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In Vitro Interactions of Antimicrobial Combinations with Fosfomycin against KPC-2-Producing *Klebsiella pneumoniae* and Protection of Resistance Development[▽]

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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_{KPC-2}. 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, 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-epoxypropylphosphonic acid) with a broad spectrum of activity against various Gram-positive and Gram-negative pathogens, including *Pseudomonas aeruginosa*, extended-spectrum beta-lactamase- and/or carbapenemase-producing *Enterobacteriaceae*, even those that are tigecycline or colistin nonsusceptible (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 multiresistant *Klebsiella pneumoniae* carbapenemase (KPC)-producing bacteria predominant nowadays has not yet been studied.

(Part of these data was presented at the 47th Infectious Diseases Society of America Annual Meeting, 2009, Philadelphia, PA [abstr. 218].)

We investigated the *in vitro* activities of fosfomycin and meropenem, gentamicin, or colistin alone and in combination against 17 unique clinical isolates of KPC-2-producing *K. pneumoniae* isolated from inpatients at the University General Hospital Attikon, Athens, Greece. MICs were determined using the BD Phoenix automated system (Becton Dickinson Diagnostic Systems, Sparks, MD). Those of meropenem (Dianippon Sumitomo Pharma, Osaka, Japan) and fosfomycin (Sigma-Aldrich, St. Louis, MO) were also evaluated with agar dilution (2), and those of colistin (sulfate salt, AppliChem GmbH, Darmstadt, Germany) were also evaluated with the Etest (AB Biodisk, Solna, Sweden). Results were interpreted in accordance with CLSI criteria (2), except for fosfomycin and colistin, for which the susceptibility breakpoints proposed by the European Committee on Antimicrobial Susceptibility Testing (www.eucast.org) were used ($\leq$32 μg/ml for fosfomycin and $\leq$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 bla_{KPC} (1), and sequencing (Eurofins MWG GmbH, Ebersberg, Germany). On the basis of these tests, all of the isolates studied carried bla_{KPC-2}. The genetic relatedness of these isolates was evaluated by 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 Mueller-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 the entry of fosfomycin into bacterial cells (2). The antibiotic concentrations used were 100 μg/ml for fosfomycin, 10 μg/ml for meropenem, and 5 μg/ml for colistin 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 of <0.05 was considered to be statistically significant. In order to evaluate the development of resistance to fosfomycin as a reason for bacterial regrowth after 24 h 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.

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TABLE 1. Clonal types of the 17 KPC-2-positive *K. pneumoniae* isolates studied, MICs of fosfomycin, meropenem, colistin, and gentamicin, ratio of the concentration of each antibiotic used in time-kill studies to the MIC, and *in vitro* interactions of the combinations tested against these isolates

Strain	Clonal type	MIC (μg/ml), ratio of concn tested[a] to MIC				Interaction[b] of FOF with (growth time[s] [h]):		
		Fosfomycin	Meropenem	Colistin	Gentamicin	Meropenem	Colistin	Gentamicin
m 4096C	C	8, 12.5	4, 2.5	0.75, 6.67	>8, <0.61	Ind	Ind	Ind
439 CII	A2	8, 12.5	64, 0.16	0.38, 13.16	≤2, ≥2.5	Syn (24)	Ind	Ind
m 4908C	D	16, 6.25	64, 0.16	32, 0.16	≤2, ≥2.5	Syn (24)	Ind	Ind
b 1013	A2	16, 6.25	64, 0.16	0.38, 13.16	4, 1.25	Syn (24)	Ind	Ind
P 908	A1	16, 6.25	32, 0.31	12, 0.42	≤2, ≥2.5	Syn (24)	Syn(24)	Ind
258	A1	16, 6.25	64, 0.16	8, 0.63	≤2, ≥2.5	Syn (24)	Ind	Ind
202	A1	16, 6.25	32, 0.31	48, 0.10	≤2, ≥2.5	Syn (24)	Ind	Ind
P 903	A1	32, 3.13	32, 0.31	16, 0.31	4, 1.25	Syn (6, 24)	Syn (6)	Ind
m 538C	A2	32, 3.13	128, 0.08	0.38, 13.16	8, 0.61	Ind	Ind	Ind
m 2573A	A1	32, 3.13	8, 1.25	6, 0.83	>8, <0.61	Ind	Ind	Ind
m 4185C	B1	32, 3.13	32, 0.31	0.5, 10	≤2, ≥2.5	Syn (24)	Ind	ND[c]
m 4362C	B1	32, 3.13	64, 0.16	0.38, 13.16	≤2, ≥2.5	Syn (24)	Ind	Ind
m 3353	B1	32, 3.13	32, 0.31	0.75, 6.67	≤2, ≥2.5	Syn (24)	Ind	ND
m 1044C	A2	256, 0.39	>256, <0.04	24, 0.21	4, 1.25	Ind	Ind	Ind
608	A2	256, 0.39	>256, <0.04	4, 1.25	≤2, ≥2.5	Ind	Ind	Ind
878 P	A2	256, 0.39	256, 0.04	0.5, 10	≤2, ≥2.5	Ind	Ind	Ind
m 3473C	B2	>256, <0.39	8, 1.25	1, 5	>8, <0.61	Syn (24)	Ind	Ind

[a] The antibiotic concentrations used in time-kill studies were as follows: fosfomycin, 100 μg/ml; meropenem, 10 μg/ml; colistin and gentamicin, 5 μg/ml. The MICs (μg/ml) for 50 and 90%, respectively, of the isolates studied (percent susceptible) were as follows: fosfomycin, 32 and 256 (76.5); meropenem, 64 and >256 (5.9); colistin, 1 and 32 (52.9); gentamicin, ≤2 and >8 (76.5).

[b] Ind, indifference; Syn, synergy. The number (%) of isolates showing synergy/total number of isolates was as follows: meropenem, 11/17 (64.7%);colistin, 2/17 (11.8%); gentamicin, 0/15 (0%). The number (%) of isolates in which resistance to fosfomycin was prevented (the denominator is the number of fosfomycin-susceptible isolates tested) was as follows: meropenem, 9/13 (69.2%); colistin, 7/13 (53.8%); gentamicin, 9/11 (81.8%).

[c] ND, not done.

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 (12). Strains representative of all four clonal types were evaluated in the present study. Multiple isolates 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 (64.7%) of the 17 isolates. This combination was bactericidal against 12 (70.6%) of the 17 isolates, 11 of which were susceptible to fosfomycin (bactericidal activity of the combination versus that of fosfomycin or meropenem alone, $P < 0.05$). The combination of fosfomycin and colistin was synergistic against 2 (11.8%) of the 17 isolates and exhibited bactericidal activity against 11 (64.7%) of them. All of these isolates were colistin susceptible, with the exception of two (608 and P 908) ($P < 0.05$ for the bactericidal activity of the combination versus that of fosfomycin and $P = 0.3$ versus that of colistin alone).

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

Representative time-kill curves are shown in Fig. 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 h 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 are scarce. Recently, fosfomycin was administered to 11 seriously ill intensive care unit 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-nonsusceptible *Escherichia coli* and *K. pneumoniae* clinical isolates, none of which carried bla_{KPC}. An additive effect and a ca. 2-fold reduction of the carbapenem MIC were 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 of the combinations studied

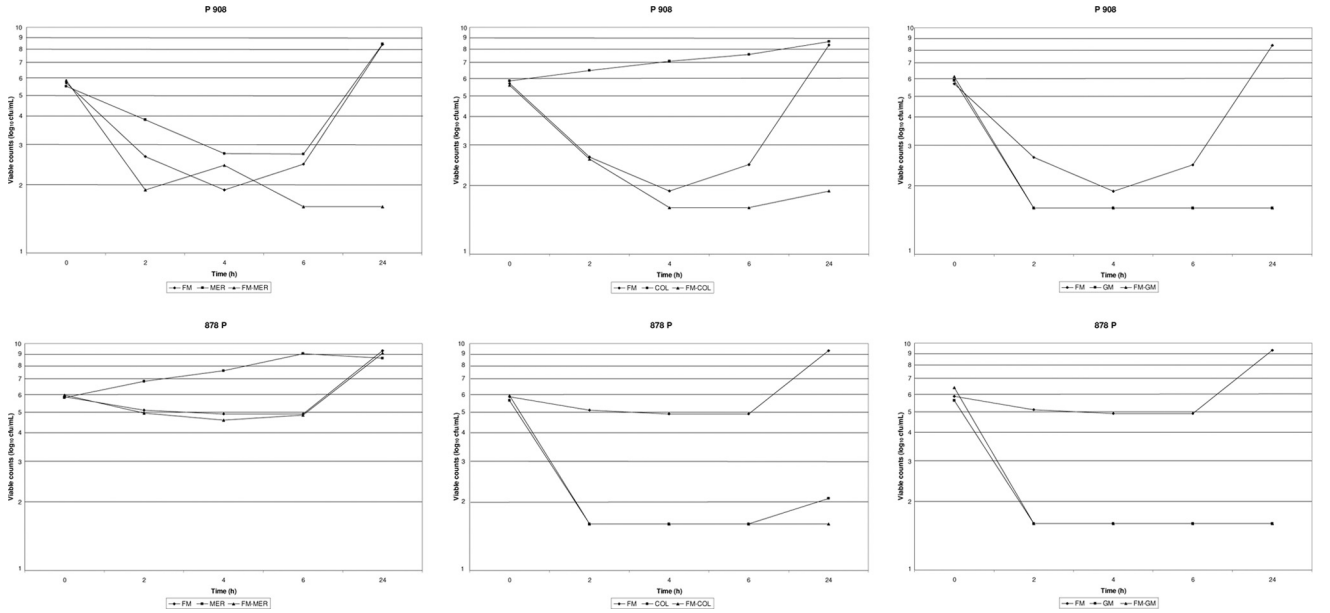

FIG. 1. Time-kill studies showing interactions of fosfomycin (FM) with meropenem (MER), colistin (COL), or gentamicin (GM) against two representative isolates (P 908 and 878 P) included in the present study.

showed improved bactericidal activity compared to fosfomycin alone and prevented the development of fosfomycin resistance in the majority of susceptible isolates.

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