ORIGINAL ARTICLE

In vitro characterization and inhibition of the interaction between ciprofloxacin and berberine against multidrug-resistant *Klebsiella pneumoniae*

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Ciprofloxacin is a quinolone antibiotic used to treat *Klebsiella pneumoniae* infections in the clinic. Previous studies have demonstrated that berberine exhibits antibacterial activity and less acquired resistance related to efflux pumps. The multidrug efflux pump *acrAB-tolC* can be stimulated to expel as much toxic material as possible from the cells, but a detrimental effect can be produced owing to an overcrowded periplasm with excess expression products, which inhibits bacterial growth. In this study, the *in vitro* antibacterial activities of ciprofloxacin in combination with berberine were evaluated and compared with those of ciprofloxacin and berberine alone by evaluating the MIC, MBC and summation fractional IC against 20 clinical multidrug-resistant *K. pneumoniae* isolates, 1 quality control bacterium and 1 induced-resistance bacterium. Susceptibility tests showed that the MIC for the combination of berberine and ciprofloxacin was 1/2 that of the individual agents or less. Antimicrobial activities of 18.18% synergy and 77.27% additivity were found. Furthermore, synergism was verified through a time-kill assay, which suggested that the synergistic antibacterial effect of the two-drug combination may, to some extent, be related to the high expression of the *acrAB-tolC* and *acrR* multidrug efflux pumps. Indeed, the expression of these genes was increased > 14-fold in the isolates affected by ciprofloxacin–berberine combination synergism.
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INTRODUCTION

Klebsiella pneumoniae is a Gram-negative opportunistic bacterium that is usually found in the human mouth, skin and intestines. This bacterium commonly causes infections of the respiratory tract, urinary tract, lower biliary tract, gastrointestinal tract and wound sites.[1] The massive use of antibiotics and invasive devices has increased the rate of nosocomial infections and multidrug resistance in *K. pneumoniae*. The US Center for Disease Control and Prevention reported that, during the past 10 years, the number of resistant strains of *Klebsiella* species has increased faster than that of other bacteria (from 1.6 to 10.4%).[2] During the past 8 years in Switzerland, the rate of infection with extended-spectrum cephalosporin-resistant *K. pneumoniae* has increased significantly, from 1.1 to 4.4%.[3] In China, the Mohnarin 2011 annual report indicated that the rate of detection of *K. pneumoniae* capable of producing extended-spectrum beta-lactamases was 50.3% in all clinical specimens.[4] Multidrug-resistant *K. pneumoniae* is a rapidly growing issue worldwide. Concerning the mechanism of resistance in *K. pneumoniae,* efflux systems have an essential role in rendering pathogens resistant to a variety of antibiotics by extruding these drugs.[5] *acrAB-tolC* has been identified as a major multidrug efflux pump and a structural component of enterobacteria.[6] In *K. pneumoniae*, *acrAB-tolC* dominates efflux-mediated resistance to fluoroquinolones.[7] Berberine is an alkaloid that is extracted from various plants used in traditional Chinese medicine.[8] Previous studies have shown that berberine exhibits activity against bacteria, viruses, yeast, parasites, fungi and *Candida albicans*.[9] Berberine is considered an antibacterial agent in both Chinese and Western medicine.[10] Some studies have indicated that berberine can inhibit the reproduction of pathogens if used in combination with other antibiotics.[11–14] Moreover, Karaosmanoglu *et al.*[15] reported that the transcription of *acrEF* was upregulated 17-fold in the presence of berberine in *Escherichia coli*, and *acrEF* was found to be highly homologous to the *acrAB* portion of the multidrug efflux system. Synergistic and additive effects against pathogenic microbes have been observed between berberine and antibiotics or antifungal drugs.[12,16–19] However, the combined effects of berberine and ciprofloxacin against multidrug-resistant *K. pneumoniae* isolates rarely appear in mechanistic studies.

In the present study, the interactions between berberine and ciprofloxacin and their activity against multidrug-resistant *K. pneumoniae* isolates were examined *in vitro,* and the effects of berberine and ciprofloxacin on the expression of *acrAB-tolC* were

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investigated in *K. pneumoniae* at the molecular level to elucidate the underlying antibacterial activity.

MATERIALS AND METHODS

Bacterial isolates

Twenty nonduplicated, multidrug-resistant clinical *K. pneumoniae* isolates (R1–R20) were collected from the Clinical Microbiology Laboratory of the First Affiliated Hospital of Guangzhou Medical University, Guangdong, China, during 2013 and 2014. The strains included ESBLs[+] that were resistant to three types of penicillin, quinolones, cephalosporins, aminoglycosides, macrolides and sulfonamides according to the Performance Standards for Antimicrobial Disk Susceptibility Tests.[20] All isolates were identified and confirmed using a Vitek-60 system (bioMérieux, Lyon-Gerland, France). The *K. pneumoniae* ATCC700603 strain was included as a quality control strain and was referred to as ATCC. All strains were stored at −80 °C and subcultured in Mueller–Hinton broth and Mueller–Hinton agar (Oxoid, Basingstoke, UK). Highly purified lines of active monoclonal strains were then selected for further study. The ATCC strain was induced by ciprofloxacin at concentrations of 0.5–256 µg ml^{-1},[21] which was then referred to as RATCC.

Antimicrobial agents

Ciprofloxacin powders (Guangzhou Nanxin Pharmaceutical, Guangzhou, China) that were obtained as gifts were diluted to concentrations of 5–120 µg ml^{-1} in ddH$_2$O. Berberine was diluted to concentrations of 10–240 µg ml^{-1} in ddH$_2$O (purity ⩾ 98%; Shanghai Yuanye Bio-Technology, Shanghai, China). Each sample was stored at −20 °C for <2 weeks before use.

Antimicrobial susceptibility testing

The macrodilution broth method was used in accordance with the methodology of the Clinical and Laboratory Standards Institute.[22] The density of the inocula of isolated colonies was standardized to 0.5 McFarland standard turbidity (~1–2 × 10^8 CFU ml^{-1}) with sterile saline, diluted with cation-adjusted Mueller–Hinton broth and adjusted to a bacterial concentration of 1 × 10^5 CFU ml^{-1} in each of several sterile 13 × 100 mm test tubes, with a final volume of 1 ml. The final concentrations were prepared in serial twofold dilutions; the ciprofloxacin solutions ranged from 0.0625 to 512 µg ml^{-1} and the berberine solution from 128 to 1024 µg ml^{-1}. The macrodilution tubes were incubated at 37 °C in an ambient air incubator for 16–18 h. Turbidity was compared by eye. Under the MIC, visible growth of the bacterium was completely inhibited. Samples were then pipetted onto Mueller–Hinton agar. After 24 h of incubation in a 37 °C forced air incubator, the inoculum with the lowest concentration of an antimicrobial agent that still demonstrated no visible colonies was considered to represent the MBC of that agent. MBC is defined here as the concentration capable of killing 99.9% of the bacterium in the final inoculum.

Checkerboard method

A checkerboard method was used to assess the antibacterial interactions between ciprofloxacin and berberine, as described by Pillai *et al.*[23] Blank control groups with no drugs were also tested. In 96-well microtiter plates containing ~1 × 10^5 CFU ml^{-1} of inoculum, ciprofloxacin at 1/8 to 1/2 the MIC was added to the wells and the concentration of ciprofloxacin was plotted on the *x*-axis. Berberine was plotted on the *y*-axis in the same way. After 16–18 h of incubation at 37 °C, the lowest possible concentration of the agents associated with no visible turbidity represented the MIC of the checkerboard results.

Synergistic effects

The fractional IC (FIC) index and the summation fractional IC (ΣFIC) were used to assess the synergistic effects of these two antibacterial agents in accordance with the checkerboard MIC results:[23] $\Sigma FIC = FIC_A + FIC_B = FIC_{CIP} + FIC_{BER} = MIC_{CIP(combine)}/MIC_{CIP(alone)} + MIC_{BER(combine)}/MIC_{BER(alone)}$. ΣFIC ⩽ 0.5 was considered synergistic and 0.5 < ΣFIC ⩽ 1 was considered to indicate an additive effect; 1 < ΣFIC ⩽ 2 indicated indifference, and antagonism of ΣFIC was indicated by values > 2.[24–27] When these values exceeded the highest fixed concentration (>512 µg ml^{-1}), the experimental concentration was changed

to the next twofold dilution (1024 µg ml^{-1}) and was used to calculate the ΣFIC.

Time-kill assay

The time-kill assay was used to evaluate the antimicrobial activity of the combinations of the two drugs from another perspective. A modified version of the guidelines for the time-kill assay was used.[28,29] Approximately 1 × 10^6 CFU ml^{-1} of an initial inoculum of five isolates (R5, R12, R15, ATCC and RATCC) was exposed to a fixed final concentration of a single drug or a drug combination in each test tube under the same conditions and time periods in duplicate. Thus, three experimental groups, including a ciprofloxacin group (⩽1/4 MIC), a berberine group (1024 µg ml^{-1}) and a ciprofloxacin ⩽ 1/4 MIC +berberine 1024 µg ml^{-1} group, as well as a blank control group (without agents) were set up. The test tubes were incubated at 37 °C. At 0, 1, 2, 4, 6, 8, 12 and 24 h, 10 µg of the bacterial cultures was removed and transferred to Mueller–Hinton agar. After 24 h, viable bacterial colonies were counted and compared with the single most active agent after 24 h of incubation with both agents. Synergy was defined as a ⩾ 2 log$_{10}$ decrease in the number of bacterial colonies after the combination treatment. Additivity was defined as a 1–2 log$_{10}$ decrease and antagonism as a ⩽ 1 log$_{10}$ increase.

Reverse transcription polymerase chain reaction

Total RNA was extracted from five multidrug-resistant clinical *K. pneumoniae* isolates (R5, R12, R15, ATCC and RATCC) using the RNeasy Protect Bacteria Mini Kit (Qiagen China, Shanghai, China) and lysozyme (Sigma, Sigma-Aldrich China, Shanghai, China) following the manufacturer's protocol. *acrA*, *acrB*, *acrR*, *tolC*, *ramA* and *marA* were detected via RT-PCR using primers to examine gene expression (Table 1). 16S ribosomal RNA was used as an internal control. The PrimeScript RT reagent kit (Takara Bio, Kyoto, Japan) was used for complementary DNA synthesis. Reverse transcription polymerase chain reaction (RT-PCR) was performed in an RT-PCR system (Applied Biosystems, CA, USA) according to the protocol provided with the SYBR Select Master Mix reagent (Applied Biosystems by Life Technologie, CA, USA), with the following standard thermal-cycling conditions: 4 min holding stage, followed by a cycling stage of 40 repetitive cycles including denaturation at 95 °C for 15 s, annealing at 58 °C for 30 s and extension at 72 °C for 30 s. The final melt curve was run at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 30 s and 60 °C for 15 min. The $2^{-\Delta\Delta CT}$ method was used to analyze the RT-PCR data, quantifying the relative differences in gene expression.[30] In the current study, four experimental groups with five strains were used, and three test groups were included: the ciprofloxacin (target), berberine (target) and ciprofloxacin+berberine (target) groups, along with one control group (the blank control (reference)). The C_T value was used to determine the fluorescence threshold cycle for the amplification of target or reference molecules, and the amplification efficiencies were kept approximately equal (the absolute value of the slope was always positive, even when it approached 0). $\Delta C_{T\ acrA}$ was determined using the formula $\Delta C_{T\ acrA} = C_{T,acrA} - C_{T,16s\text{-}RNA}$ and similar formulae. The $2^{-\Delta\Delta CT}$ equation was used to calculate the $\Delta\Delta C_T$ value ($\Delta C_{T\text{-target}} - \Delta C_{T\text{-reference}}$), which was assumed to be the relative quantified expression of variable amplified RNA.

Table 1 Primer sequences and annealing temperatures used for RT-PCR

Gene(s)	Sequence (5′ → 3′)	TM (°C)	GenBank accession no.
acrA-F	GACTTGGTTTGTTCTGATGGCG	58	CP000647.1
acrA-R	CCGTCTGGAAGAAGGGATTAACC		
acrB-F	CCAAACAGTGCAGAACACCGT	57	AJ318073.1
acrB-R	GCCACGTAGTCCGAAATATCGT		
tolC-F	ATGAGCCAGGCGGAAAACC	57	CP000647.1
tolC-R	TTCGGTATAGGAAAGCGTGTCG		
acrR-F	TATGTTCTTGAAGCGACAGTGACA	57	CP000647.1
acrR-R	TTAAGCTGACAAGCTCTCCGG		

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; TM, melting temperature.

Statistical analysis

Each experiment was performed in triplicate. Time-kill curves and RT-PCR data are expressed as the means ± SD. The differences were calculated and compared using Student's *t*-test or ANOVA. The threshold for statistical significance was set as a two-tailed *P*-value < 0.05. Data were analyzed using GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). Time-kill curves were drawn as line charts, which were plotted with time (h) on the horizontal axis and $\log_{10}$ CFU ml^{-1} on the vertical axis. The RT-PCR data are presented as a histogram. The $\Delta\Delta C_T$ value was determined in accordance with the ordinate and abscissa from the four experimental groups for the five strains.

RESULTS

Characterization of antimicrobial susceptibility

The assay to determine the *in vitro* antibacterial activity of ciprofloxacin and berberine against multidrug-resistant *K. pneumoniae* was performed via the macrodilution method and checkerboard microdilution analysis. The results, measured in terms of the MIC and MBC, are summarized in Table 2. The MICs for ciprofloxacin against *K. pneumoniae* ranged from 4 to > 512 μg ml^{-1} and the MICs for berberine were ⩾ 512 μg ml^{-1}. The MBCs for both ciprofloxacin and berberine were ⩾ the value of twofold MICs. These results demonstrated that the MICs for the combination of berberine and ciprofloxacin were at least twofold lower than those for the individual agents. When combined with sub-MICs of ciprofloxacin, the concentration of berberine was lower than that for berberine alone. The FICs of berberine ranged from 0.125 to 0.5 and the FICs of ciprofloxacin ranged from 0.125 to 1. The ΣFIC data showed that the

values for the combination of berberine and ciprofloxacin ranged from 0.375 to 1, with 4 of the 22 (18.18%) showing synergy, 17 of the 22 (77.27%) showing additivity and 1 isolate showing no difference.

In accordance with the ΣFIC data for *K. pneumoniae*, there were five typical isolates, including three clinical strains (synergy): R5, R12 and R15; one control strain (synergy): ATCC; and one induced strain (additivity): RATCC.

The results regarding the synergistic and additive interactions were reproduced using time-kill assays (Figure 1). Figure 1 shows that ciprofloxacin (⩽1/4 MIC) did not affect bacterial growth or viability after 24 h (*P* > 0.05). As anticipated for berberine alone at 1024 μg ml^{-1}, no appreciable antibacterial activity was found (0.9–1.79 $\log_{10}$ CFU ml^{-1}, *P* > 0.05). However, the antibacterial activity of ciprofloxacin was markedly enhanced in the presence of berberine, with bacterial growth being markedly reduced after 24 h when ciprofloxacin was combined with berberine at 1024 μg ml^{-1}, compared with ciprofloxacin at ⩽1/4 MIC (2.41 − 5.7 $\log_{10}$ reduction in CFU ml^{-1}, *P* < 0.05) or berberine at 1024 μg ml^{-1} alone (1.51 − 4.17 $\log_{10}$ reduction in CFU ml^{-1}, *P* < 0.05). The results showed that this combination produced synergistic/additive bactericidal effects. These results were consistent with those of the checkerboard analyses (ΣFIC data). The results of the time-kill assay showed that against R12, R5, R15 and ATCC, the combination of ciprofloxacin and berberine exerted a synergistic effect on bacterial yields ⩾ 2 $\log_{10}$ reduction in CFU ml^{-1}, with stronger antibacterial effects being observed in all groups after 24 h (*P* < 0.05). The exception was the RATCC strain, which was considered indicative of an additive effect (< 2 $\log_{10}$ CFU ml^{-1} lower than in any other group, *P* > 0.05).

An assessment of the dose-response inhibition of *K. pneumoniae* by berberine or ciprofloxacin is shown in Figure 1f. It can be seen that, compared with single agents, the curves for the berberine–ciprofloxacin combination were both shifted to the left. The dose of ciprofloxacin was at least fourfold lower, while that of berberine was at least twofold lower. Berberine alone at a concentration < 512 μg ml^{-1} exhibited no detectable antibacterial activity, and at 1024 μg ml^{-1} it showed poor antibacterial effects. Similar results were obtained at a concentration < 4 μg ml^{-1} for ciprofloxacin alone, where the growth of *K. pneumoniae* was not significantly inhibited. However, when ciprofloxacin was combined with berberine, the growth of *K. pneumoniae* was partially inhibited. Therefore, berberine functionally complemented the antibacterial activity of ciprofloxacin at low concentrations. Moreover, when combined with sub-MIC concentrations of ciprofloxacin, the concentration of berberine was reduced (< 256 μg ml^{-1}). These results indicated that the combination of berberine and ciprofloxacin can be used to reduce the use of single agents, thus supplementing the inadequate antimicrobial activity of single agents at subinhibitory concentrations.

Expression of *acrA*, *acrB*, *tolC* and *acrR* in *K. pneumoniae* in response to ciprofloxacin and berberine

The gene expression levels in the R12, R5, R15, ATCC and RATCC strains were confirmed in the control group (untreated cultures), the berberine group (1024 μg ml^{-1}), the ciprofloxacin ⩽ 1/4 MIC group and the ciprofloxacin ⩽ 1/4 MIC+berberine 1024 μg ml^{-1} group through RT-PCR analysis. For the ciprofloxacin ⩽ 1/4 MIC group, the following concentration was used: ciprofloxacin at 32 μg ml^{-1} (R12, R5, R15 and RATCC strains) and 0.0625 μg ml^{-1} (ATCC strain). The following methods were used for RNA extraction: bacteria were grown until they reached the exponential growth or stationary phase and were then exposed to ciprofloxacin, berberine and the combination of the two for 3 h. The rate of transcription of *acrAB* was

Table 2 MICs and MBCs (μg ml^{-1}) of CIP and BER alone against 20 clinical strains of multidrug-resistant *K. pneumoniae*, ATCC and RATCC[a]

Strain no.	MIC		MBC		ΣFIC[b]	Effect
	CIP	BER	CIP	BER		
R1	256	>512	512	>1024	0.625	add
R2	64	>512	128	>1024	1.5	ind
R3	512	>512	>512	>1024	0.75	add
R4	4	512	4	512	1	add
R5	128	>512	128	>1024	0.5	syn
R6	>512	>512	>512	>1024	0.75	add
R7	32	>512	32	>1024	0.75	add
R8	512	>512	>512	>1024	0.75	add
R9	512	>512	512	>1024	0.75	add
R10	256	>512	256	>1024	1	add
R11	256	>512	256	>1024	1	add
R12	256	>512	256	>1024	0.375	syn
R13	64	>512	64	>1024	0.75	add
R14	4	512	8	1024	1	add
R15	256	>512	512	>1024	0.375	syn
R16	8	>512	8	1024	1	add
R17	512	>512	>512	>1024	0.75	add
R18	128	>512	256	>1024	1	add
R19	>512	>512	>512	>1024	1	add
R20	128	>512	128	>1024	1	add
ATCC	0.5	>512	1	>1024	0.375	syn
RATCC	256	>512	256	>1024	0.75	add

Abbreviations: BER, berberine; CIP, ciprofloxacin; ΣFIC, summation fractional IC.
[a]The experimental maximum concentration of the agents (ciprofloxacin/berberine) was 512 μg ml^{-1}.
[b]FICI ⩽ 0.5, synergy (syn); 0.5 < FICI ⩽ 1, additivity (add); 1 < FICI ⩽ 2, no difference (ind). MICs beyond the maximum scale were converted to the next twofold highest concentration.
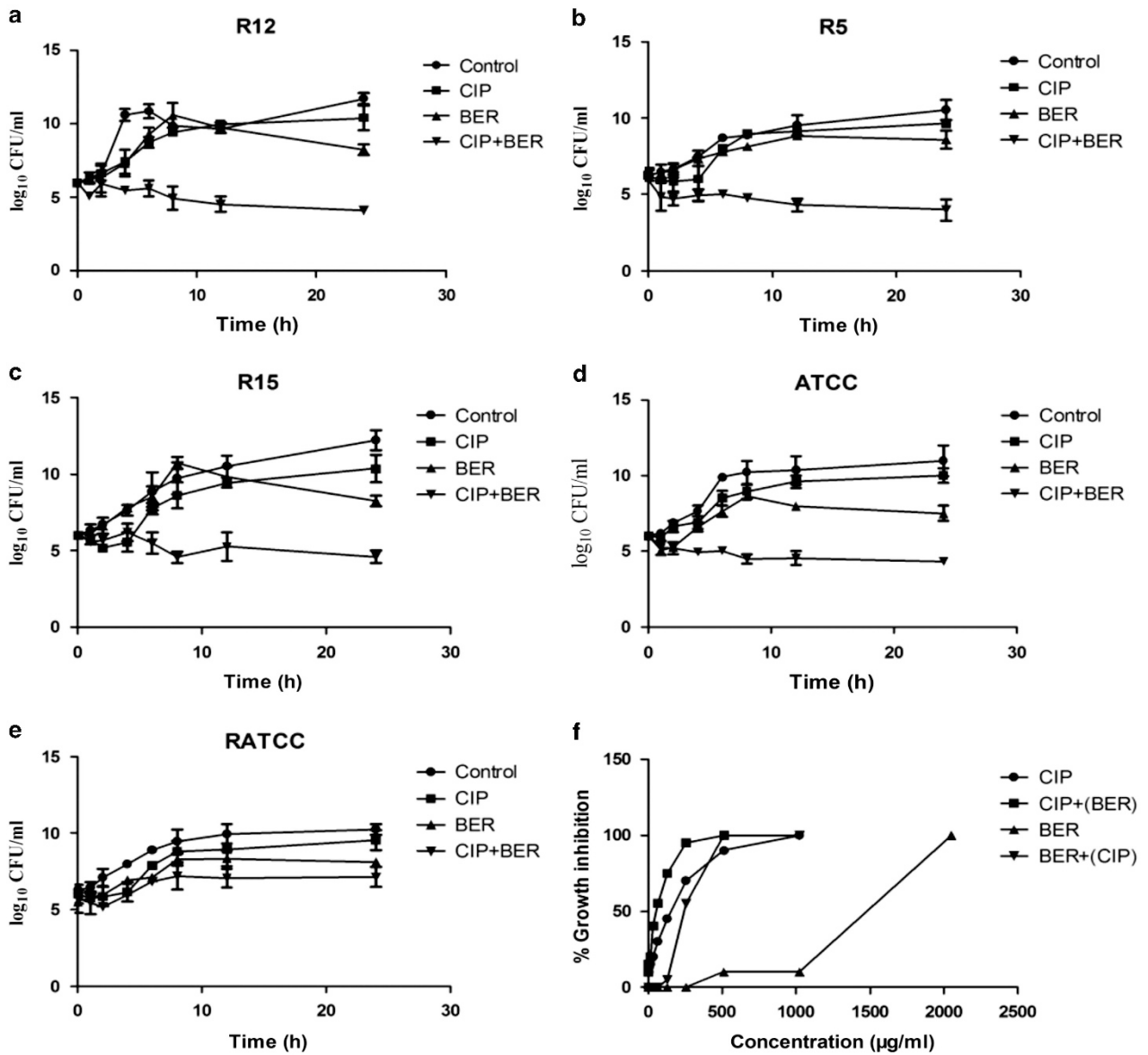
Figure 1 The time-kill curves (**a**–**e**) of *K. pneumoniae* isolates (R12, R5, R15, ATCC and RATCC); ●, growth control; ■, CIP (**a, b, c** and **e**: ciprofloxacin 32 μg ml^{-1}, **d**: ciprofloxacin 0.0625 μg ml^{-1}); ▲, BER (berberine 1024 μg ml^{-1}); ▼, CIP+BER (**a, b, c** and **e**: ciprofloxacin 32 μg ml^{-1}, **d** ciprofloxacin 0.0625 g ml^{-1} combined with berberine 1024 μg ml^{-1}). Dose–response inhibition curve of cell growth (**f**) of 22 *K. pneumoniae* isolates. ■, berberine 1024 μg ml^{-1} was combined with various concentrations of ciprofloxacin. ▲, a sub-MIC level of ciprofloxacin was combined with various concentrations of berberine.

several times higher in the stationary phase in *E. coli* and *E. amylovora* than in other phases.[31,32] The gene expression results are shown in Figure 2. All assays were inspected for single unique peaks in the melting curves; the melting temperatures were close to 89 °C. The amplification curves were linear and cycle threshold (C_T) values of nearly 18–28 indicated specific amplification. According to the concentrations of the two-drug combination in the time-kill assay, at the messenger RNA level, the analyses indicated that the expression of *acrA, acrB, tolC* and *acrR* was significantly higher than that in untreated cultures and was > 14-fold higher in four strains (R12, R5, R15 and ATCC) in the ciprofloxacin–berberine combination group (**P < 0.05). In the berberine-treated groups, the expression levels of these genes were upregulated nearly fourfold on average (*P < 0.05). However, the expression of *acrA, acrB, tolC* and *acrR* in the

ciprofloxacin-treated group was not significantly different from that in the untreated control group (*P* > 0.05). The single exception was the RATCC strain, which showed no inhibition of bacterial growth when treated with either the single or combined agents. In addition, this strain did not show inhibition of growth and did not exhibit increased transcription in the different treatment groups (*P* > 0.05).

DISCUSSION

As previously described, the development of drug resistance in *K. pneumoniae* is a serious clinical problem. Molecular biology has shown that there are several causes of drug resistance.[33–36] A combination of agents is one means of solving the problem of resistance. This study demonstrated that berberine specifically potentiates the bactericidal activity of ciprofloxacin against multidrug-
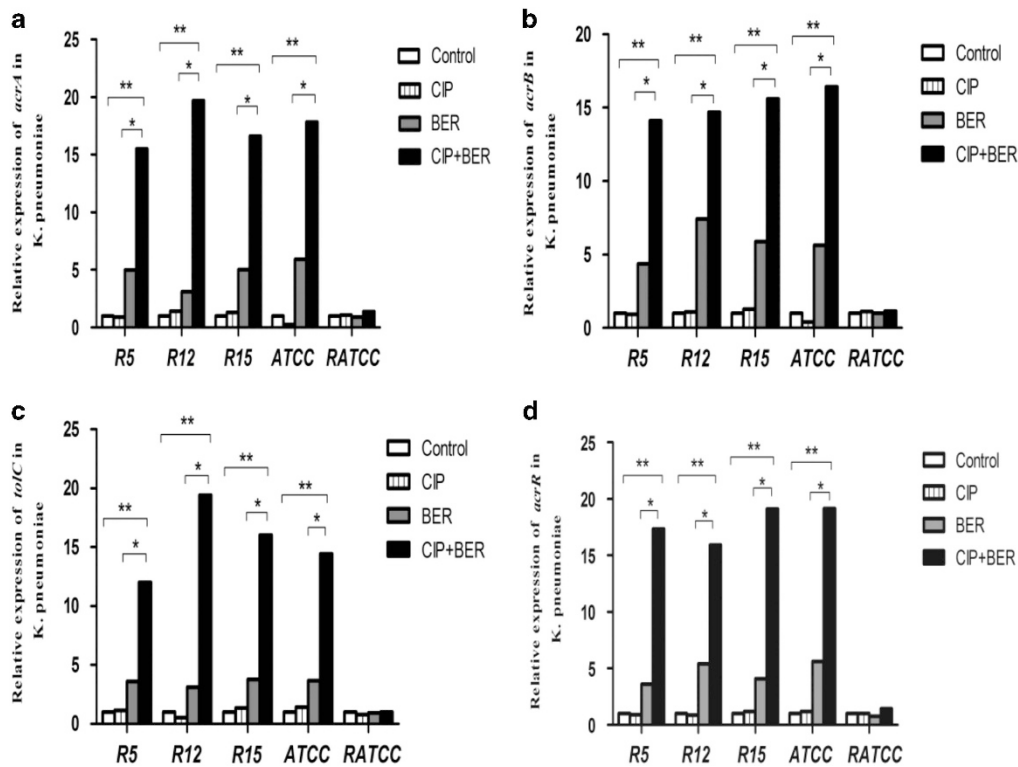
Figure 2 The relationship between the expression of *acrA, acrB, tolC* and *acrR* messenger RNA in five strains of multidrug-resistant *K. pneumoniae* (R5, R12, R15, ATCC and RATCC). (**a**) Analysis of the expression of *acrA*, (**b**) *acrB*, (**c**) *tolC* and (**d**) *acrR*. White bars indicate the control group in untreated culture; striped bars, CIP (ciprofloxacin 32 µg ml^{-1} in all cases except 0.0625 µg ml^{-1} against the ATCC strain); gray bars, BER (berberine 1024 µg ml^{-1}); black bars, CIP+BER (ciprofloxacin 32 µg ml^{-1} in all cases except 0.0625 µg ml^{-1} against the ATCC strain combined with berberine 1024 µg ml^{-1}). For the five strains not including RATCC, the expression of *acrA, acrB, tolC* and *acrR* was higher in the berberine and ciprofloxacin+berberine groups than in the controls. This effect was more pronounced in the ciprofloxacin+ berberine group (**$P < 0.05$).

resistant *K. pneumoniae*. Berberine is a plant secondary metabolite that does not produce drug resistance. In the current study, as indicated by the MIC value of ciprofloxacin in combination with berberine, the addition of berberine to ciprofloxacin reduced the required concentration of ciprofloxacin. In addition, 77.27% of the tested *K. pneumoniae* isolates showed an additive bactericidal effect.

The concentration of ciprofloxacin in the presence of berberine was decreased by 1/2 to 3/4 compared with the single agent. Synergistic effects were observed in 18.18% of isolates and the concentration of ciprofloxacin could be decreased by >3/4 through the use of the combination. Moreover, the results showed that ciprofloxacin combined with berberine resulted in bacterial growth arrest in the time-kill assay. These findings suggest that the presence of berberine enhanced bacterial susceptibility to ciprofloxacin and potentiated the inhibitory activity of ciprofloxacin. Therefore, berberine is considered an antibiotic potentiator and merits further study *in vivo*.

However, the molecular mechanism by which berberine increases the antimicrobial activity of ciprofloxacin against multidrug-resistant *K. pneumoniae* is not yet understood. It is possible that berberine increases ciprofloxacin uptake via channel formation in bacterial membranes, as observed in aminoglycosides.[37] It has long been reported that berberine is a substrate for multidrug efflux pumps.[13,38] Several efflux pumps belonging to the resistance-nodulation-division (RND) superfamily are found in multidrug-resistant Gram-negative bacteria.[39] Many types of RND family efflux systems have been characterized in *K. pneumoniae*. The *acrAB* efflux pump is homologous to the system in *E. coli*.[35,40] As reported by Karaosmanoglu *et al.*,[16] the transcription of *acrEF* is upregulated in *E. coli* after

inhibition of growth in the presence of berberine over time. In the presence of sub-MIC concentrations, compared with norfloxacin and a blank control, the transcription levels of *acrAB* and *acrR* were found to remain the same in *Erwinia amylovora* but were higher in the presence of berberine than in the blank control.[32] Accordingly, the results of the present study demonstrated that *K. pneumoniae* activity was inhibited owing to the synergistic effect of the ciprofloxacin–berberine combination, and the transcription levels of the efflux pumps *acrA*, *acrB*, *tolC* and *acrR* were upregulated. These findings suggest that regular transcription of *acrAB* may be used to identify protective mechanisms in bacteria. Some of these mechanisms may have been stimulated to expel as much berberine as possible from the cells. One of the reasons that bacterial growth is affected may be that berberine upregulates *acrAB* transcription. The inner membrane transporter *acrB* forms a complex with the periplasmic fusion protein *acrA*, which coordinates with the outer membrane channel *tolC*.[41,42] Previous studies have indicated that the inner membrane transport protein *acrB* is essential for mediating contact-dependent growth inhibition.[43] In this regard, overexpression of *acrB* might inhibit bacterial cell growth via cell-to-cell-contact-mediated mechanisms, and hence *acrB* overexpression is harmful to bacterial growth.[44] Moreover, when *acrAB* transcription is increased, the periplasm becomes overcrowded with excess expression products that can cause detrimental effects on cell fate.[45]

In summary, this study demonstrates that the combination of ciprofloxacin and berberine exhibits definite antibacterial effects that could help decrease the dose of ciprofloxacin, decrease the production of ciprofloxacin-resistant *K. pneumoniae* and reduce the incidence of

drug resistance. Berberine shows a synergistic/additive bactericidal effect that is likely to be useful for the treatment of clinical infections. Berberine may be important for the future development of antibiotics against *K. pneumoniae*, especially in multidrug-resistant and refractory infections.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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