Synergistic Activity of Fluoroquinolones Combining with Artesunate Against Multidrug-Resistant *Escherichia coli*

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Multidrug resistance (MDR) is an increasing public health concern worldwide. Artesunate (ART) has been reported to be significantly effective in enhancing the effectiveness of various β-lactam antibiotics against MDR *Escherichia coli* via inhibiting the efflux pump genes. Apart from β-lactam antibiotics, there is no report regarding the potential synergistic effects of ART combining with fluoroquinolones (FQs). In this study, we investigated whether ART can enhance the antibacterial effects of FQs *in vitro*. The antibacterial activity of ART and antibiotics against 13 animal-derived *E. coli* clinical isolates was assessed for screening MDR strains. Then the synergistic activity of FQs with ART against MDR *E. coli* isolates was evaluated. Daunorubicin (DNR) accumulation within *E. coli* and messenger RNA (mRNA) expressions of *acrA*, *acrB*, *tolC*, and *qnr* genes were investigated. The results showed that ART did not show significant antimicrobial activity. However, a dramatically synergistic activity of ART combining with FQs was obsessed with (ΣFIC) = 0.12–0.33. ART increased the DNR accumulation and reduced acrAB-tolC mRNA expression, but enhanced the mRNA expression of *qnrS* and *qnrB* within MDR *E. coli* isolates. These findings suggest that ART can potentiate FQs activity which may be associated with drug accumulation by inhibiting the expression of acrAB-tolC.

Keywords: artesunate, fluoroquinolones, MDR *E. coli*, synergistic activity

Introduction

THE EMERGENCE OF MULTIDRUG-RESISTANT BACTERIA is considered a serious public health threat by the World Health Organization. The lack of effective treatment against infections caused by such bacteria will increase the mortality rates of people and animals suffering from infectious diseases.[1] Multidrug resistance (MDR) is most commonly defined as resistance to more than three classes of antibiotics.[2]

Efflux pumps (EPs) are essential constituents of all bacterial plasma membranes, which recognize and extrude antibiotics to the environment before reaching their intended targets.[3] Overexpression of EPs has been documented in association with resistance to several antibiotic classes, including the fluoroquinolones (FQs).[4] EP-based resistance in bacteria was first described for the resistance of *Escherichia coli* to tetracyclines via overexpression of Tet proteins.[5] The overexpression of EPs can influence genes encoding the target sites of different antibiotics. The alarming increase in MDR bacteria highlights the urgent need for devising new strategies to combat bacterial infection.

Artemisinin is an active ingredient containing a sesquiterpene lactone, isolated from the traditional Chinese herb *Artemisia annua* L. (sweet wormwood). Artemisinin and its derivatives, such as artesunate (ART), dihydroartemisinin, and artemether, have been widely used against malaria. Beyond remarkable antimalarial action, artemisinin and ART have also been proved to protect sepsis-model mice from challenge with a heat-killed *E. coli*.[6,7] In recent years, several studies found that ART had no direct antibacterial activity, but encouragingly, ART could enhance the effectiveness of various β-lactam antibiotics against MDR *E. coli* and methicillin-resistant *Staphylococcus aureus*.[8,9]

FQs are broad spectrum antibiotics by inhibiting DNA gyrase and topoisomerase IV.[10] However, extensive usage of FQs has resulted in bacterial resistance, and now, two main mechanisms of FQs resistance have been reported: one is the alterations in the targets of FQs, and the other is due to decreased accumulation inside the bacteria via impermeability of the membrane and/or an overexpression of EP systems.[11] In addition, a plasmid-mediated quinolone resistance (PMQR) mechanism, namely the production of Qnr

protein capable of protecting DNA gyrase from FQs, has been proposed.[12] *qnr* genes cause only a modest decrease in quinolones susceptibility, however, they can complement other mechanisms of chromosomal resistance to reach clinical resistance level and facilitate the selection of higher level resistance, raising a threat to the treatment of infections by microorganisms that host these mechanisms.[13] Nowadays, apart from the β-lactam antibiotics, there is no report about whether ART has a synergistic effect with FQs against clinical animal-derived *E. coli* strains. If so, combining FQs with ART will be hopeful to expand the antimicrobial spectrum to reduce the emergence of resistant variants and to minimize the use of antibiotic concentration.

Under this circumstance, it is meaningful to investigate the possible synergistic effects between ART and FQs against MDR bacteria. Interestingly, we found the synergistic effects between them and then the accumulation of antibiotics in *E. coli* and the expression of *acrAB-tolC* and *qnr* genes were also determined.

Materials and Methods

Bacterial strains

Clinical isolates of *E. coli* strains used in this study were generous gifts from Dr. Yanhong Wang, who has detected the *qnr* genes, School of Veterinary Medicine, Yangzhou University, China. These isolates were collected from internal organs (the heart, liver, spleen, and intestine) of four chicken, six geese, one duck, and two pigs in 2018. *Escherichia coli* ATCC 25922 was kept in our laboratory.

Chemicals

Antibiotics were purchased from Shanghai Jizhi Biochemical Technology Co. Ltd. (Shanghai, China) and prepared as fresh stock solutions in sterile distilled water or medium on the day of testing. Daunorubicin (DNR) at the highest available purity (98%) was purchased from Med-ChemExpress (Monmouth Junction, NJ). Injectable ART dissolved in 5% $NaHCO_3$ was purchased from Guilin Pharmaceutical Co. Ltd. (Guangxi, China). Mueller–Hinton broth (MHB) and Luria–Bertani broth media were purchased from Hopebio-Technology Co. Ltd. (QingDao, China). RNA isolater Total RNA Extraction Reagent, Hi-Script II QRT SuperMix, and ChamQTM SYBR® qPCR Master Mix were purchased from Vazyme Biotech Co. Ltd. (Nanjing, China).

Determination of minimum inhibitory concentration

Minimum inhibitory concentrations (MICs) of antimicrobial agents and ART were determined by reference serial twofold broth microdilution[14] against 13 clinical *E. coli* clinical isolates. Eleven antibiotics were tested, including ampicillin, cefotaxime, cefquinome, doxycycline, kanamycin, amikacin, streptomycin, ciprofloxacin (CIP), enrofloxacin (ENR), neomycin, and colistin. Bacteria in the exponential phase of growth which were inoculated into 96-well plates were diluted in cation-adjusted MHB to reach the concentration of 5.0×10^5 CFU/mL. Results were read after 16–20 hours incubation at 37°C. Data were obtained in at least

two independent experiments, and *Escherichia coli* ATCC 25922 was used for quality control in antimicrobial susceptibility testing.[15]

Checkerboard synergy testing

Synergy testing of ART and two FQs was performed in 96-well microtiter plates by checkerboard method, which was performed in triplicate with 96-well microtiter plate using an 8-by-5 well configuration.[16] Positive growth controls (to assess the presence of turbidity) were performed in wells not containing drugs. In addition, negative growth controls were performed in a separate microtiter tray. Combination action of ART (64–1,024 μg/mL) with CIP or ENR was tested, respectively. Concentrations tested ranged from $0.0625 \times MIC$ to $2 \times MIC$ of each antibiotic. Each well was inoculated with 100 μL of a suspension of 5×10^5 CFU/mL of the test strain in a final volume of 200 μL. The checkerboard plates were then incubated for 16–20 hours at 37°C. Interactions between ART and antibiotics were determined by calculating the fractional inhibitory concentration (ΣFIC) index, which is the MIC of drug in combination divided by the MIC of drug acting alone. The formula is as follows:

$$\sum FIC = \frac{MIC\ A + B}{MIC\ A} + \frac{MIC\ B + A}{MIC\ B}$$

where MIC A + B is the MIC of ART in combination with antibiotic and MIC B + A is the MIC of antibiotic in combination with ART. Results were interpreted as follows: ΣFIC ≤0.5, synergism; 0.5 < ΣFIC <1, additive effect; 1 < ΣFIC <4, no interaction; and ΣFIC >4.0, antagonism. Data were obtained from at least two independent experiments.[17]

DNR accumulation within Escherichia coli 0501G2-H2

Given that DNR exhibited self-fluorescence, the intensity of fluorescence was used to predict the drug accumulation within *E. coli* through laser confocal scanning microscope (Leica TCS SP8, Mannheim, Germany).[18] *Escherichia coli* 0501G2-H2 at the exponential phase of growth was exposed to different concentrations of ART (128, 256, and 512 μg/mL) and DNR (40 μg/mL) at 37°C in a heated, shaking environmental chamber for 1, 3, and 6 hours. Then, the bacteria cultured in different times was centrifuged, respectively, at 3,000 rpm for 5 minutes to harvest the bacterial pellets, which

TABLE 1. PRIMERS USED IN THIS STUDY

Primer	Sequence (5′–3′)	Reference
acrA-F	CTCAAGTTAGCGGGATTA	This study
acrA-R	ACCTTTCGCACTGTCGTA	
acrB-F	CCCTGAATCTGCCCCATC	This study
acrB-R	GACCTTTGCCGTCCTTGC	
tolC-F	AAGCCGAAAAACGCAACCT	Swick *et al.*[19]
tolC-R	CAGAGTCGGTAAGTGACCATC	
qnrB-F	ATGGCTCTGGCACTCGTT	This study
qnrB-R	TGCACCCTTTCTGGCTTT	
qnrS-F	CTTGCGTGATACGACATT	This study
qnrS-R	TAGGAAAGATTACATCCAGAA	
gapA-F	CGTTAAAGGCGCTAACTTCG	Zhou *et al.*[20]
gapA-R	ACGGTGGTCATCAGACCTTC	

F, forward primer; R, reverse primer.

TABLE 2. MINIMUM INHIBITORY CONCENTRATIONS (µg/mL) OF DIFFERENT ANTIBIOTICS FOR 13 CLINICAL *ESCHERICHIA COLI* ISOLATES AND *ESCHERICHIA COLI* ATCC 25922

Antibiotics Bacteria	Source	MIC (µg/mL)											
		AMP	CTX	CEF	AMK	STR	KAN	DOX	NEO	CIP	ENR	COL	ART
0322C1-1	Chicken, liver	256	<2	<1	2	128	0.25	16	1	<0.125	0.125	1	>5,120
0313G1-1	Goose, liver	>2,048	>2,048	1,024	4	256	512	32	64	8	4	1	>5,120
0612D1-2	Duck, liver	>2,048	128	8	2	256	8	32	0.5	2	0.125	2	>5,120
0307C2-L	Chicken, liver	>2,048	2,048	1,024	2	256	256	32	32	16	8	1	>5,120
0619P1-I	Porcine, intestine	>2,048	2,048	1,024	1,024	256	1,024	64	256	64	16	2	>5,120
0220G1-1	Goose, liver	2,048	2,048	128	4	512	1,024	32	512	16	4	1	>5,120
0313P1-S	Porcine, spleen	2,048	1,024	64	2	64	1	16	1	0.5	0.25	2	>5,120
0602G1-H5	Goose, heart	128	0.5	0.125	2	128	0.125	32	0.5	0.125	0.125	2	>5,120
0501G1-H1	Goose, heart	>1,024	>1,024	1,024	2	256	1,024	16	64	32	16	1	>5,120
0501G2-H2	Goose, heart	>2,048	>2,048	1,024	4	512	1,024	2	512	32	8		>5,120
0503C4-L2	Chicken, liver	>2,048	>2,048	2,048	16	256	1,024	32	32	32	8	0.5	>5,120
0627C2-2	Chicken, liver	>2,048	>2,048	32	2	128	128	16	8	4	2	1	>5,120
0310G1-3	Goose, liver	>2,048	>2,048	1,024	2	128	64	32	32	64	16	0.5	>5,120
ATCC 25922		4	0.125	0.0625	2	4	0.5	0.5	1	0.0078	0.0039	0.5	>5,120

AMP: Susceptible, MIC ≤8 µg/mL and resistant MIC >32 µg/mL; CTX: Susceptible, MIC ≤1 µg/mL and resistant MIC >4 µg/mL; AMK: Susceptible, MIC ≤16 µg/mL and resistant MIC >64 µg/mL; KAN: Susceptible, MIC ≤16 µg/mL and resistant MIC >64 µg/mL; DOX: Susceptible, MIC ≤4 µg/mL and resistant MIC >16 µg/mL; CIP: Susceptible, MIC ≤1 µg/mL and resistant MIC >4 µg/mL; breakpoints of CEF, STR, NEO and ENR are not defined (Clinical and Laboratory Standards Institute, 2017).
AMK, amikacin; AMP, ampicillin; ART, artesunate; CTX, cefotaxime; CEF, cefquinome; CIP, ciprofloxacin; COL, colistin; DOX, doxycycline; ENR, enrofloxacin; KAN, kanamycin; MICs, minimum inhibitory concentrations; NEO, neomycin; STR, streptomycin.

TABLE 3. RESULTS OF CHECKERBOARD ASSAYS OF CIPROFLOXACIN IN COMBINATION WITH ARTESUNATE
FOR FIVE MULTIDRUG RESISTANCE ISOLATES

Strains	Source	CIP MICs (µg/mL) at different ART concentrations (µg/mL)						ΣFIC
		ART concentrations (µg/mL)						
		0	64	128	256	512	1,024	
0619P1-I	Porcine, intestine	64	64	32	8	1	1	<0.12
0220G1-1	Goose, liver	16	16	16	8	1	0.5	<0.16
0501G2-H2	Goose, heart	32	32	32	16	4	2	<0.23
0503C4-L2	Chicken, liver	32	32	32	16	8	4	<0.33
0627C2-2	Chicken, liver	4	4	2	2	0.5	0.125	<0.23

was washed with phosphate-buffered saline (pH 7.2) three times to remove DNR outside of the bacteria. After washing, the bacterial pellet was resuspended and then fixed on glass slides to observe the fluorescence of each group.

AcrA, acrB, tolC, and qnr mRNA expression

Total RNA was extracted using TRIzol reagent (Vazyme, Nanjing, China) according to manufacturer's recommendations. RNA quality and quantity were assessed by agarose gel electrophoresis and NanoDrop Spectrophotometer (Thermo Scientific, Wilmington, DE), respectively. HiScript II QRT SuperMix (Vazyme) was used to generate complementary DNA according to the protocol. Quantitative real-time PCRs (qRT-PCRs) were performed following the two-step protocol of the ChamQTM SYBR qPCR Master Mix (Vazyme) in a CFX96 Real-time system (BIO-RAD) according to the manufacturer's instructions. A no-template sample was included as a negative control in each run for each gene. gapA was chosen as an internal control to normalize the threshold cycle values of other products. Primer sequences for amplification of acrA, acrB, tolC, qnrS, qnrB, and gapA are listed (Table 1), and melt curve analysis ensured that only a single PCR product was amplified. The ΔΔCT method was used to obtain relative fold-change of target gene expression normalized by gapA compared with control samples. Each assay included three biological replicates and was performed twice.

Statistical analyses

The means of three replications and standard error (±SE) were calculated for all the results obtained, and the data were statistically analyzed by one-way of variance (ANO-VA). The $p < 0.05$ was considered to be statistically significant. All the analyses were conducted using the Prism 5.0 software.

Results

Antibiotic susceptibility testing

MICs of ART and 11 antibiotics against 13 clinical E. coli isolates are shown in Table 2. MIC of ART is >5,120 µg/mL for all tested E. coli isolates, so ART has no intrinsic antimicrobial activity against these clinical E. coli isolates as well as ATCC 25922 (Supplementary Fig. S1). Among the tested isolates, the MICs of CIP against Escherichia coli 0322C1-1, Escherichia coli 0612D1-2, Escherichia coli 1313P1-S, and Escherichia coli 0602G1-5 are less than the resistance breakpoint (i.e., 4 µg/mL). The other nine clinical isolates show different level of resistance to CIP and ENR. Among them, Escherichia coli 0619P1-I isolate showed the highest level resistance to CIP and ENR, and their MICs were 64 and 16 µg/mL, respectively.

Synergistic activity of ART in combination with FQs in checkerboard testing

To elucidate the interaction of ART combining with FQs, 5 isolates that show different level of resistance to CIP and ENR were selected from 13 tested E. coli isolates to investigate the synergistic effects with ART. A dramatically synergistic activity of ART with FQs against five E. coli clinical isolates could be observed in Tables 3 and 4, with FIC indexes <0.5. Therefore, it can be considered that ART was able to reduce CIP MICs to values equal or lower than the resistance breakpoint (i.e., 4 µg/mL), even lower than susceptibility breakpoint (i.e., 1 µg/mL) at the minimum effective concentration of ART (512 µg/mL) (Table 3).

TABLE 4. RESULTS OF CHECKERBOARD ASSAYS OF ENROFLOXACIN IN COMBINATION WITH ARTESUNATE
FOR FIVE MULTIDRUG RESISTANCE ISOLATES

Strains	Source	ENR MICs (µg/mL) at different ART concentrations (µg/mL)						ΣFIC
		ART concentrations (µg/mL)						
		0	64	128	256	512	1,024	
0619P1-I	Porcine, intestine	16	16	16	8	1	0.25	<0.16
0220G1-1	Goose, liver	4	4	4	2	0.5	0.25	<0.23
0501G2-H2	Goose, heart	8	8	8	4	1	1	<0.23
0503C4-L2	Chicken, liver	8	8	8	8	2	1	<0.33
0627C2-2	Chicken, liver	2	2	2	0.5	0.25	0.25	<0.23

Besides CIP, the MICs of ENR against the clinical isolates were also markedly reduced when combining with ART (Table 4).

ART increases accumulation of DNR within Escherichia coli 0501G2-H2

DNR (40 μg/mL) did not affect the growth of *E. coli* from the confocal microscope assay, it can be easily found that the fluorescence could be observed after ART treating for 1 hour in 512 μg/mL ART-treated group, while no fluorescence in control group and two lower concentration of ART groups. As further evidence, ART treatment for 3 and 6hours strikingly increased DNR accumulation within *Escherichia coli* 0501G2-H2 in a dose-dependent manner (Fig. 1).

ART reduces expressions of acrAB-tolC and increases expressions of qnr genes

AcrAB-tolC EP of resistance-nodulation-division (RND) family plays a dominant role in the MDR of gram-negative bacteria by reducing the accumulation of drugs. Here, the expression of *acrAB-tolC* genes were detected by qRT-PCR

and the results showed that *acrA*, *acrB*, and *tolC* mRNA expression were significantly inhibited by ART at 512 μg/mL, and the regulation of *acrB* is the most prominent within two clinical *E. coli* strains that could reduce by at least half the relative density (Fig. 2a, b). These results suggest that the inhibition of drug export could increase uptake in bacteria (reflected by DNR accumulation). In addition, *qnr* genes are responsible for low-level resistance to FQs; however, some studies dedicated that high-level resistance pattern could be linked to the presence of efflux systems.[21] In our study, ART of three concentrations upregulated the expression of *qnrS* within *Escherichia coli* 0501G2-H2, and the lower concentration, the higher expression (Fig. 2a). In the case of *Escherichia coli* 0619P1-I, only ART of 128 μg/mL increased the expression of *qnrB* (Fig. 2b). Although ART promotes the expression of qnr, it does not increase the MIC of FQs. So *qnr* gene may be not a decisive factor of ART action.

Discussion

MDR is one of the biggest public health challenges now, especially when less number of antimicrobial drugs being
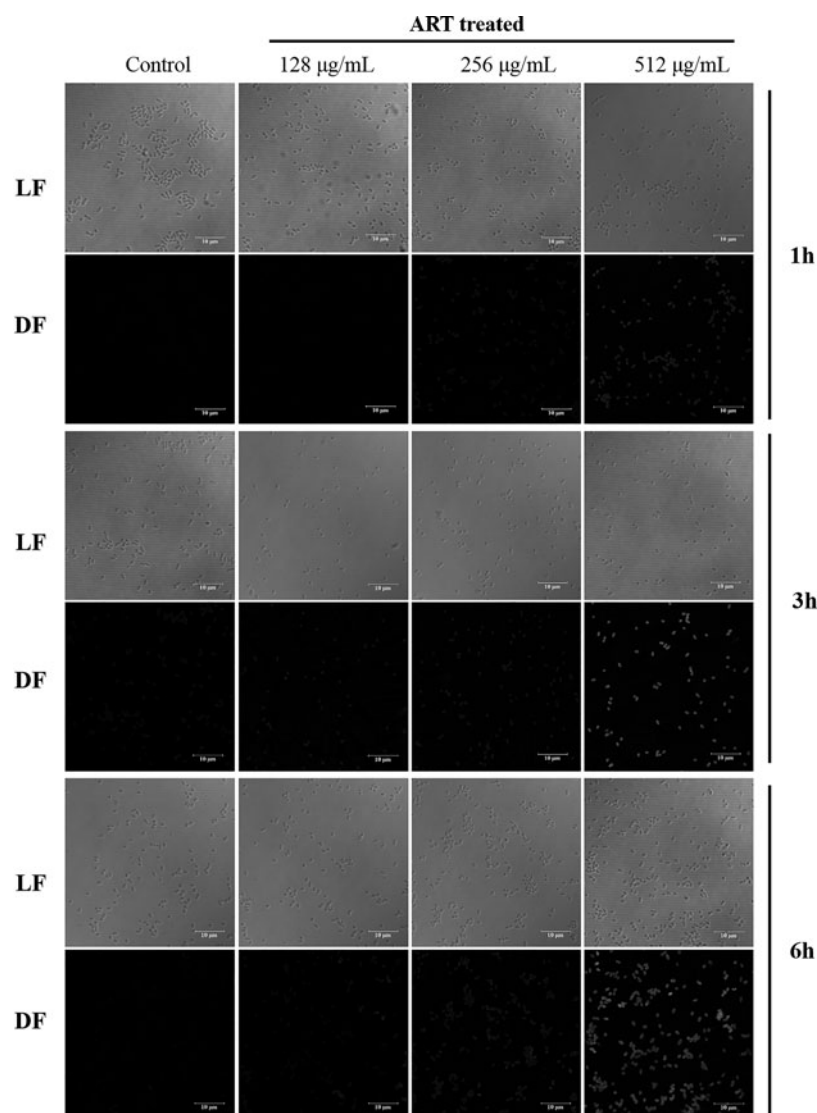

FIG. 1. DNR accumulation within clinical *Escherichia coli* 0501G2-H2. *Escherichia coli* 0501G2-H2 was treated with ART (512 μg/mL) and DNR (40 μg/mL) in the dark at 37°C for 1, 3, and 6 hours. Control group was treated with 5% NaHCO₃ and an equal dose of DNR. Quantitative determination of DNR accumulation was determined using laser confocal scanning microscope at an emission wavelength of 488 nm and an excitation wavelength of 561 nm. ART, artesunate; DF, dark field; DNR, daunorubicin; LF, light field.
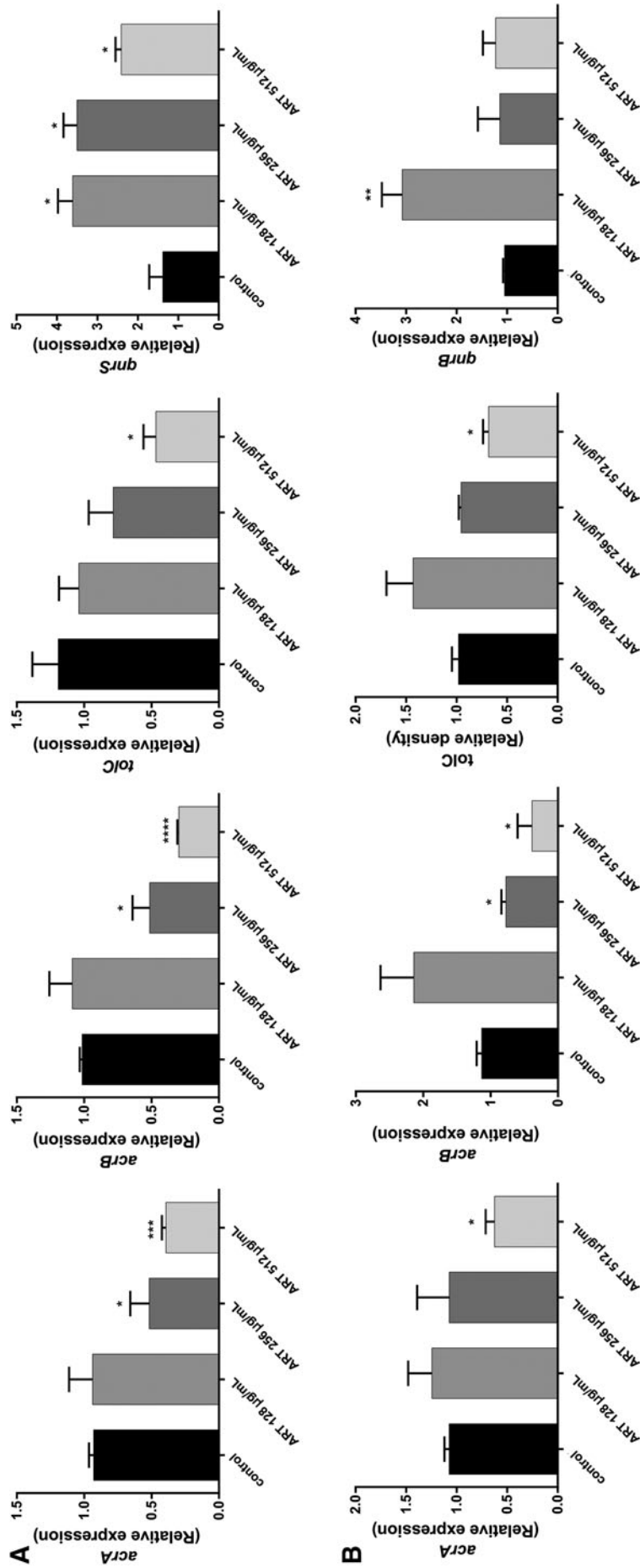
FIG. 2. *AcrAB-tolC* and *qnrS* mRNA expression within clinical *Escherichia coli* treated with ART. *Escherichia coli* 0501G2-H2 was pretreated with different concentrations of ART (128, 256, and 512 μg/mL) and the control group was pretreated with 5% NaHCO₃ in 10 mL of MHB and cultivated at 37°C and 200 rpm for 6 hours and then harvested by centrifugation at 1,200 rpm for 5 minutes. Total RNA was extracted and quantitative real-time PCR was performed. The same treatment to *Escherichia coli* 0619P1-I. **(A)** *AcrAB-tolC* and *qnrS* mRNA expression within clinical *Escherichia coli* 0501G2-H2. **(B)** *AcrAB-tolC* and *qnrB* mRNA expression within clinical *Escherichia coli* 0619P1-I. ANOVA was used to examine the differences among different treatments. *$p < 0.05$ versus control; ***$p < 0.001$ versus control, ****$p < 0.0001$ versus control. MHB, Mueller–Hinton broth; mRNA, messenger RNA.

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discovered in recent years and synthetic approaches were unable to replace natural products.[22,23] So new remedies should be devised for MDR bacterial infection that could limit, redirect, and perhaps even reverse the process of resistance evolution.[24] Encouragingly, in this study, we provided the first *in vitro* demonstration that ART can potentiate FQs activity against a collection of MDR/FQsR *E. coli* clinical isolates. Reduced antibiotics accumulation is one of the most important determinants of MDR in gram-negative bacteria.[25] In our study, ART was found to increase the DNR accumulation in a dose-dependent manner (Fig. 1), suggesting that the enhancement of ART was related to drug accumulation. This maybe mainly caused by the dampening action of ART on the expression of *acrA*, *acrB*, and/or *tolC* genes (Fig. 2).

Protein AcrA, AcrB, and TolC are major EPs of the RND family, which are expressed by gram-negative bacteria and are evidenced to relate to clinically remarkable MDR. Although there are other Acr EPs in *E. coli*,[26] only AcrAB-TolC has been found to be in overproduction by clinical isolates,[27,28] and it has been demonstrated that *acrAB-tolC* overexpression directly leads to the severe resistance to FQs of *Salmonella* clinical isolates.[29] Among multidrug *acrAB-tolC* efflux system, *acrB* was considered as the important component because the inactivation of *acrB* in high-level FQs-resistant isolates resulted in a 32-fold reduction in the MIC value.[30] Actually, another study demonstrated that ART significantly increased the antibacterial effect of β-lactam antibiotics against an *E. coli* clinical strain. In contrast, ART lost its enhancement against *Escherichia coli* AG100A lacking the gene encoding AcrB. However, after the transformation of pET28a-*acrB* into *Escherichia coli* AG100A, ART regained the enhancement.[9] Therefore, these results indicated that *acrB* may be the main target of ART action. Indeed, in our study, *acrB* mRNA expression was significantly downregulated in two ART (512 μg/mL)-treated clinical isolates (0501G2-H2, 0619P1-I), meanwhile, the expression of *acrA* and *tolC* was also in different levels of reduction.

Except EPs, several other mechanisms may also be responsible for FQs resistance, including one of the more recently reported PMQR mechanisms.[31,32] The first founded PMQR gene reducing susceptibility to CIP in gram-negatives was *qnrA*.[33] Subsequently, several classes of *qnr* genes (*qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qnrVC*) were identified that could reduce susceptibility to FQs.[34] Rezazadeh *et al.* found in their study that most *qnr*-positive isolates showed high-level resistance, however, PMQR genes are responsible for low-level resistance to FQs, it can be hypothesized that high-level resistance pattern could be linked to the presence of other mechanisms such as mutations in quinolone resistance-determining regions (QRDRs), and porin or efflux systems.[35] In our study, ART inhibits the expression of *acrAB-tolC* but not *qnr* genes. Although ART promotes the expression of *qnr*, it does not increase the MIC of FQs. Besides, we detected QRDR in gyrA and parC of the clinical *E. coli* isolates, and found that there were mutations in the tested isolates, however, no different mutations were observed in these *E. coli* isolates treated or untreated by ART (data not shown). So *qnr* genes and QRDR may not be a decisive factor of ART action in our study.

Taken together, our results suggested that ART can enhance the antibacterial effect of FQs against clinical MDR *E. coli* isolates at least, in part, by increasing antibiotic accumulation via inhibition of the multidrug EP system *acrAB-tolC* rather than *qnr* genes. These data indicated that ART may be used as a potential inhibitor of *acrAB-tolC* systems for MDR bacterial infections and further research should be considered for investigating the synergistic effects *in vivo*.

Authors' Contributions

J.L. and W.T. designed the study. S.W. and S.Y. performed the MDR clinical *Escherichia coli* screens, checkerboard synergy testing and lacer confocal scanning microscope. W.T. helped to perform the quantitative realtime PCR assays. S.W., Y.Y., and J.L. wrote the article. M.L. reviewed the article.

Disclosure Statement

The authors declare that no competing financial interests exist.

Funding Information

Supplementary Material

Supplementary Figure S1

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