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The art of antibacterial warfare: Deception through interference with quorum sensing–mediated communication

Giordano Rampioni [a], Livia Leoni [a], Paul Williams [b,*]

[a] Department of Sciences, University Roma Tre, Viale Marconi 446, 00146 Rome, Italy
[b] Centre for Biomolecular Sciences and School of Life Sciences, University of Nottingham, University Park, Nottingham NG7 2RD, UK

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ABSTRACT

Almost a century on from the discovery of penicillin, the war against bacterial infection still rages compounded by the emergence of strains resistant to virtually every clinically approved antibiotic and the dearth of new antibacterial agents entering the clinic. Consequently there is renewed interest in drugs which attenuate virulence rather than bacterial growth. Since the metaphors of warfare are often used to describe the battle between pathogen and host, we will describe in such a context, the molecular communication (quorum sensing) mechanisms used by bacteria to co-ordinate virulence at the population level. Recent progress in exploiting this information through the design of anti-virulence deception strategies that disrupt quorum sensing through signal molecule inactivation, inhibition of signal molecule biosynthesis or the blockade of signal transduction and their advantages and disadvantages are considered.

1. Introduction

Infection is essentially a war between host and pathogen that, in the context of bacterial infections, was once thought to have been won through the discovery and development of diverse clinically effective broad spectrum antibiotics. However, the current antibiotic armamentarium has lost its effectiveness as a consequence of antibiotic resistance, the emergence of multi-antibiotic resistant bacteria and the difficulties of treating chronic, biofilm-centred infections. Conventional antibiotics either kill bacterial cells or prevent bacterial growth by targeting essential biochemical processes including cell wall, protein and nucleic biosynthesis. This in turn exerts enormous selective pressures leading to the evolution of antibiotic resistance. Further erosion of the antibiotic armamentarium has occurred because the development of new antibiotic classes has lagged far behind the requirement for such new drugs. Indeed no new antimicrobials acting against novel targets have entered late stage clinical trials in recent years [1]. Consequently, there is an urgent need to consider alternative strategies likely to lead to the development of clinically useful antibacterial agents particularly in this age of 'personalized medicine'.

Since the metaphors of warfare have been used extensively to describe the pathogenesis of bacterial infections, it is instructive to reflect on "The Art of War", a seminal work on military strategy and tactics written in the sixth century B.C. by the Chinese General Sun Tzu [2]. In fact he could well have been writing about the fate of bacterial pathogens facing new growth inhibitory antibiotics when stating, *"Confront them with annihilation, and they will then survive; plunge them into a deadly situation, and they will then live"*.

The urgent need for new therapeutic approaches to treat or prevent infections caused by antibiotic resistant bacteria has stimulated research toward the discovery and development of "anti-virulence" or "anti-pathogenic" drugs. Although bacterial growth inhibition *in vitro* may require bactericidal/bacteriostatic agents, this is not necessarily the case *in vivo*. Adaptation to growth in host tissues presents the infecting bacterium with a very different set of environmental challenges. Consequently bacteria have evolved multiple virulence determinants and the ability to form biofilms that cause host damage and disease. These in turn are controlled via sophisticated regulatory mechanisms. Consequently antibacterial agents which block colonization, interfere with metabolism or attenuate virulence factors or virulence gene expression without affecting bacterial growth *in vitro* offer potential advantages. These include expanding the repertoire of drug targets, preserving the host endogenous microbiome and exerting reduced selective pressures so delaying the emergence of resistance [3,4]. In other words, anti-virulence drugs should not *"confront... pathogens... with annihilation"*, but disarm them and overthrow their defences, so that the host can clear the infection.

The development of anti-virulence compounds requires a detailed understanding of the molecular mechanisms involved in

* Corresponding author. Fax: +44 (0)115 8467951.
E-mail address: paul.williams@nottingham.ac.uk (P. Williams).

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host colonization and disease progression if they are to be exploited as potential therapeutic targets. As Sun Tzu wrote, *"What is of supreme importance in war is to attack the enemy's strategy"* [2]. The 'attack and destroy' strategy of most pathogens involves the production of adhesins to facilitate attachment to host tissues, followed by invasion of, or biofilm formation on, host cells/tissues either of which helps to protect the growing bacterial population from the host. These colonization activities are often followed by the deployment of exotoxins and tissue-degrading enzymes for combating host immune defences and the release of nutrients to 'feed' and expand the infecting bacterial 'army'.

Examples of anti-virulence compounds include inhibitors of bacterial attachment such as the 'pilicides', a family of bicyclic 2-pyridones (Fig. 1) which selectively disrupt a protein–protein interaction essential for the biogenesis of P-pili [4]. These mediate the attachment of *Escherichia coli* to bladder epithelial cells, an important stage in the development of urinary tract infections. A number of bacterial pathogens including the causal agents of typhoid fever and plague all utilize a virulence strategy involving the direct injection of proteins into human cells via a type III secretion system. High throughput screens have yielded compounds such as 2,2′-thiobis-(4-methylphenol) (Fig. 1) which is capable of inhibiting type III secretion in both *Yersinia* species and *Pseudomonas aeruginosa* [5]. In *Vibrio cholerae*, the causative agent of cholera, virstatin (4-(*N*-(1,8-naphthalimide))-n-butyric acid) (Fig. 1), blocks dimerization of the transcriptional regulator protein ToxT and so abrogates expression of the two main virulence determinants, cholera toxin and the toxin co-regulated pilus [4]. For most pathogens virulence is both multifactorial and combinatorial. In these cases one promising strategy is disruption of the "operations centre" i.e. global control systems such as quorum sensing that regulate the expression of multiple virulence determinants.

2. Quorum sensing as a therapeutic target

Quorum sensing (QS) is a cell-to-cell communication pathway that enables bacterial populations to co-ordinately re-programme gene expression in response to cell density. Briefly, in all QS systems, a signal molecule is produced and secreted (or freely diffuses) into the surrounding environment. As the bacterial population grows, the concentration of signal molecule(s) increases, until it reaches a threshold concentration at which it binds to and activates a cognate receptor protein. The perception of the QS signal molecule *via* the QS receptor triggers a physiological response in all members of the population, ultimately re-programming gene expression throughout the population. Therefore, through QS bacterial populations can modify their nature and dynamics, and act as a community to accomplish tasks that would be impossible to achieve for individual bacterial cells [6]. To defeat the enemy as Sun Tzu recommended, "*If united, separate them*" [2].

QS regulates a wide variety of physiological processes including bioluminescence, competence, antibiotic biosynthesis, motility, plasmid conjugal transfer, biofilm maturation, and the expression of key virulence factors in plant, animal and human pathogens belonging to diverse bacterial genera [6]. Indeed, many pathogens display markedly reduced virulence in infection models when their QS systems are disrupted by mutagenesis. QS also impacts on antibiotic susceptibility, either by increasing antibiotic tolerance in biofilms [7], by directly regulating antibiotic resistance genes such as *mecA* which confers methicillin resistance on *Staphylococcus aureus* [8], or by controlling the acquisition of antibiotic resistance genes by natural transformation as observed in *Streptococcus pneumoniae* [9]. Thus inhibiting QS may not only reduce virulence but also restore susceptibility to conventional antimicrobials. After all, "*In battle, there are not more than two methods of attack—the direct and the indirect; yet these two in combination give rise to an endless series of manoeuvers*" [2]. As Sun Tzu wrote, "*In the practical art of war, the best thing of all is to take the enemy's country whole and intact: to shatter and destroy it is not so good*" [2]. Consequently QS is considered a promising target for new anti-virulence agents.

Any anti-virulence strategy that is directed towards disruption of QS is commonly referred to as quorum quenching (QQ). Blocking communications within the opponent army has long been a major military tactic aimed at disrupting all possible co-operative activities. Sun Tzu warned, *"If words of command are not clear and distinct, if orders are not thoroughly understood, then the general is to blame"* [2]. A successful QQ strategy requires an in depth knowledge of the specific molecular actors and architecture of the QS system to be targeted. Indeed, "*The soldier works out his victory in relation to the foe whom he is facing*", and "*The opportunity of defeating the enemy is provided by the enemy himself*" [2].

Irrespective of their chemical and structural diversity, all QS systems reflect the classical scheme for bacterial cell-to-cell communication, in which the structure of the signal molecule contains information that is directed by a "sender" cell/organism to a "receiver" cell/organism. This common architecture provides multiple molecular targets for the action of enzymes or compounds interfering with QS-mediated cell-to-cell communication, namely (i) the biosynthesis of the signal molecule by the "sender" cell, (ii) the functionality and availability of the signal itself, and (iii) the reception/decoding of the message contained in signal molecule by the "receiver" cell (Fig. 2). As Sun Tzu stated, "*All warfare is based on deception*" [2]. Since targeting any of the three steps noted above would render bacterial cells incapable of perceiving their population size, and hence accomplishing QS-controlled tasks, it is evident that as an anti-virulence strategy, QQ is based on deception.

In the following sections, the molecular mechanisms underlying some of the best understood QS systems will be briefly described in the context of QS inhibition. Although, the development of potent, clinically effective QS inhibitors (QSIs) could have a significant impact on human health, there are also widespread opportunities

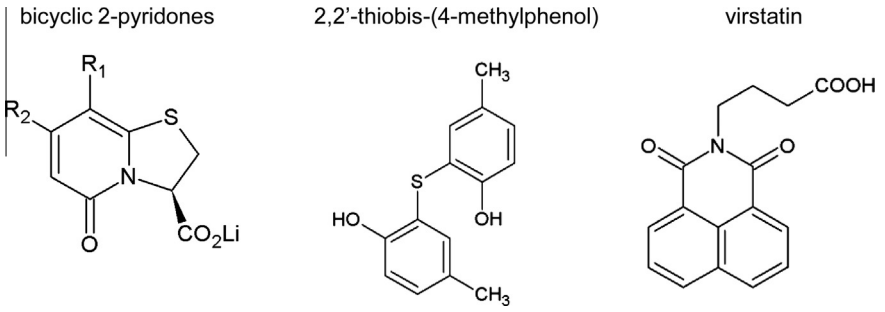

Fig. 1. Structures of exemplar anti-virulence agents which target attachment (pilicides), type III secretion (2,2′-thiobis-(4-methylphenol) and virulence gene regulation (virstatin) respectively.

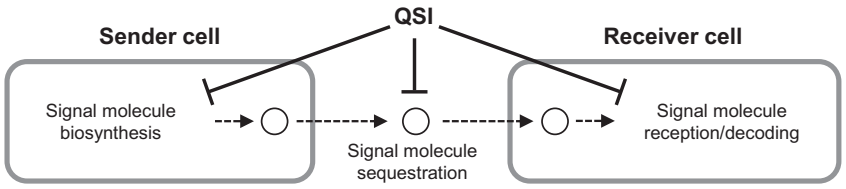

Fig. 2. Schematic showing potential targets within a generalized QS system. A QS inhibitor (QSI) may target the (A) biosynthesis of the signal molecule by the "sender" cell, (B) functionality and availability (i.e. by sequestration) of the signal molecule, or (C) reception/decoding of the signal molecule by the receiver cell.

for their exploitation in agriculture and aquaculture and against biofouling bacteria [10].

3. Targeting AHL-based QS systems

The most intensively investigated QS systems in Gram-negative bacteria employ *N*-acylhomoserine lactones (AHLs; Fig. 3A) as signal molecules [6]. Accordingly, most QQ research has been directed against these QS systems. AHL biosynthesis is typically catalysed by LuxI-family synthases which transfer an acyl group from an acylated acyl carrier protein (acyl-ACP) to the methionyl amine of *S*-adenosyl-L-methionine (SAM) after which cyclization of the methionyl moiety to homoserine lactone occurs [6,11]. The length of the acyl side chain (usually from 4 to 18 carbons), saturation and oxidation state at position 3, determine the resulting AHL structure, and thus signal-specificity. Short-chain AHLs generally freely diffuse across membranes, while there is some evidence for active efflux of AHLs with longer acyl side-chains. AHLs generally function by binding to a cognate intracellular receptor protein belonging to the LuxR-family. In most cases, the LuxR receptor-AHL complex binds to target promoters, activating gene expression (Fig. 3B) [6]. Some AHLs, notably *N*-(3-oxododecanoyl)-L-homoserine lactone ($3OC_{12}$-HSL) may also contribute to pathogenesis independent of QS by acting as immune modulators [6]. Consequently, inhibition of AHL biosynthesis may offer added benefits in the context of host-pathogen interactions.

3.1. Inactivation and sequestration of AHL signals

AHL-dependent QS can readily be disrupted by inactivation of the cognate AHL through lactonolysis at alkaline pHs or by enzymatic disruption. AHL-inactivating enzymes, either lactonases or acylases (amidases) are widely distributed in microbes and higher organisms [12]. The expression of *aiiA*, a lactonase gene from *Bacillus* in transgenic tobacco and potato plants conferred enhanced resistance to infection with the soft-rot pathogen *Erwinia* (*Pantoea*) *carotovora* [13]. Lactonases are generally active against a broad spectrum of AHL signal molecules, probably because they target the conserved homoserine lactone ring. Conversely, the AHL-inactivating acylases which cleave the AHL amide bond exhibit a marked degree of substrate specificity [14]. *P. aeruginosa* for example expresses three enzymes PvdQ, QuiP and HacB with acylase activity towards AHLs [14]. Although the physiological role of these enzymes in the context of QS modulation is not clear, overexpression or exogenous addition of recombinant PvdQ reduced expression of *P. aeruginosa* virulence-related genes and pathogenicity in a nematode infection model through inactivation of the *las* signal molecule, $3OC_{12}$-HSL. While PvdQ shows little activity against AHLs with side chains less than 10 carbons in length, Koch et al. [15] applied a structure-based design approach to modify the substrate specificity of PvdQ to confer increased activity towards shorter chain AHLs such as *N*-octanoylhomoserine lactone (C_8-HSL). This PvdQ variant strongly decreased the amount of C_8-HSL present in *Burkholderia cenocepacia* cultures, and was effective in protecting *Galleria mellonella* larvae from *B. cenocepacia* infection [15]. Interestingly, PvdQ has been processed into stable spray-freeze-dried powder suitable for inhalation to possibly treat pulmonary infections caused by *P. aeruginosa* [16].

An alternative strategy for disrupting AHL-dependent QS through chemical modification of the signal such that it no longer activates its cognate receptor has been termed "signalling confusion" [14]. For example, *Burkholderia* sp. GG4 possesses an oxidoreductase that reduces a 3-oxo-substituted AHL controlling virulence in *E. carotovora* to the corresponding, unrecognised 3-hydroxy derivative. Consequently co-cultivation of *Burkholderia* with *E. carotovora* resulted in reduced virulence in a potato infection model [17]. Furthermore, both antibody- [18] and molecularly imprinted polymer-mediated sequestration [19] of $3OC_{12}$-HSL have been employed to reduce QS-dependent virulence factor production and biofilm development in *P. aeruginosa.*

3.2. Disruption of AHL reception/decoding

To conserve the steric requirements for optimal ligand/receptor interactions, antagonists have commonly been discovered through structural modification of native agonists. Hence, AHLs, have proved useful starting templates for the design of QS inhibitors (QSIs). Indeed early investigations highlighted the potential of long chain AHLs to inhibit LuxR proteins activated by short chain AHLs in *Chromobacterium violaceum* [20]. Subsequently many studies of AHL analogues as potential QSIs have been published. Here the underlying rationale is that structural analogues of the cognate signal molecule maintain receptor binding affinity without generating productive signal-receptor complexes. Hence this class of inhibitors competitively blocks binding by the native (productive) signal. In general, AHL analogues exhibit a continuum of activities with many compounds capable of activating or inhibiting QS circuits depending on their concentration and target LuxR-type receptor. For recent reviews on the chemical structural features relevant for the rational design of AHL analogues with agonist or antagonist activities, see [21–23].

Despite the AHL specificity shown by most LuxR-like receptors, AHL analogues that competitively inhibit different LuxR proteins have been identified. Examples include compound S7 (Fig. 3C) which antagonizes the activation of LuxR (*Vibrio fischeri*), TraR (*Agrobacterium tumefaciens*) and LasR (*P. aeruginosa*) by AHLs with acyl chains of 6, 8 or 12 carbons in length respectively [24]. Compound 4606–4237 (Fig. 3C), inhibits both the intracellular LuxR protein, CviR of *C. violaceum* and the transmembrane AHL receptor, LuxN of *Vibrio harveyi* [25] while meta-bromo-thiolactone (mBTL) (Fig. 3C) antagonizes both LasR and RhlR in *P. aeruginosa*. The latter also protected *Caenorhabditis elegans* and human A549 lung epithelial cells from QS-mediated killing by *P. aeruginosa* [26]. While mBTL is a weak agonist in the absence of $3OC_{12}$-HSL and *N*-butanoylhomoserine lactone (C_4-HSL), the native LasR and RhlR signals, respectively, it has antagonist activities in the presence of the natural signal molecules, highlighting that it is not always easy to distinguish between agonist and antagonist activities [26].

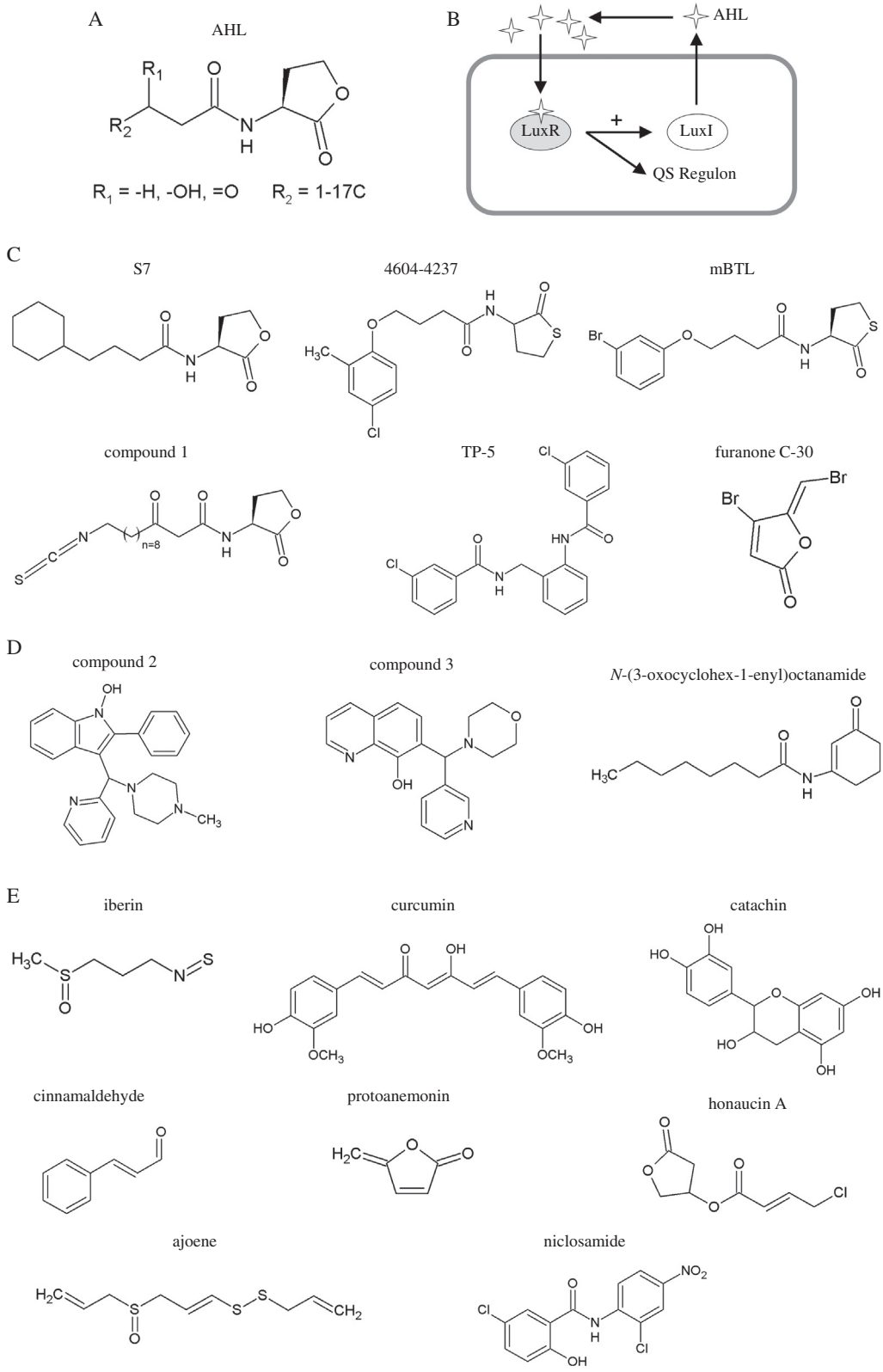

Fig. 3. Inhibition of AHL-based QS systems. (A) AHL structures. (B) Simplified schematic of a generalized QS system based on a LuxI/LuxR pair as signal synthase and receptor respectively; " + " indicates a positive feedback loop. (C) Inhibitors of AHL reception/decoding. (D) Inhibitors of AHL biosynthesis. (E) Inhibitors with unknown targets.

The mechanism of action of AHL analogues has been confirmed at the biochemical level in only a few cases. For example, three synthetic AHL antagonists with CviR prevent receptor association with target DNA, while the natural antagonist *N*-decanoyl-homoserine lactone (C_{10}-HSL) prevents a productive interaction with RNA polymerase without significantly affecting CviR DNA binding [25]. In addition, since the native ligands trigger a folding switch that enhances TraR and LuxR receptors stability and solubility,

some AHL analogues may act by compromising receptor folding [23].

Although the AHL backbone has been a useful template for antagonist discovery, there are potential drawbacks. Discovering compounds with high affinities for multiple LuxR receptors is challenging given that only slight deviations from the parent AHL structure are tolerated [10]. An alternative strategy would be to identify reactive sites within LuxR-type protein binding pockets amenable to covalent modification [23]. This has been achieved for LasR which contains an accessible cysteine residue that can be inactivated by modified AHLs containing isothiocyanate groups (compound 1, Fig. 3C) [27]. Screens of synthetic and natural compound libraries have yielded a number of interesting QSI hits. These include the triphenyl compound TP-5 (Fig. 3C), identified following *in silico* modeling of TP-1, a compound originally identified as a LasR agonist [28]. Among the earliest natural products discovered to inhibit AHL-dependent QS were furanones from the macroalga *Deisea pulchra* [7]. These structurally resemble the AHLs and although they appear to target LuxR-type proteins, their mechanism of action has not been clearly established. Although too toxic for clinical development, the synthetic brominated furanone C-30 (Fig. 3C) showed significant efficacy against *P. aeruginosa* in a murine chronic lung infection model [7].

3.3. Inhibition of AHL biosynthesis

Despite the intense efforts directed towards the discovery of AHL-receptor antagonists, much less attention has been paid to inhibitors of LuxI-type AHL synthases even though they are highly conserved and unique to bacteria. This is perhaps because of the difficulty of establishing high throughput screening assays although the recent development of a cell-free assay by Christensen et al. [11] should aid the discovery of AHL synthase inhibitors. Indeed the authors screened a library of over 12,000 compounds and identified four compounds with IC_{50s} of ~10 μM, two of which were capable of reducing AHL biosynthesis by *E. coli* strains expressing recombinant LuxI-type proteins (compounds 2 and 3, Fig. 3D) [11]. Furthermore, from a QSI screen in the rice pathogen *Burkholderia glumae*, the AHL analogue J8–C8 (*N*-(3-oxocyclohex-1-enyl)octanamide; Fig. 3D) was identified as an inhibitor of the LuxI orthologue, TofI with an IC_{50} of ~35 μM [29]. From functional and crystallographic studies J8–C8 was shown to bind directly within the TofI binding site for the acylated acyl carrier protein substrate [29].

3.4. Inhibitors of AHL-based QS systems with unknown target

A variety of natural products derived from plants and microbes have QSI activity towards AHL-dependent QS systems although the nature of the active components(s) and their mechanism(s) of action are often unknown [22,23]. Examples of natural products with QSI activity include iberin, curcumin, catachin, cinnamaldehyde, protoanemonin isolated from a *Pseudomonas* spp. and the honaucins from coral-associated cyanobacteria (Fig. 3E) [30,31]. Crude garlic extracts containing an inhibitor(s) of AHL-dependent QS were subjected to a clinical trial in *P. aeruginosa*-infected cystic fibrosis patients and although the results were not statistically significant (the patient numbers were too small), a trend towards improvement after oral garlic extract administration was noted [32]. The primary QSI in garlic has been identified as ajoene (4,5,9-trithiadodeca-1,6,11-triene 9-oxide; Fig. 3E) which promoted *P. aeruginosa* clearance in a mouse lung infection model and enhanced the killing of biofilms in the presence of tobramycin [33].

4. Auto-inducing peptide-based QS systems as targets in Gram positive bacteria

In common with Gram-negative bacteria, many Gram-positive bacteria including pathogens such as *S. aureus*, *Enterococcus faecalis* and *S. pneumoniae* employ QS to regulate virulence [23,34]. These QS systems generally rely on genetically encoded peptides often termed 'autoinducing peptides' (AIPs) as signal molecules. AIPs are expressed as inactive pro-peptides *via* canonical ribosomal synthesis, and later processed and modified to generate the active QS signal [23,34]. AIPs are not freely diffusible across membranes. AIP perception by the receiver cell is usually mediated by sensor kinases, which transduce the signal from the membrane to cognate response regulators inside the cell *via* a phosphorylation cascade.

The *S. aureus agr* QS system is a useful paradigm for AIP-dependent QS [34,35]. AIPs typically consist of seven to nine amino acids in which a central cysteine is covalently linked to the *C*-terminal amino acid carboxylate (Fig. 4A). AIP sequences are highly variable and the AIP pro-peptide encoded by the *agrD* gene is processed by the transmembrane endopeptidase AgrB. After export the mature AIP is sensed by the sensor kinase AgrC. Autophosphorylation of AgrC is followed by phospho-transfer to the response regulator, AgrA which drives the expression of the *agrACBD* operon setting up an auto-amplification loop for AIP biosynthesis. AgrA also activates target promoters to up-regulate secreted virulence factors and down regulate surface proteins involved in host cell adhesion and biofilm formation (Fig. 4B) [34,35].

4.1. Inhibition of AIP reception/decoding

S. aureus strains can be divided into four different groups depending on the AIP produced. Each AIP specifically activates its cognate AgrC receptor but inhibits non-cognate AgrC receptors by competitive antagonism [34]. In a proof of concept study, Wright et al. [36] showed that administration AIP-2 to mice infected with an *agr* group-I *S. aureus* delayed *agr* activation sufficient to block the abscess formation thus usefully pointing the way for the design of peptidomimetic inhibitors [35]. Several studies directed toward the development of cross-group inhibitors of all four *S. aureus agr* groups showed that the macrocycle is critical for AIP function, while replacement of the thiolactone moiety with a lactone or lactam group virtually eliminates cognate activation, but not cross-group inhibition. Derivatives of native AIPs with wide spectrum of antagonist activities were identified by *N*-terminal truncation and by alanine scanning [35]. For example, the *N*-acetylated trAIP-I D2A (Fig. 4C) was evolved using AIP-I as scaffold, by combining *N*-terminal truncation and Asp-5-Ala substitution. This non-native AIP inhibits all four *agr* groups at nanomolar concentrations [37]. Similar approaches have proved successful for antagonists of the *fsr* system of *E. faecalis* that is related to *agr* but employs an 11-residue cyclic peptide termed gelatinase biosynthesis-activating pheromone (GBAP) that autoinduces two pathogenicity-related extracellular proteases [38]. Recently Desouky et al. [39] developed a high through screen and identified several compounds present in actinomycetes culture supernatants capable of inhibiting both *fsr* and *agr* systems without affecting bacterial growth although their structures remain to be elucidated. Interestingly, the marine Gram negative bacterium *Photobacterium halotolerans* produces the cyclodepsipeptide, solonamide B (Fig. 4C) which inhibits *agr* expression in *S. aureus* through AgrC inhibition [40]. Furthermore, a number of non-peptidic agents have been identified as inhibitors of AgrC. These include the *P. aeruginosa* AHL signal $3OC_{12}$-HSL and a series of related 3-acyltetramic and 3-acyltetronic acids which act as negative allosteric inhibitors of AgrC with no noticeable effect on the affinity of AIP-1 for the cognate AgrC receptor [41].

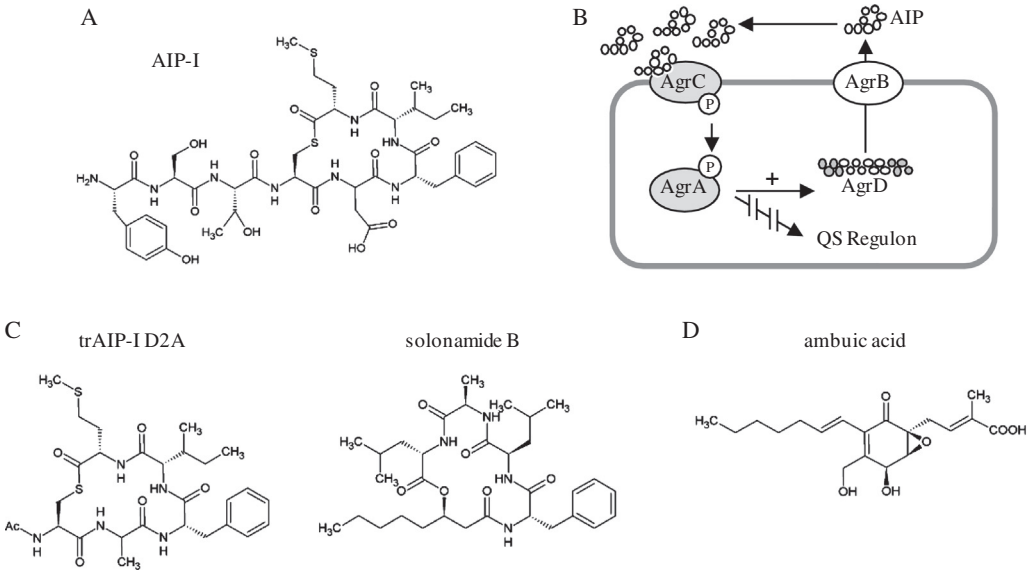

Fig. 4. Inhibition of AIP-dependent QS systems. (A) AIP-I from *S. aureus*. (B) Simplified schematic of the *agr* system of *S. aureus*; arrows labelled with " + " indicate a positive feedback loop, discontinuous arrows indicate multiple intermediate steps. (C) Inhibitors of AIP reception/decoding. (D) Inhibitor of AIP biosynthesis.

The respiratory pathogen *S. pneumoniae* employs a linear 17-mer peptide termed CSP (competence stimulating peptide) to regulate competence (DNA uptake) and virulence [9]. A CSP derivative carrying a Glu-1-Ala substitution suppressed *S. pneumoniae* virulence both *in vitro* and in a mouse lung infection model. Moreover the inhibitor blocked the ability of *S. pneumoniae* to transform into an antibiotic-resistant strain within the mouse when DNA encoding antibiotic resistance was supplied simultaneously [9]. These results illustrate the potential of CSP analogues not only to inhibit virulence but also horizontal transfer of antibiotic resistance.

4.2. Inhibition of biosynthesis and sequestration of AIPs

Although staphylococcal AIPs are relatively resistant to proteases [35] they can be sequestered *in vivo* by host proteins such as apolipoprotein B resulting in the down-regulation of *agr* and hence virulence [42]. Indeed mice deficient in plasma apolipoprotein B are more susceptible to invasive infections with *S. aureus agr* positive strains as compared with *agr* deletion mutants. Consequently AIP sequestration offers an alternative strategy for controlling *S. aureus* virulence. This has been achieved by Park et al. [43] who generated a high affinity antibody with high-affinity for AIP-IV which effectively reduced virulence factor production, prevented skin abscess in a mouse skin infection model. Furthermore, passive immunization protected mice from lethal intraperitoneal challenge with a *S. aureus* group IV strain [43].

Few AIP biosynthesis inhibitors have yet been identified although the fungal cyclohexenone metabolite ambuic acid (Fig. 4D) identified as an inhibitor of FsrB-driven GBAP synthesis in *E. faecalis*, also inhibits AIP biosynthesis in *S. aureus* and *Listeria* [44].

5. Inhibition of 2-alkyl-4-quinolone signalling in *P. aeruginosa*

In addition to the AHLs, *P. aeruginosa* employs 2-alkyl-4-quinolones (AQs) including 2-heptyl-4(*1H*)-quinolone (HHQ; Fig. 5A) and 2-heptyl-3-hydroxy-4(*1H*)-quinoline (PQS; Fig. 5A) as well as the recently identified 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde as QS signal molecules [45,46]. These QS systems are interdependent, involved in virulence gene regulation and hence are potential targets. *P. aeruginosa* strains with mutations in *pqs* biosynthetic or signal transduction genes such as *pqsA* and *pqsR* respectively are highly attenuated in experimental animal infection models (Fig. 5B) [45]. AQ biosynthesis requires the PqsABCD enzymes in a two-step pathway in which PqsD mediates the synthesis of 2-aminobenzoylacetate (2-ABA) from anthraniloyl-coenzyme A (CoA) and malonyl-CoA, followed by the decarboxylating coupling of 2-ABA to an octanoate group linked to PqsC so generating HHQ, the immediate precursor of PQS [47]. The structure of PqsD has been solved [48] and alongside the new insights obtained by Dulcey et al. [47] into the AQ biosynthesis mechanism has offered opportunities for the rational design of AQ biosynthesis inhibitors, such as the 2-benzamidobenzoic acid derivatives (Fig. 5C) [49].

Inactivation of AQ-signalling has been achieved by enzymatic inactivation of PQS using the recombinant dioxygenase, Hod from *Arthrobacter nitroguajacolicus* Rü61a. Hod converts PQS to *N*-octanoyl anthranilic acid and carbon monoxide leading to the downregulation of PQS-controlled genes and a reduction in the virulence of *P. aeruginosa* in a plant leaf infection model [50].

Several studies have focused on the discovery of inhibitors of the PQS and HHQ receptor, PqsR. Hartmann and colleagues described the synthesis of PqsR-antagonists based on the natural agonist HHQ, and the *k*-opiod receptor agonist (±)-*trans*-U50488, that induce activation of AQ-dependent QS. Hit compounds were identified with high affinity for the PqsR ligand-binding domain that strongly inhibit PqsR activity in a heterologous *E. coli*-based reporter system. However, these compounds (hydroxamic acid-derivatives and 2-amino-oxadiazoles) displayed reduced PqsR-antagonistic activity when evaluated in *P. aeruginosa* [51,52].

Recently the structure of the PqsR co-inducer binding domain has been solved both in the apo-form and as a complex with a native agonist, 2-nonyl-4(*1H*)-quinolone (NHQ) [53]. This structural information was used to guide the synthesis of a series of natural ligand-derivatives and quinazolinone (QZN) analogues as possible PqsR antagonists. 3-NH_2-7-Cl-C9-QZN (Fig. 5D) was identified as a potent inhibitor of the *pqs* QS system in *P. aeruginosa*, strongly reducing AQ and pyocyanin production, lectin expression, and biofilm development. Notably, also the crystal structure of PqsR-bound to 3-NH_2-7-Cl-C9-QZN was determined, providing valuable data for further development of QSIs targeting PqsR [53].

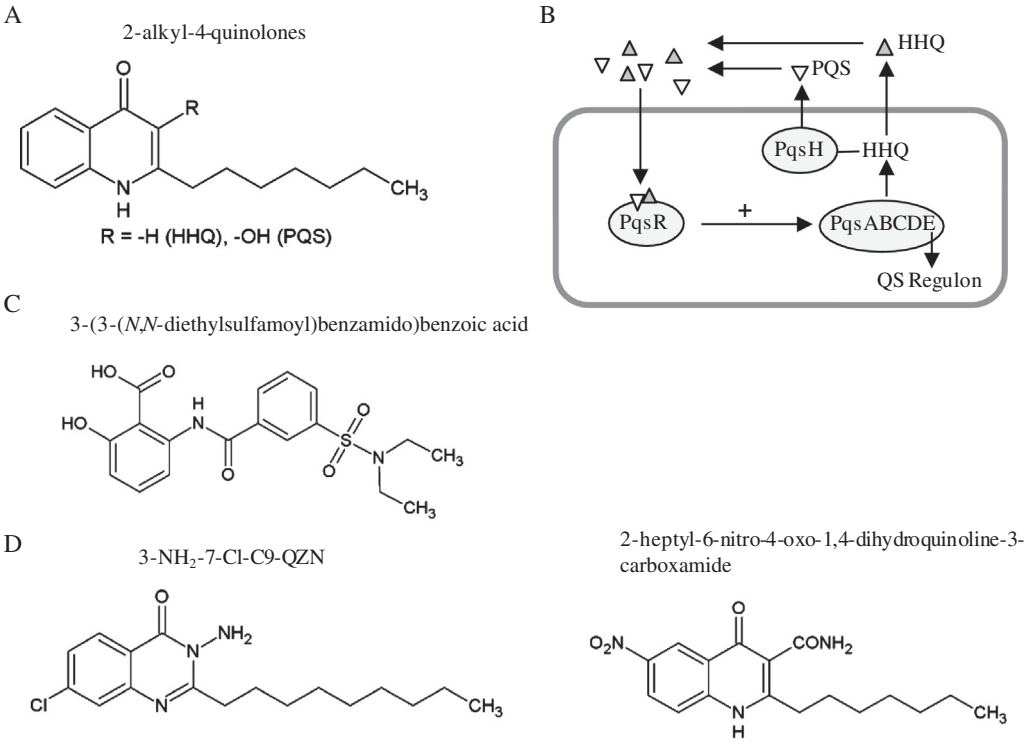

Fig. 5. Inhibition of AQ-dependent QS. (A) AQ structures. (B) Simplified schematic of *P. aeruginosa* AQ-dependent QS. Either HHQ or PQS can activate PqsR; "+" indicate a positive feedback loop. PqsE is not required for AQ biosynthesis but is the effector protein for AQ signalling. (C) Inhibitor of AQ biosynthesis. (D) Inhibitors of AQ reception/decoding.

6. Concluding remarks

Although AHL- and AIP-dependent QS systems are reasonably widespread in Gram negative and Gram positive bacteria respectively, QS systems controlling virulence that are conserved across all pathogens have not been identified. Hence the prospect of broad spectrum QSIs is unlikely. While QS systems based on autoinducer-2 (AI-2; Fig. 6) a mixture of furanones derived from 4,5-dihydroxy-2,3-pentandione (DPD; Fig. 6) are probably the most common [22], there are few examples where AI-2 signalling plays a clear central role in virulence gene regulation.

The discovery of narrow spectrum QSIs has however been simplified by the availability of biosensor strains for the rapid screening of chemical libraries. These are often heterologous organisms which incorporate a reporter gene (*e.g.*, *lacZ*, *lux* or *gfp*) that is only expressed when the appropriate QS signal is supplied exogenously [20,54,55]. Hence reporter output will be reduced when the assay includes a putative inhibitor. While this approach has proved useful for identifying inhibitors of signal receptor activity, it presents some drawbacks that should be considered. As stated by Sun Tzu, "*The general who wins the battle makes many calculations in his temple before the battle is fought*" [2]. This approach does not for example enable identification of compounds targeting key elements within the QS system other than signal reception although this can easily be remedied for QS signal biosynthesis by inclusion in a heterologous genetic background of signal synthase, receptor and target promoter coupled to a reporter gene. Alternatively, co-cultivation of the biosensor with the natural strain producing the QS signal may permit identification of compounds targeting any cellular process critical for QS response [56]. This is also relevant when considering that, in most bacteria, QS does not respond only to cell density, but integrates this information with other environmental and metabolic stimuli, ultimately controlling the QS response *via* complex pathways of transcriptional and posttranscriptional regulators [57,58]. In this context, drugs increasing the expression or activity of QS negative regulators could be endowed with QSI activity.

Moreover, hits emerging from screens employing heterologous organisms as biosensors (most frequently engineered *E. coli* or *C. violaceum*) may lack activity or even function as agonists when tested on the target pathogen [22]. Novel agonists may however still prove useful in the search for antagonists as exemplified by the HHQ analogue, 2-heptyl-6-nitroquinolin-4(*1H*)-one which acted as an antagonist in an *E. coli*-based AQ-reporter strain but as an agonist in *P. aeruginosa* as a consequence of a metabolic modification. However, a subsequent synthetic modification of this molecule resulted in a strong PqsR antagonist in *P. aeruginosa* (2-heptyl-6-nitro-4-oxo-1,4-dihydroquinoline-3-carboxamide, Fig. 5D) [59,60]. More often however, the differences in activity in heterologous versus the target bacterium relate to a lack of internalization, active efflux, modification or toxicity of the hit compound. In a recent commentary on the use of reporters for QSI screens, Coenye and colleagues highlighted the need for adequate control experiments to assess the general effects of hit compounds on both growth and the reporter gene product [61]. Indeed, the results obtained can be biased by a QS-independent effect. For

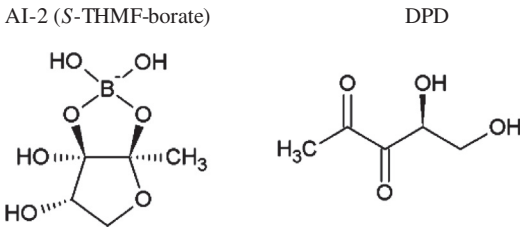

Fig. 6. Structures of AI-2 from *V. harveyi*, and its precursor, DPD.

example, pyrogallol was reported to act as a potent inhibitor of AI-2 dependent QS in *V. harveyi*. However subsequent experiments revealed that the apparent QSI activity of pyrogallol is a side effect of the peroxide-generating activity of this compound on the reporter system rather than on QS itself [61].

Despite major advances in our understanding of QS systems, their potential as targets for anti-virulence agents and the discovery of potent QSIs, it is likely to be many years before clinically safe and effective QSIs are available either as prophylactic or therapeutic agents. This is because of the many regulatory hurdles which must be overcome and also because the clinical usage of such agents will require specific diagnostics. One potential short cut is the adoption of drug-repurposing for QSI compounds with low toxicity already approved for use in humans. These can be evaluated directly in clinical trails or used as a starting point for a drug optimization program. This strategy recently resulted in identification of the established anthelmintic drug niclosamide (Fig. 3E) as a strong inhibitor of both *las* and *rhl* AHL-dependent QS systems in *P. aeruginosa*. Niclosamide inhibited virulence factor production and biofilm development and attenuated *P. aeruginosa* in the waxworm model of acute infection [62].

When considering QS inhibition as an anti-infective strategy, the appearance of strains carrying mutations in key QS genes in clinical samples should not be overlooked. For example, *P. aeruginosa lasR* and *S. aureus agr* defective mutants are frequently isolated especially in chronic infections [63,64]. This phenomenon raises concerns about the importance of QS systems in infection establishment and progression. However, a number of studies have provided convincing evidence that QS mutants behave as "social cheaters" that exploit the extracellular factors produced by the QS-proficient population, without the cost of contributing to the production of these "public goods". Notably, as the proportion of cheaters increases in a population, their relative fitness decreases, as there are fewer QS-proficient bacteria to exploit. As a consequence, the percentage of QS mutant strains in the population cannot exceed a certain limit [65–67]. These findings suggest that a QSI should decrease the fitness of the whole bacterial population in infected sites, despite the presence of QS-defective bacteria. This hypothesis is supported by *in vivo* experiments showing that co-infection with *P. aeruginosa lasR* mutants reduces mortality caused by *P. aeruginosa* wild type in a mouse burn model of infection [68].

The issue of social cheating is closely associated with the possible emergence of resistance to QSIs. Wood and collaborators reported the selection of *P. aeruginosa* mutants resistant to the QSI furanone C-30 in a growth medium containing adenosine as sole carbon source. Importantly, adenosine is intracellularly degraded by a QS-controlled enzyme; therefore, in this case, the QSI directly inhibited bacterial growth, similarly to an antibiotic [69]. A subsequent study reported that the enrichment of QSI-resistant strains in a bacterial population is an unlikely event when QS controls "public goods" (*e.g.*, extracellular enzymes used by the whole population) rather than "private goods" (*e.g.*, intracellular enzymes used by individual cells). This is because the potential benefit conferred from a small proportion QSI-resistant cells to the bacterial population is diluted with the higher proportion of QSI-sensitive cells [70]. These results may be clinically relevant for those pathogens, including *P. aeruginosa*, in which virulence mostly relies on secreted extracellular virulence factors. However, these studies highlight the importance of validating the activity of QSIs against fresh clinical isolates rather than laboratory reference strains alone.

The spectre of bacterial pathogens totally resistant to all classes of conventional antibiotics and the inability to treat simple infections is only now beginning to challenge the dogma that effective antibacterials must be broad spectrum and growth inhibitory. As Sun Tzu wrote, "*Even the finest sword plunged into salt water will eventually rust*" [2]. Consequently, anti-virulence drugs including QSIs require careful consideration and thorough evaluation.

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