Synergistic Antimicrobial Activity of Colistin in Combination with Rifampin and Azithromycin against *Escherichia coli* Producing MCR-1

Yanqin Li,^a Xiaohuan Lin,^a Xuan Yao,^a Yan Huang,^a Wenguang Liu,^a Tao Ma,^a Binghu Fang^a

^aDepartment of Veterinary Pharmacology and Toxicology, College of Veterinary Medicine, South China Agricultural University, Guangzhou, China

ABSTRACT The lack of available antibiotics is a global public health problem due to the emergence of antimicrobial resistance. Effective therapeutic regimens are urgently needed against *Escherichia coli* strains that produce the colistin resistance gene *mcr-1* and to inhibit the emergence of resistance. In this study, we assessed the antimicrobial activity of a series of concentrations of colistin-based combinations with rifampin and/or azithromycin against three strains of *Escherichia coli*, including colistin-resistant isolate MZ1501^R, isolate HE1704^R that produces MCR-1, and colistin-susceptible isolate MZ1509^S. Experiments were conducted with a medium inoculum of $\sim 10^7$ CFU/ml over 48 h. Subsequently, the *in vivo* therapeutic effect was investigated using a neutropenic mouse thigh infection model. Almost all monotherapies showed unsatisfactory antibacterial activity against *E. coli* isolates producing MCR-1. In contrast, colistin in combination with rifampin or azithromycin resulted in an obvious decrease in the bacterial burden albeit with regrowth. More obviously, synergistic antimicrobial activity of colistin-based triple-combination therapy with rifampin and azithromycin was observed, resulting in a rapid and exhaustive antibacterial effect. *In vivo* treatments confirmed these findings, where mean decreases of 0.38 to 0.90 log₁₀ CFU and 1.27 to 1.78 log₁₀ CFU were noted after 24 h and 48 h of treatment, respectively, against colistin-resistant *E. coli* strains when 5 mg/kg of body weight of colistin was combined with rifampin and azithromycin. Colistin-based combinations with rifampin and azithromycin provide a more active therapeutic regimen than monotherapy or colistin-based double combinations against *E. coli* producing MCR-1.

KEYWORDS colistin, rifampin, azithromycin, *Escherichia coli*, *mcr-1*

The rapid spread of extended-spectrum- β -lactamase-mediated bacterial resistance has resulted in a reduction in the number of clinically available and effective drugs. Colistin is considered the last line of defense against multidrug-resistant Gram-negative bacteria. However, chromosomal mutations in different genes, for example, *pmrAB*, *phoPQ*, and *mgrB*, result in the rapid development of high-level colistin resistance. Even more unfortunately, an induced-colistin-resistance gene (*mcr-1*) has been reported and has rapidly spread to a variety of bacteria (1–4) due to the transfer of this plasmid-mediated resistance gene. The *mcr-1* gene was first reported in animals and patients in their host *Enterobacteriaceae* (5), and it has also been identified in food. Limited treatment alternatives against these pathogens make such organisms an extreme public health threat, which frequently lead to low cure rates and higher mortality rates.

The increased *in vitro* bacterial effect of double- or triple-drug combinations of colistin or polymyxin B and various drugs, such as rifampin (6, 7), meropenem (8, 9), and amikacin (10, 11), has been well demonstrated in many studies. Azithromycin is the most commonly prescribed antibiotic, other than for the clinical treatment of serious

Received 2 August 2018 Returned for
modification 22 August 2018 Accepted 10
September 2018

Accepted manuscript posted online 17
September 2018

Citation Li Y, Lin X, Yao X, Huang Y, Liu W, Ma T, Fang B. 2018. Synergistic antimicrobial activity of colistin in combination with rifampin and azithromycin against *Escherichia coli* producing MCR-1. Antimicrob Agents Chemother 62:e01631-18. <https://doi.org/10.1128/AAC.01631-18>.

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Binghu Fang, fangbh@scau.edu.cn.

FIG 1 *In vitro* activities of colistin (CST) (a to c), rifampin (RIF), and azithromycin (AZM) (d to f) against colistin-resistant *E. coli* MZ1501^R (left) and HE1704^R (middle) and colistin-sensitive *E. coli* MZ1509^S (right). A growth control without drug administration was included in all tests.

Gram-negative infections because of poor diffusion across the outer membrane of most Gram-negative bacteria (12). However, disruption of the outer membrane of Gram-negative bacteria by colistin may contribute to the uptake of azithromycin. Enhanced antimicrobial activity of azithromycin against *Pseudomonas aeruginosa* in serum and bronchoalveolar lavage fluid through increased outer membrane permeability and reduced efflux of the drug has been reported (13). The immunomodulatory activity of azithromycin has also been of great interest in recent years (14). Thus, the objective of this work was to evaluate the *in vitro* synergistic effect of colistin-based combinations with rifampin and/or azithromycin against colistin-resistant *Escherichia coli* isolates producing MCR-1 and sensitive *E. coli* isolates. An *in vivo* model to verify treatment was performed using a neutropenic mouse thigh infection model. This work aimed to provide a feasible antibiotic regimen consisting of colistin, rifampin, and azithromycin against colistin-resistant *E. coli* infections.

RESULTS

Time-kill assays. Monotherapy. Time-kill curves for colistin, rifampin, and azithromycin treatments against *E. coli* strains MZ1501^R, HE1704^R, and MZ1509^S are shown in Fig. 1. Data represent the change in bacterial burden from an initial inoculum of 10^7 CFU/ml. For *E. coli* strain MZ1501^R and strain HE1704^R that produces MCR-1, the time curves for colistin concentrations of 0.5 to 4 mg/liter had a synchronous growth tendency with growth control. A colistin concentration of 8 mg/liter resulted in an early bactericidal effect (≥ 3 -log₁₀ decrease in colony counts) by 4 h but regrowth of bacteria after 6 h. Unsurprisingly, for the colistin-sensitive *E. coli* strain MZ1509^S, colistin at 1 to 8 mg/liter performed effectively. Unfortunately, all rifampin (0.5, 1, and 2 mg/liter) and azithromycin (0.5, 1, and 2.5 mg/liter) concentrations showed no evidence of inhibition of any of the strains through 48 h.

Double-combination therapy. The time curves for the addition of rifampin and azithromycin to colistin against *E. coli* MZ1501^R, HE1704^R, and MZ1509^S are shown in

FIG 2 *In vitro* activities of colistin-based double combinations with rifampin or azithromycin against colistin-resistant *E. coli* MZ1501^R (left) and HE1704^R (middle) and colistin-sensitive *E. coli* MZ1509^S (right). From top to bottom, the concentrations of colistin in combination with rifampin or azithromycin were 0.5, 1, and 2 mg/liter, respectively. A growth control without drug administration was included in all tests.

Fig. 2. The accompanying pharmacodynamics analysis is also presented in Table S1 in the supplemental material. Increased antibacterial activity against MZ1501^R and delayed recovery of growth were noticed when colistin-based combinations with rifampin or azithromycin were used, except when 0.5 mg/liter colistin was combined with 0.5 mg/liter rifampin or azithromycin. A virtual bactericidal effect was observed with a reduction of approximately 3 log₁₀ CFU/ml from the initial burden of ~10⁷ CFU/ml within 4 to 6 h when 1 or 2 mg/liter colistin was employed in combination with 0.5 to 2 mg/liter rifampin. Furthermore, the combination of 1 or 2 mg/liter colistin with 2.5 mg/liter azithromycin achieved an eradication effect (≥ 4 -log₁₀ decrease in colony counts) by 48 h, without regrowth (Fig. 2a, d, and g). Quite different from the MZ1501^R isolate, colistin-based double combinations showed weaker antimicrobial activity against HE1704^R due to simultaneous colistin and azithromycin resistance, with at most a 2-log₁₀ CFU/ml reduction (Fig. 2b, e, and h). Early antimicrobial activity against MZ1509^S was noted, where the numbers of bacteria were reduced to near the limit of detection by 4 h, followed by a quick recovery when 0.5 mg/liter colistin combined with rifampin or azithromycin was used. An eradication effect on isolate MZ1509^S was achieved by 6 h, without regrowth, when 1 or 2 mg/liter colistin was combined with rifampin or azithromycin (Fig. 2c, f, and i).

Triple-combination therapy. The *in vitro* antibacterial activity of a triple combination comprising colistin, rifampin, and azithromycin was assessed by performing time-

FIG 3 *In vitro* activities of a colistin-based triple combination with rifampin and azithromycin against colistin-resistant *E. coli* MZ1501^R (left) and HE1704^R (middle) and colistin-sensitive *E. coli* MZ1509^S (right). From top to bottom, the concentrations of colistin in combination with rifampin and azithromycin were 0.5, 1, and 2 mg/liter, respectively. A growth control without drug administration was included in all tests.

kill experiments. The results are presented in Fig. 3 and Table S2 in the supplemental material. All colistin-based simultaneous combinations of rifampin and azithromycin displayed earlier bactericidal action against MZ1501^R than the corresponding double combinations. Apart from the combinations of three drugs at low concentrations that resulted in regrowth after 8 h, triple combinations achieved bacterial eradication effects at 12 h with 0.5 or 1 mg/liter colistin and at 6 h with 2 mg/liter colistin when high concentrations of rifampin and azithromycin were used (Fig. 3a, d, and g). No evident enhancement of the antimicrobial effect against HE1704^R was observed with triple combinations with 0.5 or 1 mg/liter colistin compared with double combinations. Synergistic effects were observed at 24 h with combinations with 2 mg/liter colistin, and bacteria were undetectable at 48 h (Fig. 3b, e, and h). For MZ1509^S, triple combinations with 0.5 mg/liter colistin exhibited more-rapid antimicrobial action by 4 h than double combinations. Unsurprisingly, high concentrations of the triple combination resulted in complete eradication by 0 to 4 h (Fig. 3c, f, and i).

Pharmacokinetics validation. The plasma pharmacokinetic (PK) parameters for subcutaneously injected colistin and intragastric administration of rifampin and azithromycin are presented in Table 1. The maximum concentrations of drug in plasma ($C_{\max}$) for 2.5 and 5 mg/kg of body weight of colistin, 10 mg/kg rifampin, and 100 mg/kg

TABLE 1 Total plasma PK parameters of colistin, rifampin, and azithromycin after a single administration in infected neutropenic mice ($n = 4$)

	Mean value ± SD			
	Colistin			
PK parameter ^a	2.5 mg/kg	5 mg/kg	Rifampin at 10 mg/kg	Azithromycin at 100 mg/kg
C _{max} (mg/liter)	3.08 ± 0.65	5.93 ± 0.84	14.2 ± 2.46	4.12 ± 0.45
T _{max} (h)	0.75 ± 0.20	0.50 ± 0.20	2.00 ± 0.20	2.00 ± 0.00
AUC _{last} (mg · h/liter)	4.03 ± 0.41	10.2 ± 0.31	131 ± 2.18	31.5 ± 1.34
CL/F (ml/min/kg)	10.1 ± 0.88	7.52 ± 0.40	0.95 ± 0.14	44.6 ± 2.19
T _{1/2} (h)	0.65 ± 0.06	0.97 ± 0.11	13.2 ± 0.61	10.0 ± 0.55

^a $C_{\max}$, maximum concentration of drug in plasma; $T_{\max}$, time to maximum concentration of drug in plasma; AUC_{last}, the area under the concentration-time curve from 0 h to the last sampling time points; CL/F, body clearance divided by bioavailability; $T_{1/2}$, half-life.

azithromycin were 3.08 mg/liter, 5.93 mg/liter, 14.20 mg/liter, and 4.12 mg/liter, respectively. The time to maximum concentration of drug in plasma ($T_{\max}$) for colistin, rifampin, and azithromycin varied from 0.50 to 2.00 h. The unbound peak concentrations of colistin were approximately 0.26 and 0.50 mg/liter based on a previous report in which the unbound fraction of colistin was approximately 8.4% in neutropenic mice (15). Similarly, the free fraction of rifampin was found to be 16.2% in mice (16), and the free peak drug concentration here was calculated to be approximately 2.30 mg/liter, which was close to the concentration employed in *in vitro* time-kill tests. Given that there is a large difference in plasma protein binding among different azithromycin concentrations (the plasma protein binding of azithromycin declined from approximately 50% at 0.05 mg/liter to 12% at 0.5 mg/liter [17]), the unbound peak concentration of azithromycin may be higher than or close to the concentration used in time-kill tests.

In vivo treatment verification. To confirm the antibacterial activity of colistin-based combinations with rifampin and/or azithromycin, the *in vivo* pharmacodynamics of the administration of 2.5 and 5 mg/kg colistin by subcutaneous injection, and the accompanying intragastric administration of 10 mg/kg rifampin and 100 mg/kg azithromycin, were assessed. Regimens comprising a single agent or colistin-based double or triple combinations were assessed in a neutropenic mouse infection model by injecting the *E. coli* MZ1501^R, HE1704^R, or MZ1509^S strain into animals. The results are shown in Fig. 4 and 5. Neither colistin every 12 h nor rifampin or azithromycin every 24 h alone could inhibit *E. coli* MZ1501^R or HE1704^R over a 48-h period in this study. Slight inhibition against MZ1501^R was observed after treatment for 24 h when rifampin was added to 5 mg/kg colistin, whereas this treatment was almost useless against HE1704^R

FIG 4 *In vivo* pharmacodynamics of colistin sulfate at 2.5 and 5 mg/kg, rifampin at 10 mg/kg, and azithromycin at 100 mg/kg, alone and in colistin-based double and triple combinations, against colistin-resistant strains MZ1501^R and HE1704^R. A growth control without drug administration was included in all tests.

FIG 5 *In vivo* pharmacodynamics of colistin sulfate at 2.5 and 5 mg/kg, rifampin at 10 mg/kg, and azithromycin at 100 mg/kg alone and in colistin-based double and triple combinations against colistin-susceptible strain MZ1509^S. A growth control without drug administration was included in all tests.

over a 48-h period (0.13- to 0.4- $\log_{10}$ CFU increase). This decrease in bacterial burden did not occur in mice that experienced concurrent treatment with colistin and azithromycin against MZ1501^R and HE1704^R. Higher activity than those of double combinations was noted when colistin was combined with rifampin and azithromycin concurrently. A 0.30- to 0.60- $\log_{10}$ CFU decrease with combinations with 2.5 mg/kg colistin and a 1.27- to 1.78- $\log_{10}$ CFU decrease with combinations with 5 mg/kg colistin were observed for colistin-resistant strains MZ1501^R and HE1704^R after 48 h of therapy (Fig. 4). For colistin-susceptible strain MZ1509^S, even colistin alone at 2.5 mg/kg reduced the bacterial burden in mouse thighs. Notably, eradication of the MZ1509^S strain was observed in mice treated with double or triple combinations containing 5 mg/kg colistin for 48 h or 24 h (Fig. 5).

DISCUSSION

Polymyxins had been banished from the family of clinical antibiotics due to their many and various side-effects. The continual emergence of Gram-negative bacteria producing β -lactamases and carbapenemases, which can resist a variety of antibiotics, and the sluggish development of new drugs that possess independent target mechanisms have led to the reuse of polymyxins. However, the rapid spread of the plasmid-mediated *mcr-1* gene in recent years has dealt a devastating blow to the effective use of polymyxins. In this instance, polymyxin-based combination therapy using old drugs might be an optional therapeutic measure for drug-resistant strains.

Antibacterial activity of colistin with amikacin against *E. coli* strains coproducing NDM-5 and MCR-1 has been reported, where augmented susceptibility to colistin and depressed bacterial burdens were observed (18). Polymyxin B in combination with rifampin and meropenem against highly polymyxin B-resistant KPC-producing *Klebsiella pneumoniae* may be a viable option, since the triple-drug combination displayed early bactericidal activity against organisms within 8 h and thereafter (9). Giamarellos-Bourboulis et al. reported that the activity of colistin against *Acinetobacter baumannii* was increased in the presence of rifampin in time-kill studies (19). Bassetti et al. studied the clinical and microbiological responses and the safety of simultaneous intravenous treatment with colistin sulfomethate sodium and rifampin. No renal failure or neurotoxicity was observed among patients with normal baseline renal function, which indicated that combination treatment with colistin and rifampin was effective and safe for severe infections caused by multidrug-resistant *A. baumannii* (20).

Synergy is defined as a reduction in colony counts of ≥ 100 -fold or 2 $\log_{10}$ units for 24 h following treatment with a combination compared with the most active single agent alone and a ≥ 100 -fold decrease in colony counts from the initial burden (21). In

our study, the *in vitro* pharmacodynamics proved that clinically available concentrations of colistin, rifampin, and azithromycin alone are ineffective against *E. coli* isolates producing MCR-1. Furthermore, *in vivo* treatment with lower/routine therapeutic dosages close to the concentrations used in *in vitro* time-kill pharmacodynamics studies verified this point. The combination of colistin and high concentrations of rifampin (2 mg/liter) or azithromycin (2.5 mg/liter) against colistin-resistant *E. coli* isolates *in vitro* was found to be synergistic at one-quarter to one-half the MIC of colistin in this study. However, this double-combination treatment strategy failed to control the regrowth of strain HE1704^R. The combination of colistin and rifampin showed higher antimicrobial activity against a colistin-resistant isolate than the combination of colistin and azithromycin, but the opposite was true for the colistin-sensitive isolate. As expected, colistin in combination with both rifampin and azithromycin resulted in more-efficient antibacterial activity than colistin-based double combinations against colistin-sensitive or -resistant strains *in vitro*. More importantly, for *E. coli* strain MZ1501^R, we noted that the combination of one-eighth the MIC of colistin plus 2 mg/liter rifampin and 0.5 mg/liter azithromycin was able to stop regrowth within 8 to 12 h.

To estimate the *in vivo* pharmacodynamics of drug combinations of colistin plus rifampin and/or azithromycin, a neutropenic mouse infection model was used over 48 h in our study (to our knowledge, we believe that this is the first time that such an observation has been made). As might be expected, colistin (2.5 and 5 mg/kg) in combination with rifampin or azithromycin displayed the same effects against colistin-resistant *E. coli* strains as those observed in the *in vitro* time-kill experiments, with the bacterial burden hardly being affected (Fig. 2 and 4). A significant change in terms of the reduction of the bacterial burden was observed when the same dosage-based triple combination was employed. This was clearly the case when triple combinations containing 5-mg/kg colistin were used against *E. coli* strains, and a continuous decrease in the bacterial burden occurred in infected thighs by 0 to 48 h.

The recommended regimen of colistin methanesulfonate sodium (CMS) is 90 mg of colistin base activity (CBA) (equivalent to 3 million IU) every 8 h, following a loading dose of 270 mg of CBA in patients with normal renal function by intravenous administration. PK research with intravenous infusion of CMS at 3 million IU every 8 h for adult patients showed that the $C_{\max}$ in plasma was 2.36 mg/liter in the steady state (22), which was lower than the mean serum $C_{\max}$ values (3.34 mg/liter) at the same dose obtained in other studies (23) and close to the peak plasma concentration (3.08 mg/liter) of colistin in mice treated with 2.5 mg/kg colistin sulfate in this study. However, consistent with previous research showing that the currently used dose might not provide the most effective therapy against *Pseudomonas aeruginosa* in adult patients (23), treatment adopting colistin at 2.5 or 5 mg/kg for infection caused by colistin-resistant *E. coli* proved to be ineffective. The recommended oral dosages for rifampin and azithromycin are 10 mg/kg and 500 mg, respectively, in clinical practice. The plasma $C_{\max}$ of oral rifampin taken at 10 mg/kg once daily is approximately 10.0 mg/liter in patients (24), which is close to the $C_{\max}$ (14.2 mg/liter) measured in this study. Therefore, the pharmacodynamics of 10 mg/kg rifampin in mice can reflect the actual status in clinical practice to some extent. Previous research showed that the dose and $C_{\max}$ of azithromycin required for equivalent efficacy in murine models are considerably higher than those required in humans due to the higher rate of azithromycin clearance in mice (25). Therefore, an azithromycin dosage of 100 mg/kg was used in the *in vivo* pharmacodynamics experiments according to the conversion criterion based on body surface area in the Food and Drug Administration's *Guidance for Industry: Estimating the Maximum Safe Starting Dose in Initial Clinical Trials for Therapeutics in Adult Healthy Volunteers* (26). However, from the results of *in vivo* PK experiments, treatment using the recommended dose of azithromycin (100 mg/kg) was ineffective against *E. coli*, even if the MIC value of azithromycin was below the susceptibility breakpoint for *Enterobacteriaceae* according to Clinical and Laboratory Standards Institute (CLSI) breakpoint recommendations (27).

According to a previous report, bactericidal antibiotics can stimulate the production

TABLE 2 Antibiotic MICs and genotypes of three multidrug-resistant *E. coli* strains^a

<i>E. coli</i> strain	Genotype	MIC (mg/liter)									
		CST	PMB	RIF	AZM	AMK	CIP	AMP	A/S	CTX	IPM
MZ1501 ^R	<i>mcr-1</i>	8	8	8	8	>128	32	>128	32/16	>128	0.5
HE1704 ^R	<i>mcr-1</i>	8	8	32	128	4	2	>128	32/16	0.125	0.125
MZ1509 ^S		0.5	0.5	>128	>128	16	128	>128	32/16	4	0.5

^aCST, colistin; PMB, polymyxin B; RIF, rifampin; AZM, azithromycin; AMK, amikacin; CIP, ciprofloxacin; AMP, ampicillin; A/S, ampicillin-sulbactam; CLI, clindamycin; CTX, cefotaxime; IPM, imipenem.

of hydroxyl radicals that contribute to bactericidal antibiotic-mediated cell death (28). It remains to be seen whether synergistic antibacterial action can be explained by an increased production of hydroxyl radicals, which could be stimulated by colistin-based combination therapy with rifampin and azithromycin. In addition, colistin destroys the outer membrane of Gram-negative bacteria and thereby increases the amounts of rifampin and azithromycin in the cytoplasm, offering another explanation.

Although colistin-based combination therapy with rifampin is generally regarded as safe for multidrug-resistant *A. baumannii*-induced severe infections in the clinic (20), colistin-based combination therapy with rifampin and azithromycin may increase toxicity in the kidneys and liver. Therefore, the potential side-effects of these three drugs should be considered in future treatment strategies. In addition, carbapenems and colistin have different antimicrobial and resistance mechanisms. Therefore, the colistin-based combination with rifampin and azithromycin may be effective against colistin-resistant carbapenem-resistant *Enterobacteriaceae* (CRE) or other Gram-negative bacteria. Further investigation is necessary.

In conclusion, the bactericidal activity of the triple combination of colistin plus both rifampin and azithromycin against *E. coli* strains producing MCR-1 *in vitro* and *in vivo* has been observed for the first time. This finding might provide a promising option for combination therapy for infections caused by colistin-resistant *E. coli* strains harboring the *mcr-1* gene.

MATERIALS AND METHODS

Bacterial strains. Two strains of *E. coli* were isolated from the feces of chicken, namely, colistin-resistant isolate MZ1501^R and colistin-susceptible isolate MZ1509^S, and colistin-resistant isolate HE1704^R was isolated from the fecal samples of a breeder from the same chicken farm in this study. The susceptibilities of the selected isolates and the quality control isolate (ATCC 25922) to colistin, polymyxin B, rifampin, azithromycin, amikacin, ciprofloxacin, ampicillin, ampicillin-sulbactam, cefotaxime, and imipenem were determined in triplicate by adopting the broth microdilution method using cation-adjusted Mueller-Hinton (MH) broth (Becton, Dickinson and Company, Sparks, MD). The susceptibility breakpoints for all drugs were interpreted according to CLSI breakpoint recommendations. PCR amplification of *mcr-1* for the selected isolates was conducted according to a previously reported method (5). The PCR products underwent sequence determination by the Beijing Genomics Institute (Guangzhou, China). Nucleotide sequences were typed into BLAST to analyze identities with published sequences. The MICs and genotypes of selected isolates are shown in Table 2.

Antibiotics and media. Colistin sulfate, rifampin, and azithromycin powders were purchased from Sigma-Aldrich and hydrated with free water, methyl alcohol, and 95% ethanol, respectively. Next, all drugs were filtered for sterilization by using 0.22- μ m nylon syringe filters (Fisher Scientific, Pittsburgh, PA) before dilution with culture medium at the moment of testing (the content of methyl alcohol and 95% ethanol in the broth was less than 5%, and antibacterial tests against strains were performed concurrently with the same concentration of methyl alcohol and 95% ethanol in the broth). Cation-adjusted Mueller-Hinton broth (25.0 mg/liter Ca²⁺ and 12.5 mg/liter Mg²⁺) (Becton, Dickinson and Company, Sparks, MD) was used in all experiments.

Static time-kill assays. To determine whether the effect of colistin combined simultaneously with rifampin and azithromycin was comparable to those of colistin, rifampin, and azithromycin alone or as a combination of two antibiotics between them, a 48-h time course was needed to evaluate the rate and extent of killing. Series of drug concentrations of colistin (0.5, 1, 2, 4, and 8 mg/liter), rifampin (0.5, 1, and 2 mg/liter), and azithromycin (0.5, 1, and 2.5 mg/liter) were close to or higher than the clinically or preclinically attainable unbound plasma concentrations (15, 16, 25, 29, 30). Three-by-three (colistin at 0.5, 1, and 2 mg/liter combined, respectively, with rifampin at 0.5, 1, and 2 mg/liter or azithromycin at 0.5, 1, and 2.5 mg/liter) and three-by-three-by-two (colistin at 0.5, 1, and 2 mg/liter combined, respectively, with rifampin at 0.5, 1, and 2 mg/liter and azithromycin at 0.5 and 2.5 mg/liter) concentration procedures were performed on both strains. A single bacterial colony from a plate was incubated overnight in preprepared cation-adjusted MH broth to attain a concentration of $\sim 10^8$ CFU/ml. Next, the antibiotics

were added to a logarithmic-phase culture at $\sim 10^7$ CFU/ml, which was obtained by taking a supplement of fresh medium. At the time points of 0, 1, 2, 4, 6, 8, 12, 24, 28, 32, 36, and 48 h after incubation, 100 μ l of each sample was withdrawn for colony counting. The lower limit of quantification was $2.0 \log_{10}$ CFU/ml. A growth control was performed in each experiment.

Pharmacokinetics in the mouse model. Six-week-old specific-pathogen-free male ICR mice weighing 24 to 27 g (Laboratory Animal Center of South China Agricultural University, China) were used in this experiment. The animals were raised in accordance with national standards for laboratory animals in China (33). Thirty-six mice in each dosage regimen were separated into nine groups, in which four mice were named mice A to D. Colistin sulfate (2.5 or 5 mg/kg) with subcutaneous injection in the neck region was accompanied by intragastric administration with both rifampin (10 mg/kg) and azithromycin (100 mg/kg) in thigh-infected neutropenic mice. We referred to the dosing strategies in other reports and tried to match the *in vitro* antimicrobial concentrations (25, 29, 30). Animals were then humanely killed, and blood samples were collected from a central vein at the given time ($n = 4$ mice per time point). Drug concentrations in the plasma of all A/B/C/D mice in different groups were used to calculate the PK parameters. The experimental protocol was approved by the Committee on the Ethics of Animals of South China Agricultural University.

Analysis method. The concentrations of colistin in plasma were determined by using a previously reported method that had been modified (31). Another method for the determination of rifampin and azithromycin concentrations was developed in this study. Briefly, an aliquot of 100- μ l plasma samples was mixed with 50 μ l 0.1% formic acid in water, followed by 250 μ l methanol. After vortexing, the mixtures were centrifuged at $1,000 \times g$ for 10 min at 4 °C. The supernatants were refined by using a 0.22- μ m nylon syringe filter. Five microliters was analyzed by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The entire analysis procedure was performed in the dark due to the photosensitivity of rifampin. Concentrations of colistin (390.8/101.1 [precursor ion/product ion] for colistin A and 386.2/101.0 [precursor ion/product ion] for colistin B), rifampin (823.8/791.5), and azithromycin (749.7/591.4) were determined simultaneously under a gradient elution program involving 0.1% formic acid in water (buffer A) and 0.1% formic acid in acetonitrile (buffer B) at a flow rate of 0.20 ml/min. The gradient elution program involved 6% buffer B from 0.0 to 1 min and an increase from 50% buffer B to 70% buffer B from 3.0 to 8.0 min; the ratio of buffer A and B is maintained from 8.1 min to 10 min. The limits of quantification for colistin, rifampin, and azithromycin ranged from 0.1 to 20 μ g/liter. The rates of recovery of all drugs ranged between 84.4 and 115.0%. The inter- and intra-assay coefficients of variation were $<10\%$. Pharmacokinetic parameters for three drugs in plasma samples were obtained by submitting the concentration-time data to WinNonlin software, version 5.2.1, for noncompartmental analysis.

Neutropenic mouse thigh infection model and *in vivo* treatment. Neutropenic (defined as neutrophil counts of ≤ 100 neutrophils/mm³) mice were generated by the intraperitoneal injection of cyclophosphamide (Puboxin Biotechnology Co., Ltd., Beijing, China) 4 days before the 150-mg/kg dose and 1 day before the 100-mg/kg dose prior to thigh infection (32). Mice were infected by injecting 0.1 ml of a logarithmic-phase bacterial suspension (10^6 to 10^7 CFU/ml) intramuscularly into each thigh (four thighs in each group).

In vivo treatments were conducted 2 h after inoculation to verify the difference in antimicrobial effects between monotherapy and the drug combinations. Colistin sulfate (2.5 and 5 mg/kg) was administered by subcutaneous injection every 12 h, while rifampin (10 mg/kg) and azithromycin (100 mg/kg) were given to mice by intragastric administration every 24 h. The mice were euthanized after 24 h or 48 h of the procedure. Next, four thighs from each group were immediately separated and homogenized in 10 ml of precooled 0.9% sterile saline. The serial dilutions of thigh homogenates were plated onto MH agar plates for CFU determination and incubated overnight at 37 °C. Untreated control mice were sacrificed at the same time.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.01631-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant number 3167130015) and the Natural Science Foundation of Guangdong Province, China (grant number 2015B090901059).

We thank the Guangdong Wens Dahuanong Biotechnology Co., Ltd. (Yunfu, China), for assistance with determination of drug concentrations in mouse plasma. We also thank the native-English-speaking scientists of Elixigen Company (Huntington Beach, CA) for editing the manuscript.

REFERENCES

1. Zhao F, Feng Y, Lü X, McNally A, Zong Z. 2017. IncP plasmid carrying colistin resistance gene *mcr-1* in *Klebsiella pneumoniae* from hospital sewage. *Antimicrob Agents Chemother* 61:e02229-16. <https://doi.org/10.1128/AAC.02229-16>.

2. Webb HE, Granier SA, Marault M, Millemann Y, den Bakker HC, Nightingale KK, Bugarel M, Ison SA, Scott HM, Loneragan GH. 2016. Dissemination of the *mcr-1* colistin resistance gene. *Lancet Infect Dis* 16:144–145. [https://doi.org/10.1016/S1473-3099\(15\)00538-1](https://doi.org/10.1016/S1473-3099(15)00538-1).
3. Thanh DP, Tuyen HT, Nguyen TNT, Hao CT, Wick RR, Thwaites GE, Baker S, Holt KE. 2016. Inducible colistin resistance via a disrupted plasmid-borne *mcr-1* gene in a 2008 Vietnamese *Shigella sonnei* isolate. *J Antimicrob Chemother* 71:2314–2317. <https://doi.org/10.1093/jac/dkw173>.
4. Zeng K-J, Doi Y, Patil S, Huang X, Tian G-B. 2016. Emergence of the plasmid-mediated *mcr-1* gene in colistin-resistant *Enterobacter aerogenes* and *Enterobacter cloacae*. *Antimicrob Agents Chemother* 60:3862–3863. <https://doi.org/10.1128/AAC.00345-16>.
5. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H, Shen J. 2016. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect Dis* 16:161–168. [https://doi.org/10.1016/S1473-3099\(15\)00424-7](https://doi.org/10.1016/S1473-3099(15)00424-7).
6. Aydemir H, Akduman D, Piskin N, Comert F, Horuz E, Terzi A, Kokturk F, Ornek T, Celebi G. 2013. Colistin vs. the combination of colistin and rifampicin for the treatment of carbapenem-resistant *Acinetobacter baumannii* ventilator-associated pneumonia. *Epidemiol Infect* 141:1214–1222. <https://doi.org/10.1017/S095026881200194X>.
7. Tascini C, Tagliaferri E, Giani T, Leonildi A, Flammini S, Casini B, Lewis R, Ferranti S, Rossolini GM, Menichetti F. 2013. Synergistic activity of colistin plus rifampin against colistin-resistant KPC-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 57:3990–3993. <https://doi.org/10.1128/AAC.00179-13>.
8. Sharma R, Patel S, Abboud C, Diep J, Ly NS, Pogue JM, Kaye KS, Li J, Rao GG. 2017. Polymyxin B in combination with meropenem against carbapenemase-producing *Klebsiella pneumoniae*: pharmacodynamics and morphological changes. *Int J Antimicrob Agents* 49:224–232. <https://doi.org/10.1016/j.ijantimicag.2016.10.025>.
9. Diep J, Jacobs DM, Sharma R, Covelli J, Bowers DR, Russo TA, Rao GG. 2017. Polymyxin B in combination with rifampin and meropenem against polymyxin B-resistant KPC-producing *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 61:e02121-16. <https://doi.org/10.1128/AAC.02121-16>.
10. Bozkurt-Güzel C, Gerçek AA. 2012. Post-antibiotic effect of colistin, alone and in combination with amikacin, on *Pseudomonas aeruginosa* strains isolated from cystic fibrosis patients. *J Antibiot (Tokyo)* 65:83–86. <https://doi.org/10.1038/ja.2011.101>.
11. Fulnecky EJ, Wright D, Scheld WM, Kanawati L, Shoham S. 2005. Amikacin and colistin for treatment of *Acinetobacter baumannii* meningitis. *J Infect* 51:e249–e251. <https://doi.org/10.1016/j.jinf.2005.04.003>.
12. Nikaido H. 1998. The role of outer membrane and efflux pumps in the resistance of gram-negative bacteria. Can we improve drug access? *Drug Resist Updat* 1:93–98. [https://doi.org/10.1016/S1368-7646\(98\)80023-X](https://doi.org/10.1016/S1368-7646(98)80023-X).
13. Buyck JM, Plésiat P, Traore H, Vanderbist F, Tulkens PM, Bambeke FV. 2012. Increased susceptibility of *Pseudomonas aeruginosa* to macrolides and ketolides in eukaryotic cell culture media and biological fluids due to decreased expression of *oprM* and increased outer-membrane permeability. *Clin Infect Dis* 55:534–542. <https://doi.org/10.1093/cid/cis473>.
14. Bosnar M, Dominis-Kramarić M, Nujić K, Stupin PD, Marjanović N, Glojnaric I, Eraković HV. 2013. Immunomodulatory effects of azithromycin on the establishment of lipopolysaccharide tolerance in mice. *Int Immunopharmacol* 15:498–504. <https://doi.org/10.1016/j.intimp.2013.02.011>.
15. Cheah SE, Wang J, Nguyen VT, Turnidge JD, Li J, Nation RL. 2015. New pharmacokinetic/pharmacodynamic studies of systemically administered colistin against *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in mouse thigh and lung infection models: smaller response in lung infection. *J Antimicrob Chemother* 70:3291–3297. <https://doi.org/10.1093/jac/dkv267>.
16. Jayaram R, Gaonkar S, Kaur P, Suresh BL, Mahesh BN, Jayashree R, Nandi V, Bharat S, Shandil RK, Kantharaj E, Balasubramanian V. 2003. Pharmacokinetics-pharmacodynamics of rifampin in an aerosol infection model of tuberculosis. *Antimicrob Agents Chemother* 47:2118–2124. <https://doi.org/10.1128/AAC.47.7.2118-2124.2003>.
17. Foulds G, Shepard RM, Johnson RB. 1990. The pharmacokinetics of azithromycin in human serum and tissues. *J Antimicrob Chemother* 25:73–82. https://doi.org/10.1093/jac/25.suppl_A.73.
18. Zhou YF, Tao MT, Feng Y, Yang RS, Liao XP, Liu YH, Sun J. 2017. Increased activity of colistin in combination with amikacin against *Escherichia coli* co-producing NDM-5 and MCR-1. *J Antimicrob Chemother* 72:1723–1730. <https://doi.org/10.1093/jac/dkx038>.
19. Giamarellos-Bourboulis EJ, Xirouchaki E, Giamarellou H. 2001. Interactions of colistin and rifampin on multidrug-resistant *Acinetobacter baumannii*. *Diagn Microbiol Infect Dis* 40:117–120. [https://doi.org/10.1016/S0732-8893\(01\)00258-9](https://doi.org/10.1016/S0732-8893(01)00258-9).
20. Bassetti M, Repetto E, Righi E, Boni S, Diverio M, Molinari MP, Mussap M, Artioli S, Ansaldi F, Durando P, Orengo G, Bobbio Pallavicini F, Viscoli C. 2008. Colistin and rifampicin in the treatment of multidrug-resistant *Acinetobacter baumannii* infections. *J Antimicrob Chemother* 61:417–420. <https://doi.org/10.1093/jac/dkm509>.
21. White RL, Burgess DS, Manduru M, Bosso JA. 1996. Comparison of three different *in vitro* methods of detecting synergy: time-kill, checkerboard, and E test. *Antimicrob Agents Chemother* 40:1914–1918.
22. Plachouras D, Karvanen M, Friberg LE, Papadomichelakis E, Antoniadou A, Tsangaris I, Karaikos I, Poulakou G, Kontopidou F, Armaganidis A, Cars O, Giamarellou H. 2009. Population pharmacokinetic analysis of colistin methanesulfonate and colistin after intravenous administration in critically ill patients with infections caused by Gram-negative bacteria. *Antimicrob Agents Chemother* 53:3430–3436. <https://doi.org/10.1128/AAC.01361-08>.
23. Daikos GL, Skiada A, Pavleas J, Vafiadi C, Salatas K, Tofas P, Tzanetou K, Markogiannakis A, Thomopoulos G, Vafiadi I, Petrikos G. 2010. Serum bactericidal activity of three different dosing regimens of colistin with implications for optimum clinical use. *J Chemother* 22:175–178. <https://doi.org/10.1179/joc.2010.22.3.175>.
24. Ruslami R, Nijland HMJ, Alisjahbana B, Parwati I, Crevel RV, Aarnoutse RE. 2007. Pharmacokinetics and tolerability of a higher rifampin dose versus the standard dose in pulmonary tuberculosis patients. *Antimicrob Agents Chemother* 51:2546–2551. <https://doi.org/10.1128/AAC.01550-06>.
25. Girard D, Finegan SM, Dunne MW, Lame ME. 2005. Enhanced efficacy of single-dose versus multi-dose azithromycin regimens in preclinical infection models. *J Antimicrob Chemother* 56:365–371. <https://doi.org/10.1093/jac/dki241>.
26. Food and Drug Administration. 2005. Guidance for industry: estimating the maximum safe starting dose in initial clinical trials for therapeutics in adult healthy volunteers. Food and Drug Administration, Washington, DC.
27. Clinical and Laboratory Standards Institute. 2018. Performance standards for antimicrobial susceptibility testing, 28th ed. CLSI supplement M100. Clinical and Laboratory Standards Institute, Wayne, PA.
28. Kohanski MA, Dwyer DJ, Hayete B, Lawrence CA, Collins JJ. 2007. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 130:797–810. <https://doi.org/10.1016/j.cell.2007.06.049>.
29. Dudhani RV, Turnidge JD, Coulthard K, Milne RW, Rayner CR, Li J, Nation RL. 2010. Elucidation of the pharmacokinetic/pharmacodynamic determinant of colistin activity against *Pseudomonas aeruginosa* in murine thigh and lung infection models. *Antimicrob Agents Chemother* 54:1117–1124. <https://doi.org/10.1128/AAC.01114-09>.
30. Lyons MA, Reisfeld B, Yang RSH, Lenaerts AJ. 2013. A physiologically based pharmacokinetic model of rifampin in mice. *Antimicrob Agents Chemother* 57:1763–1771. <https://doi.org/10.1128/AAC.01567-12>.
31. Ma Z, Wang J, Gerber JP, Milne RW. 2008. Determination of colistin in human plasma, urine and other biological samples using LC-MS/MS. *J Chromatogr Anal Technol Biomed Life Sci* 862:205–212. <https://doi.org/10.1016/j.jchromb.2007.12.009>.
32. Vogelmeier B, Gudmundsson S, Turnidge J, Leggett J, Craig WA. 1988. *In vivo* postantibiotic effect in a thigh infection in neutropenic mice. *J Infect Dis* 157:287–298. <https://doi.org/10.1093/infdis/157.2.287>.
33. Standardization Administration of the People's Republic of China. GB 14925-2010. Laboratory animal: requirements of environment and housing facilities. Beijing, China.