Synergistic effects of aminoglycosides and fosfomycin on Pseudomonas aeruginosa in vitro and biofilm infections in a rat model

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Objectives: To study the in vitro and in vivo efficacy of aminoglycosides against Pseudomonas aeruginosa, either alone or in combination with fosfomycin.

Methods: Using an in vitro study to assess inhibition of the growth of P. aeruginosa, MIC90 and MIC50 values of amikacin, gentamicin, netilmicin, tobramycin and isepamicin were determined, either alone or in combination with fosfomycin, and then the fractional inhibitory concentration index was calculated. In the biofilm-infected rat model, the efficacy and effects of treatment with isepamicin and fosfomycin on infection were studied.

Results: The combinations of amikacin and fosfomycin or isepamicin and fosfomycin showed the most significant synergistic effects against P. aeruginosa as compared with other treatments. In the biofilm-infected rat model, the efficacy and effects of treatment with isepamicin and fosfomycin on infection were studied.

Conclusions: Combination of aminoglycosides and fosfomycin not only showed a positive effect in vitro but also improved the therapeutic effect in a biofilm-infected rat model. This offers an effective treatment strategy against some therapy-resistant infections.

Keywords: isepamicin, amikacin, gentamicin, netilmicin, tobramycin

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen, frequently found in clinical specimens and in the lungs of cystic fibrosis patients. Patients with underlying conditions, such as cystic fibrosis and immunosuppression, usually develop chronic infections with the formation of biofilms, which are resistant to host immune responses. The attached bacteria are much more resistant to antibiotic treatments than their non-attached, planktonic counterparts. Thus, biofilm infections are usually persistent and intractable.

Fosfomycin is an effective antibiotic agent against both Gram-positive and Gram-negative bacteria. It has been successfully used for treating uncomplicated urinary tract infections due to its excellent stability, ideal pharmacokinetic parameters, ease of administration, good tolerability and clinical efficacy. However, fosfomycin is not recommended for complicated severe infections. Instead, aminoglycosides are used extensively in clinical practice, and have activities against a broad spectrum of bacteria, including P. aeruginosa. However, with the increasing use of these drugs in clinics, resistant strains occur more frequently. Moreover, the potential nephrotoxicity and cytotoxicity caused by aminoglycosides further restrict their clinical usage.

In this study, the in vitro antibacterial activity of five aminoglycosides, either alone or in combination with fosfomycin, against P. aeruginosa was investigated. The therapeutic efficacy of the combination of isepamicin and fosfomycin against P. aeruginosa in vivo was also examined in a biofilm-infected rat model.

Materials and methods

Strains, agents and checkerboard microdilution assay

Twenty P. aeruginosa clinical isolates were used in in vitro studies and wild-type PAO1 was used in the biofilm-infected rat model.

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Fosfomycin, amikacin, gentamicin, netilmicin and tobramycin standards were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP) (Beijing, China). Isepamicin was purchased from Haizheng Pharmaceutical (Zhejiang, China). Fractional inhibitory concentration index (FICI) values were interpreted as follows: synergy, FICI < 0.5; no interaction, 0.5 < FICI ≤ 4.0; and antagonism, FICI > 4.0.

Biofilm-infected rat model and experimental design
Silica gel tubes with a formed P. aeruginosa biofilm were processed as described previously. Twenty-four male Wistar rats were intraperitoneally injected with drug and randomly divided into four groups: group F (300 mg/kg fosfomycin); group I (300 mg/kg isepamicin); group F+I (300 mg/kg fosfomycin followed by 300 mg/kg isepamicin 1 h later); and group C (saline). The drugs were administrated 24 h after the embedding of the silica gel tube, once a day for 3 days.

Assay of white blood cells (WBCs), C-reactive protein (CRP) and colony counts
Blood (1–1.5 mL) was drawn from each treated rat after euthanization and the peripheral numbers of WBCs and CRP levels were determined following the manufacturer’s guideline using a haematology automated analyser (Sysmex XE-2100; Sysmex Corporation, Japan) and an automated system for plasma protein determination (BN II; Dade Behring, USA). The colony counts of the bacteria from both tissue and silica gel tubes were determined as described previously.

Ethics
Animal experiments were performed in accordance with the regulations for the care and use of laboratory animals and were approved by the local authorities.

Statistical analysis
Variance analysis was performed using SPSS 12.0.

Results
Enhancement of antibacterial activity of aminoglycosides against P. aeruginosa by fosfomycin
The MIC\textsubscript{90}s of amikacin, isepamicin, gentamicin, netilmicin and tobramycin alone were 32, 16, 16, 16 and 8 mg/L, respectively. When fosfomycin was added at 1/16, 1/8 or 1/4 of the MIC (4–16 mg/L) to the aminoglycoside regimens, the MIC\textsubscript{90} of amikacin, isepamicin, gentamicin, netilmicin and tobramycin decreased by 64–, 32–, 8–, 8– and 2-fold, respectively. The distribution of FICI values among 20 P. aeruginosa clinical isolates is shown in Figure 1. Sixty to eighty percent of isolates had FICI values <0.5. Fifteen to twenty percent of isolates had FICI values between 0.5 and 1. Antagonistic effects were not observed. These data indicated that synergism may account for the observed improved efficiency of combinations of aminoglycosides and fosfomycin against P. aeruginosa.

Changes in numbers of WBCs, CRP levels and colony counts in the biofilm-infected rat model
To examine the effects of the drugs on the inflammation of the biofilm-infected rats, the numbers of WBCs and CRP levels were evaluated (Table 1). Before embedding or before first drug administration, there were no differences in the numbers of WBCs among all four groups. However, there were significant increases in the numbers for all groups (P<0.05) from embedding to the first drug administration. Before drug administration to 24 h after the third administration, while there were small decreases in the numbers of WBCs in both the fosfomycin and isepamicin groups (P>0.05), the count was significantly decreased with the combined treatment (F+I) (P<0.05), although it was still higher than that before embedding (P<0.05). Furthermore, at the end of the experiment before euthanasia, the numbers of WBCs of the fosfomycin and isepamicin groups were much higher than those of the combined group (P<0.05). Changes in CRP levels for all four groups followed similar trends to those seen for numbers of WBCs.

Viable bacterial counting (Table 1) showed that there was a significant decrease in colony formation by the combination treatment for 3 days both in infected tissue and in silica gel tubes as compared with the control group (P<0.05), while viable bacterial counts in both the fosfomycin and isepamicin groups were not significantly different from that of the control group (P>0.05).

Discussion
In this study we found that all five widely used aminoglycosides displayed synergistic effects against planktonic P. aeruginosa in combination with fosfomycin and showed significant decreases in their MICs. Among them, amikacin and isepamicin showed the most significant decrease and the lowest FICI. However, we only evaluated isepamicin/fosfomycin combination therapy in further animal studies, since our previous study had confirmed that the combination of isepamicin/fosfomycin can reduce formation of biofilm in vitro more efficiently than other combinations, including amikacin/fosfomycin. Furthermore, isepamicin is one of the least toxic aminoglycosides that is effective
Synergistic effects of aminoglycosides and fosfomycin

Table 1. Effects of drugs on numbers of WBCs, CRP levels and colony counts

<table>
<thead>
<tr>
<th></th>
<th>Group C</th>
<th>Group F</th>
<th>Group I</th>
<th>Group F + I</th>
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<tbody>
<tr>
<td>WBCs (×10⁹/L)</td>
<td></td>
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<tr>
<td>before embedding</td>
<td>10.38 ± 0.35</td>
<td>10.17 ± 0.29</td>
<td>10.38 ± 0.40</td>
<td>10.45 ± 0.47</td>
</tr>
<tr>
<td>before administration</td>
<td>18.90 ± 0.22⁰</td>
<td>18.86 ± 0.23⁰</td>
<td>18.82 ± 0.25⁰</td>
<td>18.93 ± 0.27⁰</td>
</tr>
<tr>
<td>before euthanasia</td>
<td>19.15 ± 0.07</td>
<td>18.33 ± 0.22⁰</td>
<td>18.03 ± 0.16⁰</td>
<td>15.65 ± 0.23⁰</td>
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<tr>
<td>CRP (mg/L)</td>
<td></td>
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<tr>
<td>before embedding</td>
<td>5.06 ± 0.11</td>
<td>5.09 ± 0.04</td>
<td>5.14 ± 0.09</td>
<td>4.98 ± 0.11</td>
</tr>
<tr>
<td>before administration</td>
<td>28.45 ± 0.08⁰</td>
<td>28.36 ± 0.12⁰</td>
<td>28.40 ± 0.10⁰</td>
<td>28.48 ± 0.13⁰</td>
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<tr>
<td>before euthanasia</td>
<td>30.18 ± 0.04</td>
<td>26.79 ± 0.17⁰</td>
<td>26.03 ± 0.14⁰</td>
<td>14.57 ± 0.19⁰</td>
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<tr>
<td>Colony counts (log cfu/mL)</td>
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<td></td>
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<tr>
<td>tissue</td>
<td>7.62 ± 0.01</td>
<td>7.41 ± 0.02</td>
<td>7.27 ± 0.02</td>
<td>5.41 ± 0.20⁰</td>
</tr>
<tr>
<td>silica gel tubes</td>
<td>7.69 ± 0.01</td>
<td>7.49 ± 0.02</td>
<td>7.34 ± 0.01</td>
<td>5.67 ± 0.09⁰</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD obtained from six rats in each group except group C, before euthanasia (four rats died before the last drug administration in this group).

*P < 0.05, as compared with the same group before embedding.

+P < 0.05, as compared with group F + I at the same time.

−P < 0.05, as compared with the same group before administration.

−P < 0.05, as compared with the control group.

against many bacteria that are resistant to other aminoglycosides. Therefore, this combination may have clinical significance and advantage.

A staggered (time-lag/sequential/step-by-step) chemotherapy strategy was adopted in our study: fosfomycin was injected intraperitoneally first and the injection of isepamicin was 1 h later. Chemotherapy using fosfomycin with other antibiotics was confirmed to have excellent therapeutic effects in both in vitro and clinical studies. It was reported that with 1 h of fosfomycin treatment, flomoxef can achieve the strongest bactericidal effect and the longest post-antibiotic effect against methicillin-resistant Staphylococcus aureus.

Four out of six rats died before euthanasia in the control group, indicating that, without any antimicrobial therapy, biofilm infection is severe and lethal. Although the control group before euthanasia (n = 2) is too small to evaluate the statistical significance, we can still conclude from the remaining data that as a single agent, neither fosfomycin nor isepamicin affected the CRP levels, the numbers of WBCs or the colony counts of the bacteria from both tissue and silica gel tubes. In contrast, the combined treatment of fosfomycin and isepamicin resulted in a good therapeutic effect. Not only was the growth of bacteria suppressed, but the inflammatory reaction indexes also indicated recovery.

Fosfomycin has been reported to be involved in some combination therapies that have an excellent therapeutic effect against biofilms. However, most of these therapies focused on the combination of fluoroquinolones and fosfomycin. Although the detailed mechanism of the synergistic effect of fosfomycin with other antibiotics remains unclear, some explanations were suggested in previous studies. First of all, fosfomycin was reported to destroy or change the outer layer construction of bacteria, thus inhibiting the first step of cell wall synthesis. As a result, the other agent can easily get into cells to produce a synergistic effect. Monden et al.¹ reported that pre-treatment with fosfomycin significantly enhanced cellular uptake of ofloxacin in biofilm cells as well as in floating cells. Impaired permeability is known to be an important resistance mechanism of P. aeruginosa biofilms, thus, the high rate of penetration into biofilms by fosfomycin may be another important factor contributing to its efficacy. Rodrı´ guez-Martı´ nez et al.⁹ have evaluated several antimicrobial agents for their activity against and penetration into P. aeruginosa biofilms, and found that ciprofloxacin, amoxicillin/clavulanic acid and fosfomycin had high penetration rates against a mature biofilm. Another study indicated that the expression level of the transport system (sn-glycerol 3-phosphate transport) that delivers fosfomycin into bacterial cells was increased under anaerobic conditions. However, fosfomycin did not react with a negatively charged bacterial glycocalyx. These observations implied that fosfomycin is able to penetrate through multilayered biofilms and is transported into cells in the stationary phase with a low growth rate. Lastly, the excellent therapeutic effect of fosfomycin combination therapies may result from its effect on the immune system. Fosfomycin directly affects lymphocyte function and modulates the secretion of several cytokines. The anti-inflammatory effect of fosfomycin has been observed both in vitro and in vivo. One study suggested that fosfomycin may modify the acute-phase inflammatory response by disturbing the cytokine cascade, this effect being independent of fosfomycin’s antibacterial activity.¹⁰

In conclusion, the use of fosfomycin in combination with aminoglycosides not only showed a positive effect in vitro but also offered an improved therapeutic effect against a biofilm-infected rat model. Current studies are underway to test other dosing regimens and pharmacokinetics. Although this is a preliminary study, we believe that such combination treatment can provide a new strategy to combat previously untreatable drug-resistant infections at a lower dosage so that the side effects caused by high dosages of aminoglycosides can be avoided.
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Transparency declarations
None to declare.

References