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Correspondence and requests for materials should be addressed to K.-G.C. (kokgan@um.edu.my)

Non-antibiotic quorum sensing inhibitors acting against *N*-acyl homoserine lactone synthase as druggable target

Chien-Yi Chang[1,2], Thiba Krishnan[3], Hao Wang[4], Ye Chen[5], Wai-Fong Yin[3], Yee-Meng Chong[3], Li Ying Tan[3], Teik Min Chong[3] & Kok-Gan Chan[3]

[1]Interdisciplinary Computing and Complex BioSystems (ICOS) research group, School of Computing Science, Claremont Tower, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK, [2]The Centre for Bacterial Cell Biology, Medical School, Newcastle University, Richardson Road, Newcastle upon Tyne, NE2 4AX, UK, [3]Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia, [4]School of Pharmacy, Ningxia Medical University, Yinchuan, P. R. China, [5]School of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham, Nottingham NG7 2RD, UK.

_N_-acylhomoserine lactone (AHL)-based quorum sensing (QS) is important for the regulation of proteobacterial virulence determinants. Thus, the inhibition of AHL synthases offers non-antibiotics-based therapeutic potentials against QS-mediated bacterial infections. In this work, functional AHL synthases of _Pseudomonas aeruginosa_ LasI and RhlI were heterologously expressed in an AHL-negative _Escherichia coli_ followed by assessments on their AHLs production using AHL biosensors and high resolution liquid chromatography–mass spectrometry (LCMS). These AHL-producing _E. coli_ served as tools for screening AHL synthase inhibitors. Based on a campaign of screening synthetic molecules and natural products using our approach, three strongest inhibitors namely are salicylic acid, tannic acid and trans-cinnamaldehyde have been identified. LCMS analysis further confirmed tannic acid and trans-cinnemaldehyde efficiently inhibited AHL production by RhlI. We further demonstrated the application of trans-cinnemaldehyde inhibiting Rhl QS system regulated pyocyanin production in _P. aeruginosa_ up to 42.06%. Molecular docking analysis suggested that trans-cinnemaldehyde binds to the LasI and EsaI with known structures mainly interacting with their substrate binding sites. Our data suggested a new class of QS-inhibiting agents from natural products targeting AHL synthase and provided a potential approach for facilitating the discovery of anti-QS signal synthesis as basis of novel anti-infective approach.

A number of bacterial phenotypes such as virulence, secondary metabolite production and biofilm maturation are controlled by cell-to-cell communication, a process commonly known as quorum sensing (QS). *N*-acylhomoserine lactones (AHLs) are employed as QS signal molecules in many Gram-negative bacteria. AHL-mediated QS systems requires two components *viz.* a signal generator (LuxI homologue) which catalyses the formation of AHLs and a response regulator (LuxR homologue) which can bind with the AHLs forming AHL-receptor complex to regulate the transcription of target QS-mediated genes[1,2]. AHL synthase catalyses the formation of an amide bond between the homoserine lactone ring from *S*-adenosylmethionine (SAM) and the acyl chain from the acyl acyl-carrier-protein (acyl-ACP). After catalysed, the product of AHL and by-products of holo-ACP and 5′-methylthioadenosine (MTA) are generated and released[3].

In *Pseudomonas aeruginosa*, two AHL-mediated LasR/I and RhlI/R and one AQ-mediated (2-alkyl-4-(1*H*)-quinolone) QS systems which work hierarchically have been extensively studied[4–5]. LasI is responsible for producing the *N*-3-oxododecanoyl-L-homoserine lactone (3-oxo-C12 HSL), which in turns activates the regulator LasR to modulate the expressions of the downstream genes including the Rhl system and the genes encoding a virulence factor, elastase. RhlI is essential to synthesize *N*-butanoyl-L-homoserine lactone (C4-HSL). The C4-HSL/RhlR complex is crucial to regulate a series of downstream genes, including *rhlI* and *phz* operon for pyocyanin synthesis. These two Lux families of QS regulatory systems are interlinked with the AQ-mediated *Pseudomonas* QS (PQS) system whereby 2-heptyl-3-hydroxy-4-quinolone (PQS) is used as a QS signal. The Las, Rhl and PQS systems are correlated in a hierarchical circuitry and exert a global impact on a range of genes in *P. aeruginosa*, covering virulence production, antibiotic resistance and programmed cell death[6–8]. Several animal

studies have suggested the important roles of QS in *P. aeruginosa* pathogenesis by showing that the QS mutants cause less tissue damage and mortality rate when compared to wild-type[5].

Bacterial QS has been considered as the therapeutic target to attenuate bacterial virulence and thus control infection by degrading QS signals or interrupting the perception of signal molecules on LuxR homologous protein[1,8]. Three types of enzymes including AHL-lactonase, AHL-acylase and AHL-oxidoreductase have been documented to cause AHL molecules degradation in distinctive pathways from one another has been documented[9–11]. In addition, there are also several small molecules that are capable of inhibiting QS. These molecules are either structurally mimics to the cognate QS signals or enzyme inhibitors interfering with the corresponding signal binding to the receptor or decreasing the receptor concentration, therefore disrupting the QS mechanism[12]. For instance, halogenated furanones produced by the red marine alga *Delisea pulchra* has been shown to bind and increase the turnover rate of LuxR homologue and hence disrupting the AHL-mediated swarming and surface colonization of pathogenic bacterium *Serratia liquefaciens*[13,14]. For the inhibition of PQS pathway, it has been reported that anthranilate, one precursor for PQS, was modified into methyl anthranilate that would act as inhibitor for PQS production resulting in reduced pathogenicity of *P. aeruginosa* including reduction in the expression of elastase[15]. Although QS is an ideal target to attenuate bacterial virulence, several studied including mathematic models and experimental evidence suggest bacteria rapidly evolve and spread the resistance against QS inhibitors (see review[16]). For example, *P. aeruginosa* PA14 is capable of resisting to a well-characterised QS inhibitor, brominated furanone C-30 by enhancing efflux of this compound[17]. Therefore, it is necessary to identify new targets for inhibiting bacterial QS.

Here we constructed two well-controlled AHL producing *E. coli* from LasI and RhlI expression plasmids, respectively. A total of 114 plant extracts and 10 pure synthetic compounds were screened for their specific inhibition on AHL synthesis. One particular compounds, trans-cinnamaldehyde, have been demonstrated their potential for inhibiting AHLs production and QS-regulated pyocyanin in *P. aeruginosa* PAO1. Molecular docking analysis with known structure LasI and EsaI suggested the inhibiting mechanism of trans-cinnamaldehyde might be the occupation of crucial substrate binding pocket for AHL production.

Results

Constructing AHL-producing *E. coli*. AHLs play important roles for bacterial communication and virulence in human pathogens. Inhibiting AHLs production is considered a useful strategy to attenuate pathogens virulent determinants. To screen for AHL synthase inhibitors, two L-arabinose-dependent expression plasmids namely pBAD-*lasI* and pBAD-*rhlI*, harbouring *P. aeruginosa* PAO1 *lasI* and *rhlI* genes were constructed and transformed into AHL-negative *E. coli* MG1655, respectively (Supplementary Fig. S1 online). The expression of *lasI* and *rhlI* genes was driven by L-arabinose-inducible promoter in the pBAD expression plasmid. The constructed plasmids in *E. coli* MG1655, MG1655 [pBAD24], MG1655 [pBAD-*lasI*] and MG1655 [pBAD-*rhlI*] were tested for their ability to produce AHLs by induction separately using (in w/v) 0%, 0.1% and 1% L-arabinose for 4 hours during the exponential phase (OD_{600} = 0.6–0.8). AHLs from the respective induced cultures were extracted and spotted on thin layer chromatography (TLC) plate. Positive controls involving the extract from exponential phase of *P. aeruginosa* PAO1, 50 pmol of synthetic C4-HSL and 3-oxo-C12-HSL were included while solvent acted as negative control were all spotted on the same TLC plate. Subsequently, the resulting TLC plate was overlaid with biosensors *E. coli* JM109 [pSB1075] and *E. coli* JM109 [pSB536] for detection of long and short chain AHLs, respectively (Supplementary Fig. S2A and Fig. S2B online). No bioluminescence was observed from the extracts without L-arabinose induction hence indicating the production of long chain AHL by *E. coli* MG1655 [pBAD-*lasI*] and short chain AHL by *E. coli* MG1655 [pBAD-*rhlI*] are tightly controlled by L-arabinose. In addition to this, high resolution LCMS analysis confirmed 3-oxo-C12-HSLand C4-HSL productions by *E. coli* MG1655 [pBAD-*lasI*] and *E. coli* MG1655 [pBAD-*rhlI*], respectively, which are well controlled by L-arabinose (Supplementary Fig. S2C online). These two AHL-producing *E. coli* were then employed for anti-QS compounds screening.

Rapid Screening for AHL Synthases inhibitors. Plants were shown to contain abundant sources of anti-bacterial or anti-QS compounds[18]. Our original aim was to screen for anti-QS natural products specific targeting bacterial AHL signal synthesis using constructed AHL-producing *E. coli*. However, after screening 114 natural products extracts from tropical plants and herbs but to no avail. Thus, we turned to screen synthetic, pure compounds from commercial sources and 10 pure compounds of plant origin were selected based on literatures with reference to their reported anti-QS activity or anti-bacterial biofilm formation. Among these 10 compounds, furanones is a well-studied analogue to AHLs, which affects the QS system in *C. violaceum*[19]. Additional studies also showed that *P. aeruginosa* biofilms pre-treated with furanones are more susceptible to antibiotic tobramycin[20]. Two 2[5H]-furanones of *Lactobacillus helveticus* were proposed to be the QS signal in this microorganism[21]. This has led to the selection of 5-ethyl-3-hydroxy-4-methyl-2[5H]-furanone, a commercially available derivative of furanones, an alternative to 2[5H]-furanones in the preliminary screening. Andrographolide and curcumin were also reported to significantly repress the QS-regulated virulence, including pyocyanin and elastase in *P. aeruginosa*[22,23]. Ellagic acid and fisetin were found to drastically attenuate the biofilm formation of *Streptococcus dysgalactiae*[24]. Salicylic acid has been shown to repress AHL production in *P. aeruginosa* as well as reduction of bacterial invasion and cytotoxicity to HCE cells[25]. Tannic acid is a type of tannin, which was originally discovered from tropical tree *Terminalia catappa* which is traditionally used as antimicrobial activity and is recently discovered for the novel anti-inflammatory effect[26]. Lastly, trans-cinnamaldehyde is an ingredient in cinnamon oil. Previous studies have suggested cinnamaldehyde and its analogs are inhibitor of AI-2 (autoinducer 2)-based QS and virulence in *Vibrio* spp.[27].

To initiate our screening, firstly the pure compounds with (3 mg/ml, final concentration) were incubated with 1% w/v L-arabinose induced *E. coli* MG1655 [pBAD-*rhlI*] for 4 hours and AHLs were extracted from the spent supernatants. AHLs extracts were then detected by *C. violaceum* CV026 which produces purple violacein in the presence of short-chain AHLs (Fig. 1A). The extracts from *E. coli* MG1655 [pBAD-*rhlI*] treated with salicylic acid, tannic acid and trans-cinnamaldehydehave been suggested no short-chain AHLs production due to no violacein production from CV026 biosensor. Based on this preliminary screening among those compounds, salicylic acid, tannic acid and trans-cinnamaldehyde were selected for further analysis as potential AHL synthase inhibitors.

LCMS analysis for detecting AHLs reduction. To verify the results involving biosensors screening, AHL extracts obtained from L-arabinose-induced *E. coli* MG1655 [pBAD-*rhlI*] culture treated with three potential AHL synthase inhibitors (with concentrations of 0.3 mg/ml and 3 mg/ml) were analysed by LCMS (Fig. 2 and Supplementary Table S1 online). C4-HSL extracted from spent supernatants of negative controls was readily detected (Fig. 2). C4-HSL was also detected from the extract treated with 2.17 mM (equivalent to 0.3 mg/ml) salicylic acid (*m/z* 172.3; 0.556 min). However, no C4-HSL was detected from the extract treated with 0.06 mM (equivalent to 0.3 mg/ml) tannic acid and 2.27 mM (equivalent to 0.3 mg/ml) trans-cinnamaldehyde in our experimental

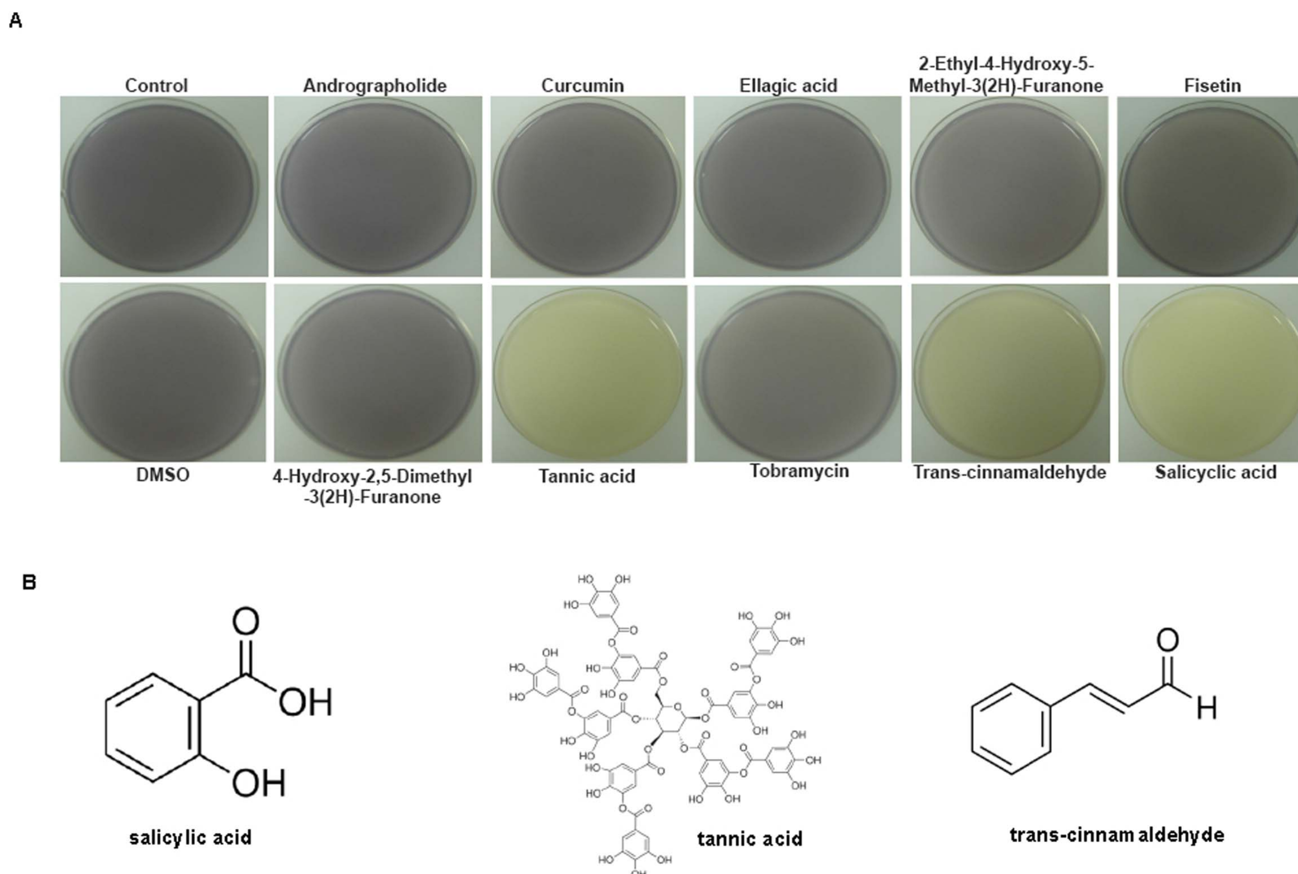

Figure 1 | AHL production of 1% (w/v) L-arabinose induced *E. coli* MG1655 [pBAD-*rhlI*] treated with 10 pure compounds with the concentration 3 mg/ml detected by *C. violaceum* CV026 (A). Salicylic acid, tannic acid and trans-cinnamaldehyde are potential AHL synthase inhibitors (B).

condition. In the screening using *C. violaceum* CV026, no detectable AHL was observed in extract treated with salicylic acid but C4-HSL was still detected by the sensitive LCMS which suggested that the salicylic acid may be able to reduce but not to completely inhibit AHL production. Similar result was also reported with AHL production being suppressed by 30 mM salicylic acid using TLC overlaying with *C. violaceum* CV026 biosensor[25]. In addition to this, previous studies have shown that salicylic acid attenuates the virulence production and biofilm formation in *P. aeruginosa* and inhibits its growth and swarming[25,28]. Our data confirmed salicylic acid with high concentration is able to repress AHL production, which resulted in the attenuation of bacterial virulence.

To further investigate whether AHL synthase inhibition of the three potential inhibitors is universal, AHLs extracted from L-arabinose induced *E. coli* MG1655 [pBAD-*lasI*] cultures were also detected by LCMS (Fig. 3 and Supplementary Table S2 online). Synthetic 3-oxo-C12-HSL was detected (*m/z* 298.3; 5.785 min) which served as standard. Detection of 3-oxo-C12-HSL from negative controls was also indicated (Fig. 3). Not surprisingly, 2.17 mM salicylic acid cannot inhibit AHL production from LasI where 3-oxo-C12-HSL was readily detected (*m/z* 298.3; 5.720 min). However, 3-oxo-C12-HSL was also detected in the extracts treated with 0.06 mM tannic acid at 5.883 min (*m/z* 298.3) and with 2.27 mM trans-cinnamaldehyde at 5.883 min (*m/z*298.3). These data suggested that tannic acid and trans-cinnamaldehyde are QS inhibitors that specifically target short-chain AHL synthase (RhlI) but not long-chain AHL synthase (LasI).

Phenotypic effects of AHL synthase inhibitors. In order to confirm tannic acid and trans-cinnamaldehyde are not targeting the transcription of promoter BAD and translation process, the third plasmid, pBAD-*lux*, was constructed with luminescent light producing genes under P_{BAD} control and transformed into *E. coli* MG1655. The resulting transformed bacteria were then incubated in LB containing 0.1% (w/v) L-arabinose with tannic acid (0.06 mM) and trans-cinnamaldehyde (2.27 mM) followed by the measurement of optical density and light output for 24 hours (Supplementary Fig. S3 online). As a result, bacterial bioluminescence was induced in presence of 0.1% w/v L-arabinose, which suggested that the *lux* operon in *E. coli* was fully functional (Supplementary Fig. S3A online). The bioluminescence of trans-cinnamaldehyde treated samples showed no difference as compared to negative controls (Supplementary Fig. S3A online). Therefore the trans-cinnamaldehyde is neither a transcriptional nor translational inhibitor. After treated with tannic acid, bacterial bioluminescence was reduced compared to controls with the output being much higher than non-induction control. The tannic acid may have effects on L-arabinose inducing transcription, protein translation or bioluminescence production with unknown mechanism. The treatment of (10% v/v) DMSO, tannic acid and trans-cinnamaldehyde did not affect the growth of *E. coli* (Supplementary Fig. S3B online). Collectively, these data confirmed the inhibition of AHL production by trans-cinnamaldehyde is not due to the growth, the transcription of pBAD promoter and protein translation. Furthermore, the growth curve of *P. aeruginosa* was measured showing no anti-bacterial effects exerted by tannic acid and trans-cinnamaldehyde (Supplementary Fig. S3C online). Therefore trans-cinnamaldehyde was selected for further characterisation of AHL synthase inhibition.

QS regulation of swarming enable optimal distribution of bacterial cells when the population is getting too large to occupy a small niche or when the nutrients in the surrounding environment is no longer to meet their needs[29]. In *P. aeruginosa*, *rhlAB* operon encoding

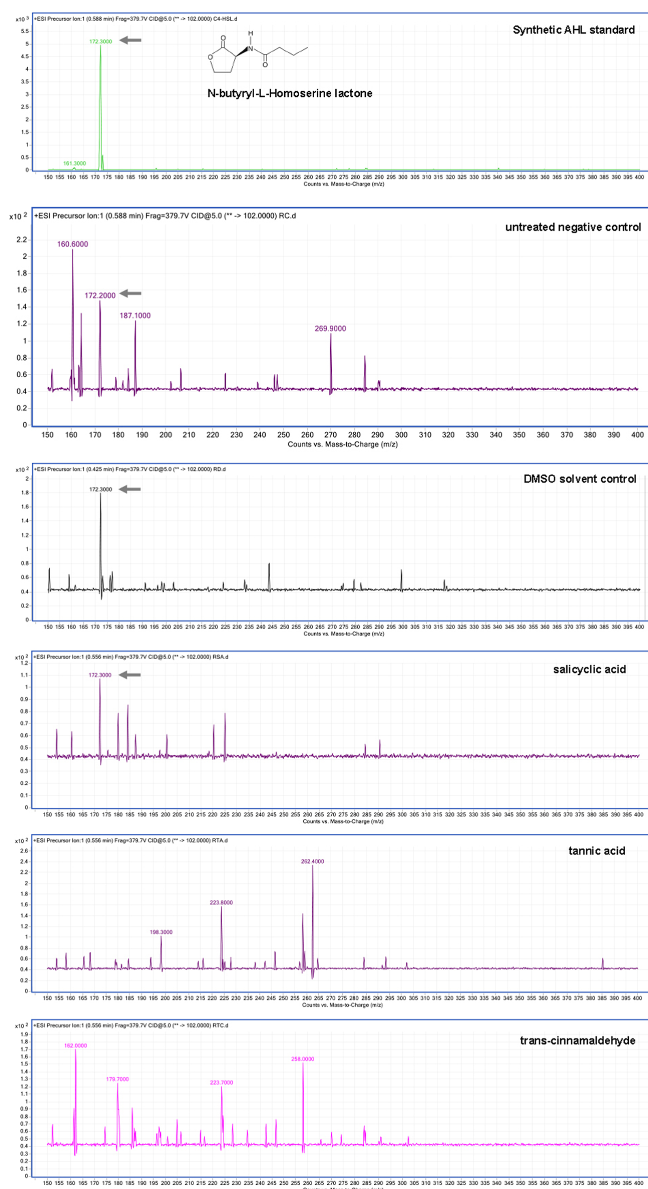

Figure 2 | LCMS analysis of C4-HSL production treated by salicylic acid, tannic acid and trans-cinnamaldehyde. Grey arrows indicate the parental ion of C4-HSL (*m/z*: 172).

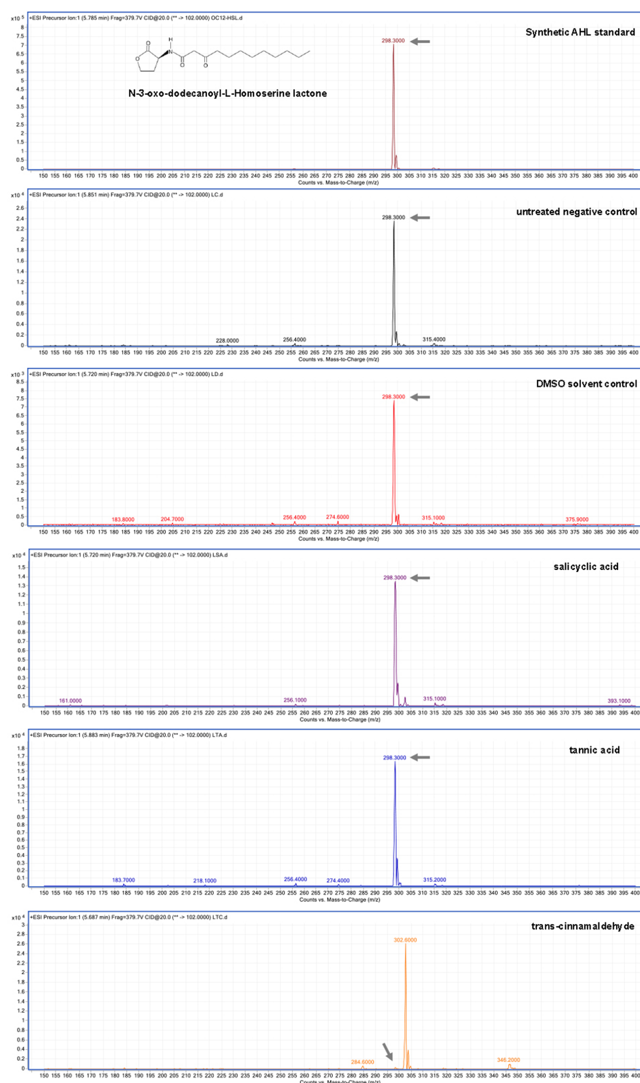

Figure 3 | LCMS analysis of 3-oxo-C12-HSL production treated by salicylic acid, tannic acid and trans-cinnamaldehyde. Grey arrows indicate the parental ion of 3-oxo-C12-HSL (*m/z*: 298).

rhamnolipid, a type of biosurfactant promoting swarming motility, is positively regulated by *rhlI/R* C4-HSL QS system[1,30]. In this study, *P. aeruginosa* PAO1 culture was spotted on the swarming agars without any solvent and inhibitor and swarming agars containing 10% v/v DMSO as solvent control and 0.3 mg/ml trans-cinnamaldehyde (equivalent to 2.27 mM). However bacterial swarming has no reduction caused by the inhibitor (Fig. 4A). This suggested the regulations of swarming are unable to be inhibited by simply reducing QS signal molecule production by trans-cinnamaldehyde.

Pyocyanin is a green phenazine pigment produced by *P. aeruginosa*, functioning as a redox active toxin and antibiotic and promoting extracellular DNA (eDNA) release for biofilm formation[31]. Pyocyanin biosynthesis in *P. aeruginosa* is mediated by two operons, *phz1* and *phz2*, which are regulated by Rhl QS system via PQS system hierarchically[4]. Therefore pyocyanin is a good indicator for investigating the effect of RhlI inhibitor. *P. aeruginosa* PAO1 cultures was treated with different concentration of trans-cinnamaldehyde for 24 hours and then the pyocyanin was extracted and determined. Pyocyanin from no treatment and 10% v/v DMSO treated cultures were also measured as negative control (Fig. 4B). The data were acquired from 4 biological replicates and normalised. No treatment of PAO1 culture was defined as 100% pyocyanin production and DMSO treated was 93.02%, which is not significant. When treated with 0.1, 0.2 and 0.3 mg/ml of trans-cinnamaldehyde, the production of pyocyanin was significantly reduced as the production are 65.54%, 63.03% and 57.94%, respectively ($P < 0.05$). These data suggested the trans-cinnamaldehyde reduced the QS-regulated pyocyanin production up to 42.06%.

Molecular docking analysis of trans-cinnamaldehyde. Although protein sequence in most well studied AHL synthase is divergent, they share four regions of conserved sequence and there are 8 residues are identical within these regions[4,31–33]. To understand the mechanism of trans-cinnamaldehyde mediated inhibition of AHL synthase, we performed the molecular docking analysis and predicted the potential trans-cinnamaldehyde binding site in AHL synthase. LasI is the counterpart of AHL synthase in *P. aeruginosa*. LasI, which produces 3-oxo-C12-HSL, shares 31% identity (47% homology) of protein sequence with RhlI and its protein crystal structure has been determined (PDB: 1RO5)[33]. Trans-cinnamaldehyde was docked into LasI successfully (Fig. 5A). Based

Figure 4 | Swarming assay of *P. aeruginosa* PAO1on semi-solid agar without inhibitors as non-treated control, agar containing with DMSO and Trans-cinnamaldehyde (A). Pyocyanin assay of *P. aeruginosa* PAO1without inhibitor as non-treated control, with DMSO as solvent control and treated with 0.1 mg/ml, 0.2 mg/ml and 0.3 mg/ml of trans-cinnamaldehyde (B).

on this docking pose, we speculated that the trans-cinnamaldehyde is "locked" deeply into the binding site and forms hydrophobic and Pi-Pi interactions with surrounding residues (Phe27, Trp33and Phe105) and one hydrogen bond with Arg30. The crystal structure study of LasI has suggested that N-terminal residue Phe27, Arg30 and Trp33 are crucial for forming an SAM binding pocket and Phe105 is a well conserved residue for acyl-chain binding tunnel[33]. Although trans-cinnamaldehyde mainly binds to the SAM binding pocket of LasI, reduced amount of 3-oxo-C12-HSL was still detected by LCMS which suggested the blocking SAM binding site may not be the only mechanism for trans-cinnamaldehyde inhibition. To further understand the binding property of trans-cinnamaldehyde to AHL synthase, we performed the second docking analysis using the other structure-known EsaI (PDB: 1KZF) which produces short chain 3-oxo-C6-HSL in phytopathogenic bacteria *Pantoea stewartii*. EsaI also containing four conserved regions and 8 crucial residues shares 23% identity (44% homology) with RhlI[34]. The successful molecular docking suggested the trans-cinnamaldehyde forms two hydrogen bonds with the backbones of Arg100 and Phe101, and forms hydrophobic and Pi-Pi interactions with surrounding residues (Phe101, Phe123 and Trp155) (Fig. 5B). Arg100 and Phe101 have been suggested to form hydrogen bonds with acyl-chain substrate in the hydrophobic core of EsaI. EsaI Phe123 and Trp155 may have no direct contact with *N*-hexanoyl chain but provide the shape and size through hydrophobic packing with other residues[34]. Thus the docking pose suggested that trans-cinnamaldehyde may bind to acyl-chain binding site in EsaI.

Discussion

The insolubility and activity lost of the purified *P. aeruginosa* LasI and RhlI expressed in *E. coli* background made these two proteins difficult to study. Therefore low copy number IPTG (isopropyl β-D-1-thiogalactopyranoside) induction expression plasmids were constructed. However, those plasmids showed loose-control of protein expression which are unsuitable for AHL biosynthesis study[35,36]. In contrast, the plasmids constructed in this study showed

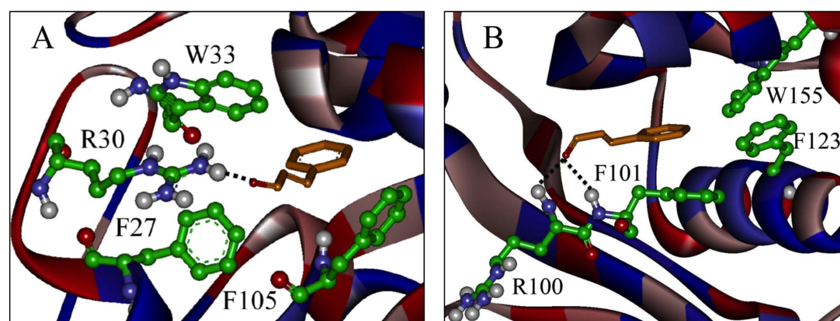

Figure 5 | The binding of trans-cinnamaldehyde with LasI (A) and EsaI (B). The protein was represented by ribbon, the carbon atoms of trans-cinnamaldehyde were coloured in orange, the carbon atoms on amino acids were coloured in green, the oxygen atoms were coloured in red and the nitrogen atoms were coloured in blue.

well-controlled AHLs production and accurate AHLs profile[37]. Therefore *E. coli* MG1655 carrying pBAD-*lasI* and pBAD-*rhlI* are ideal for the studies of AHL synthases and screening for AHL synthases inhibitors. Three hit compounds from initial screenings, salicylic acid, tannic acid and trans-cinnamaldehyde were selected. High resolution LCMS analysis suggested salicylic acid was unable to abolish AHL synthesis and bacterial luminescence measurement suggested tannic acid has potential effects on Lux protein expression or function. Therefore trans-cinnamaldehyde is the only hit compound showing AHL synthase inhibition with no bactericidal and bacteriostatic effects. Previous studies have shown that cinnamaldehyde interferes with the 3-oxo-C6-HSL based QS system in *Vibrio* spp.by decreasing the DNA-binding ability of LuxR regulator proteins[27,38]. To the best our knowledge, this is the first report showing that cinnamaldehyde interferes with AHLs production.

AHL synthase catalyses the formation of an amide bond between the homoserine lactone ring from SAM and the acyl chain from acyl-ACP. A synthetic C8-HSL analogue, J8-C8, has shown its inhibition of TofI, a C8-HSL synthase in *Burkholderia glumae* strain BGR1. X-ray crystal structure analysis showed that J8-C8 binds to TofI and occupies the binding site for the acyl-ACP providing acyl chain to TofI[39]. Interestingly, our hit compound trans-cinnamaldehyde, have no chemical structure similarity to AHLs or AHL analogues, which suggests a novel mechanism of AHL synthase inhibition.

Based on our docking analysis, trans-cinnamaldehyde interacts with LasI Phe27 and Trp33 and forms hydrogen bond with Arg30. These residues are completely conserved in LuxI family including EsaI and RhlI and form the putative pocket for SAM[32]. However we were unable to dock trans-cinnamaldehyde to potential SAM binding pocket of EsaI due to lack of highly mobile residues 16 to 28[34]. Here we proposed that the binding of trans-cinnamaldehyde to the SAM binding pocket is unable to inhibit AHL synthase activity.

AHLs vary in their acyl-chain. The selection mechanism of Acyl-ACP with different acyl-chain length has been suggested by the structure studies but the detail of acyl-chain preference in different AHL synthases is still unclear. In LasI a hydrophobic tunnel with no restriction on the length of the acyl-chain has form and Phe105 in beta-4 region is one of the conserved residues[33]. EsaI has a size restrictive hydrophobic pocket for acyl-chain binding and Arg100 and Phe101 (the conserved residue as LasI Phe105) in beta-4 region may form hydrogen bonds with acyl-chain substrate during the acylation reaction[34]. It has been suggested that the different sizes and open/closed acyl-chain binding pockets provide the explanations for Acyl-ACP selection. In our docking analysis, trans-cinnamaldehyde forms hydrophobic and Pi-Pi interactions with conserved LasI Phe105 in the open hydrophobic tunnel. In EsaI docking analysis, two hydrogen bonds forming between trans-cinnamaldehyde and two crucial residues (Arg100 and Phe101) may block completely the interaction between catalytic site and acyl-chain substrate. This may explain the trans-cinnamaldehyde is able to inhibit short-chain AHL synthase but not long-chain AHL synthase completely due to the difference of acyl-chain binding site structures. Interestingly, instead of conserved nonpolar phenylalanine in LasI and EsaI, polar tyrosine was located in the beta-4 region of RhlI. Therefore the mechanism of trans-cinnamaldehyde on RhlI is still unclear and more biological and structural analysis is needed.

In summary, plant extracts and 10 pure compounds were screened by tightly regulated LasI and RhlI expression plasmids resulting in one hit compounds showing active inhibition of AHLs production and attenuated *P. aeruginosa* PAO1 pyocyanin production without interference on the bacterial growth. Molecular docking studies proposed the potential mechanism of inhibition on AHL synthases. Our study may have facilitated the design of a new class of QS-inhibiting therapeutic agentby structure-based approaches and more structure details of the interaction between inhibitor and enzymes are required.

Methods

Bacterial strains, media and culture conditions. *Escherichia coli* DH5α was used as the cloning host and *E. coli* MG1655 was used for proteins expression and AHLs production. *E. coli* JM109 [pSB536] and *E coli* JM109 [pSB1075] are *lux*-based AHL biosensors[40,41]. *P. aeruginosa* PAO1 was cultured as AHL production positive control. All bacterial strains were routinely grown on LB (Luria-Bertani, Oxoid Ltd. UK) agar plates and in LB broth with 200 rpm shaking with appropriate antibiotics at 37°C for *E. coli* and *P. aeruginosa*PAO1 and 28°C for *C. violaceum* CV026 biosensor[42]. Antibiotics were used at the following concentrations: ampicillin, 100 mg/ml; tetracycline, 10 mg/ml (Sigma Aldrich, UK). Growth curve and luminescence was quantified in 96-well microtitre plates by using Tecan luminometer (Infinite M200Pro).

Plasmids construction. Phusion High-Fidelity DNA Polymerase was used for PCR amplification of the *lasI* and *rhlI* genes from *P. aeruginosa* PAO1 genomic DNA following the manufacturer's instructions (NEB, US). For *lasI* gene, the forward and reverse primers used were lasIFor (5′-CCTCTAGAATGATCGTACAAATTGG-3′) with XbaI engineered restriction site (underline) and lasIRev (5′-TCAAGCTTTCATGAAACCGCCAGTC-3′) with HindIII engineered restriction site, respectively. For *rhlI*, the forward and reverse primers used were rhlIFor (5′-CCTCTAGAATGATCGAATTGCTCTC -3′) with XbaI engineered restriction site (underline) and rhlIRev (5′-TCAAGCTTTCACACCGCCATCGACA-3′) with HindIII engineered restriction site respectively. The PCR products were digested with XbaI and HindIII and ligated with the same enzymes digested pBAD24 which consisted of a P_{BAD} promoter and a Shine-Dalgarno box[43]. The ligation mixtures were electroporated into *E. coli* DH5α. The resulting plasmids, pBAD-*lasI* (Supplementary Fig. S1A online) and pBAD-*rhlI* (Supplementary Fig. S1B online) were sequenced using pBADFor (5′-CTGTTTCTCCATACCCGTT-3′) followed by transformation *into E. coli* MG1655. pBAD-*lux* was constructed by ligating pBAD24 digested with KpnI and BamHI with *luxCDABE* operon from pSB401 digested by the same enzymes[41] and then transformed into *E. coli* MG1655.

AHLs extraction and detection. *E. coli* MG1655 strains harbouring expression plasmids for the AHL synthases LasI and RhlI were grown in 10 ml cultures in LB broth for overnight. Each culture was then diluted 1:50 into 10 ml of fresh LB broth and incubated until the cell density reached an optical density at 600 nm (OD_{600}) of 0.6 to 0.8 followed by adding L-arabinose (Sigma Aldrich, UK) with final concentration 0%, 0.1% or 1% (w/v) for 5-hour induction. *P. aeruginosa* PAO1 culture was grown in 10 ml LB broth for overnight without L-arabinose induction and its AHLs production was extracted to serve as positive control. At desired time

point, cell-free culture supernatant was extracted using acidified ethyl acetate (0.01% v/v glacial acetic acid in ethyl acetate). Extracts were evaporated to dryness and stored in −20°C for further analysis by *lux*-based biosensors, *C. violaceum* CV026 or LCMS as previously described[41,44]. Synthetic AHLs (Sigma Aldrich, UK) were used as LCMS standards.

QS Inhibiting Compounds. All synthetic compounds were obtained from Sigma Aldrich (UK) and dissolved in DMSO, sterilized by filtering through syringe filter (pore size of 0.2 µm) and stored at −20°C in sealed sterile tubes. DMSO (10% v/v) was added to controls in all assays to eliminate solvent effect.

Swarming Assay. The swarming plates for *P. aeruginosa* PAO1were prepared using 0.5% w/v Bacto agar, 0.5% w/v peptone, 0.2% w/v yeast extract and 1.0% w/v glucose, per 100 ml distilled water and the assay was performed as previously described[45]. The inhibitors with appropriate concentrations were seeded with 5 ml of the agar, mixed well and poured immediately on a 10 ml of pre-poured agar plate as an overlay. Two microliters of the *P. aeruginosa* PAO1overnight culture was inoculated at the center of the agar surface and the plates were incubated for 16 hours at 37°C.

Pyocyanin Assay. Pyocyanin quantification assay was performed as previous described with slight modification[46]. Overnight culture of *P. aeruginosa* PAO1 was diluted with LB medium to an OD_{600} of 0.1 and the diluted culture was supplemented with 15 µl of 0.1, 0.2 and 0.3 mg/ml (final concentration) of trans-cinnamaldehyde and incubated at 37°C for 24 hours. DMSO (10% v/v) served as negative control. The 5 ml cell culture was extracted with 3 ml of chloroform and was then mixed with 1 mL of 0.2 M hydrochloric acid. The organic layer was collected by centrifugation and was then measured at 520 nm using Tecan luminometer (Infinite M200, Männerdorf, Switzerland). The experiments were performed in quadruplicate.

Bioluminescence Assays. Bioluminescence and optical density were determined simultaneously in 96-well microtiter plates using a Tecan luminometer (InfiniteM200Pro) as previously described[45]. Briefly, overnight culture of *E. coli* MG1655::pBAD24-*lux* was diluted 1 : 100 in sterile fresh LB medium with 0.1% w/v L-arabinose, aliquoted (200 µL) and then loaded into each well containing the test QS inhibitors at desired concentrations. Bioluminescence and optical density were automatically simultaneously determined every 30 min for 24 hours. Bioluminescence is given as relative light units (RLU) per unit of optical density at 495 nm, which accounted for the influence of increased growth on the total bioluminescence. Experiments were done in triplicates and repeated three times.

Molecular docking. GOLD (v 5.1; Genetic Optimization for Ligand Docking) was used to dock trans-cinnamaldehyde into LasI (PDB: 1RO5)[47] and EsaI (PDB:1KZF)[34].The crystal structure of LasI and EsaI was treated by Amber 12 with amber ff12SBforce field[48] and all hydrogen atoms were added. GOLD was used to dock each ligand 10 times, starting each time from a different random population of ligand orientations and using the default automatic genetic algorithm parameter settings. LasI contains two binding sites: SAM (composed by Phe27, Arg30 and Trp33) and acyl-chain binding site (composed by Arg30, Trp69, Leu102, Phe105, Met125, Leu140, Thr144, Val148, Met151, Met152, Ala155, Leu157, Ile178 and Leu188) reported previously[33,39]. These two sites are adjacent to each other and were combined together as the active site for molecular docking. For molecular docking on EsaI the cavity composed by Ser98, Phe101, Phe123, Ile138, Val142, Met146, Leu150, Ser153, Trp155 and Leu176 was indicated as the binding site. In both docking studies, all torsion angles in each compound were allowed to rotate freely.

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Author contributions

C.Y.C. and Y.C. performed the molecular work. T.K., W.F.Y., Y.M.C., L.Y.T. and T.M.C. screened for anti-QS molecules and performed LCMS analysis. H.W. performed the molecular docking analysis. C.Y.C., W.F.Y. and K.G.C. developed the study strategy. C.Y.C., T.K., H.W. and K.G.C. contributed to the writing of the manuscript.

Additional information

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