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ORIGINAL ARTICLE

Efficacy of combination oral antimicrobial agents against biofilm-embedded methicillin-resistant *Staphylococcus aureus*

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Background: The combination of fusidic acid and rifampicin has a demonstrated synergistic effect against methicillin-resistant *Staphylococcus aureus* (MRSA), including planktonic and biofilm-related organisms. However, the *in vitro* efficacy of other combinations of oral anti-MRSA antibiotics in biofilm models has not been established.
Methods: The antibacterial activity of fusidic acid, linezolid, rifampicin, and minocycline against 33 biofilm-embedded MRSA isolates in low susceptibility and high resistance breakpoint concentrations was investigated using the 3-[4, 5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium-bromide staining method. The compounds were further examined to determine their antibacterial efficacies in combination. The optical density ratio (ODr) was used to evaluate the antibacterial effects of these antibiotics, and the results indicate higher survival rates of MRSA on biofilm. A biofilm-positive phenotype (determined using the crystal violet stain) was defined as an optical density $\geq$ 0.17 at 492 nm, and strong biofilm formation was defined as an optical density $\geq$ 1.0.

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Results: One-third of the MRSA isolates demonstrated weak biofilm formation, and two-thirds demonstrated strong biofilm formation. At low concentrations, linezolid alone lowered the ODr to 0.55 and was effective against biofilm-embedded MRSA ($p < 0.001$). The activity of minocycline was concentration-dependent and more effective against MRSA isolates that demonstrated weak biofilm formation. The effect of minocycline seems to be further enhanced when used in combination with either fusidic acid or linezolid at low concentrations, with the obtained results equal to those obtained with rifampicin-based regimens ($p < 0.001$). Rifampicin plus minocycline was also effective against MRSA in biofilm.
Conclusion: In comparison with monotherapy, minocycline-based combinations exhibit highly effective bactericidal effects against biofilm-embedded MRSA.

Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) causes a variety of infections, including bacteremia, septic arthritis, osteomyelitis, and artificial graft infections such as those that occur in artificial joints.[1–3] When infected artificial grafts are retained, biofilm-associated infections are very difficult to treat.[4] Consequently, long-term combination oral antibiotic therapies are needed that not only effectively treat biofilm-related infections but also demonstrate few side effects. Some *in vitro* studies on the antibacterial effects of combination therapies have been performed on biofilms.[4–12] Among these, rifampicin has a demonstrated synergistic effect against MRSA, including planktonic and biofilm-related organisms.[3,11,13] In a review of the literature, it was found that minocycline alone is highly active—even more effective than other anti-MRSA antibiotics—against biofilm-embedded MRSA isolates.[14,15] Considering the hepatotoxicity of rifampicin,[16] the development of a minocycline-based combination therapy (such as those that incorporate fusidic acid or linezolid) may be especially important for overcoming the present problem of treating biofilm-associated MRSA infections that require long-term oral antibiotics. In this *in vitro* study, we examined the efficacy of minocycline, in combination with other available oral anti-MRSA agents, for reducing the bacterial burden on biofilms.

Materials and methods

Bacterial isolates

Thirty-three MRSA isolates, including those from blood ($n = 18$), joint fluid ($n = 7$), pus ($n = 5$), and other aseptic specimens ($n = 3$), were randomly obtained from patients with clinical infections from the clinical microbiology laboratory of Chi-Mei Foundational Hospital (Tainan, Taiwan). *Staphylococcus* species were identified by colonial morphology, Gram staining, and coagulase testing. MRSA was further confirmed by tube coagulase testing and growth on a 6 μg/mL oxacillin salt-agar screening plate. The organism was stored at −70°C in Protect Bacterial Preservers (Technical Service Consultants Limited, Heywood, Lancashire, England) before use.

Antibiotics and minimum inhibitory concentrations

The antibiotics that were tested included rifampicin and minocycline (Sigma, St. Louis, MO, USA), linezolid (Pfizer, New York, NY, USA), and fusidic acid (Leo, Ballemp, Denmark). The minimum inhibitory concentrations (MICs) were determined by agar dilution, according to the recommendations from the Clinical and Laboratory Standards Institute (CLSI).[17] Interpretation criteria for susceptibility testing were based on the guidelines from CLSI or the British Society for Antimicrobial Chemotherapy (BSAC).[17,18] The inoculum was 5.0×10^5 colony-forming units (CFU)/mL. The inoculated plates were incubated in ambient air at 37°C for 24 hours. The MIC was defined as the lowest concentration of antibiotic that yielded no visible growth after overnight incubation. *S. aureus* ATCC 29213 was included in each run as the standard quality control strain.

Biofilm formation

Isolates were cultured for 1 day at 37°C in 5 mL of tryptic soy broth that was supplemented with 1% D-glucose (TSBGlc). The cultures were diluted to 1:1000 in TSBGlc, and 200 μL aliquots were added to each well of a 96-well tissue culture-treated polystyrene plate. After 24 hours of growth at 37°C, the plates were vigorously washed three times with phosphate-buffered saline (PBS) to remove any unattached bacteria and then dried for 1 hour at 60°C prior to staining with 0.4% crystal violet solution. The optical density (OD) was used as an index of bacterial adherence to the surface and biofilm formation. Experiments were performed in triplicate, the results were averaged, and standard deviations were calculated. To compensate for background absorbance, OD readings of the sterile medium with both the fixative and dye were averaged and subtracted from all of the experimental values. A biofilm-positive phenotype was defined as $OD \geq 0.17$ at 492 nm (OD_{492}). Strong biofilm formation was classified as $OD_{492} \geq 1.0$, and weak biofilm formation was classified as OD_{492} between 0.17−1.0.[19]

Biofilm staining method

A 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium-bromide (MTT) assay was performed using the method