In vitro activity of fosfomycin in combination with various antistaphylococcal substances

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Using the chequerboard technique we studied the in vitro activity of the broad spectrum antibiotic fosfomycin in combination with vancomycin, rifampicin, linezolid, quinupristin/dalfopristin, cefazolin, meropenem and moxifloxacin against two Staphylococcus epidermidis strains (ATCC 12228, DSM 3269) and five Staphylococcus aureus isolates (ATCC 29213, DSM 683, DSM 46320, GISA 323/93, MRSA 3558/00). The phenomena of ‘trailing’ and ‘skipped wells’ did not present a problem. Synergy was the most common effect of all drugs tested in combination with fosfomycin; only combination with vancomycin showed antagonism for two of seven isolates. Using a killing-curve technique fosfomycin showed cidal activity, where increasing the drug concentration above the MIC did not enhance killing velocity. Inhibitory concentrations of vancomycin plus fosfomycin against DSM 46320 caused effects identical to those observed with vancomycin alone. The combination of fosfomycin plus linezolid exerted the bacteriostatic effect found with linezolid alone. Fosfomycin plus quinupristin/dalfopristin exhibited the bactericidal effect found with fosfomycin alone (in contrast to the rapidly bactericidal effect of quinupristin/dalfopristin). Electron microscopy showed that fosfomycin given in combination with linezolid, quinupristin/dalfopristin or moxifloxacin (substances that do not cause morphological alterations when given alone) resulted in ‘cauliflower-shaped’ distortion as caused by fosfomycin alone. Our in vitro data indicate considerable potential for fosfomycin used in combination with other antistaphylococcal antimicrobials, especially linezolid or quinupristin/dalfopristin.

Materials and methods

Bacterial strains

Five Staphylococcus aureus isolates and two Staphylococcus epidermidis strains were tested. S. epidermidis ATCC 12228 and DSM 3269 were purchased commercially. The latter originated from an infected catheter tip and produced extracellular slime. Also commercially purchased were S. aureus ATCC 29213, DSM 683 (ATCC 9144) and DSM 46320; the latter is methicillin resistant (MRSA).

S. aureus GISA 323/93 was kindly provided by Dr Gabriele Bierbaum (Institut für Medizinische Mikrobiologie und Immunologie, Rheinische Friedrich-Wilhelms-Universität, Bonn, Germany) and had a vancomycin MIC...
of 8 mg/L when initially isolated. This MRSA isolate is now susceptible to vancomycin. Reversion of glycopeptide resistance has been described by Boyle-Vavra et al.

*S. aureus* Sp3558/00 is a wild strain MRSA from a tracheal secretion of an ICU patient in Innsbruck. According to Professor Dr Wolfgang Witte (Robert Koch Institut, Wernigerode, Germany) this isolate belongs to an epidemic clone found frequently in southern Germany called ‘Süddeutscher Epidemiestamm’.

**Susceptibility testing**

**Antimicrobial agents.** The following antimicrobial agents were provided by the manufacturers as powders of known potency: fosfomycin (Biochemie, Kundl, Austria), vancomycin (Eli Lilly, Giessen, Germany), rifampicin (Sigma Chemical Co., St Louis, MO, USA), linezolid (Pharmacia & Upjohn, Nerviano, Italy), quinupristin/dalfopristin (Aventis Pharma, Vitry sur Seine, France), cefazolin (Eli Lilly), meropenem (Zeneca, Wilmington, DE, USA), moxifloxacin (Bayer, Wuppertal, Germany). MIC interpretive standards were chosen according to the NCCLS criteria and are listed in Table I.

**Chequerboard technique.** Serial dilutions of fosfomycin and other drugs were made using 96-well microtitre plates. The final inoculum was approximately 10⁷ cfu/mL, the final volume per well was 200 µL. Plates were incubated in parallel in ambient air and anaerobically (AnaeroGen; Oxoid, Basingstoke, UK) at 35°C for 18 h. Definitions for synergy, antagonism and autonomy (indifference) were used as suggested by Eliopoulos & Moellering. Mueller–Hinton broth (supplemented as described above) was used, and supplemented with α-D-glucose-6-phosphate (Sigma) at a final concentration of 25 mg/L, as recommended by the manufacturer of fosfomycin (Biochemie). An MIC of ≥128 mg/L characterizes a strain as resistant, 32–64 mg/L as intermediate and ≤16 mg/L as susceptible. The NCCLS has not yet provided specific guidelines for the testing of fosfomycin; NCCLS criteria exist for urinary tract infection caused by *Escherichia coli* and *Enterococcus faecalis*, defining ≥256 mg/L as resistant and ≤64 mg/L as susceptible. Tests using the chequerboard technique were performed once only, on various days, but by the same technician.

**Killing-curve method.** Ten millilitre samples of Mueller–Hinton broth (supplemented as described above) with an initial bacterial cell count of 10⁸ cfu/mL of MRSA DSM 46320 were incubated at 37°C. For tests with cefazolin and meropenem, *S. aureus* ATCC 29213 was also used. At 0, 2, 4, 6, 8 and 24 h 500 µL aliquots were withdrawn and used undiluted for viable cell count determination; fosfomycin was diluted to a final concentration of 40 mg/L; vancomycin, 10 mg/L; linezolid, 8 mg/L; quinupristin/dalfopristin, 1 mg/L; cefazolin, 16 mg/L; meropenem, 8 mg/L; and moxifloxacin, 2 mg/L. Aliquots containing rifampicin at a concentration of 0.02 mg/L were tested after 100-fold dilution to eliminate antibiotic carry-over. The concentrations chosen were based on intermediate MIC breakpoints. Concentrations for quinupristin/dalfopristin (1 mg/L) and rifampicin (0.02 mg/L) were chosen arbitrarily after a major carry-over problem was observed with 2 mg/L rifampicin and a minor carry-over problem with 2 mg/L quinupristin/dalfopristin. Tests were performed in triplicate. Quantitative cultures were performed by logarthmic plating of 50 µL in duplicate on Mueller–Hinton agar plates using a spiral plater (Whitley automatic spiral plater; Don Whitley Scientific Ltd, West Yorkshire, UK). At present there are no established criteria to evaluate possible synergy in the killing-curve technique using two drugs that have significant activity alone.

**Electron microscopy.** Bacteria grown overnight in Mueller–Hinton broth (specimens taken from single wells of microtitre plates, as described above) were centrifuged after

<table>
<thead>
<tr>
<th>Interpretative standard</th>
<th>QC limits</th>
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<tbody>
<tr>
<td>ATCC 29213</td>
<td></td>
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<tr>
<td><strong>Fosfomycin</strong></td>
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<td>≤16</td>
<td>32–64</td>
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<tr>
<td><strong>Vancomycin</strong></td>
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<tr>
<td>≤4</td>
<td>8–16</td>
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<tr>
<td><strong>Rifampicin</strong></td>
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<td>≤1</td>
<td>2</td>
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<tr>
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<tr>
<td>≤4</td>
<td>8</td>
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<tr>
<td><strong>Quinupristin/dalfopristin</strong></td>
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<tr>
<td>≤1</td>
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<tr>
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<td>16</td>
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<tr>
<td><strong>Meropenem</strong></td>
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<td>≤4</td>
<td>8</td>
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<tr>
<td><strong>Moxifloxacin</strong></td>
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<td>≤1</td>
<td>2</td>
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*S*, susceptible; I, intermediate; R, resistant.

*Tentative breakpoints.*

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Table I. MIC (mg/L) interpretative standards and quality control (QC) limits used
Fosfomycin in combination with antistaphylococcal agents

Table II. Summarized results of the chequerboard technique

<table>
<thead>
<tr>
<th>Fosfomycin plus:</th>
<th>rifampicin</th>
<th>linezolid</th>
<th>quinupristin/dalfopristin</th>
<th>moxifloxacin</th>
<th>vancomycin</th>
<th>cefazolin</th>
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<td>S</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>I</td>
</tr>
<tr>
<td>DSM 3269</td>
<td>S</td>
<td>S</td>
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<tr>
<th>S. aureus</th>
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<td>S</td>
<td>S</td>
<td>I</td>
<td>S</td>
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</tr>
<tr>
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<tr>
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<td>S</td>
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<td>A</td>
<td>S</td>
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S, synergy; I, indifference; A, antagonism.

fixation with 2.5% glutaraldehyde in 0.15 M cacodylate buffer pH 7.3. A concentrated suspension of bacteria was pipetted on to a 0.45 μm Millipore filter (Millipore Corporation, Bedford, MA, USA) and gentle suction applied. Subsequently, organisms were washed briefly in cacodylate buffer, followed by osmification for 1 h in 1% OsO4 in distilled water and gradual dehydration with ethanol after critical point drying with CO2 (BAL-TEC CPD 030 BALZERS; Vaduz, Liechtenstein). Specimens were mounted on aluminium stubs with colloidal silver, sputtered with 10 nm Au/Pd (BAL-TEC MED 020 BALZERS; Vaduz) and examined with a Zeiss scanning electron microscope (Gemini 982; Oberkochen, Germany).

Results

Effects of antimicrobial combinations as measured by the chequerboard technique

Fosfomycin showed synergic activity against S. epidermidis (ATCC 12228 and DSM 3269), with rifampicin, linezolid, quinupristin/dalfopristin and moxifloxacin; indifference was observed with vancomycin, cefazolin and meropenem.

Fosfomycin showed synergic activity against S. aureus (ATCC 29213, DSM 683, DSM 46320, GISA 323/93 and MRSA Sp3558/00) with all drugs tested except vancomycin. The combination with vancomycin showed antagonism for two organisms (DSM 683 and DSM 46320) and indifference for the remaining three of the five S. aureus strains tested. Table II summarizes these results.

The phenomenon of ‘trailing’ (gradual increase in turbidity under the influence of decreasing fosfomycin concentrations) was not a problem in testing fosfomycin; minor growth was simply ignored when reading MICs.

The phenomenon of ‘skipped wells’ was observed twice in 49 chequerboard tests: (i) when testing S. aureus ATCC 29213 with fosfomycin plus quinupristin/dalfopristin, one well containing a fosfomycin concentration of 0.1 mg/L remained blank; and (ii) when testing MRSA Sp3558/00, one well containing fosfomycin at 0.06 mg/L plus cefazolin at 2 mg/L remained blank (Figure 1).

Anaerobic incubation increased the in vitro activity of fosfomycin. MICs of fosfomycin were one- to three-fold lower when compared with aerobic incubation.

Effects of fosfomycin given alone or in combination as measured by the killing-curve method

The effect of fosfomycin on the velocity of killing was not dependent on concentration, as shown in Figure 2 for MRSA DSM 46320.

Figure 3 shows the results for fosfomycin in combination with antistaphylococcal agents. Fosfomycin in combination with vancomycin yielded killing curves identical to those observed when the drugs were used alone. Carry-over of rifampicin led us to use an unusually low rifampicin concentration (0.02 mg/L) for the killing-curve experiment. Fosfomycin plus rifampicin exerted an effect better than rifampicin alone, similar to that observed for fosfomycin alone. Fosfomycin plus linezolid exerted a bacteriostatic effect as found with linezolid alone. Fosfomycin plus quinupristin/dalfopristin exerted a bactericidal effect as found with fosfomycin alone but not the fast killing of quinupristin/dalfopristin. Fosfomycin plus moxifloxacin exerted a bactericidal effect also found with fosfomycin alone but not the fast killing of moxifloxacin. Fosfomycin plus cefazolin and fosfomycin plus meropenem exerted bactericidal effects stronger than that of fosfomycin alone.

Against the methicillin-susceptible S. aureus ATCC 29213 fosfomycin plus cefazolin and fosfomycin plus meropenem exerted bactericidal activity comparable to that of fosfomycin alone. Figure 4 shows the results obtained for S. aureus ATCC 29213.
Electron microscopy

Overnight cultures of all seven test strains with various concentrations of the antibiotics tested (with and without fosfomycin) were photographed by electron microscopy and studied for morphological alterations. A total of 131 specimens were processed for electron microscopy. Inhibitory concentrations of fosfomycin (and the β-lactams for β-lactam-susceptible strains) caused 'cauliflower-shaped' morphological alterations of the cocci, as shown in Figure 5 for GISA 323/93 after overnight incubation with fosfomycin (8 mg/L). Rifampicin, linezolid, quinupristin/dalfopristin and moxifloxacin did not cause significant morphological changes when given alone. Combination of these drugs with fosfomycin caused morphological alterations as seen with fosfomycin alone. Figure 6 compares the profound effect of fosfomycin 2.0 mg/L plus linezolid 0.25 mg/L on GISA 323/93 with the minor effect on morphology of 1.0 mg/L linezolid alone. Figure 7 shows the profound effect of fosfomycin 0.5 mg/L plus quinupristin/dalfopristin 0.03 mg/L compared with that of 0.06 mg/L quinupristin/dalfopristin alone using S. epidermidis DSM 3269. Overnight culture with meropenem and with the combination of fosfomycin plus linezolid yielded unique spindle-shaped structures ('naviculae'). Figure 8 shows such naviculae for S. aureus.

Discussion

Data obtained from the killing-curve method showed that fosfomycin exhibits bactericidal activity similar to that of β-lactam antibiotics, where increasing the drug concentration above minimal bactericidal concentrations is not reflected in increased killing velocity. Using the chequerboard technique the majority of drugs tested showed synergic activity. Only combination with vancomycin caused antagonism for two of seven isolates (including results obtained by anaerobic incubation for five of seven strains). Hamilton-Miller15 observed that the in vitro activity of fosfomycin against staphylococci and enterococci was significantly greater under anaerobic conditions, especially against coagulase-negative staphylococci. We confirm that anaerobic incubation generally increases the in vitro activity of fosfomycin. Whether or not this phenomenon correlates with improved in vivo efficacy, especially in inflamed areas with decreased oxygen levels, remains to be seen.

Vancomycin is usually active against S. aureus and S. epidermidis, including strains resistant to methicillin.
Fosfomycin in combination with antistaphylococcal agents

Figure 3. *In vitro* effect of fosfomycin (40 mg/L), alone and in combination with antistaphylococcal agents, against the MRSA DSM 46320. Data are mean \pm S.E.M. Symbols: (a) ●, meropenem 16 mg/L; ■, without antimicrobial agents; Δ, fosfomycin 40 mg/L; ×, fosfomycin + meropenem; (b) ●, quinupristin/dalfopristin 1 mg/L; ■, without antimicrobial agents; Δ, fosfomycin 40 mg/L; ×, fosfomycin + quinupristin/dalfopristin; (c) ●, vancomycin 10 mg/L; ■, without antimicrobial agents; Δ, fosfomycin 40 mg/L; ×, fosfomycin + vancomycin; (d) ●, rifampicin 0.02 mg/L; ■, without antimicrobial agents; Δ, fosfomycin 40 mg/L; ×, fosfomycin + rifampicin; (e) ●, cefazolin 8 mg/L; ■, without antimicrobial agents; Δ, fosfomycin 40 mg/L; ×, fosfomycin + cefazolin; (f) ●, linezolid 8 mg/L; ■, without antimicrobial agents; Δ, fosfomycin 40 mg/L; ×, fosfomycin + linezolid; (g) ●, moxifloxacin 2 mg/L; ■, without antimicrobial agents; Δ, fosfomycin 40 mg/L; ×, fosfomycin + moxifloxacin.

213
This glycopeptide inhibits synthesis and assembly of the second stage of cell wall peptidoglycan polymers, resulting in a bactericidal effect. We tested its cidal activity against a methicillin-resistant strain, DSM 46320, an isolate showing antagonism with the chequerboard technique when incubated aerobically, in a kinetic model. At present there are no established criteria for assessing synergy in killing-curve studies of two drugs that both have significant activity alone. Clark et al. performed a time–kill analysis of trovafloxacin with other antibiotics using trovafloxacin at sub-MIC concentrations. We performed a time–kill analysis using inhibitory concentrations of both drugs tested, considering this to be more relevant to the in vivo situation.

For strain DSM 46320 the combination of vancomycin plus fosfomycin yielded killing curves similar to those observed with vancomycin alone. No evidence of antagonism was detected using this model.
Rifampicin is bactericidal in vivo as well as in vitro but, because of rapid development of resistance, this antibiotic is always used in combination with another agent to prevent resistance when treating serious infections. Hamilton-Miller observed synergy between rifampicin and fosfomycin against MRSA and coagulase-negative staphylococci. Our chequerboard technique results confirm Hamilton-Miller’s observations. The killing-curve method was not able to eliminate the problem of antibiotic carry-over. The killing rate observed with rifampicin at 0.02 mg/L significantly underestimates the potential of this drug, which achieves serum concentrations of ≥2 mg/L after iv administration.

To our knowledge, there are as yet no data on the in vitro activity of fosfomycin in combination with linezolid or quinupristin/dalfopristin. Linezolid is a member of a new class of antimicrobial agents, the oxazolidinones. Rybak et al. reported on the in vitro susceptibility of methicillin-resistant staphylococci to linezolid but no data on the effect of combination with fosfomycin have yet been published. Using chequerboard titration we observed synergic activity for the combination of fosfomycin and linezolid against all strains tested. In the killing-curve method the combination exerted a bacteriostatic effect as found with linezolid alone.

Quinuprin/dalfopristin is a new drug composed of two semisynthetic streptogramin molecules. The combination of these two agents has a synergic antibacterial activity in vitro and in vivo against a wide range of Gram-positive organisms, including methicillin-resistant staphylococci, but again no data on the effect of combination with fosfomycin have yet been published. Using chequerboard titration we observed synergic activity for the combination
of fosfomycin plus quinupristin/dalfopristin against all strains tested. In the killing-curve method the combination exerted a bactericidal effect as found with fosfomycin alone, although not the extremely fast cidal effects of quinupristin/dalfopristin.

Cefazolin and meropenem are β-lactams with bactericidal activity including against producers of β-lactamase, but not against methicillin-resistant strains of staphylococci. Chin & Neu21 found that fosfomycin combined with nafcillin or with cefotaxime showed synergy or partial synergy for 90% of methicillin-resistant S. aureus strains tested. Chequerboard studies of imipenem against coagulase-positive and coagulase-negative staphylococci showed high rates of synergy with fosfomycin.22 We observed synergic activity for the combinations of fosfomycin plus cefazolin and fosfomycin plus meropenem against most of the strains tested. The killing-curve method showed improved in vitro activity for the combination of fosfomycin plus a β-lactam even against MRSA DSM 46320, although methicillin resistance prohibits the use of β-lactams in vivo.

The new 8-methoxy-6-fluoroquinolone, moxifloxacin, has bactericidal activity against Gram-negative bacteria similar to that of ciprofloxacin but superior potency against Gram-positive bacteria including staphylococci.23 Blondeau24 reported MIC₉₀ of this drug for S. epidermidis and for MRSA of 2 mg/L. Hamilton-Miller15 observed synergy for the combination of ciprofloxacin and fosfomycin against MRSA and coagulase-negative staphylococci. Using chequerboard titration we observed synergic activity between fosfomycin and moxifloxacin against most strains tested. In the killing-curve method the combination exerted a bactericidal effect similar to that of fosfomycin alone, although not the extremely fast killing of moxifloxacin.

The effects of antibiotics on biofilm production by S. epidermidis are less clear than those using Pseudomonas aeruginosa.25 Our scanning electron micrographs did not show production of extracellular slime by S. epidermidis DSM 3269 grown under the test conditions described. Studying antibiotic susceptibility of four slime-producing isolates of S. aureus in biofilms developed in vitro, Amorena et al.26 found that fosfomycin, followed by rifampicin, cefazolin, vancomycin and ciprofloxacin, significantly affected biofilm cell viability.

Our electron microscopy studies revealed that fosfomycin given in combination with linezolid, quinupristin/dalfopristin or moxifloxacin (substances not causing morphological alterations when given alone) resulted in ‘cauliflower-shaped’ morphology such as that caused by fosfomycin alone. This supports the view that bactericidal fosfomycin can be given in combination with these other antistaphylococcal agents.

Our in vitro results indicate considerable potential for fosfomycin in combination with other antistaphylococcal agents, including linezolid, quinupristin/dalfopristin and moxifloxacin. This indication remains to be proven by clinical studies.

References


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