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***Novel approaches for the design and discovery of quorum-sensing inhibitors***

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Abstract

***Introduction:*** The spread of antibiotic resistance, together with the lack of antibiotics based on novel molecular scaffolds, mark the so-called "post-antibiotic era." Among the potential strategies to develop new anti-infective drugs, interference with bacterial virulence is an attractive approach. The discovery that virulence gene expression is mostly regulated by quorum sensing (QS) raised a lot of interest and prompted a lot of research aimed at finding inhibitors of this mechanism.

***Areas covered:*** This paper deals with the most recent strategies aimed at discovering new inhibitors able to disrupt the different steps of the QS system, targeting signal production, signal molecules and signal receptors. We provide an overview of the literature including research papers, mostly dealing with inhibitors of the *S. aureus* and *P. aeruginosa* QS systems, and a number of comprehensive reviews dealing with the application of the newest technologies to the field. Prospects and emerging concerns regarding the possible clinical applications are also discussed.

***Expert opinion:*** QS inhibition is a promising strategy against infections. However, despite the discovery of a huge number of QS inhibitors, with about 40 patents, the potential of QS inhibition is still to be fully assessed. The current validation methods of QS inhibitors must be optimized, and the discovery that QS disruption may favour or select more virulent strains must be investigated in depth. Given the current market-dependent situation, the possibility to develop hits into licensed drugs is likely to be made possible by joint ventures between private companies, academia and public institutions.

**Keywords:** Antibodies; autoinducers; *P. aeruginosa*; quorum sensing; receptors; *S. aureus*, virulence factors.

## 1. Introduction

Intercellular communication by means of secreted hormone-like molecules (pheromones) is an important natural feature, which in different bacterial species is involved in bioluminescence, virulence, biofilm formation, antibiotic production, competence, conjugation, swarming, motility, and sporulation [1]. For most pathogenic bacteria, the ability to implement this kind of multicellular coordination is critical for disease pathogenesis and host colonization. Quorum sensing (QS) is the term used to describe a density-dependent intercellular communication that allows a coordinated multicellular behaviour in prokaryotes, based on the constitutive production and secretion of small molecules defined quorum sensors or autoinducers (AI) [2]. When the AI concentration reaches a critical detection threshold that depends upon bacterial population density (quorum), the AI interaction with species-specific receptors triggers a signal transduction cascade leading to an alteration in gene expression. The production of a number of virulence factors involved in bacterial pathogenesis and in the formation of bacterial sessile communities called biofilms is based on this mechanism. Biofilms account for more than 80% of human infections [3] and their treatment is highly problematic, because sessile bacteria are up to 1000 times more resistant to antibiotics in comparison to their planktonic counterparts, and are less exposed to the host immune system [4].

Since the pioneering work on AI by Tomasz and Hotchkiss [5] and Nealson and co-workers, [6], several AI molecules have been identified, which may be classified as follows: i) AI-1, or N-acyl homoserine lactones (AHLs), which are produced by over 70 species of Gram-negative bacteria; ii) oligopeptides, consisting of 5-17 amino acids, which are generally produced by Gram-positive bacteria; iii) AI-2, a class of furanone-based AI derived from 4,5-dihydroxy-2,3-pentanedione (DPD), which can be produced by both Gram-positive and Gram-negative bacteria and may be considered universal interspecies signalling molecules; and iv) other small molecules that include the *Pseudomonas* quinolone signal (PQS), the *Vibrio cholerae* autoinducer (S)-3-hydroxytridecan-4-one, 3-hydroxypalmitic acid methyl ester, the AI-3 (a small AI with unknown structure, used by enterohemorrhagic *E. coli*), bradyoxetin, and other molecules that do not fall within one of the major groups [7].

QS inhibition, also called quorum quenching (QQ), is considered an attractive modality of therapeutic intervention for a series of reasons: i) it would overcome the conventional mechanisms of resistance to antibiotics; ii) a single inhibitor could have multiple effects, being able to interfere with the production of a number of virulence factors and with biofilm formation; iii) in principle, anti-virulence factors should not exert an evolutionary pressure on the targeted bacterial population, avoiding the emergence of resistant strains; iv) avoiding or limiting biofilm formation, QS inhibitors could enhance the efficacy of existing antibiotics; v) except AI-2 members, most AI are species-specific. Consequently, the narrow spectrum of activity of inhibitors should avoid the disruption of beneficial microbiota [8]. It is well known that both prokaryotic and eukaryotic organisms have evolved molecular mechanisms able to carry on QQ activity. The study of the signal degradation systems enacted by different bacteria, plants, and mammals may lead to the design of novel approaches for quenching bacterial QS [9]. Ideally, a QS inhibitor should meet the following criteria: it should be a small molecule, highly specific, non toxic for eukaryotic cells, and chemically stable [10].

QQ compounds could be used in many fields, such as human and veterinary medicine, agriculture, and aquaculture. The therapy of chronic infections, which are often due to multiresistant bacterial strains, could benefit particularly from the use of antivirulence drugs [11]. By means of *in vitro* and *in vivo* experiments, it has been shown that *P. aeruginosa* QS blockage restores the immune response efficiency favouring the clearance of bacterial biofilms [12].

QS systems generally offer three points of attack: the signal generation process, the signal molecule, and the signal receptor [13]. During the last three decades, many natural and synthetic agents belonging to the categories of small non-peptide molecules, peptides, and enzymes, have been identified as QS inhibitors [14]. A very comprehensive review on QS inhibitors has recently been published by Kalia [10]. The field is rapidly evolving, and many inhibitors are being discovered. However, clinical applications are still lacking. To the best of our knowledge, only two clinical trials on QS inhibitors have been performed. In the first one (ClinicalTrials.gov Identifier: NCT00610623), the effect of azithromycin, used as a QS blocker at subinhibitory doses for the prevention of *P. aeruginosa* ventilator-associated pneumonia was evaluated. The authors of the study concluded that the major effect of QS inhibition was the selection of more virulent strains. The second trial (ClinicalTrials.gov Identifier: NCT01201577), completed but with no study results posted, was aimed at studying the effect of orally administered combinations of prebiotics, probiotics and antibiotics on QS signalling molecules and on innate and adaptive immunity in humans.

In this article, we focus on modern approaches for the development and validation of QS inhibitors, providing the reader with a comprehensive overview of the recent literature on the subject.

## 2. Strategies targeting QS signal production

The studies aimed to hit at targeting this step are relatively few, and the field is open for further investigation. Compounds affecting the AHL biosynthesis or efflux pumps are likely to behave as QQ. Substrate analogues of AI, like butyryl-S-adenosylmethionine, holo-acyl carrier protein, sinefungin and L/D-S-adenosylhomocysteine can block AHL production *in vitro*. However, *in vivo* experiments have not been performed, because these homologues are likely to affect the central pathways of amino acid and fatty acid metabolism [15]. Small-molecule inhibitors of the AHL synthase have been identified by Chung et al. [16]. The involvement of multidrug efflux pumps in the QS of *Burkholderia pseudomallei* has been established, suggesting that this pathway could also be exploited [17].

Methylthioadenosine/S-adenosyl homocysteine nucleosidase (MTAN) is the product of the *pfs* gene, which is highly conserved across bacterial species. This enzyme is present in bacteria but not in mammals, in which its activity is performed by two different enzymes: methylthioadenosine phosphorylase and purine nucleotide phosphorylase [18]. Beyond playing a crucial role in maintaining homeostasis, MTAN is directly involved in the biosynthesis of AI-1 and AI-2 [19]. For this reason, QS inhibitors belonging to the class of MTAN transition state analogues could be used to block both AI-1 and AI-2 production in bacteria without interfering with host cell metabolism. This approach has been explored by Gutierrez *et al*. [19], and Longshaw *et al*. [20], who found that both sulphur-containing and sulphur-free MTAN transition state analogues are potent inhibitors of *E. coli* QS. Recently, the Schramm laboratory patented two different types of transition state analogues and a pharmaceutically acceptable carrier for treating bacterial infections [21]. These developments indicate the interest that MTAN has aroused as a possible target for bacterial anti-infective drug design [1].

It has been demonstrated that anthranilate (**Figure 1, 1**) is a precursor of the PQS (**Figure 1, 3**), which is a secondary metabolite maximally produced during the late stationary phase of growth. An anthranilate analogue (methyl anthranilate) **(Figure 1, 2)** inhibits the production of PQS, with a negative effect on the production of elastase by *P. aeruginosa* [22]. These data suggest that pseudomonas PQS targeting may have a therapeutic value, but it must be taken into account that *P. aeruginosa* has two other QS systems, based on N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (**Figure 2, 4)** and N-butyryl-L-homoserine lactone (C4-HSL) (**Figure 2, 5**), respectively. In this regard, it has recently been shown that eugenol (**Figure 3, 6**), the major constituent of clove extract, affects *P. aeruginosa* QS decreasing the transcriptional activation of both *las* and *pqs* systems, which together regulate the expression of numerous virulence-related genes [23]. Further studies are needed to assess the relative importance of each system on the virulence of *P. aeruginosa*, and to examine the possibility of simultaneously turning off all three systems.

## 3. Strategies targeting QS signal

Neutralization of QS signals has been extensively investigated [24]. Biological inactivation of AHLs can be achieved by enzymatic degradation or antibody-mediated inactivation of the signal molecule.

**3.1 Enzymes**

QQ enzymes can be grouped in two classes: class I includes the enzymes that break the AHL molecule, i.e. AHL-lactonase, AHL-acylase and paraoxonase, whereas class II includes enzymes that reduce carbonyl to hydroxyl, i.e. oxidoreductases [25].

About 20 AHL lactonases have been discovered to date, most of which are supposedly valuable for the biocontrol of plant diseases. Recently, Migiyama et al. reported the effect of the AiiM lactonase on a mouse model of acute pneumonia by *P. aeruginosa* [26]. The study supports the therapeutic potential of AHL lactonases in therapy, demonstrating that AiiM is a potent inhibitor of *P. aeruginosa* QS and reduces bacterial virulence. The use of this molecule could represent a new strategy to cure chronic pulmonary infections by multiresistant strains. However, the problematic issue is the way of administration: in their experiments, the authors used lactonase-overexpressing plasmid vector-transformed *P. aeruginosa* to infect mice. This technique can be a good experimental tool, but, of course, it is not of any use for therapeutic purposes. The authors suggest two possible solutions to this impasse: the genetic modification of probiotics, or the local administration of the purified AiiM protein. In both cases, the authors agree that further studies are needed to evaluate the possibility of developing AiiM lactonase into a drug molecule.

One of the best studied acylases is the PvdQ protein, a member of the N-terminal nucleophile hydrolase superfamily. This enzyme is produced by the *P. aeruginosa* PAO1 strain, suggesting that it may participate to the regulation of its own QS-dependent pathogenic potential. PvdQ behaved as a QQ *in vitro* in a number of phenotypic assays in which the *pvdQ* gene was overexpressed in *P. aeruginosa*, and its activity was demonstrated *in vivo* in a *Caenorhabditis elegans* model [27]. The development of the molecule has reached the stage of the production of a stable and inhalable powder formulation for the treatment of *P. aeruginosa* pulmonary infection [28].

Paraoxonases (PON) are a group of $Ca^{2+}$-dependent esterases with AHL lactonase activity widely conserved in mammals, but not present in chicken and fish [29]. In humans, the family comprises three members, whose genes are located on chromosome 7: PON1 and PON3, mainly expressed in liver and kidney, and PON2, expressed in various tissues but not present in plasma [30]. These enzymes are supposed to play an important role in the defence against the formation of bacterial biofilms. The protective effect of PON1 against *P. aeruginosa* infection has been demonstrated in an experimental model of transgenic *Drosophila melanogaster* expressing human PON1 [31]. Recently, by *in vitro* and *in vivo* experiments Devarajian et al. demonstrated that PON2 plays an important role in the mouse innate immune response [32].

Oxidoreductases catalyze the functional inactivation of AHLs by oxidating or reducing their acyl side chain. Two oxidoreductases have been isolated from cultivated bacteria, one from *Bacillus megaterium* CYP102A1 and the other from *Rhodococcus erythropolis* W2 [33, 34]. Recently, a novel oxidoreductase named BpiB09, able to inactivate the 3-oxo-C12-homoserine lactone, was obtained by screening a soil metagenomic library. The expression of this molecule in *P. aeruginosa* PAO1 significantly reduced pyocyanin production, bacterial motility, and biofilm formation. In addition, transformed bacteria resulted non pathogenic in a *C. elegans* model of infection [35]. The possibility to screen metagenomic libraries opens wide horizons for the discovery of new active QQ enzymes. However, their value for clinical purposes needs to be assessed. It can be expected that they will find application in the treatment of skin and airway infections, and in the inhibition of bacterial biofilm formation on catheters and prosthetic devices.

## 3.2 Antibodies

Most AHLs exert potent biochemical effects, such as induction of apoptosis and modulation of NF-κB activity, behaving as small-molecule toxins on mammalian cells. Consequently, specific antibodies can achieve the dual purpose of neutralizing both the QS activity and the toxic effect. The immunological approach includes the induction of antibodies by vaccination, and the administration of preformed mono- or poly-clonal antibodies.

Following the first demonstration of the efficacy of vaccination with a homoserinelactone-carrier protein conjugate in a mouse model of *P. aeruginosa* lung infection by Miyairi et al. [36], the possibility to produce protective antibodies has been extensively investigated, especially by the Janda laboratory at The Scripps Research Institute [37-39]. The attractivity of this approach is based on the high evolutionary conservation of AI, their extracellular distribution, and the established knowledge of antibody pharmacodynamic and kinetic properties. The chemical structure and the low molecular weight make the AI poor antigens, which could be more properly defined haptens [7]. The efficacy of the immunopharmacotherapeutic approach to the inhibition of QS has been demonstrated *in vitro* and *in vivo* against *S. aureus* and *P. aeruginosa*. Park et al. reported the production of a monoclonal antibody, AP4-24H11, elicited against the autoinducing peptide AIP-4 produced by *S. aureus* RN4850. The antibody was able to inhibit QS *in vitro*, suppressed *S. aureus* pathogenicity in an abscess formation mouse model, provided complete protection against a lethal *S. aureus* challenge [38], and its structure was subsequently further investigated in detail [40].

Very recently, Palliyil et al. reported the development of monoclonal antibodies against homoserine lactones produced by *P. aeruginosa*. These antibodies were produced by using a combination of sheep immunisation and phage antibody display library construction/selection, and were characterized by high sensitivity (100-1000 times higher than that of any published antibodies raised to the same target). Their protective effect was assessed in a slow-killing model of *C. elegans*, and in a mouse model of *P. aeruginosa* infection [41]. These findings provide a strong foundation for further investigations of the potential of AHL monoclonal antibodies in the immunopharmacotherapy of antibiotic resistant strains of *P. aeruginosa*.

## 4. Strategies targeting autoinducer-receptor interaction and/or receptor-mediated signal.

The possibility to inhibit the expression of QS signal receptors by means of antisense RNAs has been demonstrated by Hirakawa et al. [42]. For a comprehensive review on the inhibition of signal detection, the reader is referred to [43]. In the following paragraphs, we will discuss some recent insights on the QS of *S. aureus* and *P. aeruginosa* that may suggest the possibility of interfering with the receptor side of the system.

The clarification of the QS system in Gram-positive bacteria allowed the identification of molecules inhibiting different steps of the mechanism. The QS system of *S. aureus*, which is the most studied, consists of the accessory gene regulator (agr) locus, which is composed of two transcripts called RNAII and RNAIII, driven by the P2 and P3 promoters, respectively. The RNAIII transcript is the effector of the agr response, which up-regulates a number of toxins and multiple exoenzymes (proteases, lipases, and nucleases), and down-regulates the expression of numerous surface protein genes. The RNAII transcript is an operon of four genes, *agrBDCA*. AgrD is the 46 aminoacid precursor of the secreted AI, a cyclic thiolactone peptide (AIP) of 7-9 aminoacids, which is processed, cyclized and exported through the transmembrane protein AgrB. The extracellular AI binds to AgrC, the transmembrane receptor, which is a histidine kinase that following the binding phosphorylates the AgrA cytoplasmic response regulator, activating the P2 and P3 promoters. More advanced strategies targeting *S. aureus* virulence gene regulation make use of inhibitors of the sensor kinase AgrC, of the transcriptional activator AgrA, or of the RNAIII [44].

Due to allelic variations within the agr gene system, *S. aureus* can be subdivided into four agr specific groups, agr I−IV, each secreting a distinct AIP with different primary amino acid sequence. Most cross-group AIP-AgrC interactions are inhibitory, with AIPs activating their cognate receptors and competitively inhibiting non-cognate receptors. The competitive AIP inhibition constitutes a promising therapeutic approach for attenuating *S. aureus* infections.

Recently, it has been demonstrated by *in vitro* experiments that oxidized low-density lipoproteins bind all four *S. aureus* agr AIPs and antagonize agr signalling by each agr allele [45].

Molecules interfering with the *S. aureus* agr system have been obtained by a marine *Photobacterium*. Due to the structural similarity of these molecules, called solonamides, to the agr AI, the proposed mechanism of action is competitive inhibition [46]. The solonamides are the first reported natural antagonists with a structure resembling that of native *S. aureus* AI. Another natural product, hamamelitannin, obtained from *Hamamelis virginiana* (witch hazel), was identified as an inhibitor of RNAIII and δ-hemolysin production in *S. aureus* by Kiran et al [47].

Two of the three *P. aeruginosa* QS systems consist of the *lasRI* and *rhlRI* genes, where LasR and RhlR belong to the LuxR family of transcriptional regulators that specifically bind to *N*-(3-oxododecanoylhomoserine lactone (3-oxo-C12-HSL) and *N*-butanoylhomoserine lactone (C4-HSL), respectively [48]. The *las* and *rhl* systems regulate over 10% of the *P. aeruginosa* genome and are hierarchically organized. The 3-oxo-C12-HSL signal is the key factor that exerts transcriptional control over *lasI*, *rhlR*, *rhlI* and the genes of the third system, the PQS system. Despite the positive auto-regulation of *lasI*, levels of 3-oxo-C12-HSL reach a steady level long before cultures reach the stationary phase, due to the intervention of the transcriptional repressor RsaL, whose activity was originally reported in 1999 [49; 50]. It is now known that the QS systems are controlled by a complex regulatory network at both the transcriptional and post-transcriptional level, and RsaL is just one of over 15 QS regulators that have been identified so far [51]. RsaL is a DNA binding protein that competes against LasR, directly repressing both lasI and rsaL transcription by binding to the bi-directional *rsaL–lasI* promoter. In addition, Rampioni et al. observed that, independently of its effects on the production of QS signal molecules, RsaL directly represses 120 genes, many of which code for virulence and antibiotic resistance, and activates 10 genes (undefined) [50].

A recently identified novel LasR-specific antiactivator is QslA, which binds to LasR and prevents it from binding to its target promoter [52]. The investigation of bacterial autoregulation of

QS may open new horizons for the therapeutic exploitation of QS control. Studying the interaction between QslA and the LasR ligand-binding domain, Fan et al. elucidated the crystal structure of QslA and demonstrated that its mechanism of action consists in the disruption of LasR dimers by occupying the LasR dimerization interface, which is a previously unknown mechanism of QS inactivation [53].

Other QS negative regulators produced by *P. aeruginosa* are QscR, which is an "orphan" receptor activated by the 3-oxo-C12-HSL signal [54], and QteE, which reduces LasR protein stability without affecting LasR transcription or translation [55]. It has been shown that negative regulation of QS by QteE and QscR has a major impact on the absolute and relative fitness of *P. aeruginosa* [56]. The possibility to interact with these regulator systems for therapeutic purposes is currently being investigated. In a recent paper, Weng et al. report on a novel QS inhibitor, called C2, which causes a 375.4% upregulation of the *qscR* gene, resulting in the attenuation of elastase and protease activity, swarming motility and biofilm formation in the *P. aeruginosa* strain PAO1[57].

The *P. aeruginosa* PQS system may be inhibited by anthranilic acid halogenated analogues, whose development, however, has not been pursued [58; 59]. However, novel insights on the structure of PqsE, a product of the *pqs* operon, could lay the foundations for the computer-aided design of PqsE inhibitors [60]. Antagonists of the PqsR were reported for the first time in 2012 [61], and a peculiar mechanism of functional inversion of a PqsR antagonist has been recently described by Lu et al. [59]. These authors observed that compound 1 (**Figure 4, 7**), which behaves as a pure PqsR antagonist in an *E. coli* reporter gene assay, displayed a dose-dependent agonistic activity when tested in a *P. aeruginosa* functional assay. This unusual behaviour can be explained by the transformation of compound 1 into compound 2 (**Figure 4, 8**). A product of the *pqs* operon, the enzyme PqsH, which is present in *P. aeruginosa* only, carries out the transformation. The analysis of the molecular structure of the antagonist and of the chemical modification that turns it into an agonist allowed the synthesis of a small library of substituted compounds, among which compound

3 (**Figure 4, 9**) showed high potency in the *E. coli* reporter gene assay, but retained its antagonistic activity in *P. aeruginosa* without displaying any agonistic activity [59]. In a recent paper, Ilangovan et al. report the determination of the PqsR crystal structure, which allowed the visualization of the shape of the PQS-binding site. This work in conjunction with the chemical synthesis of PQS analogues resulted in the discovery of potent quinazolinone inhibitors of PqsR. These novel insights into the structure of PqsR provide further opportunities for targeting *P. aeruginosa* QS [62].

Farnesol (**Figure 5, 10**) is a sesquiterpene alcohol produced by *Candida albicans* and present in the essential oils of citrus fruits. Beyond its activity as fungal QS molecule [63], it affects PQS production by inhibiting the transcription of the pqs operon. According to the proposed model, farnesol stimulates PqsR interaction with a *pqsA* promoter fragment in the region of the PqsR binding site, causing a non-productive interaction with the *pqsA* promoter that does not lead to transcriptional activation of the *pqsA-E* operon [64]. The result is the impairment of production of pyocyanin, a pseudomonas QS-controlled virulence factor. Farnesol is an interesting molecule, because it is devoid of toxic effects, non mutagenic, and affects biofilm formation by *C. albicans*, *Streptococcus mutans*, and *Staphylococcus aureus* [65]. For an interesting discussion on the interactions between *C. albicans* and *P. aeruginosa*, the reader is referred to [66]. However, farnesol use is hampered by its low solubility in water. In a very recent paper, Bhattacharyya et al. describe a new efficient strategy for the local simultaneous delivery of farnesol and vancomycin in the treatment of multi-resistant *S. aureus* (MRSA) infections [67]. Following the observation that sufficient quantities of vancomycin and farnesol can be incorporated into sol-gel silica applied as thin films on an implant surface, they demonstrated a potent adjuvant effect of farnesol on vancomycin in the inhibition of *in vitro* MRSA experimental infection. Although in this case the mechanism of action proposed for farnesol does not involve the inhibition of QS, the technique is attractive and could be used to prevent or limit the formation of bacterial biofilms on the surfaces of catheter and joint implants.

## 5. Modern approaches based on innovative technologies.

Innovative technologies are being tested in the field of QS control, opening up possibilities that were unthinkable a few years ago.

### 5.1 Chemical methods.

Following the original discovery of the anti-QS activity of natural halogenated furanones (fimbrolides) isolated from *Delisea pulchra*, many analogues have been and are produced by chemical synthesis [68]. Traditional synthetic approaches usually yield small panels of synthetic AI analogues, some of which have proved potent QS modulators. A significant improvement of the QS disrupting activity has been recently obtained by the combination of fimbrolides with NO-donor molecules in hybrid compounds (**Figure 6, 11, 12**) [69]. The combination, which represents the first example of dual-acting molecules with high activity against QS and biofilm formation, is worth of further development. The chemical process typically requires different steps such as individual isolation, purification, and reaction optimization, which for large sets of analogues can rapidly become cumbersome. On the other hand, it usually allows the establishment of correlations between structure and activity. In this regard, Tsuchikama et al. recently reported the synthesis of a panel of analogues of (4S)-4,5-dihydroxy-2,3-pentanedione (DPD), called C4-alkoxy-HPDs, which are more potent than natural AI-2 molecules. These findings highlight how manipulation of the DPD scaffold can provide valuable tools for in-depth studies of the ligand-receptor interactions involved in AI-2-mediated QS, and lay the foundation for future chemical structure-based studies aimed at identifying and developing antagonistic analogues [70]. For a comprehensive review focused on chemical methods including combinatorial synthesis, affinity chromatography, and electrochemical sensing of QS signals, the reader is referred to [71].

### 5.2 Computer-aided investigations.

Among modern approaches, high throughput screening maintains its validity yielding hit molecules often based on new molecular scaffolds [72, 73], but *in silico* investigations are considered more convenient in terms of cost, speed of execution, and potency, being able to screen up to millions of compounds. Examples of results obtained by the use of structure-based virtual screening are the recent identification of inhibitors of *P. aeruginosa* QS from a natural-derivative database [74], and from the ChemBridge library [75]. The *in silico* approach has been used not only for screening purposes, but also for studying QS dynamics [76], and to identify bacterial species that contained both QS and aromatics degrading systems, in order to acquire information for developing novel bio-processing techniques [77].

An innovative approach is the production by Schaadt et al. of a multi-level logical model based on computational analysis of a combined regulatory and metabolic network for the three *P. aeruginosa* QS systems. The model may be used to analyse how enzyme inhibitors and receptor antagonists affect the formation of AI and virulence factors. In addition, it allows almost quantitative predictions about the effect of inhibitors of AI biosynthesis and antagonists of their corresponding receptor, and the investigation of the effect of reviewed network topologies. In the words of the authors, this approach can serve as a basis for further integrating the effect of random mutations in various parts of the network [78].

In order to get a better insight into the regulation of *S. aureus* QS system, Audretsch et al used an innovative approach to model the whole system [79]. Instead of creating a mathematical model, these authors used a Boolean network of nodes centred on the agr locus of *S. aureus*. The *in silico* node activation patterns were compared with gene activation patterns obtained from microarray, northern blot, and transcriptome data. The network can easily be manipulated and studied, and has two different steady states: one representing an invasive, toxic phenotype, and the other representing a biofilm producing phenotype. By manipulating the nodes of the simulated network, the model may be used to test theories about mutant strains and to predict the effects of QS inhibitors.

Sahner et al. have recently devised a novel approach based on the combination of *in silico* and biophysical methods (surface plasmon resonance, isothermal titration calorimetry, saturation transfer difference, and nuclear magnetic resonance) for the development of *P. aeruginosa* QS inhibitors. They demonstrated that the use of the two methods in combination represents a powerful complement to co-crystallography, and allows the rapid and efficient development of inhibitors. By using this combination technique, these authors identified an irreversible inhibitor that covalently binds to the active site of PqsD, which mediates the formation of the precursor of the *Pseudomonas* quinolone signal (PQS) [80].

### 5.3 Nano-and micro-technologies.

Nanotechnologies can be used to direct bacterial communication, by means of "nanofactory"-loaded biopolymer capsules placed at the midst of bacterial populations [81, 82]. It can be expected that this type of instruments, once developed, could be used to assist or replace the activity of the classic antibiotics, and to extend our knowledge of the QS systems and modulate them, so as to direct the microbiome to operate in a host-friendly manner. The possibility to modulate QS has also been demonstrated by means of titanium dioxide bead coated with AHL [83]. The implications of these experiments include the production of medical devices (catheters, joint and cardiac prosthetic devices) with coated surfaces able to interrupt QS signalling and to avoid biofilm formation.

The application of novel technologies derived from quite a different field like 3D printing to study bacterial interactions opens new horizons. By means of a microscopic three-dimensional (3D) printing strategy that enables multiple populations of bacteria to be organized within essentially any 3D geometry, including adjacent, nested, and free-floating colonies, it has been possible to obtain the rapid growth of fully enclosed cellular populations that release a number of biologically active molecules, including polypeptides, antibiotics, and QS signals. Using this approach, Connell et al. showed that picoliter-volume aggregates of *S. aureus* can display substantial resistance to β-lactam antibiotics by enclosure within a shell composed of *P. aeruginosa* [84]. The technique takes advantage of thermally set gelatine mixtures as a reagent for micro-3D printing, which provides the ability to print enclosures around any bacterial cell of interest suspended within the hydrogel matrix. In the words of the authors, this manufacturing strategy provides a versatile base for exploring the mechanisms involved in cell communication, which allow bacterial adaptation to the natural environment by means of social behaviour. This technology could perhaps be exploited at its best if coupled with the use of bacteria programmed to sense and destroy highly pathogenic species.

**5.4 Bacterial bio-engeneering**

In a recent paper, Gupta et al. describe the engineering of sentinel *E. coli* cells, capable of specifically detecting the QS AI molecule $3OC_{12}HSL$, produced by *P. aeruginosa*, and then to synthesize and secrete the chimeric bacteriocin CoPy, specific for PAO1 *P. aeruginosa* [85]. The engineering of *E. coli* consists of three interconnected modules: the detection module, which is activated by the PAO1 QS signal; the destruction module, which produces the toxin; and the secretion module that allows the secretion of CoPy. The effectiveness of the system was assessed by experiments in which the two bacterial species were co-cultured on semi-solid agar plates. The system has two advantages: first, it is possible to change the type of toxin produced; second, in the absence of the pathogen, the system remains in stand-by, but the production starts immediately after the detection of the pathogen.

Bacterial QS is also involved in extracellular cell death and in the action of antibiotics, through its interference in a system also called "addiction module", which can be found in many different Gram-positive and -negative bacteria. The system, which has mainly been studied in *E. coli*, consists of a pair of genes whose products are a stable toxin and an unstable antitoxin, which prevents the lethal action of the toxin [86]. In this situation, bacterial survival depends upon the continuous production of the short-lived antitoxin, to which the cell is "addicted". It has been shown that in *E. coli* the effect of some antibiotics such as rifampicin, chloramphenicol, spectinomycin, and nalidixic acid is due to the inhibition of the antitoxin production, and that this action is dependent upon the presence in the culture of high concentrations of a QS mediator called "extracellular death factor" (EDF) [87, 88]. The *E.coli* EDF is a linear pentapeptide with the amino acid sequence Asn-Asn-Trp-Asn-Asn, which is able to induce programmed cell death also in conditions of cell stress, like amino acid starvation or DNA damage. Recently, it has been shown that one hexapeptide produced by *Bacillus subtilis* and three peptides, one nonapeptide and two hexadecapeptides produced by *P. aeruginosa*, have the same effect of the *E. coli* EDF on *E. coli* cultures [89]. These findings establish the existence of a growing family of EDFs that behave as AI and exert both intra- and inter-specific activity. These molecules may provide a basis for the development of a new class of antibacterials, which could synergize with antibiotics.

## 6. Expert opinion

Despite the huge amount of molecules so far discovered that inhibit the QS mechanisms, the potential of QS inhibition as a future treatment strategy has yet to be fully assessed. In addition, there are obstacles, some obvious, others in the process of being recognized, which hinder the transformation of QS inhibitors into real drugs. The most obvious roadblock, which QS inhibitors share with antibiotics, is the lack of interest of pharmaceutical companies to bear the costs related to the development of hits into drugs that are inevitably bound to have a very limited market. This issue is critical to the future of the treatment of infectious diseases in this so-called post-antibiotic era. In accordance with what has already been proposed for the development of new antibiotics and drugs for neglected diseases, probably the best way to overcome the impasse is the union of skills and funds from private companies, academia and public institutions. In the short term, the knowledge gained so far, especially that concerning plant-derived inhibitors, could perhaps be used in the field of dietary advice and food supplements. Since the pioneering work of Givskov et al. [90] reporting the inhibitory effect of brominated furanones from the Australian macroalga *D. pulchra* on *Serratia liquefaciens* QS-mediated swarming, many plant-derived inhibitors have been identified. For a comprehensive review, the reader is referred to [91]. To improve the characteristics of natural derivatives, a number of synthetic molecules have also been developed, and more than 40 have been patented [92, 93, 15, 1]. However, we are still far from having a licensed drug available on the market, if we exclude some antibiotics, such as azithromycin, ceftazidime and ciprofloxacin, which at subinhibitory concentrations block the expression of QS-regulated virulence factors in *P. aeruginosa* [94]. In this regard, we must stress the importance of distinguishing between the direct toxic effect of a substance and its inhibiting activity on QS. To this aim, it is essential to verify the effect of QS inhibitors on bacterial viability by using sensitive methods and appropriate controls. In the case of pyrogallol, it has recently been shown that its previously highlighted QS-disrupting activity is a side effect of the peroxide production induced by this compound rather than true QS inhibition [95]. According to Defoirdt et al., many of the results obtained in experiments that make use of QS signal molecule reporter strains may be questioned, due to the possibility that the tested compounds may be toxic to the reporter strains [96]. These authors suggest that toxicity tests more stringent than those implemented so far should be used on molecules proposed as QS inhibitors.

Another emerging problem is the possibility that QS inhibitors can select more virulent strains. Resistance to QS inhibitors has been studied by means of mathematical models [97-101], and of *in vitro* experiments [102-104]. Bacterial resistance to brominated furanones is mediated by increased efflux activities. As this kind of resistance overlaps with resistance to antibiotics, the treatments with antibiotics endowed with a strong selective pressure may result in improved resistance to antivirulence compounds (and perhaps vice versa). The finding that some strains isolated from cystic fibrosis patients are resistant to QS inhibitors, corroborates this hypothesis. Additional clinical evidence of the ability of bacteria to evolve resistance to QQ compounds was provided by studying the resistance of *P. aeruginosa* isolated from urine, blood, and catheter tip specimens obtained from Mexican children [104].

Other *in vivo* and *in vitro* results reported by Kohler et al. demonstrate that QS inhibition interferes with natural selection towards reduced virulence, and therefore may increase the prevalence of more virulent genotypes [105]. Some concerns about the widespread use of QQ compounds as part of an antibacterial therapy have been raised by the possibility that targeting a specific bacterial group may predispose the patient to infection by other groups. For example, in *S. aureus*, but not in *S. epidermidis*, biofilm production is under the agr system control. In this situation, agr inhibition would favour *S. epidermidis*, suggesting that agr antagonists would not be indicated for the control of staphylococcal biofilm infections [106].

Interestingly, resistance to QQ compounds may be enhanced by the complex interplay of bacteria with bacteriophages, and it seems that QS constitutes a significant, but so far overlooked, determinant of bacterial susceptibility to phage attack [107]

The AHLs often exert toxin-like effects on mammalian cells. Hence, neutralizing antibodies would achieve the double goal of disrupting bacterial QS and protecting the host from toxicity. As discussed above, the disrupting efficacy of antibodies was demonstrated both in *P. aeruginosa* and *S. aureus* QS systems. Based on these results, and taking into account the evolutionary high conservation and extracellular distribution of AI, QS can be considered a good target for active and passive immunotherapy approaches [39]. However, here again some caution is necessary. In a recent paper, Michael-Gayego et al. report that SilCR, a QS peptidic AI produced by Group A (GAS) and G streptococci, effectively decreases bacterial virulence *in vivo*. The effect was also observed in SilCR-vaccinated mice, which developed a more severe disease than non-vaccinated ones [108]. The paradox is that SilCR is the product of a gene belonging to the streptococcal invasion locus, sil, which is a virulence factor involved in GAS spreading into deeper tissues. This is perhaps the first demonstration that antibodies directed against a bacterial component increase the severity of the disease. On the other hand, QS activation can be used to improve the antigenicity and the efficacy of an *E. coli* bacterin preparation to be used for vaccination purposes, as reported by Sturbelle et al. [109]. These examples underline both the potentials of QS exploitation for therapeutic purposes and the need to clarify all aspects of the involved mechanisms.

In conclusion, QS inhibition can be considered a promising strategy against infections, especially those associated with biofilm formation, and the discovery of new inhibitory molecules is rapidly progressing. However, to identify a new molecule as a pure QS inhibitor, it is necessary to develop more standardized methods of evaluation, with particular attention to rule out direct toxicity that may mimic a QQ effect. Moreover, concerns about the possible emergence of resistance and adverse effects resulting from the alteration of the microbiome must be properly addressed in-depth.

In our view, the current challenge in the field of QS research is not so much to find new inhibitory molecules, but to refine and deepen our knowledge on the QS-based interactions between different bacterial species and between bacteria and the host. Important issues that need further investigation are the impact of QS on the social evolution of bacteria, as well as the impact of bacterial social-driven evolution on the infection of the host. Studies performed in mice [110] and humans [111] demonstrated that cultures containing mixtures consisting of QS cooperators, i.e. autoinducer-producing cells, and cheats, i.e. cells that do not produce autoinducers but benefit of cooperator-produced autoinducers, are less virulent than cultures containing pure cooperators, suggesting that asocial cheats could be exploited to exert a potential therapeutic role [112]. An interesting discussion on bacterial cooperation and its possible relevance in the clinical field can be found at [113].

The last, but not least important aspect that must be taken into account is that so far the transformation of laboratory results into viable drugs is almost non-existent [114]. This situation is not expected to improve in the near future, because, as is the case for antibiotics, market forces are insufficient to drive the development of antibacterial drugs based on new scaffolds. The need for long-term, huge investments, and the prospect to see the novel drug indications limited to small number of cases, made many large pharmaceutical companies to quit antibiotic discovery for more profitable therapeutics [115]. The political, medical and public concern about the rising innovation gap in 2009 prompted the U.S. and European Community presidencies to establish a Transatlantic Task Force to address antimicrobial resistance, and the Infectious Diseases Society of America called for a global commitment to develop 10 novel antimicrobials by 2020 [116]. We can envisage that in the near future the synergy between new technical developments and public-private industrial partnerships will bring into being a new harvest of badly needed novel antimicrobials, including antibiotics and antivirulence agents.

Article highlights:

The increased frequency of infections caused by multiresistant strains indicates the need for new antibacterial drugs with a low selection pressure. The quenching of bacterial virulence by disrupting QS-dependent intercellular communication is one of the best options to achieve this goal.

Many natural and synthetic QS inhibitors have been identified by means of modern methods that include high throughput screenings and *in silico* structure-function analysis. About 40 molecules with QS inhibitory activity have been patented.

The possibility to interrupt QS by means of active or passive immunologic interventions is being evaluated and appears promising.

The most recent approaches in the field of QS research span from mathematical modelling of the QS systems to the combination of *in silico* and biophysical methods, to the creation of hybrid compounds by fusing fimbrolides with NO-donor molecules, and to the exploitation of the 3D printing technology to study short-distance interspecific bacterial interactions.

The impact of the social behaviour of bacteria on the microbiome and on the establishment of infections needs further investigation. Some concerns are emerging following the someway unexpected discovery that QS inhibitors can exert a selective pressure favouring the establishment of more virulent strains. This aspect should be thoroughly investigated before any QS inhibitor is licensed and put on the market.

None of the inhibitors identified to date have reached the market yet. This can be attributed in part to the necessity of further basic research and development, and in part to the lack of interest of pharmaceutical companies for the development of drugs with limited market prospects.

Conflict of interest

The authors declare no conflict of interest.

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## Figure legends

Figure 1

Chemical structures of anthranilate (1), methyl anthranilate (2), and PQS (3)

Figure 2

Chemical structures of N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) (4), and N-butyryl-L-homoserine lactone (C4-HSL) (5)

Figure 3

Chemical structure of eugenol (6)

Figure 4

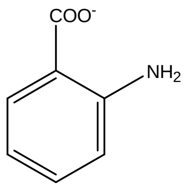
Chemical structures of two PqsR antagonists (7) and (9), and of a PqsR agonist (8) (from Lu et al., 2014)

Figure 5

Chemical structure of farnesol (10)

Figure 6

Chemical structures of examples of fimbrolides-NO-donor hybrid compounds (11), (12) (from Kutty et al., 2013)

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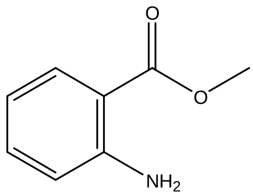


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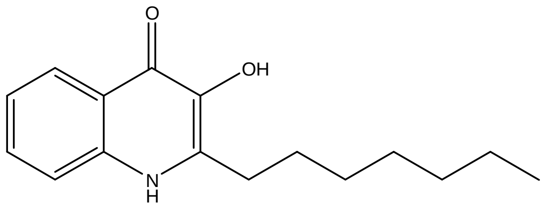


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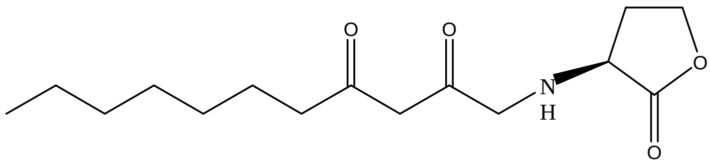
Figure 1

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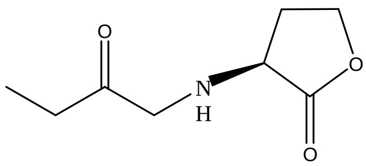


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Figure 2

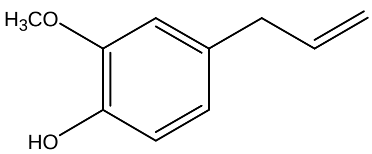


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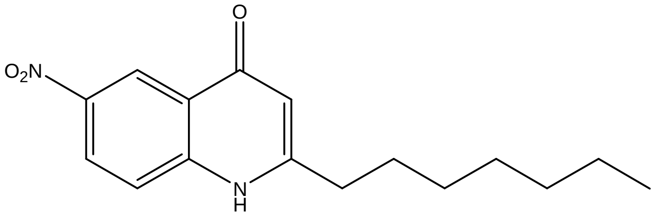
Figure 3

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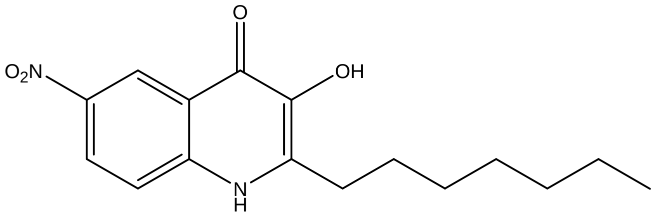


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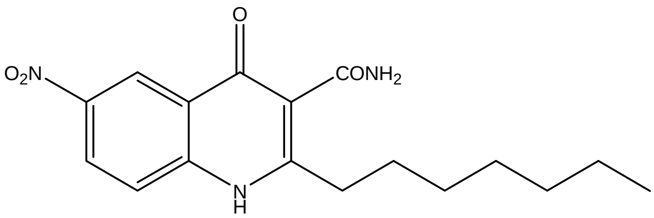


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Figure 4

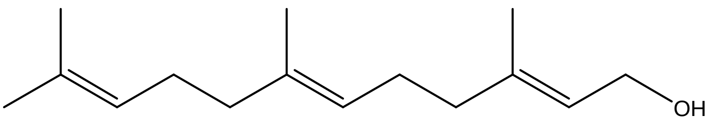


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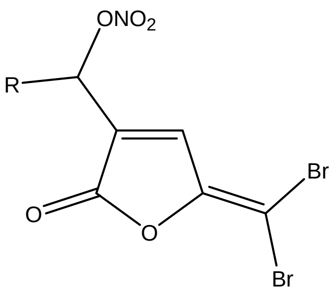
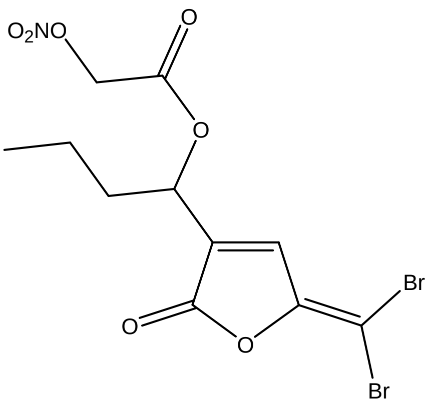
Figure 5




R=$C_3H_7$
R=$C_{11}H_{23}$




**11** **12**

Figure 6