Infection and Drug Resistance

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ORIGINAL RESEARCH

Palmatine Is a Plasmid-Mediated Quinolone Resistance (PMQR) Inhibitor That Restores the Activity of Ciprofloxacin Against QnrS and AAC (6′)-Ib-cr-Producing *Escherichia coli*

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Peng Wang[1]
Longfei Hu[1]
Zhihui Hao[2]

[1]Agricultural Bio-Pharmaceutical Laboratory, Qingdao Agricultural University, Qingdao 266109, People's Republic of China; [2]National Centre for Veterinary Drug Safety Evaluation, College of Veterinary Medicine, China Agricultural University, Beijing 100089, People's Republic of China

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Purpose: The emergence of plasmid-mediated quinolone resistance (PMQR) is a global challenge in the treatment of clinical disease in both humans and animals and is exacerbated by the presence of different PMQR genes existing in the same bacterial strain. Here, we discovered that a natural isoquinoline alkaloid palmatine extracted from traditional Chinese medicinal plants effectively inhibited the activity of PMQR proteins QnrS and AAC(6′)-Ib-cr.
Methods: In total 120 clinical ciprofloxacin-resistant *Escherichia coli* (*E. coli*) were screened for the presence of *qnrS* and *aac(6′)-Ib-cr* by PCR. Recombinant *E. coli* that produced QnrS or AAC(6′)-Ib-cr proteins were constructed and the correct expression was confirmed by MALDI/TOF MS analysis and SDS-PAGE. A minimal inhibitory concentration (MICs) assay, growth curve assay and time-kill assay were conducted to evaluate the in vitro antibacterial activity of palmatine and the combination of palmatine and ciprofloxacin. Cytotoxicity assays and mouse thigh infection model were used to evaluate the in vivo synergies. Molecular docking, gyrase supercoiling assay and acetylation assay were used to clarify the mechanism of action.
Results: Palmatine effectively restored the activity of ciprofloxacin against *qnrS* and *aac(6′)-Ib-cr*-positive *E. coli* strains in a synergistic manner in vitro. In addition, the combined therapy significantly reduced the bacterial burden in a mouse thigh infection model. Molecular docking revealed that palmatine bound at the functional large loop B of QnrS and Trp102Arg and Asp179Tyr in the binding pocket of AAC(6′)-Ib-cr. Furthermore, interaction analysis confirmed that palmatine reduced the gyrase protective effect of QnrS and the acetylation effect of AAC(6′)-Ib-cr.
Conclusion: Our findings suggest that palmatine is a potential efficacious compound to restore PMQR-mediated ciprofloxacin resistance and warrants further preclinical evaluations.
Keywords: plasmid-mediated quinolone resistance, PMQR, palmatine, QnrS, AAC(6′)-Ib-cr, inhibitor

Introduction

Quinolones are synthetic antibacterials that are extensively used clinically worldwide due to their satisfactory safety profile, oral bioavailability and broad antibacterial spectra.[1–5] However, the overuse of antibiotics in both humans and animals has created a serious quinolone-resistance problem and clinical applications of quinolones have become limited.[1,6] Quinolones inhibit the activity of bacterial type II DNA topoisomerases, DNA gyrase and topoisomerase IV that participate

Correspondence: Zhihui Hao
National Centre for Veterinary Drug Safety Evaluation, College of Veterinary Medicine, China Agricultural University, Beijing 100089, People's Republic of China
Email abplab@126.com

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in chromosomal supercoiling required for DNA synthesis, transcription and cell division.[4,7–12] High-level quinolone resistance was first observed as a result of mutations in chromosomal genes encoding DNA gyrase and topoisomerase IV, the targets of quinolone action.[7,8,13–17] These mutations lead to amino acid substitutions that structurally changed the target proteins altering quinolone binding affinity.[1,7,8] In addition, changes in efflux activity or permeability which result in decreased cytoplasm quinolone concentrations can also confer quinolone resistance.[15,18-20]

Unlike these target-specific resistance mechanisms, a novel form of quinolone resistance developed via the uptake of specific plasmids. Plasmid-mediated quinolone resistance (PMQR) is now a global problem in clinics.[21–28] PMQR determinants generally generate only low-level resistance, but nonetheless can complement the mechanisms of chromosomal resistance to reach clinical resistance levels. This facilitates the selection of additional quinolone-resistant mutants during the treatment of infections by microorganisms that host these plasmids.[22,26,29] Furthermore, the potential threats of PMQR, including the diversity of transmission (horizontally and vertically), plasticity of integrating into the chromosome, as well as co-localization with resistance genes for β-lactams and aminoglycosides, becomes a serious problem in managing quinolone-resistant pathogens.[1,25,27,29–33]

Three types of classic PMQR mechanisms have been identified in plasmids and these encode (A) the DNA type II topoisomerase protection protein Qnr,[4,34] (B) the aminoglycoside acetyltransferase AAC(6′)-Ib-cr[35,36] and (C) transferable quinolone efflux pumps such as OqxAB and QepA.[37–39] Recently, a novel ciprofloxacin-modifying enzyme gene *crpP* was detected in pUM505 plasmids, suggesting an increasing trend of PMQR mechanism.[40,41] The actions of Qnr and AAC(6′)-Ib-cr have been systematically investigated.[36,42] Qnr proteins, as pentapeptide repeat protein (PRP) family members, can bind to and protect bacterial DNA gyrase and topoisomerase IV from quinolone inhibition.[43–45] Structural predictions indicated that Qnr formed highly asymmetric rod-like dimers with the threading of pentapeptides into the β-helical fold interrupted by two varying size loops A and B that extend out from the surface.[26,42] The absence of the smaller loop A reduces the protective effect of quinolones while the absence of the larger loop B or both loops directly leads to functional inactivation.[4,46] In addition, the substitution or deletion of key individual amino acid residues in the larger loop B compromises protective activity, suggesting a pivotal role for the quinolone resistance of Qnr.[26,42,43] Currently, various types of *qnr* variant including *qnrA, qnrB, qnrC, qnrD, qnrS*, and *qnrVC* have been identified on the basis of sequence similarity.[22] AAC(6′)-Ib-cr, a bifunctional *-cr* variant (Trp102Arg and Asp179Tyr) of common aminoglycoside acetyltransferase AAC(6′)-Ib, is able to acetylate the fluoroquinolones ciprofloxacin and norfloxacin at the secondary amino nitrogen N4 on the piperazine ring.[26,36] Interestingly, the Qnr variant encoded by *qnrS* as well as *aac(6')-Ib-cr* positive plasmids is frequently detected in *Enterobacteriaceae* isolates from different countries including the United States, Japan, China and India.[47–49] The widespread dissemination of combination of these two PMQR genes is becoming a serious threat to global public health. Therefore, identification of potent inhibitors of QnrS and AAC(6′)-Ib-cr is urgently needed.

Palmatine (Figure 1A) is a naturally occurring isoquinoline alkaloid extracted from traditional Chinese medicinal plants including *Coptis chinensis* (goldthread), *Fibraurea recisa, Enantia chlorantha* (whitewood) and *Phellodendron amurense* (Amur cork tree).[50] Diverse pharmacological effects of palmatine have been reported and include neuroprotective, anti–inflammatory, antibacterial, antiviral, antioxidative and anticancer effects.[50,51] In the present study, we demonstrated that palmatine is a potent inhibitor of QnrS and AAC(6′)-Ib-cr activities and we developed molecular docking models to simulate the potential sites of action. In addition, palmatine-mediated inhibition of QnrS and AAC(6′)-Ib-cr activity exerted a synergistic effect with ciprofloxacin against quinolone-resistant *Escherichia coli* both in vitro and in vivo.

Materials and Methods

Bacterial Strains, Cells and Chemicals

A ciprofloxacin-resistant *E. coli* isolate obtained from a chicken cloacae sample (in total 110) was collected from a chicken farm in Pingdu, Qingdao, China. In total, 10 ciprofloxacin-resistant *E. coli* isolates from human urine and pig feces (5 each) were gifts of Professor Zhangqi Shen (China Agricultural University, Beijing, China). Luria-Bertani (LB) broth and agar were used for bacterial cultivation.

Murine RAW 264.7 cells were purchased from National Infrastructure Cell Line Resource (Shanghai, China). Ciprofloxacin (purity ≥98%) and palmatine (purity ≥98%) were purchased from the Solarbio Science (Beijing, China) and were dissolved in sterile water to make stock solutions at the time of use. In total 120 ciprofloxacin-resistant *E. coli* were screened for the presence of *qnrS* and *aac(6')-Ib-cr* by

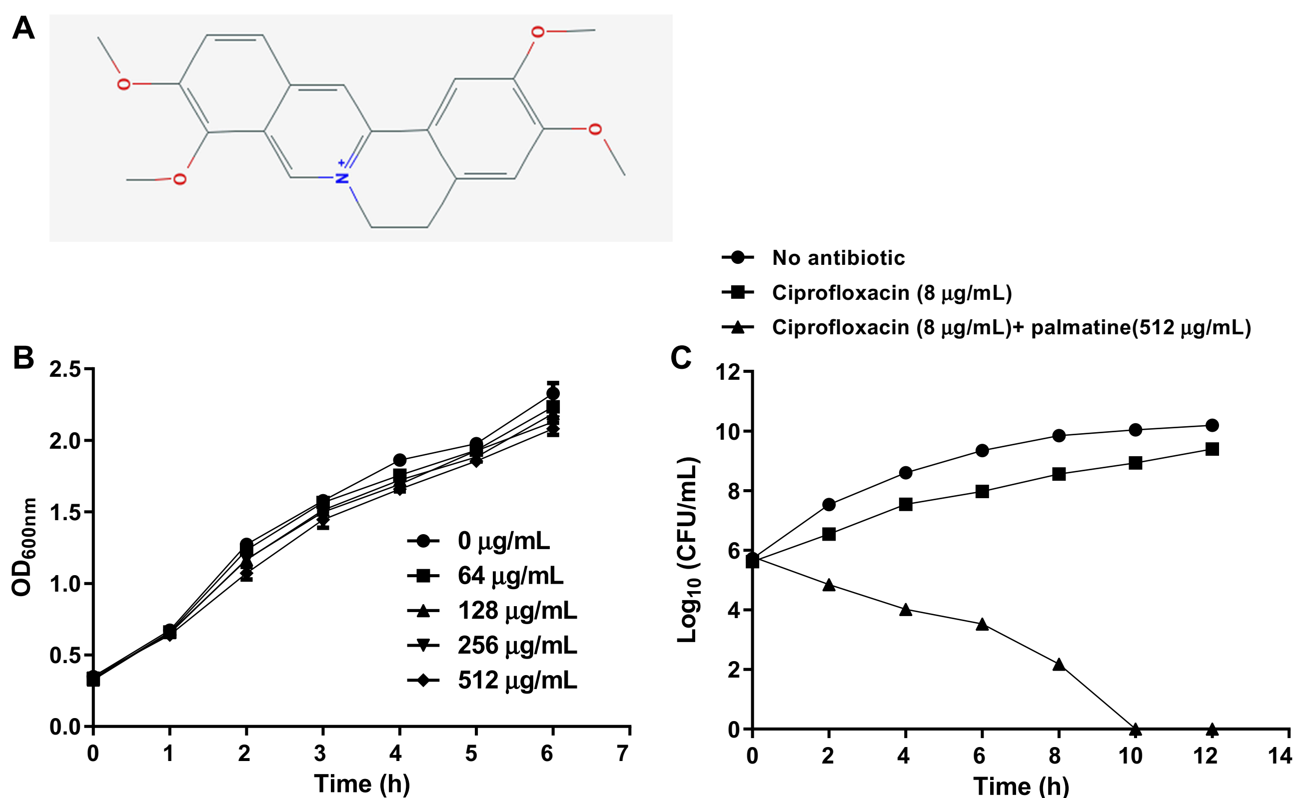

Figure 1 Effect of palmatine on ciprofloxacin-resistant *E. coli* producing QnrS and AAC(6′)-Ib-cr proteins in vitro. (**A**) Chemical structure of palmatine (**B**) Growth curves of *E. coli* 289 in the presence of the indicated concentrations of palmatine. Absorbance at 600 nm (OD_{600nm}) was measured to determine the influence of palmatine on the growth of bacteria (**C**) Time-kill curves of compounds against *E. coli* strain 289. Values are the averages of three independent experiments.

PCR using primer pairs P1 and P2, and P3 and P4 (Supplemental Table 1). PCR amplicons were detected using electrophoresis in 1% TBE agarose gels stained with GoldView I dye and were sequenced by Beijing Genomics Institute.

Cloning, Expression and Purification of Recombinant Proteins

The *qnrS* and *aac(6')-Ib-cr* genes contained in positive amplicons were cloned into plasmid expression vector pET28a using the *Nco* I-linked sense primers P5 and P7 and *Xho* I-linked antisense primers P6 and P8 for *qnrS* and *aac(6')-Ib-cr*, respectively (Supplemental Table 1). Plasmid insert identity was verified by sequencing (see above) and plasmids were designated *pET28a/qnrS* and *pET28a/aac(6')-Ib-cr*, respectively. Subsequently, in order to perform the purification of target proteins, the cDNA regions encoding *qnrS* and *aac(6')-Ib-cr* were re-amplified using the primer pairs P5 and P9 with *Nco* I and *Xho* I sites, P10 and P8 with *BamH* I and *Xho* I sites, respectively. In addition, the complete coding regions of *gyrA* and *gyrB* genes were amplified by PCR from *E. coli* ATCC 25922 using the primer pairs P11 and P12 with *Nde I* and *Xho* I sites, and P13 and P14 with *Nde I* and *Xho* I sites, respectively. PCR products were digested with the corresponding restriction enzymes and subcloned into pET28a to construct *pET28a/qnrS-His, pET28a/aac(6')-Ib-cr-His, pET28a/gyrA-His* and *pET28a/gyrB-His*. The recombinant plasmids were verified by sequencing. All PCR primers are listed in Supplemental Table 1.

For protein expression, recombinant plasmids were introduced into *E. coli* strain Transetta DE3 chemically competent cells (TransGen Biotech, Beijing, China) by transformation. Transformants were selected on LB plates containing 50 μg/mL kanamycin. Individual colonies were expanded by liquid culture in 100 mL LB with 50 μg/mL kanamycin and grown to mid-logarithmic phase. Expression was induced with 0.1 mM isopropyl- β-D-thio galactopyranoside (IPTG) for 5 h at 25°C. The cultures were then harvested by centrifugation at 4000 × g for 15 min at 4°C, suspended in 20 mL of TBS (pH 7.4) and sonicated on ice. The supernatant was separated from the residual cellular debris by centrifugation at 13,000 × g for 25 min at 4°C and analyzed using 12.5% SDS-PAGE gels stained with Coomassie Brilliant Blue R-250. After the successful expression was confirmed,

His_6-tagged recombinant proteins were further purified using Ni-nitrilotriacetic acid (Ni-NTA) resin column (GE Healthcare, Pittsburg, PA, USA) following the manufacturer's instructions. Briefly, the supernatant was filtered through a 0.45 μm filter membrane and loaded onto the Ni-NTA resin column, which was then incubated for 20 min at 4°C. The column was then washed twice with 10 mL of TBS (pH 7.4) containing 10 mM imidazole, followed by a successive wash with 10 mL of TBS (pH 7.4) containing 20 mM, 40 mM, 80 mM and 200 mM imidazole, respectively. Protein purity was assessed by SDS-PAGE as described above. The purified proteins were dialyzed at 4°C against the TBS (pH 7.4) for 72 h, during which the dialysate was changed 9 times. The identity of purified proteins was verified using MALDI/TOF MS analysis. The concentration of the recombinant proteins was determined using a BCA protein assay kit (CWBio, Beijing, China) according to the manufacturer's instructions.

Determination of Antibacterial Activity

Minimal inhibitory concentrations (MICs) of ciprofloxacin against *E. coli*, including the clinical ciprofloxacin-resistant *E. coli* strains and recombinant *E. coli* (Transetta DE3) strains producing QnrS or AAC(6′)-Ib-cr were determined in the presence and absence of palmatine using the broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines.[52,53] *E. coli* ATCC 25922 and recombinant *E. coli* (Transetta DE3) strains harboring empty vector were used as a quality control strain, respectively. The fractional inhibitory concentration index (FICI) values were calculated to evaluate the combination effect as described previously.[54] Synergistic effects were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) as follows: FICI ≤ 0.5 represents synergy; 0.5 < FICI ≤ 4 represents no interaction; and FICI > 4 represents antagonism.

Biological Assays

A growth curve assay was employed to evaluate the effects of palmatine on our test strains as previously described with slight modifications.[55] We selected *E. coli* isolate 289 that produced QnrS and AAC(6′)-Ib-cr as the experimental strain. Briefly, *E. coli* 289 was grown in LB broth at 37°C with shaking until reaching an OD 600 nm of 0.3 and were then aliquoted into 96-well plates containing palmatine at 0 (control), 64, 128, 256 and 512 μg/mL. Subsequently, *E. coli* were cultured at 37°C with constant shaking and the absorbance at 600 nm was monitored by a microplate reader over 6 h.

A time-kill assay was applied to examine the presence of synergistic effects between ciprofloxacin and palmatine. *E. coli* 289 (see above) was grown in LB broth at 37°C with shaking to mid-logarithmic-phase and diluted to 5×10^5 CFU/mL and then statically incubated with ciprofloxacin (8 μg/mL), a combination of ciprofloxacin (8 μg/mL) plus palmatine (512 μg/mL) and same volume of sterile water (control) for 0, 2, 4, 6, 8, 10 and 12 h. Subsequently, each sample was diluted in sterile PBS (pH 7.4) and plated on LB agar. Time-kill curves were established by plotting mean colony counts ($\log_{10}$ CFU/mL) versus time.[54]

Cytotoxicity Assays

Murine RAW264.7 cells were seeded into 96-well plates at a density of 1×10^4 cells per well and cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 for 24 h. Subsequently, palmatine at 0 (control), 128, 256 and 512 μg/mL were added to the culture medium and incubation was continued for 12 h. The activity of cells was measured by Cell Counting Kit (CCK) assay[56] according to the manufacturer's protocol (TransGen Biotech, Beijing, China).

Mouse Thigh Infection Model with Ciprofloxacin-Resistant *E. coli* Strains

Four-week-old male KM mice weighing 20 ± 2 g were housed in a controlled environment under a 12 h light/12 h dark cycle at a constant temperature 23 ± 2°C with humidity at 60% ± 5%. The mice had free access to standard chow and water and were acclimated to the environment for 5 days before the start of experiments. Animal experiments were approved by and conducted in accordance with the Guidelines of Experimental Animal Care issued by the Animal Welfare and Research Ethics Committee at Qingdao Agriculture University (Approved Number: SYXK (SD) 20170005).

Mice were divided randomly into four groups (n = 10 in each group) and infected with *E. coli* 289 by thigh injection as previously described.[55] Briefly, *E. coli* 289 bacterial suspensions (50 μL of 2×10^7 CFU) of early logarithmic phase cells were injected into the posterior thigh muscle. The infected mice were treated with ciprofloxacin (10 mg/kg), palmatine (50 mg/kg), a combination of both or control solvent using intragastric administration 24 h after infection. After 36 h, the infected thighs were

harvested, homogenized, diluted with sterile PBS (pH 7.4) and plated for colony counting.

Molecular Modelling and Docking

The three-dimensional (3D) structures of ciprofloxacin-resistant proteins QnrS or AAC(6′)-Ib-cr were predicted using the iterative threading assembly refinement (I-TASSER) program (https://zhanglab.ccmb.med.umich.edu/I-TASSER/). The tertiary structures were visualized with the PyMOL program. The 3D structure of palmatine (Compound CID: 19009) was obtained from the PubChem Compound database (http://pubchem.ncbi.nlm.nih.gov/) and the conformation optimized was calculated by Chem3D 16.0 software before docking. Molecular dockings between palmatine and QnrS or AAC(6′)-Ib-cr were carried out employing the Molegro Virtual Docker Version 6.0 program to gain insights into the molecular interaction mechanism as previously described.[57] LigPlot$^+$ Version V.1.4.5[58] was used to further investigate the docking model complexes.

Gyrase Supercoiling Assay

DNA supercoiling activity assays were performed to examine the effects of palmatine on QnrS activity as described previously with a minor modification.[44] In brief, relaxed pBR322 plasmids were prepared by incubation with topoisomerase I using a commercial kit according to manufacturer instructions (Novoprotein, Shanghai, China). Proteins purified as described above were used for the test assays. Gyrase holoenzyme was reconstituted by incubating recombinant GyrA and GyrB subunits on ice for 60 min. 40 μM purified recombinant QnrS proteins and 200 μM palmatine were mixed in equal volume and preincubated at room temperature for 20 mins. For each reaction, a common mixture buffer containing 17 mM Tris-HCl (pH 7.5), 6 mM $MgCl_2$, 19 mM KCl, 0.25 μg relaxed pBR322 plasmid, 8 μg/mL tRNA, 1.8 mM spermidine, 1% glycerol, 200 μg/mL BSA, 5 mM DTT and 1.4 mM ATP was prepared in advance. Simultaneously, the remaining components 2.8 nM gyrase holoenzyme, 6 μM ciprofloxacin, 2 μM purified His_6-tag proteins (gifts from Qingyun Yang, Ocean University of China), 2 μM QnrS and preincubated complexes containing 2 μM QnrS and 10 μM palmatine were added to the reaction system as different groups. The His_6-tag proteins were used as control for QnrS proteins. The mixtures were incubated at 25°C for 2 h and DNA loading buffer was added to stop the reaction. Subsequently, the result was analyzed on a 1% TBE agarose gel for electrophoresis and stained as described above. The bands of the most supercoiled isomer were digitized using the software ImageJ.

Acetylation Assays

Acetylation assays were carried out to verify the inhibitory effect of palmatine on AAC(6′)-Ib-cr using a previously described method with slight modifications.[36] In brief, 80 μM purified recombinant AAC(6′)-Ib-cr protein and 400 μM palmatine were mixed in equal volume and preincubated at room temperature for 20 mins. To eliminate the interference of palmatine on absorbance, the degree of absorbance change associated with the AAC(6′)-Ib-cr plus palmatine groups was subtracted from that associated with the palmatine alone groups. Acetylation assays were performed in 96-well plates containing 5 mM Tris-HCl (pH 7.5), 5 mM ammonium sulfate, 0.1 mM EDTA, 50 μM ciprofloxacin, 4 μM AAC(6′)-Ib-cr proteins or preincubated complexes containing 4 μM AAC(6′)-Ib-cr and 20 μM palmatine. The His_6-tag proteins (4 μM) were used as control for AAC(6′)-Ib-cr proteins. The reaction was initiated by adding 20 μM 5,5′-dithiobis-(2,2)-nitrobenzoic acid (DTNB) and 40 μM acetyl-CoA, and the absorbance at 405 nm was monitored by a microplate reader at room temperature.

Statistical Analysis

Data are presented as the mean ± SD from at least three times experiments and were analyzed using GraphPad Prism 5 (San Diego, CA, USA). Significant differences were determined by paired sample *t*-test as indicated and differences at $P<0.05$ were considered significant.

Results

Palmatine Enhances Ciprofloxacin Susceptibility of *E. coli* Containing *qnrS* or *aac(6′)-Ib-cr*

We identified 42 clinical strains of *E. coli* containing the *qnrS* or *aac(6')-Ib-cr* genes and determined the antibacterial activity of ciprofloxacin, palmatine and a combination of the two. We found a synergistic effect (FICI ≤ 0.5) in 10 of the *E. coli* test strains (Table 1). Palmatine concentrations up to 512 μg/mL had no significant effect on the growth of the *E. coli* test strain 289 that contained both *aac(6')-Ib-cr* and *qnrS* (Figure 1B). However, the activity with ciprofloxacin was synergistic (Table 1). In addition, the time-kill experiments further confirmed the synergism of the combination. Ciprofloxacin used alone had only a minor effect on the

Table 1 MIC and FICI Values of the Palmatine and Ciprofloxacin Combination Therapy for Each of the Tested Clinical Bacterial Isolates

Strains	Source	*qnrS*	*aac(6′)-Ib-cr*	Drugs	MIC (μg/mL)		FIC Index
					Alone	Combination	
II-161	Human urine	+	-	Ciprofloxacin Palmatine	128 2048	16 **(8)** 512	0.375
08-08	Pig feces	+	-	Ciprofloxacin Palmatine	64 2048	16 **(4)** 512	0.5
10-08	Pig feces	+	-	Ciprofloxacin Palmatine	8 1024	2 **(4)** 256	0.5
11-09	Pig feces	+	+	Ciprofloxacin Palmatine	64 1024	16 **(4)** 128	0.375
11-33	Pig feces	+	-	Ciprofloxacin Palmatine	4 1024	1 **(4)** 256	0.5
214	Chicken cloacae	+	-	Ciprofloxacin Palmatine	32 2048	8 **(4)** 512	0.5
227	Chicken cloacae	+	-	Ciprofloxacin Palmatine	32 2048	4 **(8)** 512	0.375
289	Chicken cloacae	+	+	Ciprofloxacin Palmatine	64 2048	8 **(8)** 512	0.375
336	Chicken cloacae	+	-	Ciprofloxacin Palmatine	16 1024	4 **(4)** 256	0.5
352	Chicken cloacae	-	+	Ciprofloxacin Palmatine	16 2048	4 **(4)** 512	0.5
ATCC 25922	Laboratory strain	-	-	Ciprofloxacin Palmatine	0.015625 1024	0.015625 **(1)** 512	1.5

Notes: All MICs were determined in triplicate. Fold changes are indicated in parentheses in bold.

reduction of *E. coli* strain 289. However, the combination of ciprofloxacin plus palmatine exerted effective killing effects on the bacteria and rapidly reduced viable counts and thoroughly killed the bacteria by 10 h (Figure 1C).

In order to test the targeted action of palmatine to ciprofloxacin-resistance genes, we generated recombinant *E. coli* strain (Transetta DE3) that produced QnrS and AAC(6′)-Ib-cr by IPTG induction and the correct expression was confirmed by MALDI/TOF MS analysis and SDS-PAGE (data not shown). *E. coli* strains producing QnrS and AAC(6′)-Ib-cr displayed 8- and 4-fold MIC increases compared with the empty vector control, respectively. However, the susceptibility of both recombinant strains to ciprofloxacin was restored when combined with palmatine (Table 2). The results indicated that palmatine possessed a potential as an inhibitor of QnrS and AAC(6′)-Ib-cr and was able to restore the antibacterial activity of ciprofloxacin.

Table 2 Effect of Palmatine on the Susceptibility of Recombinant *E. coli* to Ciprofloxacin Producing QnrS or AAC(6′)-Ib-Cr

Strains	Ciprofloxacin (μg/mL)	Combination (μg/mL)
Recombinant *E. coli* (empty vector)	0.001	0.001
Recombinant *E. coli* (QnrS)	0.008	0.001 **(8)**
Recombinant *E. coli* (AAC(6′)-Ib-cr)	0.004	0.001 **(4)**

Notes: All MICs were determined in triplicate. Palmatine in combination with ciprofloxacin was tested at 512 μg/mL. Fold changes are indicated in parentheses in bold.

Palmatine and Ciprofloxacin Synergistically Inhibit *qnrS* and *aac(6′)-Ib-cr* -Positive Bacteria in vivo

Cytotoxicity is an important parameter for evaluating the clinical application of novel molecules.[55] Palmatine did not show any significant cytotoxicity (P<0.05) at ≤512 μg/mL for 12 h when used alone (Figure 2A). We, therefore, used

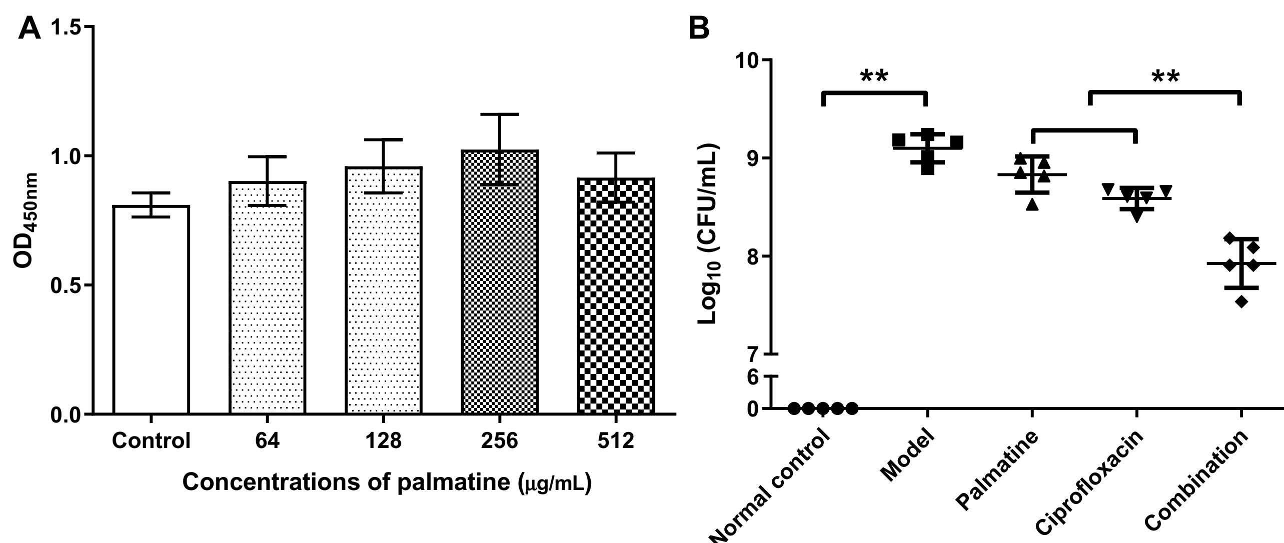

Figure 2 Effects of palmatine and ciprofloxacin combination therapy in vivo. (**A**) Palmatine cytotoxicity in murine RAW264.7 cells (**B**) Efficacy in mice intramuscularly infected with *E. coli* 289 followed by intragastric treatments of ciprofloxacin (10 mg/kg), palmatine (50 mg/kg), ciprofloxacin combined with palmatine (combination) or control solvent. Uninfected mice served as normal controls. The bacterial burden in the thighs was calculated by colony enumeration. **, p < 0.01.

a murine thigh infection model to evaluate the efficacy of palmatine plus ciprofloxacin in vivo. The combination of these compounds when administered to mice intragastrically resulted in a significant reduction of bacterial burdens compared with either compound alone (P <0.01) (Figure 2B). These results indicated a synergistic effect of palmatine and ciprofloxacin in vivo.

Determination of the Molecular Binding Mode of Palmatine with QnrS and AAC (6′)-Ib-cr

The loop B of Qnr and the amino acid substations Trp102Arg and Asp179Tyr in AAC(6′)-Ib-cr are key functional sites for quinolone-resistance activity.[6,22,26] We used molecular binding modeling to identify interactions between palmatine and QnrS and AAC(6′)-Ib-cr. We found that palmatine was bound to loop B of QnrS via different intermolecular forces (Figure 3A–C and G). The highly conserved amino acids Phe-114 and Cys-115 interacted with the drug ligand through hydrophobic interactions and hydrogen bonding, respectively. In addition, Met-112 and Tyr-113 also contributed intermolecular forces (Figure 3H). Similarly, the binding mode of palmatine with AAC(6′)-Ib-cr indicated that palmatine was localized to the binding pocket of AAC(6′)-Ib-cr (Figure 3D–F and G) and formed hydrophobic interactions with Arg-102 and Tyr-179 indicating probable molecular binding interactions (Figure 3I).

Palmatine Reduces the Gyrase Protective Effect of QnrS and Acetylation of AAC (6′)-Ib-cr

Additionally, we used gyrase supercoiling and acetylation assays to further verify the interaction of palmatine with QnrS and AAC(6′)-Ib-cr, respectively. The purified proteins QnrS, AAC(6′)-Ib-cr, GyrA and GyrB each yielded single bands of 25.8, 25.6, 99.3 and 92.3 kDa, respectively, after elution from the Ni affinity columns that matched their expected sizes (Figure 4A–D). The sequence accuracy of recombinant proteins was further verified by MALDI/TOF MS analysis (data not shown). The supercoiling activity of Qnr was effectively inhibited in the presence of ciprofloxacin (Figure 4E, lane 5, F). However, palmatine restored the QnrS gyrase activity in the presence of ciprofloxacin (Figure 4E, lane 6, F). Likewise, acetylation of recombinant AAC(6′)-Ib-cr proteins could also be suppressed by palmatine (Figure 4G). These results further confirmed the interaction of palmatine with the PMQR proteins QnrS and AAC (6′)-Ib-cr.

Discussion

Plasmids are a serious threat for dissemination of antimicrobial resistance because they can be horizontally transferred between both related and unrelated bacteria.[59] This process has seriously compromised the use of quinolones as clinical antibacterials due to the rapid variation and spread of PMQR. PMQR genes have been identified in

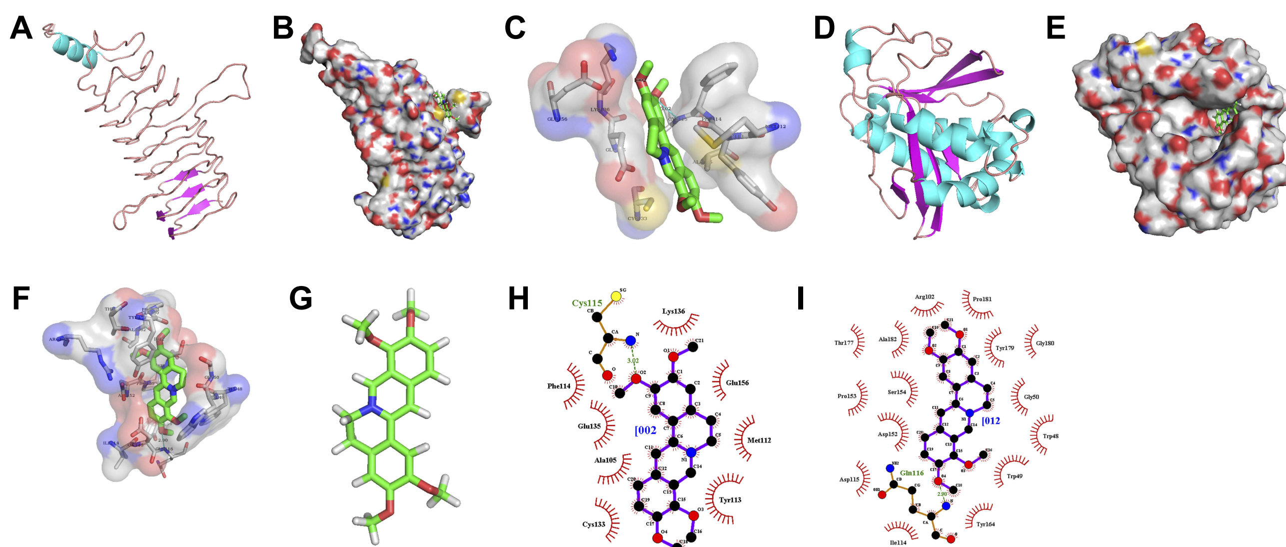

Figure 3 Molecular dockings and specific interactions between palmatine and QnrS or AAC(6′)-Ib-cr. 3D structures of (**A**) QnrS, (**D**) AAC(6′)-Ib-cr and (**G**) palmatine (see text for details). Molecular surfaces of (**B**) QnrS and (**E**) AAC(6′)-Ib-cr after docking with palmatine (green structure) and detailed views of the interactions between (**C**) QnrS and (**F**) AAC(6′)-Ib-cr. Hydrogen bonds are colored cyan and the amino acid residues involved in the non-bonded contacts are represented by transparent surfaces. Interactions between palmatine and amino acid residues at binding sites of (**H**) QnrS and (**I**) AAC(6′)-Ib-cr. Residues contacting palmatine via hydrogen bonds are colored green and the numbers represent bond lengths. Residues that provide hydrophobic interactions with palmatine are colored in black with red eyelash symbols.

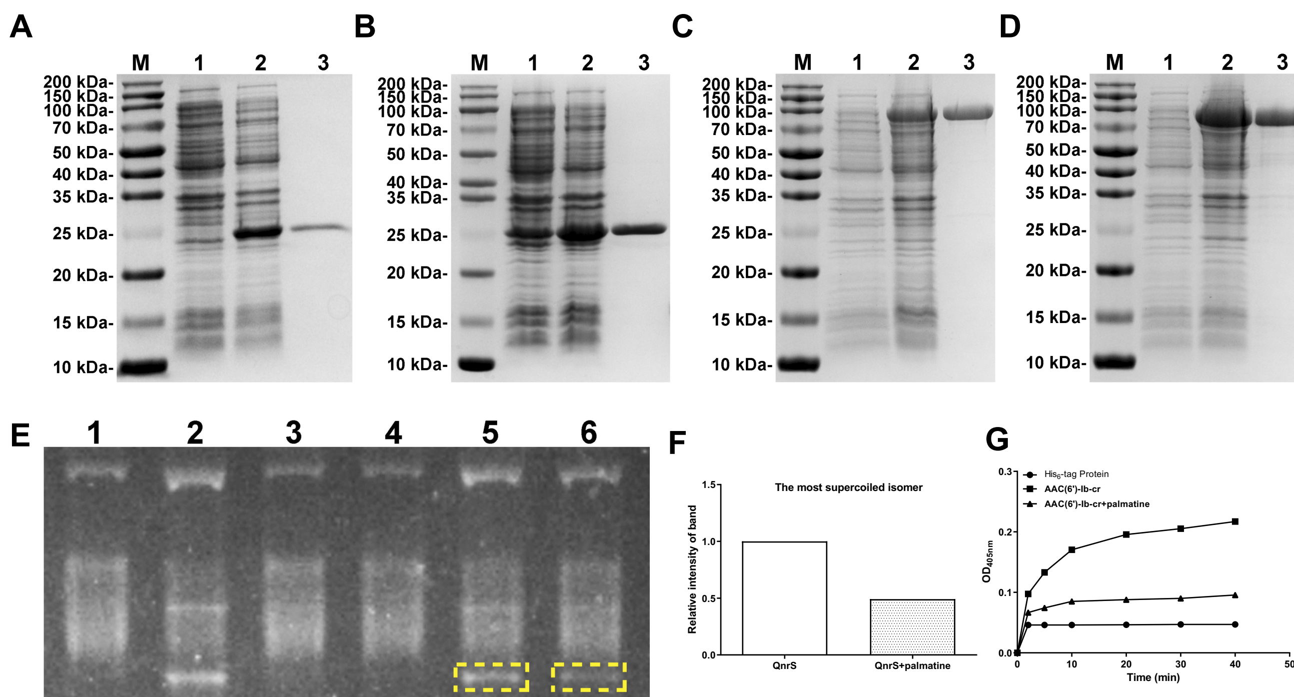

Figure 4 Interaction of palmatine with QnrS and AAC(6′)-Ib-cr. Purification of recombinant proteins visualized using SDS-PAGE for (**A**) QnrS, (**B**) AAC(6′)-Ib-cr, (**C**) GyrA and (**D**) GyrB. Lane M, protein molecular mass marker; Lane 1, total cellular extracts before induction; Lane 2, total cellular extracts after IPTG induction; Lane 3, extracts after Ni-NTA column elution (**E**) Palmatine reduces the gyrase protective effect of QnrS. 20 μL reaction mixtures were analyzed in 1% TBE gels. Reaction mixtures contained 0.25 μg relaxed pBR322 plasmid DNA (lanes 1 to 6), 2.8 nM gyrase (lanes 2 to 6), 6 μM ciprofloxacin (lanes 3 to 6), 2 μM purified His_6-tag proteins (lane 4), 2 μM purified QnrS (lane 5) and 2 μM purified QnrS that were preincubated with 10 μM palmatine (lane 6). (**F**) Digital analysis of the most supercoiled isomer (yellow-dotted box). (**G**) Palmatine reduces acetylation of AAC(6′)-Ib-cr. 50 μM ciprofloxacin was incubated in the presence of 4 μM AAC(6′)-Ib-cr protein or 4 μM AAC(6′)-Ib-cr protein that was preincubated with 20 μM palmatine, an acetyl donor (acetyl-CoA) and an indicator (DTNB). Absorbance at 405 nm was measured as an index of acetylation (see text for details). 4 μM His_6-tag proteins were used for controls.

human as well as isolates from wild and domestic animals including chickens, pigs, dogs and fish.[6] Higher levels of quinolone resistance are observed if a plasmid or strain carries two or more unrelated quinolone-resistance genes such as *qnr* and *aac(6′)-Ib-cr*.[22] In contrast, the presence of two different Qnr variants in a same strain shows no

additive effect.[26,60,61] It is noteworthy that *qnrS* (a *qnr* variant) and *aac(6')-Ib-cr* positive plasmids are frequently detected in the same strain.[49,62,63] This problem is exacerbated by the lack of effective PMQR inhibitors. Therefore, the development of PMQR inhibitors that simultaneously suppress the activity of the two different resistant proteins is of great clinical relevance.

Traditional Chinese medicines have found uses as antibiotic replacement therapies.[54,64–66] For instance, the isoquinoline alkaloid palmatine has been widely used for clinical treatments.[50,51] In this study, we found that palmatine enhanced the ciprofloxacin susceptibility of *E. coli* strains containing *qnrS* or *aac(6')-Ib-cr* and the effect was synergistic. In addition, the synergistic activity of the combination was further confirmed by time-kill assays using the strains of *E. coli* which simultaneously contained both PMQR genes. Furthermore, in vivo synergistic evidence was obtained using an in vivo mouse model. These experiments established that the bacterial burdens of experimentally infected mice using QnrS- and AAC(6′)-Ib-cr-producing *E. coli* isolates were successfully controlled with the combination therapy. We also found that that palmatine disrupted drug resistance to ciprofloxacin caused by *qnrS* or *aac(6')-Ib-cr* in vitro. These results indicated that palmatine in combination with ciprofloxacin was effective in vitro and in vivo and that palmatine may have the potential to enhance ciprofloxacin clinical effectiveness.

We also used an analytical approach to investigate the mechanism of the inhibitory action of palmatine and QnrS and AAC(6′)-Ib-cr using molecular docking modeling. Loop B of Qnr has been shown to be important for quinolone resistance and has been proposed to be conformationally flexible.[42] Our docking results demonstrated that palmatine localized to loop B of QnrS and contributed to gyrase inhibition. More specifically, the conserved Phe-114 and Cys-115 residues as well as other functionally validated Met-112 and Tyr-113 all participated in intermolecular hydrophobic and hydrogen bonding interactions. AAC(6′)-Ib-cr is the *cr* variant of AAC(6′)-Ib that promotes fluoroquinolone acetylation and inactivation.[36] The variant had two unique amino acid substitutions (Trp102Arg and Asp179Tyr) both of which are critical for acetylation activity.[67,68] We observed that palmatine docked at the AAC(6′)-Ib-cr binding pocket that included these important amino acids. To confirm this prediction, we found that palmatine reduced the gyrase protective effect of QnrS and acetylation by AAC(6′)-Ib-cr as expected. Although QnrS and AAC(6′)-Ib-cr proteins belong to different families, the existence of functional loop B and binding pocket provides interaction possibility for palmatine. Hence, in consideration of the additive effect of the presence of unrelated quinolone-resistance genes, the inhibitory activity of palmatine to QnrS and AAC(6′)-Ib-cr is particularly important. In addition, QnrS is just one member of the huge Qnr family, and it would also be interesting to know if there is a similar effect on other Qnr proteins, as might be expected.

Conclusions

Together, these data demonstrated that palmatine restored the activity of ciprofloxacin against *qnrS* and *aac(6')-Ib-cr*-positive *E. coli* isolates by inhibiting the gyrase protective effect of QnrS and acetylation by AAC(6′)-Ib-cr. The synergistic activity was observed with the combination of palmatine plus ciprofloxacin in vitro and in vivo and indicated a potential for palmatine to enhance ciprofloxacin effectiveness in the clinic. Palmatine is a natural clinically efficacious compound that shows promise as a powerful PMQR inhibitor.

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Disclosure

The authors report no conflicts of interest in this work.

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