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MINIREVIEW

# Control of Biofilm Formation: Antibiotics and Beyond

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**ABSTRACT** Biofilm-associated bacteria are less sensitive to antibiotics than free-living (planktonic) cells. Furthermore, with variations in the concentration of antibiotics throughout a biofilm, microbial cells are often exposed to levels below inhibitory concentrations and may develop resistance. This, as well as the irresponsible use of antibiotics, leads to the selection of pathogens that are difficult to eradicate. The Centers for Disease Control and Prevention use the terms "antibiotic" and "antimicrobial agent" interchangeably. However, a clear distinction between these two terms is required for the purpose of this assessment. Therefore, we define "antibiotics" as pharmaceutically formulated and medically administered substances and "antimicrobials" as a broad category of substances which are not regulated as drugs. This comprehensive minireview evaluates the effect of natural antimicrobials on pathogens in biofilms when used instead of, or in combination with, commonly prescribed antibiotics.

**KEYWORDS** biofilms, complementary medicines, integrative approach, natural antimicrobial products

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As many as 80% of pathogens that form biofilms are associated with persistent infections (1, 2). Approximately 90% of the biofilm mass is composed of extracellular polysaccharides (EPS), proteins, and DNA (3). EPS provides stability to the cells, mediates surface adhesion, and serves as a scaffold for cells, enzymes, and antibiotics to attach (4–8). *Pseudomonas aeruginosa*, associated with cystic fibrosis (9), and *Staphylococcus aureus*, which is responsible for most wound infections (10), are typical examples of persistent pathogens that form biofilms.

Cells in biofilms experience stringent growth conditions. Survival depends on their ability to mutate and exchange genetic information, e.g., through horizontal gene transfer (4, 11). Resistance to antibiotics may thus be seen as a phenotypic shift in behavior when cells adapt to a sessile lifestyle (12). This hypothesis is supported by cells developing tolerance to antimicrobial peptides and phagocytosis (13). Some staphylococci produce poly-γ-DL-glutamic acid (PGA) that binds to antimicrobial peptides and protects bacterial cells from neutrophil phagocytosis (14). Other physiological changes occur due to oxygen deprivation or nutrient deprivation, especially in deeper layers of the biofilm. Oxygen deprivation and low metabolic activity in biofilms render *P. aeruginosa* more tolerant to antibiotics (15). Rapid changes in pH between layers in a biofilm may lead to the accumulation of organic acids and the deactivation of penetrating compounds (15). Complex (polymicrobial) biofilms composed of multiple species are generally more resistant to antibiotics than biofilms composed of a single species (16, 17). The diversity and metabolic state of cells in a biofilm play key roles in antibiotic resistance. Persistent cells are generally more resistant to antibiotics,

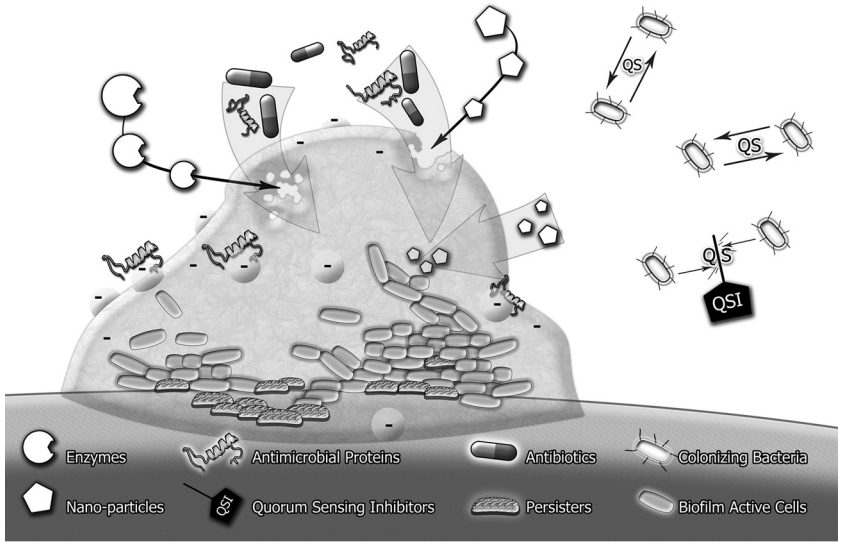


**FIG 1** Persistence of microbial pathogens in biofilms requires a sophisticated arsenal of killing machines to break their party.

and they play an important role in supporting the reestablishment of the biofilm community (8, 18, 19). Cells in dormant sections of a biofilm are usually not affected by antibiotics, as recorded with studies on $\beta$-lactams, ciprofloxacin, tetracycline, and tobramycin (9, 20).

*P. aeruginosa* showed an increase in antibiotic tolerance when the cells were immobilized in a biofilm. The efflux pump PA1874-1877 in *P. aeruginosa* was more actively expressed in biofilm-associated cells than in planktonic cells (21). Efflux pumps in *Pseudomonas* spp. are also used for secretion of biocides, such as glutaraldehyde (22). Moreover, cells with inactive efflux pumps may have diminished ability to form biofilms (23). Therefore, antimicrobial agents inactivating efflux pumps, such as thioridazine and Phe-Arg-naphthylamide (PAN) (23), might be helpful in the prevention of biofilm formation.

Failure to develop new antibiotics, combined with the spread of resistance, may result in increased morbidity and mortality, especially in health care facilities. Challenges to the discovery of alternative treatments have been mentioned in other reviews (7, 24, 25). Estrela and Abraham (26) discussed the potential of combining antimicrobial compounds with antibiotics to inhibit quorum sensing in a biofilm.

In this review, the combination of different classes of antimicrobial compounds with antibiotics to control biofilm formation is discussed and summarized (Fig. 1). This is in line with the recent approach taken by the National Center for Complementary and Integrative Health (NCCIH) of the NIH, i.e., combining conventional treatments with complementary methods to uncover "potential usefulness and safety issues of natural products" (27).

## ANTIMICROBIAL PEPTIDES: SYNERGY WITH ANTIBIOTICS WHEN AIMED AT DIFFERENT TARGETS

Antimicrobial peptides (AMPs) are naturally produced by eukaryotes and prokaryotes as a part of their innate immune/defense system (28). The unique features of many AMPs are their small size (15 to 30 amino acids), charge (often overall positive), and that they target cell membranes (28, 29). The positively charged peptides are attracted to the negatively charged cell membranes of bacteria and biofilm surfaces. Active and slow-growing bacteria in biofilms are killed by AMPs (30), and manipulation of the amino acid composition of AMPs may result in increased antimicrobial activity (31–33). One example of genetic manipulation is construction of the broad-spectrum bactericidal peptide R-FV-I16 by removing the functional "defective" sequence RR7 and

by inserting the antibiofilm sequence FV7 embedded in peptide RI16 (32). The specificity of AMPs can also be manipulated by designing specifically targeted AMPs (STAMPs) highly selective against pathogens but harmless to nonpathogenic bacteria (34, 35).

Many AMPs either form pores in the cell membrane or act as membrane perturbers (36). However, at low concentrations, AMPs may act bacteriostatically (7). de la Fuente-Núñez and coworkers have shown that AMP 1037 stimulates the swarming of *P. aeruginosa* PA2204 cells but inactivates twitching motility and biofilm formation (37). The peptide antimicrobial NA-CATH:ATRA1-ATRA1, a synthetic cathelicidin, inhibited *S. aureus* biofilm formation, and the peptide LL-37 controlled *P. aeruginosa* biofilm formation when used at levels below the MIC (38, 39). These AMPs prevented expression of the genes encoding proteins involved in biofilm formation. In *P. aeruginosa*, the downregulated genes are coding for type IV pili, rhamnolipid synthesis, quorum sensing, and the assembly of flagella (38). Some AMPs have specific antimicrobial features; for example, milk lactoferrin chelates iron and inhibits biofilm formation by *P. aeruginosa* (40). Moreover, binding of AMPs to extracellular DNA may enhance the detachment of biofilms (41).

To survive in the presence of AMPs, bacteria utilize various approaches, e.g., mutations that change the structure and charge of the cytoplasmic membrane, modification of lipopolysaccharides in the cell wall, and secretion of AMPs by specific efflux pumps, etc. (42). In addition, the *S. aureus* biofilm formation regulatory system (GraRS) plays an important role in the microorganism's resistance to AMPs (43). This resistance was reversed when AMPs were added in combination with other antimicrobial compounds. AMPs from various sources, when combined with commonly prescribed antibiotics, effectively prevented biofilm formation by *P. aeruginosa* (44–48). Moreover, STAMP G10KHc synergized with tobramycin against planktonic and biofilm-associated cells of *P. aeruginosa* (44). This peptide destabilized the cell membrane, which enhanced the penetration of tobramycin into bacterial cells. Tobramycin and AMP GL13K synergized for 67.5% eradication of *P. aeruginosa* (47). Similarly, broad-spectrum AMP tachyplesin III synergized with antibiotic piperacillin-tazobactam (TZP) against biofilm-associated *P. aeruginosa* (49). Inhalation of the amphipathic polypeptide colistin, combined with ciprofloxacin, killed biofilm-associated cells of *P. aeruginosa* and improved the lung functions of cystic fibrosis (CF) patients (46) over a 4-week treatment. Noticeably, colistin inhibited persister cells.

*In vivo* studies showed a synergistic effect on biofilms of methicillin-resistant *S. aureus* (MRSA) when nisin was combined with daptomycin/ciprofloxacin, indolicidin with teicoplanin, and cecropin(1-7)-melittin A(2-9) amide (CAMA) with ciprofloxacin (50, 51). Pretreatment of central venous catheters (CVCs) with cathelicidin peptide BMAP-28, in combination with traditional antibiotics quinupristin-dalfopristin (Q/D), linezolid (LZD), and vancomycin, reduced *S. aureus* on CVC and prevented bacteremia (52). To effectively eradicate biofilms on CVCs, the "antibiotic lock" technique (also called "intraluminal therapy") was suggested, which involves the filling of CVCs with a predetermined concentration of AMPs. A combination of the cationic peptide IB-367 and LZD in the antibiotic lock technique eradicated *S. aureus* biofilms on CVCs (53). A noticeable reduction in biofilm-associated *S. aureus* on vascular grafts was observed when sub-MIC levels of vancomycin were combined with the lipopeptides Pal-Lys-Lys-$NH_2$ and Pal-Lys-Lys (54). Some AMPs with broad antibiofilm activity, such as peptide 1018, blocked or degraded guanosine pentaphosphate [(p)ppGpp], which is essential for biofilm formation. At low concentrations, peptide 1018 inhibited biofilm formation but eradicated preformed biofilms when applied at higher concentrations (55). In a separate study, the same authors reported on the *in vivo* and *in vitro* antibiofilm activity of newly synthesized broad-spectrum D-enantiomeric AMPs (56). These peptides synergized with antibiotics in the inhibition and eradication of pathogenic biofilms of *P. aeruginosa*.

AMPs combined with conventional antibiotics may be a better alternative than antibiotics alone. The synergy of AMPs and antibiotics against biofilm-associated

pathogens should attract the attention of scientists to explore the mechanistic actions of these combinations.

## BIOFILM-DEGRADING ENZYMES: EFFECTIVE HELPERS WHEN IT COMES TO MATRIX DESTRUCTION

Adhesion to surfaces stimulates bacterial cells to produce EPS (57), which is mainly composed of polysaccharides, proteins, and nucleic acids (58). These components play a key role in cell-cell or cell-surface attachment, supporting the integrity of biofilm architecture and protecting biofilm cells from the shearing stress factors (59, 60). Enzymes could inhibit and disrupt the EPS matrix formation and then facilitate the detachment of biofilm. However, a second antimicrobial substance is required to target the detached cells (61, 62).

The biofilm-degrading enzymes DNase I, $\alpha$-amylase, and dispersin B (DspB) reduced the EPS mass and biofilm cell numbers (62–65). However, the older the *P. aeruginosa* biofilm, the more difficult it was to be dissolved by DNase I. The production of high quantities of EPS and proteolytic exo-enzymes by the mature biofilms inactivated DNase I (64). Nevertheless, purified recombinant DNase I derivative (DNase1L2), extracted from human stratum corneum, effectively controlled biofilm-associated *P. aeruginosa* and *S. aureus* (63). *Bacillus subtilis* S8-18 $\alpha$-amylase was evaluated against biofilms of a clinical MRSA strain and *P. aeruginosa* ATCC 10145 (65). Efficient biofilm inhibition and degradation of mature biofilms were reported due to the disruption of EPS. Interestingly, *B. subtilis*-derived $\alpha$-amylase was more effective in degrading the EPS in biofilms of *S. aureus* and *P. aeruginosa* than amylases from human saliva and sweet potato (66). In a different study (67), strong degradation activity of $\alpha$-amylase (82% biofilm reduction) against biofilm-associated *P. aeruginosa* was also reported. Biofilm-degrading enzymes, such as lysostaphin (68) and alginate lyase (69), showed antibiofilm activities against various pathogenic bacteria. Although these enzymes destroy and detach biofilms, biofilm reestablishment is not guaranteed.

Treatment of *S. aureus* biofilm with combinations of recombinant human DNase I (rhDNase I) and topical antiseptics (chlorhexidine gluconate and povidone iodine) reduced the number of viable cells by an additional 4 to 5 logs compared to treatment with antibiotics only (70). DNase is likely acting against biofilms by changing their texture and morphology and influencing biofilm-associated cell numbers (71). In turn, alteration of biofilm structure enhances the activity of antibiotics against biofilms of *P. aeruginosa* and *S. aureus*.

Donelli and coworkers (72) found that dispersin B (DspB), produced by *Actinobacillus actinomycetemcomitans*, alone or in synergy with cefamandole nafate, hydrolyzed the EPS of a staphylococcal biofilm, promoted antibiotic penetration, and augmented the killing of microbial cells. Furthermore, DspB synergized with triclosan against *S. aureus* biofilms formed on vascular catheters (61).

Using a continuous-flow culture, alginate lyase showed remarkable sensitization and elimination of mucoid biofilm-associated *P. aeruginosa* when administered with gentamicin (69). The enzyme lysostaphin, extracted from *Staphylococcus simulans*, killed *S. aureus* by cleaving the pentaglycine cross-bridges in the cellular membrane's peptidoglycan, and it destroyed extracellular polymeric substance matrix of lysostaphin-sensitive staphylococci (73). Furthermore, lysostaphin (15 mg/kg) combined with naficillin (50 mg/kg) effectively killed MRSA biofilms on a medical device (68). Lysostaphin has shown synergy with five of nine antibiotics, with the highest eradication of MRSA in combination with clarithromycin, which suppresses hexose polymerization (74). Early adhesion and dispersion of *S. aureus* in mature biofilms were inhibited by proteinase K (75).

Despite the high cost of production, biofilm-eradicating enzymes could possibly be used as an alternative or as a synergistic helper to antibiotics in the treatment of persistent infections.

## QUORUM-SENSING INHIBITORS: STOP TALKING, HELP KILLING

Quorum sensing (QS) regulates virulence behaviors, including biofilm formation (76). QS compounds include *N*-acyl-homoserine lactones (AHLs), produced by Gram-negative bacteria, and autoinducing peptides (autoinducer 2 [AI-2]) produced by Gram-positive bacteria. Inhibition of biofilm formation by QS quenchers or inhibitors (QSQ or QSI, respectively) may play a significant role in preventing biofilm formation by many pathogens. Therefore, enzymatic degradation of QS signals, such as lactonase, acylase, oxidoreductase, and paraoxonase, could be considered a promising approach in controlling biofilm formation (77). QSQs can also attenuate QS by blocking or shutting down the expression of QS genes in pathogens, which leads to biofilm inhibition without killing planktonic cells or influencing normal growth.

Recently, inhibition of QS and biofilm formation had been reported in several studies (78–80). The injection of RNAIII-inhibiting peptide (RIP) in rats with MRSA graft infection repressed staphylococcal RNAIII-activating protein (RAP) and *agr* QS systems, which are required for staphylococcal biofilm formation (79). In addition, usnic acid, a natural secondary metabolite of lichen, interfered with QS, which resulted in the prevention of *S. aureus* biofilm formation and changed the morphology of *P. aeruginosa* biofilms (78). A pungent oil of fresh ginger (6-gingerol) adhered to QS receptors in *P. aeruginosa* and paused biofilm maturation. Transcriptomic analysis confirmed that 6-gingerol inhibited QS-induced gene expression and the production of virulence factors (81).

Several compounds were reported to have QS-inhibiting effects, including penicillic acid, solenopsin A, catechin, ellagic acid derivatives, and curcumin (82). Rasmussen et al. (83) referred to the activity of patulin and penicillic acid, which were isolated from *Penicillium* species, as active QSI compounds that controlled QS gene expression in *P. aeruginosa*. The use of QSI alone, or in combination with antibacterials, creates an opportunity for their implementation in biofilm-controlling formulations.

Interestingly, a clearance of *P. aeruginosa* biofilm and reduction in pyocyanine production were reported when phenyl-DPD (phenyl-4,5-dihydroxy-2,3-pentanedione), an AI-2 analog, was combined with gentamicin, indicating the possible role of QS systems in biofilm maturation and/or dispersion (84). Furthermore, while lactonase from *Bacillus* spp. did not affect the growth of *P. aeruginosa*, it reduced biofilm formation (85). The perturbation of biofilm formation by lactonase increased the susceptibility of biofilms to antibiotics and significantly reduced the production of virulence factors when lactonase was used in combination with ciprofloxacin and gentamicin. It is still to be explored if QSIs from various sources possess similar antibiofilm activity.

Plant-derived QSIs often exhibit remarkable biofilm reduction ability, especially when combined with antibiotics. An *in vivo* study by Brackman and coworkers (86) assessed the activity of tobramycin against *P. aeruginosa* biofilm, and the activities of clindamycin and vancomycin against an *S. aureus* biofilm, alone and in combination with QS inhibitors (baicalin hydrate, cinnamaldehyde, and hamamelitannin). The combined treatments strengthened the antibiotics' potential (86).

The traditional Chinese medicine baicalein proteolytically digested the signal receptor (TraR protein) in *P. aeruginosa*, which is likely contributing to its antibiofilm activity as a QSQ (87). In addition, 14-alpha-lipoyl andrographolide (AL-1), an antimicrobial diterpenoid lactone from green chiretta (*Andrographis paniculata*), inhibited the Las and Rhl QS systems in *P. aeruginosa* by suppressing the transcriptional level of QS-regulated genes (88). Both baicalein and AL-1 synergized with the tested antibiotics against *P. aeruginosa* biofilm. Furthermore, fruit extract of *Lagerstroemia speciosa* (LSFE) caused the downregulation of QS genes (*las* and *rhl*) and *N*-acyl-homoserine lactones in *P. aeruginosa* PAO1 (89). Also, LSFE increased the antibiotic potential of tobramycin in *P. aeruginosa* PAO1 biofilms. A garlic extract, ajoene, inhibited or controlled QS-associated virulence factors, such as rhamnolipids, in *P. aeruginosa* (90). In the same study, ajoene synergized with tobramycin, killed *P. aeruginosa* in biofilms, and pre-

vented lytic necrosis of polymorphonuclear leukocyte (PMN) cells (91). Recently, a comprehensive *in vivo* study was performed by Christensen et al. (92), evaluating differences between single treatments (ajoene or horseradish juice extract) and combination treatment (QS inhibitors with tobramycin) of BALB/c mice in which wild-type *P. aeruginosa* were injected into the peritoneal cavity. Mice treated with a combination of the antimicrobials showed a significant decrease in the number of biofilm-associated *P. aeruginosa* cells compared to mice treated with a single formulation.

Overall, QSIs combined with antibiotics could have a great impact on future applications to prevent biofilm formation of clinically important pathogens, especially *P. aeruginosa*.

## ESSENTIAL OILS: BROAD-SPECTRUM COMPOSITIONS, MULTIPLE MECHANISMS OF ACTION IN ASSISTING ANTIBIOTICS

Essential oils (EOs) are natural antimicrobial formulations with broad-spectrum activities against bacteria, fungi, and viruses (93). EOs may inhibit ATP production and ATPase activity. Moreover, EOs disrupt membrane permeability and modify proton motive forces and membrane fatty acids, leading to the leakage of metabolites and ions. Some EOs act as QSIs by interfering with and regulating QS genes, leading to a reduction in biofilm formation and virulence factor production (see Table 1 in the review of Nazzaro et al. [94]). The ease of EO extraction, nontoxicity to the tissue culture, quick degradation in water, and positive health impacts (95–97) may increase the value of EOs as alternative antimicrobial agents.

Kavanaugh and Ribbeck (98) referred to the high biofilm-eradicating effect of three EOs, cassia, Peru balsam, and red thyme, compared to ofloxacin and gentamicin against biofilms of *Pseudomonas* and *S. aureus*. Biofilm formation was also inhibited when oregano essential oils, carvacrol and thymol, were used against *S. aureus* (99). Five of nine biofilms formed by coagulase-negative staphylococci (CoNS) strains were completely eradicated when 5% tea tree oil (TTO) was used, while the same concentration of TTO achieved complete eradication of methicillin-susceptible *S. aureus* (MSSA) and MRSA biofilm growth as microcolonies in glycocalyx during 1 h of treatment (100). The antimicrobial function of TTO was attributed to the disruption of the hydrophobic phospholipid bilayers in the cell membrane.

Few studies focused on the antimicrobial combinations of EOs and antibiotics. EOs modify the tolerance of bacterial cells to antibiotics (reviewed by Yap et al. [101]). In this regard, synergistic activity was reported when *Pelargonium graveolens* essential oil was used in combination with norfloxacin against two strains of *S. aureus* (102). In the same study, EOs increased the norfloxacin uptake by bacterial cells. This may reduce the side effect(s) of antibiotics. Moreover, the antibiofilm potential of several EOs, including eugenol, cinnamaldehyde, citral, and geraniol, had been elucidated (103). Three essential EOs, cinnamon (*Cinnamomum zeylanicum*), TTO (*Melaleuca alternifolia*), and palmarosa (*Cymbopogon martini*), synergized with ciprofloxacin against preformed biofilm of *P. aeruginosa* (104).

The antimicrobial tolerance of *P. aeruginosa* was effectively controlled when two antimicrobials that targeted more than one component of the bacterial cell (105) were combined in a single formulation. The targets included DNA synthesis (106) and the cytoplasmic membrane (107). EOs from *Origanum vulgare* L., carvacrol and thymol, were identified as putative efflux pump inhibitors facilitating the uptake of antibiotics, norfloxacin, erythromycin, and tetracycline (108).

More *in vitro* and *in vivo* studies are required to verify the safety and efficacy of EOs as drug resistance modulators, alone or in combination, with conventional antibiotics.

## NANOPARTICLES: NEW GENERATION OF ANTIBIOTIC HELPERS

Various nanoparticles (NPs) are often reported as having an inhibitory effect against planktonic and biofilm cells. This activity is related to ATP-associated metabolism, permeability of the outer membrane, and the generation of hydroxyl radicals that are induced by bactericidal compounds (109). Silver nanoparticles (Ag NPs) at concentra-

tions of 100 mg/ml showed antibiofilm activity by causing a 4-log reduction in *P. aeruginosa* cell growth (110). Moreover, a 95% inhibition in biofilm formation by *P. aeruginosa* was noted when Ag NPs were used as an antibiofilm agent for 24 h (111). A synergism was noticed when NPs were combined with antibiotics against *S. aureus*, leading to disruption of the biofilm architecture and modulation of the antibiotic resistance of pathogens. Ag NPs were reported to inhibit QS and prevent biofilm formation by *S. aureus* (112). In the same study, a synergistic antibiofilm effect was noticed when Ag NPs were combined with chloramphenicol and gentamicin. The antimicrobial activity of Ag NPs is influenced by their net charge and their ability to diffuse through a biofilm (113).

Recently, Gurunathan et al. (114) generated new cost-effective Ag NPs prepared by combining silver ions with leaf extract of *Allophylus cobbe*. These NPs showed higher antibacterial and antibiofilm activities against *P. aeruginosa* and *S. aureus* when combined with ampicillin and vancomycin than when using NPs or antibiotics alone. The interaction of $Ag^+$ with the bacterial cell membrane disrupted membrane permeability, inhibiting respiratory enzymes and thus production of reactive oxygen species (ROS) (115). It had been suggested that at higher production levels of ROS, cellular membranes become more damaged, leading to increased ampicillin and vancomycin uptake (115).

In an attempt to improve the bactericidal activity of NPs, Habash and coworkers (116) evaluated different sizes of citrate-capped Ag NPs, alone and in combination with aztreonam, against *P. aeruginosa*. Ten-nanometer capped Ag NPs synergized with aztreonam, efficiently disrupting the biofilm structure of *P. aeruginosa*.

A combination of antibiotics (ampicillin, oxacillin, and penicillin) with selenate NPs (SeNPs) was more effective (94%) in disrupting and inhibiting MRSA biofilms than the antibiotics alone (117).

Overall, the antimicrobial potential of NPs compounds may depend on their sizes, charges, and stability in order to enhance antibiotics and control biofilm. However, the safe consumption of NPs must be established before using them in pharmaceutical formulation as antibacterial agents.

## CONCLUSION: COMPLEMENTARY APPROACH MAY BE A SOLUTION THAT WORKS WITHOUT ABANDONING "OLD-TIMER" ANTIBIOTICS

In this minireview, the attempt was made to bring the reader's attention to the abovementioned challenges, illustrating them with two representative organisms from the groups of Gram-positive and Gram-negative pathogens. With the goal of provoking and inspiring the reader's interest to the topic of this review, we consider it important to mention another target, the so-called "Gram-variable" pathogens, such as *Gardnerella vaginalis*, one of the major contributors to a multimicrobial infection known as bacterial vaginosis (118). Similar to discussed representatives of two groups of infectious bacteria, *G. vaginalis* biofilms are effectively controlled by combinations of DNase (enzyme) and metronidazole (antibiotic) (119). Subtilosin (AMP) inhibited biofilms of *G. vaginalis* when combined in synergistic formulations with antibiotics (metronidazole or clindamycin) or naturally derived substances, such as lauramide arginine ethyl ester (120, 121). Also, thymol had been found to interfere with the adhesion of *G. vaginalis* to human vaginal cells (122), and the combination of thymol and eugenol showed synergistic activity against newly established and matured *G. vaginalis* biofilm, reducing the microbial adhesion to the human vaginal epithelial cells (122). Moreover, in the *in vivo* study, a synergistic activity between thymol and eugenol vaginal douche was reported to reduce the recurrence rate of bacterial vaginosis (BV) infection (123). All of these are sound examples of the complementary approach's validity and significance when applied to traditional and unorthodox pathogens.

Finding an effective strategy to control biofilm formation remains a challenge (Table 1). Antibiotic resistance and the recurrence of infections reflect the failure of conventionally used antibiotics in the treatment of biofilm-associated persistent infections. Alternative methods for biofilm prevention and/or eradication are urgently required to

**TABLE 1** Mode of action, advantages, and limitations of the reviewed antibiofilm agents

| Antimicrobial compound | Proposed mode(s) of action on biofilm[a] | Advantage(s)[b] | Limitation(s) |
|---|---|---|---|
| Antimicrobial peptides | Cationic AMPs interact with anionic biofilm surface (37) | Relative selectivity, broad-spectrum activity, cationic and amphipathic properties, disruption of bacterial cell membrane with low frequency, and slow emergence of bacterial tolerance (28, 29) | Development of resistance to AMPs via modification of bacterial lipopolysaccharides or using efflux pumps (43) |
| | AMPs inactivate the twitching motility of *P. aeruginosa* and inhibit biofilm formation (37) | Bactericidal activity against slow-growing bacteria within biofilm (30) | Susceptibility of some AMPs to proteolytic enzymes as well as their high cost of purification and sequences (140) |
| | Disrupt expression of biofilm formation essential genes, downregulate expression of type IV pili, rhamnolipid, quorum-sensing, and flagellar assembly genes (38) | Antimicrobial activity improved and cytotoxicity reduced by modifying and hybridization the sequences of primary amino acids (31, 32) | Insufficient selectivity of STAMPs (140) |
| | Bind to eDNA and accelerate detachment of the biofilm (41) | | Possible hemolytic effect and potential cytotoxic effect (140) |
| Biofilm-degrading enzymes | D*egradation* of extracellular matrix components (polysaccharides, protein, and eDNA) (63) | Efficient inhibition of biofilm formation, disruption of EPS production, dispersing preformed biofilm (64, 66)<br>Reduces no. of biofilm-viable cells (70) | High quantities of EPS and proteolytic exoenzymes by mature biofilm counteract the enzymatic activity (64) |
| Quorum-sensing inhibitors | Inhibition of cell-to-cell or cell-to-surface attachment | Inhibition of phosphorylation of the TRAP protein by QSI; TRAP regulates expression of virulence factors (biofilm formation, essential proteases, toxins) and their regulator, *agr* (79) | Lack of bactericidal activity (85) |
| | Inhibition of binding of QS signals to receptor proteins, antagonizing quorum-signal biosynthesis, or degrading QS signals (136) | QSIs possess high species specificity and effectively act against certain pathogens (138) | Reported toxicity of and resistance to QSI (136) |
| Essential oils | Attack cellular ATP and ATPase, acting on cytoplasmic enzyme and membrane proteins/fatty acids leading to the leakage of metabolites and ions (94) | Have a broad-spectrum activity against a wide range of pathogenic microbes (93) | Some EOs produce oxidative stress and possess toxic properties, inducing killing activity against eukaryotes (141) |
| | Anti-quorum-sensing activity by downregulation of QS genes leading to reduced virulence factor production and biofilm formation (94)<br>Putative efflux pump inhibitors facilitating the uptake of antibiotics (108) | Have been used as ethnomedicine against bacterial infection and cancer for a long time (95, 96)<br>Simple extraction, nontoxic to tissue culture, quick degradation in water, and positive health impacts (97)<br>Biofilm inhibition is noticed at the sub-MICs of many tested EOs, such as *Thymus vulgaris* EO (103) | Increased albumin level and skin irritation (see review by Patel et al. [141]) |

(Continued on following page)

**TABLE 1** (Continued)

| Antimicrobial compound | Proposed mode(s) of action on biofilm[a] | Advantage(s)[b] | Limitation(s) |
|---|---|---|---|
| Nanoparticles | Interference with ATP-associated metabolism, change in outer membrane permeability, and generation of hydroxyl radicals (109) | New technique, simple method, cost-effective compounds, and deliver strong antimicrobial activity (114) | Antimicrobial potential of NPs depends on size, charge, stability, and biocompatibility (116) |
| | Inhibition of quorum sensing and prevention of biofilm formation (112) | As antimicrobial carriers (139), have high stability in biological environment and high carrier capacity, possibility to incorporate both hydrophilic and hydrophobic molecules, and viability using different courses (oral, parenteral, and inhaled) of administration | Cytotoxicity (139) |
| | Changing of bacterial protein profile and modification of pathogenesis by interaction with bacterial DNA (137) | Design NPs to ensure release of an efficient drug concn from matrix (139) | |

[a]eDNA, extracellular DNA.
[b]TRAP, target of autoinducer RNAIII-activating protein.

**TABLE 2** *In vivo* and *in vitro* studies of natural antibiofilm agents in combination with antibiotics for combating biofilm-associated pathogens

| Natural antibiofilm compound[a] | Antibiotics in combination | Interaction activity | Study type(s) | Biofilm-associated pathogen(s) | Reference(s) |
|---|---|---|---|---|---|
| Antimicrobial peptides | | | | | |
| G10KHc (STAMP) | Tobramycin | Synergistic effect | *In vitro* | *P. aeruginosa* | 44 |
| Tachyplesin III | TZP | AMPs enhance antibiotic activity | *In vivo* and *in vitro* | *P. aeruginosa* | 45 |
| Colistin | Tobramycin, aminoglycoside | AMPs enhance antibiotic activity | *In vivo* and *in vitro* | *P. aeruginosa* | 46 |
| GL13K | Tobramycin | AMPs enhance antibiotic activity | *In vitro* | *P. aeruginosa* | 47 |
| LL-37, CAMA, melittin, defensin, magainin-II | Ceftazidime, tobramycin, ciprofloxacin, doripenem, piperacillin, colistin | Synergistic effect | *In vitro* | *P. aeruginosa* | 48 |
| Indolicidin, CAMA, nisin | Daptomycin, linezolid, teicoplanin, azithromycin, ciprofloxacin | Synergistic effect | *In vitro* | MRSA | 50, 51 |
| Cathelicidin BMAP-28 | Q/D, linezolid, vancomycin | AMPs enhance antibiotic activity | *In vivo* and *in vitro* | *S. aureus* | 52 |
| Protegrin IB-367 | Linezolid | AMPs enhance antibiotic activity | *In vivo* and *in vitro* | *S. aureus* | 53 |
| Pal-Lys-Lys-$NH_2$ or Pal-Lys-Lys-soaked graft | Vancomycin | AMPs enhance antibiotic activity | *In vivo* and *in vitro* | *S. aureus* | 54 |
| LAE, subtilosin | Clindamycin, metronidazole | Synergistic effect | *In vitro* | *G. vaginalis* | 120 |
| Citropin 1.1 | Rifampin, minocycline | AMPs enhance hydrophobic activity of antibiotics | *In vivo* and *in vitro* | *S. aureus* | 132 |
| BMAP-27, BMAP-28 | Tobramycin | AMPs enhance antibiotic activity | *In vitro* | *S. aureus, P. aeruginosa* | 133 |
| LL-37 | Tobramycin | AMPs enhance antibiotic activity | *In vivo* | *P. aeruginosa* | 134 |
| Biofilm-degrading enzymes | | | | | |
| Dispersin B | Triclosan | Synergistic effect | *In vivo* and *in vitro* | *S. aureus* | 61 |
| Lysostaphin | Nafcillin | Lysostaphin enhances antibiotic activity | *In vivo* and *in vitro* | MRSA | 68 |
| Alginate lyase | Gentamicin, ceftazidime | Alginate lyase enhances antibiotic activity | *In vitro* | *P. aeruginosa* | 69 |
| rhDNase I | Povidone iodine, chlorhexidine gluconate, benzalkonium chloride | rhDNase I enhances antibiotic activity | *In vitro* | *S. aureus* | 70 |
| DNase I, RNase A, proteinase K | Ampicillin, cefotaxime, rifampin, levofloxacin, azithromycin | DNase I enhances antibiotic activity | *In vitro* | *P. aeruginosa, S. aureus* | 71 |
| Dispersin B | Cefamandole nafate | Synergistic effect | *In vitro* | *S. aureus* | 72 |
| Lysostaphin | Clarithromycin, levofloxacin, linezolid | Synergistic effect | *In vitro* | MRSA and MSSA | 74 |
| Proteinase K | Streptomycin, gentamicin, ampicillin | Proteinase K enhances antibiotic activity | *In vitro* | *S. aureus* | 75 |
| DNase I | Metronidazole | DNase I enhances antibiotic activity | *In vitro* | *G. vaginalis* | 119 |
| Quorum-sensing inhibitors | | | | | |
| Patulin, penicillic acid | Tobramycin | QSIs enhance antibiotic activity | *In vitro* | *P. aeruginosa* | 83 |
| Phenyl-DPD | Gentamicin | QSI enhances antibiotic activity | *In vitro* | *P. aeruginosa* | 84 |
| Lactonase | Ciprofloxacin, gentamicin | Lactonase enhances antibiotic activity | *In vitro* | *P. aeruginosa* | 85 |
| Baicalin hydrate, cinnamaldehyde, hamamelitannin | Tobramycin, clindamycin, vancomycin | QSIs enhance antibiotic activity | *In vivo* and *in vitro* | *P. aeruginosa, S. aureus* | 86 |
| Baicalein | Ampicillin | Synergistic effect | *In vitro* | *P. aeruginosa* | 87 |
| 14-Alpha-lipoyl andrographolide | Azithromycin, ciprofloxacin, streptomycin, fosfomycin, erythromycin, gentamicin | Synergistic effect | *In vitro* | *P. aeruginosa* | 88 |
| LSFE | Tobramycin | LSFE enhances antibiotic activity | *In vitro* | *P. aeruginosa* | 89 |
| Ajoene | Tobramycin | Synergistic effect | *In vivo* and *in vitro* | *P. aeruginosa* | 90 |

(Continued on following page)

**TABLE 2** (Continued)

| Natural antibiofilm compound[a] | Antibiotics in combination | Interaction activity | Study type(s) | Biofilm-associated pathogen(s) | Reference(s) |
|---|---|---|---|---|---|
| Essential oils | | | | | |
| *P. graveolens* essential oil | Norfloxacin | Synergistic effect | *In vitro* | *S. aureus* | 102 |
| Thyme oil, eugenol | Penicillin, ampicillin, cloxacillin, cephalothin, methicillin, novobiocin, vancomycin | EOs enhance antibiotic activity | *In vitro* | *S. aureus* | 103 |
| TTO (terpinen-4-ol) | Ciprofloxacin | Synergistic effect | *In vitro* | *P. aeruginosa* | 104 |
| Nanoparticles | | | | | |
| Ag NPs | Chloramphenicol, gentamicin | Synergistic effect | *In vitro* | *S. aureus* | 112 |
| Green Ag NPs | Ampicillin, vancomycin | NPs enhance antibiotic activity | *In vitro* | *P. aeruginosa* and *S. aureus* | 114 |
| Citrate-capped Ag NPs | Aztreonam | Synergistic effect | *In vitro* | *P. aeruginosa* | 116 |
| Selenate NPs | Ampicillin, oxacillin, penicillin | NPs enhance antibiotic activity | *In vitro* | MRSA | 117 |
| Other antimicrobial agents | | | | | |
| Chitosans | Streptomycin | Chitosan enhances antibiotic activity | *In vitro* | *S. aureus* | 127 |
| Buffered chitosan sponge | Vancomycin, amikacin | Chitosan enhances antibiotic activity | *In vivo* | *S. aureus*, *P. aeruginosa* | 128 |
| Antibiotic-loaded chitosan microspheres | Tetracycline | Chitosan enhances antibiotic activity | *In vitro* | *P. aeruginosa* | 129 |
| Nitric oxide | Tobramycin | Nitric oxide enhances antibiotic activity | *In vitro* | *P. aeruginosa* | 130 |
| Cis-2-decenoic acid | Daptomycin, vancomycin | C2D enhances antibiotic activity | *In vitro* | MRSA | 131 |
| Ultrasonic exposure | Gentamicin | Ultrasonication increased transport of gentamicin | *In vitro* | *P. aeruginosa* | 135 |

[a]LAE, lauramide arginine ethyl ester.

modify the traditional treatments. The ability of several novel natural antimicrobial compounds to efficiently control biofilm formation on biotic and abiotic surfaces has been identified. Compared to the activity of each one individually, stronger antibiofilm activity (synergistic or enhancement) was reported when traditional antibiotics were used in combination with alternative antimicrobials reviewed here, or when used in the presence of other recently reported compounds, such as chitosan (124–129), nitric oxide (130), and cis-2-decenoic acid (131). The potency of antimicrobial combinations is ultimately determined by the synergy of interacting antimicrobials, where each one of them is acting on different targets (Table 2 and Fig. 1).

Overall, the beneficial properties of the complementary approach in controlling biofilms of health-threatening bacteria prove it to be a promising strategy that could be used in personal care and pharmaceutical applications.

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