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In vitro evaluation of dual carbapenem combinations against carbapenemase-producing Enterobacteriaceae

Laurent Poirel[1]*†, Nicolas Kieffer[1]† and Patrice Nordmann[1,2]

[1]*Medical and Molecular Microbiology 'Emerging Antibiotic Resistance' Unit, Department of Medicine, Faculty of Science, University of Fribourg, Fribourg, Switzerland;* [2]*Hôpital Fribourgeois-Hôpital Cantonal, Fribourg, Switzerland*

*Corresponding author. Tel: +41-26-300-9582; E-mail: laurent.poirel@unifr.ch
†These authors contributed equally to this work.

Objectives: This study aimed to analyse the *in vitro* activity of dual combinations of carbapenems against *Klebsiella pneumoniae* producing the main carbapenemase types.

Methods: MIC values of the carbapenems, imipenem, meropenem, ertapenem and doripenem were determined alone and in dual combinations for 20 clinical *K. pneumoniae* isolates producing representative carbapenemases, i.e. OXA-48 ($n=6$), NDM-1 ($n=4$), NDM-1+OXA-48 ($n=2$) and KPC-2 ($n=8$). MICs were also determined for *Escherichia coli* recombinant strains with or without permeability defects producing NDM-1, OXA-48 or KPC-2. *In vitro* synergy combination testing was performed using the microdilution and chequerboard techniques. Fractional inhibitory concentration indexes were calculated to determine whether the combinations were synergistic, indifferent or antagonistic.

Results: All carbapenemase producers were resistant to the tested carbapenems, with most isolates showing MICs of carbapenems >32 mg/L. None of the combinations was antagonistic. For KPC producers, synergistic combinations were observed with imipenem/ertapenem (5/8 isolates), imipenem/doripenem (4/8), imipenem/doripenem (4/8), meropenem/doripenem (3/8) and ertapenem/doripenem (3/8), while no synergy was observed with meropenem/ertapenem. For OXA-48 producers, synergies were observed with imipenem/ertapenem and with imipenem/meropenem for both isolates tested. Notably, combining imipenem with a non-carbapenem β-lactam (cefalotin) did not give any synergistic result. No synergy was observed for all NDM-1 and NDM-1+OXA-48 producers. Time–kill assays confirmed most of the data obtained by chequerboard testing.

Conclusions: The data strongly support the hypothesis that dual carbapenem combinations might be effective against serine-β-lactamase producers (KPC, OXA-48). The imipenem-containing combinations appeared to be the most efficient.

Introduction

MDR Enterobacteriaceae are increasingly reported and currently represent one of the main threats to public health.[1] Among the MDR bacteria, emergence of *Klebsiella pneumoniae* isolates resistant to carbapenems is of particular importance, since they are mostly the source of nosocomial infections in severely ill patients.[2] Resistance to carbapenems in *K. pneumoniae* is mainly due to the production of carbapenem-hydrolysing β-lactamases such as the KPC type (Ambler class A), IMP, VIM and NDM types (class B) and OXA-48 (class D).[3] KPC producers are mainly identified in the USA, South America, Greece and Italy,[4] VIM producers mainly in Greece and Italy[5] and IMP producers in Japan and Australia.[3] NDM producers have been identified mainly from India, Pakistan, Bangladesh, the Middle East and the UK, and subsequently from many countries worldwide, but often with a link with the Indian subcontinent.[6] Finally, OXA-48 producers are mainly reported in Europe (France, Belgium, Spain), Turkey and North African countries.[7] Most of the carbapenemase producers are resistant to all β-lactams, including carbapenems, to most aminoglycosides, and to fluoroquinolones, fosfomycin and sulphonamides, remaining mostly susceptible to very few antibiotics (tigecycline and colistin).

Antibiotic combination schemes including a carbapenem and colistin, tigecycline, fosfomycin or an aminoglycoside might be efficient against carbapenemase-producing *K. pneumoniae*, with combination therapies being proved to be more efficient than monotherapies.[8]

However, colistin and tigecycline raise some concern about toxicity and poor diffusion in the urinary tract, respectively. Furthermore, frequent colistin use may select for colistin resistance, as exemplified by the current situation with KPC producers in Italy.[9]

Therefore, pioneer studies performed using animal models of infections or experimental treatments of patients have suggested the use of carbapenem combinations for treating infections

caused by carbapenemase producers. The rationale of combining carbapenems (and in particular ertapenem with another carbapenem) would be that ertapenem may bind to the active site of the carbapenemase with high affinity, and therefore may prevent the hydrolysis of the other carbapenem molecule, this drug combination presumably being more effective.[10,11] The mode of action of these kinds of combinations would be somewhat similar to that of amoxicillin and the β-lactamase inhibitor clavulanic acid, for example.

Therefore our objective was to analyse *in vitro* the activity of dual carbapenem combinations against different types of carbapenemase-producing *K. pneumoniae*.

Materials and methods

A collection of 20 clonally unrelated carbapenemase-producing *K. pneumoniae* isolates was included in the study. They produced the three main types of carbapenemases: KPC-2 ($n=8$), NDM-1 ($n=4$) and OXA-48 ($n=6$). Two isolates co-produced two carbapenemases, i.e. NDM-1 and OXA-48. These isolates were of worldwide origin (India, USA, Canada, Colombia, France, Israel, Turkey, Sultanate of Oman, Kuwait and Morocco), have been previously fully characterized for their β-lactamase content and have been selected for their resistance to carbapenems (Table 1). In addition, each carbapenemase gene was separately cloned into the same plasmid pCR®-Blunt II-TOPO® (Invitrogen, Illkirch, France) and expressed in two recipient strains: *Escherichia coli* TOP10 (WT) and *E. coli* HB4 (exhibiting permeability defects since it lacks porins OmpC and OmpF[12]).[13] The MICs for all the isolates of cefalotin and the four carbapenems, namely doripenem, ertapenem, imipenem and meropenem, were determined by using the broth microdilution technique as recommended by the CLSI guidelines.[14] Carbapenem breakpoints established by the CLSI for Enterobacteriaceae are as follows: doripenem, ≤1/≥4 mg/L; ertapenem, ≤0.5/≥2 mg/L; imipenem, ≤1/≥4 mg/L; and meropenem, ≤1/≥4 mg/L. Chequerboard synergy testing was performed as described previously[15,16] using combinations of two carbapenems or a combination of cefalotin and imipenem as a control. Chequerboard synergy testing was performed in duplicate with the 20 carbapenemase-producing *K. pneumoniae* isolates, and in addition with the 3 *E. coli* TOP10 and the 3 *E. coli* HB4 recombinant strains. Fractional inhibitory concentration (FICs) indexes were calculated according to the formula ΣFIC=FIC of drug A+FIC of drug B, where FIC of drug A or B=MIC of drug A or B in combination divided by the MIC of drug A or B alone.

Table 1. Features of clinical isolates and recombinant strains

Isolates	Country of isolation	Carbapenemases	MICs (mg/L)				Associated β-lactamases
			IPM	MEM	DOR	ETP	
K. pneumoniae clinical isolates							
K301	Colombia	KPC-2	>512	1024	512	>1024	SHV-11
K315	France	KPC-2	512	64	32	128	SHV-11
K23	Colombia	KPC-2	256	512	256	512	SHV-11
K322	France	KPC-2	128	128	32	256	SHV-12
K317	France	KPC-2	128	128	32	128	SHV-12+OXA-9
K302	Israel	KPC-2	64	64	32	128	SHV-11+TEM-1+OXA-9
HMA284	Canada	KPC-3	64	64	16	128	none
YC	USA	KPC-2	32	64	16	128	SHV-11+SHV-12+TEM-1+OXA-9
BIC	France	OXA-48	64	64	32	256	none
11978	Turkey	OXA-48	64	32	32	128	SHV-2a+SHV-11+OXA-47+TEM-1+OXA-1
52	France	OXA-48	16	32	8	64	ND
43	France	OXA-48	16	4	4	8	ND
E212	France	OXA-48	8	32	16	256	ND
ALI	Kuwait	OXA-48	8	16	4	8	SHV-28
4N14	France	NDM-1	128	128	128	64	SHV-11+TEM-1+CTX-M-15+OXA-1+OXA-9
OMA2	Oman	NDM-1	16	64	64	128	SHV-11+OXA-1
NAS	France	NDM-1	64	64	32	128	CTX-M-15+TEM-1+SHV-11+OXA-1
6560	Morocco	NDM-1	16	32	32	64	CTX-M-15+TEM-1+SHV-5+OXA-1+OXA-9
T45	Turkey	NDM-1+OXA-181	512	512	256	512	ND
C93	Turkey	NDM-1+OXA-181	512	512	256	512	ND
Recombinant *E. coli*							
clone 1		KPC-2	128	32	16	32	none
clone 2		OXA-48	1024	256	128	1024	none
clone 3		NDM-1	64	32	16	32	none
clone 1b		KPC-2	16	16	8	16	none
clone 2b		OXA-48	4	1	1	2	none
clone 3b		NDM-1	8	8	4	8	none

IPM, imipenem; MEM, meropenem; DOR, doripenem; ETP, ertapenem; ND, not determined.

Interpretation of the results was based on the following: FIC values of ≤ 0.5 indicate synergy, FIC values of >0.5 to 4 indicate no interaction and FIC values of >4 indicate antagonism.[17]

Time–kill assays were performed as described previously.[18,19] Killing effects were quantified by standard time–kill assays using effective carbapenem concentrations as suggested by chequerboard experimental data (Table 2). Log kill was determined by microdilution on MH agar after 2, 4, 6, 9 and 10 h of incubation with carbapenems alone or in combination. Synergies were considered when a $\log_{10}$ kill difference of ≥ 2 was observed for carbapenem combinations compared with single carbapenem treatments at a given timepoint. Corresponding data are summarized in Table 2.

Results

Determination of MICs of carbapenems for the 20 carbapenemase producers showed that all isolates were resistant to doripenem (MICs ranging from 4 to 256 mg/L), ertapenem (8 to >1024 mg/L), imipenem (8 to >512 mg/L) and meropenem (4–1024 mg/L). The two isolates co-producing NDM-1 + OXA-48 showed very high MICs of all carbapenems (Table 1). The *E. coli* HB4 recombinant strains were highly resistant to all carbapenems, whereas *E. coli* TOP10 showed lower MICs, as expected (Table 1). All strains were resistant to cefalotin.

Interestingly, *in vitro* synergistic activity was noticed with the combinations imipenem/meropenem ($n=8$ isolates), imipenem/ertapenem ($n=8$), imipenem/doripenem ($n=4$), doripenem/meropenem ($n=3$) and doripenem/ertapenem ($n=3$), but not with the ertapenem/meropenem combination (Table 1). At least one synergistic combination was systematically observed with all KPC producers, whereas it was obtained for only four out of the six OXA-48 producers. No synergy was found for all combinations of all NDM-1 producers, including those co-producing OXA-48. Overall, our results showed that many combinations resulted in mainly no interaction (Table 1). However, no antagonism (defined by an FIC >4) was noticed with any of the six combinations tested.

When analysing the distribution of significant synergies obtained during this duplicate experiment, the best results were obtained with all combinations including imipenem either for KPC and OXA-48 producers, and also with the ertapenem/doripenem combination, but only for the KPC producers.

In order to evaluate whether the dual carbapenem synergy observed with KPC or OXA-48 producers might be attributed to the type of carbapenemase produced or rather to the specific genetic background of the host strain, a comparative evaluation was conducted with different *E. coli* isogenic backgrounds (either exhibiting a WT susceptibility pattern or permeability defects) producing the carbapenemase KPC-2, OXA-48 or NDM-1. Accordingly, *E. coli* TOP10 and *E. coli* HB4 recombinant strains were respectively supposed to exhibit low and high MICs of carbapenems (Table 1). Synergistic activities were identified only against the KPC-2 producer, whereas no synergy was found against either OXA-48 or NDM-1. Three out of the six combinations tested (imipenem/meropenem, imipenem/ertapenem and imipenem/doripenem) were found to be synergistic against the KPC-2-producing strain when *E. coli* HB4 was used as background, and only one (imipenem/doripenem) when *E. coli* TOP10 was used as background.

It was noteworthy that all of the combinations showing synergistic activity included imipenem. In order to evaluate whether the synergies observed could indeed be attributed to the dual carbapenem combination, and not to imipenem only, the imipenem/cefalotin combination was also tested in isolates for which synergies had been observed with combinations including imipenem, but no synergistic effect was observed.

Time–kill assays confirmed many of the synergies evidenced by the chequerboard assays. This was particularly true for imipenem/doripenem and ertapenem/doripenem (Table 2). The synergies obtained through time–kill assays were very significant for some specific combinations, as exemplified with the meropenem/doripenem combination against a KPC-2-producing strain and the imipenem/ertapenem combination against an OXA-48-producing strain (Figure 1).

Discussion

This study showed that dual combinations of carbapenems might be synergistic against carbapenem-resistant and carbapenemase-producing *K. pneumoniae* isolates. We found evidence that the efficacy of the dual carbapenem combinations depended on the type of combination, those including imipenem or doripenem being the most efficient. These results disagree with the hypothesis that ertapenem might be the carbapenem molecule of choice to partially inactivate the carbapenemase activity. Synergies were frequently obtained with KPC producers, and to a lesser extent with OXA-48 producers (both enzymes being serine β-lactamases), but not with NDM (a metallo-β-lactamase) producers. We showed that synergies were more likely to occur with clinical isolates showing high rather than moderate MICs of carbapenems.

Overall, our results agree with the observations made by Giamarellou *et al.*,[20] who noticed a successful outcome (and presumably synergistic activity) of the doripenem/ertapenem combination when treating a single patient infected with a KPC-2-producing *K. pneumoniae*, and with Ceccarelli *et al.*,[21] who reported the clinical success of the same combination against a KPC-3-producing *K. pneumoniae*. Our results are also consistent with those published by Bulik and Nicolau,[22] who demonstrated in a chemostat and in an *in vivo* murine thigh infection model that the doripenem/ertapenem combination had enhanced efficacy compared with either agent alone. By contrast, our results do not confirm the time–kill study performed by Oliva *et al.*,[23] who showed that synergistic activity might be obtained *in vitro* with ertapenem/meropenem against KPC-producing *K. pneumoniae*, since no synergistic activity was noted here for any of the isolates tested with that combination.

The results of the present study justify additional *in vivo* investigations. In view of our results, an imipenem-containing dual carbapenem therapy may be efficient, in the context of treating an infection caused by a carbapenem-resistant and carbapenemase-producing *K. pneumoniae*. It remains to be determined whether the dual carbapenem combinations are more efficient than combinations of a carbapenem and a non-β-lactam molecule, such as colistin, fosfomycin or tigecycline.[24] However, since replacement of one of the carbapenems by a cephalosporin negatively interferes with the synergistic

Table 2. MICs, FIC variations and chequerboard synergy testing for carbapenemase-producing isolates

		FIC variations						Chequerboard synergy testing[a]						Concentrations of carbapenems (mg/L)[b]			
Isolates	Carbapenemases	IPM+MEM	IPM+ETP	DOR+IPM	MEM+DOR	ETP+DOR	ETP+MEM	IPM+MEM	IPM+ETP	DOR+IPM	MEM+DOR	ETP+DOR	ETP+MEM	IPM	MEM	DOR	ETP
K. pneumoniae clinical isolates																	
K301	KPC-2	0.75–0.75	0.5–0.5	0.63–0.63	0.75–0.75	0.63–0.63	0.63–0.63	NI/NI	SYN/SYN	NI/NI	NI/NI	NI/NI	NI/NI	256	/	/	512
K315	KPC-2	0.53–0.56	0.5–0.5	0.5–0.5	0.75–0.75	0.75–0.75	0.75–0.75	NI/NI	SYN/SYN	SYN/SYN	NI/NI	NI/NI	NI/NI	2[c]	/	1[c]	8[c]
K23	KPC-2	0.38–0.38	0.38–0.38	0.38–0.38	0.5–0.62	0.75–0.75	0.75–0.75	SYN/SYN	SYN/SYN	SYN/SYN	SYN/NI	NI/NI	NI/NI	32	128	64	128
K322	KPC-2	0.5–0.5	0.51–0.53	0.56–0.56	0.5–0.5	0.75–0.75	1–1	SYN/SYN	NI/NI	NI/NI	SYN/SYN	NI/NI	NI/NI	4[c]	4[c]	2[c]	/
K317	KPC-2	0.75–0.75	0.5–0.5	0.63–0.63	0.63–0.63	0.31–0.38	1–0.75	NI/NI	SYN/SYN	NI/NI	NI/NI	SYN/SYN	NI/NI	4[c]	/	2[c]	4[c]
K302	KPC-2	0.5–0.38	0.38–0.56	0.5–0.38	0.63–0.5	0.5–0.38	0.53–0.56	SYN/SYN	SYN/NI	SYN/SYN	NI/SYN	SYN/SYN	NI/NI	8[c]	4[c]	4[c]	8[c]
HMA284	KPC-3	0.53–0.56	0.56–0.56	0.75–0.75	0.75–0.75	0.5–0.5	0.63–0.63	NI/NI	NI/NI	NI/NI	NI/NI	SYN/SYN	NI/NI	/	/	2[c]	8[c]
YC	KPC-2	0.5–1	0.53–0.56	0.38–0.5	1–0.75	0.75–0.75	1–0.75	SYN/NI	NI/NI	SYN/SYN	NI/NI	NI/NI	NI/NI	2[c]	8[c]	2[c]	/
BIC	OXA-48	0.5–0.63	0.63–0.5	0.75–0.56	1–0.63	0.75–0.75	0.75–0.56	SYN/NI	NI/SYN	NI/NI	NI/NI	NI/NI	NI/NI	1[c]	1[c]	/	2[c]
11978	OXA-48	1–0.5	0.75–0.63	0.56–0.51	0.63–0.63	0.75–0.75	0.75–0.75	NI/SYN	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	16	16	/	/
52	OXA-48	0.5–0.75	0.63–0.5	1–1	0.56–0.56	0.75–0.75	0.53–0.56	SYN/NI	NI/SYN	NI/NI	NI/NI	NI/NI	NI/NI	1[c]	4[c]	/	16
43	OXA-48	0.53–0.53	0.52–0.52	0.56–0.56	0.75–0.75	0.53–0.56	0.63–0.63	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
E212	OXA-48	1–1	1.5–1.5	0.75–0.75	0.75–0.75	1–1	1–1	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
ALI	OXA-48	0.5–0.5	0.5–0.5	0.75–0.75	0.75–1	1–0.75	0.75–1	SYN/SYN	SYN/SYN	NI/NI	NI/NI	NI/NI	NI/NI	2[c]	1[c]	/	2[c]
4N14	NDM-1	1–1	1–1	0.75–0.75	1–1	0.75–0.75	0.75–0.75	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
OMA2	NDM-1	1–0.63	0.75–0.75	0.75–0.63	0.56–0.56	0.63–0.56	0.75–1	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
NAS	NDM-1	0.75–0.75	0.75–0.63	1.5–1	1–0.75	0.75–1	1–1	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
6560	NDM-1	0.56–0.63	0.63–0.63	0.75–0.75	1–0.75	1–1	0.75–0.75	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
T45	NDM-1+OXA-181	0.63–1	0.75–0.75	0.63–0.75	1–1	1–1	1–1	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
C93	NDM-1+OXA-181	0.75–0.75	0.63–1	1–1	1–1	0.75–0.75	1–1	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
Recombinant *E. coli*																	
clone 1	KPC-2	0.63–0.5	0.63–0.5	0.5–0.31	0.75–0.75	0.63–0.63	1–1	NI/SYN	NI/SYN	SYN/SYN	NI/NI	NI/NI	NI/NI	8[c]	4[c]	4[c]	8[c]
clone 2	OXA-48	1–0.75	0.75–0.63	1–1	0.63–0.63	0.56–0.53	0.75–0.75	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
clone 3	NDM-1	0.75–0.75	1.0–1.0	0.75–0.75	0.75–1	0.75–0.75	1–1	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
clone 1b	KPC-2	0.75–0.75	0.75–0.75	0.5–0.5	0.75–0.75	0.75–1	0.75–0.75	NI/NI	NI/NI	SYN/SYN	NI/NI	NI/NI	NI/NI	4[c]	/	2[c]	/
clone 2b	OXA-48	1–1	0.75–0.75	0.75–1	1–1	1–0.75	0.75–0.75	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/
clone 3b	NDM-1	1–1	1–1	0.75–1	1–0.75	1–1	0.75–0.75	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	NI/NI	/	/	/	/

IPM, imipenem; MEM, meropenem; DOR, doripenem; ETP, ertapenem; SYN, synergy; NI, no interaction.
Dark grey shading, confirmed by time–kill assay; light grey shading, not confirmed.
[a]Results represent interpretations of the minimum and maximum FIC values; two separate experiments were performed, hence the two results obtained (shown separated with a slash).
[b]Concentrations for which the synergy was obtained by using chequerboard analyses.
[c]Values that are compatible with the clinically utilized doses.

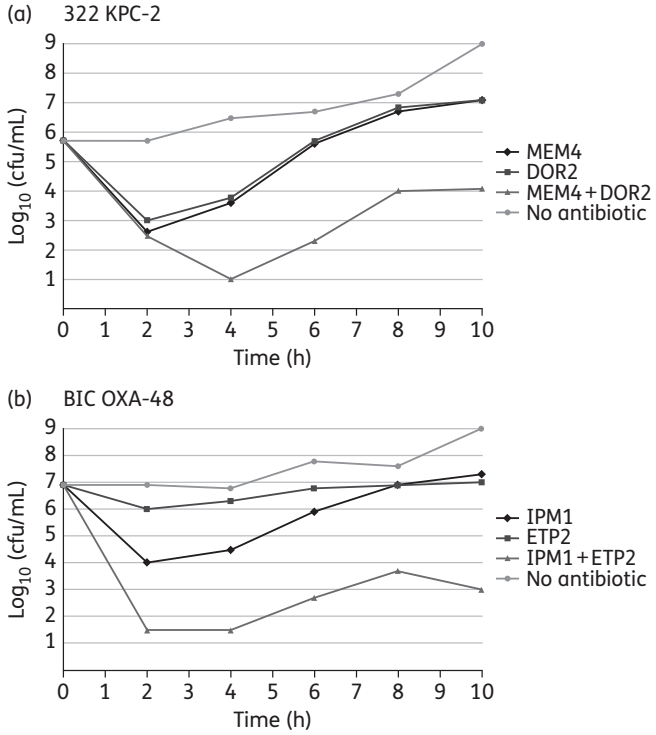

Figure 1. Time–kill curves of various carbapenem combinations against a strain of *K. pneumoniae* producing KPC-2 (a) and a strain of *K. pneumoniae* producing OXA-48 (b). Each line represents a carbapenem either alone or combined with another carbapenem. MEM4, meropenem at 4 mg/L; DOR2, doripenem at 2 mg/L; IPM1, imipenem at 1 mg/L; ETP2, ertapenem at 2 mg/L.

effect, this suggests that such an effect can be obtained only with two carbapenem molecules.

We might ultimately consider also that a triple combination with two synergistic carbapenems and another non-β-lactam antibiotic would be an efficient alternative. This kind of strategy might be thus evaluated at least *in vitro*.

Regarding the OXA-48 producers, successful synergies were observed for some clinical *K. pneumoniae* isolates, but not with recombinant *E. coli* strains. However, it must be noted that the FIC values of the imipenem-based combinations obtained for either the clinical isolates or the recombinant strains were not very different, being close to the cut-off value, which is 0.5 for defining synergy. Therefore, we cannot rule out that slight synergy may exist against OXA-48, but not as efficient as against the KPC producers.

It remains intriguing why none of the dual combinations was synergistic against NDM-1 producers. This might be related to the mechanism of action of metallo-β-lactamases, which differs significantly from that of serine-based carbapenemases.[25] Treatment of infections caused by metallo-β-lactamase producers may therefore remain particularly challenging since even the β-lactam/β-lactamase inhibitor combination ceftazidime/avibactam is not efficient in these cases. Overall, our study provides additional knowledge on potential treatment strategies aimed at eradicating MDR strains. As recently highlighted by Thomson,[26] knowledge regarding double-carbapenem therapies must be extended and we believe this present study will significantly contribute to this goal.

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Transparency declarations

None to declare.

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