Quorum Sensing Inhibitory Activities of Oridonin in *Pseudomonas Aeruginosa*

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Abstract. *Pseudomonas aeruginosa* is an opportunistic pathogen, and it can produce cell-associated and extracellular virulence factors. Several of these virulence factors have been demonstrated to be regulated by quorum sensing (QS). Disabling QS system with anti-infective agent is considered as a potential strategy to prevent bacterial infection. *Rabdosia rubescens* has been used as antibacterial agents for many centuries in China. In this study, Oridonin, the major active components of *Rabdosia rubescens,* was tested for QS inhibition in *Pseudomonas aeruginosa*. QS inhibitory activity is demonstrated by reduction in pyocyanin (58.4%), rhamnolipids (64.3%), elastase (58.6%), and protease (49.1%) in *Pseudomonas aeruginosa* PAO1 at 125 µg/ml (MIC) concentration. Biofilm formation by *Pseudomonas aeruginosa* PAO1 was reduced considerably (40.3-57.7%) over control. These findings suggest that Oridonin might be a potent Quorum Sensing Inhibitor (QSI) and anti-biofilm agent in the treatment of *Pseudomonas aeruginosa* infections.

1 Introduction

Bacterial infection has been a major problem on the medical treatment. With the introduction of natural antibiotics such as penicillin, antibiotic derivatives and synthetic antibiotics, they have opened a new era in the treatment of bacterial infections[1]. However, with the widely abuse of antibiotics, drug resistance is becoming a severe problem, at the same time, the emergence of multi-resistant bacteria and mutation time of drug resistance also become more and more short[2], so it is urgent to find new targets of antibacterial agents and pathways to treat diseases.

Quorum Sensing (QS) found in Gram-negative and Gram-positive bacteria. Bacteria could produce some signaling molecules, called autoinducers (AI), that can coordinate the physiological activities as well as the expression of virulence factors in the pathogenesis[3]. The attenuation of virulence factors and pathogenicity of bacteria through interfering QS is a possible alternative to killing or inhibiting growth of pathogenic bacteria. Many researchers have indicated that phytochemicals inhibit virulence factor production and biofilm formation by interfering auto-induce signaling molecules in quorum sensing system.

P. aeruginosa is an opportunistic pathogenic bacterium, which can be easily detected in the skin and lung infections. QS plays a significant role in the regulation of P. aeruginosa virulence expression such as biofilm, rhamnolipid, pyocyanin, elastase, and protease[4]. Bacterial biofilm development depends on release of extracellular polymeric compounds. Rhamnolipid promotes biofilm formation and diffusion, Elastases and proteases play key roles in early invasion and disintegration of host cells[5, 6].

Rabdosia rubescens, a Traditional Chinese Medicine (TCM), has been used in reducing fever, antidiarrhea. It has a significant effect on acute laryngitis and suppurative tonsillitis, and also has a good effect on chronic bronchitis and chronic pharyngitis[7]. The main active component of *Rabdosia rubescens* is oridonin, a natural diterpene carvacene organic compound. In this work, we aim to study the potential of oridonin as quorum sensing inhibitor.

2 Methods

2.1 Bacterial strains and culture conditions

P. aeruginosa PAO1 was routinely cultured in Luria–Bertani (LB) broth at 37°C with shaking. Bacterial culture with an optical density of 0.5 at 600nm was used for all studies.

2.2 MIC Determination

Minimum inhibitory concentration (MIC) was determined by the broth micro-dilution method (Clinical and Laboratory Standards Institute, CLSI 2015) by using 96-

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well microtiter plates. Serial two-fold dilutions method was used in this assay, 100μl of Oridonin(Sigma) solution and 100 μl of LB broth were placed into the first well of a microtiter plate and mixed evenly, extra 10μl PAO1 cultures was added in every well and incubated at 37°C. The minimum concentration with no visible bacterial growth was defined as MIC.

2.3 Pyocyanin Assay

Oridonin solution was mixed well with overnight grown PAO1 cultures and incubated at 37°C for 24h. Pyocyanin was extracted with chloroform and vortexed vigorously. Then, the mixed cultures were centrifuged at 10000rpm for 10min. The chloroform layer was re-extracted with 0.2mol/l HCl. After the same centrifugation, 200μl of the HCl layer solution were transferred to 96-well plates and the absorbance was read at 570nm[8].

2.4 Rhamnolipids assay

Overnight grown PAO1 culture was adjusted to OD600 0.5 by PPGAS broth (20 mM NH4Cl, 20 mM KCl, 1.6 mM MgSO4, 0.5% Glucose, 1.0% Peptone, 120 mM Tris-HCl every 1000 mL, pH 7.2). Oridonin solution was mixed with PAO1 cultures to make the test concentrations, incubated at 37°C for 24h. After centrifugation (8000rpm for 10min at room temperature), the supernatant was adjusted to pH2.0, and extracted with ethyl acetate twice, evaporate to dryness in a drying chamber. The sediment was re-dissolve in ddH2O. The resulting solution was mixed completely with sulfuric acid (800μl, 60% (v/v)) and orcinol (1.7%, 100μl). 200μl of solution were transferred to 96-well plates and the absorbance was read at 421 nm[9].

2.5 Protease Activity Assay

Overnight grown PAO1 culture was adjusted to OD600 0.5 by LB broth. Oridonin solution was added to PAO1 cultures to make the test concentrations, incubated at 37°C for 24 h. PAO1 cultures were centrifuged (10000 rpm for 10 min at room temperature) and then filtered through 0.22 μm filter membrane. Culture supernatant (150μl) were added to 250μl of 2% (w/v) azocasein-Tris-HCl solution, stored at 4°C for 4 hours, Trichloroacetic acid(10%,1.2ml) were added to terminate the reaction and then centrifuged at 10000 rpm for 10 min. Finally, 200 μL of solution were transferred to 96-well plates and the absorbance was read at 440 nm[10].

2.6 Elastase Assay

Oridonin was added to PAO1 cultures to make the test concentrations, incubated at 37°C for 24h. PAO1 cultures were centrifuged (6000 rpm for 10 min at room temperature) and then filtered through 0.22 μm filter membrane. Culture supernatant(200μl) was mixed with Congo red elastin solution (800μl, 2%, pH7.5), incubated at 37°C for 4h, centrifuged at 10000 rpm for 10 min. 200 μL of solution were transferred to 96-well plates and the absorbance was read at 495 nm[11].

2.7 The semiquantitative of Biofilm

Sterilized coverslip as adhesive carrier (2cm × 2cm) was placed in a sterilized 6-well microtiter plate. Oridonin solution(2ml) and PAO1 culture(20μl) were added in each well, incubated at 37°C for 7days. Within the seven days, bacteria that did not form biofilm was removed by sterilized PBS (pH7.8) every day (each coverslip was washed twice with 2ml of sterilized PBS). Two milliliters of pentanediol was used to fix, after 20 min, then the same way to deal with pentanediol. Crystal violet (0.5%,2 ml) was added and incubated at room temperature for 15 min. Coverslips rinsed with distilled water until there was no purple liquid. To quantitate, 2 ml of 95% (v/v) ethanol was added in each well to re-dissolve the crystal violet that closely combined with the coverslip. Finally, 200 μL of solution was transferred to 96-well plates and the absorbance was read at 570 nm[12].

2.8 Statistical Analysis

All experiments were performed in triplicate and the data obtained from the experiments were presented as mean values with or without standard deviation and the differences between control and test were analyzed using Student's t-test

3 Results

3.1 MIC of Oridonin against *P. aeruginosa* PAO1

The influence of oridonin on PAO1 growth was examined by MTT method. This assay was aimed to rule out any antibacterial properties of oridonin that may inhibit growth of PAO1. The results showed that oridonin inhibited PAO1 growth at the concentration of 250μg/ml (Table 1), while there was little effect on the growth of bacteria below 125μg/ml. The MIC of oridonin against PAO1 was 125μg/ml.

Table 1. MIC of Oridonin to Pseudomonas Aeruginosa (mean± SD)

Oridonin(μg/ml)	2000	1000	500	250	125	62.5	31.3	15.6
Bacterial Concentration[a]	0.22 ±0.02*	0.31 ±0.02*	0.48 ±0.03*	0.92 ±0.02*	1.22 ±0.04**	1.23 ±0.04**	1.23 ±0.03**	1.22 ±0.03**

[a]Concentrations were expressed as the absorbance at 600nm, Data represent mean and standard deviation of three independent experiments. *p⩽0.05, **p⩽0.01

3.2 Effects of oridonin on QS-Regulated virulence factors in PAO1

Table 2 shows the dose-dependent effect of oridonin on virulence factors in PAO1. Oridonin reduced variously pyocyanin production in PAO1 at different concentration in a dose-dependent manner, with 58.4% (MIC), 39.4% (1/2MIC) and 27.7% (1/4 MIC) reductions, respectively. The result shows oridonin reduced rhamnolipids production by 32.0%, 60.5 % and 64.3% at MIC, 1/2 MIC, 1/4 MIC, respectively

A significant concentration-dependent decrease (25.9–40.9%) of protease production was observed in the culture supernatant of PAO1 compared to control. Oridonin reduced protease activity by 25.9%, 30.0 % and 40.9% at MIC, 1/2 MIC, and 1/4 MIC, respectively (Table 2).

Elastase activity decreased substantially at sub-inhibitory concentrations (31.3-125 μg/ml) of oridonin with 42.4-58.6% reduction compared to the control. The results showed that there was significant difference between the experimental groups and the negative group ($P < 0.05$).

3.3 Effect on Biofilm Formation

Sub-MICs were tested for biofilm inhibition in PAO1 using crystal violet assay. Sub-MICs of oridonin decrease biofilm Formation of PAO1. Biofilm formation was significantly inhibited by 40.3%, 48.4%, and 57.7% at 31.3, 62.5, and 125 μg/ml concentrations, respectively.

Table 2. QSI Activity of Oridonin in Pseudomonas Aeruginosa (mean± SD)

Oridonin(μg/ml)	Pyocyanine[a](IR[b])	Rhamnolipids[c](IR[b])	Protease[d](IR[b])	Elastase[e] (IR[b])	Biofilms[f] (IR[b])
Control	0.137 ±0.025	0.824±0.032	0.220±0.019	0.494±0.027	0.397±0.040
31.3	0.099 ±0.022(27.7)	0.560±0.024(32.0)	0.163±0.015(25.9)	0.284±0.022(42.4)*	0.238±0.033(40.3)*
62.5	0.084 ±0.023(39.4)*	0.325±0.018(60.7)*	0.155±0.012(30.0)*	0.246±0.021(50.3)*	0.206±0.027(48.4)*
125	0.058 ±0.017(58.4)**	0.294±0.017(64.3)**	0.113±0.012(40.9)*	0.204±0.018(58.6)**	0.169±0.016(57.7)**

[a]Pyocyanin concentrations were expressed as the absorbance at 520nm.[b]Inhibiting Rate. [c]Rhamnolipids concentrations were expressed as the absorbance at 421nm.[d]Protease activity is expressed as the absorbance at 440nm. [e]Elastase activity is expressed as the absorbance at 495nm.[f]Biofilm formation is expressed at 470 nm after incubation with crystal violet. Data represent mean and standard deviation of three independent experiments. Values in parentheses indicate percent reduction over control. *p≤0.05, **p≤0.01

4 Discussion

Pseudomonas aeruginosa is one of the pathogens that can escape the treatment of various antibiotics. It is widely distributed in nature, normal skin, intestinal and respiratory tract, which is the main cause of respiratory tract infection, urinary tract infection, septicemia, osteomyelitis and skin infections[13]. QS enables the *P. aeruginosa* to synthesize and release a large numbers of extracellular virulence factors, such as pyocyanin, rhamnolipids, proteases, etc. Some research has found that an obvious reduction was detected in the secretion of virulence factors, biofilm formation, invasiveness of infected hosts when QS system was blocked[14] .

Pyocyanin can be easily found in the sputum of cystic fibrosis (CF) patients and it can cause detrimental effects toward lung epithelial cells[15]. Pyocyanin formation is regulated by a complex synchrony of rhlR-rhlI and lasR-lasI whereby interferences in these systems led to the deficiency of pyocyanin formation[16]. Our study shows pyocyanin production has a steady decrease with the increasing concentrations of oridonin.

Rhamnolipids are vital microbial derived surfactants produced by *P. aeruginosa* and under the control of Las and Rhl systems. They are important to bacteria motility. The rhlA mutant which lacks rhamnolipids production completely loses its swarming ability. Furthermore, many studies showed that QS positively regulates rhamnolipids production. Rhamnolipids are made up of two fatty acid molecules and rhamnose residues, which is controlled by Rhl system, mainly by rhlAB synthetic gene cluster expression[17]. It can decrease surface tension and biofilm development. Our result shows oridonin reduced rhamnolipids production by 32.0-64.3% without interfering the growth of PAO1. Similar reduction in pyocyanin production was recorded in literature[18].

The hydrolytic enzymes such as protease and elastase are known to affect the host cell proteins in the infected tissues and facilitate bacterial invasion and growth. LasI/LasR system regulates the genes responsible for lasA(protease) [19]. In the present study, preincubation with oridonin created dose-dependent inhibition of elastase

and protease production. These data corroborated with the literature where, elastase activity and proteolytic activity of *P. aeruginosa* was decreased by plant extracts and essential oils [20]

Quorum sensing are known to play significant role in biofilm formation. P. aeruginosa biofilm can effectively resist the attack of antibiotics and immune system. We found that oridonin at Sub-MICs inhibited the biofilm biomass by 40.3-57.7% significantly ($P \leqslant 0.05$) with no significant growth inhibition on PAO1.

Based on our data and available literature, it could be assumed that oridonin might have inhibited the QS-systems in *P. aeruginosa*, as a potential candidate for exploiting as anti-infective agent in modern phytomedicine.

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