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Staphylococcal Biofilms: Challenges in the Discovery of Novel Anti-infective Agents

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Staphylococci can induce a wide spectrum of infectious diseases that are associated with remarkable morbidity and mortality [1]. In fact, community and hospital-acquired methicillin resistant *Staphylococcus aureus* (MRSA) is a major health problem that has created a pressing need for novel therapeutic options [2]. Importantly, pathogenic staphylococci have not only an amazing ability to acquire resistance to antibiotics, but also to form biofilms, bacterial communities that grow on surfaces and are surrounded by a self-produced polymer matrix. This latter characteristic is likely the most important virulence factor of staphylococci in the development of the chronic form of infectious diseases in humans such as otitis media, osteomyelitis, endophtalmitis, urinary tract infections, acute septic arthritis, native valve endocarditis, burn or wound infections and cystic fibrosis associated infections [3-9].

Staphylococcal biofilms are particularly dangerous because they are more resistant to host immune defence systems and have a significantly increased tolerance to antibiotics [10]. The polymer matrix of a biofilm retards the rate of antibiotic penetration of antibiotics such that the expression of genes within the biofilm that mediate resistance can be induced [3]. This is compounded by the fact that bacterial cells growing in biofilm show increased horizontal gene transmission, which can facilitate the spread antibiotic resistance traits [11].

The recurrence of some staphylococcal infections in the hospital setting has been attributed to the existence of specialized persister cells [12]. In *S. aureus* biofilms, although these persister cells do not grow in the presence of an antibiotic, they also do not die. When the drug is removed, the persister cells will give rise to a normal bacterial colony. It has been observed that *S.aureus* in biofilms is 100–1000 times less susceptible to antibiotics than equivalent bacterial populations of single cells (planktonic) [13]. Although conventional antibiotics can be effective against planktonic cells, there are currently no therapies that effectively target staphylococcal biofilms.

Staphylococcal biofilms are also responsible for many biomaterial-associated infections (BAI). Together, the Gram-positive pathogens *S. aureus*, *S. epidermidis* and *Enterococcus faecalis* represent more than 50% of the species isolated from patients with medical device-associated infections [15]. *S. aureus* is often the cause of metal-biomaterial infections, while *Staphylococcus epidermidis* is seen more often in polymer associated infections [14].

The continual increase in the use of medical devices is associated with a significant risk of infectious complications, septic thrombophlebitis, endocarditis, metastatic infections, and sepsis [17,18]. Biofilm associated infections of indwelling medical devices are usually resolved after replacement of the device but involve a prolonged hospital stay and increased healthcare costs. In fact, the treatment of catheter-related bloodstream infections that arise during intensive care unit stays in four European countries (France, Germany, Italy, UK), has an estimated cost of € 163.9 million [16]. If one considers that an increasing number of elderly patients require indwelling medical devices like artificial knees and hips, it becomes clear that a new generation of anti-infective agents effective in the prevention or eradication of biofilms is needed [19].

There is undoubtedly an urgent need for novel treatments, strategies and anti-staphylococcal biofilm agents. In this field of preclinical research, three different approaches are followed: (i) screen based strategies, (ii) target-based strategies, and (iii) biofilm matrix targeting strategies. The screen based strategies involved screening novel compounds (synthetic or natural) for inhibition staphylococcal biofilms through direct effects on bacterial growth and viability. The hope is that these screens will identify agents that may serve as alternatives to conventional antibiotics. Target-based strategies, on the other hand, focus on identifying or developing compounds that specifically target pathways that are essential for staphylococcal biofilm formation. The biofilm matrix targeting strategies instead, are aimed at identifying enzymes that target staphylococcal biofilm matrix.

The first approach has led to the identification and characterization of a number of small synthetic organic molecules with anti-biofilm properties. For instance, a collection of 2-aminoimidazole/triazole were synthesized and screened for anti-biofilm activity and found to inhibit biofilm formation against *Acinetobacter baumannii*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. One such compound in this library demonstrated the most potent inhibitory effect against *S.aureus* biofilm formation that has been displayed by any 2-aminoimidazole derivative [20]. Furthermore, a recent study that looked at the anti-staphylococcal biofilm activity of pyrrolomycins C, D, F1, F2a, F2b, F3 naturally produced by *Actinosporanfium vitaminophylum* and *Strepyomyces ssp*., and of the synthesized related compounds I, II, III, found that some of the tested compounds were active at the lowest screening concentration of 0.045 μg/mL [22].

Slow-growing and non-dividing bacteria, such as the aforementioned persister cells, exhibit tolerance to many antibiotics and represent a reservoir for recurrent biofilm infections. Importantly, the novel porphyrin antibacterial agents, XF-70 and XF-73, which have rapid membrane-perturbing activity against *S. aureus*, were also active against growth-attenuated cells. These results support the hypothesis that membrane-active agents may be particularly effective in eradicating slow- or non-growing bacteria and suggest that XF-70 and XF-73 could be utilized to treat staphylococcal infections where the organisms have a slow rate of division, such as biofilm-associated infections of prosthetic devices [21].

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In order to adequately assess the utility of any of these new compounds as antibiofilm agents, their toxicity against human cells must be evaluated and their selectivity indexes (ratio of cytotoxicity as IC_{50} to anti-biofilm concentration) determined. A selectivity index value >200 can be considered a good "safety margin" for a compound that will be used for potential therapeutic development as antimicrobial agent [23]. In fact, only those compounds that show good selectivity indexes can be considered promising inhibitors for developing novel agents against staphylococcal biofilms.

An interesting class of antimicrobial agents are the antimicrobial peptides (AMPs), which have different modes of action than those of conventional antibiotics. AMPs are small molecules with a broad antimicrobial spectrum. AMPs from different natural sources such as terrestrial or marine animals have been studied for their anti-staphylococcal biofilm activity. An example of a recently identified AMP is PSN-1, a novel 19 amino acid antimicrobial peptide of the phylloseptin family, isolated from the skin secretion of the waxy monkey frog, *Phyllomedusa sauvagei*. PSN-1 displayed broad-spectrum activity against a range of planktonic organisms with a high potency (MIC 5μM) against *Staphylococcus aureus*. In a specific bioassay *S. aureus* grown as a biofilm, the minimal biofilm eradication concentration (MBEC) was found to be of the same high potency (5μM) [24].

Some authors have focused on marine invertebrates as a source of new antimicrobial agents. AMPs from marine invertebrates display broad antimicrobial spectra, even against human pathogens. Indeed, many of these organisms are not fouled so they must possess an effective defense strategy to prevent bacterial adhesion. The 5kDa peptide fraction of the cytosol from coelomocytes (5-CC), the effector cells of *Paracentrotus lividus* (sea-urchin), showed antibiofilm properties against staphylococcal biofilms of reference strains *Staphylococcus epidermidis* DSM 3269 and *Staphylococcus aureus* ATCC 29213. The antimicrobial efficacy of 5-CC against biofilms of clinical strain *S. epidermidis* 1457 was also tested. At a sub-MIC concentration (31.7 mg/mL) of 5-CC the formation of young (6h old) and mature (24h old) staphylococcal biofilms was inhibited. The biological activity of 5-CC could be attributed to three novel antimicrobial peptides belonging to the sequence segment 9-41 of a beta-thymosin [25].

The second approach, aimed at developing target-based agents, represents a rational strategy for discovering staphylococcal biofilm inhibitors. Quorum-sensing (QS) is a complex regulatory mechanism in biofilm formation. It is a process that is dependent on the release of chemical signals called autoinducers. These autoinducers allow bacteria to assess the density of the local bacterial population and coordinate the expression of a wide array of genes and phenotypes including bacterial virulence and pathogenesis [26]. Among Gram-positive species, QS autoinducers are unmodified or post-transcriptionally modified peptides, cyclic thiolactone peptides (AIPs) [27]. It has been proposed that novel compounds with close sequence similarity to AIPs may function as antagonists and may provide an alternative way of treating *S.aureus* infections [26].

The *agr* (accessory gene regulation) system of *S.aureus* is a model for QS systems in Gram-positive bacteria. The *agr* QS system consists of a four-gene operon (*agrB, agrD, agrC, agrA*) that synthesizes and secretes AIPs. AIPs bind to and activate *agrC*, a membrane bound histidine sensor kinase (the major environmental sensory system in prokaryotic cells), which in turn phosphorylates and activates *agrA*, a transcription factor that regulates the production of the effector molecule RNAIII. Interestingly, a mechanism that interferes with biofilm formation in *S. aureus* involves the heptapeptide RNAIII-inhibiting peptide (RIP). This heptapeptide inhibits biofilm formation of *S.aureus* in vivo [28], possibly by blocking the *agr* dependent QS system [29]. In fact bone cement containing RIP has been patented as a means to prevent the colonisation and the development of biofilm on bone cement implant [30].

It is important to note, however, that the *agr* system might not be RIP's primary target. In fact, it has also been reported that inhibition of the *agr* system increases biofilm formation [31]. RIP appears to have an effect on biofilm formation, and as such, its structure is an interesting subject for modelling studies aimed at the identification of other biofilm inhibitors. Through structure based virtual screening using RIP as a template, hamamelitannin, a RIP non peptide-analogue derived from the bark of *Hamamelis virginiana* (witch hazel), that prevents device-associated MRSA infections in a concentration dependent manner was identified [32]. This work represents a clever variation of the structure-based screening approach in which the molecule used for modelling studies was not the target of a desired inhibitor, but itself an inhibitor [33].

The third approach consists in targeting the extracellular polymeric substance (EPS) matrix of bacterial biofilms. EPS is a complex mixture of components that can consist of polysaccharides, proteins, nucleic acids and/or lipids [34]. Purified N-acetylglucosaminidase, dispersin B, produced by the Gram-negative periodontal pathogen *Actinobacillus actinomycetemcomitans*, can dissolve mature biofilms produced by *Staphylococcus epidermidis* as well as some other bacterial species [35] by degrading polysaccharide intercellular adhesin (PIA) [36]. PIA is involved in the majority of staphylococcal biofilm associated infections and thus could be considered an ideal target for anti-biofilm drugs [37]. One recent study reported the synthesis of new polymeric matrices that can bind dispersin B alone or in combination with an antibiotic molecule, cefamandole nafate (CEF). These functionalized polyurethanes were able to adsorb a significant amount of dispersin B, which was able to exert its hydrolytic activity against the exopolysaccharide matrix produced by staphylococcal strains. When microbial biofilms were exposed to both dispersin B and CEF, a synergistic action became evident. Thus these polymer-dispersin B-antibiotic systems may be promising, highly effective tools for preventing bacterial colonization of medical devices [17]. Finally, a gel preparation containing dispersin B in combination with the disinfectant Triclosan has been marketed for the treatment of wound and skin infection and for disinfection of medical devices [38]. The downfall of this system is that, unfortunately, dispersin B cannot be used for the treatment of systemic biofilm-mediated infections, due to the immunogenic properties of bacterial enzymes [33]. The failure of conventional antibiotic therapy against bacterial biofilms can be attributed to the different mode of growth of pathogens as biofilms as opposed to planktonic pathogens. A chemotherapeutic approach that combines conventional antibiotics and novel anti-biofilm agents could be a new strategy for the treatment of biofilm associated infections. Some of the recently discovered inhibitors mentioned in this article show antibiofilm activity and selective toxicity, which make them good candidates for potential therapeutic development as effective chemical countermeasures against staphylococcal biofims.

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