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In vitro investigation of synergy among fosfomycin and parenteral antimicrobials against carbapenemase-producing Enterobacteriaceae

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ABSTRACT

Intravenous fosfomycin is undergoing clinical development in the United States for treatment of complicated urinary tract infections (cUTIs) and may be prescribed as a component of dual antibiotic regimens against carbapenemase-producing Enterobacteriaceae (CPE). Fosfomycin, aztreonam, cefepime, ceftazidime, ceftazidime/avibactam, ceftolozane/tazobactam, meropenem, piperacillin/tazobactam, and tobramycin minimum inhibitory concentrations (MICs) were determined by gradient diffusion strip (GDS) against CPE isolates ($N = 49$). The GDS cross method was used to assess antibiotic interactions between fosfomycin and the aforementioned parenteral antibiotics. The resultant fractional inhibitory concentration index was used to classify interactions. Fosfomycin-containing combinations were evaluated only if nonsusceptible to the second agent. The fosfomycin MIC_{50} was ≥1024 mg/L by GDS. Synergy or additivity was detected in 80 (22%) fosfomycin-containing combinations. Antagonism was not observed. Ceftolozane/tazobactam most frequently displayed synergy [8 (16.3%) isolates]. When CPE are isolated, clinical laboratories should consider performing GDS synergy tests to identify favorable antibiotic interactions.

1. Introduction

Fosfomycin is a broad-spectrum antibiotic available as an intravenous (IV) formulation, fosfomycin disodium, for treatment of various systemic infections in several European countries and Japan (Falagas et al., 2016; Grabein et al., 2017). In the United States, fosfomycin is approved for treatment of uncomplicated urinary tract infection, but it is available only as an oral formulation (Kaye et al., 2017, Monurol Prescribing Information, 2007). However, IV fosfomycin is now being investigated in U.S. clinical trials to support its use in the treatment of complicated urinary tract infections (NCT02753946).

Infections caused by multidrug-resistant (MDR) pathogens such as carbapenemase-producing Enterobacteriaceae (CPE) pose therapeutic challenges. Due primarily to acquisition of multiple broad-spectrum resistance mechanisms, a single agent (e.g., β-lactam) is unlikely to provide adequate empiric coverage of these pathogens in serious systemic infections (Kollef et al., 2011; Pogue et al., 2015). Fortunately, worldwide clinical use of fosfomycin has remained infrequent since its original discovery in 1969 (Grabein et al., 2017), and thus, the compound has retained activity against many MDR pathogens, including some CPE (Falagas et al., 2010,2016;. Furthermore, favorable pharmacokinetic properties such as negligible protein binding and high tissue penetration allow for a potential utility in combination antibiotic regimens for the treatment of MDR pathogens even in cases of deep-seated infection (Grabein et al., 2017).

Pharmacodynamic optimization of antimicrobial regimens, which may include the use of combination therapy with 2 or more agents, is especially important in the treatment of CPE and other MDR Gram-negative pathogens (Kollef et al., 2011; Monogue et al., 2016). The gradient diffusion strip (GDS) cross method is an *in vitro* method used to investigate antibiotic interactions in terms of the fractional inhibitory concentration index (FICI), and it has been used to examine the activity of dual antibiotic combinations against MDR organisms such as *Acinetobacter baumannii* (Nageeb et al., 2015), Enterobacteriaceae (Monogue et al., 2017; Samonis et al., 2012; White et al., 1996), *Neisseria gonorrhoeae* (Wind et al., 2015), *Pseudomonas aeruginosa* (Samonis et al., 2012; White et al., 1996), and *Stenotrophomonas maltophilia* (Church et al., 2013). An anticipated role for IV fosfomycin in the U.S. will be as a component of dual antibiotic therapy when prescribed for CPE infections, especially those outside of the urinary tract. Thus, the purpose of the present study was to characterize antibiotic interactions between fosfomycin and nonsusceptible antibiotics (aztreonam, cefepime, ceftazidime, ceftazidime/avibactam, ceftolozane/tazobactam, meropenem, piperacillin/tazobactam, and tobramycin) among a genotypically diverse population of CPE isolates utilizing the GDS cross method.

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2. Materials and methods

2.1. Bacterial isolates and antimicrobial susceptibility testing

In total, 49 CPE isolates were studied, of which 37 were acquired from the FDA-CDC Antimicrobial Resistance Bank (Atlanta, GA) and 12 were selected from the Center for Anti-Infective Research and Development isolate library. Fosfomycin, aztreonam, cefepime, ceftazidime, ceftazidime/avibactam, ceftolozane/tazobactam, meropenem, and tobramycin MICs were determined by Etest® (bioMérieux, Durham, NC) in accordance with the manufacturer's instructions. Fosfomycin MICs were determined in duplicate, and in cases of discordance, the procedure was performed until a modal MIC was evident. MIC Test Strip (Liofilchem®, Waltham, MA) was used to determine piperacillin/tazobactam MICs due to lack of availability of the Etest product. For MIC and synergy assessments, *P. aeruginosa* ATCC 27853 was used as a quality control organism, which is appropriate for extended spectrum β-lactamase (ESBL)–producing Enterobacteriaceae according to the manufacturer's instructions.

2.2. Antibiotic interaction assessments

For assessments of antibiotic interaction using GDS methodology, isolates were prepared in accordance to the manufacturer's instructions for antimicrobial susceptibility testing of single agents. Each bacterial inoculum was prepared to a concentration of 1 to 5 × 10^8 CFU/mL from a second subculture, which was confirmed by performing serial dilutions and colony counts on agar media (BD™ Trypticase™ Soy Agar with 5% Sheep Blood, Franklin Lakes, NJ). MICs of the agents in combination were determined by the GDS cross method (Pankey et al., 2013) and used to calculate the FICI of each fosfomycin–antibiotic combination. Briefly, the bacterial suspension was streaked onto Mueller Hinton II agar (BD™ BBL™, Franklin Lakes, NJ) with a sterile swab to create a bacterial lawn; for each fosfomycin-antibiotic combination tested, antibiotic gradient strips were crossed at a 90° angle at the respective MICs for each agent. To calculate the FICI, the MIC of each antibiotic in combination was divided by the MIC of the antibiotic alone, and the results were added together. In the presence of high off-scale MICs, the value of the next-highest $\log_2$ dilution was applied (e.g., meropenem MIC of ≥32 mg/L converted to 64 mg/L). Antibiotic interactions were defined as synergistic (FICI ≤0.50), additive (FICI >0.50 to ≤1.00), indifferent (FICI >1.00 to ≤4.00), or antagonistic (FICI >4.00) (Hall et al., 1983). To allow translation of the interaction assessments to challenging clinical scenarios that occur with isolation of extensively drug-resistant Enterobacteriaceae, only isolates that were nonsusceptible (CLSI, 2018) to the second antibiotic were assessed for synergistic potential with fosfomycin.

3. Results

3.1. Bacterial isolates and antimicrobial susceptibility testing

Isolates selected for study contained at least 1 carbapenemase (i.e., KPC, NDM, or OXA) as shown in Table 1. Three *K. pneumoniae* isolates co-produced carbapenemases from different Ambler classes (e.g., NDM and OXA-48-like). Class B VIM enzymes were present in 3 *K. pneumoniae* isolates. In addition, 17 (34.7%) CPE were AmpC-positive, and truncated porins (i.e., OmpC, OmpF, or OmpK) were detected in 16 (32.7%) isolates.

The fosfomycin $MIC_{50/90}$ was 96/≥1024 mg/L by GDS methodology. MICs were lowest for *Citrobacter* spp. ($N = 2$) and *Escherichia coli* ($N = 8$), ranging 0.75 mg/L to 4 mg/L. MICs were highest for *Enterobacter cloacae* ($N = 4$) and *Klebsiella* spp. ($N = 35$), both with MIC_{50} ≥ 1024-mg/L. Fosfomycin MICs for 11 *fosA*-positive *K. pneumoniae* isolates were also high (MIC_{50} ≥ 1024 mg/L), ranging 96 to ≥1024 mg/L. The phenotypic profiles of all CPE are described in Table 2.

Table 1
Select resistance mechanisms of carbapenemase-producing Enterobacteriaceae isolates.

Mechanism	*N* (%)			
	Citrobacter spp. ($N = 2$)	*E. coli* ($N = 8$)	*E. cloacae* ($N = 4$)	*Klebsiella* spp. ($N = 35$)[a]
KPC	1 (50.0)	2 (25.0)	3 (75.0)	16 (45.7)
NDM	1 (50.0)	6 (75.0)	1 (25.0)	14 (40.0)
OXA-CP	0 (0.0)	0 (0.0)	0 (0.0)	5 (14.3)
VIM	0 (0.0)	0 (0.0)	0 (0.0)	3 (8.6)
ESBL[b]	1 (50.0)	5 (62.5)	3 (75.0)	31 (88.6)
fosA	0 (0.0)	0 (0.0)[c]	0 (0.0)	11 (44.0)[d]

ESBL = extended spectrum β-lactamase; KPC = *K. pneumoniae* carbapenemase; NDM = New Delhi metallo-β-lactamase; OXA-CP = OXA-type carbapenemase.
[a] Includes *K. pneumoniae* ($N = 34$) and *K. ozaenae* ($N = 1$).
[b] ESBL-positive isolate contained CTX-M-15 and/or SHV-12.
[c] Presence of *fosA* mutation not assessed in 2 *E. coli* isolates; fosfomycin GDS MICs were 1 mg/L and 2 mg/L for these strains.
[d] Presence of *fosA* mutation not assessed in 10 *K. pneumoniae* isolates; among these 10, a total of 4 isolates displayed fosfomycin MICs ≥64 mg/L (high off-scale GDS MICs ≥1024 mg/L were observed in 3 of these 4 strains).

3.2. Antibiotic interaction assessments

Among 364 total fosfomycin–antibiotic combinations assessed, the most frequent type of interaction observed was indifference (284/364, 77.8%, Table 3). This observation is reflected in the geometric mean FICI for each fosfomycin–antibiotic combination as each value is >1.00. Synergy with fosfomycin was observed most frequently with ceftolozane/tazobactam (C/T), and additivity was observed most frequently with aztreonam.

An example of synergy between fosfomycin and aztreonam is shown in Fig. 1. Antagonistic activity between fosfomycin and any of the 8 antibiotics tested was not present in any CPE isolate assessed.

When results were categorized by carbapenemase type, fosfomycin plus aztreonam was the only combination to display synergy (4/24, 16.7%) among isolates positive for Ambler class B enzymes (i.e., NDM and VIM), as shown in Fig. 2. Furthermore, additivity with aztreonam was present in 8 (33.3%) isolates (Fig. 2).

Among KPC-positive isolates, synergy with C/T was observed in 7 (31.8%) isolates, and additivity was observed with C/T in 10 (45.4%) isolates. All of the isolates that displayed synergy or additivity with C/T were KPC-positive/NDM-negative, except 1 that was OXA-232–positive/NDM-negative with FICI equal to 0.42 (Fig. 2). Among *fosA*-positive isolates, aztreonam and C/T were synergistic or additive with fosfomycin in 5 (45.5%) and 3 (27.3%) isolates, respectively. Among isolates with truncated porins, additivity was observed in 8 (47.1%) isolates in combination with aztreonam, and both C/T and meropenem produced synergistic or additive results in 7 (38.9%) isolates.

3.3. Restored susceptibility

There were 2 instances of restored susceptibility of the second agent in a single isolate. In this KPC-2–, CTX-M-15–positive *K. pneumoniae* isolate, the C/T MIC was 4 mg/L alone *versus* 2 mg/L in combination with fosfomycin, and the ceftazidime MIC was 8 mg/L alone *versus* 3 mg/L with fosfomycin.

4. Discussion

In this *in vitro* study of carbapenemase-producing Enterobacteriaceae ($N = 49$), synergy between fosfomycin and other broad-spectrum antibiotics was infrequently observed. However, rates of additivity that signified an MIC-lowering effect for both agents in the combination were more prevalent among all 8 antibiotics tested in combination with fosfomycin. Therefore, additivity between fosfomycin and another antibiotic may be an important observation when either MIC approaches the susceptibility breakpoint. Furthermore, we observed restoration of

Table 2
Phenotypic profiles of CPE isolates.

Organism	MIC_{50} (range) (mg/L)				
	FOF	ATM	FEP	CAZ	
Citrobacter spp.[a] (*N* = 2)	2, 4	≥256	≥256	≥256	
E. coli (*N* = 8)	1 (0.75, 2)	≥256 (1.5, ≥256)	≥256	≥256	
E. cloacae (*N* = 4)	≥1024 (0.75, ≥1024)	≥256	≥256	≥256 (128, ≥256)	
Klebsiella spp.[b] (*N* = 35)	≥1024 (8, ≥1024)	≥256	≥256	≥256 (12, ≥256)	
	CZA	C/T	MEM	TZP	TOB
Citrobacter spp.[a]	2, ≥256	≥256	≥32	≥256	24, ≥256
E. coli	≥256 (2, ≥256)	≥256 (24, ≥256)	≥32	≥256	32 (0.38, ≥256)
E. cloacae	2 (1.5, ≥256)	128 (6, ≥256)	≥32	≥256	≥256 (16, ≥256)
Klebsiella spp.[b]	≥256 (0.19, ≥256)	≥256 (3, ≥256)	≥32 (32, ≥32)	≥256	≥256 (0.75, ≥256)

Data presented as a single MIC value if identical among all isolates. MIC = minimum inhibitory concentration; ATM = aztreonam; FEP = cefepime; FOF = fosfomycin; CAZ = ceftazidime; CZA = ceftazidime/avibactam; C/T = ceftolozane/tazobactam; MEM = meropenem; TZP = piperacillin/tazobactam; TOB = tobramycin.
[a] Data presented as range only.
[b] Includes *K. pneumoniae* (*N* = 34) and *K. ozaenae* (*N* = 1).

susceptibility of 2 antibiotics in a KPC-producing isolate, a finding of utmost interest to clinicians who encounter extensively drug-resistant CPE infections in practice.

Among all CPE assessed, the most active combination was fosfomycin plus ceftolozane/tazobactam. Tazobactam irreversibly binds the active site of serine β-lactamases (Humphries et al., 2017; van Duin and Bonomo, 2016); however, KPCs effectively hydrolyze tazobactam, thereby offsetting any relevant inhibitory effect (Papp-Wallace et al., 2010). One plausible explanation for our observation of synergy between C/T and fosfomycin is that the weak, irreversible binding of tazobactam to serine carbapenemases may be sufficient to allow ceftolozane to inhibit *in vitro* growth in the presence of fosfomycin. However, fosfomycin plus C/T should be investigated using other *in vitro* methods to fully elucidate the mechanism of synergistic activity against CPE.

Although we observed an overall low rate of synergy and additivity between ceftazidime/avibactam (CZA) and fosfomycin, this finding is not unexpected as avibactam is generally ineffective *in vitro* in the presence of metallo-β-lactamases (van Duin and Bonomo, 2016), which were prevalent in the isolates assessed (Table 1). Consistent with reliable activity of CZA against KPCs (van Duin and Bonomo, 2016), there was only 1 KPC-positive isolate that was CZA-nonsusceptible (MIC = 32 mg/L). *In vitro* CZA susceptibility for this KPC-3–, OXA-9–, TEM-1–, SHV-11–positive *K. pneumoniae* isolate would be anticipated (Zasowski et al., 2015); however, there have been reports of resistance-conferring KPC-3 mutations observed following CZA treatment (Shields et al., 2017). Indifference was observed between fosfomycin and CZA against this isolate, which also contained a *fosA* mutation. Fosfomycin plus CZA should be explored further for synergistic potential in CZA-susceptible Enterobacteriaceae.

To the best of our knowledge, this is the largest antibiotic interaction assessment of fosfomycin conducted in Enterobacteriaceae to date. Berçot and colleagues used checkerboard methodology to assess interactions between fosfomycin and 2 agents, colistin and tigecycline, against only 9 NDM-1–positive Enterobacteriaceae. Despite most of these isolates having susceptible MICs, synergy was rarely observed (Berçot et al., 2011). However, in the present study, exactly half of all NDM- or VIM-positive isolates that were aztreonam-nonsusceptible displayed synergy or additivity in combination with fosfomycin. This observation is in agreement with known stability of aztreonam to hydrolysis by NDM (Wenzler et al., 2017). We hypothesize that, in combination with fosfomycin, other mechanisms that contribute to aztreonam nonsusceptibility may become less important, thereby allowing adequate inhibition of NDM-positive isolates. Overall, aztreonam was synergistic or additive in combination with fosfomycin in more than a third of all aztreonam nonsusceptible isolates. These observations are concordant with those of Hickman and colleagues, who described a fosfomycin-susceptible (MIC 16 mg/L) but aztreonam-resistant (MIC 64 mg/L) *K. pneumoniae* isolate that produced synergistic results by checkerboard methodology (FICI 0.23), and 1 *E. coli* isolate with a similar phenotypic profile that produced additive results (FICI, 0.58) (Hickman et al., 2014). Moreover, we observed instances of synergy between meropenem and fosfomycin which has also been described in 11 of 17 (64.7%) KPC-2–positive *K. pneumoniae* isolates assessed by time-kill methodology (Souli et al., 2011).

Our observations of macrocolonies present in the fosfomycin inhibition ellipse (Fig. 1) has previously been reported; Kaase and colleagues documented this phenomenon in 44 (41.1%) carbapenem-resistant Enterobacteriaceae (CRE) isolates studied (Kaase et al., 2014). The authors commented that it may be prudent to ignore such resistant subpopulations because fosfomycin is likely to be used in combination with other agents against CPE which has been shown to deter development of fosfomycin resistance (Kaase et al., 2014; Souli et al., 2011). Further guidance relative to characterization of the resistant macrocolonies would be required prior to adoption of this reading technique by clinical laboratories. For this reason,

Table 3
Antibiotic interactions of various antimicrobials with fosfomycin.

	Nonsusceptible antibiotics tested in combination with FOF								
	ATM	FEP	CAZ	CZA	C/T	MEM	TZP	TOB	Total
Synergy, *N*	4	2	3	0	8	1	1	2	21
Synergy, %	8.3	4.1	6.1	0.0	16.3	2.0	2.0	4.4	5.8
Additive, *N*	13	5	8	3	11	10	2	7	59
Additive, %	27.1	10.2	16.3	11.5	22.5	20.4	4.1	15.6	16.2
Indifferent, *N*	31	42	38	23	30	38	46	36	284
Indifferent, %	64.6	85.7	77.6	88.5	61.2	77.6	93.9	80.0	77.8
FICI, geometric mean	1.25	1.42	1.38	1.56	1.15	1.37	1.68	1.31	NA
FICI, range	0.38–2.00	0.22–2.00	0.27–2.00	0.75–2.50	0.31–2.00	0.38–2.00	0.39–2.00	0.41–2.00	NA

MIC = minimum inhibitory concentration; ATM = aztreonam; FEP = cefepime; FOF = fosfomycin; CAZ = ceftazidime; CZA = ceftazidime/avibactam; C/T = ceftolozane/tazobactam; MEM = meropenem; TZP = piperacillin/tazobactam; TOB = tobramycin; FICI = fractional inhibitory concentration index; NA = not applicable.

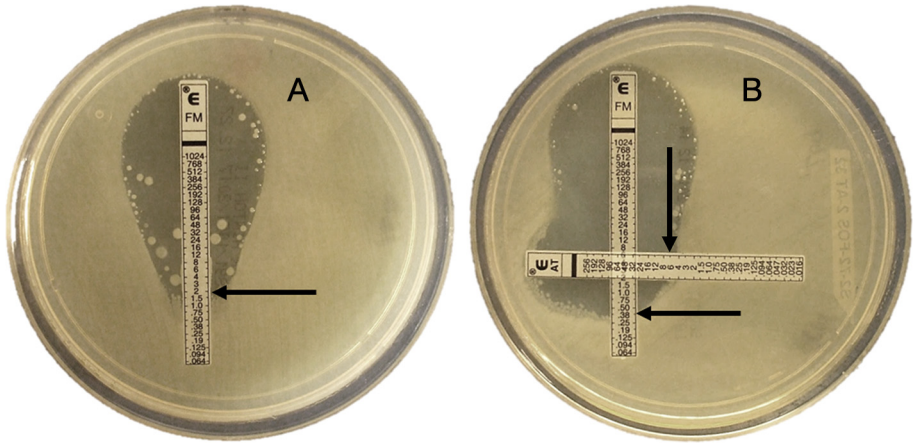

Fig. 1. Example of synergistic interaction. Plate A contains *E. coli* with fosfomycin MIC of 2 mg/L alone as marked with an arrow (1–5 colonies ≤5 mm from strip may be ignored), while plate B depicts MIC-lowering effect in combination with aztreonam (fosfomycin MIC = 0.38 mg/L, marked with a horizontal arrow) and synergistic interaction (FICI = 0.38). Plate B also demonstrates a reduction of the aztreonam MIC in the presence of fosfomycin (6 mg/L, marked with a vertical arrow) compared with the MIC alone (32 mg/L).

we consistently and conservatively read all MICs according to the manufacturer's instruction (i.e., read the MIC where numerous macrocolonies are completely inhibited, though up to 5 colonies can be ignored).

This study had several limitations. First, discordance among synergy testing methods that define interactions in terms of FICI (e.g., checkerboard method, GDS methods), as well as discordance between FICI methods and the gold-standard time-kill assessments, has been described (Pankey et al., 2013; White et al., 1996). Reported concordance rates between the GDS cross method and time-kill analyses are variable in the literature (Bonapace et al., 2000; Pankey et al., 2013; White et al., 1996), and it is unknown if any 1 method is superior when assessing Enterobacteriaceae isolates. Regardless, we found that the cross method allowed for testing of up to 8 different fosfomycin–antibiotic combinations among almost 50 isolates. Second, others have cautioned that the GDS cross method may prohibit visualization of borderline antagonism as 1 of the strips is placed on top of the other (Pankey et al., 2013). However, we found that inhibition ellipses could be visually extrapolated to be in close proximity to the point of intersection (i.e., within a single 2-fold dilution) without difficulty, indicating indifference *versus* antagonism. Lastly, the European Committee on Antimicrobial Susceptibility Testing issued a warning relative to the use of piperacillin/tazobactam GDS. However, we did not observe any issues among multiple quality control tests conducted. Thus, we believe the low percentage of synergy or additivity observed with piperacillin/tazobactam is a function of the highly resistant isolates (i.e., $MIC_{50} \geq 256$-mg/L) assessed.

5. Conclusions

With rates of CPE colonization and infection increasing around the globe (van Duin and Doi, 2017), clinical microbiologists, epidemiologists, and practitioners should be encouraged by our observations of synergy or additivity between fosfomycin and other parenteral antibiotics in nearly a quarter of all CPE isolates assessed. We advocate for consideration of synergy testing of isolated CPE in order to identify MIC-lowering effects that may aid in treatment decisions. It is our

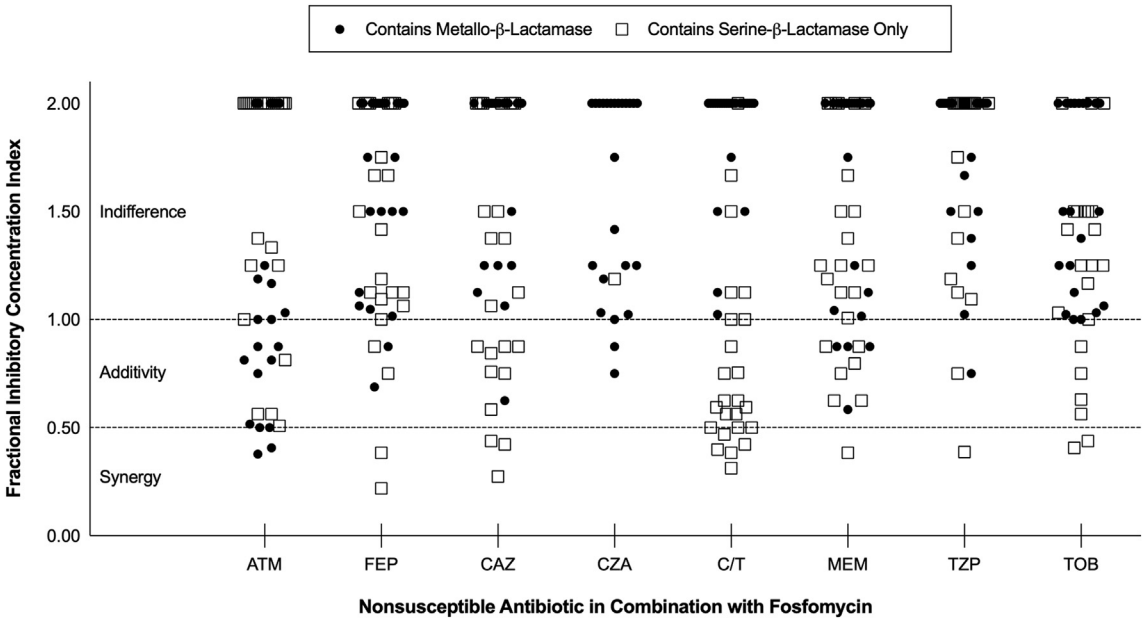

Fig. 2. Antibiotic interaction assessment results. Among all fosfomycin–antibiotic combinations tested ($N = 364$), indifference occurred most frequently. Aztreonam (ATM) was the only synergistic antibiotic in combination with fosfomycin in metallo-β-lactamase–positive isolates. Only serine-β-lactamase–positive isolates displayed synergy or additivity with ceftolozane/tazobactam (C/T). There was 1 data point for CZA where FICI = 2.50 (indifference) in a metallo-β-lactamase–positive isolate (not shown).

opinion that the most time-efficient process entails crossing GDS at MICs derived from reference antimicrobial susceptibility testing methods, and using the same inoculum, the GDS MIC of each agent alone should be determined simultaneously for use in FICI calculations. In this manner, a general assessment of the *in vitro* potency of antibiotic combinations would be available approximately 1 full day earlier than the traditional GDS cross method (Avery and Nicolau, 2018a, 2018b). Pragmatic trials testing these methods are likely needed prior to widespread, routine adoption of the technique in the clinical laboratory. In the present study, the antibiotic interaction assessments conducted support fosfomycin plus C/T and fosfomycin plus aztreonam for treatment of CPE infection, though further study is required to confirm *in vitro* and *in vivo* activity, as well as clinical efficacy in treatment of complicated urinary tract infections.

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Conflicts of interest

D.P.N. has acted as a consultant, member of the Speakers Bureau, or grant investigator for Zavante Therapeutics, Inc., Nabriva Therapeutics plc, Allergan plc, and Merck & Co., Inc.

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