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Quorum Sensing Inhibitors as Anti-Biofilm Agents

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Abstract: Biofilms are microbial sessile communities characterized by cells that are attached to a substratum or interface or to each other, are embedded in a self-produced matrix of extracellular polymeric substances and exhibit an altered phenotype compared to planktonic cells. Biofilms are estimated to be associated with 80% of microbial infections and it is currently common knowledge that growth of micro-organisms in biofilms can enhance their resistance to antimicrobial agents. As a consequence antimicrobial therapy often fails to eradicate biofilms from the site of infection. For this reason, innovative anti-biofilm agents with novel targets and modes of action are needed. One alternative approach is targeting the bacterial communication system (quorum sensing, QS). QS is a process by which bacteria produce and detect signal molecules and thereby coordinate their behavior in a cell-density dependent manner. Three main QS systems can be distinguished**:** the acylhomoserine lactone (AHL) QS system in Gram-negative bacteria, the autoinducing peptide (AIP) QS system in Gram-positive bacteria and the autoinducer-2 (AI-2) QS system in both Gram-negative and -positive bacteria. Although much remains to be learned about the involvement of QS in biofilm formation, maintenance, and dispersal, QS inhibitors (QSI) have been proposed as promising antibiofilm agents. In this article we will give an overview of QS inhibitors which have been shown to play a role in biofilm formation and/or maturation.

Keyword: Quorum sensing, quorum sensing inhibition, biofilm, antibiofilm.

INTRODUCTION

Biofilms are microbial sessile communities characterized by cells that are attached to a substratum or interface or to each other, are embedded in a self-produced matrix of extracellular polymeric substances and exhibit an altered phenotype compared to planktonic cells [1]. Biofilms are estimated to be associated with 80% of microbial infections [2]. Biofilm formation occurs in different steps. A conditioning film is formed by the adsorption of organic and inorganic nutrients and influences initial attachment of bacteria [3]. After an initial attachment stage, adhered bacteria will produce an exopolysaccharide matrix and will adhere more firmly. The biofilm will then undergo maturation, thereby obtaining a complex three-dimensional structure of biofilm cells, matrix and water channels. The biofilm cells will alter their physiological process in response to the conditions present in the biofilm [4]. In addition, cells can actively or passively detach from the biofilm or the surface, disperse and colonize other surfaces and/or environments or return to a planktonic state [5].

It is currently common knowledge that growth of micro-organisms in biofilms can enhance their resistance to antimicrobial agents. This may be due to a decreased penetration of antibiotics, a decreased growth rate of the biofilm cells and/or a decreased metabolism of bacterial cells in biofilms [6]. In addition, the presence of persister cells and the expression of specific resistance genes in biofilms may contribute to this tolerance [6]. As such antimicrobial therapy often fails to eradicate biofilms from the site of infection. For this reason, innovative anti-biofilm agents with novel targets and modes of action are needed.

One alternative approach is targeting the bacterial communication system (quorum sensing, QS). QS is a process by which bacteria produce and detect signal molecules and thereby coordinate their behavior in a cell-density dependent manner [7]. Three main QS systems can be distinguished**:** the acylhomoserine lactone (AHL) QS system in Gram-negative bacteria, the autoinducing peptide (AIP) QS system in Gram-positive bacteria and the autoinducer-2 (AI-2) QS system in both Gram-negative and -positive bacteria (Fig. **1**). Many Gram-negative bacteria use AHL signalling molecules (Fig. **1**) which are produced by a LuxI-type synthase and are perceived by a DNA-binding LuxR-type transcriptional activator [7]. The QS system of Gram-positive bacteria typically consists of signalling peptides (Fig. **1**) such as Agr and RNA-III activating/inhibiting peptides (RAP/RIP) in *Staphylococcus aureus*, and a two-component regulatory system made up of a membrane-bound sensor and an intracellular response regulator [8]. A third QS system is shared by many Gram-positive and Gram-negative bacteria and is based on a mixture of interconvertible molecules collectively referred to as AI-2 (Fig. **1**) [7, 9]. A key enzyme in the production of AI-2 is LuxS. LuxS catalyzes the cleavage of S-ribosylhomocysteine to homocysteine and 4, 5-dihydroxy-2, 3-pentanedione (DPD). DPD will subsequently undergo spontaneous rearrangements and modifications, forming a mixture of molecules, collectively called AI-2. Although LuxS is encoded in many sequenced bacterial genomes, AI-2 receptors and signal transduction systems have only been described in *Vibrio* spp., in *Salmonella enterica* serovar Typhimurium and in *Escherichia coli* [9-10]. In *Vibrio* spp., binding of AI-2 to LuxP, a periplasmic AI-2 receptor associated with the LuxQ sensor kinase, results in the production of LuxR and ultimately changes in gene expression. In *S. enterica* serovar Typhimurium and *E. coli* AI-2 is first transported into the cell prior to initiating a signalling cascade [9].

Multiple reports have discussed the involvement of QS in biofilm formation and conflicting conclusions have been drawn regarding the importance of QS in bacterial biofilm formation [11-21]. These inconsistencies may be the result of the use of different biofilm models and/or different bacterial strains. Although much remains to be learned about the involvement of QS in biofilm formation, maintenance, and dispersal, QS inhibitors (QSI) have been proposed as promising antibiofilm agents. QS inhibition can be achieved by inhibiting signal synthesis or direct degradation of the signal, inhibition of binding of the signal molecule to the receptor and/or inhibition of the signal transduction cascade. In this article we will give an overview of QSI which have been shown to play a role in biofilm formation and/or maturation. These will be organised based on their target and/or chemical structure.

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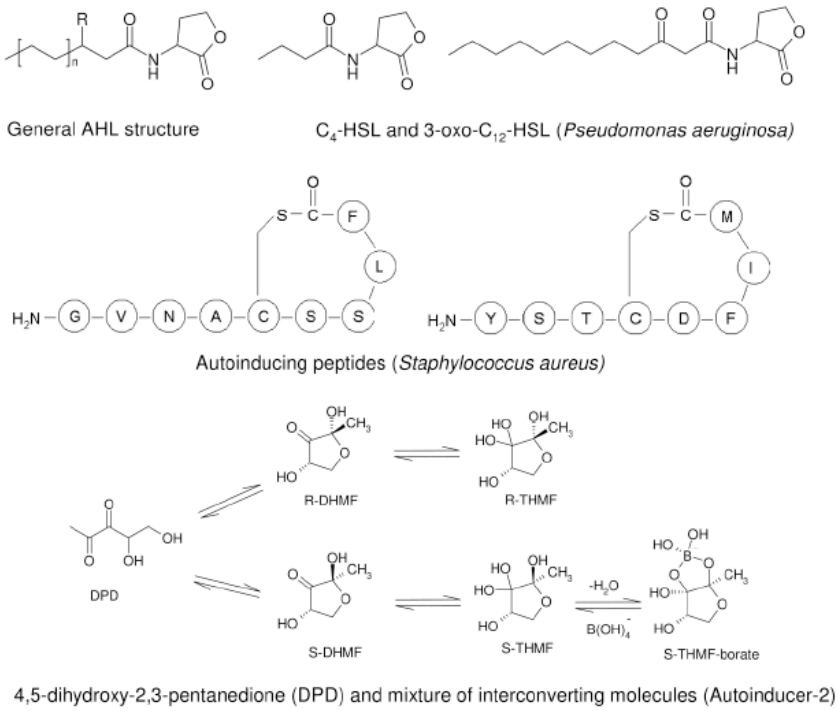

Fig. (1). QS signal molecules.

QSI TARGETING SIGNAL SYNTHESIS AND THEIR EFFECT ON BIOFILMS

Several reports have indicated that mutations affecting signal synthesis have an effect on biofilm formation. In the case of AHL synthesis for example, *Pseudomonas aeruginosa lasI* mutants, that cannot synthesize 3oxoC12-HSL, form flat unstructured biofilms in a flow cell. Furthermore *Burkholderia cenocepacia* K56-2 *cepI* and *B. cenocepacia* J2315 *cepI* and *cciI* mutants are defective in biofilm formation [15-18, 22]. Mutation of *ahyI* and *swrI*, genes coding for AHL signal synthesis enzymes, resulted in the formation of immature biofilms lacking microcolonies in *Aeromonas hydrophila* and *Serratia liquefaciens*, respectively [23]. In addition, deletion of *luxS*, a gene coding for a key enzyme in AI-2 production affect biofilm formation in several *Vibrio* spp, *Streptococcus* spp, *Staphylococcus* spp. [19-21]. Finally, the loss of AIP and RNAIII production affects biofilm formation in *S. aureus* [20]. As such, blocking signal production or degrading the signal might be promising strategies.

AHL signal molecules are formed when an acyl-carrier protein-bound fatty acyl derivative is transferred to the amino group of S-adenosyl-methionine (SAM) by a LuxI family protein. Given the nature of this reaction and the precursors involved, inhibitors of SAM or fatty acid biosynthesis may be used as AHL QSI. S-adenosyl-homocysteine (SAH), sinefungin, 5-methylthioadenosine (MTA), various SAM analogs and the SAM biosynthesis inhibitor cycloleucine inhibit AHL production [24-25]. The antibiotic azithromycin interferes with C_4-homoserine lactone (C_4-HSL) and 3-oxo-C_{12}-HSL synthesis in *P. aeruginosa* and thereby reduces bacterial adhesion to polystyrene surfaces [26]. In addition, treatment with azithromycin significantly improved clearance of biofilms in a mouse model of chronic pulmonary *P. aeruginosa* infections [27].

AI-2 synthesis involves two major enzymatic steps. First, adenine is removed from SAH by 5'methylthioadenosine nucleosidase (MTAN) (encoded by *pfs*), resulting in the production of S-ribosyl-homocysteine (SRH). Next, SRH is cleaved by LuxS to form DPD and homocysteine. In addition, MTAN is also involved in the AHL QS system, and LuxS and MTAN are only found in bacteria, making them attractive targets. Several inhibitors of LuxS and MTAN have been described. S-anhydroribosyl-L-homocysteine and S-homoribosyl-L-cysteine block the initial and final steps of the LuxS reaction mechanism, respectively [28-29]. Based on these molecules, Shen and colleagues [30] synthesized several more potent LuxS inhibitors. Different peptides capable of inhibiting LuxS have also been developed [31-32]. Starting from immucillin and DADMe-immucillin, several other MTAN inhibitors (e.g. BuT-DADMe-immucillin-A and p-Cl-PhT-DADMe-immucillin-A), active in pico- and femtomolar concentrations, were developed [33-35]. Finally, several peptides bearing homology to the C-site of LuxS affected AI-2 production and biofilm formation of *Edwardsiella tarda* [32].

Proteins involved in peptide signal synthesis and post-translational modifications of the peptide in Gram-positive bacteria are also interesting targets, but to date no inhibitors specifically targeting these proteins have been reported. In contrast, different linear peptide inhibitors targeting type-I signal peptidase SpsB reportedly reduce AIP-I production [36-37]. However, most of these signal synthesis inhibitors have not yet been evaluated for their effect on biofilms.

QUORUM QUENCHING OF SIGNALLING MOLECULES

Once synthesized, QS signal molecules can be enzymatically degraded (quorum quenching, QQ) so as to prevent their accumulation and subsequent activation of the QS system. AHL degradation can occur in four different ways. AHL lactonases and AHL acylases hydrolyze the HSL ring and the amide bonds of the AHL molecule, respectively. The first hydrolysis is identical to pH-mediated lactonolysis and can be reversed by acidification, while the second hydrolysis is irreversible. AHL oxidases and AHL reductases do not degrade the AHL molecule but modify it and change its activity. Several bacterial species, including *Bacillus* spp., *P. aeruginosa*, *Acinetobacter* spp., *Bosea* spp., *Delftia acidovorans*, *Sphingomonas* spp., *Agrobacterium tumefaciens*, *Arthrobacter* spp.,

Klebsiella pneumonia and *Sphingopyxis* spp. produce enzymes capable of degrading AHLs [38-43]. In addition, AHL degrading enzyme activity can also be detected in eukaryotes. Several plants and root associated fungi, including *Hordeum vulgare*, *Lotus corniculatus* and *Pachyrhizus erosus* can degrade AHL signal molecules [44-46]. In addition, paraoxonases found in mammalian serum and in several human cell lines show AHL degrading activity and these enzymes can be used to interfere with biofilm formation of pathogenic bacteria [47-52].

To date no specific enzymatic quenchers of AIP or AI-2 QS signals have been described. However, these two signals can be affected by the immune system. Recently, anti-autoinducer monoclonal antibody affected AIPs produced by *S. aureus* and AI-2 produced by *S. enterica* serovar Typhimurium [53-54]. As such these strategies might also be used to target biofilm formation in AI-2 and AIP producing strains.

AHL SIGNAL ANALOGUES AND COMPOUNDS TARGETING THE AHL RECEPTOR

Many studies have focussed on developing analogues of the native AHL signal molecule in which the acyl side chain or the lactone moiety was modified [55-60]. Only a handful of studies have focused on alterations of the central amide moiety [61-63]. Several of these compounds were shown to affect biofilm formation. AHL analogues in which the lactone ring was replaced by a cyclopentyl or a cyclohexanone ring significantly affected biofilm formation of *Serratia marcescens* and *P. aeruginosa* [64-65]. In addition, AHL in which the amide function was replaced by a triazolyldihydrofuranone (Fig. **2**) showed biofilm eradicating as well as biofilm inhibitory activity against *B. cenocepacia* and *P. aeruginosa* [63]. Finally, phenylpropionyl homoserine lactones and phenoxyacetyl homoserine lactones, analogues with aromatic groups on the acyl-side chain, inhibited *P. aeruginosa* biofilm formation [57, 66].

Next to compounds resembling AHL, several unrelated compounds are shown to block AHL QS and thereby affect biofilm formation of AHL producing strains. Some of these compounds originated from natural extracts. For example, bergamottin and dihydroxybergamottin isolated from grapefruit juice and extracts from South Florida plants inhibited AHL QS and affect biofilm formation of *P. aeruginosa* [67-68]. Rasmussen and colleagues [69] observed that extracts of *Penicillium* species and garlic contained QS inhibitory compounds. Using liquid chromatography -mass spectrometry penicillinic acid and patulin were identified to be responsible for the QS inhibitory effect of the *Penicllium* species. In addition, allicin (Fig. **2**), ajoene (Fig. **2**), a cyclic thioacetal and cyclic disulfide were identified to be responsible for the QS inhibitory effect of the garlic extract. Patulin, ajoene and garlic extracts increased biofilm susceptibility of *P. aeruginosa* biofilms toward tobramycin treatment and resulted in an increased clearance of *P. aeruginosa* in an *in vivo* pulmonary infection model [69-71]. Similarly, different polyphenolic compounds including baicalin hydrate (Fig. **2**) and epigallocatechin were observed to block AHL QS [72-73]. In addition, these compounds were shown to have no effect on adhesion but affected biofilm formation of *B. cenocepacia*, *Burkholderia multivorans* and *P. aeruginosa* at later stages of biofilm development and maturation [72-74]. Baicalin hydrate increased *B. cenocepacia* and *P. aeruginosa* biofilm susceptibility towards tobramycin in different *in vitro* biofilm model systems and the combined use of baicalin hydrate and tobramycin reduced the microbial load in the lungs of BALB/c mice infected with *B. cenocepacia* more than tobramycin alone [74].

AI-2 AND DPD ANALOGUES AND QSI TARGETING AI-2 RECEPTOR

DPD derivatives including Ac_2-DPD, alkyl-DPD, carbonate-DPD, trifluoro-*S*-THMF-borate and structures resembling DPD such as laurencione and 4-hydroxy-5-methyl-3-(2H)-furanone (MHF) can activate the AI-2 QS system, while several diol-containing compounds (including pyrogallol), boronic acids and sulfones have been shown to be potent antagonists of AI-2-LuxP binding [60, 75-80]. In addition, oxazaborilidines (heterocyclic hydrated complexes containing a negatively charged boron atom), phenothiazine and an adenosine derivative with a *p*-methoxyphenylpropionamide moiety at C-3 (LMC-21) affect the AI-2 QS system [60, 81-82]. Although these compounds have been studied for their effect on AI-2 QS, only a limited number has been investigated for an effect on biofilm formation. Ursolic acid, Isobutyl-DPD and phenyl-DPD inhibited *E. coli* and *P. aeruginosa* biofilm formation and resulted in a removal of preformed biofilms [83-84]. In addition, 4-methoxycarbonyl-phenylboronic acid and LMC-21 affected biofilm biomass in *Vibrio anguillarum* and *Vibrio vulnificus*, without affecting the number of cells [85].

SIGNAL ANALOGS AND QSI TARGETING QS-RECEPTOR IN GRAM POSITIVE BACTERIA

Several AIP-analogs have also been developed. First, AIP of one group can block the AgrC receptor of another group, e.g. *S. aureus* group-I AIPs can be inhibited by AIP-IV. Truncated analogs of AIP (Tr-AIP-I, Tr-AIP-II and Tr-AIP-IV) and N-acetylated Tr-(Ala)-AIP-I inhibited QS in different *Staphylococcus* spp. [86-87]. Additionally, several aminobutyric acid analogs and 4-substituted phenoxybutyryl analogs of AIP-I, peptomers derived from Tr-AIP-I and truncated AIP-II analogs with glycine insertion, N-methylation, and alterations to the thioester linker were developed [86-87]. In addition, solonamide A and B produced by *Photobacterium* spp. and the cyclic dipeptides, cyclo(L-Tyr-L-Pro) and cyclo(L-Phe-L-

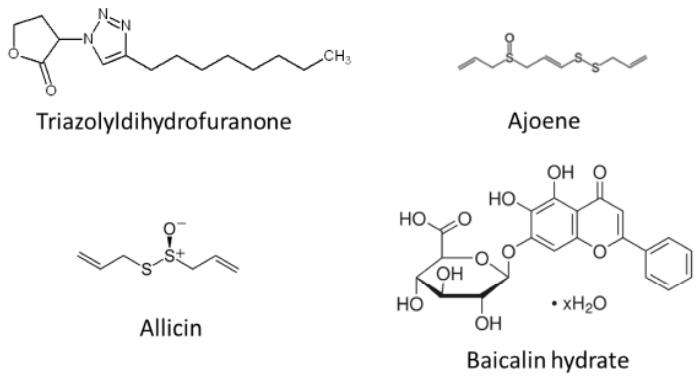

Fig. (2). Selection of QSI targeting AHL QS system.

Pro) produced by *Lactobacillus reuteri* RC-14 can interfere with *agr* [88-89]. However currently, little experimental data documenting the antibiofilm properties of these compounds are available.

In contrast, one of the most extensively investigated QS inhibiting peptides is the RNAIII inhibiting peptide (RIP). RIP, several RIP analogs and the non-peptide analog hamamelitannin (Fig. **3**) interfere with the RAP/TRAP QS system [90-91]. Several groups have presented evidence for the anti-biofilm effect of RIP and/or RIP analogs against *Staphylococcus* spp. RIP loaded into polymethylmethacrylate beads prevented *Staphylococcus* spp. biofilm formation in an subcutaneous graft and vascular graft rat model [90]. In addition, RIP, RIP analogs and hamamelitannin were shown to increase biofilm susceptibility towards antibiotics [74, 90-92].

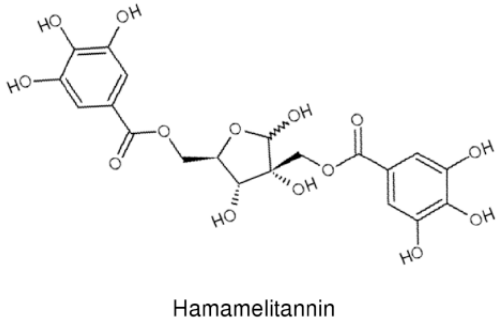

Fig. (3). Hamamelitannin. As a non-peptide analog of RIP, hamamelitannin can block QS in *Staphylococcus* spp. and thereby affect biofilm formation and biofilm susceptibility in this species.

BROMINATED FURANONES AND CINNAMALDEHYDE ANALOGS

The QS system can also be blocked at the level of the signal transduction cascade. Among the most extensively investigated compounds are a natural furanone compound, (5Z)-4-bromo-5-(bromomethylene)3-butyryl-2(5H)-furanone and cinnamaldehyde (Fig. **4**). The natural furanone or fimbrolide was first isolated from the red algae *Delisea pulchra*, while cinnamaldehyde originates from the bark of the cinnamon tree. To date different natural fimbrolide analogs and several synthetic furanones have been discovered and synthesized [93- 99]. These differ mostly in acetyl side chain and halogens substituent. In addition, several cinnamaldehyde analogs have been described [100-101]. The structural elements critical for QS inhibitory effect of cinnamaldehyde analogs were observed to include an α, β unsaturated acyl group capable of reacting as Michael acceptor connected to a hydrophobic moiety and a partially negative charge [101].

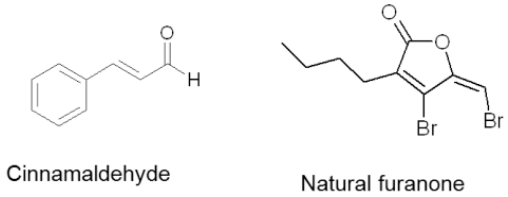

Fig. (4). Cinnamaldehyde and furanone. Both compounds can block AI-2 and AHL type QS systems in bacteria and thereby affect biofilm formation and biofilm susceptibility towards antibiotics.

Furanones and cinnamaldehyde were both shown to affect different types of QS system [100-103]. Both compounds inhibit AI-2 QS in *Vibrio harveyi* by decreasing the DNA binding ability of a response regulator LuxR which is located at the base of the signal transduction cascade [100, 103]. Both compounds are also shown to block different AHL QS systems, probably by displacing AHL from its receptor [73, 102, 104]. In addition, the natural furanone covalently modifies and inactivates LuxS and accelerates LuxR turnover, thereby blocking AI-2 and AHL QS, respectively [105-106].

Halogenated furanones have been shown to affect biofilm formation in *P. aeruginosa*, *E. coli*, *B. subtilis*, *S. epidermidis*, *Phorphyromonas gingivalis*, *S. enterica* serovar *Typhimurium*, *Streptococcus* spp. and *Vibrio* spp. [22, 95-97, 107-109]. Cinnamaldehyde was shown to affect biofilm formation of *B. cenocepacia*, *B. multivorans*, *P. aeruginosa, E. coli* and *Vibrio* spp. [73, 101, 110]. Both compounds were also shown to increase biofilm susceptibility towards antibiotic treatment [74, 111].

Although several studies have demonstrated the QS inhibitory effects of furanones on biofilms, the toxicity of these compounds will probably limit their use [95, 112]. In contrast, cinnamaldehyde is widely used as a flavouring agents in food and beverages.

CONCLUSION

It is currently common knowledge that growth of microorganisms in biofilms can enhance their resistance to antimicrobial agents. Multiple reports have discussed the involvement of QS in biofilm formation and conflicting conclusions have been drawn regarding the importance of QS in bacterial biofilm formation. These inconsistencies may be the result of the use of different biofilm models and/or different bacterial strains. Furthermore, many known QSI are cytotoxic and several fundamental mechanisms by which the different QS systems exert their regulatory functions and are inhibited by QSI are still poorly understood. In addition, it is still unknown whether these compounds would be useful in humans too. As such more research is needed to investigate the involvement of QSI in biofilm formation, maintenance, and dispersal, and to develop several non-toxic more active QSI before they can be used into practice. Despite this, QSI have been shown to be promising antibiofilm agents and can be of great value in the future treatment of bacterial infections.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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