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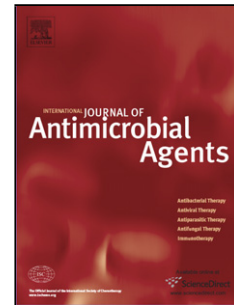
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Activity of tigecycline alone and in combination with colistin and meropenem against *Klebsiella pneumoniae* carbapenemase (KPC)-producing Enterobacteriaceae strains by time–kill assay

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ABSTRACT

Antibiotic combinations including tigecycline have not been studied against *Klebsiella pneumoniae* carbapenemase (KPC)-producing pathogens. Tigecycline alone and combined with colistin and meropenem was tested against eight genetically unrelated KPC-producing clinical strains of Enterobacteriaceae (four *K. pneumoniae*, two *Escherichia coli*, one *Enterobacter cloacae* and one *Serratia marcescens*) by time–kill assay. Tigecycline displayed a concentration-independent bacteriostatic activity in seven strains and bactericidal activity in one strain. Colistin showed bactericidal activity at 4× the minimum inhibitory concentration (MIC) in three strains and was bacteriostatic for the remaining strains and concentrations. Meropenem was bactericidal in three strains and bacteriostatic in five strains. The tigecycline + meropenem combination was not bactericidal against the four *K. pneumoniae* strains and was non-synergistic against all eight strains. Tigecycline + colistin was bactericidal against all strains at most time intervals and concentrations and was also synergistic at 1× and 2× MIC against most strains up to 4–8 h and at 4× MIC up to 24 h against all strains. These findings suggest that, at most drug concentrations, tigecycline, colistin and meropenem as single agents do not exhibit efficient bactericidal activity against most of the KPC-producing strains. Tigecycline alone might be a therapeutic option for infections caused by KPC-producers when bacteriostatic activity is adequate or combined with colistin when bactericidal activity is necessary. Additional in vivo tests are warranted to assess better the killing kinetics of tigecycline combinations against KPC-producers.

1. Introduction

Since the beginning of the last decade, *Klebsiella pneumoniae* carbapenemase (KPC)-producing Enterobacteriaceae have been increasingly detected in the USA and subsequently in several regions worldwide [1,2]. KPC enzymes confer various levels of resistance to all β -lactams, including carbapenems [2]. Moreover, bla_{KPC} genes are easily transferable and are often linked with various non β -lactam resistance determinants, further compromising the therapeutic alternatives for clinically significant infections [1–3]. Clinical reports have already documented that hospital infections due to KPC-possessing Enterobacteriaceae are commonly associated with increasing therapeutic failure and mortality [1,2,4].

Beside these observations, studies dealing with antimicrobial treatment of KPC infections and clinical outcomes are based on a limited number of case patients and therefore the optimum treatment has not been well established [4]. In this respect, in vitro studies examining antibiotic combinations and well controlled clinical trials are needed to ascertain the optimum treatment of KPC infections. Susceptibility testing data suggest that treatment of infections caused by KPC-producers commonly requires the use of tigecycline or colistin as last-resort drugs; meropenem in many cases also retains phenotypic activity against KPC-producers and is considered as a possible alternative [2,4,5]. Tigecycline, a glycylicycline antibiotic approved by the US Food and Drug Administration (FDA) for the treatment of complicated intra-abdominal infections and skin and skin-structure infections in adults, has been found to be active against

Enterobacteriaceae regardless of the presence of carbapenemases [6]. Its action is mediated by the inhibition of bacterial protein synthesis and is mainly bacteriostatic. The in vitro activity of tigecycline against KPC-producers has been only scarcely tested [2,7], whilst antibiotic combinations including tigecycline have not been studied previously in these bacteria. To assess further the in vitro efficacy of tigecycline alone or combined with colistin and meropenem, a time–kill study of KPC-producing clinical isolates belonging to several Enterobacteriaceae species was undertaken. The purpose of this study was to provide data regarding effective antibiotics or antibiotic combinations against KPC-producers that could be of value to clinicians treating KPC infections at the bedside.

2. Materials and methods

2.1. Bacterial strains

The strains tested comprised eight genetically confirmed KPC-producing enterobacterial clinical strains (four *K. pneumoniae*, two *Escherichia coli*, one *Enterobacter cloacae* and one *Serratia marcescens*) recovered from patients hospitalised in five tertiary care hospitals located in four Greek regions (two hospitals in the broad region of Athens and one hospital each in Thessaloniki, Larissa and Serres). Polymerase chain reaction (PCR) and sequencing assays showed that all microorganisms produced KPC-2 carbapenemase. The *K. pneumoniae* and *E. coli* strains were randomly selected among those representing different clonal types of KPC-possessing pathogens. The non-

carbapenemase-producing *E. coli* strain ATCC 25922 was used as a control in all assays.

2.2. Susceptibility testing

Tigecycline, colistin and meropenem minimum inhibitory concentrations (MICs) were determined by broth macrodilution [8], which is normally applied as a reference method for isolates to be tested by killing curve assays. For tigecycline, the FDA recommendation was used (susceptible, ≤ 2 $\mu\text{g/mL}$; and resistant, ≥ 8 $\mu\text{g/mL}$), and for colistin the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint for Enterobacteriaceae (susceptible, ≤ 2 $\mu\text{g/mL}$; and resistant, ≥ 4 $\mu\text{g/mL}$) were used [9]. For meropenem, the Clinical and Laboratory Standards Institute (CLSI) breakpoints modified in June 2010 [8] were used. Colistin was not tested against the *S. marcescens* clinical strain as this species exhibits intrinsic resistance to colistin.

2.3. Time–kill curve analyses

Time–kill curves were performed in triplicate by inoculating 5×10^5 colony-forming units (CFU)/mL of the test organisms into 3 mL of fresh cation-adjusted Mueller–Hinton broth. Antibiotics (tigecycline, colistin and meropenem as single agents, tigecycline + colistin and tigecycline + meropenem) were added at concentrations of 1 \times , 2 \times and 4 \times MIC for each strain [10]. Aliquots were removed at 0, 2, 4, 6, 8, 16 and 24 h post inoculation, serially diluted and plated on

Mueller–Hinton agar plates for enumeration of viable colonies. Antimicrobial carry-over was controlled by widely streaking the transferred aliquot over the agar plate and observing for possible inhibition of growth at the site of the initial streak. Bactericidal activity was defined as a $\geq 3 \log_{10}$ reduction in the total CFU/mL from the original inoculum. Bacteriostatic activity was defined as maintenance of or $< 3 \log_{10}$ reduction in the total CFU/mL from the original inoculum [8]. Time–kill curves were constructed by plotting mean colony counts versus time. Synergy was defined as $\geq 2 \log_{10}$ decrease in the number of CFU/mL between the combination and the most active compound. Synergy time–kills are usually read after 24 h of incubation; however, it is believed that assessment of viability at earlier time intervals may also have clinical significance [9], as the responsiveness according to the time of exposure may represent the dosage intervals used in patients. Thus, all time periods were evaluated. Indifference was defined at 24 h as a $\pm 1 \log_{10}$ kill to $< 2 \log_{10}$ compared with the most efficient agent alone [11].

3. Results

Macrodilution MICs were 0.25–4 $\mu\text{g/mL}$ for tigecycline, 0.5–1 $\mu\text{g/mL}$ for colistin and 2–16 $\mu\text{g/mL}$ for meropenem (Table 1). Using previous CLSI criteria, four strains were considered as susceptible to meropenem, having MICs of 2–4 $\mu\text{g/mL}$, but by applying the updated CLSI criteria [8] all strains were classified as non-susceptible. The mean \log_{10} CFU/mL changes from the initial bacterial

concentrations with the antibiotics tested as single agents and in combinations during the time–kill assays are presented in Table 1.

Tigecycline alone displayed a concentration-independent bacteriostatic effect in seven strains, with moderate re-growth after 8–16 h of incubation. In the remaining one *K. pneumoniae* strain, tigecycline was bactericidal at 16 h, with subsequent re-growth. Meropenem alone had a concentration-independent bactericidal activity at 24 h in three strains (one each of *E. coli*, *E. cloacae* and *S. marcescens*). It is of note that in both *E. coli* strains with low meropenem MICs the responsiveness to meropenem varied considerably at 1× and 2× MIC, with the one strain being effectively killed and the second strain remaining unaffected at 24 h, implying a potential for heteroresistance. In the four *K. pneumoniae* strains, bacteriostatic activity was maintained for 8 h, with significant re-growth observed. Colistin as a single agent showed bactericidal activity at 8–16 h in only three of the seven strains tested (one each of *E. coli*, *K. pneumoniae* and *E. cloacae*) only at 4× MIC. Furthermore, consistent re-growth was observed in these strains beyond 8–16 h, causing a loss of bactericidal activity. For the remaining clinical strains and concentrations used, colistin caused a bacteriostatic effect, with re-growth after 6–8 h.

Tigecycline + colistin exerted bactericidal activity against all strains at most time intervals and concentrations tested, although a degree of re-growth was observed in some strains at 16–24 h. In addition, this combination was

synergistic at 1× and 2× MIC against most organisms at 4 h and 8 h, and at 4× MIC the synergism was sustained at 24 h against all strains compared with either tigecycline or colistin alone. In contrast, tigecycline + meropenem was non-synergistic against all strains and was also not bactericidal against the four *K. pneumoniae* strains. The non-KPC-producing control strain did not exhibit significant differences in its responsiveness to the antibiotics tested compared with the study strains. The time–kill profiles obtained with 4× MIC of tigecycline, colistin and tigecycline + colistin are presented in Fig. 1.

4. Discussion

KPC-producers cause severe hospital infections, commonly requiring combination therapies [2,4,5,7]. However, there is a lack of information regarding the possible bactericidal activity of existing antimicrobials, alone or in combination, against KPC-producing bacteria.

The results of the present study have shown that, at most drug concentrations tested, tigecycline, colistin and meropenem as single agents do not exhibit efficient bactericidal activity against KPC-producing Enterobacteriaceae. Similar to these findings, previous reports have shown that tigecycline has bacteriostatic activity against Enterobacteriaceae exhibiting resistance to several unrelated antimicrobial classes [12,13]. A single study testing tigecycline alone against KPC-producing *K. pneumoniae* isolates also showed bacteriostatic activity [7], whilst similar results have been exerted by tigecycline against one metallo-β-

lactamase-producing *K. pneumoniae* isolate [10]. Colistin has also been found to exert concentration-dependent bactericidal activity against KPC-producing *K. pneumoniae* isolates [7]. However, in the current study colistin exposure was followed by re-growth in all strains, including those exhibiting an initial bactericidal activity, possibly explaining why colistin when used alone commonly exhibits low clinical success rates [4]. However, the latter literature review [4] revealed that the outcomes were improved when colistin was combined with other antibiotics and this is also suggested by the time–kill experiments in this study. With regard to meropenem, its bactericidal activity against only three of the eight strains tested supports the clinical data of carbapenem-susceptible KPC infections treated by carbapenem monotherapy, which frequently leads to clinical and microbiological failures, being successful in only 40% of cases [4,14].

The in vitro effect of tigecycline combined with other agents against KPC-producers has not been tested previously. The present study has shown a lack of synergism using the combination of tigecycline + meropenem. It should be noted that this combination is usually considered as a therapeutic alternative against multidrug-resistant Gram-negative bacilli. In contrast, the combination of tigecycline + colistin was found here to be bactericidal against all strains. This result supports previous findings that this combination was bactericidal against *K. pneumoniae* isolates [15] as well as initial clinical observations that tigecycline + colistin succeeded against most KPC infections [4]. It was also synergistic at 1× and 2× MIC at 4 h and 8 h for most organisms tested, whilst synergy was

achieved at multiple time points and was sustained after 24 h at 4× MIC, although such a concentration is hardly achievable in serum. The results of this study indicate that tigecycline combined with colistin at appropriate dosage intervals might be a therapeutic option for infections due to multidrug-resistant KPC-producers when bactericidal activity is necessary, such as in bacteraemia, endocarditis or other severe infections. However, additional in vivo tests are warranted to assess better the performance of tigecycline combinations with antibiotics that may have different pharmacokinetic and pharmacodynamic parameters against KPC infections.

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None.

Competing interests

None declared.

Ethical approval

Not required.

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Fig. 1. Time–kill kinetics performed in fresh cation-adjusted Mueller–Hinton broth at 4× the minimum inhibitory concentration (MIC) of (a) tigecycline, (b) colistin and (c) tigecycline + colistin against the study clinical strains and the control strain *Escherichia coli* ATCC 25922. CFU, colony-forming units.

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Table 1

Tigecycline, meropenem and colistin minimum inhibitory concentrations (MICs) of the tested strains and mean changes from initial bacterial concentrations following incubation for 24 h with tigecycline, colistin, meropenem, tigecycline + colistin and tigecycline + meropenem during time–kill assays at 1×, 2× and 4× MIC

Antimicrobial agent/combination	<i>Escherichia coli</i> 1	<i>E. coli</i> 2	<i>Enterobacter cloacae</i> 1	<i>Serratia marcescens</i> 4	<i>Klebsiella pneumoniae</i> 1	<i>K. pneumoniae</i> 2	<i>K. pneumoniae</i> 3	<i>K. pneumoniae</i> 4	<i>E. coli</i> ATCC 25922
Tigecycline MIC (µg/mL)	0.5	0.25	1	4	0.5	1	1	2	0.25
Mean change (log ₁₀ CFU/mL)									
1× MIC	-1	-2.2 ^a	-0.9	-1.3	-0.6	-1.6	-1.7	-2	-0.4
2× MIC	0	0	0	-0.2	0	0	-2.9	-1.6	-1.6
4× MIC	-1.8	-0.7	-1.3	-0.5	-0.6	-0.7	-1.8	-0.7	-2.1
Meropenem MIC (µg/mL)	2	2	16	4	4	16	16	8	0.06
Mean change (log ₁₀ CFU/mL)									
1× MIC	-3.5 ^b	0	-3 ^b	-2.8	0	0	0	-0.3	-5.6 ^b
2× MIC	-5.6 ^b	0	-5.6 ^b	-3.3 ^b	0	0	0	0.6	-5.6 ^b
4× MIC	-4.3 ^b	-2.9	-2.9	-3.6 ^b	-0.6	-0.2	0	0	-5.6 ^b

Tigecycline + meropenem									
Mean change (log ₁₀ CFU/mL)									
1× MIC	-0.6	-1.6	-1.6	-2.9	-0.6	-0.9	-1	-1	-0.7
2× MIC	-0.9	-2 _{b,c}	-2	-2.6	-0.7	-1	-1	-0.9	-0.8
4× MIC	-3.3 ^b	-3.2 _b	-2.6	-2.6	-1.5	-1.3	-1.6	-1.3	-3.8 ^b
Colistin MIC									
Colistin MIC	0.5	0.5	1	N/A	0.5	1	0.5	0.5	0.5
Mean change (log ₁₀ CFU/mL)									
1× MIC	-0.3	-0.2	-0.3	N/A	-0.3	-0.2	-0.5	-0.3	-0.3
2× MIC	0.1	0.1	0	N/A	0	0.1	0.1	0.1	-2.5
4× MIC	0	-2.7	0.6	N/A	-2.7	0	0.6	0	-5.6 ^b
Tigecycline + colistin									
Mean change (log ₁₀ CFU/mL)									
1× MIC	-1.5	-1.4	-2.2	N/A	-0.3	-1	-1.9	-1.2	-0.8
2× MIC	-1.5	-2.6 _c	-2.6 ^c	N/A	-1	0.1	-2.3	-2.2	-2.4
4× MIC	-4.3 ^{b,c}	-5.6 _{b,c}	-3.6 ^{b,c}	N/A	-5.6 ^{b,c}	-5.6 ^{b,c}	-5.6 ^{b,c}	-5.6 ^{b,c}	-5.6 _{b,c}

CFU, colony-forming units; N/A, not applicable (as *S. marcescens* is intrinsically resistant to colistin).

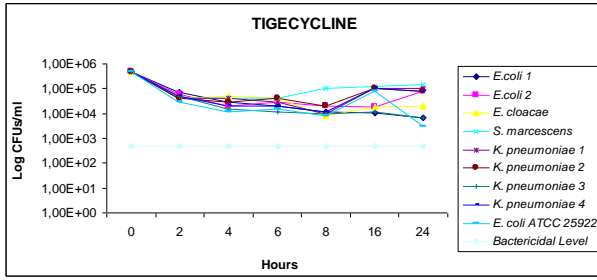
^a <3 log₁₀ reduction in CFU, implies bacteriostatic effect.

^b $\geq 3 \log_{10}$ reduction in CFU, implies bactericidal effect.

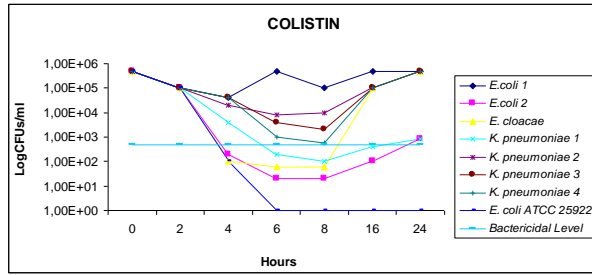
^c $\geq 2 \log_{10}$ reduction in CFU of a drug combination at 24 h compared with the most active drug, implies synergism.

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(a)



(b)



(c)

