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REVIEW

Quorum sensing inhibitors: a patent review (2014–2018)

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ABSTRACT

Introduction: Quorum sensing (QS) is a cell density-dependent phenomenon in which specific pathways are activated after autoinducers (AIs) outside the microorganism reach a threshold concentration. QS creates a positive feedback loop that induces a cascade of gene expression and causes biofilm formation, virulence and sporulation. QS signals are diverse, acyl-homoserine lactone (AHL), AI peptide (AIP) and AI-2 are three major categories of QS signals. QS inhibitors (QSIs) can disrupt or prevent the formation of biofilm and reduce virulence while exerting less selective pressure on the bacteria, suggesting that QSIs are potential alternatives for antibiotics.

Areas covered: This review summarized the pertinent patents on QS inhibition available from 2014 to 2018. The authors analyze these patents and provided an overview of them and their potential applications.

Expert opinion: The main strategy for QS inhibition is to use the analogues of various QS signals to block downstream signal transducers. The inactivation of signal molecules or the stimulation of the immune response is also attractive strategies to inhibit QS. However, additional clinical trials are needed to assess their efficacy in mammals. In sum, QS inhibition can reduce the virulence of bacteria without affecting their growth or killing them and the reduced pressure may minimize the increasingly resistance.

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KEYWORDS
Quorum sensing; inhibitor; antibacterial; biofilm formation; acyl-homoserine lactones; autoinducer peptides; autoinducer-2

1. Introduction

Quorum sensing (QS) is a cell density-dependent process of cell-to-cell communication by which cells measure population density and trigger appropriate responses [1]. The phenomenon of QS was first reported in the marine bacterium *Vibrio fischeri* by Hastings et al. in 1977 [2]. Autoinducers (AIs) are specific signaling molecules that play a crucial role in QS and consist of three different classes according to their structure and specific functions. The main QS signal molecules are acyl-homoserine lactones, (AHLs, AI-1), AIPs (autoinducing peptides) and autoinducer-2 (AI-2) while AI-3, cholerae AI-1 (CAI-1), Pseudomonas quinolone signal (PQS) are also common [3]. Certain bacteria even use host epinephrine or norepinephrine as QS signals [4]. In general, Gram-positive bacteria produce AIPs as their AIs, while Gram-negative bacteria use AHLs as their signaling molecules, which bind directly to regulate gene expression without additional processes [5]; AI-2 acts as AI in both Gram-positive and Gram-negative bacteria.

Genes regulated by QS include those involved virulence factor production and biofilm production. Over 80% of bacterial infections in humans involve the formation of biofilms, which is the basis of multiple chronic infections [6]. Biofilms allow bacteria to create a microenvironment that enable the bacteria to attach to the host surface and evade host immune responses and even exclude antibiotics [7].

A number of research groups are actively developing quorum sensing inhibitors (QSIs) that include small molecule and macromolecular agents, which can be divided into different classes based on their mechanisms and structures. In this review, the QSIs published from 2014 to 2018, and their potential applications for treating bacterial infection are summarized.

2. Las/AHL system

2.1. Introduction

The Las system is present in Gram-negative bacteria, with that in *Pseudomonas aeruginosa* being a classic example. The Las system regulates the expression of multiple virulence factors and consists of two main molecules [8,9]: LasI, as synthase protein, and LasR, a transcriptional regulator [10]. LasI, causes a rapid increase in the synthesis of 3-oxo-C12-HSL [11], which results in an amplification of the signal by self-induction. LasR interacts with 3-oxo-C12-HSL, activating 3-oxo-C12-HSL as a transcription factor, and also binds to target genes, regulating transcription [11,12]. However, it has also been reported that QS mediated by Las may inhibit the production of glucose-rich exopolysaccharide that is necessary for biofilm matrix building [13]. Activated LasR induces the transcription of *rhlR* and *rhlI* and initiate the downstream synthesis of corresponding proteins which activates the transcription of a subset of genes. Additionally, PQS system and Vfr system could also get involved in this QS signal pattern as Figure 1 illustrated [14]. And the patents concerning this system was summarised in Table 2.

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Article highlights

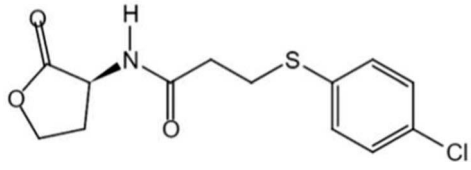
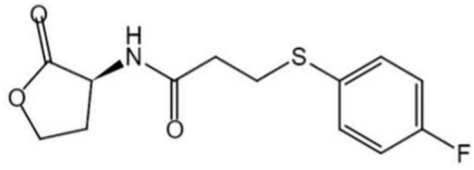
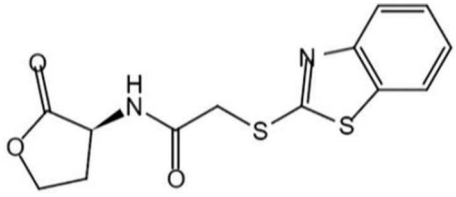
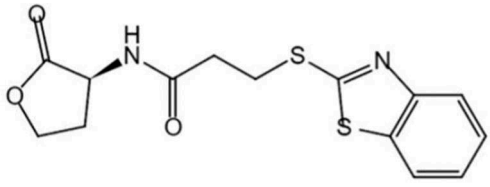
- This review summarized the patents concerning QS inhibition from 2014 to 2018 and gave an overview of their development and application.
- The majority of these inhibitors are based on antagonizing receptors and quenching signal molecules. Some inhibitors could also promote the immune response of the host.
- However, few clinical experiments of these inhibitors were applied assess their actual efficacy and toxicity *in vivo*. The mechanisms of some inhibitors are still unclear and more profound studies should be implemented in the future to unravel the exact mechanisms of the processes of QS pathway.

This box summarizes key points contained in the article.

2.2. AHLs containing aromatic rings

As mentioned before, AHLs act in the QS signal pathways of Gram-negative bacteria. It has been hypothesized that AHL analogues may act as competitive inhibitors and numerous QSIs were designed based on this idea. The structures of AHLs vary in different species but they share the key features of a lactonized homoserine ring and a hydrocarbon tail linked by an amide bond. Recently, Wu et al. prepared AHLs containing aromatic rings that were to be tested as QSIs. Wu et al. measured their activities *in vitro* using furanone C30 and naphthol as positive controls for the inhibition study testing the *P. aeruginosa* LAS and PQS systems, respectively. Among these analogues, four compounds demonstrated the best activity compared with the positive controls in both tests, making them a potential candidate for further development (Table 1) [15,16].

Table 1. IC_{50} for four most potent aromatic AHL analogues prepared by Wu et al. that act on the *P. aeruginosa* Las and PQS systems.

Compund	QSIS-lasI IC_{50}(μM)	PQSI IC_{50} (μM)
	5.2	93.9
	7.4	198.4
	10.3	185.4
	10.8	194.4
Furanone C30 (MeOH as negative control)	17.4	/
Naphthol (CH_3CN as negative control)	/	384.0

2.3. Non-lactone and heterocyclic compounds

Blackwell et al. proposed that several libraries of non-lactone carbocyclic and heterocyclic AHL analogues could act as antagonists or agonists of bacterial QS. Although the structures of these compounds do not possess a lactone ring, they still exhibited antagonistic or agnostic activity toward LasR and RhlR with elongated half-life periods. For instance, the compounds J18 and RN15 exhibited agnostic activity toward RhlR to the extent of 86% and 88%, respectively. However, the antagonist activity of certain compounds was not satisfactory. Compound RN2, at a concentration of 10 μM, only inhibited 28% of RhlR activity in *Escherichia coli* JLD271/pJN105R2/pSC11-*rhlI* in the presence of 10 μM BHL, a natural substrate

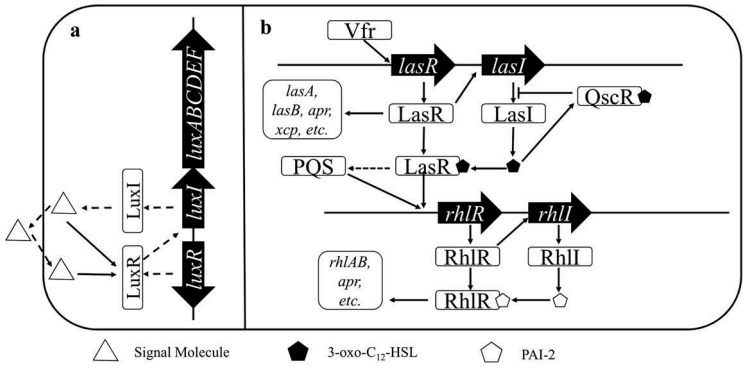


Figure 1. (a) In this LuxI/LuxR QS paradigm, the AI is generated by LuxI and diffuses freely in and out of cells and stimulates the transcriptional regulator LuxR to form a positive feedback. (b) QS system acts as a mediator regulatory pathway between the las and rhl systems and regulates many genes also controlled by las and rhl systems including those for virulence-factors and biofilm formation. LasR and RhlR inhibitors could prevent the gene regulation of the two Lux-type receptors.

Table 2. Summary of inhibitors that modulate the Las/AHL system.

Inhibitor molecule/compound	Molecular target	Biological effect	References
Compounds containing a benzene ring or a heterocyclic ring	LasR receptor	Attenuate the virulence of *P. aeruginosa* and increase its susceptibility	Gifskov et al., 2014 [92]
Farcalindiol	Las system and rhl system based on AHL signaling molecules	Prevention and treatment of microbial infections	Yu et al., [117]
Genetically engineered bacterial phages	AHL	Encoding the quenching polypeptide and destroying the autoinducer AHL to inhibit QS of *P. aeruginosa* and *E. coli*	Pei et al., [49],
N-sulfonyl homoserine lactone derivatives	LasR receptor	Inhibition of QS of multiple Gram-negative bacteria	Li et al. (2014)[53]
AHL substituted with benzoheterocycle	Las system and *pqs* system	Inhibition of *P. aeruginosa las* system and *pqs* system	Wu et al. (2017)[15]
AHL substituted with benzene ring	Las system and *pqs* system	Inhibition of *P. aeruginosa las* system and *pqs* system	Wu et al. (2017)[16]
Combination of several small molecule regulators	Las , RhlR and PqsR system	Multiple QS regulatory effect, particularly for modulating QS in *Pseudomonas* and *Pasteurella*	Blackwell, Welsh et al. (2017)[94]
Non-lactone carbocyclic and heterocyclic compounds	LasR receptor	Inhibition of Gram-negative bacteria, especially the las system of *P. aeruginosa*	Blackwell, McInnis et al. (2017)[17]
Gold(I) thiolate complex	Rhl, *pqs, las* system	Inhibition of QS in *P. aeruginosa*	Givskov et al. [19],
(5R,7R)-7-(4-(benzyloxy)phenyl)-5-(2-chlorophenyl)-4,5,6,7-tetrahydro-[1,2]-triazole-[1,5-a]-pyrimidine-2-amine	LasR	Inhibition of QS of *P. aeruginosa*	Xiong et al. (2017) [20]
N-acylated homoserine lactase	AHL signaling molecule	Catalyzing hydrolysis of AHL to quench AHL signaling molecules to inhibit *P. aeruginosa*	Ruan et al. [48],
Non-lactone carbocyclic and heterocyclic compound	RhlR	Inhibition of QS of Gram-negative bacteria	Blackwell et al. (2017) [18].

Table 3. Summary of Lux system inhibitors.

Inhibitor	Target	Biological Effect	References
Coumarin	RhlR	Downregulation of the expression of elastase, lectins, siderophores and Xcp, and production of hydrogen cyanide and rhamnolipids.	Deryabin et al. [29].
	LuxR	Downregulation of the expression of LuxI	
	LasR	Downregulation of expression of siderophores, RhlR, catalase, haemolysin, exotoxin A, Xcp and elastase.	
	CviR	Downregulation of expression of violacein protein	
Scopoletin	RhlR	Downregulation of expression of elastase, lectins, hydrogen cyanide, rhamnolipids, siderophores and Xcp.	Deryabin et al. [29].
	LuxR	Downregulation of LuxI	
	LasR	Downregulation of expression of siderophores, RhlR, catalase, haemolysin, exotoxin A, Xcp and elastase.	
	CviR	Downregulation of expression of violacein protein	
4606–4237	LuxN	Downregulation of expression of LuxR	Bassler et al. [30]
	CviR	Downregulation of expression of violacein protein	
CTL	LuxN	Downregulation of expression of LuxR	Bassler et al. [30]
	CviR	Downregulation of expression of violacein protein	
CL	LuxN	Downregulation of expression of LuxR	Bassler et al. [30]
	CviR	Downregulation of expression of violacein protein	
C450-0730	LuxN	Downregulation of expression of LuxR	Bassler et al. [37]
12	LuxO	Downregulation of expression of Qrrs	Bassler et al. [38]
32	CqsS	Upregulation of expression of HapR, and Downregulation of expression of TcpA	Bassler et al. [39]
8	CqsS	Upregulation of expression of HapR, and Downregulation of expression of TcpA	Bassler et al. [38]

of RhlR. Moreover, these compounds displayed greater selectivity toward RhlR than LasR and the compounds J18 and RN36 exhibited 255-fold and 105-fold greater selectivity, respectively. In addition, Blackwell et al. revealed that the lactone carbonyl's hydrogen bonds are not indispensable because compounds 2 and 3, which lack hydrogen bond substitutions, demonstrated the ability to modulate the LasR system. In the thiolactone compound library that was prepared, the inhibition ratio of compound 18 reached 80% in *E. coli* and 99% in *V. fischeri*, respectively (Figure 2) [17,18].

2.4. Gold(I) thiolate complexes

Givskov et al. proposed that gold(I) thiolate complexes, specifically auranofin, could act as inhibitors of *P. aeruginosa*. Auranofin demonstrated comprehensive inhibitory activity towards *P. aeruginosa* because all three QS signal pathways in *P. aeruginosa*, namely, *las, rhl*, and *pqs* were inhibited, maximizing the potency of this gold compound. For example, auranofin inhibited Vfr, which is known to control Type III secretion and an electrophoretic mobility shift assay verified the inhibition of Vfr binding to the *lasR* promotor [19].

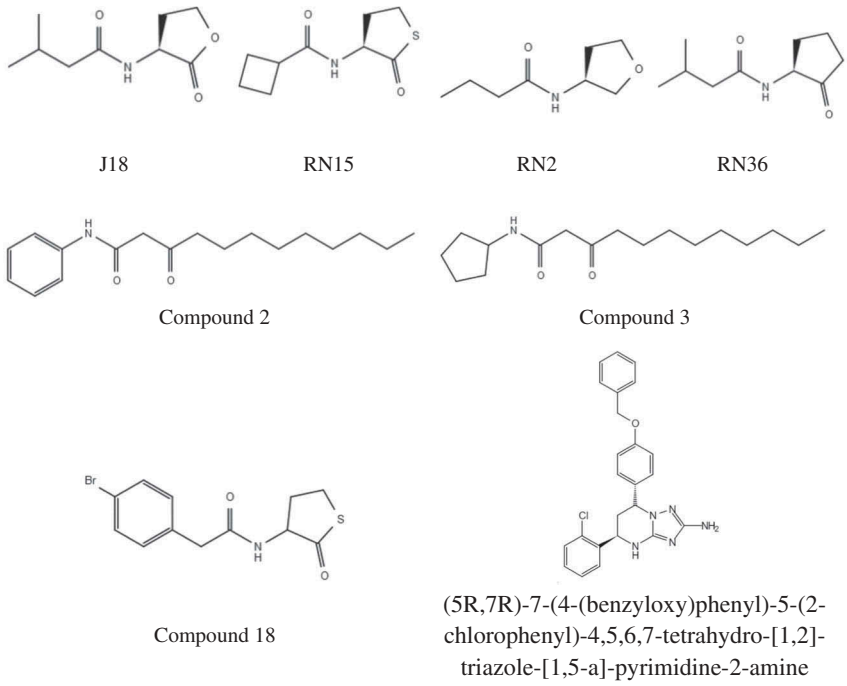


Figure 2. The representatives of Las/AHL inhibitors.

2.5. Pyrimidine derivative

Since the Las system is activated first after the population of bacteria reaches a threshold, the inhibition of LasR will block various downstream signals, making LasR an ideal target for QS inhibition. Thus, Xiong et al. obtained a derivative of pyrimidine, namely, (5R,7R)-7-(4-(benzyloxy)phenyl)-5-(2-chlorophenyl)-4,5,6,7-tetrahydro-[1,2]-triazole-[1,5-a]-pyrimidine-2-amine, by using LasR as the target with the assistance of virtual screening. It was reported that this derivative down-regulates LasR, LasB, and LasI in *P. aeruginosa* (Figure 2) [20].

3. Lux system

3.1. Introduction

Over a 100 Gram-negative bacteria, strains communicate with the engagement of Lux homologs genes [21], with *Vibrio harveyi* being a classic example [22]. LuxI is an autoinducer synthase that mediates the interaction between S-adenosylmethionine and an acyl carrier protein, leading to the formation of N-(3-Oxohexanoyl)-L-homoserine lactone, which functions as an autoinducer [23,24]. LuxR regulate 625 genes and coregulate 77 genes with AphA [25]. AHLs are taken back into the cell and interact with LuxR at a high external concentration. LuxR will be capped from degrading while it is forming, but free LuxR is degraded inside the cell [26]. Moreover, the LuxR-AHL complex can bind to the Lux promoter region and then initiate other QS-regulated functions [27]. LuxO is dephosphorylated by nitric oxide (NO) through a NO-responsive channel, and then feeds into the quorum-sensing system at LuxU (Figure 1) [28]. Additionally, the patents about this system was summarised in Table 3.

3.2. LuxR-type receptor antagonists

The majority of AHL-based QS systems have been termed LuxI-type synthases and LuxR-type receptors. The primary AHL-dependent system comprises the LuxI and LuxR genes that encode AHL synthetase and the AHL-responsive transcriptional activator such as LasI/LasR, and CviI/CviR, respectively.

Deryabin et al. described coumarin and its derivative – scopoletin as LuxR, LasR and RhlR inhibitors (Figure 4). These researches also demonstrated that the antagonistic effect of coumarin and its derivatives could be enhanced when combined with aminoglycoside antibiotics such as kanamycin, gentamycin, and amikacin [29].

Bassler et al. reported that 4-(4-chloro-2-methylphenoxy)-N-(2-oxotetrahydrothiophen-3-yl) butanamide (compound 4606–4237) (Figure 4), as well as and some of its derivatives, are LuxN and CviR inhibitors and tested it against *E. coli*. Its structure was altered by substituting a halogen atom at the para position to the phenyl on the aromatic ring and eliminating the methyl moiety from the ortho position. These changes strengthened the anti-effect (compound CTL, IC_{50} decreased from 2.2 to 1.1 μM) (Figure 4). When the sulfur was replaced with an oxygen atom in the thiolactone head group, the potency of the antagonist CL increased by nearly 10-fold over that of the original 4606–4237 molecule, giving an IC_{50} value of 377 nM. Thus, CL is the most potent synthetic antagonist in the series (Figure 4) [30].

It was found that *Delisea pulchra* produces furanone compounds that bind to LuxR, thus suppressing the effect of AHLs [31]. Furanone 45 and furanone C-30 has been reported to be potent inhibitors against QS [32,33]. Recently, Sun et al. proposed that another furanone is a QSI and measured its activity against *P. aeruginosa*. It was found that compound 8, with the minimal inhibitory

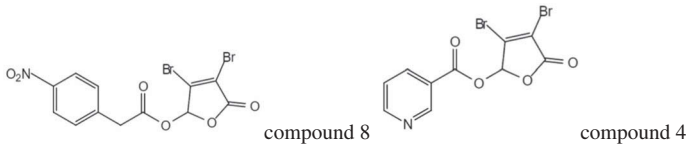


Figure 4. Structures of Lux system inhibitors.

concentrations (MICs) of 32, 64 and 32 μg/mL against of ATCC27853, ATCC9027, and PAOA strains respectively, was the most potent inhibitor among the compounds synthesized, and more than 80% of the biofilm was inhibited in the three strains when the concentration of compound 8 is the quarter of the MIC (Figure 3) [34]. In addition, Kim et al. synthesized a series of brominated furanone derivatives and evaluated their inhibitory effects on *Fusobacterium nucleatum*. It was found that the QS of *F. nucleatum* was inhibited without significant cytotoxicity toward the host, making them possible candidate drugs for oral and dental infection [35]. Guo et al. synthesized five furanone derivatives and assessed their biological activities against *P. aeruginosa* and crop diseases. Compound 4 exhibited the greatest potency with a MIC value of 32 μg/mL against *P. aeruginosa* ATCC9027 (Figure 3) [36].

3.3. Specific antagonists of the luxN receptor

Lee et al. reported a group of LuxN receptor-specific antagonists, the scaffolds of which consists of benzoheterocycle rings or phenoxy acetamides, which are dissimilar to that of AHLs. Compound C450-0730 (Figure 4) (IC_{50} = 2.7 μM) represents one of these specific antagonists. It is notable that these are the first antagonist molecules that target an AHL membrane-bound sensor kinase. These inhibitors block LuxN competitively, preventing LuxN from activating the transcription in the cascade [37].

3.4. LuxO inhibitors

Bassler et al. proposed that 5-thio-6-azauraci derivatives, varying only by their side chains are the primary of the inhibitors of LuxO. The activities of these compounds are highly dependent on the alkyl side chain's structural features. Increasing the steric bulk by introducting of a tert-butyl carbinol side chain led to a three-fold enhancement in potency, and the hydrophobic terminal tert-butyl moiety was optimal within the series. These compounds inhibited the ATPase activity of LuxO, preventing the transcription of the Qrr sRNAs, which is controlled by LuxO [38].

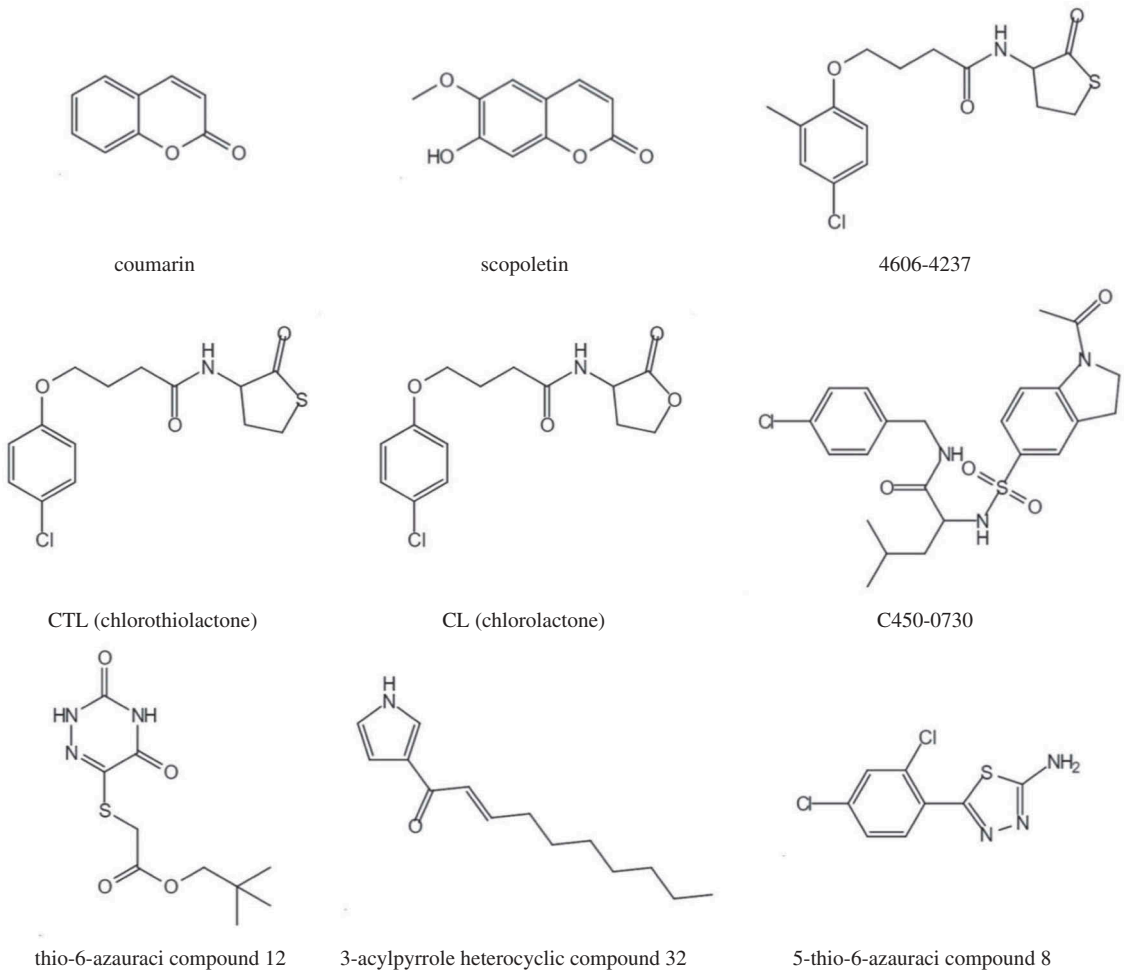


Figure 3. Representative of furanone QSIs.

3.5. CqsS receptor agonist

The primary QS autoinducer in *Vibrio cholerae* is known as CAI-1, which is the small molecule, (S)-3-hydroxytridecan-1-one. It regulates the production of the transcription factor HapR and the virulence factor TcpA when it binds to its receptor CqsR. Bassler et al. designed a series of CqsS receptor agonists, based on Ea-CAI-1, the prodrug of CAI-1, using a 3-acylpyrrole heterocyclic motif as the central group due to its chemical stability. The most effective compound is compound 32 (EC_{50} = 3.8 nM) (Figure 4). The synthesized agonists caused *V. cholerae* cells to exhibit high cell density behaviors. For example, the QS system represses virulence and biofilm traits. While these agonists affected the QS behaviors of *V. cholerae* and other pathogenic bacteria that utilize *V. cholerae*-type QS circuits, such as *Legionella* species, none of these agonists showed significant activity toward the *V. harveyi* CqsS receptor [39]. In addition, Bassler et al. reported a second kind of CqsS receptor agonists that shared no structural homology to the native CAI-1 autoinducer, but possessed other types of heterocycles instead. The compounds exhibited inhibitory activity toward *V. cholerae* (Compound 8) (Figure 4) [38].

The Lsr system promotes AI-2 up-take [40] The Lsr transporter is an ABC transporter encoded by the *Lsr* operon [41]. LsrB is responsible for AI-2 internalization. Following internalization, AI-2 is phosphorylated by LsrK, modified by LsrF and LsrG. Finally, it binds to and inactivates LsrR, initiating expression of the Lsr system [41,42]. Ryu et al. constructed a dual vector to co-express the HPr and LsrK proteins. Under osmotic pressure shock or heat shock, the yield of LsrK increased significantly, providing a possible method to inhibit QS [43].

The QseBC two-component system is present in *E. coli* and *Salmonella typhimurium*, and modulates motility in the presence of specific quorum-sensing signals [44]. The two main components of the QseBC two-component system are, QseC, a histidine sensor kinase, and QseB, a response regulator [45]. QseC senses AI-3/catecholamine to augment its phosphorylation state, and the β-adrenergic antagonist suppresses the QseC signal [4,46]. Sperandio et al. developed thiazole derivatives and assessed their inhibitory effects on *S. typhimurium*. Notably, these inhibitors will disrupt the QseC signal communication rather than killing the bacteria directly. Among these compounds, CF325, CF326 and CF334 demonstrated efficacy against *S. typhimurium* virulence. In addition, compounds CF331 and CF345 illustrated the greatest modulation of the virulence such bacteria as Enterohemorrhagic *Escherichia coli* (EHEC) (Figure 5) [47].

3.6. Biological methods

In addition to mimicking the structure of QS signal molecules, the quenching of these molecules could also block the downstream pathway and inhibit QS. This strategy has also been used widely in the research on the quenching of QS signals. Several natural enzymes or modified enzymes have been discovered that exhibited satisfactory activity, and many of them were AHL lactonases which hydrolyze the lactone ring in AHL molecules [41]. Ruan et al. used pET32a as a vector to express AiiK, a homologous enzyme of AHL lactonase from *Kurthia huakuii* LAM0618, in *E. coli* BL21 to degrade AHL and thus treat *P. aeruginosa* infection. Moreover, AiiK retains 86% of its activity after incubating at 45℃ for two hours, and the enzyme is resistant to chymotrypsin, trypsin and proteinase K, suggesting its stability. Upon quenching the QS signal with this enzyme, it was observed that the expression of extracellular protease and pyocyanin was lowered [48].

In addition to incubating bacteria with the lactonase AiiK directly, engineered phage could also be used to infect bacteria and play the role of a live inhibitor. Pei et al. used engineered T7aiiA phage, whose recombinant genome contained the sequence of *Bacillus anthracis* AHL lactonase, an inhibitor against Gram-negative bacteria. To construct the recombinant phage, the wide-type T7 DNA genome was inserted by the AHL lactonase *aiiA* gene into the 3′ end of the 10B gene with a Φ10 promoter. The AHLs of *Agrobacterium tumefaciens* and *P. aeruginosa*, but not *Chromobacterium violaceum*, were significantly more degraded compared with the control group [49]. Several organisms are known to produce AHL degrading enzymes such as AHL acylase, AHL lactonases and AHL oxidoreductases, which are implicated in inhibiting various components of QS-controlled pathogenicity. Zhang et al. reported that *Muricauda olearia*

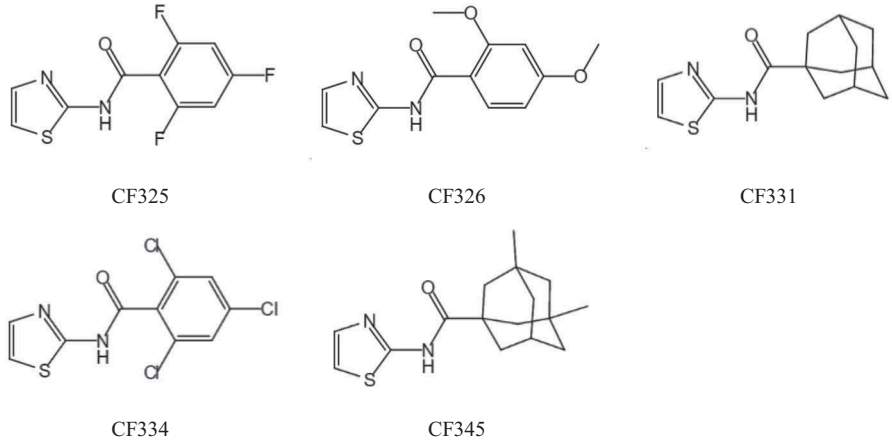


Figure 5. Structures of Qse system inhibitors.

LM001 produced MomI, a type of AHL lactonase that exhibited an anti-effect against *Aeromonas hydrophila* and reported its amino acid sequence [50]. Chen et al. reported that *Acinetobacter schindleri* degrades AHLs efficiently when it utilizes AHLs as its only carbon source, and demonstrated its inhibitory effect on *Dickeya dadantii* [51]. María et al. reported that *Tenacibaculum* 20J produced Aii20J, a type of AHL lactonase, that possessed a high degrading efficiency and described the genetic engineering method that was used to obtain it [52].

4. AI inhibitor

4.1. Amides

Li et al. discovered a new series of amide compounds that are AHL analogues to modulate QS in bacteria. It is notable that both QS antagonistic and agonistic activity of QS is possible depending on the substitutions on the parent skeleton. In particular, 4-bromo-N-(2-oxoxazolidin-3-yl)benzamide (compound 12) exhibited partial agonism-antagonism activity with an IC_{50} of 7.85 ± 1.71 μM [53].

4.2. Piperitone

Fujinaka et al. revealed that 0.01% piperitone inhibits 90.3% of *V. harveyi* with no significant effect on *Porphyromonas gingivalis, Fusobacterium nucleatum, Actinomyces viscosus* and *Streptococcus mutans*. It has been speculated that piperitone may be used to treat dental infection [54].

4.3. Saccharides

Xylitol has been widely used to prevent dental diseases. Hong et al. found that such pentoses as D-ribose not only enhances the effect of xylitol, but also reduces tolerance. The composition of two carbohydrates were reported in this patent, and the carbohydrates were found to inhibit AI-2 and reduce the expression of *gbpC* and *dblB* which are genes that promote bacterial adhesion, and thus weaken the biofilm of dental bacteria such as *S. mutans* or *Streptococcus sobrinus* [55].

4.4. Polypeptide

Wang et al. designed a polypeptide with the sequence of HSIRTGSKKPVPIIY that binds to the putative receptor of the LuxS/AI-2 system and blocks AI-2 from entering the bacteria. The growth of various bacteria, namely, *Streptococcus suis, Staphylococcus aureus, E. coli, Salmonella, Listeria monocytogenes, P. aeruginosa* was shown to be inhibited by the polypeptide although its effect on *P. aeruginosa* was slightly inferior compared to the other strains. Moreover, it exhibited little haemolytic activity as well as cytotoxicity towards mice spleen cells [56].

5. Agr system

5.1. Introduction

The Agr system is also an essential component of QS. Agr systems exists in Gram-positive bacteria, with the system in S. aureus being a classic example. The Agr locus consists two transcripts, RNA II and RNA III [57]. AgrD produces Pro-AIP which is then processed, modified and transported out by AgrB. AgrC is then activated and binds to AIP while AIP accumulates in the extracellular environment and reaches a certain threshold level when the bacterial density is high [58]. AgrC and AgrA together constitute a two-component system, which activates RNAII and RNAIII after itself is activated [59]. RNAII is transcribed to agr operon while RNAIII decreases the expression of surface adhesins and increases the production of capsule, toxins, and proteases 41 (Figure 6 and Table 4).

5.2. Agr system inhibitor

The goal of developing AgrC inhibitors is to treat the infections of specific microorganisms, such as *S. aureus*, by reducing the formation and the expression of virulence factors such as histolytic enzymes, immune escape factors and pore-forming toxins which are regulated by the *agr* QS system.

5.2.1. Apicidin

Apicidin is a fungal metabolite that inhibits histone deacetylase (HDAC) and thus exhibits antiparasitic activity [60]. Pearce et al. found that apicidin and its derivatives target the P3 promotor or the RNA-III sequence and mediate the inhibition of the agr-P3 reporter gene (Figure 7). It is remarkable that although apicidin acts on HDAC, its inhibitory effect toward *S. aureus* is independent of this mechanism because histones are absent in this strain. Pearce et al. found that apicidin could even inhibit QS in Methicillin-resistant *Staphylococcus aureus* (MRSA) and abate its pathogenesis without killing the bacterium directly. Furthermore, upon determining the quorum quenching activity of all four *agr* types using the P3-YFP reporter strains AH1677 (type I), AH430 (type II), AH1747 (type III), and AH1872 (type IV), it was found that type II and IV were the most sensitive and resistant to treatment with apicidin, exhibiting the IC_{50} values of 2.4 ± 0.2 μM and 69.9 ± 4.9 μM, respectively, indicating that the activity of apicidin depends on the type of *agr* [61].

5.2.2. Agr cyclopeptide derivatives

Polypeptides are the most promising category of antagonists that act on the Agr system. One strategy to utilize them is to modify the natural peptide signal molecule. Blackwell et al. proposed that a cyclopeptide acts as a pan-group or group-selective inhibitor of the AgrC receptor. They also discovered that AgrC-1 agonist strongly inhibit the biofilm growth better effect than the natural AIP-1 [62]. Blackwell et al. also developed a series of AIP analogues to inhibit AgrC of *S. epidermidis* and alleviate its virulence. For instance, the compound n3LF (Figure 7) suppressed AgrC-IV activity, exhibiting an IC_{50} of 352 nM [63,64]. Shimizu et al. synthesized another cyclic peptide containing the sequence CFWAH (Figure 7). The cyclopeptide

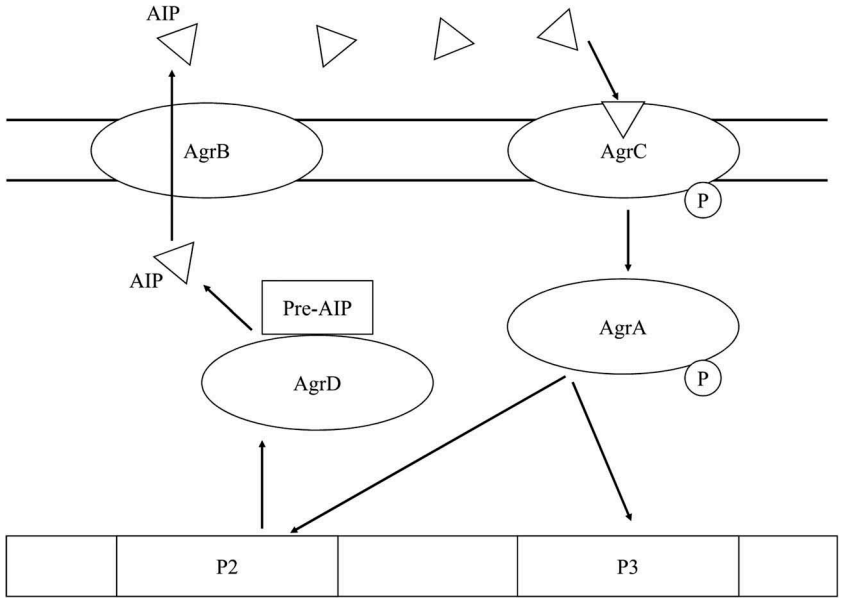


Figure 6. Agr system mechanism.

Table 4. Summary of Agr system inhibitors.

Inhibitor	Molecular target	Biological effect	References
Apicidin	AgrC-P3	Attenuate the virulence of *S. aureus*	Pearce et al. [61]
Antisense deoxyribozyme	RNAIII	Attenuate the virulence of *S. aureus*, especially MRSA	Hou et al. [67]
PP7-VLP containing modified AIP and VIP	AgrC	Induce antibody to inhibit AgrC and attenuate the virulence and positive feedback regulation of *S. aureus*	Hall et al. [68]
Cyclopeptide	AgrC	Attenuate the virulence of *S. aureus*, especially *S. epidermidis*.	Blackwell et al. (2017) [62]
A cyclic peptide containing the sequence Cys-Phe-Trp-Ala-His and/or oa broth f *C. butyricum*	Toxin gene	Attenuate the virulence of *Clostridium*	Shimizu et al. [65]
Peptidomimetics	AgrC	Attenuate the virulence of *S. aureus*	Blackwell et al. [63]
AIP mimetics	AgrC	Attenuate the virulence of *S. aureus*	Blackwell et al. [64]
Polyhydroxyanthraquinone	Four alleles of AgrABCD	Attenuate the virulence of S. aureus	Hall et al. [66]

from the broth of *Clostridium butyricum* had a desired effect in terms of controlling the QS and virulence of *Clostridium welchii*. This specific compound suppressed VirS, regulated the expression of virulence genes and inhibited the downstream signal of the VirR/VirS system. In addition, AgrD from *C. butyricum* inhibited the production of *Clostridium* toxins [65].

5.2.3. Anthraquinone

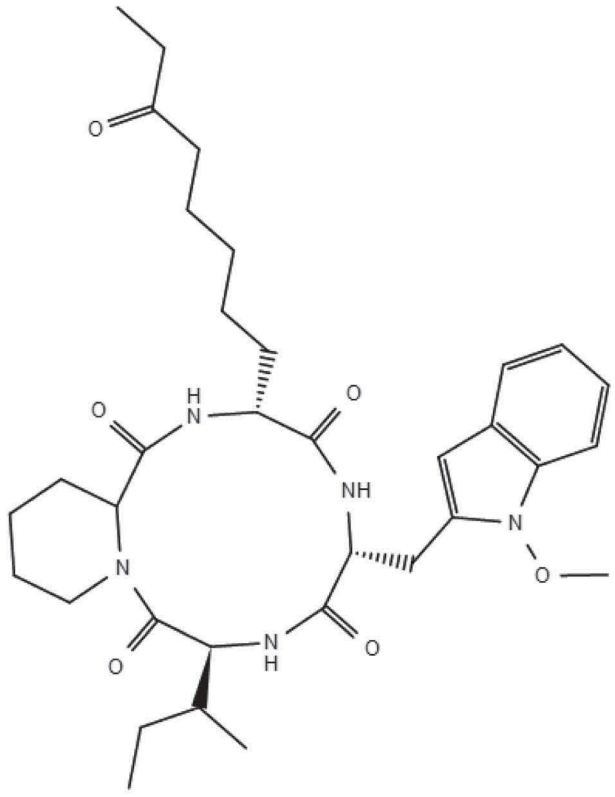
Hall et al. revealed that polyhydroxyanthraquinones, such as ω-hydroxy emodin (OHM) (Figure 7) may be the active antagonist of AgrA in certain microorganisms, such as MRSA. OHM inhibited the QS in *S. aureus* via all four *agr* alleles and reduced the expression of virulence factors and the signal molecule AIP. Moreover, the compound promoted immune cells to kill pathogens and limited the expression of inflammatory factors, making a positive contribution to the host [66].

5.2.4. Antisense DNA

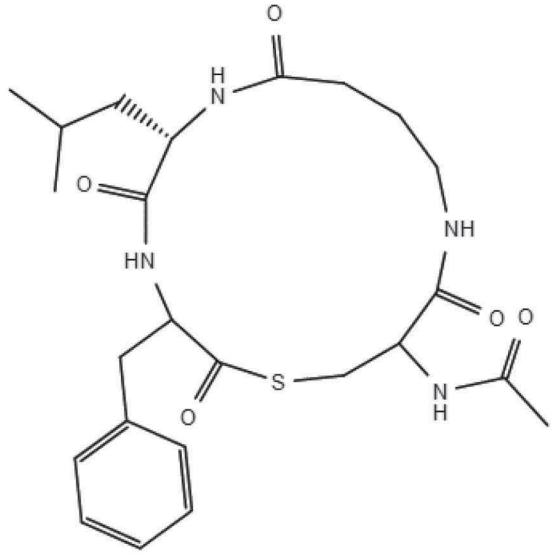
Since the transcription of RNAIII increases the expression of toxins and virulence factors, inhibiting the biological function of RNAIII could also attenuate the harmful effect of bacteria toward the host. Hou et al. proposed another strategy to treat MRSA with antisense DNA, and seven DNAzymes were screened out using RNA Structure 4.5^{TM}. Among these DNAzymes, DNAzyme23, possessing the sequence 5′-TTAAACAACTCAGGCTAGCTACAACGACAA-3′, demonstrated the greatest activity by inhibiting the expression of RNAIII and the secretion of α-toxin [67].

5.2.5. Agr vaccine

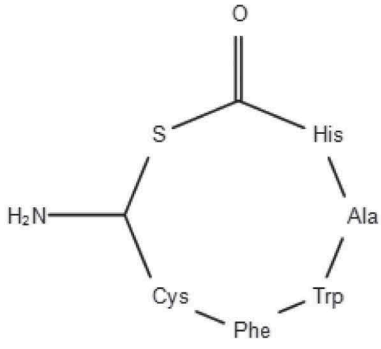
Since the molecular weight of AIP is insufficient to cause an immune reaction directly, Hall et al. transformed the virus-like particle (VLP) of the virus to present the antigen of AIP. Specifically, Hall et al. designed PP7-VLP by inserting the sequence of AIP into the single strain of PP7. When this engineered particle was administered to the host, the corresponding antibody was induced to repress the recognition site of AIP on AgrC, thus interfering with the Agr system. The experimental animal's infection was alleviated significantly after treatment with the engineered particle [68].



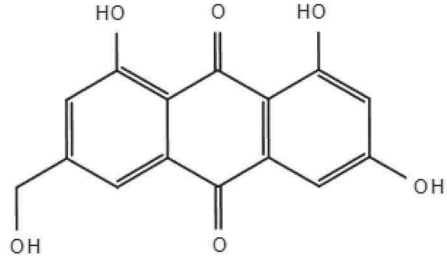
Apicidin



n3LF



Cyclic CFWAH



OHM

Figure 7. Agr system inhibitors.

5.3. Agonist

It has been reported that the *agr*-negative genotype of *S. epidermidis* will enhance the biofilm formation on polymer surfaces through the regulation of AtlE. Thus, the agonist of *agr* system may also exert the activity of inhibiting biofilm formation [69]. Blackwell et al. found that the agonist of AgrC-1 exhibited potent inhibition on *S. epidermidis* growth. The cyclopeptide skeleton of the agonist is identical to that of the antagonist, but it possesses different side chains. However, the development of the antagonist still dominates over agonist due to the potential dual function of the agonist [62].

6. DSF system

6.1. Introduction

The DSF system is present in *Xanthomonas campestris*, and is utilized by the bacterium via a two-component system [70]. It has been reported that the *rpf* gene is the necessary for the synthesis of DSF. *rpf*F encodes a bifunctional protein that dehydrates and thiolyses the substrate, and *rpf*C, and *rpf*G encode the two-component signal transduction system that involved in the sensing and transduction of the DSF signal [71]. In previous studies, DSF was believed to promote phenotypic changes via the second messenger c-di-GMP [72], but

a natural DSF turnover mechanism has recently been identified in *X. campestris* [73]. DSF signaling regulates virulence factors such as biofilm formation and extracellular polysaccharide [74].

6.2. Inhibition methods

Xanthomonas is a phytopathogen that infects at least 124 kinds of monocotyledons and 268 kinds of dicotyledons. It has been proposed that *Burkholderia anthina* HN-8 (GDMCC NO.60289) inhibits *Xanthomonas* , *Burkholderia cepacia* and *P. aeruginosa* and thus control plant diseases [75]. *Acinetobacter lactucae* QL-1 (CCTCC NO.M2017487) can use DSF as its only carbon source and degrade 2 mM of the signal molecule within 15 hours [76]. As aforementioned, the degradation of the signal molecule blocks QS signal transduction efficiently. Deng et al. separated an enzyme FadD1 from *P. aeruginosa* that degrades the DSF in *X. campestris* pv. Campestris and BDSF in *B. cepacia*. Thus, this enzyme may be used to control plant diseases caused by *X. campestris* or *B. cepacia* and reduce potential agricultural economic loss [77].

7. Other inhibitors

7.1. Chemical substances

7.1.1. Indole

Kushmaro et al. revealed that 2-(indolin-2-yl)-1*H*-indole (DIV), di(1*H*-indol-3-yl) methane (DIM) and 1,1′-biindole (NN) inhibit bacterial biofilm formation and disrupt existing *P. aeruginosa* biofilm (Figure 8) [78].

7.1.2. Amide

Wang et al. prepared and purified 2-methyl-N-(2′-phenethyl)-butyramide and 3-methyl-N-(2′-phenethyl)-butyramide from the fermentation broth of *Oceanobacillus* sp XC22919 and found that their inhibitory effects on QS is concentration-dependent. Specifically, the violacein production of *C. violaceum* CV026 was decreased, and the expression of virulence factors, such as pyocyanin, elastase, proteolytic enzyme and organism membranes, was reduced in *P. aeruginosa* PAO1. However, bacterial growth was not affected [79]. Parry et al. found that 4-(4-chlorophenyl)-5-methylene-pyrrol-2-one and 5-methylene-4-(p-tolyl) pyrrol-2-one can be used as a PqsR antagonist and inhibit alkylquinolone dependent QS in *P. aeruginosa* [80]. Copp et al. found that some salicylamides, namely, niclosamide and nitazoxanide, prevent, reduce or eliminate the formation of bacterial biofilm produced by some Gram-positive bacteria such as *Staphylococcus, Listeria*, and *Bacillus*. The antibacterial effect could be further enhanced if these drugs were administered with efflux pump inhibitor [81].

7.1.3. 2-Aminoimidazole

Yang et al. found 2-aminoimidazole derivatives inhibit the QS of *Acinetobacter baumannii* and *E. coli*. However, the compounds failed to inhibit the QS of *P. aeruginosa* ATCC27853 (Figure 9) [82].

7.1.4. Pyrimidinedione derivatives

Song provided a pyrimidinedione compound and demonstrated that this compound inhibited the biofilm of *Streptococcus*

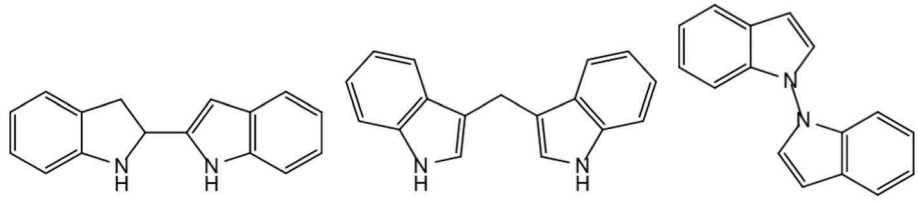


Figure 8. Structures of DIV, DIM and NN.

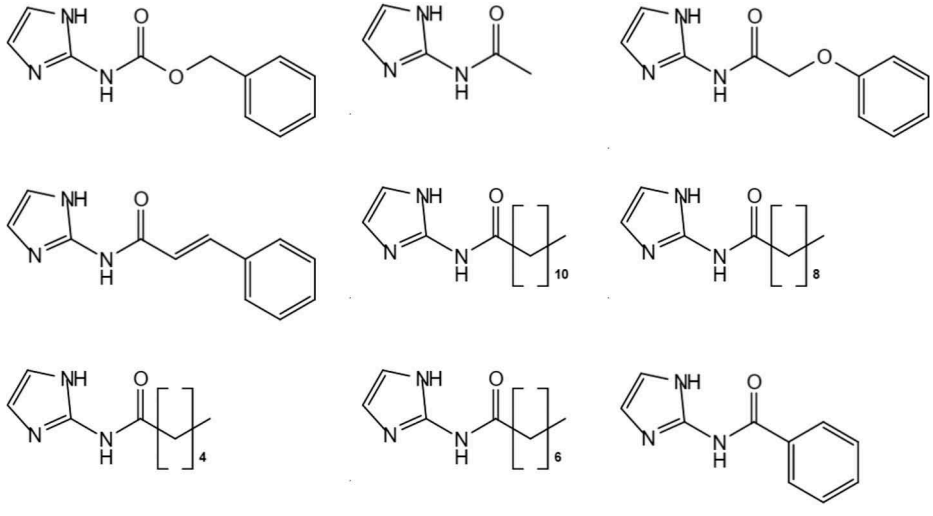


Figure 9. Examples of 2-aminoimidazole inhibitors.

pneumoniae, Chlamydia pneumoniae or *Legionella pneumoniae* with a concentration-dependent effect (Figure 10). It was be found that only 1 μM of the compound was sufficient to inhibit the biofilm formation of *S. pneumoniae* [83].

7.1.5. Phenazine derivatives

Huigens et al. proposed that several phenazine derivatives may act via a microbial warfare strategy, such as increasing the reactive oxygen species in, near or around microbes like *S. aureus* and *Staphylococcus epidermidis*, to induce a significant toxic reaction in them and remove biofilm with little toxicity and hemolysis in mammalian (Figure 10) [84].

7.1.6. 5-Aryl-2-aminoimidiazole

Hooyberghs et al. proposed that 5-aryl-2-aminoimidiazole is also an attractive skeleton for the development of QSIs (Figure 10). Several compounds exhibited an inhibitory effect on microorganisms, such as *Candida albicans* SC5314 or a mixture of *C. albicans* and *S. epidermidis*, making them potential agents for the disinfection of medical devices [85].

7.1.7. Iron complexes

Ala'aldeen et al. reported that several iron complex compounds acted as biofilm inhibitors and enhanced the health of poultry and livestock when they were added to the fodder. For instance, Fe-Tyr and Fe-DOPA can disrupt a mature biofilm of EPEC-ΔcsrA (EPEC: Enteropathogenic *E. coli*). It was found that 100 μM of Fe-DOPA was sufficient to disperse the biofilm. The addition of ferric citrate to rear bull calves at an early age also resulted in statistically relevant performance benefits [86,87].. Newman et al. found that pyocyanin demethylase and/or a derivative was capable of degrading phenazine which is of great significance for virulence and competitive adaptability. The substrate forms a complex with iron following enzyme catalysis to limit the concentration of iron and thus interfere with the QS of pathogens like *P. aeruginosa* [88]. Huigens et al. designed halogenated quinoline derivatives that act via an iron-dependent mode of action and inhibit and reduce the formation or growth of a biofilm. The compound HQ-1 was found to inhibit MRSA with a MIC of 0.78 μM (Figure 10) [89].

7.1.8. Efflux pump inhibitor

Dreier et al. invented a series of efflux pump inhibitors that combine with other antimicrobial agents to enhance their efficacy. They synthesized more than 500 compounds with the skeleton below and measured their efficacy on inhibiting QS of *P. aeruginosa* PAO1 and *E. coli* ATCC25922. It was found that almost half of these compounds demonstrate the pleasing inhibitory effect. For instance, compounds 18 and 25 (Figure 10), at a concentration of 25μM, can inhibit *P. aeruginosa* PAO1 and *E. coli* ATCC25922 growth to no more than 10% that of the control group [90]. Mincer et al. discovered another efflux pump inhibitor with the structure of 3,4-dibromopyrrole-2,5-dione, which is separated from *P. aeruginosa* and *Pseudoalteromonas piscicida*. This compound inhibits the efflux pump function of bacteria that possess resistance nodulation cell division pumps [91].

7.1.9. Molecule docking

With the development of computer-assisted drug design, the searching for new lead compounds and potential molecules becomes much more efficient. Givskov et al. used virtual screening and molecular docking with the reference of the signal molecules and existing inhibitors. As a result, 22 QSI primary candidates were selected out. To determine the efficacy of this 22 compounds, their inhibition activity against *P. aeruginosa lasB-gfp*(ASV) and *E. coli lasB-gfp*(ASV) were tested. It demonstrated that green fluorescent protein expression in the *P. aeruginosa* strain was inhibited by five compounds (C1, F1, G1, H1, F2) (Figure 11) without affecting cell growth. It is notable that C1 and G1 can inhibit QS with the nanomolar range of IC_{50}. However, only G1 showed satisfactory activity against LasR in *E. coli* strain. Although G1, as a LasR inhibitor, has higher binding specificity to LasR than RhlR, G1 could also inhibit RhlR in the absence of LasR, suggesting its complex mechanism. Moreover, F1 can inhibit both the *rhl* and *pqs* system in a LasR-independent manner with the inhibition rate of 61.7% and 63.1%, respectively [92].

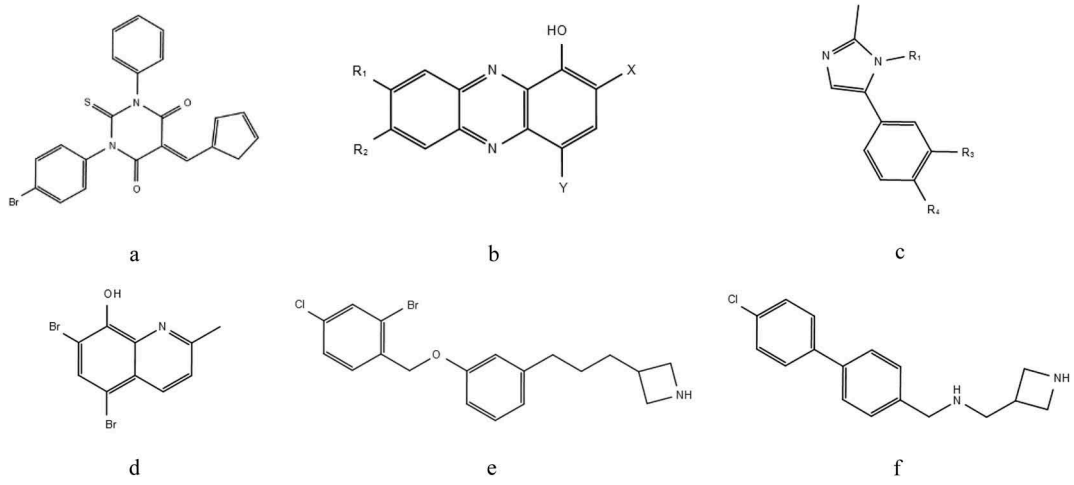


Figure 10. Structure of (a) Pyrimidinedione (b) Phenazine (c) 5-aryl-2-aminoimidiazole (d) HQ1, (e) compound 18, and (f) compound 25.

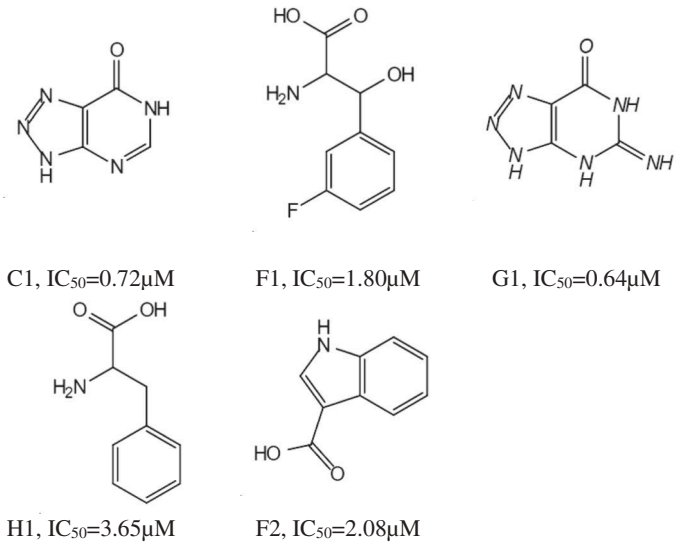


Figure 11. Structure and IC50 of compound C1, F1, G1, H1 and F2.

7.1.10. Cocktail

It is known that there are various of QS signal pathway in some specific species like *Pseudomonas* and *Burkholderia* and it is plausible that the combination of the inhibitor toward different pathways may be synergistic in controlling biofilm. As a result, Blackwell et al. used the cocktail of LasR, RhlR, and PqsR inhibitors to maximize the inhibitory effect (Figure 12) and each compound can inhibit their target to the extent of 85%. It was found that LasR antagonist and RhlR antagonist will inhibit the production of pyocyanin but not rhamnolipid and elastase B in *P. aeruginosa*. The PqsR antagonist also failed to inhibit rhamnolipid production. Interestingly, the RhlR agonist compound 3 can inhibit pyocyanin production through Rhl-mediated suppression of *pqs* [93], but rhamnolipid and elastase B production will also increase inevitably. By using the cocktail of LasR antagonist and RhlR antagonist, the inhibition activity against rhamnolipid improved significantly while this cocktail will lose activity under the circumstance of phosphate- and iron-depleted media. However, the combination of LasR and PqsR antagonist with RhlR agonist failed to improve activity. Blackwell et al. demonstrated that the cocktail of an RhlR antagonist and a PqsR antagonist was the best choice. In addition, the culture media could affect the inhibitory effect of these inhibitors because RhlR antagonist could not attenuate the production of pyocyanin in SCFM2 media [94].

7.2. Biological method

By using the modified protein degradation tag mf-ssrA derived from the *Mesoplasma florum* tmRNA system, the target protein in synthetic and endogenous bacterial systems can be degraded by mf-Lon or its analogues [95]. Alarcon et al. created a bifunctional ligand with a quorum-sensing-peptide-binding region and a protease-binding region to induce the degradation of the target protein in bacteria and inhibit biofilm formation [96]. Greenberg et al. discovered that antisense morpholino oligomers can interfere with the expression of genes relevant to antibiotic resistance or biofilm formation, resulting in the repression of QS system in bacteria [97]. Opatowsky et al. designed a peptide based on the N′ loop extension of the periplasmic subunit in the phosphate specific transfer system (PstS). It possess the sequence formula $Xaa_{(n)}$-Ala-Ile-Asp-Pro-Ala-Leu-Pro-Glu-$Xaa_{(n)}$ and targets and meddle with PstS to inhibit QS [98]. McLean et al. reported that lamellar bodies comprising phosphatidylcholine, sphingomyelin, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and cholesterol can modulate QS to inhibit or reduce biofilm production to assuage bacterial infection [99]. Kim et al. constructed a vector that can self-induce the expression of protein by sensing cell concentration with no additional inducer. When using the *Salmonella* host cell containing the construct of the present invention, a drug can be expertly produced and delivered within the body, thereby maximizing a therapeutic effect [100]. Patton et al. discovered an enzyme that converts the substrate to release hydrogen peroxide and a suitable substrate, such as raw hone, disrupts the formation of biofilm [101]. In summary, these biological methods are also attractive for their specificity and efficacy. However, the oral availability of these methods may become the major obstacle for further development.

7.3. Extracts of natural plant and nature compounds

Over millions of years of coevolution pressure, miscellaneous organisms have evolved natural defence strategies to ameliorate the potential infection of bacteria. Thus, the extract or

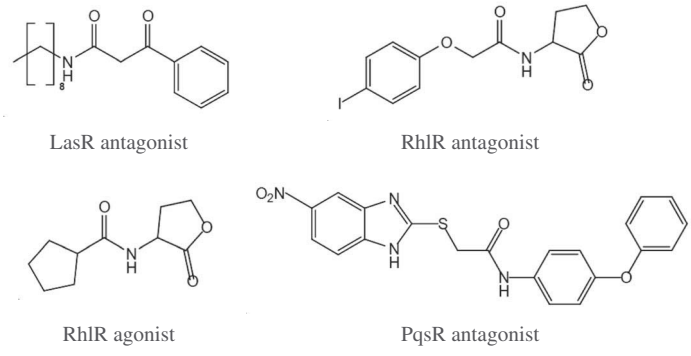


Figure 12. Blackwell et al. proposed cocktail to inhibit QS comprehensively.

natural product of some organisms may possess the potential to inhibit biofilm formation and QS.

Zhao et al. found that a pharmaceutical composition of marine and herbal organisms, namely, *Syngnathus* sp, *Ecklonia kurome, Sophorae flavescentis* Radix, *Lonicerae Japonicae* Flos, *Isatidis* Folium, *Phellodendri chinensis* Cortex and *Caryophylli* Flos, demonstratedd the potential of treating various bacterial infection with little resistance [102]. Li et al. discovered that an extract of *Moutan* Cortex disrupted the QS system of *Pseudomonas fluorescens*, which kept fishery products fresh [103]. Sugiyama et al. found that a mixture of polypeptides and lipids originating from *Lactobacillus plantarum* SN35N inhibited TSST-1 production of *S. aureus* [104]. Tufenkji et al. illustrated that phenolic-rich maple syrup extract, combined with antibiotics also possesses antimicrobial potential [105]. Other extracts from plants such as *Carex dimorpholepis* [106], *Cercis chinensis* [107], *Citrus junos* [108], *Myristica fragrans* [109], *Schinus terebinthifolia* [110], *Vaccinium macrocarpon* [111], *Styrax paralleoneurus*, and *Styrax tonkinensis* [112] can inhibit biofilm formation and interfere with QS to control the growth of microorganisms. Jia et al. found that stilbenoids, namely, resveratrol, piceatannol and oxyresveratrol inhibit *C. violaceum* [113] and *P. aeruginosa* QS [114]. The bis-benzyl-tetrahydro-isoquinoline compounds from *Plumula nelumbinis* were also shown to inhibit the QS of different strains of *P. aeruginosa* [115]. In addition, hamamelitannin, a natural compound found in *Hamamelis virginiana* HAM analogue has been reported to be a potential QSI in 2007, but it had several drawbacks. As a result, Coenye et al. developed some 250 analogues of hamamelitannin that can also disrupt bacterial QS [116].

Falcarindiol is a polyacetylene compound found in *Umbelliferae* and *Araliaceae* plants that exhibited a potent inhibitory effect on Gram-positive bacteria, such as *S. aureus*, and an attenuated effect on Gram-negative strains. Yu et al. disclosed that falcarindiol could inhibit QS under sub-inhibitory concentrations without killing the microorganisms directly. They revealed that 12.5 μM of falcarindiol was sufficient to inhibit the production of the PQS signal in *P. aeruginosa* which regulates the expression of downstream virulence genes, and 20 μM of falcarindiol inhibited 60% of biofilm formation in *P. aeruginosa*. It was also reported that the *V. harveyi* emission behavior was also suppressed with 26 μg/mL falcarindiol [117].

7.4. Composition

Song et al. reported that the composition of *Baillus pumilus* CCTCC NO. M 2,013,240 and some immunopotentiator, namely, vitamin C, *Astragali* Radix, *Eucommiae* Cortex, *Codonopsis* Radix, *Angelicae sinensis* Radix, *Lonicerae japonicae* Flos, *Bupleuri* Radix, *Prunus mume* Fructus, *Punica granatum* Cortex and *Schisandrae chinensis* Fructus, inhibited QS and made fish more resistant to bacterial infection [118]. Adding the strain or culture of *V. harveyi* DIR21 MCC0081 in the field of fishery is also reported by Kumar to quench QS [119]. Sambasivam et al. invented a composition to disrupt the existing biofilm and hinder microbial reproduction to minimize the wound infection chance. The composition thereof is QSI, iron chelating agent, antimicrobial agent. For instance, nitropyridine N-oxide (0.1%), EDTA (0.5%) and silver nitrate (0.5%) DI water solution can be used to clean and disinfect the wound. In some cases, extracellular polymer disrupting agent can also be added optionally [120].

7.5. Noncanonical methods

Charles et al. revealed that the gold complex auranofin, which was commonly prescribed as the drug to treat rheumatoid arthriti, showed potential in inhibiting biofilm formation and development and disrupting the existing biofilm. Its mechanism is forming a complex with selenium and thus block and disrupt selenium metabolism [121]. Naik et al. administered sufficient sorbent material, such as phyllosilicate, to the environment to modulate the flora of bacteria by adsorbing or catalyzing the QS signal molecules. It is proposed that this method could be used to prevent the spoilage of food and vibriosis in fish [122]. Locock et al. demonstrated that guanylated polymethacrylate or a random copolymer of 2-guanidinoethyl methacrylate and methyl methacrylate can inhibit or kill the biofilm of *S. aureus* and *C. albicans* [123]. Schüwer et al. developed a series of isosorbide compounds and measured their inhibitory activities on *P. aeruginosa*. It revealed that the composition of 20 mM isosorbide digallate ester and 20 mM tetracycline made the biofilm of *P. aeruginosa* undetectable. However, the gallic acid may undermine the inhibitory effect when prescribed with the drugs above [124]. Wright et al. reported that chronic sinusitis could be treated with the composition that comprises phenol, phenolic, or polyphenolic compounds as a contact or topical application to sinus membranes or tissue. These phenols were reported to be effective on inhibiting and disrupting *P. aeruginosa* biofilm [125]. Choi et al. used D-galactose to treat oral bacterial diseases by inhibiting *F. nucleatum, P. gingivalis* and *T. forsythia* biofilm without affecting bacterial growth [126]. O'neil et al. proposed that cystamine can be used to fight infection caused by fungi or bacteria, such as *P. aeruginosa*, by preventing biofilm formation or destroying existing biofilm. Moreover, antibiotics or arginine-rich peptides can be applied optionally to enhance the effect of cystamine [127].

8. Expert opinion

The discovery of penicillin marks the milestone for the fighting with bacterial infection. The rapid development of miscellaneous categories of antibiotics promoted the sanity and health of mankind significantly. However, the increasingly stains of drug-resistant bacterial under huge selectivity forces researchers to develop new strategies to control the bacterial infection and one way to achieve this goal is the inhibitor against QS system.

In this review, the patents related to QS inhibition from 2014 to 2018 were summarized. Not only may chemical substances be used to antagonize the signal molecule and proteins involved in signal transduction, but biological enzymes may also be used to quench the signal. It was found that the majority of these inhibition methods inhibited the formation of biofilms and reduced the virulence of bacteria without killing them directly, reducing selective pressure. However, since

little experiment was carried out on human beings, the clinical application of these inhibition strategies may be limited.

Moreover, since there is a countless number of substances in an organism, it is uncertain whether these inhibitors will interfere with other off-target pathways and cause side effects. Although AI-2-mediated QS is shared by both Gram-positive and Gram-negative bacteria, some bacteria, such as *X. campestris*, use other QS systems. In addition, there are at least three different pathways present in *P. aeruginosa*. The complexity of these signals should be given more attention to develop more specific inhibitors.

The exact mechanisms of certain steps of the QS systems are also ambiguous, and further studies should be performed to pave the road for the development of inhibitors. It is also notable that some molecules in this review were screened out with the assistance of computer software. More molecules may be found in the future with the structural analysis of the protein involved in QS. Besides, the mechanism of some inhibitors in this review is uncertain, and more profound research of them may reveal some unprecedented drug design strategies.

In sum, QSIs may become a potential alternative to antibiotics and exhibited a reduced selective pressure on bacteria. More detailed studies on the biological processes of QS and drug-receptor interactions should be pursued in future drug discovery.

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Declaration of interest

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