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Non-conventional therapeutics against *Staphylococcus aureus*

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I. Introduction

Staphylococcus aureus is one of the most important human pathogens, causing a variety of disease including skin and soft tissue infections, osteomyelitis, endocarditis, surgical site infections, pneumonia, and sepsis. In recent decades, the treatment of staphylococcal infections has become increasingly difficult as the prevalence of multi-drug resistant strains continues to rise. Penicillin-resistant *S. aureus* emerged in the 1940s, followed by the appearance of methicillin-resistant *S. aureus* (MRSA) in 1961 (1, 2). Subsequent introduction of new antibiotics has been followed by reports of resistance (3). With increasing mortality rates and medical costs associated with MRSA and other drug resistant strains, there is an urgent need for alternative therapeutic options (4). Therefore, considerable effort has been put forth to identify and develop novel *S. aureus* treatment strategies as alternatives to conventional antibiotics.

II. Prevention and Disruption of Biofilm Formation

Biofilms are multicellular, three dimensional aggregates of bacteria embedded in a matrix composed of polysaccharides, extracellular DNA, proteins and/or lipids and are formed as an adaptation to environmental stress. *S. aureus* biofilms are notorious for causing chronic infections due to their ability to adhere to living tissues and implanted medical devices (artificial heart valves, catheters, and joint prosthetics, etc.), as well as their inherent recalcitrance to antibiotics (5–7). These biofilm-related infections lead to increases in morbidity, mortality, and healthcare costs, with infected devices often requiring surgical removal. Yet, antibiotic resistance is adaptive due to the fact that biofilm-associated resistant bacteria revert to their planktonic susceptible phenotype as they disperse from the established biofilm (8). Thus, considerable effort has been put forth to identify effective antimicrobials that specifically treat *S. aureus* biofilms.

The biofilm extracellular matrix serves as a protective physical barrier that shelters the resident bacteria against antibiotics and host immune defenses. Therefore, approaches to disrupt the matrix by enzymatically degrading the chemical components have been investigated. DNase I-mediated degradation of extracellular DNA appears to be effective in disrupting early *S. aureus* biofilms and treatment with trypsin or proteinase K disrupts the protein components of the biofilm matrix (9–12). Likewise, dispersin B, a glycoside hydrolase produced by the periodontal pathogen *Actinobacillus actinomycetemcomitans*, is able to breakdown the polysaccharide components of staphylococcal biofilms and can promote antibiotic penetration, resulting in synergistic killing when combined with the antibiotics cefamandole nafate or triclosan (13–15). However, a number of clinical strains are capable of forming polysaccharide-independent biofilms and thus *S. aureus* susceptibility to dispersin B can vary widely among strains (16, 17). Additional glycoside hydrolases, α-amylase and cellulase, and lysostaphin, a glycine endopeptidase produced by *Staphylococcus simulans* that cleaves the pentaglycine bridge in the staphylococcal cell wall, have also been shown to significantly reduce matrix biomass of *S. aureus* biofilms *in vitro* (18, 19). Although these *in vitro* results are promising, the application of exoenzymes as therapeutic drugs may be limited due to the possibility of protein-induced inflammatory responses in the host, toxicity, or immunity. Alternatively, these enzymes could be employed in an approach similar to an “antibiotic lock” where a high concentration is applied to catheter lumens to prevent catheter-associated *S. aureus* infections (12, 20). The efficacy of this strategy was demonstrated when implanted jugular vein catheters in mice pre-instilled with lysostaphin provided complete protection against *S. aureus* infection compared to untreated catheters (21).

The release of planktonic cells has been shown to result in increased susceptibility to antimicrobials, thus combining molecules that induce biofilm dispersal with traditional antibiotics could be another viable strategy to eradicate *S. aureus* infections (22). One such candidate is *cis*-2-decenoic acid, a fatty acid produced by *Pseudomonas aeruginosa,* that causes an increase in planktonic bacteria released by *S. aureus* biofilms (23). Although the mechanism by which this occurs is not understood and further studies are needed to confirm these findings, it does suggest *cis*-2-decenoic acid could be utilized as a dispersal agent. Moreover, *S. aureus* produces a number of endogenous dispersal agents, including the surfactant-like molecules phenol soluble modulins (PSMs). PSMs are intrinsically inflammatory and cytolytic for neutrophils, therefore repurposing PSMs into therapeutic dispersal agents seems questionable. However, due to the fact that PSMs are key to proper biofilm formation (24, 25), interference with PSM production or secretion could prove to be an effective approach to inducing dispersal of *S. aureus* biofilms and enhancing antibiotic killing (26).

Targeting bacterial iron metabolism through the use of chelators and gallium-based therapeutics has been demonstrated to effectively disrupt staphylococcal biofilms (27). Iron is crucial for a variety of cellular processes including DNA synthesis, energy production, respiration, and biofilm formation and thus is a potential target for anti-staphylococcal therapeutics (28). Due to their structural similarity, gallium is able to serve as an iron analog. Applying a ‘Trojan Horse’ strategy, gallium complexes are imported into the cell through bacterial iron uptake systems, where once inside, gallium competes with iron by binding to

iron- dependent enzymes and molecules. This results in disruption of vital iron-dependent activities including respiration, DNA synthesis, biofilm production, and bacterial proliferation (27). Gallium nitrate [$Ga(NO_3)_3$] has been shown to be effective at reducing bacterial biofilms *in vitro* and mice treated with gallium maltolate had significantly lower bacterial burdens 48 hours post treatment in a burn wound model of *S. aureus* infection (29, 30). However, not all gallium-based molecules exhibit antimicrobial effects. Conjugation of gallium to the *S. aureus* siderophore staphyloferrin A failed to effectively inhibit MRSA (31).

Recently a combination therapy of synthetic gallium-based heme analogs and a metal chelator have shown promise as effective antimicrobials against *S. aureus* biofilms. Heme bound to hemoglobin is the most abundant source of iron within the host and is the preferred iron source for *S. aureus* (32). The metalloporphyrin gallium-protoporphyrin IX (GaPP) is capable of mimicking heme, thus facilitating its uptake (33). Once inside the cell, GaPP can be substituted for heme in heme-containing enzymes, including cytochromes, catalases, and peroxidases, disrupting vital cellular processes (33). *In vitro* studies indicate treatment with the iron chelator deferiprone and GaPP results in significant reduction of MSSA and MRSA biofilms (34). Similar antimicrobial activity was also observed against biofilms formed by small colony variant *S. aureus* strains, which are linked to increased antibiotic tolerance and resistance (35). Moreover, this combination therapy has the ability to potentiate antibiotic-mediated killing, thus combining current antimicrobials with gallium could be a promising strategy for treatment of biofilm infections (36).

Although current data indicate GaPP-mediated treatments may prove to be efficacious for abolishing *S. aureus* biofilms, the possibility of cytotoxicity due to inference with host iron metabolism should not be entirely discounted. Loss of cell viability and increased lactate dehydrogenase production, a biomarker for cellular cytotoxicity and cytolysis, have been observed in a number of mammalian cell lines when exposed to high concentrations of GaPP (33, 34, 37). However, concentrations of GaPP that induced cytotoxicity were considerably higher than those needed to significantly inhibit *S. aureus* biofilms (33, 35). Moreover, no health effects or changes in behavior were observed in mice given a single intraperitoneal dose of GaPP (25–30 mg/kg), followed by a daily dose (10–12 mg/kg) of GaPP given for an additional four days (33). This suggests that with optimized dosing of GaPP-based therapeutics, host toxicity could be avoided without compromising GaPP antimicrobial activity.

Antimicrobial peptides (AMPs) have been increasingly recognized for their anti-biofilm properties. AMPs are typically small, cationic peptides that exhibit a range of antimicrobial and immunological properties. One of the first recognized AMPs with antimicrobial activity against *S. aureus* biofilms was the human cathelicidin peptide, LL-37 (38). This peptide displays bactericidal activities against a wide range of Gram positive and Gram negative pathogens by disrupting the bacterial membrane (39). LL-37 synthetic derivative OP-145, when integrated into a medical device coating, was shown to prevent *S. aureus*-induced implant associated infections in rabbits (40). Moreover, OP-145 has been successfully used in a clinical Phase 2 trial for the treatment of chronic otitis media (41). Another LL-37 derivative, SAAP-148 demonstrated significant efficacy against MRSA where treatment led

to complete eradication of established biofilms *in vitro*, as well as *in vivo* in a murine wound model (42). Furthermore, co- treatment of AMPs IB-367 or BMAP-28 with antibiotics was shown to be highly effective at treating catheter-associated infections, suggesting AMPs could also be used to potentiate antibiotic killing of *S. aureus* biofilms (43, 44).

Another innovative attempt to effectively eradicate *S. aureus* biofilms includes the use of small-molecule inhibitors. A variety of small molecules with activity against *S. aureus* biofilms *in vitro* have been identified, including aryl rhodanines, D-amino acids, benzimidazole, and metal chelators (12, 45–47). An inhibitor of the essential *S. aureus* protein RnpA (RNP1000) significantly reduced the number of biofilm bacteria in an *in vitro* catheter model, as well as protected against lethal systemic *S. aureus* infection in mice (48). These results are encouraging and suggest small molecules that exhibit strong anti-biofilm activities *in vitro* could be potent antimicrobials. However, very few small molecule biofilm inhibitors have been tested in animal models and thus the ability of these compounds to treat *S. aureus* infections is not yet well defined.

III. Inhibition of Virulence by Targeting Quorum Sensing

Virulence factor production in *S.* aureus is regulated by quorum sensing (QS), a cell to-cell communication mechanism bacteria use to regulate gene expression in response to cellular density. The *S. aureus* QS system is under the control of the accessory gene regulator (*agr*) system and activation of the *agr* system by an accumulation of auto-inducing peptide (AIP) leads to activation of the *agr* regulatory network that controls expression of virulence factors by RNAIII, the major effector for downstream virulence expression and biofilm dispersal (10, 49–51). Inhibiting QS would prevent the production of QS-regulated toxins such as delta-toxin, staphylococcal enterotoxin C, and Panton-Valentine leukocidin, thus restricting *S. aureus'* ability to evade the host immune system, kill host cells, and disseminate (52). Moreover, targeting virulence systems like QS, rather than systems critical for bacterial survival, may exert less selective pressure for the development of resistance as compared to traditional antibiotics.

A number of synthetic and natural QS quenchers have been evaluated for their efficacy against MSSA and MRSA. Biaryl hydroxyketones were shown to successfully inhibit QS by preventing the interaction between the AgrA transcriptional regulator and the P3 promoter, which drives the transcription of the RNAIII master virulence regulator (53). Follow up studies with synthesized biaryl hydroxyketones demonstrated compound F12 was capable of reducing MRSA-induced rabbit erythrocyte hemolysis by 98% *in vitro* (54). In a *Galleria mellonella* insect larvae infection model, F12 treatment led to increased larval survival from 12 hours in untreated controls to 42 hours and combining biaryl hydroxyketones with β-lactam antibiotics cephalothin or naficillin, both of which MRSA is resistant to, further increased larval survival (55). However, in a murine wound infection model, compounds F12 and F1 promoted only modest increases in wound healing and there were no significant differences in wound bacterial burdens between treatment groups (55). This suggests the success of biaryl hydroxyketones to treat MRSA may be highly dependent on the infection model utilized and therefore it has yet to be proven that biaryl hydroxyketone inhibition of QS will be an effective drug development strategy.

Additional ArgA-targeting molecules include the synthetic small molecule savarin and the natural product ω-hydroxyemodin (OHM). Savirin, which is capable of blocking *S. aureus* QS, attenuates *S. aureus* in a murine skin lesion infection model (56). Importantly, extensive passage of *S. aureus* in the presence of savarin does not lead to the development of resistance. OHM, a polyhydroxyanthraquinone isolated from the fungus *Penicillium restrictum,* successfully reduced inflammatory cell recruitment and cytokine production and promoted bacterial cell clearance in a murine model of *S. aureus* skin infection (57, 58). Both molecules show promise for skin and soft tissue infections, however it is unknown if these molecules will be effective in other infection models.

Another promising QS inhibitor is ambuic acid, a fungal small molecule metabolite that selectively inhibits *S. aureus* AIP production (59). Treatment with ambuic acid resulted in decreased lesion size and reduced weight loss in a murine model of *S. aureus* skin and soft tissue infection (59). Furthermore, plant-derived quorum sensing inhibitors such as hamamelitannin and its associated derivatives, ajoene, and cinnamaldehyde exhibit potent killing against *S. aureus* biofilms alone or in combination with antibiotics (60–66).

Pursuing additional components of the staphylococcal QS regulatory network could be an alternative option for development of QS-targeted therapeutics, however further research into the molecular mechanisms of QS regulation is needed. Another biofilm and virulence regulatory locus, staphylococcal accessory regulator (*sar*), is a promising target as it is thought to perform an opposing role to *agr* in *S. aureus* biofilm formation (45, 67–69). Overexpression of *sar* can inhibit biofilm production in some *S. aureus* strains; however, other studies demonstrate *sarA* facilitates the expression of *agr* (67, 68). Although it is clear the *agr* and *sar* systems are important regulators of *S. aureus* biofilm activities, a better understanding of the roles and relationships between *agr* and *sar* and how modulators of *agr* or *sar* affect *S. aureus* QS signaling, virulence factor production, and biofilm formation will be necessary to advance the development of drugs targeting QS.

IV. Bacteriophage-Based Therapy

Bacteriophages (phages) have evolved to be the ultimate bacteriocidal agents. Phages are viruses that infect bacteria and multiply via a lytic cycle in which the phage particle attaches to the host, injects its genomic material, manipulates the host machinery resulting in intracellular phage multiplication. The cycle is complete when the bacterial cell is lysed, releasing multiple phage progeny. The antimicrobial power of lytic phages against staphylococcal infections was recognized as early as the 1920's, however with the discovery of antibiotics, phage therapy quickly fell out of favor in western medicine (70–72). Yet with the rise of multi-drug resistant bacteria, the use of phage-based therapies as an alternative to antibiotic treatment has garnered a renewed interest from the medical and research communities.

A number of factors make phage therapy an attractive therapeutic strategy for *S. aureus* infections. Highly conserved components of the cell wall, such as teichoic acids, serve as phage receptors in *S. aureus;* thus, the likelihood of developing resistance to this type of therapy is reduced and strains that do develop resistance often exhibit a reduction in

virulence or fitness (73). The highly specific nature of phages results in only targeted bacteria being infected and subsequently killed, which prevents the disruption of the resident microbiota and morbidities associated with microbiota dysbiosis (74, 75). Moreover, phage therapy eliminates the potential for toxicity that is associated with many antibiotics. Additionally, many phages are capable of targeting multiple strains, including both MRSA and methicillin-susceptible *S. aureus* (MSSA) (76–80).

The efficacy of phage therapy has been explored for a wide range of *S. aureus* diseases including skin and soft tissue infections, sepsis, pneumonia, and osteomyelitis utilizing animal models with relatively good success (76, 79–88). An early report examining *S. aureus* skin infections in rabbits demonstrated simultaneous subcutaneous administration of LS2a phage and *S. aureus* prevented abscess formation in 88% of the rabbits treated (81). Abscess size and bacterial burdens were also shown to significantly decrease compared to untreated infected controls in a dose-dependent response (81). A similar outcome was observed in skin lesions of mice infected with MRSA and injected intraperitoneally with SATA-8505 phage, however in this case lesion size failed to decrease despite a reduction in bacterial burdens (79). Phages have also been shown to protect against lethal doses of *S. aureus* (76, 82). Matsuzaki, *et. al* demonstrated intraperitoneal treatment with φMR11 phage led to complete protection against a *S. aureus* systemic infection, whereas untreated mice exhibited a mortality rate greater than 90% 24 hours post infection (76). Phage levels rapidly increased within the blood stream and remained high until 6 hours post infection, coinciding with a drop in bacterial burdens below detectable limits (76). Moreover, phage therapy has been shown to be effective against chronic *S. aureus* infections. Intravenous injection of M^{sa} phage suspension into mice that were systemically infected with a low dose of *S.* aureus 10 days *a priori*, resulted in a significant drop in organ bacterial counts to below the limit of detection compared to infected untreated control mice (82). Combined, these studies provide clear evidence that phages are able to multiply and kill *S. aureus in vivo,* leading to disease reduction. *S. aureus* biofilms on indwelling medical devices and in the sinonasal cavity are notoriously difficult to eradicate, thus some phage studies have focused specifically on the treatment of biofilms. Multiple groups have demonstrated that lytic phages are capable of significantly reducing biofilm biomass *in vitro*; however, it is still unclear if this is the case *in vivo* (89–91). Recently Drilling, et. al. described a significant reduction in *S. aureus* biofilm mass in the frontal sinuses of sheep that were flushed with a cocktails of *S. aureus* specific phages compared to those treated with heat-inactivated viruses (92, 93). Moreover, bacteriophage treatment significantly reduced *S. aureus* colonization of an in-dwelling catheter compared to controls in a rabbit model (94). Although these results are encouraging, questions of the efficacy of phage therapy for biofilm-associated infections, particularly in relation to biofilms in less accessible body sites, such as those coating joint prosthetics or artificial heart valves, have not been extensively addressed. In a single study using a rat orthopedic implant infection model, local injection of phage significantly decreased *S. aureus* colony forming units and biofilm thickness on the implant as compared to the control (95). Pretreating the surface of such devices with a coating of phages may also prevent *S. aureus* medical device colonization and subsequent disease. Studies examining phage-coated orthopedic implants in mice saw a significant reduction in bacterial adherence to the device (96) and bacterial load in adjoining tissues (97). Combined, these studies suggest phage

therapy could be applicable towards a number of *S. aureus* biofilm-associated diseases and phage prophylaxis could help prevent *S. aureus* infections of indwelling medical devices.

A few attempts have been made to turn phages into drug delivery systems to increase the efficacy of *S. aureus* treatments (98–102). Bacteriophage 75 complex was used to administer a photosensitizer to *S. aureus* cells, which significantly enhanced MRSA and MSSA killing when exposed to red light *in vitro* (102). Additional reports describe the use of phages to transfer the antibiotic chloramphenicol to *S. aureus* cells, however bacterial growth was only partially inhibited due to limitations of drug-loading capacity caused by the drug's hydrophobicity (100, 101). Although the concept of manipulating phages into highly specific drug transfer systems is appealing, additional research is needed to further develop this strategy and determine if it could be applicable to the wide breadth of *S. aureus* disease presentations.

At this time, clinical use of therapeutic phages is limited to European countries and the former Soviet Union (71, 103–105). No formal regulations or standards for phage therapy in these countries exist, thus well documented clinical trials including robust controls are lacking (104). Therefore, it is difficult to come to any definitive conclusions, as well as to confidently assess the risks associated with these treatments in humans (106, 107). Nevertheless, multiple reports have described positive clinical outcomes associated with phage therapy for a wide range of *S. aureus* diseases in humans, including respiratory, circulatory, orthopedic, and soft tissue infections (71, 105, 108–110). These clinical reports, taken together with the growing body of literature on *in vitro* and *in vivo* studies, demonstrate phage therapy could be a feasible strategy for treating *S. aureus* infections.

However, it should be noted that phage therapy is not without potential pitfalls. Due to the high specificity of phages, one significant drawback can be a narrow spectrum of sensitive strains. This issue can be circumvented by selecting polyvalent phages, i.e. those with the capability to infect a large set of strains within a species or combining multiple phages into a therapeutic cocktail (78, 111, 112). Additionally, the health risks associated with *S. aureus* phage therapy are poorly defined. In most cases no adverse effects have been reported, however it is not unforeseeable that a sudden influx of phage or the release of bacterial toxins due to lysis could stimulate a robust, inflammatory response (70, 103, 113). Immune induction could also lead to the production of antibodies and subsequent clearance of phages, significantly reducing the efficacy of the treatment (114). Moreover, as viruses are replicating biological agents, it would be extremely difficult to standardize commercial production for clinical use. Nonetheless, the potential of phage therapy may outweigh the drawbacks in the face of increasing staphylococcal antibiotic resistance and therefore warrants continued consideration.

V. Staphylolytic Enzymes as Therapeutics

The antimicrobial potential for lytic enzymes was first appreciated by Alexander Fleming upon the discovery of the eukaryotic-derived cell wall hydrolase, lysozyme, however, more recently the staphylolytic enzyme lysostaphin, an endopeptidase that cleaves the pentaglycine crosslinking bridges of peptidoglycan, has garnered much attention as a

potential antimicrobial agent (115). Since identification of the enzyme, a growing body of literature indicates lysostaphin is effective at targeting MSSA, MRSA, and vancomycin-resistant *S. aureus,* as well as *S. aureus* biofilms (19, 116–120). Treatment with lysostaphin systemically or as a material coating has shown promise for eradication of *S. aureus* infections *in vivo* using several animal models (121–123). Moreover, application of lysostaphin reduced *S. aureus* nasal carriage in humans with no reported toxicity. This suggests lysostaphin treatment may also be an effective decolonization strategy (124).

Recombinant phage-derived lysins have also demonstrated to be highly effective antimicrobials *in vitro* and *in vivo* (125). During the lytic phage cycle, viral peptidoglycan hydrolases (endolysins) are produced to facilitate the release of progeny virions by degrading the bacterial cell wall (126). Phage endolysins are particularly attractive as alternative antimicrobial candidates due to a high degree of species and strain specificity (127). Additionally, endolysins have evolved to bind and cleave highly conserved structures in the cell wall without necessitating intracellular transport of the enzyme, thus decreasing the potential for resistance development and avoiding mechanisms that play a role in conventional antibiotic resistance (e.g., active efflux from the cell) (128). Staphylococcal endolysins can differ significantly at the amino acid sequence level, which is also reflected in their diversity of enzymatic and antibacterial properties (129, 130). Combinations of endolysins have been shown to provide a synergistic treatment effect and would also help decrease the chance of resistance development (131–133). Moreover, recombinant endolysin proteins have the potential to be mass-produced for clinical use.

A variety of endolysins and their anti-staphylococcal activity have been characterized, with many identified as being highly effective at clearing *S. aureus in vivo* (128). MV-L, originally derived from the staphylococcal phage ϕMR11, was the first phage endolysin tested in an animal model. These early studies demonstrated MV-L is capable of killing multiple strains of *S. aureus in vivo*, including those with vancomycin and methicillin resistance (134). Since then, additional endolysins with potent lytic activities against drug-resistant strains of *S. aureus* have been identified, including LysK, an endolysin derived from bacteriophage K, and LysK derivatives PlyGH15, ClyH, ClyS, CHAPk, and SAL-1 (83, 135–139). Unlike MV-L where lytic activity is limited to only *S. aureus* strains, LysK has a much broader spectrum of antimicrobial activity that includes the ability to lyse coagulase-negative staphylococci (112). CHAPk, an engineered, truncated version of LysK, has an even broader lytic spectrum that includes members of *Streptococcus* and *Micrococcus* genera (137).

Numerous reports indicate endolysins have the potential to be highly effective against skin and soft-tissue *S. aureus* infections. Intranasal treatment with MV-L successfully eliminated *S. aureus* in the nares of mice and similar nasal decolonization outcomes were observed in mice administered CHAPk orally or intranasally (134, 137). Intranasal inoculation with the engineered endolysin fusion protein ClyS resulted in a 2-log reduction in colony forming units 1 hour post infection of mice intranasally infected with MRSA (136). In a murine skin infection model, bacterial loads were significantly reduced when ClyS was topically applied as compared to mice treated with mupirocin, a commonly prescribed antibiotic for the topical treatment of *S. aureus* skin infections, and untreated controls (140). Additionally,

endolysin MR-10 combined with the antibiotic minocycline significantly reduced the mortality rate and healing time in a murine burn wound model (141).

Endolysins also hold promise as effective therapeutics against more severe *S. aureus* infections. Mice infected intraperitoneally with MRSA were 100% rescued when MV-L was administered intraperitoneally 30 minutes post infection. Similar outcomes were observed in mice systemically infected with MRSA and treated intraperitoneally with endolysins phi11, LysK, 80α WMY, and 2638A (129). After 2 days, 100% of the endolysin-treated mice survived, where only 25% of vehicle-treated mice survived (129). LysGH15 and SAL-1 have also been shown to be effective against systemic MRSA infections (83, 138). Moreover, intravitreal injection of the engineered endolysin Ply187 significantly reduced bacterial burdens in the eye and preserved retinal function in a murine model of endophthalmitis (50). However, delays in treatment time can significantly reduce the efficacy; thus, endolysin-based therapies may need additional optimization to ensure they are effective against the most severe *S. aureus* diseases (134).

Recent attempts have been made to enhance the stability and delivery of endolysins utilizing nanoparticles. Nanoparticles containing CHAPk and lysostaphin in the thermoresponsive polymer Poly(N-isoporopylacrylamide) allowed for the controlled release of the enzymes upon reaching 37°C (142). Moreover, complexing LysK in polycationic polymers enhanced enzyme stability and lytic activity (143). Nanotechnology could prove to be an effective way to enhance endolysin-based therapies and ensure stability at both storage and physiological temperatures.

Notably, the first generation of staphylococcal phage endolysin-based antimicrobial products is already on the market and clinical trials are underway for endolysin-based drugs. Staphefekt XDR.300 is an antiseptic solution that is effective against MSSA and MRSA on human skin and incorporated into a series of creams and gels sold under the Gladskin brand name by the company Micreos Human Health BV (Netherlands). These skin products are for the treatment of skin conditions with an infectious component, such as acne, rosacea, eczema, and skin irritation and contain the active ingredient Staphefekt. SAL200 is a therapeutic formula containing the endolysin SAL-1, a well characterized homolog of LysK derived from the *S. aureus* phage SAP-1 (138, 144). It is the first to have undergone a good laboratory practice (GLP) compliant safety evaluation including single and repeated dose toxicity and organ function studies in rats and dogs, as well as further pharmokinetics and safety testing in monkeys (145, 146). SAL200 has been shown to be well tolerated with limited side effects observed in these studies. Recently, SAL200 was intravenously administered to healthy male humans as part of a Phase 1 clinical trial (147). No serious adverse effects were observed for any of the participants, however there were reports of mild headache, fatigue, and myalgia (147). Additionally, a Phase 2 clinical trial is underway for CF-301, an antistaphylococcal endolysin derived from a prophage originally isolated from *Streptococcus suis* (148, 149). Previous work has demonstrated CF-301 to be highly effective at eradicating *S. aureus* biofilms, including biofilms enriched for the more resistant small-colony variants, and was more effective than antibiotics for the treatment of septicemia in a murine infection model (150, 151). In light of these developments, endolysin-based therapies are likely to be clinically applied in the near future.

VI. RNA Guided Nucleases

The natural bacterial defense system known as clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR associated (Cas) genes enables bacteria to recognize and degrade foreign DNA and can serve as an effective, programmable tool for genome editing (152, 153). The Cas9 endonuclease found in the Type II CRISPR/Cas system uses a 20 nucleotide small RNA guide to specify the site of DNA cleavage (154). Recent studies have demonstrated that re-programing Cas9 to target bacterial genomic sequences can result in effective cell killing (155, 156). Therefore, it may be possible to create highly specific, programmable antimicrobials by exploiting the CRISPR system.

Only recently has the antimicrobial power of CRISPR/Cas systems been tested experimentally (157–159). Bikard, et. al. generated phagemids encoding the packaging site and *rinA, terS,* and *terL* genes from the staphylococcal φNM1 bacteriophage with the CRISPR/Cas9 system that were capable of selectively killing *S. aureus* strains depending on the guide RNA sequence provided (158). Application of a phagemid containing RNA-guided Cas9 specific to the methicillin resistance gene, *mecA,* to a mixed culture of MRSA and MSSA strains resulted in a significant reduction in the proportion of MRSA from 50% (pre-treatment) to 0.4% (post-treatment), with no differences observed in treated MSSA cells or either strain treated with nonspecific Cas9 targets (158). Similar outcomes were observed when phagemids were topically applied in a murine skin colonization model. Mice colonized with a mixture of kanamycin-resistant and kanamycin-sensitive *S. aureus* saw a significant reduction in kanamycin-resistant but not kanamycin-sensitive cells when treated with RNA-guided Cas9 targeting the kanamycin resistance gene *aph* (158). These results suggest programming Cas9 nuclease to be a sequence-specific antimicrobial could be an effective treatment strategy, particularly against drug-resistant *S. aureus* infections, or as a decolonization strategy to selectively eliminate *S. aureus* without disturbing the rest of the host's microbiota.

Effective drug delivery remains a significant hurdle to towards implementation of CRISPR-based antimicrobials (160). As mentioned in the previous section, bacteriophage delivery systems are associated with a number of drawbacks, not limited to reduced host range, poor penetration to areas of infection, and possible adverse health effects. A few attempts have been made to circumvent these issues, including genetically modifying phage tail protein sequences to increase host range, and conjugating the CRISPR/Cas9 system to nanoparticles to eliminate the use of the virus altogether (159, 161). Kang et. al. described a non-viral delivery system where CRISPR/Cas9 machinery was covalently modified with the cationic polymer branched polyethylenimine to form a CRISPR nanocomplex (159). These nanocomplexes significantly reduced growth of MRSA strains *in vitro* compared to the native CRISPR/Cas complex, however their efficacy against *S. aureus* infections *in vivo* remain untested (159). Additional studies examining the use of nanoparticles and other alternative delivery systems are warranted. Future efforts will need to focus on engineering and refining CRISPR/Cas antimicrobial delivery systems, as well as validating these strategies utilizing animal models.

VII. Photodynamic therapy

Photodynamic therapy (PDT) is a treatment method combining photosensitizers, visible light, and oxygen to induce cell death. Photosensitizers accumulate in the targeted cells, and, upon illumination with light of a specific wavelength, become activated from a ground state to an excited state. The energy produced during excitation is either transferred to a cellular substrate and then to oxygen to form several reactive oxygen species (Type I Mechanism), or directly to molecular oxygen to form a highly reactive singlet oxygen (Type II Mechanism) (162). Various biomolecules are affected during this process, specifically proteins, nucleic acids, and unsaturated lipids, resulting in irreversible damage and cell death (163, 164). In some cases, the direct mechanism of cytotoxicity has been investigated which, depending on the photosensitizer and its subcellular location, can be attributed to inactivation of enzymes, damage to the cell membrane, or indirect damage to the chromosome (164–168).

Historically PDT was applied to treat various forms of cancer, however over the past two decades PDT has emerged as an alternative modality for the treatment of localized microbial infections. The non-selective nature of PDT and non-specific damage triggered by reactive oxygen species ensures that the development of antimicrobial resistance is unlikely (169–171). Perhaps even more importantly, the effectiveness of PDT against *S. aureus* is independent of a strain's antibiotic resistance profile; thus PDT treatments can be applied to treat both MSSA and MRSA (172). Moreover, since PDT is exclusively used to treat localized infections, the risk of host cell toxicity and disruption of the microbiota is greatly minimized compared to the use of systemic antibiotics.

A key factor dictating the success of PDT-mediated treatment of *S. aureus* is the selection of a suitable photosensitizer. Consideration should include the propensity of the photosensitizer to preferentially target bacterial cells over host cells, solubility, a long light wavelength absorption band, and high generation of reactive oxygen species (173). Most antimicrobial photosensitizers tested are organic, aromatic dyes, namely porphyrins, chlorines, phthalocyanine, Rose Bengal, phenothiazines, and acridines (174). Although a number of photosensitizers have been approved for use in humans, only a select few have been applied clinically to treat microbial infections. These include methylene blue, toluidine blue O, neutral red, PP904 phenothiazium dye, and protoporphyrin IX formed from the porphyrin precursor 5-aminolevulinic acid (ALA) or the ALA-methyl ester, methyl aminolevulinate (MA) (175).

Depending on the light source, duration of exposure, and photosensitizer used, PDT can be highly effective at reducing both MSSA and MRSA numbers *in vitro* (166, 172, 176–180). For example, combining 50 μg/mL toluidine blue O and 15 minute exposure to 632.8 nm HeNe laser resulted in complete eradication of eight MRSA isolates and PDT treatment with aluminum disulfonated phthalocyanine was shown to effectively inactivate 16 epidemic MRSA strains (181, 182). Similar outcomes were observed for the treatment of *S. aureus* biofilms, where PDT treatment with the photosensitizer hypericin significantly reduced biofilm viability 92–99% in all 22 MRSA strains tested (180).

Effectiveness of PDT has also been explored for a number of animal models of localized *S. aureus* infections. In parallel with what is observed *in vitro,* PDT can be highly successful at eradicating *S. aureus* within the host; however, this is greatly dependent on the photosensitizer, light source, and duration of exposure chosen (183). Nonetheless, PDT has been shown to effectively deplete bacterial levels and decrease wound healing time in superficial skin infections using murine skin abrasion and burn wound models, as well as to reduce bacterial burdens in deeper soft tissue abscesses (184–189). Using fiber optic light delivery systems, PDT treatment has also been quite effective against *S. aureus* osteomyelitis in rats (190–193). Administration of the photosensitizer toluidine blue and a red diode laser resulted in an immediate bacterial reduction of 97% within the bone tissues of *S. aureus* infected rats that was maintained for at least 30 days following treatment (190); moreover, significantly less bone destruction was observed when rats were treated with either toluidine blue or another photosensitizer, Na-Pheophorbide, and the corresponding laser lights (190, 193). Additionally, increased accumulation of neutrophils and bacterial clearance was observed in mice treated with PDT in a murine *S. aureus* septic arthritis model (194, 195).

Despite the non-specific nature of PDT's ROS-induced killing, PDT sensitivity and resistance levels can vary widely among *S. aureus* strains. A recent study comparing MSSA and MRSA strain susceptibilities to PDT inactivation using protoporphyrin arginate, toluidine blue O, and ALA found resistance to PDT was independent of antibiotic resistance or virulence profile (196). Moreover, the study also demonstrated that the same bacterial strain could be categorized as PDT sensitive or resistant depending on the photosensitizer used (196). Coupling PDT treatment with ALA and antibiotics has been shown to enhance bacterial killing compared to PDT alone, however this synergistic effect is not necessarily enough to overcome strain differences in PDT resistance (197–199). Although the mechanism that confers strain-dependent resistance to PDT has not yet been fully elucidated, polymorphic differences in the *agr* locus and Agr system functionality have been shown to correlate with *S. aureus* sensitivity to PDT (200, 201). Continued research into the molecular markers that predict strain responses to photo-inactivation will aid in the development of more effective treatment modalities in the future. One significant issue with PDT-based therapies is the potential for ROS to inflict harm onto neighboring host cells. Thus, a significant challenge in PDT development is to identify mechanisms in which pathogenic bacteria are efficiently inactivated without damaging the surrounding host tissue. Improving the selectivity of photosensitizers has been an area of intense research within the antimicrobial PDT field. Modification of photosensitizers via antibody conjugation, attachment of peptides, and use of bacteriophage delivery systems have been used to enhance the specificity of antimicrobial PDT (102, 202–204). Moreover, targeting bacterial-specific structures has also been proposed. The addition of two phenothiazinium photosensitizers (EtNBS-COOH) to the side chains of cephalosporin resulted in an enzymatically-activated photosensitizer, whereby activation was reliant on the cleavage of the lactam ring by beta-lactamase (205). This novel approach of targeting the antimicrobial resistance mechanism itself resulted in very little non-specific photosensitizer uptake by host cells *in vitro* (205). Small-molecule activation of the coproporphyrinogen oxidase (CgoX), an enzyme essential for heme biosynthesis and specific to gram positive organisms, induced accumulation of the phototoxic heme precursor coproporphyrin III in *S. aureus*, and upon

light exposure led to a reduction of bacterial burdens *in vitro* and in a murine model of skin and soft tissue infections (206).

Nanotechnology has also been applied to enhance the efficacy of antimicrobial PDT by improving photosensitizer solubility, photochemistry, photophysics, and targeting of the pathogen (207, 208). Covalent conjugation of a photosensitizer to a nanostructure or encapsulation in engineered nanoparticles, such as liposomes, micelles, chitosan nanoparticles, and carbon nanotubules, have been proposed to heighten PDT-mediated killing of microbes (207). Nanostructures with cationic charges have been shown to increase the specificity of PDT photosensitizers due to increased binding to the negatively charged microbial membranes (209). Many photosensitizers are insoluble and tend to aggregate, thus nanoparticle-based delivery helps to improve the lethality of PDT via increasing the concentrations of photosensitizer absorbed by the targeted bacterial cells (207, 208, 210). Moreover, some nanoparticles, such as gold, can potentiate PDT by exerting a photo-thermal effect when exposed to light or, as is the case with silver, have intrinsic antimicrobial properties of their own (208, 211). Additional advantages of using nanoparticles include increased photosensitizer resistance to photobleaching and inactivation, generation of higher concentrations of locally produced ROS resulting in more damage to the targeted bacteria, and low immunogenicity (207, 212).

Another chief obstacle for the advancement of PDT technologies is the limited penetration of light into tissues. Several approaches have been employed to overcome this limitation. Major advancements in fiber optics and microendoscopic technology have allowed PDT to be used with interstitial, endoscopic, intraoperative, or laparoscopic light delivery systems (213). This allows light to be delivered to almost any body site in a minimally invasive manner. Moreover, optical clearing with harmless substances such as glycerol that match the refractive index of tissues can dramatically reduce the effects of tissue scattering of light during PDT (214). Light application with a two-photon short-pulsed laser has also been shown to result in deeper photo-penetration of tissues compared to traditional continuous wave lasers or light sources.

At this time, antimicrobial PDT is predominately applied to treat dental and dermatological infections, however clinical use of antimicrobial PDT has been documented for the treatment of oral infections, acne vulgaris, burn wound infections, and skin ulcers (175). Yet, clinical trials testing the efficacy of PDT specifically for the treatment of *S. aureus* infections in humans are few. In a randomized, double-blind, placebo-controlled Phase 2 trial by Mannucci, et. al., patients with chronic leg ulcers or chronic diabetic foot ulcers that were treated with a gel containing the photosensiziter RLP086 and 689nm red light had a significant reduction in total microbial load, with no significant adverse effects reported compared to the placebo-treated group (215). Additionally, a clinical trial in Vancouver, Canada found surgical patients that received intranasal PDT during preoperative care had significantly reduced levels of nasal carriage and a 42% reduction in post-surgical site infection rate compared to a four-year historical average (216). This study led to the development and commercial release of the PDT– based decolonization system MRSAid (Ondine Biomedical, Inc., Vancouver, Canada), which has been approved for clinical use in Canada and is pending approval in the European Union. In the United States, a clinical trial

sponsored by the University of Rochester investigating PDT-based treatment of deep tissue bacterial abscesses is expected to be completed by the end of 2018 (217). With the rapid pace at which PDT technologies continue to evolve, it is expected more clinical trials and PDT-based products will appear in the near future.

VIII. Antibodies & Antibody Conjugates

S. aureus expresses many immune evasion and virulence factors that may be potential candidates for antibody therapies and vaccine development. Unfortunately, previous efforts in immunotherapies have failed, due in large part to the functional redundancies of these evasion factors, which is further aggravated when only one antigen is targeted. There have been a number of failed passive immunotherapies against different targets including lipoteichoic acid (Pagibaximab), clumping factor A (Veronate), capsular polysaccharide (Altastaph), and α-hemolysin (Salvecin, MEDI4893) (218–221). Currently the development of a promising human monoclonal antibody therapy for the treatment of *S. aureus* bacteremia, 514G3, is ongoing. 514G3 was isolated from the immune repertoire of a healthy human donor and targets the Staphylococcus Protein A (SpA) (222). In early 2017, 514G3 completed a double-blind, placebo-controlled, Phase 1/2 clinical trial of more than 50 patients in the hospital setting (223). Another monoclonal antibody treatment against *S. aureus*, ASN100, was developed by Arsanis, Inc., and targets α-hemolysin, Panton-Valentine leukocidin, leukocidin ED, leukocidin GH, and γ-hemolyins AB and γ-hemolyins CB (224, 225). ASN100 was developed in particular to treat patients on respirators who are at risk of developing *S. aureus* pneumonia. End-points of a Phase 1 trial were met and a Phase 2 clinical trial has been scheduled (226).

IX. Summary

Unfortunately, antibiotic-resistant microorganisms continue to become more and more prevalent, threatening public health and placing a significant economic burden on the healthcare system. To eliminate drug-resistant infections, novel and effective therapeutic options are desperately needed. Many innovative strategies for alternative drug development are being pursued, including disruption of biofilms, bacteriophage-derived antimicrobials, anti-staphylococcal vaccines, and light-based therapies. While many compounds and methods still need further study to determine their feasibility, some are quickly approaching clinical application and may be available in the near future.

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