound infection, and indwelling device infections). Free-floating or nonadhered biofilm aggregates are present in *S. aureus* knee joint infections and infections of the cystic fibrosis lung (8).

In the United States, chronic wounds affect approximately 2% of Americans, of which the most common are vascular ulcers, diabetic ulcers, and pressure ulcers (9). A total of 93.5% of chronic leg ulcers are infected with *S. aureus* (10). Bacterial biofilms are the leading cause of treatment failure in chronic wounds and usually require surgical intervention, such as debridement in conjunction with antibiotic therapy (11). With risk factors for chronic wounds, such as cardiovascular disease, diabetes, and obesity, rising globally, there is an urgent need for improved treatment strategies. It also represents an enormous financial health care burden, with Medicare cost estimates for chronic wound treatments now upward of \$30 billion annually (11).

Surgical implant infections and osteomyelitis respond so poorly to antibiotics that therapy is routinely combined with surgical intervention (12, 13). Additionally, *S. aureus* can cause tissue abscesses comprising a dense *S. aureus* community encapsulated by fibrin and immune cells (14). The enclosed *S. aureus* communities share similarities with biofilms but have distinct genetic requirements (15). Encapsulation serves to contain the infection, but host defenses may fail to resolve the abscess (14) which likely contributes to the high infection relapse rates (20–50%) associated with skin and soft tissue infections (SSTIs) (16, 17) and chronic osteomyelitis (18). Antibiotic tolerance of *S. aureus* within abscess communities undoubtedly contributes to infection relapse, but the phenomenon has not been thoroughly studied (14).

A crucial criterion for antibiotic efficacy is that the drugs reach high enough concentrations at the site of infection to reduce pathogen burden or to halt pathogen replication so that the immune system can clear the infection. Antibiotics tend to be effective at micromolar levels, namely, 100 to 1,000 times higher than most host-targeted drugs (19). This greatly decreases the therapeutic window of antibiotics, increases the chance of toxicity, and compromises the clinical efficacy in any niche where antibiotic penetration is impeded. Biofilms act as a physical barrier and to some extent can impede or slow the penetration of glycopeptides, β -lactams, and phenicols (Table 1) (20–22). Previous studies have indicated that penetration of positively charged aminoglycosides, such as tobramycin, into biofilm is impeded by negatively charged components of the matrix, such as extracellular DNA and certain components of polysaccharides (23). Fluoroquinolones, macrolides, rifampicin, linezolid, and daptomycin penetrate well into biofilms (20, 21, 24–27).

In addition to impeded penetration of some drugs, biofilms harbor high numbers of persister cells which are frequently implicated in treatment failure (28–33) (Fig. 1a). Persister cells are a subpopulation of antibiotic-tolerant cells that transiently survive antibiotic challenge. Antibiotic tolerance of biofilms has long been associated with metabolic quiescence due to poor nutrient and oxygen availability within the biofilm (26). Persister cell formation has been strongly associated with ATP depletion in *S. aureus*, so the presence of high numbers of persisters within the biofilm is not surprising (33–35). But, low ATP is not an essential requirement for antibiotic tolerance; direct inhibition of cellular processes, including cell wall synthesis or protein synthesis, can induce antibiotic tolerance while maintaining high ATP (34–36).

TABLE 1 Penetration of antibiotics into host cells and biofilms

Antibiotic class	Antibiotic example(s)	Host cell penetration ^a	Biofilm penetration ^a
Macrolide	Azithromycin, telithromycin, erythromycin, clarithromycin	Efficient (59, 62, 145)	Efficient (146, 147)
Aminoglycoside	Gentamicin, tobramycin	Poor (26, 59)	Impeded/retarded penetration into biofilms (26, 148)
Oxazolidinone	Linezolid	Conflicting data on accumulation (59, 149)	Efficient (150)
β -lactam	Penicillin V, nafcillin, ampicillin, oxacillin	Poor penetration and low accumulation (53, 56–59, 61)	Impeded (21)
Glycopeptide	Vancomycin	Poor (59, 61, 62)	Penetration is retarded but can reach therapeutic levels within the biofilm (20, 151, 152)
Ansamycin	Rifampicin	Efficient (60–62)	Efficient (20, 27)
Quinolone	Ciprofloxacin, levofloxacin, moxifloxacin	Efficient (59, 61, 145)	Efficient (26, 153, 154)
Cephalosporin	Cefazolin, cefuroxime, cefotaxime	Poor (59, 61)	Impeded (21)
Tetracycline	Minocycline	Moderate (58, 59, 61)	Efficient (22, 155)
Lipopeptide	Daptomycin	Poor (59)	Efficient (24, 25)
Lincosamide	Clindamycin	Temp dependent; moderate penetration at 37°C (58, 59)	Limited data on penetration (156)

^aReferences are in parentheses.

Elucidating mechanisms of persister formation has traditionally involved testing the capacity of genetic mutants to survive antibiotic challenge *in vitro*. Mutants of the *agr* quorum sensing system *agrCA* and *agrD* displayed increased persisters to levofloxacin in stationary phase *in vitro*. Conversely, an *RNAIII* mutation had decreased persisters under the same conditions (37). Phenol-soluble modulins (PSM) mutants formed fewer persisters to fluoroquinolones and gentamicin *in vitro* but had no impact on cell wall-acting antibiotics (37, 38). In addition, persisters under *in vitro* conditions were associated with reduced tricarboxylic acid (TCA) cycle activity (39, 40) and reduced membrane potential (39, 41). It is evident that a single pathway for antibiotic tolerance does not exist and not all pathways will lead to multidrug tolerance. We have yet to learn which of these mechanisms are relevant in the context of infection.

More recently, *S. aureus* antibiotic tolerance and persister formation have been examined in more physiologically relevant environments. A study by Ma et al. reported that there are mechanisms of persister formation that are specifically activated in the biofilm. Specifically, deleting the single type II toxin-antitoxin (TA) module *mazEF* showed decreased survival to vancomycin in an *in vitro* biofilm model. This phenotype was confirmed *in vivo* with a *mazEF* mutant displaying fewer persisters to vancomycin in a deep-seated biofilm infection model in mice (42–44). Interestingly, the *mazEF* mutant was also associated with increased pathogenicity and mortality, and the authors

FIG 1 Persisters and penetration in antibiotic treatment failure. Two primary challenges to antibiotic-mediated eradication of *S. aureus* infection are antibiotic-tolerant bacteria and poor antibiotic penetration to the infection site. Biofilms (A) and host cells (B) represent physical barriers to efficient penetration of antibiotics. However, even when antibiotics are able to penetrate these barriers, antibiotics may still fail due to the bacteria being in an antibiotic-tolerant state. Numerous mechanisms, including toxin-antitoxin modules, interactions with immune cells, and the stringent response, have been implicated in the formation of antibiotic-tolerant cells in different niches. Schematic credit: created with BioRender and modified by Patrick Lane, ScEYence Studios.

concluded that the mutant was unable to transition from an acute infection into a chronic infection often associated with increased antibiotic tolerance. Importantly, we previously reported that a mutant devoid of three type II TA modules (*mazEF*, *axe1/txe1*, and *axe2/txe2*) had no contribution to persister formation under broth culture conditions (34). This major disparity in the contribution of TAs to persister formation under biofilm and planktonic conditions emphasizes the fact that numerous pathways to the persister state exist and their importance is dependent on environmental conditions. For this reason, stress response pathways leading to persister formation in biofilm-related infections are likely to be distinct from the pathways leading to persister formation in nonbiofilm-associated infections, such as bacteremia. It also highlights the salience of modeling physiologically relevant conditions *in vitro*.

Macrophages. In *S. aureus* bacteremia, *S. aureus* is engulfed by macrophages within minutes of entering the bloodstream (45). Although macrophages exhibit an array of bactericidal assaults designed to kill invading pathogens, subpopulations of *S. aureus* reside, survive, and even proliferate within macrophages (45, 46). Infected macrophages may function as “Trojan horses,” shielding *S. aureus* from antibiotic and immune-mediated clearance, facilitating *S. aureus* dissemination to other tissues, and contributing to infection recurrence and secondary infections (45–48). Poor penetration of antibiotics into host cells (Table 1) on its own does not explain the ability of intracellular *S. aureus* to withstand antibiotic treatment, spurring recent efforts to elucidate a role for the host response in the formation of *S. aureus* persister cells (47–50).

S. aureus resides in multiple cell types, including macrophages, endothelial cells, epithelial cells, osteoblasts, osteoclasts, and neutrophils, and several classes of drugs fail to effectively penetrate host cells (51–53). Penetration of vancomycin, the front-line antibiotic prescribed for methicillin-resistant *S. aureus* (MRSA) bacteremia, is excluded from macrophages, likely due to its high molecular weight ($1,449.2 \text{ g mol}^{-1}$) and hydrophilicity (45, 54, 55). Although β -lactams may diffuse well into host cells, they do not accumulate well, presumably due to their acidity (56–58). Similarly, the highly polar aminoglycosides exhibit poor penetration into host cells (57, 59). Conversely, macrolides, rifampicin, and fluoroquinolones diffuse and accumulate well in host cells (Table 1) (60, 61). However, both macrolides and rifampicin exhibit poor killing efficacy intracellularly (62). In fact, only 5 out of 16 antibiotics (representing 7 antibiotic classes) tested in a comparative study caused a reduction in *S. aureus* burden following internalization by macrophages (62). Intracellular efficacy was consistently lower than extracellular efficacy for all antibiotics tested.

It appears that multiple pathways may contribute to antibiotic tolerance within host cells. We recently found that macrophage-derived reactive oxygen species (ROS) produced by activated macrophages during oxidative burst led to the formation of *S. aureus* antibiotic-tolerant persister cells. Exposure to ROS collapsed tricarboxylic acid (TCA) cycle activity, reducing ATP levels, and forcing *S. aureus* into a metabolic state that is incompatible with antibiotic killing (50). Furthermore, *S. aureus* recovered from mice deficient in the production of macrophage-derived ROS were more susceptible to antibiotic killing than *S. aureus* recovered from wild-type mice, supporting the *in vitro* findings (50).

Another study suggests that antibiotic pressure induces the formation of stable but reversible antibiotic-tolerant persister cells in a variety of host cell types, including unstimulated macrophages, keratinocytes, osteoblasts, epithelial cells, and monocytes, and that antibiotic-induced persister cells represent a major reservoir for secondary infection (48). Stringent response-deficient mutants of *S. aureus* formed fewer persisters in unstimulated macrophages (48). In addition, intracellular *S. aureus* persisters had induced expression of several stress response pathways, including the stringent response, cell wall stress, SOS, and heat shock responses that conferred multidrug tolerance (48). Clearly, drug penetration as well as multiple intracellular stress stimuli contribute to the recalcitrance of intracellular *S. aureus* populations to antibiotics (Figure 1b).

SOLUTIONS

Most conventional bactericidal antibiotics kill by corrupting ATP-dependent cellular processes, fluoroquinolones kill by preventing the religation step during DNA synthesis (63), aminoglycosides produce toxic peptides during translation (64), β -lactams induce a toxic futile cycle of cell wall synthesis and degradation (65), and glycopeptides prevent the cross-linking step of peptidoglycan synthesis (66). We now appreciate that any stress encountered during infection which slow or stop the processes targeted by antibiotics will likely lead to antibiotic tolerance. *S. aureus* can achieve antibiotic tolerance through multiple distinct mechanisms, as follows: activation of TA modules in biofilms (67), host-produced ROS in macrophages (50), nutrient limitation and activation of the stringent response during interaction with host cells (48), and likely numerous pathways that have yet to be identified. Therefore, we suggest that treatment failure should be studied in the context of a particular infection site, and strategies to overcome treatment failure may need to be niche specific. The logical but arduous task of improving antibiotic therapy of recalcitrant *S. aureus* infections is to first identify the persister reservoirs *in vivo*, determine how they formed, and strategize to eradicate them.

Antipersister drugs. Antipersister antibiotics kill independently of the metabolic state of the cell and are more effective against biofilms than conventional antibiotics (68–70). Membrane-acting agents are attractive antipersister drugs as they exert rapid bactericidal activity, and in theory, they target persisters and growing cells indiscriminately. In addition, membrane-acting drugs often interact with multiple targets in the membrane which reduces the probability of resistance development (71). Daptomycin, a lipopeptide antibiotic, inserts into the cell membrane and disrupts fluid membrane microdomains. Daptomycin has potent activity against recalcitrant populations of *S. aureus*, including biofilms and persister cells (72), and in combination with linezolid, daptomycin is the leading therapy recommended to treat persistent MRSA bacteremia or vancomycin failure (72). However, despite its antipersister activity, its efficacy *in vivo* is limited by infection types; daptomycin is inactivated by surfactant and is therefore ineffective in the treatment of pneumonia or cystic fibrosis-associated infection (73). When prescribed, its efficacy against biofilm-associated infections is questionable, and treatment failure occurs in ~20% cases (74–76). Tolerance to daptomycin is thought to arise from structural modifications to the membrane during infection which disrupt interaction with the drug (71, 74). In addition, mutants deficient in *agr* release phospholipid decoys which bind and inactivate daptomycin (77). This has major clinical relevance as *agr* mutants arise frequently in invasive *S. aureus* infections (78–80). This finding emphasizes that some pathways induce multidrug tolerance, while others may be specific to a single drug. Understanding the distinct mechanisms by which tolerance arises to individual drugs may lead to improved therapeutics. For instance, it was shown that a FASII inhibitor prevented phospholipid release from *S. aureus* cells leading to improved daptomycin killing (81).

Membrane-acting agents can also potentiate aminoglycosides (70, 82). Aminoglycosides are a broad-spectrum antibiotic that require proton motive force (PMF) for uptake into the cell. Following uptake, aminoglycosides bind to the 30S ribosomal subunit promoting tRNA mismatching which leads to the production of toxic peptides and cell death (64). This class of antibiotics is very effective against respiring cells which maintain a PMF at sufficient levels to facilitate drug uptake. However, aminoglycosides have limited utility against tolerant populations that maintain a PMF below the threshold required for drug uptake, such as anaerobically growing cells, small colony variants, and metabolically indolent cells within a biofilm (36, 70, 83). Pairing membrane-acting agents with aminoglycosides can increase drug uptake and sensitize recalcitrant *S. aureus* populations. Importantly, research has repeatedly shown that once the penetration barrier is overcome, aminoglycosides are highly bactericidal, even against persister cells, suggesting protein synthesis is sufficiently active in persisters for its corruption to lead to cell death (70, 84, 85). Rhamnolipids, biosurfactants produced by *Pseudomonas aeruginosa*, permeabilize the *S. aureus* membrane to

allow PMF-independent diffusion of tobramycin into the cell (70, 86). This combination of tobramycin and rhamnolipids rapidly sterilized anaerobically growing cells and small colony variants and increased efficacy against biofilms by $\sim 1,000$ -fold. In another study, synthetic retinoids which are analogues of vitamin A that target lipid bilayers showed impressive activity alone and in combination with gentamicin in a mouse model of chronic MRSA (70, 82). Despite the promise of membrane-acting agents, identifying novel agents that specifically interact with bacterial membranes and exhibit low toxicity against host cells is challenging.

Another avenue of interest is the use of antimicrobial peptides (AMPs) to treat recalcitrant *S. aureus* infections. AMPs are a diverse class of naturally occurring defense peptides produced by all organisms, with activity against bacteria, viruses, and fungi (87, 88). Cationic AMPs usually consist of 10 to 50 amino acids which form positively charged, hydrophobic structures that are soluble in water, with very little secondary structure. With a high ratio of hydrophobic amino acids and an overall positive charge, AMPs selectively bind and disrupt the negatively charged bacterial cell membranes or cell walls, which can cause lysis and cell death (89). Due to their mechanism of action, it is unsurprising that AMPs have shown efficacy against biofilms; derivatives of LL37 eradicated MRSA biofilms on thermally wounded human skin equivalent models (90), and several studies have demonstrated efficacy against *S. aureus* infections *in vivo* (91–93). Synthetic cationic peptide nanoparticles containing a hydrophobic cholesterol core form micelles in the *S. aureus* membrane. When administered intraperitoneally in rabbits, these nanoparticles were able to cross the blood brain barrier and suppress *S. aureus* growth in a meningitis model (94).

AMPs can also be used to potentiate conventional antibiotics. The human β -defensins and CAP18 produced by epithelial cells synergize with β -lactams and improve killing of *S. aureus in vitro* (95). The frog-produced Citropin 1.1 displayed antibiofilm efficacy alone and improved the activity of rifampicin and minocycline in a central venous catheter rat model of *S. aureus* infection (96). In addition to their bactericidal activity, AMPs also recruit immune cells to the site of infection and modulate cytokine production (97). The role of AMPs in immune homeostasis as well as high production costs will likely hinder their therapeutic development (98). In addition, resistance to AMPs is conferred by any mutation that reduces the negative charge of the membrane, thus decreasing drug interaction (99). This is thought to be a general strategy used by gut bacteria to tolerate host-produced AMPs but also presents an insight into the mechanism that resistance could occur if AMPs were to be developed as therapeutics.

Another potent antipersisters drug class is the acyldepsipeptides (ADEPs). ADEPs bind to the ClpP peptidase in *S. aureus*. Under normal conditions, ClpP degrades proteins in conjunction with the ClpC and ClpX unfoldases in an ATP-dependent manner. ADEPs cause the catalytic core of ClpP to expand, resulting in the formation of a dysregulated protease that digests bacterial proteins in an ATP-independent manner (69). Development of ADEPs was abandoned by Bayer in the 2000s due to the high frequency of resistance; mutants of the nonessential *clpP* arise at a frequency of 10^{-6} . However, *clpP* mutants display decreased virulence and increased susceptibility to other antibiotics (69, 100). Indeed, ADEP combined with rifampicin led to impressive *in vivo* activity against biofilms in a murine deep-seated *S. aureus* infection (69). Recently, modifications to the ADEP structure has led to improved biological activity and metabolic stability (101, 102). ADEPs also have activity against *Bacillus*, *Streptococcus*, and *Enterococcus* bacteria, and they currently remain in preclinical development (100, 103).

Despite the promise of antipersisters drugs, eradication of the population *in vivo* remains arduous, and antipersisters activity alone may be insufficient to eradicate some recalcitrant infections. This is evidenced in a mouse catheter model where, following 7 days of treatment, daptomycin cleared less than 10% of *S. aureus* biofilms (104). In this scenario, it is possible that other factors such as drug penetration or membrane composition were impeding therapy. Additionally, the capacity of many antipersisters drugs to pass into host cells and target intracellular persisters has yet to be explored.

Improving antibiotic penetration. Evidence of *in vivo* reservoirs of *S. aureus* poorly accessed by antibiotics has fueled several innovative strategies for improving drug delivery to tackle this problem.

Ultrasound. Ultrasound technology, traditionally used in medical imaging, uses sound waves that travel through soft tissues and fluids. When combined with intravenously administered microbubbles, ultrasound applications can be expanded to improve drug delivery. Upon exposure to an ultrasound wave, gas-filled microbubbles will oscillate; at low pressures, bubbles continuously expand and contract; and at higher pressures, the bubbles violently collapse. Ultrasound pressures can be exploited to create fluid movements around the bubble to induce shear stress to nearby biological structures, such as biofilms (105). A disadvantage of microbubbles is their short half-life following injection and their relatively large size (1 to 4 μm in diameter) (106), which may hamper penetration into biofilms. Maintaining microbubbles in the more stable liquid phase (nanodroplets) is hypothesized to improve penetration into biofilms due to their smaller size (0.1 to 0.4 μm in diameter) (107). Upon ultrasound activation, nanodroplets expand into microbubbles, creating shear stress from inside the biofilm (108). This experimental treatment strategy has successfully improved antibiotic efficacy against *S. aureus* biofilms *in vitro* (107, 108), improved transdermal drug delivery (109), and transiently permeabilized the blood brain barrier (110); in theory, this method could be expanded to improve drug penetration to any niche that is vascularized. In addition, it has the potential for rapid clinical translation, as ultrasound is already used routinely in wound debridement, some nanodroplet formulations are variants of FDA-approved ultrasound contrast agents that are already in clinical practice (111), and the low-pressure ultrasound settings required can be achieved with existing ultrasound hardware at pressures below the FDA set limits for diagnostic imaging (107).

Liposomes and nanoparticles. Technological advances in nanoscience have yielded promising results in drug delivery using nanoparticles. Research has been dominated in the area of cancer therapeutics, but nanoparticle-bound-antibiotics is a growing research area. Pharmacokinetic data assess the length of time specific doses of antibiotics remain in the body. Dosing regimens desired to achieve clinical efficacy are decided based on pharmacokinetics, toxicity, and *in vitro* susceptibility data. For most infections, antibiotics must penetrate out of the blood and into host tissues to achieve efficacy. This is dependent on many factors, including initial concentration in the serum, blood flow to the tissue, penetration barriers such as host membranes, as well as effects of disease on these factors (112). Encapsulating drugs in liposomes, artificial spherical nanoparticle phospholipid vesicles, can drastically affect the pharmacokinetic properties and is a promising strategy for improving drug delivery. Drugs encapsulated in phospholipid, a component of the host membrane, interact with host cells and other biological structures, such as bacterial biofilms, to facilitate drug delivery. The aliphatic composition of phospholipids facilitates incorporation of hydrophilic drugs into the hydrophilic aqueous space or hydrophobic drugs into the hydrophobic membrane (112). An advantage to this strategy is improving the pharmacokinetics of antibiotics and potentially decreasing toxicity. An obstacle of liposomes is their osmotic fragility which can be improved by incorporating polyethylene glycol into the outer membrane. This has been shown to improve blood circulation times (113, 114).

Encapsulating front-line anti-*Mycobacterium tuberculosis* drugs (isoniazid, pyrazinamide, and rifampicin) in liposomes significantly increased their therapeutic index (115). Similar strategies have been applied to *S. aureus*; flucloxacillin, rifampicin, and vancomycin encapsulated in liposomes all improved killing of *S. aureus* cells internalized in macrophages and embedded in biofilms (45, 55). It is interesting to consider the potential of encapsulating antipersisters drugs in liposomes as a dual approach for improving antibiotic efficacy against biofilms (Fig. 2).

Other types of nanoparticles, such as polymeric nanoparticles, also serve as excellent drug carriers. Polymeric nanoparticles are polymer-based nanoparticles which can protect drugs from degradation, improve pharmacokinetics, and achieve localized high concentrations of drug at the site of infection. Formulations can be modified to

FIG 2 A combined approach for improving antibiotic efficacy against *S. aureus* biofilms. (A) Biofilms display remarkable tolerance to antibiotics. Susceptible cells at the biofilm periphery die (dead *S. aureus*), while less metabolically active cells within the biofilm are tolerant to conventional antibiotics (tolerant *S. aureus*). Failure to eradicate the biofilm leads to relapse in infection following removal of the antibiotic. (B) Encapsulating drugs in liposomes will improve penetration of some conventional antibiotics that do not penetrate well through the biofilm matrix. This strategy is limited, as it does not improve killing of antibiotic-tolerant persister cells (tolerant *S. aureus*) and leads to relapse. (C) Targeting biofilms with antibiotics which kill persister cells (antipersister drug) improves efficacy, but if drug penetration is impeded into the biofilm, some cells will remain viable following drug treatment and lead to relapse. (D) Encapsulating antipersister drugs in liposomes improves penetration of drugs that are active against persister cells. Combining strategies to improve antibiotic efficacy against biofilms will improve the chances of eradicating all cells within a biofilm and prevent relapse of infection following removal of the antibiotic. Schematic credit: created with BioRender and modified by Patrick Lane, ScEYence Studios.

tailor drug release and maximize the amount of time the drug is maintained above the MIC (116, 117). This strategy is proposed to increase the therapeutic potential of the drug while minimizing the chance of resistance development.

Antibody-based antibiotic therapy. The ability of intracellular *S. aureus* to evade both antibiotic and immune-mediated killing, as well as seed secondary infections, suggests that sensitizing intracellular populations to antibiotics is a crucial strategy for improving efficacy. Antibody-antibiotic conjugate (AAC) therapy has proven more effective than antibiotic treatment alone in eradicating intracellular *S. aureus* (118). AAC is composed of an anti-*S. aureus* antibody linked to a rifamycin antibiotic using a protease-sensitive linker (118). *S. aureus* is opsonized by the anti-*S. aureus* antibody and phagocytosed by immune cells. Upon internalization, the linker is cleaved by host cell proteases, releasing the antibiotic in close proximity to and, importantly, in the same compartment as the bacteria (118). The AAC therapy was shown to effectively kill *in vitro* persister cells, internalized *S. aureus*, and, in conjunction with vancomycin treatment, prevented internalized *S. aureus* from causing secondary infection in mice (118). The superior potency, efficacy, and specificity of the AAC therapy, particularly in combination with vancomycin treatment, render AAC therapy a viable option for solving the following two major problems associated with treatment failure: antibiotic-tolerant bacteria and poor antibiotic penetration into host cells. Despite the promise of this approach, high production costs may hinder the translation of this therapy to the clinic (119).

Metabolic priming. As persister cells are frequently in a state of metabolic quiescence, coercing them out of that state by providing metabolites is an established means of sensitizing persisters to antibiotics. Allison et al. found that addition of carbon metabolites sensitized *Escherichia coli* and *S. aureus* to aminoglycoside antibiotics (84). Interestingly, metabolites that stimulated the beginning steps of glycolysis (i.e., glucose, mannitol, and fructose) induced the most rapid and efficacious killing of persisters, while metabolites that stimulated the end of glycolysis, as well as the pentose phosphate and the Entner-Doudoroff pathways, resulted in less potentiation (84). Supplementing these metabolites increased PMF, facilitating aminoglycoside uptake without requiring resumption of growth (84). This treatment strategy was effective under both aerobic and anaerobic conditions, against *S. aureus* and *E. coli* biofilms, and in a murine chronic catheter-associated urinary tract infection model (84). Subsequently, a similar phenomenon was described for *P. aeruginosa* where addition

of TCA cycle intermediates similarly stimulated respiration and increased PMF, sensitizing *P. aeruginosa* to aminoglycoside antibiotics (85). Stalling or shunting the TCA cycle was associated with increased tolerance (85). In *S. aureus*, decreased TCA cycle activity was shown to contribute to rifampicin tolerance following ROS exposure (50). Upon addition of excess glucose, rifampicin susceptibility was restored, presumably by circumventing the TCA cycle generating ATP through substrate-level phosphorylation (50). However, the feasibility of metabolic priming to sensitize *S. aureus* persisters in patients remains to be seen. It is likely that the specific limiting nutrient(s) would need to be identified for a given niche of interest, and it is therefore unlikely that a single molecule will serve as a universal persister resuscitation signal. Additionally, the nutrients available for resuscitating persisters are limited, as any nutrient easily metabolized by host cells would be difficult to deliver at sufficiently high levels to the pathogen populations.

Immunomodulation. In addition to directly targeting the bacteria, it may be possible to target host pathways that induce antibiotic tolerance during infection. High levels of ROS are associated with an M1-like proinflammatory macrophage response (50, 120–122). Thus, in the case of ROS-dependent induction of antibiotic tolerance, modulation of the immune response toward an anti-inflammatory M2-like response where ROS production is decreased (120, 122, 123) may improve antibiotic efficacy. Indeed, the use of antioxidants was shown to increase antibiotic efficacy in a murine bacteremia model of *S. aureus* infection (50). Additionally, corticosteroids, such as dexamethasone, are commonly used in combination with antibiotics to treat bacterial infections (124, 125). The effects of corticosteroids are largely immunosuppressive, although effects on innate immune cells, such as macrophages and monocytes, are complex (126–130). In an *S. aureus* arthritis model, coadministration of dexamethasone and antibiotics significantly decreased disease severity and resulted in rapid resolution of infection compared with antibiotic treatment alone (131). Other studies using human macrophages have found that treatment of macrophages with dexamethasone increases phagocytosis, as well as killing of *S. aureus* (132).

In contrast, chronic and recurrent *S. aureus* biofilms effectively evade macrophage-mediated killing by skewing the macrophage response to an alternatively activated M2-like state (133–136). Treatment of *S. aureus* tissue and catheter-associated biofilms with either EP67, a C5 α receptor agonist, or classically activated M1-like macrophages decreased biofilm bacterial burden compared with antibiotic treatment alone (135). However, the effect(s) of classically or alternatively activated macrophages in combination with antibiotic treatment outcome in biofilm infections is unclear.

Despite the promise of targeting the host to increase antibiotic efficacy, immunomodulation carries with it its own set of risks and challenges. The initial proinflammatory immune response is critical for controlling initial *S. aureus* burden, thus bringing into question when an immunosuppressive treatment should be administered (50, 137, 138). In addition, the benefits of activating or suppressing the immune response may be niche and infection type dependent. In the case of corticosteroids, treatment outcome may be adversely affected by patient age and comorbidities (139, 140). Similarly, stimulating an immune response may lead to tissue damage and cytotoxicity associated with systemic inflammation (138). Despite potential adverse effects, immunomodulation remains an exciting and promising strategy for combating antibiotic treatment failure, but more research is required to explore its promise and limitations.

SUMMARY

Research in *E. coli* and more recently in *S. aureus* has underscored that there is not a singular mechanism of persister formation, and consequently, there is not a single “cure” for the problem. Therefore, understanding the nature of treatment failure to each drug for each indication is crucial for improving therapy. Although the examination of multidrug tolerance in broth culture has led to many important foundational discoveries, we need to now consider the barriers to the efficacy of clinically relevant

antibiotics during infection. We must determine where *S. aureus* persisters are located and how they form in relevant murine models and eventually in patients. Murine models are a powerful resource which have afforded us immeasurable insight into the nature of *S. aureus* infections, but they have important limitations (141); some receptors of key *S. aureus* toxins present in humans are absent in mice (142), and neutrophils from mice do not express defensins, the potent antimicrobial peptide expressed by human neutrophils (143). The examination of antibiotic efficacy using human primary cells and the recently developed humanized mouse lines must be encouraged in order to bridge the gap from mouse relevance to human relevance (144).

We must continue drug discovery and development efforts with a focus on efficacy against persister cells. We must develop improved delivery methods to allow antibiotics to reach recalcitrant populations. Admittedly, many of the solutions proposed here are costly and may currently suffer from commercial viability issues. However, there is a critical need to improve antibiotic therapies; Medicare is burdened with costs of over \$30 billion annually for chronic wounds alone; and risk factors for chronic bacterial infections, such as obesity and diabetes, are increasing at an alarming rate (11). Perhaps government investment in these novel therapies will be less expensive in the long run as they promise to improve killing of problematic subpopulations of *S. aureus* and get us closer to the ultimate goal—efficient and reliable eradication of *S. aureus* infection.

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