

ORIGINAL ARTICLE

In vitro activity of fosfomycin in combination with colistin against clinical isolates of carbapenem-resistant *Pseudomonas aeruginosa*

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The shortage of effective antibiotics against carbapenem-resistant *Pseudomonas aeruginosa* (CRPA) poses a public health threat. Combination treatment may represent a good choice for treating infections caused by CRPA. The aim of this study was to evaluate the *in vitro* efficacy of fosfomycin in combination with colistin against clinical CRPA isolates. Eighty-seven isolates were collected from three hospitals in China. The checkerboard method and time-kill assay were used to assess the interactions between fosfomycin and colistin. The fosfomycin/colistin combination displayed synergistic and partial synergistic activity against 21.84% and 27.59% of the isolates, respectively. Antagonism was not observed. In combination, the colistin MIC values were $\leq 0.5 \mu\text{g ml}^{-1}$ for 91.95% of the isolates. This result differed significantly from those obtained using a single agent treatment (The colistin MIC values were $\leq 0.5 \mu\text{g ml}^{-1}$ for only 25.29% of the isolates). In addition, the time-kill assay demonstrated that the fosfomycin/colistin combination treatment exerted bactericidal effects against five isolates and that the regrowth observed after colistin monotherapy was prevented. In summary, the combination of fosfomycin and colistin demonstrated synergistic activity against the CRPA isolates tested in this study. Furthermore, fosfomycin may potentially widen the therapeutic window of colistin, suggesting that this combination could be applied clinically to control infections caused by CRPA.

The Journal of Antibiotics (2015) 68, 551–555; doi:10.1038/ja.2015.27; published online 25 March 2015

INTRODUCTION

Pseudomonas aeruginosa (PA) is an important pathogen that causes serious healthcare-associated infections. Carbapenem is the mainstay for the empirical treatment of suspected *P. aeruginosa* infections due to its excellent antimicrobial activity.¹ However, outbreaks and the increased prevalence of carbapenem-resistant PA (CRPA) have been continuously reported in hospitals worldwide, resulting in high morbidity, mortality, and medical costs.² CRPA pathogens can confer resistance not only to most clinically available β -lactam agents but also to other classes of antibiotics such as aminoglycosides, fluoroquinolones, and co-trimoxazole.³ Consequently, this problem renders the empirical choice of an appropriate antimicrobial treatment very difficult.⁴

Colistin, or polymyxin E, is a cationic polypeptide antibiotic that was discovered >60 years ago. Because of its nephrotoxicity and neurotoxicity, the use of colistin has been discontinued since the 1970s.⁵ Currently, colistin is typically used to treat infections of multidrug-resistant *P. aeruginosa* (MDR-PA).⁶ However, antibiotic resistance and heteroresistance have also been associated with colistin treatment, particularly if the drug is used as a monotherapy or at a high dosage.⁷ This finding highlights the importance of investigating

rational combinations of colistin and other antibiotics for the treatment of CRPA infections.⁸ Fosfomycin, a phosphonic acid derivative discovered in Spain in 1969, exhibits bactericidal activity against a wide range of bacteria, including Gram-negative bacteria (GNB) and some Gram-positive bacteria. In recent years, the drug has evoked interest as a treatment for both urinary and systemic infections arising from GNB that are resistant to traditional agents because it appears to retain antimicrobial activity against a substantial percentage of these isolates.⁹

In this study, we investigated the *in vitro* activity of the combination treatment of colistin and fosfomycin against CRPA isolates. We further determined whether such a combination could potentially be used in the clinic for treating CRPA infections.

MATERIALS AND METHODS

Bacterial strains

Eighty-seven non-duplicate clinical isolates of CRPA were collected between February 2012 and March 2013 from three hospitals in Beijing, China (PLA General Hospital 22 strains, Beijing Hospital 27 strains, Navy General Hospital 38 strains) in this study. Sixty-five strains were collected from sputum, 13 from urine, 4 from wound sites, 3 from catheters, and 2 from blood. The

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Received 31 May 2014; revised 9 February 2015; accepted 21 February 2015; published online 25 March 2015

identification and antimicrobial susceptibility profiles of the isolates were determined with the VITEK-2 compact system using the cards ID-GNB and AST-GN09 (BioMérieux, Marcy l'Etoile, France). Quality control was assessed using the strain *P. aeruginosa* ATCC 27853 (ATCC, Manassas, VA, USA).

Antibiotics and other materials

Fosfomycin powder (96.5 % purity) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Colistin sulfate powder with a potency of 23 584 U mg⁻¹ and D-glucose-6-phosphate disodium salt hydrate (G-6-P) were purchased from Sigma-Aldrich (St Louis, MO, USA). Mueller Hinton agar (MHA) and cation-adjusted Mueller II broth (CA-MHB) were purchased from Becton, Dickinson (Franklin Lakes, NJ, USA). Fosfomycin and colistin were diluted in sterile water and used as prepared stock solutions of 2048 µg ml⁻¹ and 1024 µg ml⁻¹, respectively.

MIC determination and synergistic activity testing

The disk agar diffusion method was further used to verify antibiotic susceptibility with Oxoid discs (Oxoid, Wade Road, UK) containing imipenem (10 µg), meropenem (10 µg), amikacin (30 µg), gentamicin (10 µg), ceftazidime (30 µg), cefepime (30 µg), aztreonam (30 µg), ciprofloxacin (5 µg) or piperacillin-tazobactam (100 µg/10 µg) placed on MHA, according to the Clinical and Laboratory Standards Institute's (CLSI) recommendations.¹⁰ The MIC values for fosfomycin were determined by the agar dilution method supplemented with 25 µg ml⁻¹ G-6-P, and the MIC values for colistin were evaluated using the broth microdilution method, according to the CLSI's recommendations.¹⁰ *P. aeruginosa* ATCC 27853 was included in each method as the quality control strain. The MIC was defined as the lowest drug concentration that inhibited the visible growth of colonies. CRPA was defined as those isolates that were resistant to imipenem, according to the CLSI.¹⁰ MDR-PA isolates were defined as those that were non-susceptible to one or more agents in three or more antimicrobial categories.¹¹ The experiments were performed in triplicate on separate days.

Synergy testing was performed in 96-well microplates with CA-MHB containing the tested agents at two-fold dilutions dispensed in a checkerboard pattern. The concentration ranges of these antibiotics were determined according to the MIC values obtained in the preliminary susceptibility tests described above. Fosfomycin supplemented with G-6-P (25 µg ml⁻¹) was prepared in a checkerboard conformation. Fifty microliters of each drug and 100 µl of the bacterial suspensions were added to the 96-well microplates. The final concentration of the inocula was approximately 1.5 × 10⁵ CFU per ml in each well. The MIC values of colistin determined using *P. aeruginosa* ATCC 27853 were similar in CA-MHB with and without G-6-P. The experiments were performed in triplicate on separate days. The interaction between the combinations of two study agents was determined according to the fractional inhibitory concentration index (FICI). The FICI for a given isolate was calculated as follows: FICI = (MIC of fosfomycin in combination/MIC of fosfomycin alone) + (MIC of colistin in combination/MIC of colistin alone). Synergy, partial synergy, additive, indifference, and antagonism were defined by FICI indices of ≤ 0.5, 0.5 < FICI < 1, FICI = 1, 1 < FICI ≤ 2, and FICI > 2, respectively.¹² The cumulative inhibition ratio (CIR) was defined as the percentage of isolates that were inhibited at a certain antibiotic concentration.

Time-kill curves

Twenty-four-hour time-kill assays were performed in duplicate to evaluate the combination of fosfomycin and colistin against five isolates (PA-1-5), for which synergy was demonstrated using the checkerboard method. Each isolate was treated with each antibiotic alone and in combination at its respective 1/2 × MIC and 1 × MIC values. The bacterial strains were inoculated at ~5 × 10⁵ CFU per ml into tubes containing freshly prepared CA-MHB supplemented with antibiotics, for a final volume of 10 ml and were incubated at 37 °C. A tube without antibiotic was included in each experiment as a growth control. Aliquots were obtained from each tube at 0, 2, 4, 8, 12, and 24 h after inoculation and were serially diluted and/or used as undiluted samples for determining viable counts. The diluted samples (50 µl) were plated onto duplicate MHA plates, and then the plates were incubated at 37 °C for 18 h in

ambient air. The number of colonies that formed were counted, and the total bacterial log₁₀ CFU per ml of the original samples was calculated. Synergy was defined as a ≥ 100-fold or 2-log₁₀ decrease, whereas antagonism was defined as a ≥ 100-fold or 2-log₁₀ increase, in colony counts at 24 h induced by the treatment combination, compared with the most active single agent.¹³ Bactericidal activity of the single antibiotics or combinations was defined as a ≥ 1000-fold or 3-log₁₀ reduction relative to the initial inocula after 24 h of incubation.¹⁴

RESULTS

Susceptibility

The antibiogram results demonstrated that 100% of the isolates were resistant to imipenem, 100% were resistant to ceftazidime and cefepime, 73.56% were resistant to meropenem, 51.73% were resistant to piperacillin-tazobactam, 45.98% were resistant to ciprofloxacin, 41.38% were resistant to gentamicin, 40.23% were resistant to aztreonam, and 39.08% were resistant to amikacin. Approximately 67% of the isolates were MDR-PA.

The MIC values for colistin and fosfomycin ranged from 0.125 µg ml⁻¹ to 4 µg ml⁻¹ and 8 µg ml⁻¹ to 512 µg ml⁻¹, respectively. The colistin MIC values of 25.29% (22/87) of the isolates were ≤ 0.5 µg ml⁻¹. The MIC₅₀ and MIC₉₀ values for fosfomycin were 64 µg ml⁻¹ and 256 µg ml⁻¹, respectively, whereas those of colistin

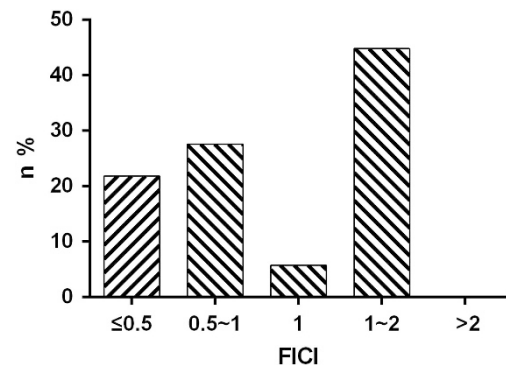


Figure 1 Fractional inhibitory concentration index (FICI) for the combination treatment of fosfomycin and colistin against carbapenem-resistant *Pseudomonas aeruginosa* (n=87). Synergy, partial synergy, additive, indifference, and antagonism were defined by FICI indices of ≤ 0.5, 0.5 < FICI < 1, FICI = 1, 1 < FICI ≤ 2, and FICI > 2, respectively.

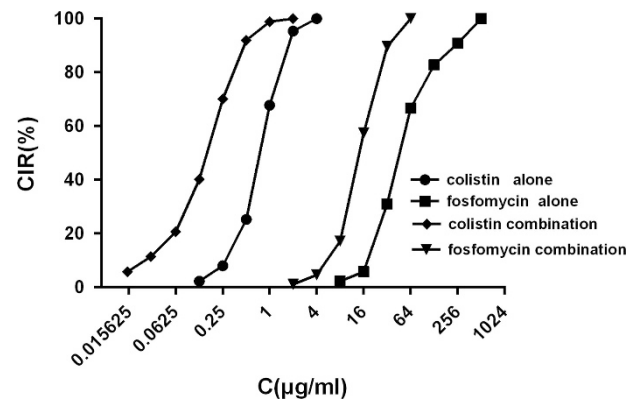


Figure 2 The cumulative inhibition ratio (CIR) of fosfomycin and/or colistin against carbapenem-resistant *Pseudomonas aeruginosa* (n=87). The CIR was defined as the percentage of isolates that were inhibited at a certain antibiotic concentration, C, concentration.

were $1 \mu\text{g ml}^{-1}$ and $2 \mu\text{g ml}^{-1}$, respectively. Of the isolates analyzed, 95.40% (83/87) were susceptible to colistin.

Synergistic activity of the combination treatment

As shown in Figure 1, the drug combination displayed synergistic and partial synergistic activity in 21.84% (19/87) and 27.59% (24/87) of the isolates, respectively. For the other isolates, the combination treatment demonstrated additive or indifferent activity. Antagonism was not observed in this study. The MIC values of colistin and fosfomycin in combination ranged from $0.015 \mu\text{g ml}^{-1}$ to $2 \mu\text{g ml}^{-1}$ and $2 \mu\text{g ml}^{-1}$ to $64 \mu\text{g ml}^{-1}$, respectively. The MIC₅₀ and MIC₉₀ were $0.25 \mu\text{g ml}^{-1}$ and $0.5 \mu\text{g ml}^{-1}$ for colistin, respectively, and $16 \mu\text{g ml}^{-1}$ and $64 \mu\text{g ml}^{-1}$ for fosfomycin, respectively. The colistin MIC values were $\leq 0.5 \mu\text{g ml}^{-1}$ for 91.95% (80/87) of the isolates. The CIR curves for fosfomycin and/or colistin against CRPA are displayed

in Figure 2. The CIR curves for both antibiotics were shifted to the left when the drugs were used in combination, compared with each drug alone, which suggests the potential utility of this combination.

Time-kill assay

The five CRPA isolates studied were MDR-PA with different MIC values for fosfomycin and colistin. As shown in Figure 3, the time-kill assays indicated that neither of the two agents demonstrated notable bactericidal activity against any of the five isolates when tested alone. Colistin monotherapy demonstrated bacteriostatic activity against PA-1, PA-2, PA-3, and PA-4 at $1 \times \text{MIC}$ within 4 h, but considerable regrowth occurred after 4 h. However, when colistin was combined with fosfomycin, the regrowth did not occur, even after 24 h of incubation (Figure 3a–d). The combination of fosfomycin and colistin demonstrated bactericidal activity within 12 h at both $1/2 \times \text{MIC}$ and

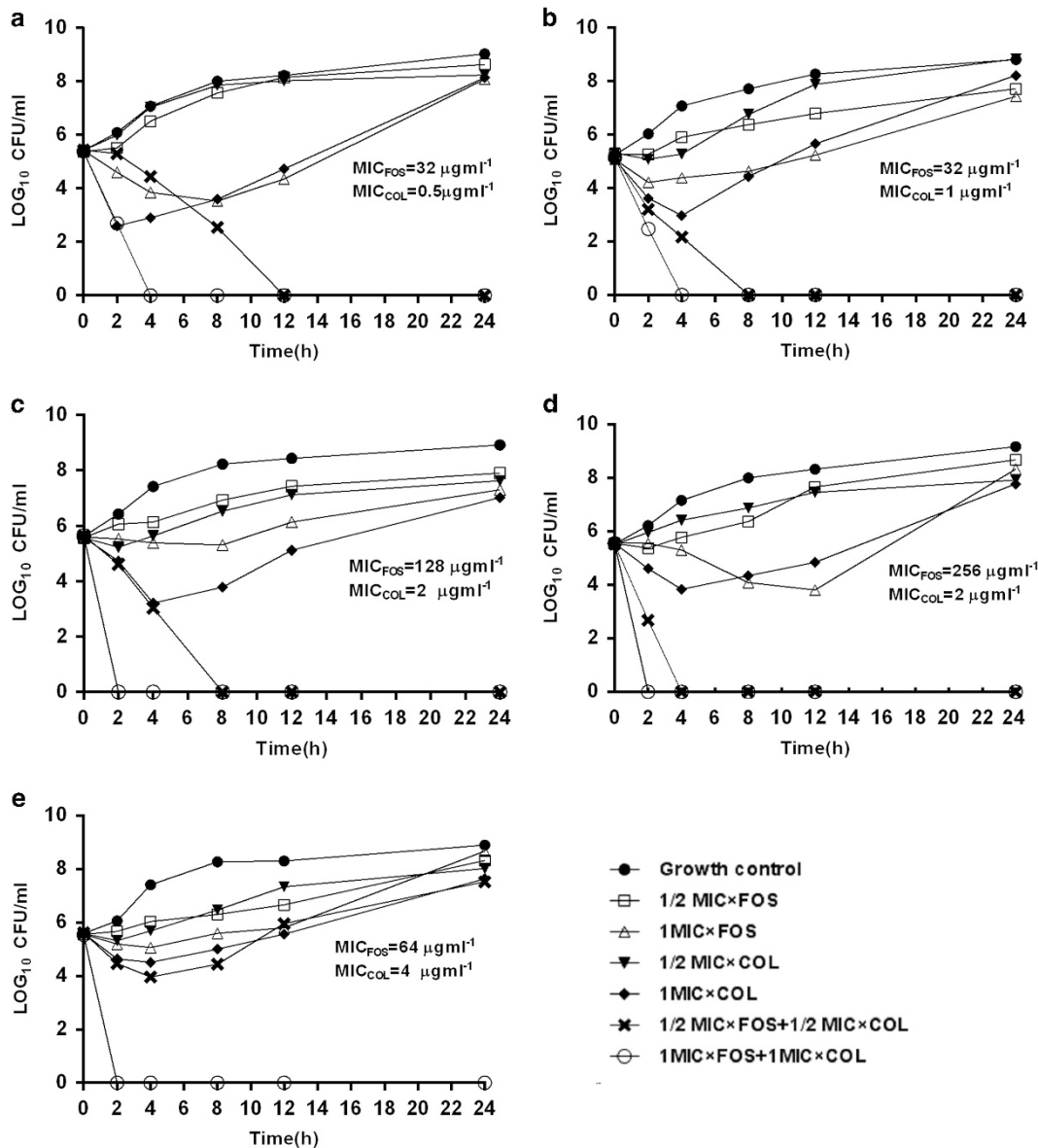


Figure 3 The time-kill curves of fosfomycin and/or colistin against carbapenem-resistant *Pseudomonas aeruginosa*. The *in vitro* time-kill experiments were performed in duplicate, and the mean values were plotted. For the duplicate experiments, similar time-kill results were obtained. (a) *P. aeruginosa*-1 (PA-1); (b) *P. aeruginosa*-2 (PA-2); (c) *P. aeruginosa*-3 (PA-3); (d) *P. aeruginosa*-4 (PA-4); (e) *P. aeruginosa*-5 (PA-5). COL, colistin; FOS, fosfomycin.

1 × MIC for PA-1, PA-2, PA-3, and PA-4 (Figure 3a–d), whereas the combination exhibited synergistic bactericidal activity for PA-5 only at 1 × MIC (Figure 3e).

DISCUSSION

The treatment of infections caused by *P. aeruginosa* is often difficult due to the natural and acquired resistance of this pathogen to several antibiotics. The emergence and global spread of CRPA is a major therapeutic and epidemiological challenge that leaves few available therapeutic options. In this study, all CRPA isolates were resistant to the antibiotics tested, including ceftazidime, aztreonam, and ciprofloxacin, etc., to various degrees. However, the majority of the tested isolates were susceptible to colistin, indicating that this drug may be a viable alternative for treating infections with CRPA.^{7,15} However, a limitation of colistin treatment has been its inability to achieve adequate plasma concentrations, especially for infections caused by organisms with MIC values >0.5 µg ml⁻¹.¹⁶ Our study demonstrated that the colistin MIC values for most of the evaluated isolates were ≤0.5 µg ml⁻¹ in the combination therapy. Therefore, combination therapies may provide alternative treatment strategies and improve treatment outcomes.

Fosfomycin has demonstrated low toxicity against a number of infections and has a role in the treatment of infections caused by MDR-PA. Some studies have also reported that fosfomycin improves treatment outcomes, prevents antibiotic resistance, and reduces the nephrotoxicity induced by aminoglycoside or vancomycin.^{17–20} In this study, colistin and fosfomycin displayed synergistic and partial synergistic effects in 49.43% of the isolates, and no antagonism was observed. The results were partially consistent with a previous study by Samonis *et al.*,²¹ in which 15 MDR-PA isolates were tested. Based on the CIR curves, we observed a notable difference between single agent and combination treatment against CRPA isolates. In combination therapy, the fosfomycin and colistin MIC values for most of the isolates were significantly lower than the plasma concentrations that can be achieved for both drugs.^{22,23} Thus, fosfomycin and colistin could be used in combination when treating CRPA infections.

The time-kill assay results were consistent with those of the checkerboard method. Colistin alone at 1/2 × MIC could not inhibit bacterial growth, and at 1 × MIC produced minimal initial killing of PA-1, PA-2, PA-3, and PA-4 within 4 h. This result suggests that the bactericidal activity of colistin is concentration dependent. However, the bacteriostatic activity of colistin was not sustained, and regrowth occurred rapidly after 4 h of treatment. The heteroresistance of the isolates might contribute to the observed regrowth following colistin monotherapy.²⁴ When colistin was combined with fosfomycin, the treatment's bactericidal activity was substantially enhanced, and the regrowth that occurred with colistin monotherapy was prevented. Rao *et al.*²⁵ have demonstrated that increasing the C_{max} of colistin monotherapy could not completely eliminate the bacteria population, but the addition of a synergistic second antimicrobial may sustain the initial killing achieved by a colistin front-loaded regimen. Enhanced bacterial killing was particularly evident for combinations at 1/2 × MIC against PA-1, PA-2, PA-3, and PA-4, and no viable bacteria was detected at 24 h (Figure 3). The time-kill assay demonstrated that the co-administration of fosfomycin possesses the potential to increase the therapeutic index of colistin.

The limitation of this study was the lack of molecular characterization and fingerprinting of the CRPA isolates. Epidemiological typing could provide additional insight into the resistance mechanisms of CRPA.

In conclusion, the combination of fosfomycin and colistin displayed significant synergistic activity against most of the tested CRPA isolates. The presence of fosfomycin enhanced and prolonged the bactericidal effects of colistin. Fosfomycin may widen colistin's therapeutic window, especially when treating an infection caused by organisms for which an MIC of >0.5 µg ml⁻¹ is required. Further research is needed to clarify the potential utility of the combination treatment of colistin and fosfomycin *in vivo* and in the clinic.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

ACKNOWLEDGEMENTS

This study was supported by the Major National Science and Technology Special Projects for New Drugs (No.2012ZX09303004) and the Beijing Municipal Natural Science Foundation (No.7122167).

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