

# Sulbactam enhances the *in vitro* activity of sitafloxacin against extensively-drug resistant *Acinetobacter baumannii*

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**Abstract.** The present study aimed to determine the *in vitro* activities of sulbactam and sitafloxacin against extensively-drug resistant *Acinetobacter baumannii* (XDR-*A. baumannii*). A total of 50 strains of XDR-*A. baumannii* were isolated from clinical specimens. Broth microdilution assay was applied to determine the minimum inhibitory concentration (MIC) for sulbactam and sitafloxacin. Microdilution checkerboard method was used to determine the *in vitro* activity of this antimicrobial combination. Accordingly, the fractional inhibitory concentration (FIC) and FIC index (FICI) were calculated. Time-kill study was also carried out for four strains with different susceptibilities to determine the bactericidal activities of individual or combined use of sitafloxacin and sulbactam. Isolates with MICs of sitafloxacin  $\leq 2$  mg/l were considered to be susceptible to sitafloxacin. The susceptibility rate for sitafloxacin was 92% originally. When combined with sulbactam, this rate increased to 96%. Microdilution checkerboard results indicated that, when tested in combination, sulbactam/sitafloxacin exhibited marked synergistic and partial synergistic effects on 16 and 50% of the 50 strains, respectively. Time-kill assay suggested that sulbactam enhanced the bactericidal activity of sitafloxacin and the combination induced a synergistic effect. For strains that were not susceptible to sitafloxacin, the bactericidal activities of the combination of sitafloxacin and sulbactam at a sub-MIC concentration were impaired. However, this impairment could be overcome with the increase of the concentration to 1X MIC. The present study demonstrated that sulbactam

enhanced the *in vitro* antimicrobial activity of sitafloxacin against XDR-*A. baumannii*.

## Introduction

*Acinetobacter baumannii* is an opportunistic pathogen that can cause a broad array of infections including pneumonia, skin and soft tissue infection, meningitis, urinary tract infection and blood stream infection, especially in immunocompromised patients (1). This ubiquitous organism can be detected in a wide range of environments, including hospitals and other care facilities, and can survive for a prolonged period of time on both biotic and abiotic surfaces (2). *A. baumannii* has been designated as a 'red alert' human pathogen due to its extensive antimicrobial resistance (3). Bacterial strains can be classified as multidrug-resistant (MDR), extensively drug-resistant (XDR) and pandrug-resistant (PDR). XDR is defined as non-susceptibility to at least one agent in all but two or fewer antimicrobial categories (4). Therefore, treatment options for XDR-*A. baumannii* infections are limited. When one agent is routinely used in clinical practice, the susceptibility of *A. baumannii* to this drug may markedly decrease (5,6). Given unpredictable or suboptimal pharmacodynamics and concerns of resistance emerging along with therapy, antimicrobial combinations may provide improved treatment options (7). Therefore, a search for novel agents and their efficient combinations is required. In the present study, the *in vitro* antimicrobial activity of sitafloxacin, a new fluoroquinolone, and the combined effect of sitafloxacin and sulbactam were detected against clinical isolates of XDR-*A. baumannii*.

## Materials and methods

**Bacterial isolates.** XDR-*A. baumannii* strains were isolated from clinical specimens collected in three tertiary hospitals affiliated to Shandong University (Qilu Hospital, Jinan Central Hospital and Shandong Provincial Qianfoshan Hospital; all in Jinan, China) from November 2014 to December 2015. For patients from whom *A. baumannii* strains were isolated more than once, only one strain from each patient was included. VITEK<sup>®</sup> 2 microbial analysis instruments were used to identify the XDR-*A. baumannii* isolates (bioMérieux, Inc., Marcy l'Etoile, France). The Kirby-Bauer method (8) was applied to re-evaluate the strains to meet the criteria for

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XDR-*A. baumannii* (4). As a result, a total of 50 strains were included, of which 36 strains were from sputum, 5 from lavages, 3 from blood, 3 from skin wounds or surgical incisions of skin, 2 from cerebrospinal fluid and 1 from urine. ATCC 25922 and ATCC 27853 were used as quality controls (American Type Culture Collection, Manassas, VA, USA). The Ethics Committee of Qilu Hospital of Shandong University approved the present study (approval no. KYLL-2017-612). All patients provided written informed consent.

**Broth microdilution assay.** Mueller-Hinton (MH) powder was purchased from Thermo Fisher Scientific, Inc. and dissolved according to the manufacturer's protocol. Isolated colonies of *A. baumannii* strains were maintained in 10 ml fresh MH broth and shaken in a thermo-incubator at 37°C overnight. Suspensions with a turbidity that matched the 0.5 McFarland standard [ $1.5 \times 10^8$  colony-forming unit (CFU)/ml] were further diluted to obtain the final bacterial counts of  $2-8 \times 10^5$  CFU/ml. Sitafoxacin and sulbactam were obtained from Beijing Biodee Biotechnology Co., Ltd. (Beijing, China). To determine minimum inhibitory concentration (MIC) values, broth microdilution method was carried out as described in Clinical and Laboratory Standards Institute (CLSI) guidelines (9). Susceptibility was also determined for each isolate using these criteria. The serially diluted drugs were at concentrations of 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125 and 0 µg/ml. When MICs were higher than 128 µg/ml, drug arrays of 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25 and 0 µg/ml were further prepared. Following the addition of bacterial suspensions, 96-well plates were incubated overnight in ambient atmosphere at 37°C. MIC values were determined by the concentrations of drugs at which bacterial growth was visibly inhibited.

**Microdilution checkerboard assay.** Following determination of MICs of sulbactam and sitafoxacin for each strain, another set of dilution series was prepared for the two agents: 8X MIC, 4X MIC, 2X MIC, 1X MIC, 0.5X MIC, 0.25X MIC, 0.125X MIC and 0 µg/ml. Sitafoxacin was added by column, while sulbactam was added by row in 96-well plates. Bacterial suspensions at concentrations of  $2-8 \times 10^5$  CFU/ml were subsequently added and incubated overnight at 37°C. Fractional inhibitory concentration index (FICI) values were calculated as follows:  $FICI = \text{MIC value of sulbactam combined with sitafoxacin} / \text{MIC value of sulbactam applied alone} + \text{MIC value of sitafoxacin combined with sulbactam} / \text{MIC value of sitafoxacin applied alone}$ . FICI values were interpreted as follows:  $\leq 0.5$ , synergy;  $>0.5$  to  $<1$ , partial synergy; 1, addition;  $>1$  to  $<4$ , indifference; and  $\geq 4$ , antagonism (10). The above steps were performed in triplicate and average values were used as the final results. The software we used in this study was Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

**Time-kill assay.** Time-kill assay was conducted for four representative strains with different susceptibilities to sitafoxacin and sulbactam. Bacterial suspensions were prepared following the aforementioned steps and turbidity was adjusted respectively. Thereafter, drugs alone or in combination were added into the suspensions and incubated at 37°C. Time-kill curves for individual or combined sulbactam and sitafoxacin were plotted at 0.5X MIC and 1X MIC,

respectively. Drug concentrations were selected according to MIC levels instead of a fixed concentration and the four representative strains were different from each other in their susceptibilities towards the two agents. 1X MIC were chosen to guarantee the antimicrobial activity, while 0.5X MIC were chosen to see whether synergistic effects could be achieved at sub-MIC levels. Samples were removed at 0, 1, 2, 4, 6, 12 and 24 h. Aliquots (100 µl) were serially diluted with cold and sterile PBS. Bacterial counts were determined by plating three spots of 10 µl of appropriate dilutions (1:100) on MH agar plates and incubation at 35°C for 18-24 h. Time-kill curves were subsequently constructed by plotting the mean colony counts ( $\log_{10}$  CFU/ml) vs time. The bactericidal activities of drug combination were defined as a 3  $\log_{10}$  CFU/ml (99.9%) reduction compared with the most efficient drug at 24 h. Synergy was defined as a 2  $\log_{10}$  CFU/ml decrease between the combination and the most active agent alone at 24 h (11). The drug combination was considered to be antagonistic with a  $\geq 2$   $\log_{10}$  increase in counts. In addition, the combination was considered to be indifferent if there was a  $<2$   $\log_{10}$  increase or decrease in colony count compared with the most active drug alone. The experiment was performed in duplicate to ensure reproducibility. The experiment was continued for  $>24$  h.

## Results

*Combined use of sitafoxacin and sulbactam exhibits an increased inhibitory effect on XDR-A. baumannii strains compared with the individual use of either.* To test the effects of sitafoxacin and sulbactam against the XDR-*A. baumannii* strains, MICs and susceptibility rates were determined. Given that CLSI breakpoints are not reported for sitafoxacin, CLSI breakpoints for other fluoroquinolones were used to assess the susceptibility of pathogens to sitafoxacin. Isolates with MICs of sitafoxacin  $\leq 2$  mg/l were provisionally considered to be susceptible to sitafoxacin (12). CLSI breakpoints were not available for the use of sulbactam alone, and, therefore, breakpoints of ampicillin/sulbactam against *Acinetobacter spp* (susceptible,  $\leq 8/4$  µg/ml; intermediate,  $16/8$  µg/ml; and resistant,  $\geq 32/16$  µg/ml) were used as interpretation criteria instead (13). The data indicated that the lowest concentration of the antibiotic at which 50% of the isolates were inhibited ( $MIC_{50}$ ) and the lowest concentration of the antibiotic at which 90% of the isolates were inhibited ( $MIC_{90}$ ) for sitafoxacin decreased and the susceptibility rates increased when the drug was combined with sulbactam. Similarly, the  $MIC_{50}$  and  $MIC_{90}$  for sulbactam decreased and the susceptibility rates increased when it was combined with sitafoxacin (Table I). MIC values were determined by the concentrations of drugs at which bacterial growth was completely inhibited. The results suggested that the combined use of sitafoxacin and sulbactam exhibited a greater inhibitory effect on XDR-*A. baumannii* strains compared with the individual use of either.

*Sitafoxacin and sulbactam induce a synergistic or partial synergistic effect on the majority of the 50 strains.* To further examine the inhibitory effect of sitafoxacin and sulbactam against XDR-*A. baumannii* strains, FICI was calculated.

Table I. MIC values of sitafloxacin and sulbactam against XDR-*A. baumannii* isolates.

Antimicrobial agent	MIC range ( $\mu\text{g/ml}$ )	MIC <sub>50</sub> ( $\mu\text{g/ml}$ )	MIC <sub>90</sub> ( $\mu\text{g/ml}$ )	Rate of susceptibility (%)
Sitafloxacin				
Alone	0.125-16	1	2	92
Combined	0.016-8	0.5	2	96
Sulbactam				
Alone	2-256	32	64	16
Combined	0.016-64	8	32	48

MIC, minimum inhibitory concentration; MIC<sub>50</sub>, the lowest concentration of the antibiotic at which 50% of the isolates were inhibited; MIC<sub>90</sub>, the lowest concentration of the antibiotic at which 90% of the isolates were inhibited.

Table II. Distribution of FICI values for the combination of sitafloxacin and sulbactam against XDR-*A. baumannii* isolates.

Sulbactam/sitafloxacin	Synergy (FICI $\leq 0.5$ )	Partial synergy (FICI 0.5-1)	Addition (FICI 1)	Indifference (FICI 1-4)	Antagonism (FICI $\geq 4$ )
Number of isolates	8	25	8	9	0
Rate	16%	50%	16%	18%	0

FICI, fractional inhibitory concentration index.

The data indicated that sitafloxacin and sulbactam induced a synergistic effect in 16% of the 50 strains. In addition, sitafloxacin and sulbactam induced a partial synergistic effect on 50% of the 50 strains. Importantly, sitafloxacin and sulbactam exhibited no antagonistic effect on any strain (Table II). The results indicated that sitafloxacin and sulbactam exhibited synergistic or partial synergistic effects on the majority of the 50 strains.

*Majority of the 50 isolates are not susceptible to sulbactam and are susceptible to sitafloxacin.* According to the susceptibility status of the 50 isolates to sulbactam and sitafloxacin, they were divided into the following four groups: i) Susceptible to both sulbactam and sitafloxacin (SS); ii) non-susceptible to sulbactam and susceptible to sitafloxacin (NS); iii) susceptible to sulbactam and non-susceptible to sitafloxacin (SN); and iv) non-susceptible to either sulbactam or sitafloxacin (NN). The data indicated that isolates in the SS group accounted for 14% of all strains, those in the NS group accounted for 78%, those in the SN group accounted for 2% and those in the NN group accounted for 6% (Table III). The results suggested that the majority of the 50 isolates were not susceptible to sulbactam and were susceptible to sitafloxacin.

*Combined use of sitafloxacin and sulbactam at the concentration of 1X MIC exhibits bactericidal activity against all four isolates at 24 h, and synergistic effect on the four selected isolates.* To conduct the time-kill studies, four representative strains were randomly selected from the four groups shown in Table III, respectively. Sulbactam and sitafloxacin displayed a synergistic effect on the strain selected from the SS group, with a FICI value of 0.375. Sulbactam and sitafloxacin

Table III. Distribution of strains susceptible and non-susceptible to sitafloxacin and sulbactam.

Number of isolates	Sulbactam	
	Susceptible	Non-susceptible
Sitafloxacin		
Susceptible	7 (14%)	39 (78%)
Non-susceptible	1 (2%)	3 (6%)

had FICI values of 0.75 and 0.675 for strains selected from the NS and SN groups, respectively. Furthermore, sulbactam and sitafloxacin had an FICI value of 1.5 for the strain selected from the NN group. Time-kill curves for individual or combined use of sulbactam and sitafloxacin were plotted at 0.5X MIC and 1X MIC, respectively. Time-kill assays indicated that sitafloxacin exhibited a rapid bacteriostatic effect at the concentration of 1X MIC, regardless of the susceptibility status of the four isolates. Combined use of sitafloxacin and sulbactam at the concentration of 0.5X MIC exhibited a bactericidal effect for the strains from the SS and NS groups, and synergistic effect for all strains but that from NN group. Furthermore, combined use of sitafloxacin and sulbactam at the concentration of 1X MIC exhibited bactericidal activity, and synergistic effect against all four isolates at 24 h regardless of the susceptibility status (Figs. 1-4; Table IV). Combined use of sitafloxacin and sulbactam at the concentration of 1X MIC completely removed the strains from SS and NS groups with no regrowth after 24 h.

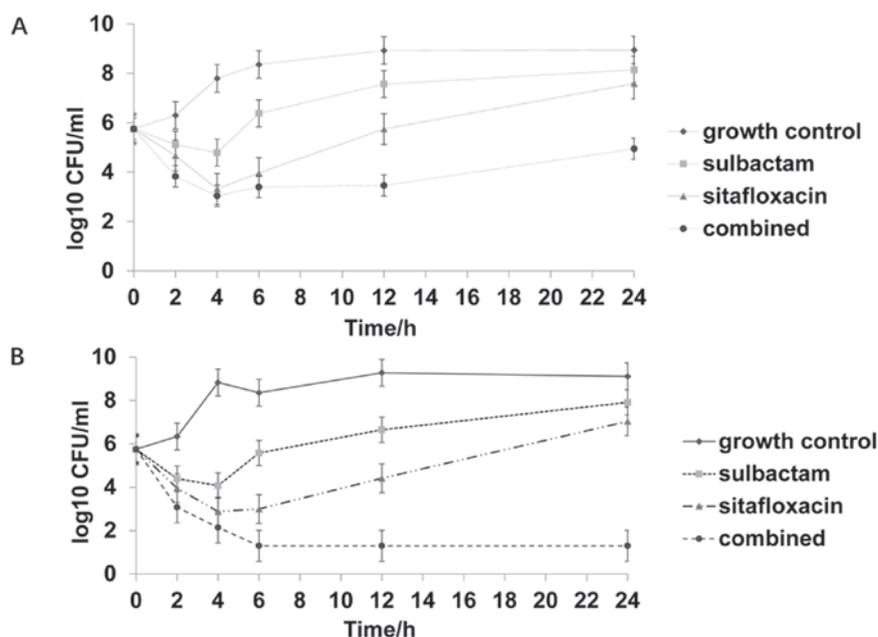


Figure 1. Time-kill curve of one strain susceptible to both sulbactam and sitafloxacin. (A) 0.5X MIC sulbactam, 0.5X MIC sitafloxacin, and the combination of 0.5X MIC sulbactam and 0.5X MIC sitafloxacin. (B) 1X MIC sulbactam, 1X MIC sitafloxacin, and the combination of 1X MIC sulbactam and 1X MIC sitafloxacin. MIC, minimum inhibitory concentration; CFU, colony-forming unit.

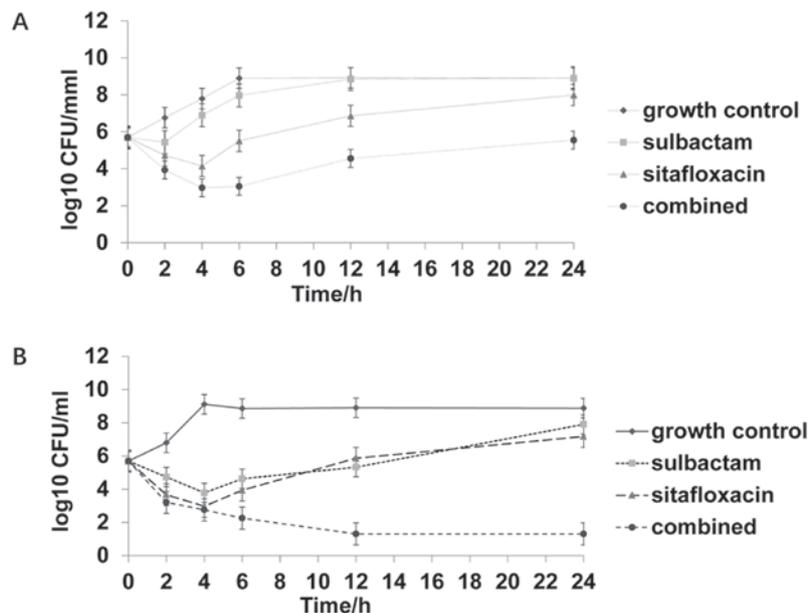


Figure 2. Time-kill curve of one strain not susceptible to sulbactam and susceptible to sitafloxacin. (A) 0.5X MIC sulbactam, 0.5X MIC sitafloxacin, and the combination of 0.5X MIC sulbactam and 0.5X MIC sitafloxacin. (B) 1X MIC sulbactam, 1X MIC sitafloxacin, and the combination of 1X MIC sulbactam and 1X MIC sitafloxacin. MIC, minimum inhibitory concentration; CFU, colony-forming unit.

## Discussion

*A. baumannii* is one of the most common causes of nosocomial infection in Asia (8). The risk factors of *A. baumannii* infection include long stay in hospital or intensive care unit, mechanical ventilation, invasive operation, antibiotic exposure, multiple infections, diabetes and COPD (14). *A. baumannii* can form biofilms, which can survive in various implants (catheterization, endotracheal intubation and deep vein catheterization) for a long time (15). Patients often merge with basic diseases,

multiple infections or invasive operations. Their immunity is weak and requires longer treatment time compared with other infectious diseases. Therefore, the clinical treatment of *A. baumannii* infection is usually longer. Chinese experts recommend combination therapy for the treatment of drug resistant *A. baumannii* (16). This pathogen harbors multiple resistance mechanisms and reduces therapeutic choices to a limited set of active antibiotics (16). With the rise of MDR, XDR and PDR strains globally (17-20), there is a requirement for the development of safe and effective therapeutic strategies.

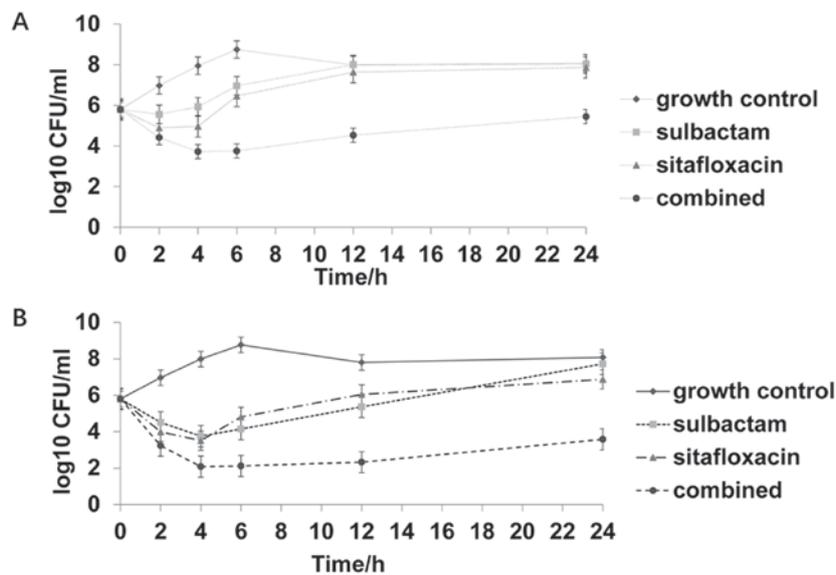


Figure 3. Time-kill curve of one strain not susceptible to sitafloxacin and susceptible to sulbactam. (A) 0.5X MIC sulbactam, 0.5X MIC sitafloxacin, and the combination of 0.5X MIC sulbactam and 0.5X MIC sitafloxacin. (B) 1X MIC sulbactam, 1X MIC sitafloxacin, and the combination of 1X MIC sulbactam and 1X MIC sitafloxacin. MIC, minimum inhibitory concentration; CFU, colony-forming unit.

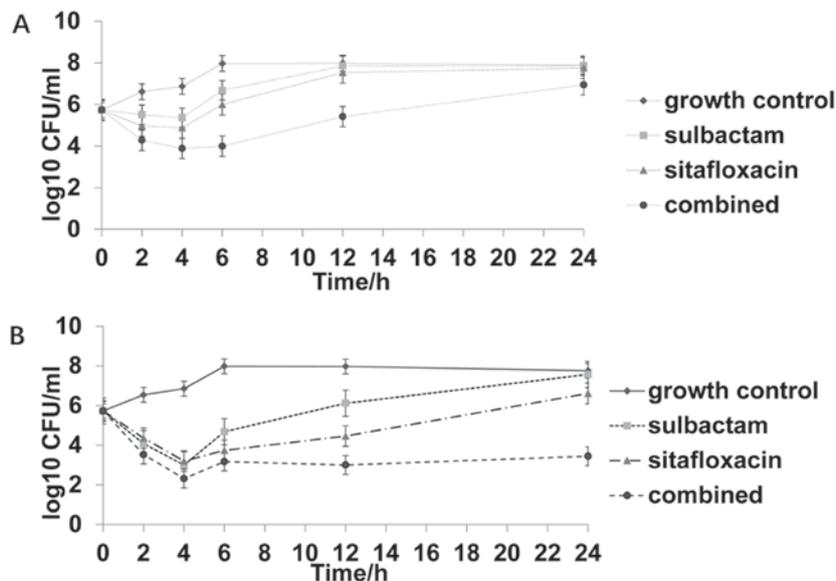


Figure 4. Time-kill curve of one strain not susceptible to either sulbactam or sitafloxacin. (A) 0.5X MIC sulbactam, 0.5X MIC sitafloxacin, and the combination of 0.5X MIC sulbactam and 0.5X MIC sitafloxacin. (B) 1X MIC sulbactam, 1X MIC sitafloxacin, and the combination of 1X MIC sulbactam and 1X MIC sitafloxacin. MIC, minimum inhibitory concentration; CFU, colony-forming unit.

Fluoroquinolones have a broad-spectrum antimicrobial activity and are commonly used in clinical practice (21). At present, the majority of nosocomial isolates of *A. baumannii* are resistant to fluoroquinolones (22-24), and, therefore, fluoroquinolones are not ideal for the empirical treatment of *A. baumannii*-associated infections. A new fluoroquinolone antibiotic, sitafloxacin, has demonstrated a good *in vitro* activity against pathogens that are resistant to other fluoroquinolones (25,26). Sitafloxacin has been reported to exhibit acceptable antimicrobial effects against carbapenem-resistant *A. baumannii* and the respective susceptibility rates were 91.4 and 58.9% according to two separate reports (12,27). Sulbactam exhibits affinities for penicillin-binding proteins

and inhibits bacterial cell wall synthesis (28,29). Sulbactam is active against *A. baumannii* and has been clinically used for infections caused by this organism (30). In addition, sulbactam exhibits synergistic effects with other antibiotics (31).

In the present study, sitafloxacin induced a promising antimicrobial activity against XDR-*A. baumannii* with a susceptibility rate of 92%. Furthermore, combined use of sitafloxacin with sulbactam resulted in a susceptibility rate of 96%. Sitafloxacin and sulbactam exhibited synergistic effects in 16% of the 50 strains. In addition, sitafloxacin and sulbactam have shown partial synergistic effect on 50% of strains. None of the strains exhibited antagonistic effects. However, Odds (32) proposed alternative criteria for interpretation of

Table IV. Results of time-kill assay for the four strains under different drug concentrations.

Results	Strains under a drug concentration of 0.5X MIC	Strains under a drug concentration of 1X MIC
Bactericidal activities		SS, NS, SN, NN
Synergy	SS, NS, SN	
Indifferent	NN	

SS, susceptible to sulbactam and sitafloxacin; NS, non-susceptible to sulbactam and susceptible to sitafloxacin; SN, susceptible to sulbactam and non-susceptible to sitafloxacin; NN, non-susceptible to sulbactam and sitafloxacin.

FICI values. The authors suggested that researchers submitting research articles containing FICI data should interpret synergy as  $FICI \leq 0.5$ , antagonism as  $FICI > 4.0$  and no interaction as  $FICI > 0.5-4.0$ . Additionally, these authors argued that their proposed criteria would encourage conservative interpretation of results (32). According to the suggestions by Odds (32), sitafloxacin and sulbactam exhibited no interaction in 84% of XDR-*A. baumannii* strains included in the present study. The time-kill assay was performed to further investigate the bactericidal activity of the two drugs and their combination (33,34).

Time-kill assays performed in the present study indicated that sitafloxacin induced a rapid bacteriostatic effect at the concentration of 1X MIC, regardless of the susceptibility status of the four isolates. Combined use of sitafloxacin and sulbactam at the concentration of 0.5X MIC only exhibited bactericidal effects for the strains susceptible to sitafloxacin. In addition, combined use of sitafloxacin and sulbactam at the concentration of 1X MIC exhibited bactericidal activity against all four isolates at 24 h. The strains from the SS and NS groups were completely removed with no regrowth after 24 h when sitafloxacin and sulbactam are combined at the concentration of 1X MIC. Combined use of sitafloxacin and sulbactam at the concentration of 0.5X MIC exhibited a synergistic effect for all strains except for the NN group strain. In addition, synergistic effect was achieved for all four isolates, regardless of the susceptibility status, when both drugs are used at the concentration of 1X MIC. The results of the present study indicated that the combination of sitafloxacin and sulbactam was more efficient at eliminating isolates that were susceptible to sitafloxacin compared with isolates that were non-susceptible to sitafloxacin.

Sitafloxacin and sulbactam combination could be a promising alternative treatment for XDR-*A. baumannii* infection. However, *in vitro* experimental results do not necessarily correspond with clinical efficacy (35), which may be the result of the metabolism of the agents and the discordant redistribution of different agents in target tissues (24). Still, *in vitro* experiments provide a convenient way to screen compounds to propose combinations that could be synergistic *in vivo*.

In conclusion, the present study indicated that sulbactam enhanced the bactericidal activity of sitafloxacin and this combination revealed synergistic or partial synergistic effect in the majority of cases. For strains that were non-susceptible to sitafloxacin, the bactericidal activities of the combination of sitafloxacin and sulbactam were impaired at a concentration that is lower than its MIC. However, this impairment was overcome by increasing the concentration to 1X MIC. In future investigations, insights should be gained into the clinical

impact of the combination of sitafloxacin and sulbactam, and the possible benefits associated with the application of this combination.

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### Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Authors' contributions

The final version of the manuscript has been read and approved by all authors, and each author believes that the manuscript represents honest work. NX, GW and QX collaborated to design the study. NX, GW, YL, XD and FC were responsible for performing experiments. NX, GW and YL analyzed the data. All authors collaborated to interpret results and develop the manuscript.

### Ethics approval and consent to participate

All procedures performed in the current study were approved by the Ethics Committee of Shandong University (Jinan, China). Written informed consent was obtained from all patients or their families.

### Patient consent for publication

Written informed consents for publication of any associated data and accompanying images were obtained from all patients or their parents, guardians or next of kin.

### Competing interests

The authors declare that they have no competing interests.

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