Screening for novel quorum-sensing inhibitors to interfere with the formation of *Pseudomonas aeruginosa* biofilm

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INTRODUCTION

*Pseudomonas aeruginosa*, an opportunistic human pathogen, may cause acute infections in hospitalized patients, can be isolated from the environment, particularly from soil and water, and it regularly contaminates medical devices (Stover et al., 2000). It is also the predominant cause of chronic lung infection in cystic fibrosis patients (Frederiksen et al., 1997) and has recently been recognized as one of the main causes of chronic wound infections (Gjødsbol et al., 2006). *P. aeruginosa* can infect patients by producing a wide range of virulence factors, the expression levels of which are tightly regulated. Key to this regulation is cell density-dependent cell-to-cell signalling, which is termed quorum sensing (QS) (Rumbaugh et al., 2000). As in many other bacteria, QS controls secretion of virulence factors (Mittal et al., 2006), biofilm formation (Waters et al., 2008) and the exchange of DNA (Fuqua & Winans, 1996) in *P. aeruginosa*. The biofilm mode of growth is recognized as an important bacterial trait that is relevant to infections (Costerton et al., 1994). Many infections involve the formation of bacterial biofilms, which are bacterial communities that settle and proliferate on surfaces and are covered by exopolymers (Lewis, 2007). Once established, biofilms are difficult to eradicate and become a source of secondary infection (Jones et al., 2009). Moreover, bacteria embedded in biofilms are more tolerant than planktonic cells of antibiotics (Donlan & Costerton, 2002; Drenkard, 2003). The dose of antibiotics needed in this situation will often exceed the highest deliverable dose, which makes efficient treatment impossible.

QS, as a regulatory mechanism, enables bacteria to make collective decisions with respect to the expression of a specific set of genes that involve the production, release and subsequent detection of chemical signalling molecules, such as *N*-acylhomoserine lactones (AHLs) that are commonly used by Gram-negative bacteria. When the concentration of AHLs reaches a certain threshold level, binding to a receptor molecule (for example, LuxR) is promoted and the activated LuxR–AHL complex forms dimers or polymers, which, in turn, act as transcriptional regulators of target genes in the QS regulon (Parsek & Greenberg, 2000; Vannini et al., 2002). *P. aeruginosa* uses AHLs to coordinate the expression of a battery of virulence genes and induction of biofilm formation via a cascade of
regulatory events (Passador et al., 1993), and so the QS system is involved in establishing infections as well as elevating tolerance to antibiotics (Drenkard, 2003).

The traditional approach for the treatment of infectious diseases is to kill or inhibit the growth of bacteria using antibiotics, which has selected for resistance to these drugs, and this has particularly been the case in P. aeruginosa. In response to the rise of antibiotic resistance, the continued development of new drugs and the judicious use of our current arsenal of antibiotics is required (Bergstrom et al., 2004). In this context, the development and use of QS-inhibition-based drugs to attenuate bacterial pathogenicity is attractive (Boyen et al., 2004). In this context, the development and use of QS-inhibition-based drugs to attenuate bacterial pathogenicity is attractive (Boyen et al., 2009; Jones et al., 2009). Indeed, because of their anti-biofilm effects, some quorum-sensing inhibitors (QSIs) like patulin and garlic extracts have even been found to make P. aeruginosa more susceptible to antibiotics, for example, tobramycin (Hentzer et al., 2003; Rasmussen et al., 2005a, b).

Like all plants, traditional Chinese medicinal herbs grow in an environment that has high bacterial density and so they have co-existed with QS bacteria during their evolution. Accordingly, the herbs may have evolved protective mechanisms against bacterial infections, and may even produce QSIs, which reduce the pathogenic capability of bacteria (Zhu et al., 1998). Components of some traditional Chinese medicines (TCMs), derived from these herbs, have been identified to be effective in the treatment of various inflammatory and infectious diseases such as gastritis, stomatitis and pneumonia (Ma et al., 2005). Thus, it was considered interesting to screen compounds from known TCMs to test whether they have QSI activity.

The discovery of inhibitors for a target protein can be greatly facilitated by using computer-aided drug design. In particular, virtual database screening approaches offer the potential to identify novel chemical entities with a high probability of binding to a target protein (Furci et al., 2007). The 3D structures of transcriptional regulators from Agrobacterium tumefaciens, Escherichia coli and P. aeruginosa involved in QS have been elucidated (Vannini et al., 2002; Yao et al., 2006; Bottomley et al., 2007). On the basis of the TraR crystal structure from the plant-associated bacterium A. tumefaciens, mutational analyses indicated that TraR is directly involved in the transcriptional activation in response to QS (Vannini et al. 2002). As an autoinducer-dependent transcriptional activator of plasmid conjugal transfer genes, TraR positively regulates interbacterial conjugal transfer of the Ti plasmid (Zhu & Winans, 2001). The complex of TraR with the QS ligand N-(3-oxo-octanoyl)-homoserine lactone binds to promoter elements, called tra boxes, upstream of at least five different tra operons on the Ti plasmid and activates gene expression. A similar protein in P. aeruginosa is LasR, which controls the expression of virulence and biofilm-associated genes. The amino acid residues in the LasR and TraR active sites show significant identity (70 %) and the 3D model of the LasR active site closely resembles the X-ray structure of the TraR active site (Müh et al. 2006). Thus, inhibitors of TraR may have evolved in plants to combat the plant pathogen A. tumefaciens, but they may also inhibit LasR in the human pathogen P. aeruginosa. Accordingly, our approach was to first identify putative inhibitors of A. tumefaciens TraR based on structure analysis in combination with computer-aided drug design and biological assays, and then to test any potential QSIs for their ability to inhibit biofilm formation and antimicrobial drug sensitivity.

### METHODS

#### Compound preparation.

All the compounds (purity ≥98 %) used in this study were purchased from the Guangdong Institute for Drug Control (Guangzhou, China) and dissolved in DMSO. The QS signal molecule N-(3-oxo-octanoyl)-L-homoserine lactone (3-oxo-C8-HSL, AHL) was purchased from Sigma. Information on the six active components extracted from TCMs is listed in Table 1.

#### Ligand docking.

The 3D structure of the A. tumefaciens QS transcriptional activator protein TraR, obtained from the Protein Data Bank (PDB ID code 1H0M), was used in the DOCK program. Only chain A was used for the model; the other chains (B, C, D, E, F, G) and all the water molecules were removed. In the DOCK package, the binding pocket was described by spheres. Each sphere touched the molecular surface at two points and had its radius along the surface of one of the points. The spheres were generated by the program SPHGEN, which is distributed as an accessory of DOCK. To define the ligand-

<table>
<thead>
<tr>
<th>Compound number</th>
<th>Compound name</th>
<th>Molecular mass (Da)</th>
<th>TCM</th>
<th>Docking score (kcal mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3-Oxo-C8-HSL</td>
<td>30</td>
<td>Rheum palmatum L.</td>
<td>-46.20</td>
</tr>
<tr>
<td>C1</td>
<td>Rhein</td>
<td>284.21</td>
<td>Rheum officinale Baill.</td>
<td>-7.78</td>
</tr>
<tr>
<td>C2</td>
<td>Chrysophanol</td>
<td>254.23</td>
<td>Peucedanum decursivum (Miq). Maxim</td>
<td>-2.96</td>
</tr>
<tr>
<td>C3</td>
<td>Nodakenetin</td>
<td>408.40</td>
<td>Lithospermum erythrorhizon Sieb.</td>
<td>10.96</td>
</tr>
<tr>
<td>C4</td>
<td>Shikonin</td>
<td>288.31</td>
<td>Rheum palmatum L.</td>
<td>-15.46</td>
</tr>
<tr>
<td>C5</td>
<td>Emodin</td>
<td>270.23</td>
<td>Fraxinus chinensis Roxh.</td>
<td>-20.16</td>
</tr>
<tr>
<td>C6</td>
<td>Fraxin</td>
<td>370.31</td>
<td></td>
<td>-28.94</td>
</tr>
<tr>
<td>Control</td>
<td>Halogenated furanone 30</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
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binding pocket in chain A of TraR, 3-oxo-C8-HSL, the autoinducer of TraR, was used as a ligand to select spheres. The spheres within 10 Å of the ligand were selected. The sphere-selector module was used for sphere selection.

On the basis of the reported application of TCMs for the treatment of diseases, 46 compounds from these TCMs were selected to construct a database for screening potential novel TraR (and so QS) inhibitors. Then, the 46 compounds and halogenated furanone C30 (a known QS inhibitor control) were rigidly docked into the TraR binding pocket to see whether they might competitively inhibit QS ligand binding. The box size was set at 6 Å, the grid space was 0.3 Å and the energy cut-off distance was 9999 Å. Maximal orientations were set at 30 000. Default settings were used in docking. Before docking, the sybyl program-package (Sybyl 6.9; Tripos Associates) was used to add hydrogen atoms and charges to the protein. All of the hydrogen atoms of the protein were added and refined in the presence of explicit solvent with a progressive energy-minimization protocol using AMBER7 FF99 force field. The termination gradient was 0.01 kcal mol⁻¹ and the maximal iteration was 1000 (Sybyl 6.9; Tripos Associates). Docking studies were performed with the DoCK 5.3.0 program (Meng et al., 1993).

**Bacterial strains and culture conditions.** *P. aeruginosa* PAO1 was routinely cultured in Luria–Bertani (LB) medium at 37 °C with 160 r.p.m. agitation in a shaking incubator. Medium was solidified with 1.2 % agar when required. *Stenotrophomonas maltophilia* GIM1118 was cultured in medium (peptone 2.5 g, beef extract 1.5 g, NaCl 2.5 g, agar 10 g, ddH₂O 500 ml, pH 7.2) at 37 °C with 160 r.p.m. agitation in a shaking incubator. The *traR* gene (encoding TraR) was amplified from the plasmid pCF218 of *A. tumefaciens* (Zeng et al., 2006). The plate was cultured at 37 °C for 3 days without shaking. The cover glasses were recovered, washed gently with sterile distilled water, Gram stained, and examined under a microscope.

**Assay of TraR degradation in E. coli.** The assay of TraR degradation in *E. coli* was performed by the method described previously with further modification (Zeng et al., 2008). *E. coli* BL21(DE3)(pETtraR) was incubated in LB broth at 37 °C with shaking until the OD₆₀₀ of cells reached 0.3. Then, 400 μM isopropyl-β-d-thiogalactoside was added, followed by further incubation at 37 °C for 4 h. Subsequently, 1.5 ml cultures were harvested by centrifugation (10 000 g) at 4 °C for 2 min. The cells were washed three times with an equal volume of sterile distilled water. The cells were resuspended in 50 μl sterile distilled water containing 100 μg ampicillin ml⁻¹ and 100 μg chloramphenicol ml⁻¹ together with different test compounds (up to 30 mM), DMSO vehicle (4 %, v/v) or AHL (10 mg ml⁻¹), and incubated at 30 °C for up to 4 h. Protein extracts were then produced from these cells, which were examined by SDS-PAGE.

**RESULTS**

**Screening for TCM compounds with potential anti-QS activity**

The Dock 5.3.0 program was applied to screen for putative novel QS inhibitors of *A. tumefaciens* TraR from the database of compounds found in TCMs constructed by us. The results suggested that 9 compounds from the 46 candidates might be potential inhibitors of the QS system; i.e. they might potentially bind to the TraR protein and inhibit binding of the QS ligand, the AHL. In this study, six of these compounds with different docking scores were further selected for bioassay (Table 1). The predicted orientation of the potential QSI, compound 5 (emodin), bound to TraR was generated using the Chimera program (Pettersen et al., 2004) (Fig. 1). The interactions between compound 5, the halogenated furanone C30 (a known QSI) and TraR are depicted in Fig. 2.

**Assay of P. aeruginosa growth**

Antibacterial activity was tested by paper disc diffusion assay. *P. aeruginosa* was incubated in LB, 100 μl of this culture (10⁻⁴ c.f.u. ml⁻¹) was spread onto an LB plate and three filter paper discs (8 mm in diameter) were placed on the plate. Different solutions, including 20 μl test compound (0.2, 2, 20, 200 mM) plus 2 μl ampicillin (1.2 mg ml⁻¹), 20 μl DMSO plus 2 μl ampicillin (1.2 mg ml⁻¹) and 20 μl test compound (0.2, 2, 20, 200 mM) plus 2 μl sterile water, were loaded onto the discs, respectively. The plate was cultured at 37 °C for 24 h and growth inhibition zones were measured (Choo et al., 2006).

**Assay of biofilm formation by P. aeruginosa and S. maltophilia**

The biofilm assay was performed by the method described previously with further modification (You et al., 2007). Briefly, 1 ml *P. aeruginosa* or *S. maltophilia* cells, which were cultured overnight and diluted to an OD₆₀₀ of 1.0, were mixed with 200 μM of the different test compounds made up in 1 % (v/v) glycerol in 2 ml microfuge tubes that were incubated at 37 °C for 3 days without shaking. The liquid in each tube was then removed and the bacteria adhering to the sides of the tubes were washed with sterile distilled water three times and air-dried. The remaining biofilm was stained with 1 ml 0.5 % (w/v) crystal violet solution for 20 min and the excess dye in tubes was removed by washing with sterile distilled water three times. The quantitative analysis of biofilm was measured by adding 1 ml 95 % (v/v) ethanol into the tube and waiting for 15 min. Then, the A₅₇₀ nm of dissolved crystal violet was determined. To view biofilm formation under a microscope, cover glasses were put into 50 ml conical flasks containing bacteria, in the presence or absence of test compounds (200 μM), and incubated at 37 °C for 3 days without shaking. The cover glasses were recovered, washed gently with sterile distilled water, Gram stained, and examined under a microscope.

The diameter of the inhibition zones was bigger in the presence of both compound 5 and ampicillin than in the presence of ampicillin alone. The results showed that whilst...
emodin had little direct effect on the growth of *P. aeruginosa* at a concentration of 0.2, 2 and 20 mM, and only a small activity against *P. aeruginosa* at 200 mM, emodin appeared to make *P. aeruginosa* more susceptible to ampicillin, an antibiotic to which *P. aeruginosa* is resistant.

**Effect of putative QSI compounds on biofilm formation of *P. aeruginosa* and *S. maltophilia***

The results showed that compounds 2, 3, 4 and 5 could inhibit biofilm formation of *P. aeruginosa* and *S. maltophilia*. Of the four compounds, compound 5 (emodin) showed the most potent inhibitory activity (Fig. 3a). Further assays for the effect of compound 5 on biofilm formation of *P. aeruginosa* and *S. maltophilia* were carried out at different concentrations of compound 5. The results indicated that compound 5 at 20 μM could significantly inhibit biofilm formation (*P*<0.05; Fig. 3b). Almost all of the *P. aeruginosa* and *S. maltophilia* cells incubated with compound 5 (200 μM) detached and dispersed from the glass surface. In contrast, the control cells adhered to the glass surface, continued to proliferate, and also congregated together (Fig. 3c, d).

**Effect of putative QSI compounds on the degradation of TraR protein in vivo***

The intensity of the SDS-PAGE band was related to the concentration of the corresponding protein expressed in *E. coli*. The results showed that the concentration of TraR protein expressed in *E. coli* BL21(DE3)(pETtrar) decreased after being treated with compound 5 for 4 h (Fig. 4a). Further studies were performed to examine the effect of different concentrations of compound 5 on the degradation of TraR (Fig. 4b). TraR concentration decreased with increased concentration of compound 5. When the concentration of compound 5 reached 30 mM, the concentration of TraR decreased rapidly within 0.5 h. The effect of AHL on the degradation of TraR was also monitored. The results showed that the degradation of TraR was inhibited in the presence of AHL (Fig. 4c).

**DISCUSSION***

Inhibition of the bacterial QS system, rather than a bactericidal or bacteriostatic strategy, might be applied in many fields such as medicine, agriculture and food technology. This approach is very attractive because it is not directly involved in the inhibition of bacterial growth and does not impose harsh selective pressure for the development of resistance. Accordingly, there is a particular interest in finding new chemical entities that inhibit bacterial QS (Zeng *et al.*, 2008; Ganin *et al.*, 2009). A number of virtual screening techniques have been developed and have become an integral part of drug discovery, and are available in the literature to identify potent drug leads. In this study, such techniques have been
applied to explore the effects of novel QS inhibitors extracted from TCMs on the inhibition of bacterial QS.

To date, the only known anti-QS compounds of non-bacterial origin are halogenated furanones from the red alga Delisea pulchra (Manefield et al., 1999). Anti-QS activity has also been noted in a number of traditional medicinal plants (Adonizio et al., 2006). Rhubarb (named Dahuang in Chinese), a medicinal plant, displays diverse pharmacological activities such as bacteriostatic, antiviral, antifungal and antitumour activities (Huang & Zhen, 2001; Kim et al., 2004; Basu et al., 2005; Huang et al., 2005). Emudin, one of the free anthraquinone compounds extracted from rhubarb, is the major active constituent (Ma et al., 2008) that inhibits the expression of the tumour necrosis factor alpha (TNF-α) gene (Chen et al., 2008) and tumour metastasis in vitro and in vivo (Kwak et al., 2006). It also promotes the apoptosis of human breast cancer BCap-37 cells (Huang et al., 2008). Some studies have also reported the effect of emodin on cell death in human prostate, lung, liver, cervical and blood cancer cells (Cha et al., 2005; Su et al., 2005; Jing et al., 2006; Fu et al., 2007; Muto et al., 2007). In this study, compound 5 (emodin) and ampicillin acted jointly against P. aeruginosa more effectively than either of them did alone, suggesting that compound 5 enhanced the activity of ampicillin against P. aeruginosa. The results were similar to the marked synergies between the compounds extracted from TCMs and antibiotics reported by Fujita et al. (2005) and Chang et al. (2007).

It has been well documented that biofilm growth and formation is a severe problem in medicine and industry (Costerton et al., 1995). The strategy to interrupt the QS system has been increasingly regarded as an important method to control the formation and growth of biofilm (Lynch et al., 2002). A QS-deficient biofilm of P. aeruginosa was almost entirely eliminated with antibiotics compared with the wild-type biofilm, in which only cells in the top layer were killed (Bjarnsholt et al., 2005). Shih & Huang (2002) obtained similar results by comparing wild-type biofilm and QS-deficient biofilm treated with kanamycin. In this study, compound 5 (emodin) inhibited the adhesion of P. aeruginosa to a glass surface and biofilm formation (Fig. 3). The results were consistent with the findings of Rasmussen & Givskov (2006). It is likely that compound 5 (emodin) penetrated into the biofilm and interfered with bacterial intercellular communications, by which the QS system of P. aeruginosa was disturbed and repressed. Moreover, previous studies have found that

### Fig. 3. Effect of different putative QSI compounds on biofilm formation of P. aeruginosa and S. maltophilia. (a) Treatment of P. aeruginosa (white bars) and S. maltophilia (black bars) with various compounds at 200 µM; (b) concentration-dependent effect of emodin (0–200 µM) on biofilm formation of P. aeruginosa (white bars) and S. maltophilia (black bars); (c) micrograph of a P. aeruginosa biofilm; (d) micrograph of a P. aeruginosa biofilm following treatment with 200 µM emodin. Bar, 10 µm.
S. maltophilia addition, the results also showed that compound 5 can inhibit growth, was able to inhibit the adhesion and biofilm (Fig. 3). In summary, emodin was shown to significantly inhibit biofilm formation in P. aeruginosa, and induce proteolysis of a known AHL-binding protein, and could be used as a potential QS inhibitor for the control of biofilm formation and growth.

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