In vitro efficacy of fosfomycin-containing regimens against methicillin-resistant Staphylococcus aureus in biofilms

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Received 7 October 2011; accepted 18 November 2011

Objectives: To compare the in vitro antibacterial efficacy of antistaphylococcal antibiotics in combination with fosfomycin or rifampicin, using a biofilm model.

Methods: The antibacterial activities of fusidic acid, linezolid, vancomycin, teicoplanin, rifampicin, minocycline, fosfomycin and tigecycline, individually and in fosfomycin or rifampicin combinations, were measured against planktonic or biofilm-embedded methicillin-resistant Staphylococcus aureus (MRSA) with susceptible and resistant breakpoint concentrations (SBCs and RBCs, respectively), using the MTT-staining method and by counting the number of cfu in the biofilms.

Results: Linezolid alone at its SBC, and fosfomycin, linezolid, minocycline and tigecycline at their RBCs, exhibited killing effects on biofilm-embedded MRSA (P<0.0001). Of the eight fosfomycin combinations studied, fosfomycin combined with linezolid, minocycline, vancomycin or teicoplanin at their respective SBCs, exhibited enhanced antibacterial activities (P<0.0001) when compared with the control group, and outperformed rifampicin combinations (P<0.01). The killing effects of fosfomycin combinations at their respective RBCs were better than those at their respective SBCs (P<0.05). Significantly enhanced killing effects were observed with fosfomycin in combination with vancomycin or teicoplanin, compared with vancomycin or teicoplanin alone. For 10 randomly selected MRSA isolates, the results of colony counting in biofilms were comparable with those of the MTT-staining method.

Conclusions: Fosfomycin enhanced the activities of linezolid, minocycline, vancomycin and teicoplanin. These combinatorial treatments were even better than rifampicin combination regimens, and may provide therapeutic advantages in catheter-related or prosthetic joint infections.

Keywords: combination effect, biofilm-embedded MRSA, S. aureus

Introduction

Methicillin-resistant Staphylococcus aureus (MRSA) is a common nosocomial pathogen, especially in intensive care units.1–3 MRSA isolates are increasingly recognized as community-acquired pathogens4–6 and lead to a variety of infections, including bacteremia, infective endocarditis, septic arthritis, osteomyelitis and prosthetic joint and artificial graft infections,7,8 which can cause significant morbidity and mortality. Artificial graft infections are difficult to cure and require long-term antibiotics. Therefore, treatment failure and recurrent diseases are common in patients whose infected grafts are not removed.

Recently, there has been increasing evidence indicating the poor efficacy of vancomycin in treating MRSA infections. Two factors may contribute to the failure of vancomycin treatment in MRSA infections. First, MICs of vancomycin for clinical MRSA isolates have steadily increased in recent years (from 0.25 mg/L to 2.0 mg/L9–11), making it difficult to treat MRSA infections. Therefore, several combination regimens have been proposed, to overcome the therapeutic shortcomings of vancomycin.1,14–17
Notably, treatment failure may be related to biofilm formation. Several reports have focused on the in vitro antimicrobial effects of combination therapies for biofilm-embedded microorganisms.

Rifampicin has been a constituent of the combinations active against MRSA, and as a component in antibiotic therapy directed against the biofilms formed by these organisms. Given the hepatotoxicity of rifampicin, the development of alternative regimens will be important in treating biofilm-associated MRSA infections. The effects of combined antimicrobial agents other than glycopeptides on biofilm-associated organisms have not been completely elucidated. Fosfomycin was initially FDA-approved for Escherichia coli and Enterococcus urinary tract infections. Previous studies have characterized the antibacterial activity of fosfomycin against MRSA in vitro, and fosfomycin-containing combination regimens have been shown to treat MRSA infections effectively. The effects of fosfomycin combined with vancomycin or other agents have also been investigated using experimental endocarditis or meningitis models. A fosfomycin combination with ciprofloxacin exhibited a synergistic effect on Pseudomonas aeruginosa in biofilms. Therefore, fosfomycin combination regimens may overcome the therapeutic barriers to treating biofilm-associated MRSA infections. However, no in vitro studies of the effects of fosfomycin on biofilm-embedded MRSA have been published. The effects of several anti-MRSA agents, alone or in combination with fosfomycin, on bacterial colony counts in biofilms were evaluated in the current study.

Methods

Bacterial strains

A total of 33 MRSA isolates from clinical specimens, including blood, joint fluid and other aseptic specimens, were randomly obtained from the clinical microbiology laboratory of the Chi-Mei Foundational Hospital. Staphylococcus species were identified based on colony morphology, Gram's stain morphology and coagulase test results. MRSA was further identified by Staphylococcus aureus phenotype was defined as OD$_{492}$ nm at 0.17. The experiments were performed in triplicate, the results were averaged, and the standard deviations were calculated.

Antibiotics

Eleven drugs with potent anti-MRSA activities were tested for MICs using the agar dilution method according to the CLSI recommendations. Standard powders of vancomycin, rifampicin, minocycline and trimethoprim/sulfamethoxazole were purchased from Sigma (St Louis, MO, USA). The following reagents were kindly provided by their manufacturers: linezolid, clindamycin and tigecycline from Pfizer (New York, NY, USA); teicoplanin from Sanofi-Aventis (Bridgewater, NJ, USA); fosfomycin from Ercros (Barcelona, Spain); fusidic acid from Leo (Ballemp, Denmark); and ciprofloxacin from Bayer (Leverkusen, Germany). The interpretation criteria of the susceptibility results were based on the CLSI or BSAC guidelines.

Glucose-6-phosphate (25 mg/L) was used for the susceptibility testing of fosfomycin. The inoculum of each isolate contained $10^8$ cfu/mL. The inoculated plates were incubated at 37°C for 24 h. MTT was then added, and the suspension was kept at 37°C for 2 h. MTT was then replaced with 100 μL of PBS and incubated at room temperature for 15 min. Viable bacteria reduces tetrazolium to the water-soluble, purple formazan form. The colour intensity of formazan was determined for background absorbance, the OD of a sterile medium with fixative and dye was recorded and subtracted from the results. A biofilm-positive phenotype was defined as OD $>0.17$ at 492 nm. The experiments were performed in triplicate, the results were averaged, and the standard deviations were calculated.

Biofilm formation

The strains were cultured for one day at 37°C in 5 mL of tryptic soy broth supplemented with 1% d-glucose (TSBGlc). The cultures were diluted 1:1000 in TSBGlc, and 200 μL of the final solution was added to each well of a 96-well tissue culture-treated polystyrene plate. After 24 h of growth at 37°C, the plates were washed vigorously three times with PBS to remove unattached bacteria and dried for 1 h at 60°C prior to staining with 0.4% Crystal Violet. The optical density (OD) was obtained as an index of adherent bacteria and biofilm formation. To compensate for background absorbance, the OD of a sterile medium with fixative and dye was recorded and subtracted from the results. A biofilm-positive phenotype was defined as OD $>0.17$ at 492 nm. The experiments were performed in triplicate, the results were averaged, and the standard deviations were calculated.

Biofilm MTT-staining method

The MTT assay was performed using the method of Kairo et al. with minor modifications. Briefly, after antibiotic treatment, the wells were emptied and washed three times with PBS. Then, 100 μL of PBS containing 1% MTT (Sigma, St Louis, MO, USA) was added, and the suspension was kept at 37°C for 2 h. MTT was then replaced with 100 μL of DMSO and incubated at room temperature for 15 min. Viable bacteria reduces tetrazolium to the water-soluble, purple formazan form. The colour intensity of formazan was determined using a microplate spectrophotometer at 540 nm. Higher OD values correlated to higher numbers of viable MRSA isolates in the biofilm.

Minimum biofilm eradication concentration (MBEC)

The MBE assay was performed using a 96-well ELISA platform as previously described. In brief, the assay involved biofilm formation on plastic pegs on the lid of the MBE plate. These biofilms were exposed to antibiotics for 24 h, placed in a second 96-well plate containing fresh Mueller–Hinton broth, and incubated overnight. The MBE was the lowest dilution that prevented bacterial regrowth after antibiotic treatment.

Time–kill studies

Among the 33 MRSA clinical isolates examined, 8 genetically unrelated isolates with different PFGE patterns (data not shown) were randomly selected for the time–kill study. Time–kill experiments were performed according to the CLSI methodology. In brief, bacterial suspensions were diluted to inocula of $\sim 1 \times 10^8$ cfu/mL in 25 mL of fresh Mueller–Hinton broth. The concentrations of drugs other than tigecycline were adjusted to the susceptible breakpoint concentrations (SBGs) recommended by CLSI and BSAC (for fusidic acid only). Specifically, the following concentrations were used: rifampicin, 1 mg/L; fosfomycin, 64 mg/L; minocycline, 4 mg/L; fusidic acid, 1 mg/L; linezolid, 4 mg/L; vancomycin, 2 mg/L; and teicoplanin, 8 mg/L. For tigecycline, the FDA-approved susceptible interpretative criterion of (0.5 mg/L) was used. Each drug, alone or in combination with rifampicin or fosfomycin, was tested by time–kill studies. Bacterial counts were measured at 24 h by counting the colonies in 10-fold serially diluted 100 μL aliquots plated on nutrient agar (BD Biosciences, Franklin Lakes, NJ, USA) and incubated at 37°C. Synergy was defined as a $>100$-fold decrease in cfu/mL between the combination and its most active constituent after 24 h, with the number of surviving organisms in the presence of the combination $>100$-fold cfu/mL below the starting inoculum. Bacteriostatic and bactericidal activities were defined as $<1000$-fold and $>1000$-fold reductions in cfu/mL relative to the starting inoculum, respectively, at 24 h. All experiments were performed twice.
Killing effects of antimicrobial agents in the biofilms

The biofilms of each isolate were prepared in 24-well culture plates (as above). The media in the well was removed by aspiration, and the biofilm in each plate was treated with various concentrations of individual antibiotics (fusidic acid, linezolid, vancomycin, teicoplanin, rifampicin, minocycline, fosfomycin or tigecycline), or in combination with fosfomycin or rifampicin. The antibiotic-containing medium was gently aspirated after 24 h and washed with PBS three times. Fresh antibiotic-containing medium was added to the wells. At 1, 3 and 5 days after the antibiotic treatment, bacterial viability was determined by the MTT assay.24 For each drug, the experiment was performed at two drug concentrations: a (low) SBC and a (high) resistant breakpoint concentration (RBC), based on the recommendations of the CLSI31 (or BSAC33 for fusidic acid). For tigecycline, we arbitrarily set 1 mg/L as the RBC. The RBCs for other antibiotics were 2 mg/L for fusidic acid, 256 mg/L for fosfomycin, 8 mg/L for linezolid, 16 mg/L for vancomycin, 32 mg/L for teicoplanin, 4 mg/L for rifampicin and 16 mg/L for minocycline.

To quantify the degree of the inhibition of biofilm-embedded MRSA by the tested antibiotics, OD ratios (ODrs) were calculated (OD values in the presence of drugs divided by those in the absence of drugs). Likewise, the average OD_{560} (formazan concentration) of the 33 MRSA isolates treated with an antibiotic, alone or in combination, was compared with that of the matched control strain on the 5th day of incubation with antibiotics, as shown in Figure 3. Based on these antibiotic agents were excluded from further in vitro tests.

Results

The susceptibility, MIC_{50}, MIC_{90}, MBEC_{50} and MBEC_{90} of different antibiotics for planktonic or biofilm-embedded MRSA isolates are shown in Table 1. Because the MICs of clindamycin, trimethoprim/sulfamethoxazole and ciprofloxacin were high, these antibiotic agents were excluded from further in vitro tests.

When 8 MRSA isolates at an inoculum of 1×10^6 cfu/mL were incubated with rifampicin at its SBC, the inhibitory activity lasted for 24 h and the colony count decreased to 10^2–10^3 cfu/mL (Figure 1). When cultured with fosfomycin, there was no inhibitory effect. However, with rifampicin or fosfomycin combinations, all colony counts decreased to 10^2–10^3 cfu/mL (Figure 1). The inhibitory activity of both groups was almost the same at 24 h in our time–kill study results. A synergistic effect was only found when fosfomycin was added in combination with minocycline, fusidic acid or vancomycin at their SBCs.

To check the MIC changes for MRSA isolates in the biofilm model after fosfomycin or rifampicin monotherapy, three fosfomycin-susceptible MRSA isolates were randomly selected. After 5 days of fosfomycin treatment at the SBC, the fosfomycin MICs of the three isolates had not changed. However, the rifampicin MICs of biofilm-embedded MRSA isolates significantly increased, from 0.0156 to >4 mg/L, after 5 days of rifampicin monotherapy at the SBC.

The ODrs of the biofilms of each of the 33 MRSA isolates after treatment with individual antibiotics at their RBCs for 1, 3 and 5 days are shown in Figure 2. Monotherapy with linezolid, teicoplanin, minocycline, fosfomycin or tigecycline showed a gradual decline in OD. In addition, the ODrs gradually decreased for all the fosfomycin combinations, as shown in Figure 3. Based on these results, we chose OD at the fifth day as the endpoint for the in vitro experiments.

At its SBC, linezolid caused a significant reduction in colony count in the biofilm (OD 0.55, P < 0.0001) (Table 2). The combinations of fosfomycin with linezolid, vancomycin, teicoplanin or minocycline decreased the colony counts of biofilm-embedded

<table>
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<tr>
<th>Drugs</th>
<th>MIC_{50}, mg/L</th>
<th>MIC_{90}, mg/L</th>
<th>susceptibility, %</th>
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<td>27</td>
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<td>100</td>
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<tr>
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<td>Vancomycin</td>
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were applied for multiple comparisons. The statistical significance was set at P value <0.05.
MRSA (ODrs 0.14–0.24, \(P\), \(0.0001\)). However, the in vitro killing effects of the combinations of fosfomycin with linezolid, vancomycin, teicoplanin or minocycline were similar to each other, but were better than those of the equivalent rifampicin combination regimens (\(P\), \(0.01\)) (Table 2).

Eight antibiotics were tested at their RBCs (Table 2). Linezolid, minocycline, fosfomycin or tigecycline alone significantly reduced the colony counts of MRSA in the biofilm after 5 days of treatment (\(P\), \(0.0001\)). All of the fosfomycin combination regimens greatly reduced the growth of MRSA in the biofilm (ODr 0.06–0.20). The ODr of fosfomycin, combined with vancomycin or teicoplanin, was 0.06 and 0.07, respectively. The in vitro killing effects of the combinations of fosfomycin with linezolid, vancomycin, teicoplanin or fusidic acid, were better than those of the equivalent rifampicin combination regimens (\(P\), \(<0.01\)). Not surprisingly, the ODrs of each combination at the RBCs were significantly lower than those at the SBCs (\(P\), \(0.05\)).

To further investigate the in vitro antibacterial effects of combination regimens against MRSA in a biofilm, 10 of the 33 MRSA isolates were randomly selected for colony counting. Compared with the controls, linezolid or minocycline alone at their SBCs induced a 100-fold decrease in colony count (Figure 4). Furthermore, fosfomycin combined with linezolid or minocycline at their SBCs enhanced the killing effects, compared with linezolid or minocycline alone (\(P\), \(<0.05\)). These results are consistent with our ODr studies, which revealed the potential enhanced capacity of fosfomycin in combination with either drug. Moreover, fosfomycin in combination with vancomycin or teicoplanin greatly decreased the colony count (100- to 1000-fold compared with vancomycin, teicoplanin or fosfomycin monotherapies, \(P\), \(<0.0001\)).

In the current study, the killing effects of fosfomycin- and rifampicin-based combinations against planktonic MRSA in the time–kill studies or the biofilm studies were similar. The effects of fosfomycin combinations in the biofilm model were better than those of rifampicin combinations. There were no significant differences between the two methods used to assess the in vitro killing effects of fosfomycin in combination with linezolid, vancomycin, teicoplanin or fosfomycin alone (\(P\), \(0.09\)). The results of the two methods were highly concordant, with a correlation coefficient of 0.731 (\(P\), \(=0.0003\)).

**Discussion**

The present study revealed significant differences in the in vitro activities of antimicrobial agents against planktonic and
biofilm-embedded MRSA isolates. Linezolid at its SBC effectively reduced the colony count of biofilm-embedded MRSA. Fosfomycin, minocycline and tigecycline at their RBCs were also effective, based on the results of the MTT method. High concentrations of antibiotics were necessary to treat biofilm-related infections, according to a previous study. In addition, our results indicate that using RBCs as opposed to SBCs improves the killing effects of the antibiotics we tested. These results imply that the killing effects of fosfomycin, minocycline and tigecycline on biofilm-embedded MRSA are concentration- and dose-dependent, and as such, high doses should be used to prevent treatment failure and resistance.

According to one literature review, when fosfomycin was given at a dose of 2 g, serum $C_{\text{max}}$ reached 202 mg/L, which far exceeds the SBC of fosfomycin that we used in this study (64 mg/L). The enhanced killing effect of fosfomycin was noted at SBC in our results. Clinically, high concentrations of antibiotics are probably toxic and not feasible. Therefore, fosfomycin taken at recommended doses as part of a combination regimen may be practical.

In the present study, treatments with fosfomycin in combination with linezolid, vancomycin, teicoplanin or minocycline at their SBCs exhibited enhanced antibacterial activities against biofilm-embedded MRSA. This combinatorial effect appears better than that for the rifampicin combinations tested in our study. Using the more sensitive colony counting method, treatment with fosfomycin in combination with vancomycin or teicoplanin exhibited dramatically enhanced effects compared with vancomycin, teicoplanin or fosfomycin alone, which were
infective. The combined effects of these treatment regimens may be synergistic. The in vitro results suggest that fosfomycin in conjunction with a glycopeptide may be a feasible alternative to treat MRSA-associated biofilm infections.

Several mechanisms have been proposed to explain why only some antibiotics are active against biofilms. Antibiotics of low molecular weights are likely to diffuse more efficiently into the biofilms. The low molecular weight of fosfomycin (194.1 Da) may partially explain its enhanced antibacterial activity against biofilm-related organisms. Another possible explanation for the enhanced activity may be the combined effects of two agents with different mechanisms of antibacterial action. When the treatment duration was taken into consideration, the killing effect of antibiotic treatment for 5 days was higher than for 1 day, with the exception of rifampicin alone, as shown by this study. Therefore, it is likely that longer treatment and high doses may lead to a better therapeutic outcome in the treatment of biofilm-related infections, such as catheter-related or prosthetic joint infections.

The MTT staining method for evaluating the antibacterial activities of antimicrobial agents in the biofilm model has been used in many previous studies. The results of MTT staining and cfu counting in the biofilm model agreed with our previous study. The MTT method provided rapid results within a few hours. Though there might be a concern that antibiotics increase the MTT signal in the biofilms, the influence of the drugs will be minimized by the PBS wash (three times) before the measurement of OD. In our current study, the MTT method was used as a screening method for the quantification of viable biofilm-embedded bacteria. The MTT method and the colony-counting method should be used for accurate evaluation of the effects of treatment regimens on biofilm-embedded bacteria.

In the present study, regimens of fosfomycin combined with linezolid, minocycline, vancomycin or teicoplanin exhibited enhanced antibacterial activity against MRSA isolates in biofilms, compared with the rifampicin-based combinations. Thus, fosfomycin combinations may be clinically effective alternatives for treating patients with biofilm-associated MRSA infections. These results are important for catheter-related or prosthetic joint infections for which the foreign bodies cannot be removed. Interest in the step-down approach to antimicrobial therapies has recently increased because of the increasing prevalence of vancomycin-intermediate or even vancomycin-resistant S. aureus. Developing effective combination therapies for treating MRSA biofilms is critically important to avoid the overuse of the super anti-MRSA drug daptomycin, and in light of the continuous elevation of the vancomycin MIC.

In conclusion, we demonstrated enhancement of the antibacterial activities of several anti-MRSA antibiotics, including linezolid, vancomycin, teicoplanin and minocycline, in combination with fosfomycin against MRSA in biofilms. Animal experiments and clinical studies are required to validate the incorporation of fosfomycin combination regimens into the clinical management of MRSA biofilm infections.

Acknowledgements

We wish to acknowledge Po-Ren Hsueh (the microbiologist and infectious disease specialist of Taiwan University Hospital) for his critical review of this article prior to submission and the members of the Research Laboratory of Infectious Diseases of the Chi-Mei Medical Center for their assistance in the statistical analyses of these data.

Funding

This study was supported by grants from the Chi-Mei Medical Center Research Foundation (CMFHR9901), the National Science Council Taiwan (NSC 96–2628-B-006-004-MY3) and the Department of Health, Executive Yuan (DOH100-TD-B-111-002).

Transparency declarations

None to declare.

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