In Vitro Activity of Eravacycline in Combination with Colistin Against OXA-type Carbapenemase Producing *Klebsiella pneumoniae* Isolates

OXA-tipi Karbapenemaz Üreten *Klebsiella pneumoniae* İzolatlarına Kolistin-Eravasiklin Kombinasyonunun in vitro Sinerjik Etkinliğinin Değerlendirilmesi

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ABSTRACT

Objective: The synergistic activity of eravacycline in combination with colistin on Oxacillinase (OXA) producing carbapenem-resistant *Klebsiella pneumoniae* isolates (CR-Kp) was evaluated in this study.
Methods: Minimum inhibitory concentrations of meropenem were determined by the broth microdilution method. All strains screened for carbapenemase activity by PCR. Chequerboard assay and time-kill analysis were used to assess potential synergy.
Results: Synergistic activity was found in 40 % of the strains by chequerboard assay. No antagonism was detected. Comparing to colistin alone at subinhibitory concentrations synergistic and bactericidal activity was observed when it combined with eravacycline. A Similar activity was also observed in colistin-resistant CR-Kp isolates.
Conclusion: Our results indicate that Eravacycline and colistin combination may be a potential therapeutic option for the treatment of CR-Kp infections.

Keywords: Eravacycline; Colistin; Synergy; *Klebsiella pneumoniae*; Time-kill assay

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ÖZET

Amaç: Bu çalışmanın amacı oksasilinaz (OXA) üreten karbapenem dirençli *Klebsiella pneumoniae* izolatlarında (CR-Kp) eravasiklin ile kolistin kombinasyonunun in vitro sinerjik etkinliğinin değerlendirilmesidir.
Yöntem: Meropenem minimum inhibitör konsantrasyonları (MIK) sıvı mikrodilüsyon yöntemi ile belirlendi. Meropenem dirençli tüm izolzatlar karbapenemaz aktivitesi için polimeraz zincir reaksiyonu ile değerlendirildi Eravasiklin ve kolistin arasındaki sinerjik ilişki dama tahtası sinerji testi ve zamana bağlı öldürme yöntemleri ile değerlendirildi.
Bulgular: Dama tahtası sinerji testi ile izolatların % 40'ında sinerji saptanmış, antagonizma saptanmamıştır. Kolistin-Eravasiklin kombinasyonu ile subterapötik kolistin konsantrasyonlarında sinerjik ve bakterisidal etkinlik saptanmıştır. Benzer bir sinerjinin etkinliğin karbapenem ve kolistin dirençli izolatlarda da olduğu gösterilmiştir.
Sonuç: Sonuçlarımız, eravasiklin ve kolistin kombinasyonunun CR-Kp enfeksiyonlarının tedavisi için potansiyel bir terapötik seçenek olabileceğini göstermektedir.

Anahtar Sözcükler: Eravasiklin; Kolistin; Sinerji; *Klebsiella pneumoniae*; Zamana bağlı öldürme

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INTRODUCTION

Carbapenem-resistant *Klebsiella pneumoniae* (CR-Kp) isolates are currently one of the most important nosocomial pathogens and mainly affect critically ill pati. Carbapenem resistance rates in *K. pneumoniae* (Kp) isolates range from 0% to 65% according to geographical regions (3, 4). Compared with carbapenem sensitive *Kp,* infections due to CR-Kp are associated with higher mortality (1, 2, 5). Appropriateness of initial antibiotic treatment is one of the major factors affecting mortality (6). Because CR-Kp isolates are often resistant to most antibiotics, the available therapeutic options are limited to colistin, polymyxin B, fosfomycin, tigecycline, selected aminoglycosides and some novel beta-lactams beta-lactamase inhibitor combinations (ceftazidime-avibactam and meropenem-varborbactam). However, a significant increase in resistance rate against polymyxins, aminoglycosides, and fluoroquinolones was noted for Kp isolates (2, 4, 7, 8). Therefore, new and effective therapeutic options are required for the treatment of CR-Kp infections.

Eravacycline is a novel fluorocycline that belongs to the tetracycline class of antimicrobials and has potent activity against many gram-negative organisms, including resistant to other classes (9). For Enterobacteriaceae, the MIC_{90} (*minimum inhibitory concentrations)* is usually ≤ 2.0 mg/L, including carbapenem-resistant organisms (9). But, limited clinical evidence exists form CR-Kp related infections (10). Colistin is often included in the treatment of CR-Kp infections to enhance the clinical efficacy of combination therapies (2, 11).

Novel therapeutic combinations should be evaluated because the effectiveness of the current combination regimens is still uncertain (11).

The aim of this in vitro study is to evaluate the synergistic activity of eravacycline in combination with colistin against CR-Kp isolates.

METHODS

Bacterial Strains

CR-Kp strains isolated from blood and lower respiratory tract specimens in different critically ill patients were used in this study. Identification was performed by MALDI-TOF MS (Bruker Biotyper; Bruker Daltonics, Bremen, Germany). During testing, the isolates were cultured from frozen stocks with 5% sheep blood agar by following guidelines from the Clinical and Laboratory Standards Institute (12). All strains were incubated at 35°C before testing.

Investigation of Carbapenemase Resistance

Minimum inhibitory concentrations of meropenem were determined for all strains by the broth microdilution method (12). MIC values of 4 mg/L and above are taken as limit values for meropenem resistance (13). All strains were screened for carbapenemase genes by PCR. Eight of the most common carbapenemase genes (*bla* $_{OXA-23}$, *bla* $_{OXA-48}$, *bla*$_{OXA-51}$, *bla* $_{OXA-58}$, *bla* $_{NDM}$, *bla* $_{IMP}$, *bla* $_{VIM}$, and *bla* $_{KPC}$) were screened by an in-house multiplex PCR test (14-20). The oligos used for the amplification of the genes as shown in Table 1.

Table 1. Oligos used for amplification

Oligos	**5' → 3'**	Amplicon Size (bp)	References
OXA, NDM, VIM, IMP, KPC			
OXA-23	GATCGGATTGGAGAACCAGA ATTTCTGACCGCATTTCCAT	501	14,16
OXA-48	TTGGTGGCATCGATTATCGG GAGCACTTCTTTTGTGATGGC	733	14,15
OXA-51	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	353	14
OXA-58	AAGTATTGGGGCTTGTGCTG CCCCTCTGCGCTCTACATAC	599	14
NDM	GTAGTGCTCAGTGTCGGCAT GGGCAGTCGCTTCCAACGGT	476	17
VIM	GTGTTTGGTCGCATATCGC CGCAGCACCAGGATAGAAG	380	18
IMP	GGAATAGAGTGGCTTAATTCTC CCAAACCACTACGTTATC	624	18
KPC	ATGTCACTGTATCGCCGTC TTTTCAGAGCCTTACTGCCC	893	19

Abbreviations: OXA, Oxacillinase; NDM, New Delhi metallo-lactamase; VIM, Verona integron-encoded metallo-β-lactamase; IMP, Imipenem-hydrolyzing β-lactamase; KPC, Klebsiella pneumoniae carbapenemase

Drugs

Eravacycline (Lot number: 26030) and colistin (Lot number:16647) were provided by Med Chem Tronica (Sweden) as laboratory-grade powders. All drugs were dissolved with dHO_2, 6,4 mg/ml stock solutions were prepared for eravacycline and colistin. All stock solutions were stored at – 20 °C throughout the study.

Minimum Inhibitory Concentration And Fractional Inhibitory Concentrations (FIC)

Minimum inhibitory concentrations of eravacycline and colistin were determined for all strains by the broth microdilution method (12). The data obtained from broth microdilution tests were used to calculate synergy. The chequerboard microdilution panel method was used for MIC determination of the eravacycline and colistin combination.

Using 96-well U-bottom microplates, graded concentrations of antibiotics were mixed. Each antimicrobial agent was prepared to a fixed volume of 45 µL (up to a total of 90 µL volume for two antimicrobial agents), and 10 µL of bacterial suspension was added to each well. The final concentration of the test strains was a 5×10^5 CFU/ mL in a total final volume of 100 µL in each well.

The plates were incubated for 16-24h at 35°C and the presence of inhibition of microbial growth was determined visually. Eravacycline and colistin synergy were studied at least 2 times with the chequerboard method in all strains. The fractional inhibitory concentration index (FICI) was calculated with the formula:

FICI = MIC AB / MIC A + MIC BA / MIC B

The results of combination tests according to the FIC index were interpreted as follows: Synergy, FICI < = 0.5; No interaction, FICI >0.5 and ≤4; Antagonism, FICI >4 (21).

Time-Kill Assay

Two strains that showed a synergistic activity on assay were randomly selected for time-kill assay. Muller-Hinton broth was inoculated with the bacterial suspension to reach a final inoculum of $5x10^5$ CFU / mL. Colistin and eravacycline concentration were tested alone and in combination at 1X, 0.5X, 0.25X MIC. The final volume of each strain/drug concentration was 5 mL. 0,1 mL aliquots at 0 hr, 3hr, 6hr and 24 hr for determination of visible count by serial dilution plating.

All plates were incubated for 18 to 24 hr at 35 °C. Bacterial colonies were counted manually and followed by the calculation. The limit of detection for the assays was 10 CFU/Ml (22). Bacterial concentration of < 10 CFU/mL were counted as 1.0 $\log_{10}$ CFU/mL. A synergistic effect was defined as a ≥ 2 $\log_{10}$ decrease in Cfu/mL compared with its more active constituent. Bactericidal activity was defined as a ≥ 3 $\log_{10}$ decrease in CFU/mL.

This in vitro study was conducted with the approved of the local ethics committee.

RESULTS

Ten CR-Kp strains were enrolled in this study. Five of ten strains were also resistant to colistin. All isolates were found to have *bla*$_{OXA-48}$. The characteristics of ten CR-Kp isolates included in chequerboard analysis were shown in Table 2.

Table 2. Characteristics of CR-Kp isolates

Isolate (Kp)	Isolation Date	Isolation Sample	Carbapenemase Activity (OXA)	MIC (mg/L) Range MEM
Kp-1	2015	Blood	OXA-48	8
Kp-2	2015	Endotracheal Aspiration	OXA-48	32
Kp-3	2018	Blood	OXA-48	16
Kp-4	2012	Blood	OXA-48	16
Kp-5	2016	Endotracheal Aspiration	OXA-48	4
Kp-6	2019	Endotracheal Aspiration	OXA-48	16
Kp-7	2014	Endotracheal Aspiration	OXA-48	16
Kp-8	2013	Blood	OXA-48	16
Kp-9	2012	Endotracheal Aspiration	OXA-48	64
K-10	2019	Blood	OXA-48	16

Abbreviations: Kp, *K.pneumoniae*; OXA, Oxacillinase; MIC, Minimum Inhibitory Concentration; MEM, Meropenem

MIC values ranged between 0.5 to 8 and 0,25 to 64 mg/L for eravacycline and colistin, respectively. In chequerboard analysis, a synergistic effect was observed in 40% of the isolates. No antagonism was observed.

Synergistic activity was detected even in both colistin and carbapenem-resistant strains. The MIC values of eravacycline, colistin, and minimum FIC values are summarized in Table 3.

Table 3. Synergistic activity of eravacycline in combination with colistin against CR-Kp isolates

Isolate (Kp)	MIC (mg/L) Range				FIC_{min}	Interpretation
	Alone		In Combination			
	Eravacycline	Colistin	Eravacycline	Colistin		
Kp -1	4	0.5	1	0.125	0.50	SYN
Kp -2	2	0.25	1.0	0.125	1.00	ADD
Kp -3	8	0.25	0.03	0.25	1.12	IND
Kp -4	1	0.5	0.06	0.5	1.06	IND
Kp -5	0.5	0.25	0.12	0.25	1.25	IND
Kp -6	2	64	0.25	8	0.25	SYN
Kp -7	4	64	0.50	1	0.14	SYN
Kp -8	2	16	0.12	2	0.25	SYN
Kp -9	1	64	0.50	16	0.75	ADD
Kp -10	1	64	0.25	32	0.75	ADD

Abbreviations: Kp, *K.pneumoniae*; MIC, Minimum Inhibitory Concentration; FIC_{min}, Smallest Total Fractional Inhibitory Concentration; ADD, Additive; IND, Indifference; SYN, Synergy

Time-kill analysis was performed toμ better evaluate the synergy between eravacycline and colistin in two strains. Eravacycline alone at 1X MIC concentration was not bactericidal for any of the strains. Colistin alone at 1 X.

MIC was rapidly bactericidal, achieving a 3 log decrease in CFU/mL by 3 h of exposure, and the bactericidal activity was sustained up to 24 h against the two strains tested.

Eravacycline and colistin combination at 1XMIC concentration was rapidly bactericidal but no synergic activity was detected for both strains because of the bactericidal activity of colistin.

Colistin alone at 0.5 and 0.25X MIC were achieved 3 log decrease in CFU/mL by 3 h of exposure against Kp-6 isolates. However, this activity was not sustained up to the 6 and 24 h time points.

When the colistin was tested alone at 0.5X and 0.25X MIC against Kp-1 isolate, no significant decrease was detected. Eravacycline tested alone at 0.5 and 0.25X MIC was not bactericidal for any of the strains. When eravacycline and colistin combination at the sub-inhibitory concentration for both antibiotics (0.5X MIC) was rapidly bactericidal, and the bactericidal activity was sustained by 24 h. Comparing to colistin alone at 0.5X MIC concentration, synergistic activity was observed at 3h for isolate KP-1, 6 h for isolate KP-6 and activity was sustained up to 24 h. Also, synergistic activity was observed at 0.25X MIC concentration in the KP-6 isolate which was colistin-resistant and continued by 24 h. The synergy was not observed against Kp-1 strain at 0.25X MIC. Time-kill analysis results were plotted in figure 1,2,3 and 4.

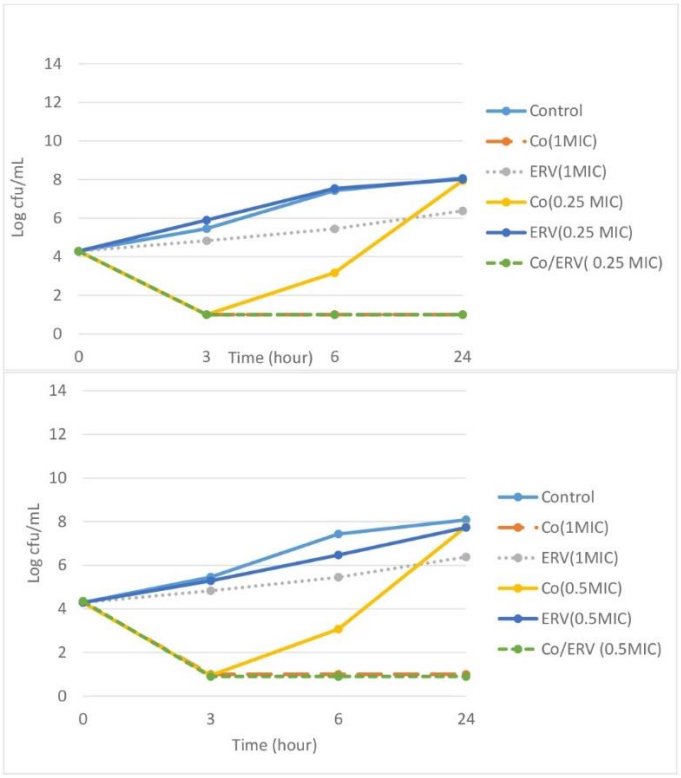

Figure 1. Time-kill assay Colistin (Co)(0.25, x MIC, 0.5 x MIC, 1 x MIC), Eravacycline (ERV) (0,25 x MIC, 0,5 x MIC, 1 x MIC) and Colistin/Eravacycline (Co/ERV) for *K.pneumoniae* Isolates (KP-6)

DISCUSSION

Our study demonstrated that eravacycline has synergistic and additive activity in combination with colistin. The combination of eravacycline and colistin may be a potential treatment option for OXA-type carbapenemase-producing CR-Kp infections.

Eravacycline is, a novel fluorocycline, active against gram-negative, gram-positive and anaerobic bacteria except *Pseudomonas aeruginosa* (23-26). In-vitro studies performed with eravacycline suggest that it may be a potential therapeutic option for CR-Kp infections. In these studies, the MIC 90 value of eravacyline in Carbapenem-resistant Enterobacteriaceae (CRE) isolates ranged from 0.5 to 2 mg/L (25, 27, 28). In vitro activity of eravacycline was demonstrated against a different type of carbapenemase-producing strains (24, 29).

In a study by Livermore et al., the MIC value of eravacycline ranged from 0.13 to 4 mg/L in OXA producing Enterobacteriaceae isolates (29).

In our study, eravacycline MIC values ranged from 0.5 to 8 mg/ L for OXA-48 producing CR-Kp strains.

Despite the conflicting results for their clinical efficacy, combination treatment is widely used to maximize bacterial killing and minimize bacterial resistance (2, 30). Colistin is widely used in combination therapies, but the optimum combination regimen is still uncertain (2, 11, 30). Previous in-vitro studies have shown a potential synergistic relationship between colistin and tigecycline(31-33). It has been shown that a similar synergistic relationship may be between colistin and doxycycline or minocycline(33). In our study, chequerboard analysis showed that colistin and eravacycline combination had 40% synergistic activity. No antagonism was observed. These expected results obtained with an tetracycline group antibiotic are thought to be important because of the high potency of eravacycline compared to other tetracyclines, especially tigecycline(34).

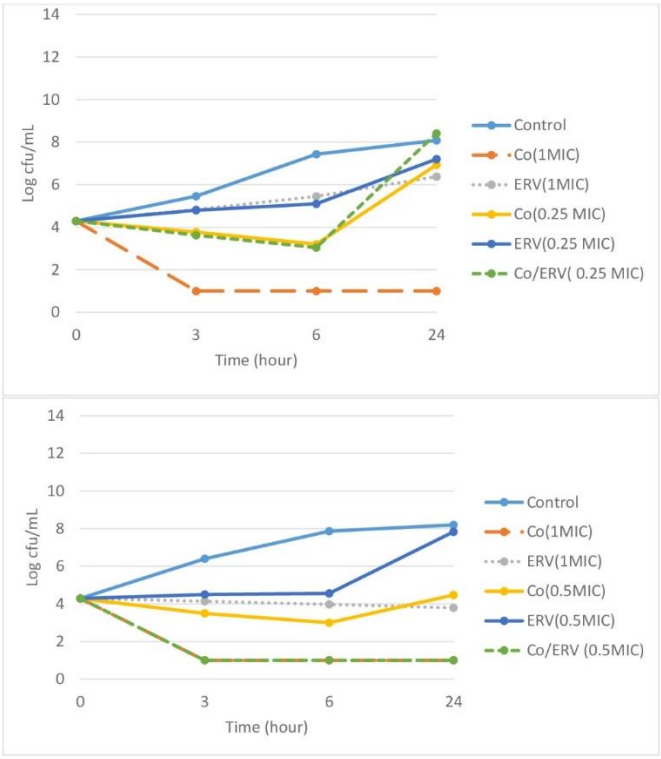

Figure 2. Time-kill assay Colistin (Co)(0.25 x MIC, 0,5 x MIC, 1 x MIC), Eravacycline (ERV) (0.25 x MIC, 0,5 x MIC, 1 x MIC) and Colistin/Eravacycline (Co/ERV) for *K.pneumoniae* Isolate (KP-1)

In vitro synergy results may differ according to the used method(35). Therefore, it is recommended to evaluate the synergistic activity with different methods (36). In our study, the time-kill analysis was used to further assess the potential synergy of eravacycline in combination with colistin against two randomly selected strains. Comparing to colistin alone at subinhibitory concentrations, synergistic and bactericidal activity was observed when it combined with eravacycline. This activity was sustained by 24 hours. An important point was the detection of synergistic and bactericidal activity at the 1/4 concentration of the MIC levels of each drug, even in colistin-resistant isolates. Furthermore, antagonism was not detected between two drugs in the time-kill analysis.

In conclusion, the combination of eravacycline with colistin showed an in vitro synergistic activity against OXA-type carbapenemase-producing CR-Kp isolates, despite high MICs value of colistin. The result of this study indicates that eravacycline and colistin combination may be a potential therapeutic option for the treatment of CR-Kp related infections.

This preliminary in vitro results indicated that eravacycline and colistin combination could be evaluated with clinical studies.

Conflict of interest
No conflict of interest was declared by the authors.

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