In vitro and in vivo activity of fosfomycin alone and in combination with rifampin and tigecycline against Gram-positive cocci isolated from surgical wound infections

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Abstract

Complicated skin and soft tissue infections constitute a heterogeneous group of severe disorders, with surgical site infections being the most common hospital-acquired ones. The aim of our study was to investigate the synergistic and bactericidal activities of antimicrobial combinations of fosfomycin with rifampin and tigecycline against Enterococcus faecalis, Enterococcus faecium and methicillin-resistant Staphylococcus aureus (MRSA) clinical isolates, and also to evaluate their in vivo effects in a mouse wound infection model. In in vitro studies, the combinations of fosfomycin with rifampin and tigecycline were both synergistic. These synergies were confirmed in in vivo studies: the drug combinations showed the highest antimicrobial effects compared to monotherapy. In conclusion, the efficacy of fosfomycin combinations, also confirmed in our in vivo model, may suggest new directions in the treatment of infected skin and a possible alternative way to control bacterial skin infection.

Complicated skin and soft tissue infections (cSSTIs) constitute a heterogeneous group of severe disorders, with surgical site infections (SSIs) being the most common hospital-acquired ones. A SSI was reported in 5.6% of patients following a surgical procedure in developing countries [1]. They constitute an escalating healthcare cost due to the need for additional surgical intervention, identification of the bacteria responsible for infection and drainage of infected material, prolonged hospital stay and antimicrobial therapy [2]. Moreover, cSSTIs (10%) are the third most frequent focus for severe sepsis or septic shock, after pneumonia (55–60%) and abdominal infections (25%) [3].

The aetiological agents of cSSTIs also commonly comprise an array of organisms, often with multidrug-resistant (MDR) phenotypes. In Europe, the most frequently isolated Gram-positive pathogen in cSSTIs is Staphylococcus aureus, including methicillin-resistant S. aureus (MRSA) and methicillin-susceptible S. aureus [4]. MRSA strains are prevalent in hospitals and have acquired the mecA gene (coding for the low-affinity binding protein PBP-2a) on genetic cassettes called SCCmec, conferring resistance to almost all of the beta-lactams, including methicillin, flucloxacillin, carbapenems and cephalosporins, with ceftobiprole being the exception [5]. In previous studies, it was shown that the rates of MRSA remain high in Italy (in the period 2012–2014 38% of S. aureus isolates were methicillin-resistant), even if they appear to be stabilizing [6, 7].

Furthermore, a number of other pathogens are linked with cSSTIs in specific epidemiological or clinical situations. In particular, enterococci, which have usually been considered to be low-grade pathogens, have emerged as an increasingly important cause of nosocomial infection, principally due to their intrinsic resistance to antibiotics and the rising number of severely ill patients [8]. In recent years, enterococci have shown an increasing range of antimicrobial resistance to, among other treatments, aminoglycosides, penicillins, chloramphenicol, tetracycline and glycopeptides [9, 10], which reduces therapeutic choices to a very limited set of active antibiotics. Among enterococci, the most important pathogenic agents are Enterococcus faecalis and Enterococcus faecium. One supplementary table and one supplementary figure are available with the online version of this article.
faecium, which are responsible for 80–90% of human enterococcal infections and are more likely to be resistant to commonly used antimicrobial agents [10].

The emergence and spread of MDR bacteria, which are currently responsible for 700 000 deaths annually [11], have been facilitated by selective pressure induced by the intensive use of antibiotics in both hospitalized patients and out-patients, by the greater movement of people and by the use of antimicrobials in breeding [12]. The massive increase of MDR bacterial infections is making cSSTIs progressively more challenging to treat [13], and there is growing evidence to suggest that combined antimicrobial therapy may be effective against these infections [14].

Fosfomycin, discovered over four decades ago, has attracted renewed interest as an agent that is active against MDR and extensively drug-resistant pathogens [15]. Fosfomycin is a broad-spectrum antibiotic that inhibits bacterial cell wall biogenesis by inactivating the initial step involving phosphoenolpyruvate synthetase. The fosfomycin mode of action is unique, so this drug does not cause cross-resistance with other antibiotics and can be administered in combination with some other antimicrobial agents [16].

Clinical data reporting drug interactions of fosfomycin with other agents are lacking, so to identify new combination regimens we examined the antibacterial activity of fosfomycin when combined with other antimicrobial agents against a large number of Gram-positive cocci. Moreover, we investigated its in vivo effects in an animal model of staphylococcal and enterococcal infected wounds.

We collected a total of 45 nonduplicate clinical isolates obtained from specimens from patients who underwent surgical treatment from July 2008 to December 2012, including 15 strains of Enterococcus faecalis, Enterococcus faecium and MRSA, respectively. The strains were identified by the VITEK 2 system (bioMérieux, Marcy-l’Etoile, France) and the antimicrobial susceptibilities are shown in Table S1 (available in the online version of this article). MRSA ATCC 43300 and E. faecalis ATCC 29212 were used as control strains and in in vivo experiments. The minimum inhibitory concentrations (MICs) for fosfomycin, rifampin (Sigma-Aldrich, St Louis, MO, USA) and tigecycline (Wyeth Pharmaceuticals, Inc., Philadelphia, PA, USA) were determined by a broth microdilution method with cation-adjusted Mueller–Hinton broth (Becton Dickinson Italia, Milan, Italy) following the procedures outlined by the Clinical and Laboratory Standards Institute (CLSI) [17] and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [18]. All of the isolates were susceptible to tigecycline and fosfomycin, but some isolates were resistant to rifampin, i.e. n=2 E. faecalis, n=2 E. faecium and n=2 S. aureus (Table 1). Tigecycline was the most active antibiotic for each species considered, showing lower MICs values than the other drugs.

The synergy of the antibiotic combinations was evaluated by a checkerboard titration method [19], using 96-well polypolypropylene microtitre plates, calculating the fractional inhibitory concentration (FIC) as the MIC of drug A or B in combination/the MIC of drug A or B alone, and the FIC index (FICI), adding the FIC values. The FICIs were interpreted as follows: ≤0.5, synergy; >0.5 and <4, indifferent; and ≥4, antagonistic [20]. The checkerboard assays showed high rates of synergism for all of the enterococcal strains when fosfomycin was combined with rifampin or tigecycline (75 % for E. faecalis strains and 73 and 67 % for E. faecium isolates, respectively). The same combinations also showed synergistic action against S. aureus: the fosfomycin/rifampin combination was synergistic for all of the tested strains, while the fosfomycin and tigecycline combination was also active in 75 % of the isolates. Interestingly, no antagonism was observed (Table 2).

These synergisms were also confirmed in time–kill experiments with E. faecalis ATCC 29212 and MRSA ATCC 43300. Each antibiotic was tested alone and in combination (½ MIC), inoculating 1×10^6 c.f.u. ml^{-1} in tubes containing Mueller–Hinton broth (Liofilchem, Roseto degli Abruzzi, Italy) and determining viable counts by serial dilutions at 0, 3, 6 and 24 h. Combinations of fosfomycin with rifampin and rifampin were synergistic after 24 h in both Staphylococcus and Enterococcus, denoting a decrease of c.f.u. ml^{-1} to zero (Fig. S1).

Taking the in vitro results into consideration, we then tested the antimicrobial combinations on an infected wound mouse model. Adult male BALB/c mice weighing 40 to 50 g were used for all the experiments. All of the animals were housed in individual cages under constant temperature (22 °C) and humidity, with a 12 h light/dark cycle (with lights on at 06.30 a.m. and off at 06.30 p.m.), and had access to as much chow and water as desired throughout the study. For each strain, we used a total of 84 animals divided into 7 groups (each composed of 12 mice) that included: (i) a not-infected group that did not receive any treatment; (ii) an infected group that did not receive any treatment; (iii) 3 groups that received intraperitoneal treatment singly with fosfomycin (75 mg kg^{-1}) [21], rifampin (10 mg kg^{-1}) and tigecycline (1.5 mg kg^{-1}); (iv) a group that received intraperitoneal fosfomycin (75 mg kg^{-1}) plus rifampin (10 mg kg^{-1}); and (v) a group that received intraperitoneal fosfomycin (75 mg kg^{-1}) plus tigecycline (1.5 mg kg^{-1}). Mice were anaesthetized by an intramuscular injection of ketamine (50 mg kg^{-1} of body weight) and xylazine (8 mg kg^{-1} of body weight) and the hair on the back was shaved and the skin cleansed with 10 % povidone/iodine solution. Using a 1.0×2.0 cm template, one full-thickness wound was established through the panniculus carnosus on the back subcutaneous tissue of each animal [22]. A small gauze was placed over each wound and then inoculated with 1 ml of 5×10^6 c.f.u. of control strains. The pocket was closed by means of skin clips [23]. This procedure resulted in a local abscess at 24 h. One wound was created per animal. The animals were returned to individual cages and thoroughly
examined daily. After 24 h, in the control animals the wound was opened, the gauze was removed for quantitative bacterial culture and treatment was initiated. Intraperitoneal treatments were administered daily for 7 days. The animals were euthanized and a 1 × 2 cm area of skin, including the wound, was excised aseptically. Skin samples were homogenized in 1 ml phosphate-buffered saline (PBS) using a stomacher. Quantization of viable bacteria was performed by culturing serial dilutions (0.1 ml) of the bacterial suspension on blood agar plates. All of the plates were incubated at 37°C for 48 h and evaluated for the presence of bacteria. The organisms were quantized by counting the number of cfu/s per plate. The limit of detection for this method was approximately 10 cfu/ml. Toxicity was evaluated on the basis of the presence of any drug-related adverse effects, behavioural alterations and local signs of inflammation. For staphylococcal infection, the mean bacterial numbers of the infected-but-untreated group (6.7 ± 0.2 × 10⁷ cfu/ml) were significantly higher than those recovered from all of the treated groups. Specifically, all single drugs reduced bacterial numbers, but tigecycline, confirming the in vitro data, was the most effective treatment (3.1 ± 0.3 × 10⁷ cfu/ml⁻¹). The greatest bacterial inhibition was obtained in the groups that received antibiotic combinations (1.6 ± 0.1 × 10⁴ cfu/ml⁻¹ for fosfomycin/tigecycline and 2.1 ± 0.4 × 10⁴ for fosfomycin/rifampicin), confirming the in vitro data. All of the results are reported in Table 3.

For enterococcal infection, we observed the same pattern of results. All groups treated with single drugs showed a statistically significant reduction of cfu/ml compared to the control group. Similarly to the staphylococcal group infection, the two groups treated with drug combinations showed the highest effect in the inhibition of the bacterial load.

Antibiotic resistance is a serious problem in the management of CSSTIs, and therefore the selection of an optimal antimicrobial regimen must take into account each individual patient’s comorbidities and drug-specific safety profiles, and, last but not least, cost considerations. Fosfomycin has attracted significant attention in recent years due to its broad-spectrum activity, and in particular its potential use as an adjunct for the treatment of invasive MRSA infection, although, as a monotherapy, fosfomycin could lead to resistance [24]. Furthermore, fosfomycin has low and stable resistance rates worldwide, despite having been in clinical use for more than 20 years in some countries [25]. The synergism of fosfomycin with other antibiotics such as vancomycin, teicoplanin, linezolid or daptomycin against MRSA and Enterococcus spp. has been previously reported [26].

Although our data showed good in vitro and in vivo activity for tigecycline, rifampin and fosfomycin against enterococci and MRSA isolates, there is evidence to indicate that the initial use of drug combinations can provide a greater spectrum of activity compared with single drugs. Our results

<table>
<thead>
<tr>
<th>Species</th>
<th>Combination</th>
<th>MIC range</th>
<th>FICI results, % (n)</th>
<th>Synergism</th>
<th>Indifference</th>
<th>Antagonism</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. faecalis</td>
<td>Fosfomycin/rifampicin</td>
<td>0.5–32</td>
<td></td>
<td>75 (12)</td>
<td>25 (4)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Fosfomycin/tigecycline</td>
<td>0.5–32</td>
<td></td>
<td>75 (12)</td>
<td>25 (4)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Fosfomycin/rifampicin</td>
<td>8</td>
<td></td>
<td>73 (11)</td>
<td>27 (4)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Fosfomycin/tigecycline</td>
<td>8</td>
<td></td>
<td>67 (10)</td>
<td>33 (5)</td>
<td>–</td>
</tr>
<tr>
<td>S. aureus</td>
<td>Fosfomycin/rifampicin</td>
<td>8</td>
<td></td>
<td>100 (16)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Fosfomycin/tigecycline</td>
<td>8</td>
<td></td>
<td>75 (12)</td>
<td>25 (4)</td>
<td>–</td>
</tr>
</tbody>
</table>

Table 1. MIC range and MIC₅₀ and MIC₉₀ of each species for fosfomycin, rifampin and tigecycline

All values are reported in µg ml⁻¹
confirmed the activities of antibiotic combinations in severe infections caused by MDR Gram-positive bacteria [2].

In the in vitro study, the combination of fosfomycin and rifampicin displayed the greatest antibacterial activity against MRSA compared to other combinations, and it was shown to be synergistic for all of the tested strains, in spite of the presence of some isolates that are resistant to rifampin. A previous study also disclosed the effectiveness of fosfomycin in combination with meropenem or colistin against resistant isolates [26]. In the time–kill assay the fosfomycin/rifampin combination resulted in a complete eradication of bacteria after 24 h, confirming the initial results.

Rifampin could be an attractive antimicrobial agent in the treatment of complicated infections, given that it is active against MRSA, has very good bioavailability and is well tolerated [27]. However, due to the rapid development of resistance, it should not be used as a monotherapy [28]. In view of our data, it is possible that, when used in combination with other agents, the rate of rifampicin resistance is not clinically significant, suggesting that the evolution of antimicrobial resistance is reduced compared with that for monotherapy. A synergistic effect of rifampicin and fosfomycin has been observed in vitro in previous studies showing the best activity against MRSA cage-associated infections [29].

The fosfomycin/tigecycline combination presented in vitro synergies of 75% in MRSA. The combination with fosfomycin was found to be less synergistic than the fosfomycin/rifampin combination, as previously reported [28], but this could be explained by the greater activity of tigecycline compared with the other antibiotics [30–32]. However, time–kill studies demonstrated the activity of fosfomycin/tigecycline, with results comparable to those for the fosfomycin/rifampin combination.

Moreover, we reported that, in the in vitro study, either rifampicin or tigecycline in combination with fosfomycin appeared to be active against E. faecalis and E. faecium strains (synergies between 67 and 75%). These combination regimens were more active than single drugs, even when in vitro testing confirmed susceptibility to the respective antimicrobials. The synergistic activity between the two combinations was observed in vitro among all of the enterococcal isolates, which is consistent with the results of previous studies [33].

The in vitro observations were confirmed when we performed experiments on an animal model of surgical wound infection with staphylococcal and enterococcal strains. In the in vivo model, antibiotic combinations were also more effective than single treatment, with a reduction of c.f.u. ≥2 log. Interestingly, our data indicate that fosfomycin, when combined with either rifampicin or tigecycline, was effective in healing infected wounds, although in vitro studies the fosfomycin/tigecycline combination was less effective.

This study emphasizes the importance of antibiotic combinations in infections due to nosocomial isolates of Gram-positive cocci. MDR bacteria have both complicated and decreased the options for the efficient treatment of cSSTI. Moreover, our results revealed that fosfomycin combined with rifampicin or tigecycline is synergistic against MDR Gram-positive bacteria. The efficacy of fosfomycin combinations, also confirmed in our in vivo model, may suggest new directions in the treatment of infected skin due to Gram-positive MDR bacteria.

Table 3. Quantitative culture of excised tissues after drug administration in staphylococcal and enterococcal wound infection

<table>
<thead>
<tr>
<th>Treatment</th>
<th>c.f.u./ml</th>
<th>S. aureus ATCC 43300</th>
<th>E. faecalis ATCC 29212</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>&lt;10</td>
<td>6.7 × 10^2 ± 0.2 × 10^7</td>
<td>7.8 × 10^2 ± 1.4 × 10^7</td>
</tr>
<tr>
<td>Infected untreated</td>
<td></td>
<td>6.2 × 10^4 ± 0.9 × 10^4</td>
<td>6.1 × 10^4 ± 1.2 × 10^4</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td></td>
<td>6.5 × 10^5 ± 1.1 × 10^4</td>
<td>7.7 × 10^5 ± 1.2 × 10^4</td>
</tr>
<tr>
<td>Rifampicin</td>
<td></td>
<td>3.1 × 10^3 ± 0.3 × 10^3</td>
<td>3.8 × 10^3 ± 0.9 × 10^3</td>
</tr>
<tr>
<td>Tigecycline</td>
<td></td>
<td>2.1 × 10^3 ± 0.4 × 10^3</td>
<td>5.0 × 10^3 ± 0.8 × 10^3</td>
</tr>
<tr>
<td>Fosfomycin/rifampin</td>
<td></td>
<td>1.6 × 10^3 ± 0.1 × 10^3</td>
<td>3.6 × 10^3 ± 0.4 × 10^3</td>
</tr>
</tbody>
</table>

a. Treatment with single antibiotics showed significant c.f.u./ml reductions compared to group without treatment (*P<0.001).
b. Treatment with antibiotic combinations showed significant c.f.u./ml reductions compared to single antibiotic treatment (*P<0.001).


