In vitro interactions of antimicrobial combinations with fosfomycin against KPC-2-producing Klebsiella pneumoniae and protection of resistance development

Maria Souli*, Irene Galani, Stefanos Boukovalas, Michael George Gourgoulis, Zoi Chryssouli, Kyriaki Kanellakopoulou, Theofano Panagea, Helen Giamarellou¹

4th Department of Internal Medicine, Athens University School of Medicine, University General Hospital “Attikon”, 1 Rimini Str, 124 62 Chaidari, Greece
¹Current affiliation: 6th Department of Internal Medicine, Diagnostic and Therapeutic Center of Athens “Hygeia” 4 Erythrou Stavrou Str and Kifissias avenue, 151 23 Maroussi, Greece

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Running title: fosfomycin combinations against KPC-producers

*Correspondence:
Maria Souli
1 Rimini Str, 124 62
Chaidari, Greece.
Tel : ++321055831984
Fax : ++32105326446
Email: msouli@med.uoa.gr
Abstract

Using time-kill methodology, we investigated the interactions of fosfomycin with meropenem or colistin or gentamicin against 17 genetically distinct *Klebsiella pneumoniae* clinical isolates carrying *bla*<sub>KPC-2</sub>. Synergy was observed with meropenem or colistin against 64.7 and 11.8% of tested isolates while the combination with gentamicin resulted in indifference. All studied combinations showed improved bactericidal activity, as compared to fosfomycin alone and prevented the development of fosfomycin-resistance in 69.2, 53.8 and 81.8% of susceptible isolates, respectively.
Fosfomycin is a phosphonic acid derivative (cis-1,2-epoxypropyl phosphonic acid) with a broad spectrum of activity against various Gram-positive and Gram-negative pathogens including *Pseudomonas aeruginosa*, ESBL- and/or carbapenemase-producing Enterobacteriaceae even those that are tigecycline or colistin non-susceptible (4,5,6). Unfortunately, resistance develops rapidly when fosfomycin is used as monotherapy (3) therefore combinations with other antimicrobials are preferred in clinical practice for the treatment of serious infections. Nevertheless, the potential advantage of such combinations against the predominant nowadays, multiresistant KPC-producing *Klebsiella pneumoniae* has not yet been studied.

We investigated the in vitro activities of fosfomycin and meropenem or gentamicin or colistin alone and in combination against 17 unique clinical isolates of KPC-2-producing *K. pneumoniae* isolated from inpatients in the University General Hospital “Attikon”, Athens, Greece. MICs were determined using BD Phoenix automated system (Becton Dickinson Diagnostic Systems, Sparks, MD, USA). Those of meropenem
(Dianippon Sumitomo Pharma, Osaka, Japan) and fosfomycin (Sigma-Aldrich, St Louis, MO, USA) were also evaluated with agar dilution (2) and those of colistin (sulphate salt, AppliChem GmbH, Darmstadt, Germany) with Etest (AB Biodisk, Solna, Sweden). Results were interpreted in accordance with CLSI criteria (2) except for fosfomycin and colistin for which the breakpoints proposed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; www.eucast.org) were used (susceptibility, ≤32 for fosfomycin and ≤2 µg/ml for colistin). All isolates were screened for the production of a KPC enzyme with the imipenem-boronic acid disk synergy test (15), PCR using primers specific for \( \text{bla}_{KPC} \) (1) and sequencing (Eurofins MWG GmbH, Ebersberg, Germany). On the basis of these tests all studied isolates carried \( \text{bla}_{KPC-2} \). The genetic relatedness among these isolates was evaluated with Pulsed Field Gel Electrophoresis (PFGE) analysis of chromosomal restriction fragments obtained after SpeI cleavage (14). In vitro interactions between fosfomycin and meropenem or gentamicin (AppliChem) or colistin were tested using time-kill methodology (13) in cation-adjusted Muller Hinton II broth (Becton Dickinson) supplemented with 25 µg/ml glucose-6-phosphate (AppliChem), which was required for induction of the transport system of hexose monophosphate necessary for entry of fosfomycin into bacterial cells (2). Antibiotic concentrations used were 100 µg/ml for fosfomycin, 10 µg/ml for meropenem and 5 µg/ml for
coli
tin and gentamicin, as these concentrations represent the steady state 
of the respective antibiotic achievable in human serum during treatment 
(7,10,11). The lower limit of detection was 1.6 log_{10} CFU/ml. Synergy, 
 antagonism, indifference and bactericidal activity were defined as 
previously reported (13). Fisher’s exact test was used to compare 
 proportions of killing activity in two by two tables. A P-value <0.05 was 
considered to be statistically significant. In order to evaluate for the 
development of resistance to fosfomycin as a reason for bacterial re-
growth after 24 hours of incubation with fosfomycin alone or in 
combination, viable colonies were submitted to susceptibility testing in 
comparison with the respective wild type strain using agar dilution. This 
evaluation was performed only for isolates that were initially susceptible 
to fosfomycin.

The results are depicted in Table 1. These strains were collected from 
September 2007 until July 2009 during an outbreak of KPC-2 producing 
*K. pneumoniae* in our institution. Four major clonal types were identified 
by PFGE during this outbreak (24). Strains representative of all four 
clonal types were evaluated in the present study. More than one isolate of 
the most common subtypes (A1, A2 and B1) were included because of 
differences in the susceptibility phenotype or the *bla* gene content of the 
isolates (data not shown). The clonal nature of the KPC-producing
K. pneumoniae outbreak in our hospital precluded from testing a larger number of isolates that were genotypically diverse.

The combination of fosfomycin and meropenem exhibited synergy against 11 of the 17 (64.7%) isolates. This combination was bactericidal against 12 of the 17 (70.6%) isolates, 11 of which were susceptible to fosfomycin (bactericidal activity of the combination vs fosfomycin or meropenem alone, p<0.05). The combination of fosfomycin and colistin was synergistic against 2 of the 17 (11.8%) isolates and exhibited a bactericidal activity against 11 of the 17 isolates (64.7%). All of these isolates were colistin-susceptible with the exception of two isolates (608 and P908), (bactericidal activity of the combination vs fosfomycin, p<0.05 and vs colistin alone, p=0.3).

The combination of fosfomycin and gentamicin exhibited an indifferent effect against all tested isolates and was not able to suppress the growth of any of the gentamicin-resistant isolates. It was bactericidal against all gentamicin-susceptible and gentamicin-intermediately susceptible ones (12 of 15, 80%) (bactericidal activity of the combination vs fosfomycin alone, p<0.05 and vs gentamicin alone, p=1.0).

Representative time-kill curves are shown in Figure 1.
Repeat MIC determination was done for 13 *K. pneumoniae* isolates that were initially susceptible to fosfomycin. All isolates developed resistance to fosfomycin after 24 hours of incubation with fosfomycin alone. A clone resistant to fosfomycin was selected in 4 isolates (4/13, 30.8%) after incubation with fosfomycin and meropenem and in 6 isolates (6/13, 46.2%) after incubation with fosfomycin and colistin. The latter were all colistin-resistant isolates. In 2 isolates (2/11, 18.2%) a clone resistant to fosfomycin was selected after incubation with fosfomycin and gentamicin. These isolates were all gentamicin-resistant.

Clinical studies have shown that combinations with fosfomycin achieved an overall cure rate of >80% against serious infections caused by multidrug resistant pathogens but data specifically concerning KPC-producers is scarce. Recently, fosfomycin was administered to 11 seriously ill ICU-patients in combination with colistin or gentamicin and resulted in a successful clinical response in all of them (8). Fosfomycin in combination with a carbapenem was evaluated against 18 ertapenem-non-susceptible *E.coli* and *K. pneumoniae* clinical isolates, none of which carried a *bla*<sub>KPC</sub>. An additive effect and a ca. 2-fold reduction of carbapenem MIC was noted (9). Fosfomycin combinations have not been previously evaluated against KPC-producing *K. pneumoniae*. Our
experiments showed that fosfomycin resulted in synergy with meropenem or colistin against 64.7 and 11.8% of isolates, respectively. All studied combinations showed improved bactericidal activity as compared to fosfomycin alone and prevented the development of fosfomycin-resistance in the majority of susceptible isolates.
157 References

Emergence of carbapenem-resistant Klebsiella species possessing the
class A carbapenem-hydrolysing KPC-2 and inhibitor-resistant TEM-30

162 2. CLSI. 2009. Performance standards for antimicrobial susceptibility
testing; 19th informational supplement. CLSI document M100-S19.
Clinical and Laboratory Standards Institute, Wayne, PA.

Woodford. 2006. Mutators among CTX-M β-lactamase-producing
Escherichia coli and risk for the emergence of fosfomycin resistance. J.

fosfomycin against blaKPC-containing Klebsiella pneumonia isolates,
including those nonsusceptible to tigecycline and/or colistin. Antimicrob.
Agents Chemother. 54:526–529.

174 5. Falagas, M. E., A.C. Kastoris, A. M. Kapaskelis, and D. E.
Karageorgopoulos. 2010. Fosfomycin for the treatment of multidrug-
resistant, including extended-spectrum β-lactamase producing,


Figure 1.

Time-kill studies showing interactions of fosfomycin (FOF) with meropenem (MEM) or colistin (CST) or gentamicin (GEN) against two representative isolates (P 908 and 878 P) included in the present study.
Table 1. Clonal types (A-D) of the 17 KPC-2 positive *K. pneumoniae* isolates studied, MICs of fosfomycin, meropenem, colistin and gentamicin, the ratio of the concentration of each antibiotic used in time-kill studies/MIC and in vitro interactions of the studied combinations against these isolates

<table>
<thead>
<tr>
<th>Strain</th>
<th>Clonal type</th>
<th>MIC (µg/ml) [Concentration tested/MIC]</th>
<th>Interaction of FOF with (Time of growth)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FOF MEM CST GEN</td>
<td>MEM CST GEN</td>
</tr>
<tr>
<td>m 4096C</td>
<td>C</td>
<td>8 [12.5] 4 [2.5] 0.75 [6.67] &gt;8 [&lt;0.61]</td>
<td>Ind Ind Ind</td>
</tr>
<tr>
<td>439 CII</td>
<td>A2</td>
<td>8 [12.5] 64 [0.16] 0.38 [13.16] ≤2 [≥2.5]</td>
<td>Syn (24h) Ind Ind</td>
</tr>
<tr>
<td>m 4908C</td>
<td>D</td>
<td>16 [6.25] 64 [0.16] 32 [0.16] ≤2 [≥2.5]</td>
<td>Syn (24h) Ind Ind</td>
</tr>
<tr>
<td>b 1013</td>
<td>A2</td>
<td>16 [6.25] 64 [0.16] 0.38 [13.16] 4 [1.25]</td>
<td>Syn (24h) Ind Ind</td>
</tr>
<tr>
<td>P 908</td>
<td>A1</td>
<td>16 [6.25] 32 [0.31] 12 [0.42] ≤2 [≥2.5]</td>
<td>Syn (24h) Syn (24h) Ind</td>
</tr>
<tr>
<td>258</td>
<td>A1</td>
<td>16 [6.25] 64 [0.16] 8 [0.63] ≤2 [≥2.5]</td>
<td>Syn (24h) Ind Ind</td>
</tr>
<tr>
<td>202</td>
<td>A1</td>
<td>16 [6.25] 32 [0.31] 48 [0.10] ≤2 [≥2.5]</td>
<td>Syn (24h) Ind Ind</td>
</tr>
<tr>
<td>Strain</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
<td>MIC&lt;sub&gt;90&lt;/sub&gt; (µg/ml)</td>
<td>MIC&lt;sub&gt;50&lt;/sub&gt; (µg/ml)</td>
</tr>
<tr>
<td>----------</td>
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<td>--------------------------</td>
</tr>
<tr>
<td>P 903 A1</td>
<td>32 [3.13]</td>
<td>32 [0.31]</td>
<td>16 [0.31]</td>
</tr>
<tr>
<td>m 538C A2</td>
<td>32 [3.13]</td>
<td>128 [0.08]</td>
<td>0.38 [13.16]</td>
</tr>
<tr>
<td>m 2573A A1</td>
<td>32 [3.13]</td>
<td>8 [1.25]</td>
<td>6 [0.83]</td>
</tr>
<tr>
<td>m 4185C B1</td>
<td>32 [3.13]</td>
<td>32 [0.31]</td>
<td>0.5 [10]</td>
</tr>
<tr>
<td>m 4362C B1</td>
<td>32 [3.13]</td>
<td>64 [0.16]</td>
<td>0.38 [13.16]</td>
</tr>
<tr>
<td>m 3353 B1</td>
<td>32 [3.13]</td>
<td>32 [0.31]</td>
<td>0.75 [6.67]</td>
</tr>
<tr>
<td>m 1044C A2</td>
<td>256 [0.39]</td>
<td>&gt;256 [&lt;0.04]</td>
<td>24 [0.21]</td>
</tr>
<tr>
<td>608 A2</td>
<td>256 [0.39]</td>
<td>&gt;256 [&lt;0.04]</td>
<td>4 [1.25]</td>
</tr>
<tr>
<td>878 P A2</td>
<td>256 [0.39]</td>
<td>256 [0.04]</td>
<td>0.5 [10]</td>
</tr>
<tr>
<td>m 3473C B2</td>
<td>&gt;256 [&lt;0.39]</td>
<td>8 [1.25]</td>
<td>1 [5]</td>
</tr>
</tbody>
</table>

**MIC<sub>50</sub>** (µg/ml) | 32 | 64 | 1 | ≤2

**MIC<sub>90</sub>** (µg/ml) | 256 | >256 | 32 | >8

**Percentage susceptible (%)** | 76.5 | 5.9 | 52.9 | 76.5
<table>
<thead>
<tr>
<th>No of isolates showing synergy</th>
<th>11/17 (64.7%)</th>
<th>2/17 (11.8%)</th>
<th>0/15</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevention of resistance to fosfomycin&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9/13 (69.2%)</td>
<td>7/13 (53.8%)</td>
<td>9/11 (81.8%)</td>
</tr>
</tbody>
</table>

MIC, minimum inhibitory concentration; Ind, indifference; Syn, synergy; MIC<sub>50</sub>, minimum inhibitory concentration inhibiting 50% of the studied isolates; MIC<sub>90</sub>, minimum inhibitory concentration inhibiting 90% of the studied isolates; FOF, fosfomycin; MEM, meropenem; CST, colistin; GEN, gentamicin; ND, not done

<sup>a</sup> Antibiotic concentrations used in time-kill studies were fosfomycin, 100 μg/ml; meropenem, 10 μg/ml; colistin and gentamicin, 5 μg/ml

<sup>b</sup> The denominator was the number of fosfomycin-susceptible isolates tested
Figure 1

P 908

Time (h)
Viable counts (log 10 cfu/mL)
FM
MER
FM-MER

10
987654321

Time (h)
Viable counts (log 10 cfu/mL)
FM
GM
FM-GM

10
987654321

Time (h)
Viable counts (log 10 cfu/mL)
FM
COL
FM-COL

10
987654321

Time (h)
Viable counts (log 10 cfu/mL)
FM
GM
FM-GM

10
987654321