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ORIGINAL ARTICLE

In vitro effects of tigecycline in combination with colistin (polymyxin E) and sulbactam against multidrug-resistant *Acinetobacter baumannii*

Wentao Ni[1], Junchang Cui[1], Beibei Liang[2], Yun Cai[2], Nan Bai[2], Xuejiu Cai[1] and Rui Wang[2]

The lack of active antimicrobial agents against multidrug-resistant (MDR) *Acinetobacter baumannii* has posed great threat to the public health. Combination therapies with antibiotics owning different antimicrobial mechanisms have been proposed as good options for treating MDR *A. baumannii* infections. This study was aimed to investigate the *in vitro* effects of tigecycline in combination with colistin and sulbactam against MDR *A. baumannii*. A total of 70 strains from two hospitals in China were examined in the study. The checkerboard method was used for determining synergistic activity of different antibiotic combinations. Tigecycline/colistin combination displayed synergistic and partial synergistic activity in 24.3% of the isolates, whereas the tigecycline/sulbactam combination showed synergistic and partial synergistic activity in 64.3% of the isolates. Neither of the combinations showed antagonism in this study. In addition, for evaluating the ability of combinations on resistance prevention, mutant prevention concentrations (MPCs) of tigecycline, colistin, sulbactam alone and tigecycline in combination with colistin and sulbactam were studied against MDR *A. baumannii*. Compared with tigecycline used alone, combination therapies could achieve lower MPCs of tigecycline. However, when the MPCs of dual-drug therapy were in conjunction with clinical pharmacokinetic profiles, combinations may not strictly curb the occurrence of resistance at current dosage regimen. In summary, this study suggested that combination therapy was a good option for treating MDR *A. baumannii* infections. But the finding that combination with these drugs at current dosage regimen may not prevent emergence of resistance warranted further studies on dosage of combined antibiotics required for achieving resistance prevention.
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INTRODUCTION

Acinetobacter baumannii has been frequently involved in serious nosocomial infections with high morbidity and mortality in recent years.[1] These pathogens can develop mechanisms of resistance to most available antibiotics, including β-lactams, fluoroquinolones, tetracyclines, aminoglycosides and even carbapenems, making treatment extremely intractable.[2] Now, only a few antibiotics are still available for us to fight with these multidrug-resistant (MDR) *A. baumannii*. Tigecycline, the first commercially available member of glycylcyclines, has appealing *in vitro* activity against MDR *A. baumannii*.[3] Also, colistin, an 'old drug' replaced because of its severe nephrotoxicity and neurotoxicity, has been reused in clinic.[4] In addition, sulbactam, a β-lactamase inhibitor usually used for enhancing the activity of β-lactams, is also found to be an active compound for killing MDR *A. baumannii*.[5]

However, as they have been widely prescribed to control MDR bacteria infections, drug resistance is increasingly emerging around the world. Reports from Asia and Europe showed that the resistance rates of tigecycline ranged from 27.5–78%.[6] Anthony[7] even found the evolution of resistance in one *A. baumannii* isolate after 14 days of treatment with tigecycline, alerting the high risk for emergence of resistance during long-term monotherapy. For colistin, the numbers of heteroresistant strains is growing,[8] and one report from a tertiary care hospital in Korea showed that the resistance rates had reached 30.6%.[9]

These evidences suggested that using these drugs as monotherapy may lead to extensive resistance and result in clinical treatment failure. Therefore, combination therapies with antibiotics owning different antimicrobial mechanisms have been proposed as good options for treating MDR *A. baumannii* infections.[10] In this study, the *in vitro* effects of tigecycline combined with colistin and sulbactam against 70 MDR *A. baumannii* clinical strains was tested. The objective was not only to focus on the synergistic activity of antimicrobial combinations, but also to evaluate the ability of

[1]Department of Respiratory Diseases, Chinese People's Liberation Army General Hospital, Beijing, China and [2]Department of Clinical Pharmacology, Chinese People's Liberation Army General Hospital, Beijing, China
Correspondence: Professor J Cui, Department of Respiratory Diseases, Chinese People's Liberation Army General Hospital, No. 28, Fuxing Road, Haidian District, Beijing 100853, China.
E-mail: guoguoyoumeng@163.com
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combinations on resistance prevention when taking their clinical pharmacokinetics into consideration.

MATERIALS AND METHODS

Antimicrobial agents

Tigecycline was obtained from Wyeth Pharmaceutical (Wyeth Pharmaceutical, Philadelphia, PA, USA). Colistin was purchased commercially from Sigma-Aldrich (St Louis, MO, USA). Sulbactam standards were obtained from the National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, Beijing, China). Colistin and sulbactam solutions in sterile water were prepared and stored at −80 °C. For tigecycline, stock solution in sterile water was freshly prepared on the day of use.

Microorganisms and susceptibility testing

A total of 70 nonduplicate clinical strains of MDR *A. baumannii* that were resistant to more than three antimicrobial classes routinely used were collected between June 2010 and July 2011 from two hospitals of Beijing, China. The minimal inhibitory concentrations (MICs) of tigecycline, colistin and sulbactam were determined by agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) recommendations.[11] In brief, Mueller-Hinton agar (Difco, Franklin Lakes, NJ, USA) plates containing a series of twofold concentration increments of each agent were prepared according to specifications. Then, approximate 10^4 CFU bacterial cells were inoculated by an autoclaved replicator and incubated at 37 °C for 24 h. The MIC was defined as the lowest drug concentration that inhibited the visible growth of colonies.

Synergistic activity testing of combinations by checkerboard method

The checkerboard method previously described was used for determining *in vitro* synergistic activity of antibiotic combinations.[12] Briefly, the concentration ranges of tested drugs were prepared based on the MIC values obtained in the preliminary test. Then, 50 µl of each drug and 100 µl bacterial suspension were added into 96-well microdilution plates. The final concentration of inoculums in each well was $\sim 5 \times 10^5$ CFU per ml. The strains were cultured at 37 °C for 24 h.

Interaction between combinations was determined according to fractional inhibitory concentration index (FICI). The FICI was calculated with the following formula: FICI = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). FICI was interpreted as previously defined:[13] synergy, FICI $\leqslant 0.5$; partial synergy, $0.5 < \text{FICI} < 1$; additive, FIC = 1; indifference, $1 < \text{FICI} \leqslant 2$; and antagonism, FICI > 2.

Effectiveness on resistance prevention of combinations by mutant prevention concentration (MPC) determinations

A method based on MPC theory was used to test the effectiveness of combinations on resistance prevention. The MPC represents a concentration threshold above which no single-step drug-resistant mutant strains can be selected and the mutant selection window (MSW) is a concentration range approximately extended from MIC to MPC, within which drug-resistant mutant strains are more likely to be selected for.[14] Therefore, if an addition of a second drug can reduce the MPCs (narrow or even close to MSW) of tigecycline in MDR *A. baumannii*, it means that the combination can effectively restrict the selection of drug-resistant mutant strains.

The MPCs of drugs alone and in combinations were determined by a modified method described by Zhao and Drlica[15] and Zhanel *et al.*[16] Strains were inoculated on agar plates and grown at 37 °C for 24 h and then bacterial cells were moved in 400 ml Mueller-Hinton broth (Difco). After culturing overnight with shaking at 37 °C, the growth was centrifuged (4000 *g* for 5 min) to yield high-density cultures containing cells of $\sim 3 \times 10^9$ CFU per ml. Samples (100 µl) were inoculated onto Mueller-Hinton agar (Difco) plates containing a series of twofold dilutions of either tigecycline, colistin or sulbactam alone and of tigecycline in combination with different concentrations of colistin or sulbactam. MPCs of tigecycline alone and combinations were determined as the lowest drug or drug combination concentrations that no colonies were recovered of after 72 h of incubation at 37 °C.

RESULT

Susceptibility

All 70 isolates were susceptible to colistin and the MIC values ranged from 0.125 to 1 µg ml^{-1}. There are no CLSI susceptibility breakpoints for tigecycline and sulbactam against *A. baumannii* at present. The MICs of tigecycline and sulbactam ranged from 0.125 to 2 µg ml^{-1} and from 4 to 32 µg ml^{-1} respectively. The MIC_{90} of colistin, tigecycline and sulbactam was 0.5, 2 and 32 µg ml^{-1}, respectively.

Synergistic activity testing of combinations

By the checkerboard method, the tigecycline/colistin combination displayed synergistic and partial synergistic activity in 24.3% of the isolates (Figure 1), whereas the results of tigecycline/sulbactam combination seemed to be better, with 64.3% of the isolates showing synergistic and partial synergistic activity and 10% showing indifference. Neither of the combinations showed antagonism in this study. In addition, the cumulative inhibition ratio of tigecycline is shown in Figure 2. The curves of the cumulative inhibition ratio were shifted to left after adding another drug. For the tigecycline/sulbactam combination, the curve moved more to the left, suggesting a better synergistic effect.

Effectiveness on resistance prevention of combinations

The MPC distributions of tigecycline, colistin and sulbactam alone in 30 clinical strains of MDR *A. baumannii* are listed in Table 1. The MPC_{90} for the three drugs was 32, >128 and 128 µg ml^{-1}, respectively. For tigecycline and colistin, the ratios of MPC_{90}/MIC_{90} (defined as selection index) were 16 and >256, which indicated quite a wide MSW. Although the MPCs of sulbactam were high, the ratio of MPC_{90}/MIC_{90} was 4, meaning a narrower MSW.

When tigecycline was used in combination with colistin and sulbactam, a decrease in MPCs of tigecycline could be found (Table 2). With an addition of colistin at 2 µg ml^{-1}, the MPCs decreased by only one time in 5/9 strains, and with an addition of a more aggressive dosage of colistin at 8 µg ml^{-1}, the MPCs could decrease 4–8 times, ranging from 1 to 8 µg ml^{-1}. For the tigecycline/sulbactam combination, the decrease of MPCs was found in 4 strains after combining with sulbactam at 16 µg ml^{-1}. When the concentration of sulbactam was increased to 64 µg ml^{-1}, 8–32 times decrease

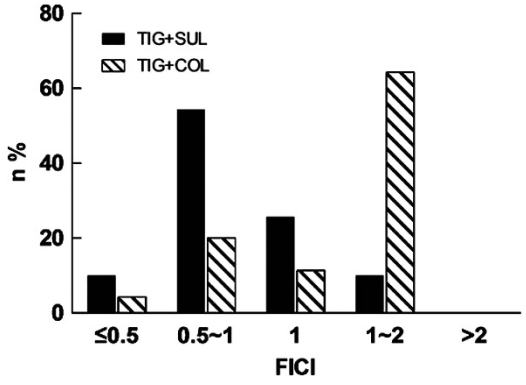

Figure 1 Fractional inhibitory concentration index (FICI) of tigecycline (TIG) combined with colistin (COL) and sulbactam (SUL) in multidrug-resistant *Acinetobacter baumannii* (*N* = 70).

could be obtained and even in 4 of 9 strains, the MPCs could reduce below the MIC of tigecycline.

DISCUSSION

Most *A. baumannii* infections could be treated successfully with traditional antibiotics such as broad-spectrum β-lactams, cephalosporins and carbapenems before 1970s.[17] However, nowadays, infections caused by MDR *A. baumannii* have been increasingly emerging worldwide. In Asia, *A. baumannii* was responsible for 13.6% of nosocomial pneumonia and nearly 82% strains were MDR strains.[18] Because of limited options, colistin and tigecycline have been introduced to control these infections. However, extreme drug-resistant *A. baumannii* that can develop resistance against all available antibiotics including these two drugs has been reported.[19] Therefore, combination therapies with two or more effective drugs are attracting more attention.

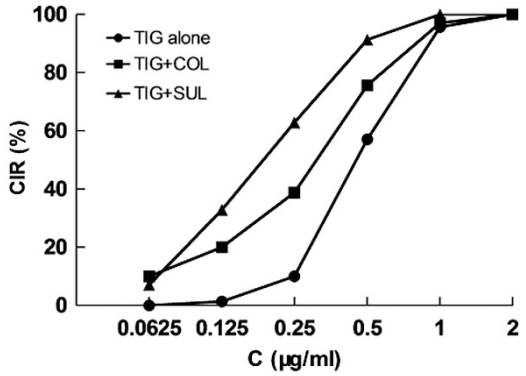

Figure 2 The cumulative inhibition ratio (CIR) of tigecycline (TIG) against multidrug-resistant *Acinetobacter baumannii* ($N=70$). C, concentration, COL, colistin; SUL, sulbactam.

Table 1 MPC distribution for three antimicrobials alone against multidrug-resistant *Acinetobacter baumannii* ($n=30$)

Test agent ($\mu g\,ml^{-1}$)	*4*	*8*	*16*	*32*	*64*	*128*	*>128*	MPC_{90}	MPC_{90}/MIC_{90}
TIG	2	8	17	3				32	16
COL				1	6	3	20	>128	>256
SUL				1	2	20	7	128	4

Abbreviations: COL, colistin; MIC, minimal inhibitory concentration; MPC, mutant prevention concentration; SUL, sulbactam; TIG, tigecycline.

There have been several studies on evaluating the synergistic activity of different combinations with tigecycline. Dizbay *et al.*[20] have determined that tigecycline/colistin combination displayed synergistic activity in 18 of 25 (72%) extensive drug-resistant *A. baumannii* strains by the E-test method. However, other studies observed an indifferent activity by the same method.[10] In tests performed by checkerboard method, the synergistic activity of tigecycline/colistin combination was found in 2/24 (8.3%) strains.[21] Our studies got a similar result, with merely 3/70 (4.3%) strains showing synergy. For tigecycline/sulbactam combination, Deveci *et al.*[22] reported synergistic effects in 2/10 (20%), and indifference in 5/10 (50%) isolates by the checkerboard method. In our studies with more strains, the results seemed to be better, with 45/70 (64.3%) of the isolates showing synergy and partial synergy activity and only 10% showing indifference.

Besides simply focusing on enhancing the activity of bacterial killing, evaluation of combination therapy on resistance inhibiting is also extremely important as drug-resistant strains are emerging so rapidly. Although the MICs of tigecycline and colistin to MDR *A. baumannii* were low, the MPCs of both were quite high, ranging from 4 to 32 $\mu g\,ml^{-1}$ and from 32 to >128 $\mu g\,ml^{-1}$, respectively. Hence, at current dose regimen, pharmacokinetic profiles for both agents can easily fall inside the MSW, leading to selectively enrich drug-resistant mutant strains.[23,24] Zhao and Drlica[15] pointed that with antibiotics individually having high MPCs, combination therapy provided a feasible method to curb the emergence of resistance. Because combination therapy using two drugs acting with different antimicrobial mechanisms would require susceptible bacteria to acquire two concurrent mutations for growth. Zhanel *et al.*[16] have demonstrated that combinations of levofloxacin and a second antibiotic could prevent the emergence of levofloxacin-resistant *Pseudomonas aeruginosa*. In addition, Cai *et al.*[25] also showed that colistin combined with levofloxacin or tobramycin could perform more effectively in decreasing resistant mutation frequencies in *A. baumannii*.

Table 2 MICs and MPCs of tigecycline alone or in combination with colistin and sulbactam against multidrug-resistant *Acinetobacter baumannii* ($n=9$)

		MPC ($\mu g\,ml^{-1}$)						
Isolate no.	*MIC* ($\mu g\,ml^{-1}$)	*TIG*	*TIG+COL*[a]	*TIG+COL*[b]	*TIG+COL*[c]	*TIG+SUL*[d]	*TIG+SUL*[e]	*TIG+SUL*[f]
Ab2	1	8	8	4	1	4	1	0.5
Ab10	2	16	16	4	2	16	4	1
Ab18	1	32	16	8	4	16	8	4
Ab24	1	16	16	16	4	8	8	1
Ab26	0.5	16	8	4	4	16	8	0.5
Ab28	2	16	8	8	2	16	8	2
Ab31	0.5	16	16	8	4	16	8	1
Ab40	1	16	8	8	4	16	8	0.5
Ab65	2	32	16	8	8	8	2	0.5

Abbreviations: COL, colistin; MIC, minimal inhibitory concentration; MPC, mutant prevention concentration; SUL, sulbactam; TIG, tigecycline.
[a]COL, 2 $\mu g\,ml^{-1}$.
[b]COL, 4 $\mu g\,ml^{-1}$.
[c]COL, 8 $\mu g\,ml^{-1}$.
[d]SUL, 16 $\mu g\,ml^{-1}$.
[e]SUL, 32 $\mu g\,ml^{-1}$.
[f]SUL, 64 $\mu g\,ml^{-1}$.

In our study, we evaluated whether combination therapy of tigecycline with colistin and sulbactam could inhibit or reduce selections of tigecycline-resistant mutants of MDR *A. baumannii*. Our results showed that with an addition of colistin at $2\,\mu g\,ml^{-1}$ or sulbactam at $16\,\mu g\,ml^{-1}$, the MPCs of tigecycline did not decrease remarkably. Dramatic decreases in MPCs of tigecycline could be observed only when high concentration of colistin ($8\,\mu g\,ml^{-1}$) or sulbactam ($64\,\mu g\,ml^{-1}$) were combined. After intravenous administration of 720 mg (9 MU) colistin methanesulfonate every 24 h, the average 24-h concentrations and the C_{max} of colistin in serum was $<8\,\mu g\,ml^{-1}$, ~ 2 and $5.63\,\mu g\,ml^{-1}$, respectively.[26] Despite that C_{max} of sulbactam in serum (1.5 g every 12 h) could reach $>90\,\mu g\,ml^{-1}$,[27] for a β-lactam inhibitor with very short half-life (nearly 1 h), it will likely not be possible to maintain an unbound average concentration of $64\,mg\,l^{-1}$ sulbactam in patients with normal renal function. Therefore, these data showed that both colistin and sulbactam combinations may not appear feasible to strictly curb the occurrence of resistance at current dosage regimen.

In conclusion, we tested the *in vitro* effectiveness of tigecycline in combination with colistin and sulbactam against 70 MDR *A. baumannii* strains. In contrast to tigecycline/colistin combination, tigecycline/sulbactam combination had better synergistic and partial synergistic activity *in vitro*. Moreover, we demonstrated that an addition of colistin or sulbactam could lower the MPCs of tigecycline in MDR *A. baumannii*. But the concentrations effectively restricting the selection of resistant mutants were much higher than that at current dosage of combination therapies. This indicated that an incremental dosage might be required for achieving good bacterial killing as well as resistance prevention. As the antibiotic concentrations in our study were static and *in vitro* study may not completely simulate *in vivo* conditions, such as the virulence of bacteria and the host immune response, further investigations using ideally animal models are needed to confirm our findings.

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