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REVIEW

Antimicrobial Peptides and Small Molecules Targeting the Cell Membrane of *Staphylococcus aureus*

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SUMMARY Clinical management of *Staphylococcus aureus* infections presents a challenge due to the high incidence, considerable virulence, and emergence of drug resistance mechanisms. The treatment of drug-resistant strains, such as methicillin-resistant *S. aureus* (MRSA), is further complicated by the development of tolerance and persistence to antimicrobial agents in clinical use. To address these challenges, membrane disruptors, that are not generally considered during drug discovery for agents against *S. aureus*, should be explored. The cell membrane protects *S. aureus* from external stresses and antimicrobial agents, but membrane-targeting antimicrobial agents are probably less likely to promote bacterial resistance. Nontypical linear cationic antimicrobial peptides (AMPs), highly modified AMPs such as daptomycin (lipopeptide), bacitracin (cyclic peptide), and gramicidin S (cyclic peptide), are currently in clinical use. Recent studies have demonstrated that AMPs and small molecules can penetrate the cell membrane of *S. aureus*, inhibit phospholipid biosynthesis, or block the passage of solutes between the periplasm and the exterior of the cell. In addition to their primary mechanism of action (MOA) that targets the bacterial membrane, AMPs and small molecules may also impact bacteria through secondary mechanisms such as targeting the biofilm, and downregulating virulence genes of *S. aureus*. In this review, we discuss the current state of research into cell membrane-targeting AMPs and small molecules and their potential

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mechanisms of action against drug-resistant physiological forms of *S. aureus*, including persister cells and biofilms.

KEYWORDS antimicrobial peptides, biofilm, persister, small molecules, *Staphylococcus aureus*

INTRODUCTION

Staphylococcus aureus is a Gram-positive human pathogen carried by approximately one-third of the human population. Globally, the incidence of *S. aureus* cases ranges from 10 to 30 cases per 100,000 people per year (1), with hospital mortality ranging from 15% to 40% (2, 3). The death rate associated with *S. aureus* bacteremia is higher than those associated with AIDS, tuberculosis, and viral hepatitis combined (4). The Centers for Disease Control and Prevention (CDC) reports that methicillin-resistant *S. aureus* (MRSA) is a common cause of skin, wound, pneumonia, and bloodstream infections (5). Almost half of *S. aureus* infections acquired in hospitals in the United States are methicillin-resistant (6) and MRSA infection rates are increasing in the community (7, 8).

In this review, we discuss the concept of using AMPs and small molecules to disrupt bacterial membranes or proteins integral to membrane function in *S. aureus*. Furthermore, we outline how membrane targeting AMPs and small molecules are effective against the persister and biofilm forms of *S. aureus*.

THE NEED FOR ANTIMICROBIAL AGENTS THAT TARGET THE BACTERIAL MEMBRANE

Drug resistance, tolerance, persistence, heteroresistance and biofilm formation contribute to the treatment challenges associated with *S. aureus* (9). The term "resistance" refers to the ability of bacteria to grow despite antibiotic treatment, regardless of the length of time they are exposed to the antibiotics (10, 11). Antibiotic resistance is caused by various molecular mechanisms, such as modified drug targets or efflux pumps (11, 12). In comparison, when bacteria become resistant to antibiotics, a higher antibiotic concentration is required to treat a resistant strain than a susceptible strain. Generally, antibiotic resistance can be classified as either natural/intrinsic or acquired resistance. Natural/intrinsic resistance occurs when certain structures are missing or present, resulting in antibiotic ineffectiveness. Acquired resistance results from mutations of chromosomal genes or horizontal gene transfers (12).

Tolerance allows bacteria to endure a more extended period of antibiotic treatment by remaining dormant (13). During dormancy, the bacteria stay unaffected by many types of antibiotics (β-lactams and quinolones) that target the growth and functioning of the bacteria (14, 15). The development of tolerant phenotypes occurs through genetic characteristics, stochastically, or in response to environmental factors in Gram-positive and Gram-negative bacteria (12). It is critical to note that tolerance is only associated with bactericidal antibiotics, not with bacteriostatic antibiotics (16).

In addition to tolerance, persistence increases the duration of treatment for a subpopulation, even if the population is clonal (17). In simple terms, "persister" refers to a subpopulation of tolerant cells that can withstand antibiotic treatment for longer in comparison to slow-growing dying cells by entering a metabolically repressed state (nonmultiplying cells) (17, 18). Most antibacterial agents are only effective against growing cells, and persisters can survive longer without being genetically resistant by not growing (12). Importantly, most currently prescribed antibiotics are ineffective against bacterial persisters, and *S. aureus* persisters are tolerant to antibacterial agents, including gentamicin (protein synthesis inhibitor), ciprofloxacin (DNA replication inhibitor), vancomycin (cell wall synthesis inhibitor), and daptomycin (18–26). Generally, persister cells make up a smaller fraction of the population (less than 1%) and are killed more slowly than susceptible cells (16). An isogenic bacterial population has been shown to show an increase in persister levels when exposed to nutrient limitations, antibiotic exposure, acidic pH, high cell numbers, heat shock, and oxidative stress (16). A study on *S. aureus* ATCC 55585 proved that the strain was completely tolerant to fluoroquinolone and ciprofloxacin, suggesting that stationery cells may be equivalent to the

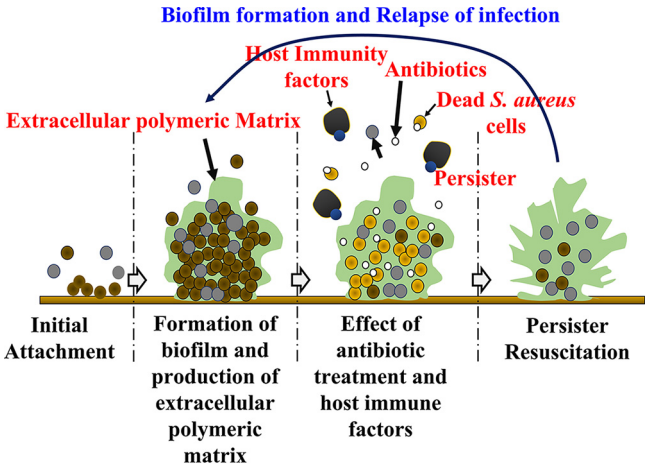


FIG 1 *S. aureus* infection model of relapsing biofilms. Figure adapted based on Conlon et al. (216) and Lister et al. (217).

persister of *S. aureus* (21). In studies using clinical staphylococcal isolates, persister formation was demonstrated in the presence of antibiotics like penicillin, tetracycline, clindamycin, chloramphenicol, cefoxitin, erythromycin, co-trimoxazole, gentamicin, ciprofloxacin, and moxifloxacin (27–29).

Distinct to persistence, heteroresistance occurs when isogenic subpopulations of bacteria display differing levels of susceptibility to an antibiotic, mostly at genetic levels (30). In practice, heteroresistance is defined when the highest inhibitory effect of an antibiotic is at least 8 times greater than the highest non-inhibitory concentration and is associated with the selection of more resistant subpopulations (30). Most variations in antibiotic resistance in bacteria, such as *S. aureus*, are caused by mutations or duplications of resistance genes and regulation factors (30). For example, in MRSA, mutations in the *mecA* gene and differences in penicillinase expression can cause heteroresistance to β-lactams such as methicillin (31, 32). MRSA heteroresistance may also be mediated by transcription regulators such as the *Sar* and the *Sigma B* operons (33, 34), and the SOS (save our souls) response *lexA/recA* gene regulators (35). The mechanism underlying the heteroresistance of MRSA to glycopeptides (vancomycin) is unclear (30) and may be associated with higher gene mutation rates of the *agr* gene locus (accessory gene regulator) (36). Also, VISA strains carry mutations in *vraSR*, *walRK*, and *rpoB* operons (37–39) and a mutation in *rpoB*, but not *graR*, is associated with heteroresistance to vancomycin in *S. aureus*, resulting in a thickened cell wall and reduced cell surface negative charge (40, 41).

Biofilm tolerance to drugs and other toxins is, at least in part, due to impaired antibiotic penetration (42, 43). Also, studies demonstrated that cells released from biofilms are less susceptible to antibiotics than planktonic cells (44, 45). Importantly, one of the physiological reasons for differential antibiotic susceptibility in the biofilm-dispersed cells is attributed to the retention of extracellular polymeric matrix, which may allow a varied antibiotic response compared to standard planktonic cells (46, 47). Apart from impairing the penetration of some drugs, biofilms harbor many persister cells that are often involved in the failure of treatments (48–50) (Fig. 1).

To improve antistaphylococcal treatment, there is a need to identify agents that overcome resistance and maintain activity against tolerant and persister cells and biofilms (20, 51, 52). The cell membrane of *S. aureus* is a crucial structure for cell survival and consists of the cytoplasmic plasma membrane (CM) and lipoteichoic acids (LTA) attached to a thick layer of peptidoglycan (53). Nearly one-third of proteins in the cell membrane are involved in bacterial metabolism, and damaged membranes impair homeostasis and reduce the possibility of drug resistance (54). Mehta et al. (55) hypothesized that a membrane-permeabilizing drug may also act as a chemosensitizer that enhances the activity of other antibacterial agents (55).

TABLE 1 Major families of AMPs primarily targeting the *S. aureus* membrane

Structural class	No. of peptides (n = 115)	Eminent examples of AMP families
α-helical	48	Dermaseptin-B2, aurein 2.5, magainin 2, melittin, buforin II, LL-37, chrysophsin-1
β-sheets	10	Lactoferricin B, baboon theta-defensin-1, human neutrophil peptide-1, gomesin, protegrin 1, tachyplesin I, and kalata B1
$\alpha + \beta$	6	Human beta-defensin 1, thrombocidin-1, porcine beta-defensin 2, CXCL10, actinomycesin
Non-$\alpha\beta$	3	Pyrrhocoricin, penisin, and tridecaptin A1

Consequently, the use of membrane-targeting small molecules and antimicrobial peptides (AMPs) has become a focus of antimicrobial chemotherapy (54, 56). These strategies could shorten the treatment period, reduce disease relapses, target bacterial persisters, and reduce antibiotic resistance. Antimicrobial agents that target the structure of bacterial membrane bilayers have become increasingly interesting because the membrane is essential and highly conservative (54).

MEMBRANE-ACTIVE AMPs AGAINST *S. AUREUS*

Antimicrobial Peptides

AMPs have evolutionarily conserved roles in the innate immune system, providing the first line of defense against microbial infection in all major phyla of plants, animals, and prokaryotes (57). Most AMPs are composed of <50 amino acids and are 40 to 60% hydrophobic and positively charged (average net charge of +3) (57) and exhibit broad-spectrum antibacterial activity against bacteria, fungi, viruses, and even cancer cells (58–60). In addition to their antimicrobial activity, AMPs may facilitate the healing of wounds, and modulate the immune system (57).

AMPs can be classified based on structure, function, or target (61). More specifically, based on their structure, AMPs are classified as α-helix, β-sheets, both $\alpha+\beta$ peptides, and non-$\alpha\beta$ (Table 1), describing major families of AMPs primarily targeting the *S. aureus* membrane (Fig. 2). AMPs are also classified based on their biological functions (antibacterial, antifungal, antiviral, antiparasitic, antiprotist, anticancer, etc.). According to their potential

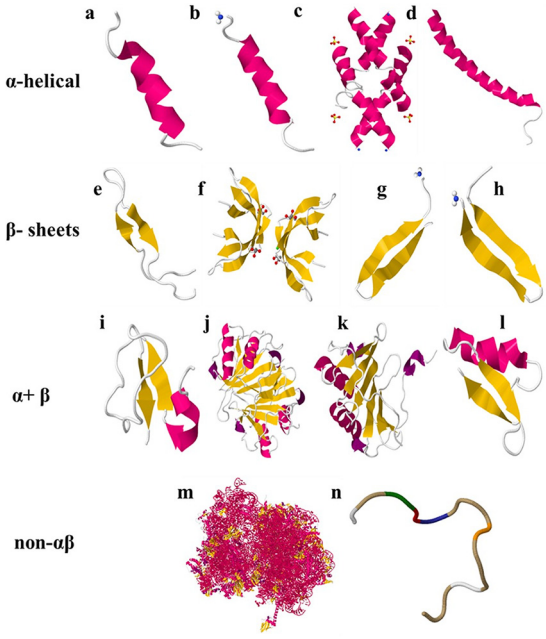


FIG 2 Examples for different types of AMPs based on their structure. (a) Aurein 2.5 (PDB ID: 6GS9); (b) Magainin 2 (PDB ID: 2MAG); (c) Melittin (PDB ID: 2MLT); (d) LL-37 (PDB ID: 2K6O); (e) Lactoferricin B (PDB ID: 1LFC); (f) Human neutrophil peptide-1 (PDB ID: 3GNY); (g) Gomesin (PDB ID: 1KFP); (h) Protegrin 1 (PDB ID: 1PG1); (i) Human β-defensin 1 (PDB ID: 1E4S); (j) Thrombocidin-1 (PDB ID: 1NAP); (k) CXCL10 (PDB ID: 1O80); (l) Actinomycesin (PDB ID: 2RU0); (m) Pyrrhocoricin (PDB ID: 5HD1); and (n) Tridecaptin A1 (PDB ID: 2N5Y). All the images were obtained from the RCSB Protein Data Bank.

targets, AMPs are classified as membrane-targeting or cell wall-targeting (loss of cellular integrity), or acts on intracellular functions, including ribosome, DNA, or RNA (61).

Physicochemical Factors Regulating the Activity of AMPs against *S. aureus* Membrane

Several factors determine the action of AMPs, including the charge, hydrophobicity, conformation, length, and amphipathicity (61). The net charge in AMPs plays a critical role in determining the bacterial targets and the majority of positively charged AMPs target bacterial membranes (62). Such positively charged amino acids in AMPs interact with negatively charged phospholipids of bacterial membranes through electrostatic interactions (62). This interaction allows the hydrophobic amino acids in the AMPs to interact further with the bacterial membrane (62).

At physiological pH, the net positive charge of cationic AMPs ranges from $+2$ to $+11$ (61). In contrast, at physiological pH, anionic AMPs have a net negative charge of -1 and -8 and are usually composed of 5 to 70 amino acid residues (57). Following this electrostatic binding, the interaction between the hydrophobic amino acids in the AMPs and the hydrophobic tail of the membrane phospholipids allows the AMPs to enter deeper inside the bacterial membrane (62). Short anionic AMPs like DDDDD (pentaD) and surfactant-associated anionic peptides (SAAPs), such as those found in the pulmonary fluids of sheep and cattle, have broad-spectrum activity (63–65). However, unlike cationic AMPs that primarily target bacterial cell membranes, anionic AMPs act by constricting the ability of bacteria to absorb zinc ions (64).

After the initial electrostatic binding of the charged amino acids, the bacterial membrane permeability by the AMPs depends on the hydrophobic amino acids of the AMPs (66, 67). Hydrophobic-hydrophilic interactions between the hydrophobic amino acid of AMPs and the hydrocarbon chain of the lipids orient in various ways to form membrane deformations (66, 67) (also described below under Mechanism of Action of Membrane-Active AMPs and Small Molecules against *S. aureus*).

To induce effective membrane binding on different bacteria, AMPs must contain specific contents of hydrophobic amino acids (66, 67). For example, AMPs targeting Gram-positive bacteria, particularly *S. aureus*, requires peptides with higher hydrophobic content than AMPs selectively acting against Gram-negative bacteria (68, 69), and using an *in silico* approach based on database filtering technology, Mishra et al. found that AMPs specifically targeting *S. aureus* membranes require 61% of hydrophobicity (68). Members of our group also found that a 56 to 70% hydrophobicity is required by a short α-helical C18, and sapecin B-derived SD-8 membrane-active peptide to work against *S. aureus* (70, 71).

The antistaphylococcal activity of many membrane AMPs is enhanced by substituting high hydrophobic amino acid residues for low hydrophobic or charged amino acid residues (70, 72, 73) (Fig. 3). A balance of both the hydrophobic and charged amino acids is critical for maintaining the antimicrobial activity of AMPs (74). However, in AMPs with a higher number of positive charges that facilitate enough initial membrane electrostatic interactions, the additional charged amino acids can be substituted for hydrophobic amino acids to enhance antimicrobial effects. Similarly, the antistaphylococcal activity of many membrane AMPs is enhanced by substituting high hydrophobic amino acid residues for low hydrophobic or charged amino acid residues (70, 73, 74). For example, tetra-F2W peptides with arginine exchanged with tryptophan (72), and cecropin-4 peptides with glycine (70), demonstrated enhanced antimicrobial potency compared to their low hydrophobic peptide counterparts. When hydrophobic amino acids are swapped for neutral amino acids, such as tetra-F2W peptides with arginine exchanged with tryptophan and cecropin-4 peptides with glycine substituted with leucine, enhanced antimicrobial potency is demonstrated. Similarly, replacing a less hydrophobic amino acid residue with a higher hydrophobic amino acid in peptides like DFTamp1 (valine or isoleucine replaced with leucine) (68), and C18G (valine replaced with leucine) (73) also increased their antimicrobial potency against *S. aureus*.

The charged and hydrophobic amino acids in peptides determine how natural AMPs act on membranes (74). Identifying any preferential amino acid selection in natural membrane-active AMPs can guide the design of antistaphylococcal peptides. For the purposes of this review, we analyzed the amino acid composition of naturally occurring membrane-

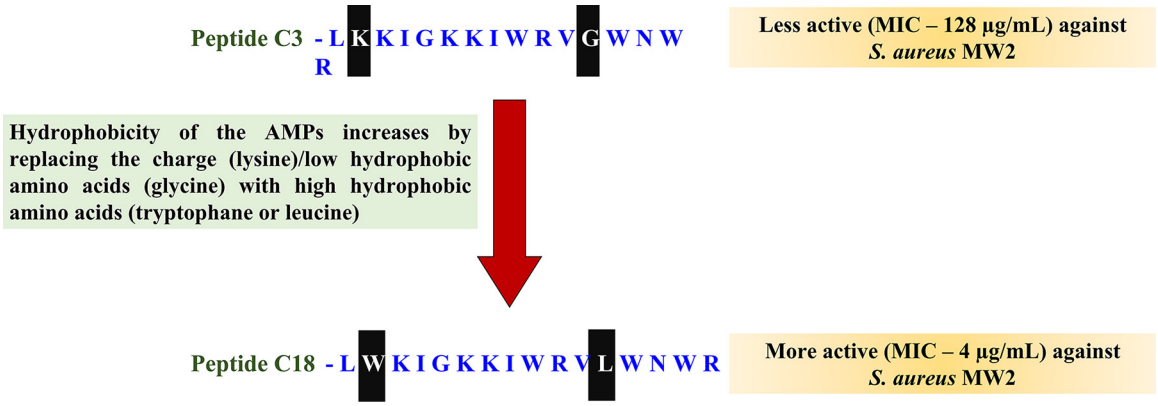


FIG 3 Replacing charged (lysine) or low hydrophobic amino acids (glycine) with high hydrophobic amino acids (tryptophane or leucine) enhances the antistaphylococcal activity of Cecropin-4-derived AMPs. The figure is adapted from Peng et al. (70).

active AMPs from the Antimicrobial Peptide Database (maintained at the University of Nebraska Medical Center, Omaha) (61). The database contains about 3,425 AMPs, of which 1,658 are effective against *S. aureus* (57). Based on the amino acid composition of the 115 AMPs specifically targeting the *S. aureus* membrane (Fig. 4), lysine was preferred over arginine as a charged amino acid (lysine occurs at 11.31%, while arginine was 6.87%) and the average hydrophobicity of all peptides was 56%. In addition, a total of 307 membrane-active AMPs exhibit broad-spectrum activities, including activity against

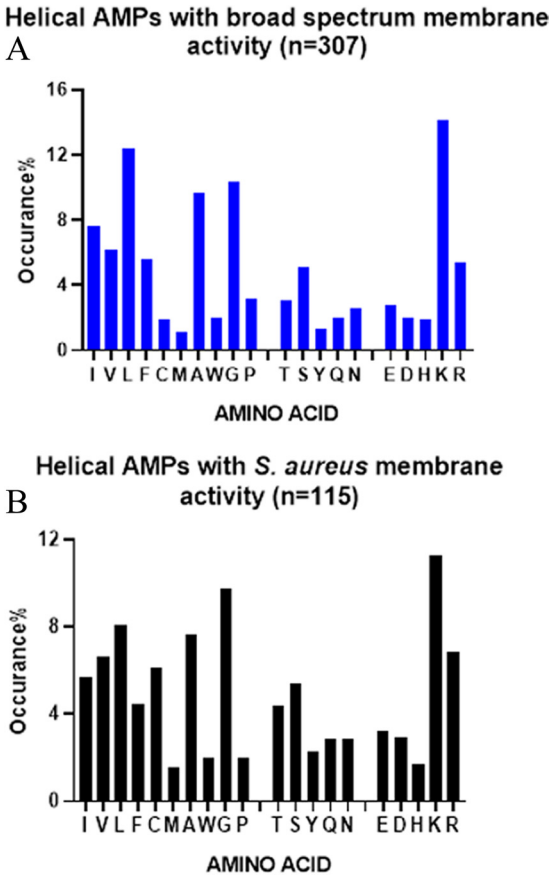


FIG 4 Distribution of amino acid residues in α-helical AMPs (determined from the antimicrobial peptide database). Each amino acid is denoted with a single letter: hydrophobic (I, V, L, F, C, M, A, W, G, P), neutral (T, S, Y, N, Q), and charged residues (E, D, H, K, R). (A) Broad spectrum membrane activity. (B) *S. aureus*-specific membrane activity.

TABLE 2 Membrane-active small molecules against *S. aureus*

Compound name	Type of Molecules	MIC (μg/mL)	Organism	References
Dialkyl cationic amphiphiles	Peptide mimics	0.5−4	*S. aureus* and MRSA	84
Structural organophosphorus aminopyrimidines	Peptide mimics	4	MRSA	85
Polyphenylglyoxamide-based small molecular peptidomimetics	Peptide mimics	2.9−5.6	*S. aureus*	183
Honokiol/magnolol amphiphiles	Peptide mimics	0.5−2	MRSA clinical isolates	113
Aryl-based synthetic mimics of antimicrobial peptides (SMAMPs)	Peptide mimics	3.13	*S. aureus*	88
Hydantoin derivatives	Peptide mimics	0.5−3.12	MRSA	89
L-lysine-based lipidated biphenyls	Peptide mimics	3.1	*S. aureus* ATCC and MRSA	90
Dimeric lysine alkylamides	Peptide mimics	0.75−1.5 1.5−3 3−6	MRSA	81
Cationic deacetyl linezolid derivatives	Peptide mimics	2−4	MRSA Clinical isolates	92
Acetanilide	Peptide mimics	1	MRSA	91
Porphyrin antibacterial agent	Repurposed drug	4	*S. aureus* SH1000	191
NH125 (1-hexadecyl-2-methyl-3-(phenylmethyl))-1H-imidazolium iodide	Repurposed drug	1	MRSA-MW2 BAA-1707	25
Veterinary drug oxyclozanide	Repurposed drug	4	MRSA	96
Bithionol-anthelmintic drug	Repurposed drug	0.5−2	MRSA-MW2 BAA-1707 and VRSA1	7
PQ401 (diaryl urea)	Repurposed drug	4	MRSA and VRSA	98
α-mangostin	Natural molecules	0.78−3.125	*S. aureus* ATCC 29213 and MRSA clinical isolates	105
α-mangostin (hydrophobic scaffold of xanthone)	Natural molecules	0.5−3	*S. aureus* DM4001 clinical isolates	106
Nonpeptidic amphiphilic xanthone derivatives of α-mangostin	Natural molecules	1.56−3.125	MRSA clinical isolates	108
Nisin + Cinnamaldehyde	Natural molecules	0.1875 + 0.375	*S. aureus* ATCC 29213	103
Licochalcone E	Natural molecules	4	*S. aureus* ATCC 29213 and MRSA T144	109
Licochalcone C		4		
Licochalcone A Glabrol		2 2		
Balsacone C	Natural molecules	3–11.6	Clinical isolates of MRSA and Methicillin sensitive *Staphylococcus aureus*	111
Natural berberine-hybridized benzimidazole derivative 2,4-dichlorobenzyl	Natural molecules	0.008–0.470	*S. aureus* ATCC 29213 and MRSA N315	110

S. aureus (57). In this group of peptides, lysine was also preferred to arginine, and the average hydrophobicity of all peptides was 60%.

Interestingly, the preferential amino acid composition of membrane-active AMPs regulates peptide binding toward different lipids and the distinction in the binding affinities of these membrane-active AMPs with various membranes forms the basis of cell selectivity (75). More specifically, the membrane selectivity of an AMP results from its affinity for more anionic bacterial membranes (75). Notably, mammalian membranes contain neutral lipids such as cholesterol, which help stabilize phospholipid bilayers and AMP interactions, resulting in less cytotoxicity (57, 76).

Peptidomimetics

Peptidomimetics (or peptide mimics) are small protein chains synthesized by modifying the existing structure of a peptide (77) (Table 2). Successful peptidomimetics in clinical trials, including PMX 30063 (78) and LTX-109 (79), are hampered by complex synthesis protocols and membrane specificity (78, 80). A successful synthesis constitutes the AMP or small molecules with low toxicity and higher membrane specificity against *S. aureus*. The development of membrane-active drugs has been significantly enhanced by designing and optimizing cationic amphipathic mimics, which overcome these weaknesses (81). Cationic amphipathic mimics were developed and optimized to overcome weaknesses, such as stability or selectivity, to contribute significantly to the development of membrane-active agents against *S. aureus* (82). Most membrane-active peptide mimics contain amino groups in their molecules that form a hydrophilic area, which is crucial to their antibacterial action (83).

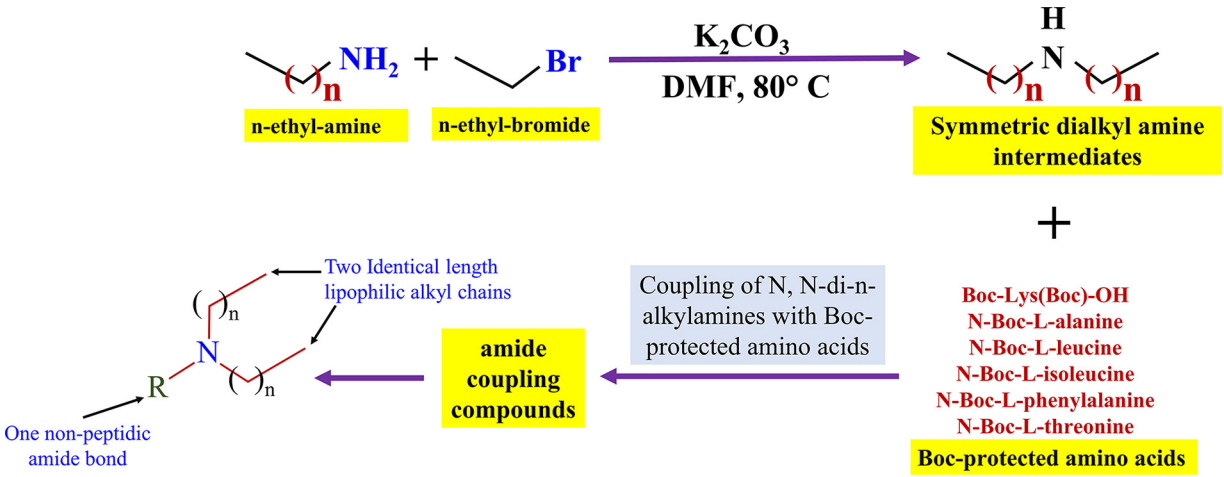


FIG 5 Changing the secondary amine in a series of dialkyl cationic amphiphiles by adding two identical-length lipophilic alkyl chains and one nonpeptidic amide bond to improve the membrane-targeting efficacy against *S. aureus*. The figure is adapted from Zhang et al. (84).

Several amine compounds with membrane-targeting efficacy include alkylamines, an aromatic group containing amines, and quaternary amines in the chemical composition (83). Changing the secondary amine in a series of dialkyl cationic amphiphiles bearing two identical-length lipophilic alkyl chains and one nonpeptidic amide bond improves the membrane targeting efficacy against *S. aureus* (84) (Fig. 5). A two-nitrogen aromatic aminopyrimidine scaffold is composed of two electron-accepting C = N groups and an electron-donating NH_2 moiety. This structure interacts with biomolecules such as DNA, RNA, enzymes, and receptors with high affinity through intermolecular supramolecular interactions (85). Supramolecular interactions occur when small molecules interact noncovalently to form molecular assemblies (86). The supramolecular interaction class is characterized by its noncovalent character. Van der Waals forces, hydrogen bonds, metal-ligand coordination, and electrostatic interactions are examples of such supramolecular interactions (86).

Moreover, organophosphorus functional groups may affect heterocycle reactivity, regulate key biological functions, and bind to a variety of biological targets. In organic chemistry, heterocycles are rings of atoms where one or more atoms are different from carbon. A heterocycle is often formed by combining oxygen, nitrogen, and sulfur, but it can also contain selenium, boron, silicon, arsenic, and phosphorus (87). By combining the aminopyrimidine and organophosphorus fragments with DNA-targeting membrane-active potencies, a series of novel structures known as organophosphorus aminopyrimidines were effective against *S. aureus* cell membrane (85). In a study by Li et al. (85), aminopyrimidine and organophosphorus structures were combined with DNA-targeting, membrane-active potencies, leading to a series of novel organophosphorus structures and aminopyrimidines which were effective against *S. aureus* cell membrane (85).

Small molecule structures with a central aromatic core exhibit membrane-targeting mechanisms against *S. aureus* (80). Considering the importance of an aromatic core in the structure of membrane-active molecules, synthetic mimics of antimicrobial peptides (SMAMPs) based on aryl groups and tuned aromatic groups were synthesized (88). Despite its complex mechanism, hydantoin still acted on the membrane and other potential targets, such as DNA and ribosomes (89). The complex mechanism of hydantoin is due to the high molecular weight (normally >1,000 Da), and structural complexity has made the synthesis tedious. Notably, compounds with optimal antibacterial activity require a balance between the hydrophobicity of the peptidomimetics with the bacterial cell membrane. For example, L-lysine-based lipidated biphenyls optimized for hydrophobicity demonstrated bactericidal properties associated with membrane-active nature (90). Also, Niu et al. (81) used a dimerization strategy to design dimeric lysine alkylamides that mimic the structure of AMPs. These AMP-mimics had well-balanced charged and hydrophobic core regions and demonstrated

potent bactericidal activity and membrane disruption against MRSA, and some of these derivatives were highly selective to bacteria over mammalian cells (81).

Structure-activity relationships (SARs) indicated that an amphiphilic structure is essential for broad-spectrum activity, particularly against Gram-negative bacteria. Hence, the alkyl chain length was most important for antibacterial activity, whereas different hydrophilic amino acid residues (lysine and arginine) did not significantly influence their antibacterial activity (83). SAR analysis indicated that compounds with more positive charges are more potent against *S. aureus* and exhibit less toxicity toward mammalian cells (83). Wang et al. (91) used acetanilide to synthesize a new class of structurally unique para-aminobenzene sulfonyl oxadiazoles (91). Changing the structural moieties (for example, changing secondary amine, combining aminopyrimidine and organophosphorus fragments, and adding hydrophobic N-alky sulfonyl group in the existing AMPs) may increase active membrane targeting potency, reduce toxicity, and enhance selectivity against the *S. aureus* membrane (92, 93). The increased activity and reduced toxicity of peptidomimetics could make them superior antibacterial candidates compared to linear peptides (94).

MEMBRANE-TARGETING SMALL MOLECULES

Similar to peptidomimetics, researchers have reported small molecules that target the cell membrane of *S. aureus*. Some of these small molecules are chemical or structurally modified from the original structure of natural molecules or repurposed drugs to enhance the efficacy against *S. aureus*. Repurposing drugs for new uses have become increasingly popular (95) because clinically approved drugs have been tested for toxicity and safety, and their pharmacological properties are well understood (95). There are several membrane-active repurposed drugs tested against *S. aureus* (Table 2).

Other membrane-targeting small molecules, including oxyclozanide (96), NH125 (1-Hexadecyl-2-methyl-3-[phenylmethyl]-1H-imidazolium iodide) (25), bithionol (97), and PQ401 (98), showed potent bactericidal activity against MRSA. For example, NH125 kills *S. aureus* by inhibiting WalK, an essential histidine kinase for Gram-positive bacteria (25, 99), and has membrane-targeting efficacy against *S. aureus* persisters (25). Bithionol demonstrated dose-dependent membrane permeability, altered membrane fluidity and permeabilized *S. aureus* membrane (97). More recently, Kim et al. (98) also identified a diaryl urea compound, PQ401, which inhibits MRSA from killing the invertebrate model host *Caenorhabditis elegans* and causes membrane permeabilization of MRSA (98). Bithionol and retinoids showed significant synergism with gentamicin (97, 100). Most likely, synergism works better because gentamicin diffuses more easily through cell membranes of *S. aureus* that are damaged by retinoid or bithionol.

Notably, some of these membrane-acting agents, such as bithionol and retinoids, act additively or synergistically with established clinical antibiotics (97, 100). Most likely, synergism works better because gentamicin diffuses more easily through cell membranes of *S. aureus* that are damaged by retinoid or bithionol (101). The combination of antibiotics with membrane-active compounds could also reduce the required dose of both agents, thereby decreasing any potential dose related side effects (100). Moreover, combining two or more antibacterial agents increases antibacterial efficacy and delays the development of antibiotic resistance (102). The combination therapy could be dose-dependent; nisin and cinnamaldehyde in combination exhibited membrane damage against *S. aureus* (103), but the combination therapy also leads to toxicity in some cases (104).

Small molecules derived from natural products have also demonstrated activity against MRSA membranes (Table 2). An example is α-mangostin (xanthone derivative), a small organic compound with a molecular weight of 410.46 g/mol that resembles the antimicrobial properties of the cationic antimicrobial peptide meta-phenylene ethynylene (mPE) (105). α-mangostin could induce rapid bacterial membrane disruption and alter the proton motive force within the cell, as well as effects the structural of cytoplasmic membrane (105). It is the strong hydrophobic interaction of α-mangostin with lipid alkyl chains that is responsible for its rapid penetration into the cell (105). Isoprenyl groups conjugated to short lipid tails triggered penetration into the hydrophobic region of the membrane.

Isoprenyl groups are therefore expected to reduce penetration barriers. In addition, the presence of isoprenyl groups further increased the hydrophobicity of α-mangostin, which enhanced its tendency to partition into membranes (105). In another study, Koh et al. (106) utilized the hydrophobic scaffold of xanthone derivatives to identify three components (hydrophobic xanthone core, lipophilic chains, and cationic amino acid structure) that mimicked an antimicrobial cationic peptide. Notably, even changing the structural characteristics of α-mangostin did not change the toxicity and selectivity against mammalian cells (106). Moreover, Koh et al. (107) performed an extended analysis of structure-activity relationships (SAR) of nonpeptidic xanthone derivatives to identify more compounds with lower hemolytic activity and more membrane selectivity (107). In summary, this study reports the synthesis of 46 amphiphilic xanthone derivatives categorized into cationic moieties, lipophilic chains, and triarm functionalization (108).

Combinations of membrane-targeting natural compounds such as nisin and cinnamaldehyde increased the membrane damage of *S. aureus*, compared to nisin alone (103). In a recent study, glabrol showed rapid killing ability by disrupting the membrane and dissipating the proton motive force of *S. aureus* (109). Similarly, natural molecules like berberine-hybridized benzimidazole derivative 2,4-dichlorobenzyl (110), balsacone C (dihydrochalcone extracted from *Populus balsamifera*) (111), 7-thiazoxime quinolones (112) and honokiol/magnolol (113) were reported to bind and alter the integrity of the *S. aureus* cell membrane. Notably, attaching a cationic AMP fragment to the structure of honokiol/magnolol improved the interaction with the negative charge of the bacterial cell membranes and improved the insertion of the molecule into the phospholipid bilayer membrane of *S. aureus* (113).

MOA OF MEMBRANE-ACTIVE AGENTS AGAINST *S. AUREUS*

The direct killing of bacteria by AMPs and small molecules with antimembrane activity is primarily accomplished by targeting the membrane or interfering with intercellular processes such as protein or nucleic acid synthesis, as well as other metabolic targets (62). However, several mechanisms contribute to the antimicrobial effect of AMPs and small molecules, apart from the direct killing of microbes (114). To identify and develop potential therapeutic candidates, it is essential to understand the mechanism of action (MOA) of these AMPs and small molecules (69). Even though this review focuses on the interaction between AMPs and small molecules and the bacterial membrane, it should be noted that AMPs and small molecules can also interfere with pathogen intercellular processes such as protein or nucleic acid synthesis, as well as other metabolic targets (62). Although in several membrane-active molecules, such as colistin and daptomycin, the mechanisms of actions are debatable (115, 116), understanding their MOA will help design better peptides and small molecules with antipersister and antibiofilm properties. For example, as the peptide-to-lipid ratio changes, AMPs such as BP100 can adopt multiple models of interactions (117). Peptide BP100 is an 11 residue cecropin-melittin hybrid peptide that adopts α-helical amphipathic conformation upon binding to phosphatidylglycerol containing lipid unilamellar vesicles (LUVs) (117). Circular dichroism and dynamic light scattering studies point to peptide and/or vesicle aggregation modulated by higher peptide: lipid ratio (117). Peptide BP100 releases the fluorescent entrapped dye gradually from LUVs when incubated at a low peptide-to-lipid ratio, indicating membrane perturbations (117). However, high ratios of peptide-to-lipid caused rapid loss of vesicle contents, suggesting vesicle disruptions. At high concentrations of peptides, membrane lipid clustering occurs, giving rise to peptide-lipid patches that eventually adopt a carpet-like mechanism (117). As the molecular mechanism of membrane interactions with AMPs has been thoroughly investigated, we will focus on AMP mechanisms in this section and compare them with the small molecules.

Cell Membrane as a Target for AMPs

AMPs are mostly cationic in nature and can interact effectively with the negatively charged lipoteichoic acid-rich membranes of Gram-positive bacteria (69). During this interaction, the hydrophilic domain of the peptide interacts with the charged groups in the phospholipid, resulting in the formation of an amphiphilic structure, while the hydrophobic portions of the peptide interact with the membrane phospholipid bilayer (69). In this regard, the composition

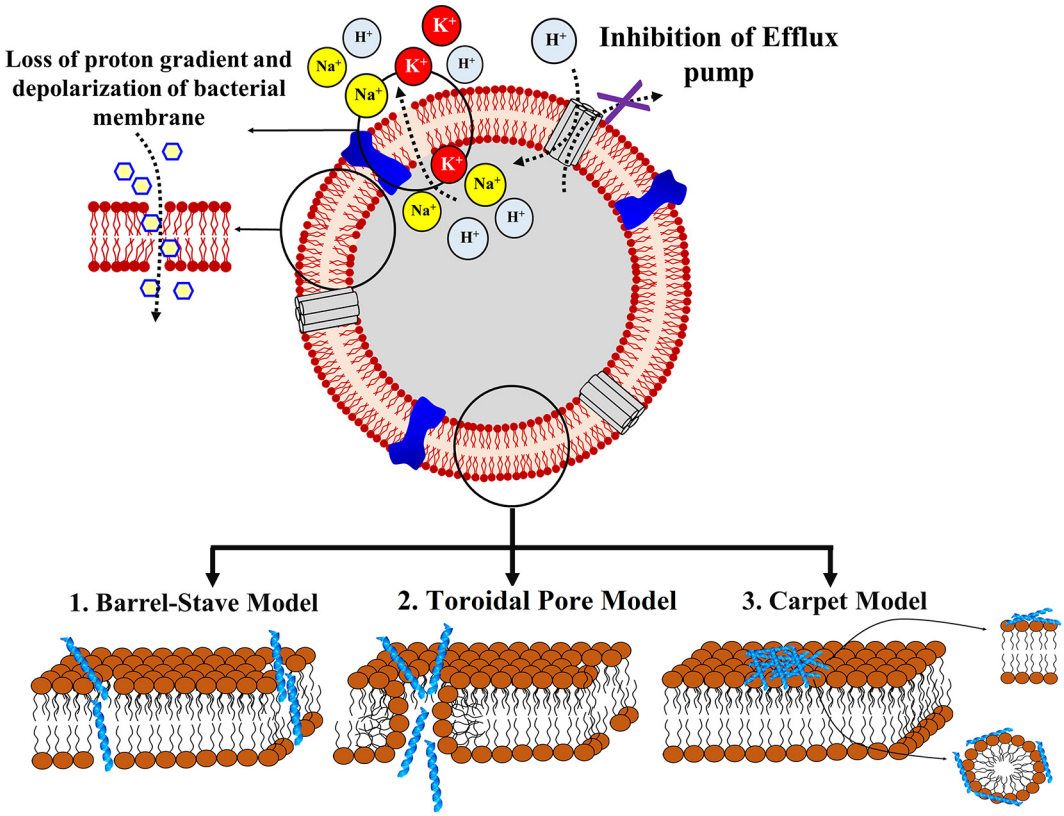


FIG 6 Mechanism of action of small molecules and AMPs on *S. aureus* membrane describing barrel-stave model, toroidal pore model, and carpet model. Figure adapted based on Brogden et al. (63).

of the *S. aureus* cell membrane makes it an ideal target for AMPs (118), as cardiolipin and phosphatidyl glycerol are both anionic lipids and cationic AMPs can effectively bind to the anionic membrane counterpart to disrupt bacterial cell membranes (118). Cardiolipin makes up 42% of the staphylococcal cell membrane, and phosphatidyl glycerol makes up the remaining 58% (118). Once the AMPs accumulate on the bacterial surface to the desired concentration, they destroy the bacterial membrane through classical and nonclassical membrane targeting. As discussed below, the classical model includes barrel-stave, toroidal pore, and carpet models (63) (Fig. 6), while the nonclassical model of membrane targeting includes the agglutination model (119), sinking raft models (120), and leaky slit models (121).

Classical models of membrane targeting. According to the barrel stave model, AMP monomers align themselves along the membrane (122) (Fig. 6). When threshold concentrations are reached, AMP oligomers insert further along the hydrophobic core in an orientation perpendicular to the lipid bilayer (122). The interaction of AMPs with the cell membrane of Gram-positive bacteria causes transmembrane pores. Notably, the amphipathic (α-helix, β-sheet) configuration of the peptide is crucial for pore formation, in which the hydrophobic side faces the membrane lipid, and the hydrophilic side faces the inner barrel wall to form the channel lumen (63, 123). Moreover, to span the entire lipid bilayer, which is 10 nm thick, AMPs need to be at least 22 residues (α-helical) or 8 residues (β-sheet) long. These membrane pores result in the release of cytoplasmic contents, and when the penetration is severe, the bacterial membrane ruptures, killing the bacteria (124). Alamethicin (125), pardaxin (126), and protegrin (127) are examples of AMPs using the barrel stave model as MOA (Fig. 6).

In the toroidal pore model (also known as the wormhole model; Fig. 6), initial AMP adsorption is followed by inserting the AMP perpendicularly inside the lipid bilayer, which induces membrane phospholipid molecules to bend inwards (128). Peptide-peptide interactions are absent, and instead, a transitory lipid-peptide molecule is formed, called a "toroidal pore" (129). As a result, these AMPs destabilize the bacterial membrane by causing thickness changes (hydrophilic and hydrophobic arrangement of lipids is disturbed) or

by aggregating and collapsing phospholipid heads that lead to increased membrane permeability. Magainin 2 (128) and melittin (130) are peptides that exhibit this MOA.

In the carpet model, AMPs are first adsorbed parallel to the lipid bilayer and accumulate until a threshold concentration is reached (131) (Fig. 6). As a result, a "carpet" is formed on the surface of the bacterial membrane, which results in the disassembly of the membrane (131). During this process, peptide-peptide interactions between the monomers create a transmembrane channel. In contrast, AMPs destroy membrane integrity by breaking them into small micelles, like aurein 1.2 (132), cecropin (70), indolicidin (133), and human cathelicidin LL-37 (134), which exhibit this MOA.

Compared to the MOA of AMPs, much less is known about the MOA of chemical compounds that target the membrane. Small molecules that cannot span the entire phospholipid membrane layer are believed to work via a carpet model. Such small molecules can first act through permeabilization mechanisms in which they induce small pores in membranes, or act through other destructive mechanisms (75, 118). Some small molecules may enter the cell, resulting in cell machinery and nutrient leaks (75, 118). Other small molecules may act through depolarization of the membrane. For the depolarization of the membrane, compounds disrupt bacterial membrane electronic gradients by forming ion-conducting pores, increasing ion permeability, or acting as ion carriers (75). The change in membrane permeability affects the proton motive force within the bacterial cell, which drives ATP synthesis and other transporters across the membrane, leading to the death of the cell or the ability of other molecules to kill it (135, 136).

Nonclassical models of membrane targeting. The nonclassical models include agglutination, sinking rafts, and leaky raft models. Thanatin is a peptide that acts via the bacterial cell agglutination model (119). After the initial electrostatic interaction, thanatin forms a micellar complex with the peptidoglycan of Gram-positive bacteria, which induces agglutination and phagocytosis (119).

The "sinking raft" model suggests that the MOA of shorter peptides, such as mastoparsan, cannot be reconciled with an orientation perpendicular to a membrane plane (120) (Fig. 7A). According to this model, the antiparallel orientation of the diamer with its monomer forms a trimer on the membrane surface and sinks into the outer bilayer leaflet (120). During this process, the helices bend slightly, sinking deeper in the middle, so that their hydrophilic terminals remain initially in contact with water (120). A combination of relative rotation and downward movement leads to the helices moving deeper and hydrophobic residues in the trimer are in contact with lipid acyl chains (120). In addition to the formation of trimer hydrophobic residues, hydrophilic faces of helices line and form a cavity (120).

Peptides that follow the "leaky slit" MOA are oriented perpendicular to the membrane, but instead of forming a circular pore, they aggregate side-by-side to form an amphipathic ribbon array (121) (Fig. 7B). In this model, the peptide hydrophobic residues face the hydrocarbon chains of the bilayer, and the hydrophilic residues are oriented toward the charged phospholipid heads. The interaction between the charged phospholipid heads of the membrane and the exposed hydrophilic amino acids from the peptides (e.g., plantaricin A) forms an aggregate, as the hydrophilic face cannot be sealed with opposing contacting bilayers (121, 137). Consequently, lipids adopt a highly positive curvature, causing the membrane to bend inwards to form a pore (121). This leaky slit arrangement of the peptide with the membranes is expected to be highly permeable to solutes and difficult for the cell to repair (121).

Other Antimicrobial Effects of Membrane-Active AMPs and Small Molecules

Membrane-targeting AMPs can also downregulate the expression of key virulence and biofilm genes in *S. aureus*. Several cationic AMPs, such as C18, NK-18, and LL-37, can directly bind to the DNA and AMPs are known to be regulators of gene expression (70, 138, 139). More specifically, C18 decreased the expression of *fnb-A* and *clf-1* that control the adhesion, colonization, and invasion (70). Additionally, human β-defensin 3 inhibits biofilm formation by reducing the expression of biofilm-producing genes *icaA* and *icaD* and increasing the expression of *icaR* (140).

AMPs and other membrane-active molecules interact with other components of the cell envelope before encountering the plasma membrane. The bacterial cell envelope is a

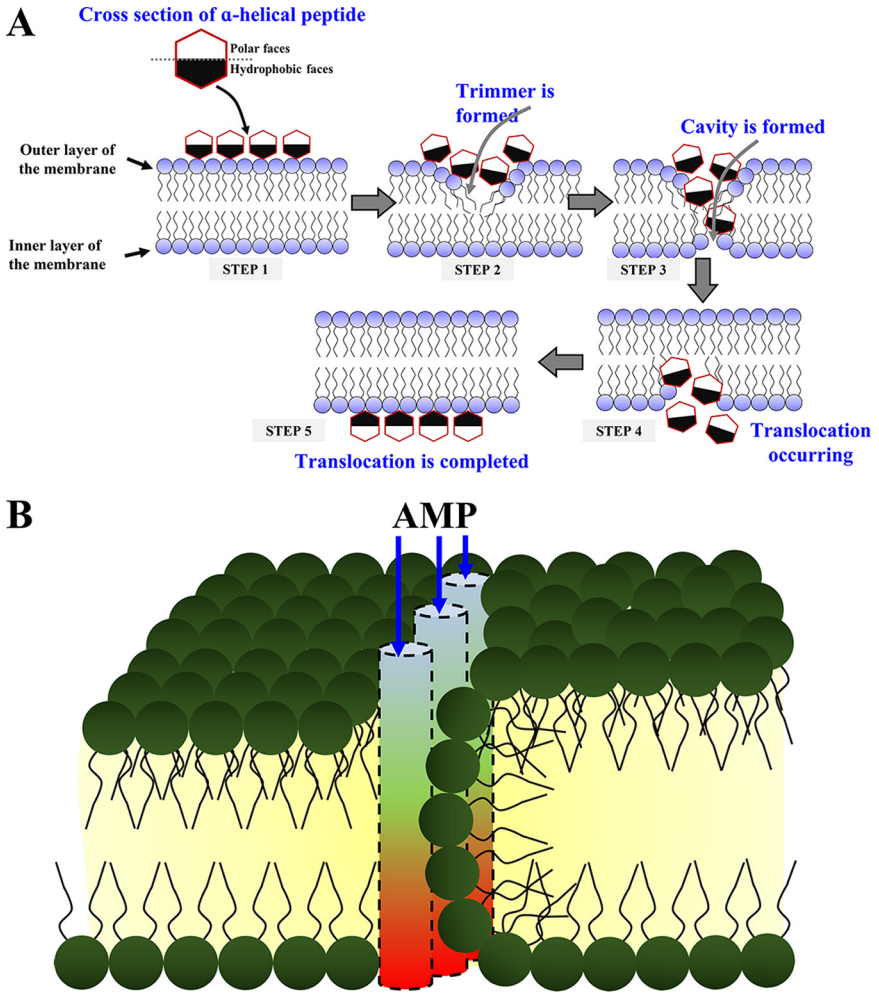


FIG 7 (A) Sinking raft model. This model illustrates how peptide trimers are inserted and translocated across the lipid bilayer. In the cross sections of α-helical peptides, the darker half-circles represent hydrophobic faces, and the lighter half-circles represent polar faces. The polar angle of δ-lysin is 180°. In step 1, the peptide forms a trimer on the phospholipid surface of the outer membrane. During step 2, the peptide sinks into the outer leaflet of the bilayer. In steps 3 and 4, a cavity is formed, which is the most unstable intermediate state. Finally, in step 5, translocation is completed symmetrically, and the trimer emerges on the inner membrane. The figure was based on the sinking raft model of δ-lysin (120, 218) (B) The "leaky slit" model is used to explain the membrane-damaging action of AMPs such as Plantaricin A (121).

dynamic, multilayered structure that protects it from unpredictable and hostile conditions (53, 141). In *S. aureus* the bacterial cell envelope is composed of capsular polysaccharides, the cell wall, teichoic acid polymers, and the phospholipid membrane (53). The polysaccharide microcapsules produced by *S. aureus* and their inhibition of complement cascades can impede opsonization (142). In contrast, USA300, that is an important MRSA clone, apparently lacks the microcapsule (143). Gram-positive organisms do not have an outer membrane, but their peptidoglycan layers are thicker than Gram-negative organisms (53, 141). Generally, the peptidoglycan network is composed of up to 80 layers of alternating disaccharide *N*-acetylglucosamine and *N*-acetyl muramic acid (144). A pentapeptide chain of the sequence L-alanyl-D-glutamyl-diaminopimelyl-L-lysyl-D-alanyl-D-alanine interconnects the disaccharides and an inter bridging peptide of the sequence GGGGG links two disaccharide pentapeptide moiety (141) (Fig. 8). The glycosyltransferase MurG transfers *N*-acetylglucosamine residue from uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) to lipid I (145). The consequent product, lipid II, is then translocated to the periplasmic side of the membrane before being incorporated into the growing peptidoglycan network by MurJ (146). Teichoic acid is an important component of the cell envelope, which plays crucial role in cell envelope physiology and pathogenesis (147–149). Other significant cell

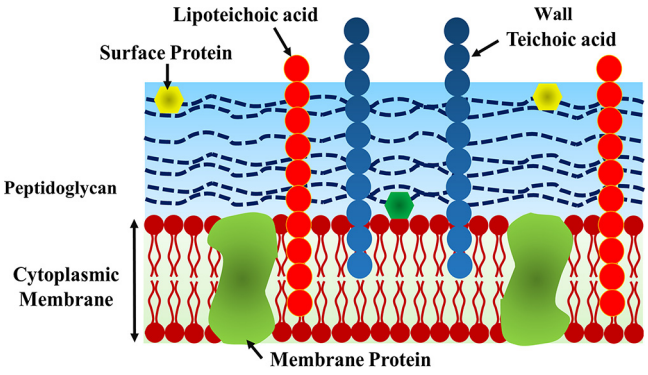


FIG 8 The complex cell wall structure of *S. aureus* is composed of a thick peptidoglycan layer, lipoteichoic acid, surface protein, and membrane protein. Figure adapted based on Epand et al. (219).

envelope components in *S. aureus* include capsular polysaccharides attached covalently to peptidoglycan and extracellular polysaccharides forming an amorphous outer layer (150, 151).

Several membrane-active AMPs and small molecules seem to also affect peptidoglycan biosynthesis (Table 3). A peptidoglycan targeting strategy commonly used by selective small molecules and AMPs is to specifically bind to components of the peptidoglycan synthesis machinery, such as lipid II, to inhibit or destroy the cell wall (152). AMPs affecting the inhibition of cell wall synthesis are known to act in very low concentrations (153). For example, the peptide nisin inhibits peptidoglycan synthesis at low concentrations by binding to the lipid II molecule, while at higher concentrations it works by targeting the bacterial cell membranes (153). Nisin is a bacteriocin AMP classified as a type A (I) lantibiotic with several unusual amino acids (154). The FDA has approved Nisin as a generally regarded as safe (GRAS) peptide that is widely used as a food preservative (155). Human α-defensin, including the

TABLE 3 Examples of cell wall-targeting AMPs and small molecules with impacts on phospholipid membranes

Compound	Action on cell wall	Cell wall references	Action on cell membrane	Cell membrane references
Nisin	Nisin's A/B-ring forms a 1:1 stoichiometric cage with lipid II's pyrophosphate moiety and blocks further peptidoglycan synthesis	220	Membrane permeabilization of bacterial cells	221
Human α-defensin	The HNP-1 peptide bound to lipid II with high affinity (binding constant Kd-2 $\times$ 10^6 M-1), and reduced levels of lipid II in bacterial membranes resulted in a significant reduction in bacterial killing	156	*In vitro* channel forming ability in planar lipid bilayer membranes	222
Ramoplanin	Forms a 2:1 stoichiometric complex with lipid II that inhibits peptidoglycan biosynthesis at the periplasmic transglycosylation step	223	Membrane depolarization of *S. aureus*	224
RWRWRW-NH_2	Delocalizes essential peripheral membrane proteins involved in cell wall biosynthesis and respiration	158	Membrane depolarization of *B. subtilis*	202
CP29, Bac2A-NH2, and CP11CN	Causes laminar mesosomes-like structures arising from the septa and cell wall	203	Membrane depolarization and permeabilization of *S. aureus*	225
Lysines conjugated lipidated biphenyls (Compound 2)	Blocks peptidoglycan biosynthesis	90	Depolarize the cell membrane of *S. aureus* and leakage of intracellular K^+ ions	90
2-[1-[(2-chlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl] ethanamine and 2-[1-[(3,4-dichlorophenyl)methyl]-2-methyl-5-methylsulfanylindol-3-yl] ethanamine	Interacts with lipid II substrate via pyrophosphate motif	226	Showed membrane depolarization and disruption activity against negatively charged lipid vesicles	226

human neutrophil peptide-1, binds to lipid II to kill *S. aureus* (156). Macrocyclic depsipeptide, such as ramoplanin, inhibits peptidoglycan biosynthesis at the periplasmic transglycosylation step (157). Other than targeting the peptidoglycans, some linear AMPs (i.e., RWRWRW-NH_2) target the respiration process in bacteria and delocalize the peripheral membrane proteins MurG and disrupts the integrity of the cell wall (158).

ACTIVITY AGAINST *S. AUREUS* BIOFILM AND PERSISTERS

AMPs and Small Molecules that Affect *S. aureus* Biofilms

Biofilms are targeted by multiple mechanisms like disruption of quorum sensing (QS) (152), suppression of the cellular stress response (159), downregulation of virulence/biofilm genes (70, 160), and direct disruption of the biofilm matrix (152). Biofilms can render conventional antibiotics ineffective (161). Within biofilms, polysaccharide intercellular adhesins provide a stable, hydrated matrix that binds cells together in a three-dimensional (3D) structure. The recognition of microbial surface components recognize adhesive matrix molecules determines the initial attachment of *S. aureus* to form biofilm on the host cell surface (162, 163). Several components play an active role in biofilm attachment, including elastin binding protein (ebpS), laminin-(eno), fibronectin-binding protein (fnbA and fnbB), fibrinogen binding protein (fib), and aggregation factor (clfA and clfB) (164). In particular, the *agr* system plays a significant role in the initial attachment and disassembly of biofilms by repressing adhesins and stimulating dispersal factors (165). Coordination of these factors is essential to ensure progress through the three stages of biofilm formation and the development of *S. aureus* chronic infections (163).

Disruption of Quorum Sensing by Membrane-Active AMPs and Small Molecules

There are limited examples of inhibition of QS by cationic AMPs and are mostly seen in Gram-negative bacteria (166). A few studies have demonstrated that membrane-active AMPs inhibit or target *S. aureus* QS (152). Recently, the Cec4-derived C18 peptide was found to downregulate the expression of *agrA* (70). Also, in a recent study, BCp12, a milk-derived AMP, is predicted to target the *S. aureus* cytoplasmic membrane, and also affects the QS system by downregulating *agrA*, *agrB*, and *agrC* (167, 168). A lack of *agr* increases biofilm formation because RNAIII reduces surface adhesin expression and increases capsule, toxin, and protease production (169). Surface adhesins of *S. aureus* are essential for the initial attachment of biofilm cells and intercellular adhesion during the biofilm maturation process (170, 171).

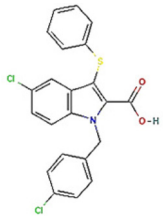
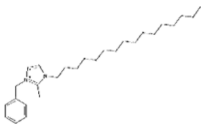
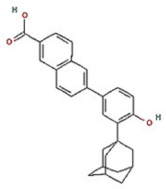
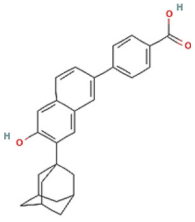
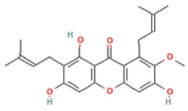
Suppression of the Cellular Stress Response by Membrane-Active AMPs

Under starvation conditions, bacteria produce biofilms, and their proficiency for transferring resistance genes increases and are thus more resistant to antibiotics (152, 159, 172). To coordinate the stringent response, guanosine pentaphosphate and its active moiety (ppGpp) are synthesized as small signaling nucleotides called alarmones, which bind to and change the specificity of RNA polymerase (159). A ppGpp network generally plays a role in "triggered persistence," which occurs when specific signals, such as starvation and nutrient stress, trigger bacterial persistence (173). A loss of ppGpp will therefore not push the bacteria toward dormancy or induce persistence and would make the situation difficult for the antimicrobials to work (174, 175). *RelA* and *SpoT* are homologous proteins that control ppGpp levels, and mutants lacking both *relA* and *spoT* (or *rsh*) were defective in forming biofilms (159, 176). Interestingly, the *S. aureus* membrane-active AMP 1018 caused a complete loss of ppGpp in *S. aureus* (159). Upon overexpression of *relA* synthase, peptide IDR-1018 could no longer inhibit *S. aureus* biofilms, and genetic blocking of ppGpp synthesis eradicated 2-day mature biofilms (159).

Disruption of the Biofilm Matrix by Membrane-Active AMPs and Small Molecules

Several AMPs, including tryptophan-rich horine (177), DFTamp1 (68), temporins-1Ola (178), WWW motif-based peptides (72), and LL-37-derived GF-17 and 17BIPHE2 (134, 179) target the cell membrane, reduce the biomass of established *S. aureus* biofilms, and kill the bacterial cells within the biofilm. Mechanistically, membrane-active AMPs weaken the biofilm matrix by forming pores within lipid components (180). Some AMPs such as the Lf-KR-12

TABLE 4 Membrane-active small molecules against the persister and biofilm of *S. aureus*

Membrane-active small molecules/AMPs	Structure of Small molecules/AMPs	*S. aureus* strain	Activity against *S. aureus* persister	Activity against *S. aureus* biofilm	References
nTZDpa – nonthiazolidinedione peroxisome proliferator-activated receptor gamma partial agonist		MRSA-MW2 BAA-1707	32 μg/mL reduction ~2-log and 64 μg/mL eradicated ~5 $\times$ 10^7 CFU/mL MRSA persister in 2 h	16 μg/mL eradicated ~10^7 CFU/mL MRSA MW2 within 2 h	187
NH125 – WalK inhibitor		MRSA-MW2 BAA-1707	5 μg/mL eradicated 5 $\times$ 10^7−10^8 CFU/mL *S. aureus* persister within 4 h		25
CD437 – synthetic retinoid		MRSA-MW2 BAA-1707	At 8–10$\times$ MIC eradicated, the persister of *S. aureus* within 1–4 h	90% MRSA persister biofilm killing at 16$\times$ MIC	100
CD1530 – synthetic retinoid		MRSA-MW2 BAA-1707	At 8–10$\times$ MIC eradicated, the persister of *S. aureus* within 1–4 h	100% MRSA persister biofilm killing at 32$\times$ MIC	100
α-mangostin – natural xanthone		MRSA-MW2 BAA-1707	Eradicated 99.99% of *S. aureus* persister within 30 min of treatment	Disrupted (96%) and inhibited (90%) the biofilm of *S. aureus*	105

peptide (180) and esculentin-1a (181), can disrupt biofilms and alter membrane potential leading to further membrane rupture.

Nisin A and lacticin Q are bacteriocins that form stable pores on *S. aureus* membranes and inhibit biofilm formation (182). Similarly, membrane-active small molecules like lipidated biphenyls (90), 2,4-dichlorobenzyl derivative (110), phenylglyoxamide (183), NH125 (25), retinoids (100), and alpha mangostin (160) disrupt the mature/preformed biofilm of *S. aureus* (Table 4). Even though the AMPs and small molecules described in this section depolarize the *S. aureus* membrane and ultimately rupture it, genetic basis or proteomic evidence is still lacking.

AMPs and Small Molecules against *S. aureus* Persisters

Hurdle et al. (54) proposed that membrane perturbation is a more profitable mechanism of action than nonmembrane-targeting antibiotics to eliminate persister cells because of the possibility of developing resistance (54). For example, membrane-active AMPs, including

TABLE 5 *In vivo* analysis of membrane-active small molecules and AMPs against *S. aureus*

Membrane-active small molecules and AMPs	Effect against *in vivo* model	Effective concn against *S. aureus*	Animal model	References
nTZDpa	90% survival	16 μg/mL	*C. elegans*	187
PQ401	100% survival	2 μg/mL	*C. elegans*	98
Small cationic molecule	5.3-log MRSA biofilm reduction (>99.99%)	40 mg/kg	Murine model of superficial skin wound infection	194
L-lysine-based lipidated biphenyls	Reduced bacterial burden (*P* value 0.0001)	200 mg/kg	Murine model of skin infection	90
Xanthones derivatives	MRSA reduction by 2.56 logs (99.7%) and 3.03 logs (99.9%)	3 mg/mL	Mouse model of corneal infection	106
Bithionol + gentamicin	90% MRSA persister killed	30 mg/kg of bithionol + gentamicin	Mouse thigh infections	97
Cationic peptide DFT561	*S. aureus* USA300 LAC decreased by 1.8 logs (liver) and 1.4 logs (kidney)	5 mg/kg	Neutropenic mouse model	185
AMP is based on the cationic structure of honokiol/magnolol amphiphiles	Reduction in bacterial loads in the liver, spleen, and kidney in 3 days	5 and 10 mg/kg	Murine sepsis model	113

C10KR8d (184), horine and verine (177), DFT561d (185), SAAP-148 (186), and small molecules, including CD437 (100), and nTZDpa (187) showed least change in MICs after several serial passages against *Staphylococcus aureus*.

Persister cells are not actively dividing (188), and membrane disruptors can kill bacteria that are not actively dividing, unlike other antibiotics that require active division of bacteria to be effective (189). AMPs like frog-skin AMP temporin G (TG) (190), cecropin-4 derived AMPs (70), short cationic AMPs (varine and horine) (177), and SAAP-148 derived from LL-37 (186) eliminated *S. aureus* persisters. Interestingly, cationic AMPs show greater potential to eliminate *S. aureus* persisters. It is therefore likely that cationic AMPs will be effective at eliminating nondividing and antibiotic-tolerant persister *S. aureus* bacteria or potentiating other antibacterial agents. Several other findings support that membrane-active small molecules could kill nondividing bacterial persisters effectively (97, 187). Small molecules such as XF-73 (191), NH125 (25), lipidated biphenyls derived from L-lysine (90), nTZDpa (187), retinoids (CD437 and CD1530) (100), and bithionol (97) seem to exhibit rapid killing of persisters. A hydrophobic interaction occurs between the aromatic backbones and lipid tails of membrane-active small molecules with the hydrophilic carboxylic acid and phenolic hydroxyl moiety binding heads of *S. aureus* lipid bilayers (100) (Table 4). In this interaction, the lipid bilayers of the membrane are permanently damaged (100). Notably, changing the structure of nTZDpa by modifying three polar branch groups or the carboxylic acid moiety of the aryl thioether improved the efficacy against stationary-phase and persister *S. aureus* cells (192).

EFFICACY IN MAMMALIAN ANIMAL MODELS

The selectivity and toxicity of membrane-active AMPs and small molecules to mammalian cells are a major hurdle in moving these agents to clinical practice. Nevertheless, the local application of membrane-active drugs is already proved in animal models (Table 5). For example, mouse skin infection models were implemented in testing small molecules and AMPs like LTX-109 (79), 1% HT61 gel (193), small cationic molecule (194), and L-lysine-based lipidated biphenyls (90) against *S. aureus* superficial skin wound infection and dermal infections. Studies also evaluated membrane-active AMPs and small molecules in an MRSA corneal infection model (106) and the mouse thigh that mimic human deep-seated chronic infections caused by the MRSA persister model (97). Regarding systemic infection models, Mishra et al. (185) tested the *in vivo* toxicity and efficacy of a cationic peptide DFT561 in a neutropenic mouse model. DFT561 demonstrated 90% killing of MRSA and reduced MRSA load in the liver, spleen, and kidney of mice (185). Also, using a sepsis model, Guo et al. (113) found a significant reduction in MRSA with cationic AMP-linked honokiol/magnolol amphiphiles.

DRAWBACKS AND CHALLENGES ASSOCIATED WITH AMPS

A critical factor that affects the binding of cationic AMPs to the bacterial membrane is the presence of salt and serum (195). The antimicrobial effect of AMPs is often reduced by a process known as the "charge shielding effect," which involves competitive binding between cations in salts and AMPs (195). However, the charge-shielding effect can be reduced or nullified by adding more numbers of charged amino acids to the peptides (180). AMPs, such as the cecropin-derived AMP C18 (+6 net charge) and the arginine-rich decamer peptides D5 (+8 net charge) and D6 (+9 net charge) maintain high antimicrobial efficacy even in the presence of monovalent or divalent salt cations (196).

Several AMPs, including LL-37, TP4, SAAP-159, and H1α, lose their antimicrobial potency in the presence of serum (197). The interaction between LL-37 and serum albumin could reduce the ability of the peptide to bind to pathogens (186). Recently, LL-37-derived KR-12 lipopeptides interacted with serum albumin, hemoglobin, serotransferrin, and apolipoproteins in mouse serum (184). Interestingly, the D-form of KR12 lipopeptides demonstrated reduced serum binding compared to its L-form (184).

Also, AMPs such as ARVA and NATT (short synthetic AMPs discovered through a combinatorial library-based approach) (198), have poor solubility and host cell toxicity and are susceptible to proteolysis (199). Using synthetic molecular evolution, Starr et al. (199) achieved high solubility and host cell selectivity of AMPs by applying selection pressures during initial AMP screening. Similarly, by using computational simulation techniques, Pandey et al. (200) designed new nisin mutants with more solubility at physiological pH.

Moreover, linear AMPs are more susceptible to proteolysis by the host proteases (185). However, the derivatives of AMP Pep05 containing unnatural residues such as L-2,4-diaminobutanoic acid (Dab), L-2,3-diaminopropionic acid (Dap), l-homoarginine, 4-aminobutanoic acid (Aib), and l-thienylalanine, have improved stability toward trypsin, plasma proteases, and secreted bacterial proteases (201). D-analogs of DFT561 (185) and KR12 lipidated version had more enzymatic stability to proteolytic degradations (184).

BACTERIAL SYSTEM INVOLVED IN SUSCEPTIBILITY TO MEMBRANE-ACTIVE AGENTS

Several mechanisms protect bacteria from membrane-targeting antimicrobial peptides (AMPs) and small molecules (202). Among these mechanisms are regulatory systems that modify the surface charge of the bacterial cell membrane to reduce binding to cationic AMPs, breaking down AMPs with secreted exoproteases, and using AMP transporters to remove AMPs (203, 204). In the case of *S. aureus*, the GraRS regulatory system plays a key role in bacteria cell surface charge-mediated deactivation of membrane-targeting compounds (205). In MRSA, the expression of two key determinants of net positive surface charge (encoded by *mprF* and *dlt*) depends on the cotranscription of *graR* and *vraG* genes (205). The MprF protein helps the synthesis and movement of lysyl-PG to the outer leaflet of the bacterial membrane, increasing the net-positive surface charge (206). Similarly, the *dlt* operon reduces net negative surface charges in *S. aureus* by transferring extra D-alanine to teichoic acids (207). Other regulatory systems in *S. aureus* are Aps and BraRS (208–210). The Aps system is composed of a two-component histidine kinase/response regulator (ApsR, ApsS), a third protein of unknown function (ApsX) and is thought to be a widespread mechanism for sensing and regulating AMPs in bacteria (208–210). The BraRS system is quite specific to *S. aureus* (210). The membrane charge of *S. aureus* also affects the effectiveness of antibacterial agents such as moenomycin and vancomycin (211).

S. aureus cleaves membrane-active AMPs by expressing two types of proteinases: a metalloproteinase called aureolysin and a glutamyl endopeptidase called V8 protease (203, 212). Aureolysin inactivates cationic AMPs, including LL-37, by cleaving specific residues involving Arg19-Ile20, Arg23-Ile24, and Leu31-Val32, while the V8 protease hydrolyzes the Glu16-Phe17 peptide bond, making the C-terminal fragment resistant to further degradation (212). Contrary to AMPs, synthetic chemical compounds lack peptide bonds, so proteases cannot cleave them. Also, in *S. aureus*, the PmtA-D proteins form an ABC transporter (213). While, structurally, the Pmt transporter is composed of two membrane proteins (PmtA and C)

and two ATPases (PmtB and D) (213), functionally, they are associated with exporting human-derived AMPs such as hBD3 and LL-37 (214, 215).

CONCLUSIONS

Membrane-targeting small molecules and AMPs such as colistin, daptomycin, gramicidin, and nisin indicate that membrane-active compounds have a potential role in combating *S. aureus* infections. However, the toxicity, serum binding, stability, and product cost of membrane-active medications remain major hurdles to the wider adoption of these agents. Studies have described the MOA of AMPs using classical and nonclassical membrane-targeting models, but to date, no clear MOA has been described for small molecules. The gap in understanding how membrane-active compounds work against *S. aureus* needs to be bridged to enhance drug development and application in clinical trials.

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