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Inhibition of *Pseudomonas aeruginosa* quorum sensing by subinhibitory concentrations of curcumin with gentamicin and azithromycin

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ABSTRACT

Objectives: *Pseudomonas aeruginosa* quorum sensing (QS) circuits regulate virulence factors and coordinate bacterial pathogenicity. This study aimed to investigate the inhibitory activity of subinhibitory concentrations of curcumin with azithromycin and gentamicin against *P. aeruginosa* QS-related genes and virulence factors.

Methods: The minimum inhibitory concentrations (MICs) and synergistic activity of curcumin with azithromycin and gentamicin against *P. aeruginosa* PAO1 were determined using broth microdilution and checkerboard titration methods, respectively. The activity of sub-MICs (1/4× and 1/16× MIC) of curcumin on the QS signal molecules was assessed using a reporter strain assay. The influence of sub-MICs of curcumin, azithromycin and gentamicin alone and in combination on motility and biofilm formation was also determined and was confirmed by RT-PCR to test the expression of the QS regulatory genes *lasI*,*lasR*, *rhlI* and *rhlR*.

Results: Addition of curcumin drastically decreased the MIC of azithromycin and gentamicin. Curcumin showed synergistic effects with azithromycin and gentamicin. Treated PAO1 cultures in the presence of curcumin showed a significant reduction of signals C12-HSL and C4-HSL ($P < 0.05$). Sub-MICs (1/4× and 1/16× MIC) of curcumin, azithromycin and gentamicin alone and in combination significantly reduced swarming and twitching motilities as well as biofilm formation. Expression of QS regulatory genes *lasI*, *lasR*, *rhlI* and *rhlR* using 1/4× MIC of curcumin, azithromycin and gentamicin alone and in combination was decreased significantly compared with untreated PAO1.

Conclusions: These results indicate that a combination of sub-MIC of curcumin with azithromycin and gentamicin exhibited synergism against *P. aeruginosa* QS systems.

1. Introduction

Pseudomonas aeruginosa is an opportunistic and nosocomial human pathogen that can cause cystic fibrosis, urinary tract infections, burn infections and many other conditions, especially in immunocompromised patients [1]. The emergence of multidrug-resistant (MDR) *P. aeruginosa* has become a serious problem in healthcare settings in developing countries [2]. Dissemination of antimicrobial resistance genes by horizontal transfer is currently thought to play a major role in development of MDR strains. These MDR isolates are associated with increased mortality and costs owing to prolonged hospitalisation, the need for surgery and prolonged treatment with antimicrobials [2,3]. Some reports have demonstrated that treatment with subinhibitory concentrations of antimicrobials may influence bacterial virulence factors such as adherence, cell surface hydrophobicity, biofilm formation, sensitivity to oxidative stress and motility [4–6].

Macrolides have many important biological characteristics, including antibacterial, antifungal and immunomodulatory activities. Azithromycin is not approved for the treatment of *P. aeruginosa* infections and there are no published breakpoints for this species. Azithromycin minimum inhibitory concentrations (MICs) for *P. aeruginosa* range from 8 μg/mL to 512 μg/mL depending on the strain and the testing procedure. Early studies showed that a sub-MIC of azithromycin suppressed motility and the production of several virulence factors, including proteases, pyocyanin, exotoxin A, phospholipase C and extracellular polysaccharides in *P. aeruginosa*. These antibacterial and antivirulence activities are based on the interaction of azithromycin with the ribosome, so that tightly interwoven effects on bacterial viability

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and production of virulence factors are hardly distinguishable in vivo. Furthermore, a previous study has shown beneficial effects of azithromycin in the treatment of patients with *P. aeruginosa* infections [7].

Quorum sensing (QS), a cell-to-cell density-dependent communication system, plays an important role in the control of virulence factors, antimicrobial resistance and biofilm formation [8,9]. *Pseudomonas aeruginosa* has three distinct QS systems, including Las, Rhl and MvfR (PqsR), mediated by small diffusible signalling molecules called autoinducers, namely 4-hydroxy-2-alkylquinolines and the *N*-acyl homoserine lactones *N*-(3-oxododecanoyl)-l-homoserine lactone (C12-HSL) and *N*-butanoyl-l-homoserine lactone (C4-HSL) [9,10]. These signalling compounds are involved in the production of exoenzymes and the regulation of virulence factors, bacterial adhesion and biofilm formation [9].

As such, quorum-sensing inhibitors (QSIs) diminish *P. aeruginosa* pathogenesis and microbial virulence and could have a major impact on the control and treatment of a wide range of acute and persistent infections [11,12]. Some natural and chemically synthesised compounds have been reported to have QSI activity [10].

Curcumin or diferuloylmethane [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is a natural component of the *Curcuma longa* rhizome [13]. Curcumin has been shown to exhibit antioxidant, anti-inflammatory, antiviral, antibacterial, antifungal, and anticancer activities and thus has a potential against various malignant diseases, diabetes, allergies, arthritis, Alzheimer's and other chronic diseases. These effects are mediated through the regulation of various transcription factors, growth factors, inflammatory cytokines, protein kinases and other enzymes [14]. Looking at the antibacterial activity of curcumin, combination of this compound with antimicrobial therapy can be useful for treatment of resistant *P. aeruginosa*. Previous investigations have been carried out on the biological activities of curcumin, but the combination effect of this natural product with different antimicrobials on the QS system in *P. aeruginosa* has not been demonstrated. Therefore, the present study aimed to investigate the inhibitory activity of sub-MICs of curcumin with azithromycin and gentamicin against *P. aeruginosa* QS-related genes and virulence factors.

2. Materials and methods

2.1. Bacterial strains, growth media and conditions

Pseudomonas aeruginosa PAO1 was used as the wild-type strain for assay of the QSI activities of curcumin, azithromycin and gentamicin. The reporter strains *P. aeruginosa* pME3846 (*rhlI-lacZ*; Tc^r) and *Escherichia coli* MG4/pKDT17 (*lasB*:*lacZ plac-lasR*; Ap^r) were used for the assay of QS signal molecules. The QS-deficient *P. aeruginosa* PAO-JP2 (Δ*lasI*:Tn*10*, Tc^r; Δ*rhlI*: Tn*501-2*, Hg^r) double mutant was used as negative control. Cultivation and enrichment of strains were carried out with Luria Bertani broth/agar (Merck, Frankfurter, Germany) at 37 °C for 16–18 h. All strains were preserved at −70 °C in trypticase soy broth (Merck) containing 20% (v/v) glycerol (Merck).

2.2. Materials

Curcumin (Merck), azithromycin and gentamicin (Sigma, St. Louis, MO) were purchased and stock solutions were prepared according to Clinical and Laboratory Standards Institute (CLSI) criteria [15]. The final concentration of azithromycin and gentamicin stock solutions was 10 mg/mL. A fresh stock solution of curcumin was prepared in dimethyl sulfoxide (DMSO) (Merck) with a final concentration of 1 mg/mL.

2.3. Minimum inhibitory concentration determination of curcumin, azithromycin and gentamicin

MICs of curcumin, azithromycin and gentamicin were determined using the broth microdilution method according to CLSI guidelines [15]. Concentrations below the MIC were considered as subinhibitory concentrations. Two-fold serial dilutions of curcumin, azithromycin (512, 256, 128, 64, 32, 16, 8, 4, 2 and 1 mg/L) and gentamicin (128, 64, 32, 16, 8, 4, 2, 1, 5, 2.5 and 1.25 mg/L) were prepared. Diluted components were inoculated with 0.1 mL of PAO1 overnight culture containing 5×10^6 CFU/mL and were incubated at 37 °C for 24 h. The MIC was calculated as the lowest concentration that inhibited visible growth of the organism.

The synergistic effect of antimicrobials in combination with curcumin was determined by the chequerboard titration method as previously described [16]. The fractional inhibitory concentration (FIC) was calculated as follows:

FIC of curcumin (FIC_a) = concentration of curcumin in combination/MIC of curcumin alone;

FIC of antimicrobials (FIC_b) = MIC of antimicrobials (gentamicin, azithromycin) in combination/MIC of antimicrobials alone.

The fractional inhibitory concentration index (FICI) of the two compounds in the combination was calculated as follows:

$$FICI = FIC_a + FIC_b$$

The interaction was defined as follows: FICI ≤ 0.5, synergism; FICI ≥ 4, antagonism; FICs > 0.5 and ≤1, additive; and 1 < FICs < 4, indifference.

2.4. Estimation of quorum-sensing signal molecules

Supernatant was extracted from overnight cultures of *P. aeruginosa* PAO1 grown in the presence or absence of sub-MICs of curcumin (1/4× and 1/16× MIC), with strain PAO-JP2 used as a negative control. The levels of *N*-acyl homoserine lactones were assessed in treated and untreated supernatant of PAO1 strain by determining β-galactosidase activity using *E. coli* MG4/pKDT17 for the detection of C12-HSL and *P. aeruginosa* pME3846 for C4-HSL as described previously [9]. All assays were performed in triplicate.

2.5. Motility assays

Swarming and twitching motilities were assayed on agar plates containing specialised medium with or without sub-MICs of azithromycin and gentamicin alone and in combination with curcumin as described previously [17].

2.5.1. Swarming

Nutrient broth (8 g/L) (Merck) supplemented with 5% glucose (Merck) was prepared. Azithromycin, gentamicin and curcumin were added at concentrations of 1/4× and 1/16× MIC in combination and alone. The medium was solidified by addition of 0.5% (w/v) Bacto® agar (Merck). Treated and untreated plates were inoculated with 2 μL of the diluted PAO1 culture and were incubated at 37 °C for 16 h.

2.5.2. Twitching

Luria–Bertani agar plates (1% Bacto® agar) with and without sub-MICs (1/4× and 1/16× MIC) of components were prepared. Overnight cultures were stabbed with a sterile toothpick through the agar layer to the bottom of the Petri dish. The plates were then incubated at 37 °C for 48 h. The ability of bacteria to adhere on the