Daptomycin, Fosfomycin, or Both for Treatment of Methicillin-Resistant Staphylococcus aureus Osteomyelitis in an Experimental Rat Model

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The in vivo activities of daptomycin, fosfomycin, and a combination of both antibiotics against a clinical isolate of methicillin-resistant Staphylococcus aureus (daptomycin MIC, 0.25 μg/ml; fosfomycin MIC, 0.5 μg/ml) were evaluated in a rat model of osteomyelitis. A total of 37 rats with experimental osteomyelitis were treated for 4 weeks with either 60 mg/kg of body weight of daptomycin subcutaneously once daily, 75 mg/kg fosfomycin intraperitoneally once daily, a combination of both drugs, or a saline placebo. After the completion of treatment, animals were euthanized, and the infected tibiae were processed for quantitative bacterial culture. Bone cultures were found to be positive for methicillin-resistant S. aureus in 9 of 9 (100%) animals of the placebo group, in 9 of 9 (100%) animals treated with daptomycin, in 1 of 10 (10%) fosfomycin-treated rats, and in 1 of 9 (22.2%) rats comprising the combination group. Results of bacterial counts in the bone samples were expressed as log10 CFU/g of bone and analyzed by using the Mann-Whitney U test followed by Bonferroni’s multiple-comparison test. Based on bacterial counts, treatment with daptomycin was significantly superior to placebo, although it remained inferior to treatment with fosfomycin. No synergistic or antagonistic effect was observed for the combination therapy. No development of resistance against daptomycin or fosfomycin was observed after the 4-week treatment period.

The emergence of glycopeptide tolerance and resistance to vancomycin represents a major concern in the treatment of deep-seated infections, including osteomyelitis, since those drugs have been the reference standard therapy for complicated methicillin-resistant Staphylococcus aureus (MRSA) infections for decades (31). Daptomycin (DAP) is a cyclic lipopeptide antibiotic with rapid in vitro bactericidal activity against a broad range of Gram-positive bacteria, including isolates resistant to methicillin or vancomycin (7). In laboratory studies on the effectiveness of DAP for the treatment of MRSA osteomyelitis, DAP was as effective as vancomycin (19, 34).

Clinical experience with DAP treatment of human osteomyelitis is limited, but case reports and retrospective analyses support its efficacy and safety for the treatment of osteomyelitis (11). Although these reports are encouraging, the emergence of antimicrobial resistance associated with the prolonged administration of DAP or preceding treatment with glycopeptides should be considered seriously (11). There remains an urgent need for new or old antibiotics with high efficacy and favorable side effect profiles for long-term therapy against bacteria with multiple-drug resistance.

Fosfomycin (FOF) is a well-tolerated bactericidal agent with longstanding sensible clinical use in a wide range of patient populations (30). FOF displays broad-spectrum activity against various Gram-positive and distinct Gram-negative bacteria, including difficult-to-treat pathogens such as MRSA, DAP-resistant S. aureus, penicillin-resistant pneumococci, and extended-spectrum-β-lactamase-producing Enterobacteriaceae (10, 14). FOF has unique pharmacological characteristics and penetrates well into osseous tissue (38). It has proved to be clinically useful for the treatment of acute and chronic osteomyelitis (21, 30). In a previous study, we have shown that FOF is highly effective in the treatment of experimental MRSA osteomyelitis (29). However, in the clinical routine, FOF is used mainly in combination with other classes of antibiotics because of the synergism frequently observed and the concern about the development of resistance (10). The clinical relevance of these findings, however, has been questioned recently (3, 20, 40).

Data on the combination of DAP and FOF are limited, and little is known about its effectiveness in osteomyelitis. The aim of the present study was to evaluate the efficacy of such treatment in an experimental rat model of chronic MRSA osteomyelitis.

The study was presented in part at the 50th Interscience Conference on Antimicrobial Agents and Chemotherapy, Boston, MA, 12 to 15 September 2010 [28a] and at the 14th Scientific Meeting of the European Society of Chemotherapy/Infectious Diseases, Vienna, Austria, 8 to 11 December 2010 [29a].
TSB and incubated for 5 h at 37°C. A bacterial inoculum containing 1 × 10^8 to 5 × 10^10 CFU/ml was prepared, and the number of viable organisms was retrospectively confirmed from plate counts made before and after the surgical procedure.

**Biochemical susceptibility testing.** MICs of DAP and FOF for MRSA were determined by using a broth microdilution method with cation-adjusted Mueller-Hinton broth (CAMHB) (75 mg/liter Ca^2+ - 25 mg/liter Mg^2+), according to CLSI guidelines (5a). For the testing of FOF activity, CAMHB was supplemented with glucose-6-phosphate (Sigma-Aldrich) at a final concentration of 25 mg/liter (4).

**Experimental osteomyelitis.** Infection of bone was established by using a modification of a model described previously by Zak et al. (44). In total, 50 male Sprague-Dawley CD rats (Charles River WIGA GmbH, Sulzfeld, Germany) weighing 350 to 400 g were used. Each animal was anesthetized with ketamine and xylazine, and the left hind leg was shaved and disinfected with polyvinyl pyrrolidine-iodine. The proximal medial surface of the tibia was surgically exposed, and a hole (0.1-cm diameter) was made in the medullary cavity using a high-speed drill (Multipro; Dremel, Racine, WI). A 20-μl sample of the MRSA inoculum containing 1 × 10^8 to 5 × 10^10 CFU/ml was injected into the bone. Drill holes were sealed with sterile bone wax (Ethicon Sutures Ltd., Peterborough, Ontario, Canada) to avoid a leakage of the inoculum, and the incisions were closed with sutures. Sclerosing agents to facilitate the onset of osteomyelitis were not used in this study.

**Antibiotic treatment regimens.** After an infection period of 4 weeks, animals with radiographically confirmed osteomyelitis of the tibia were randomly assigned to one of the following four treatment groups: (i) DAP alone, (ii) FOF alone, (iii) the two drugs in combination, and (iv) saline placebo. DAP powder (Cubist Pharmaceuticals, Lexington, MA) was dissolved in sterile water and administered subcutaneously at a dose of 60 mg/kg of body weight once daily. FOF powder (Sandoz, Kundl, Austria) was dissolved in sterile water and administered subcutaneously at a dose of 75 mg/kg of body weight once daily. All treatments were administered daily for 4 weeks.

The rationale for the treatment regimens was to achieve serum levels of drug mimicking those obtained in humans based on the literature (5, 22–24, 32). Twelve hours after the completion of antimicrobial therapy, the rats were radiographed again and then euthanized with a lethal dose of thiopental.

**Bacteriological counting.** The infected tibiae were aseptically removed, weighed, and pulverized. Sterile physiological saline (10 ml) was added to each specimen, and the bone suspensions were vigorously vortexed. Serial 10-fold dilutions were prepared, and bacterial counts were made by plating 20 μl of each dilution onto sheep blood agar plates. After incubation for 24 h at 37°C, the colonies of MRSA were counted, and results were expressed as log_{10} CFU/g of bone. The entire remaining specimen was placed into 10 ml TSB and incubated for 48 h in 5% CO₂ at 37°C. The bone marrow was removed aseptically with a sterile bone wax (Ethicon Sutures Ltd., Peterborough, Ontario, Canada) to avoid leakage of the inoculum, and the incisions were closed with sutures. Sclerosing agents to facilitate the onset of osteomyelitis were not used in this study.

**Histopathology.** For the histological confirmation of chronic osteomyelitis, bone specimens from four further animals underwent methylmethacrylate or classic embedding. Semithin sections were colored with Giemsa stain; thin sections were stained with hematoxylin and eosin.

**Statistics.** Experimental results were plotted and analyzed using GraphPad Prism, version 5.02 (GraphPad Software Inc.). The Mann-Whitney U test followed by Bonferroni’s multiple-comparison test was used to assess the significance of bacterial clearance and weight variances between the study groups. Differences between the groups were deemed statistically significant if the P value was < 0.05.

**RESULTS**

**Experimental osteomyelitis.** Six out of 50 rats died during anesthesia and were not further analyzed. Four weeks after infection with MRSA, 41 of the 44 infected rats (>93%) had radiographically confirmed localized proximal osteomyelitis of the tibia.

The histology of all embedded specimens from 4 of the 41 rats demonstrated chronic osteomyelitis with osseous destruction, limited inflammatory cortical ruptures, isolated intramedullary microabcesses, some small colonies of cocci and bone repair with osseous remodeling, granulation tissue, and bone marrow fibrosis (Fig. 1).

The quantitative results of antibiotic treatment of experimental MRSA osteomyelitis are shown as median log_{10} CFU/g of bone in Fig. 2. In the control group [(n = 9), the median bacterial count (minimum to maximum) was 6.67 log_{10} CFU/g of bone (4.66 log_{10} to 7.35 log_{10} CFU/g)]. Treatment with DAP [(n = 9)] resulted in a median bacterial count of 5.13 log_{10} CFU/g of bone (3.37 log_{10} to 6.00 log_{10} CFU/g), which was significantly superior to that of the control group (P = 0.001). Treatment with FOF (n = 10) resulted in sterilized bones in 9 out of 10 animals; the bacterial count in the remaining animal was 3.25 log_{10} CFU/g of bone. Treatment with the combination of DAP and FOF (n = 9) resulted in sterilized bones in eight out of nine animals; the bacterial count in the remaining animal was 3.82 log_{10} CFU/g of bone.

The results of treatment with FOF at 75 mg/kg of body weight once daily and with the combination of FOF at 75 mg/kg of body weight once daily and DAP at 60 mg/kg of body weight once daily were thus significantly superior to results for the placebo group and the DAP group (P = 0.001). The combination of FOF and DAP was not superior to FOF alone (P = 1.0).

Radiographic evaluation revealed no significant improvement in any treatment group. There were no significant differ-
ences in the severities of osteomyelitis among the four treatment groups before or after the antibiotic treatment (data not shown).

**MICs and development of drug resistance.** DAP and FOF showed *in vitro* activity against MRSA 4409/07 with MICs of 0.25 and 0.5 μg/ml, respectively.

Isolates of *S. aureus* obtained from all rats still infected at the end of the 4-week treatment period were tested for antibiotic sensitivity to DAP and FOF. The MICs were within 1 dilution of the pretreatment MIC, indicating no emergence of resistance with monotherapies or combination therapy.

**Adverse events.** When used as monotherapy, both study drugs were well tolerated. In the drug combination group, all animals developed diarrhea beginning 1.5 to 2 weeks after the commencement of antibiotic treatment. The median weight gains (minimum to maximum) during the 4-week treatment period for the animals in the control group, the DAP group, the FOF group, and the combination group were 45 g (32 to 68 g), 44 g (25 to 65 g), 53 g (26 to 98 g), and 19 g (~5 to 32 g), respectively. The median weight gain for the combination group was significantly lower than those for the other groups (*P* < 0.005).

**DISCUSSION**

Combination therapy with FOF has been shown to enhance the antimicrobial effects of various antibiotics against MRSA (25). For the combination of DAP and FOF, a high percentage of synergism against Gram-positive bacteria has been found, but indifference has also been described (9). However, an *in vitro* evaluation of possible synergism between antibiotics may not necessarily be predictive of results observed for animals or humans. For example, in a rat model of endocarditis, Rice et al. found previously that there was no difference between the number of valves sterilized by DAP alone and the number sterilized by DAP plus FOF, despite the synergistic bactericidal activity found by *in vitro* time-kill studies (32).

The discrepancy between *in vitro* results and observations of animal studies is not surprising, because in deep-seated infections such as endocarditis or osteomyelitis, the concentrations of antibiotics at the target sites are difficult to determine and may differ considerably from serum levels. Furthermore, *in vitro* results cannot reflect additional immunomodulatory properties of antibiotics, such as those demonstrated previously for FOF (22).

The rat model of chronic *S. aureus* osteomyelitis is well established and has been extensively used for the evaluation of antibiotics (6). In most of the models, the onset of osteomyelitis is facilitated by additional tissue manipulation with sclerosing agents or by the incorporation of foreign bodies, fibrin glue, or agar gel (6). In the present study, the absence of additional tissue manipulation provided the best possible analogy with human disease. This is of particular importance for the evaluation of antimicrobial therapy, because sclerosing agents impair bone marrow circulation and consequently may hamper drug delivery to the target site in the treatment phase. In our study, osteomyelitis was reliably induced with an infection rate of >93%. Considering the absence of preceding tissue manipulation, the high infection rate in our model was most probably due to the high pathogenicity of the MRSA strain used. In addition, the use of a high-speed drill, which was shown previously to facilitate infection due to local heat necrosis, may have contributed to the high infection rate (33). Nevertheless, high-speed drills are widely used in orthopedic and trauma surgery, and thus, the use of such a drill did not adulterate the clinical situation.

In the present study, the osteomyelitis model was used to evaluate the efficacy of a combination of FOF and DAP in osseous tissue. We have previously shown that FOF monotherapy is highly effective in experimental MRSA osteomyelitis (29): when administered at a dosage of 150 mg/kg, FOF eradicated MRSA in seven out of nine rats. To evaluate a possible synergistic effect between DAP and FOF *in vivo*, the dosage of FOF in the present study was lowered to 75 mg/kg once daily.

DAP was used at a dosage of 60 mg/kg once daily. For the therapy of osteomyelitis in humans, DAP treatment regimens of 4 to 6 mg/kg once daily are currently used, although the use of a higher dose (>6 mg/kg) may improve outcome rates (15). For rats, Sakoulas et al. demonstrated that the subcutaneous administration of DAP at 25 and 40 mg/kg once daily resulted in maximum drug concentrations and total drug exposures in serum that approximated those observed for humans given 4 and 6 mg/kg intravenously once daily (37).

Nevertheless, in the present study, DAP treatment was of only moderate benefit compared with the results from the placebo group. This is remarkable, because in several studies with animal models of MRSA osteomyelitis, DAP was shown to be as effective as vancomycin (18, 19, 34), the drug of choice for the treatment of MRSA osteomyelitis.

Like glycopeptides, DAP has a large molecular size, and the level of plasma protein binding is high, at ~92% (42). These characteristics impair the plasma-to-tissue drug exchange rate, resulting in delayed or incomplete drug equilibrium between compartments. In line with this, previous experimental data on DAP concentrations in infected bone were discouraging. For example, Mader et al. previously evaluated daptomycin in a rabbit model of MRSA osteomyelitis (19). In rabbits, the half-life of DAP is similar to that observed for humans, and no adjustment of DAP dosing is necessary (19). After a single subcutaneous dose of 4 mg/kg, DAP was undetectable in uninfected bone, and total concentrations in infected bone were as low as 0.5 mg/liter at 60 min.

In contrast, by using a microdialysis technique, Traumuller et al. found previously that free DAP in plasma equilibrates completely with soft tissues and bone tissue within 2 h and that inflammation did not affect concentrations of DAP at the target site (42). However, it should be noted that there is substantial variability in the reported bone penetration of antibiotics in different studies because of the widely differing methods used for sample preparation and drug analysis (16).

The debatable penetration of DAP into osseous tissue raises important clinical issues in relation to recent reports linking the development of bacterial resistance to subinhibitory concentrations of antibiotic at the target site (12). However, in the present study, at the end of the 4-week treatment period with DAP monotherapy, no emergence of resistance had occurred. Our findings strongly suggest that spontaneous DAP resistance *in vivo* is rare, even in deep-seated infections such as osteomyelitis, and are consistent with previous reports of a low frequency of spontaneous DAP resistance in *S. aureus* (7, 17, 37). Similarly, several reports have suggested that the development
of resistance to DAP may be linked to a prolonged preceding administration of vancomycin or other glycopeptide antibiotics (8, 27, 36), although this issue is discussed controversially (1, 35).

Surprisingly, FOF treatment in our study, even in a low-dose regimen, resulted in the sterilization of MRSA-infected bone in 9 out of 10 rats. Thus, FOF treatment was so effective that it was impossible to demonstrate a possible synergistic effect when combined with DAP. It is noteworthy that no antagonistic effect was observed.

In contrast to the results of the microbiological evaluation in our study, radiology results revealed no significant differences in the severities of osteomyelitis between the treatment groups. This is not surprising, because radiographic results reflect the rate and extent of bone reconstruction and remodeling, which in osteomyelitis always lag behind bacterial clearance (2, 43).

The absence of emerging resistance against FOF after 4 weeks of low-dose therapy is another key finding of the present study. FOF has been used in combination and as monotherapy in a wide range of clinical settings for several decades (30), but data from in vitro studies have shown that a drawback of FOF is that it allows the emergence of resistant clones at a high frequency (13, 25). However, recent studies have demonstrated that the development of chromosomal resistance to FOF entails a biological cost that reduces the virulence and biological fitness of pathogens (3, 20, 40).

In the present study, no development of increased resistance could be detected in samples of S. aureus recovered after FOF treatment. Considering the relatively long treatment period of 4 weeks, our data support the observation that the development of clinically relevant FOF-resistant strains in vivo is rare (10, 26, 39).

Nevertheless, this finding is in clear contradiction to those of a study by Thauvin et al., where FOF was evaluated in experimental MRSA endocarditis in rats. The rate of emergence of resistance was 36% when FOF was used as a single agent at a dosage of 500 mg/kg of body weight once daily for 6 days (41). No resistance developed when FOF was combined with pefloxacin. Thus, high concentrations at the target site and a short treatment period were not beneficial in avoiding resistance to FOF in vivo. However, the study period of 6 days was presumably too short to observe the effects of reduced vitality in bacteria with chromosomal resistance to FOF. The assumption of a fitness loss in FOF-resistant bacteria may thus serve as a conclusive explanation for the lack of development of FOF resistance after the 4-week treatment period in the present study.

The limitations of the study should be noted. First, all experiments were performed with the same single clinical strain of MRSA. Second, osteomyelitis is a heterogeneous disease with a great variety of disease presentations and pathophysiologies, and no single model encompasses all aspects of osteomyelitis (28). Thus, the data obtained in the present study should not be extrapolated to more subacute or chronic models of osteomyelitis or to models with other anatomic locations of the disease.

In summary, in our study of experimental MRSA osteomyelitis in rats, DAP was significantly superior to placebo, although the benefit was moderate. FOF, even in a low-dose regimen, was highly efficacious. The combination of the two drugs was not advantageous, although no antagonistic effect was observed. After prolonged therapy, an emergence of resistance to either of the drugs was not detected.

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