

# Effects of Efflux Pump Inhibitors on Colistin Resistance in Multidrug-Resistant Gram-Negative Bacteria

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**We tested the effects of various putative efflux pump inhibitors on colistin resistance in multidrug-resistant Gram-negative bacteria. Addition of 10 mg/liter cyanide 3-chlorophenylhydrazone (CCCP) to the test medium could significantly decrease the MICs of colistin-resistant strains. Time-kill assays showed CCCP could reverse colistin resistance and inhibit the regrowth of the resistant subpopulation, especially in *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*. These results suggest colistin resistance in Gram-negative bacteria can be suppressed and reversed by CCCP.**

Colistin is regarded as the last resort antibiotic for infections caused by multidrug-resistant (MDR) and extensively drug-resistant (XDR) Gram-negative bacteria. However due to colistin's widespread use, resistant strains are increasingly being isolated in the clinic (1). Resistance to colistin has been reported to be only chromosomally mediated. However, very recently, the finding of a transferable plasmid-mediated colistin resistance gene, *mcr-1*, suggests the possibility of quick acquisition of resistance (2, 3). If the *mcr-1* gene is similar to the case of *NDM-1*, colistin-resistant bacteria may soon become endemic in the world.

Therefore, it is of paramount importance to prevent the dissemination of colistin resistance in an era that lacks new antibiotics against resistant Gram-negative pathogens. Besides, the finding and development of agents that effectively reverse resistance may be a promising strategy. Efflux pump inhibitors (EPIs) are potential agents in this category, and there have been initial reports on EPIs. A recent study showed that the addition of cyanide 3-chlorophenylhydrazone (CCCP) could decrease the MICs of colistin in *Acinetobacter baumannii* (4), but whether this phenomenon is strain specific or CCCP can reverse colistin resistance in Gram-negative bacteria deserves further investigation, and whether other commonly used EPIs have the same effects remains unknown. In order to answer these questions, we evaluated the effect of various putative EPIs on resistance to colistin in multidrug-resistant Gram-negative bacteria.

Nonduplicate colistin-susceptible and colistin-resistant clinical isolates of *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, and *Stenotrophomonas maltophilia* that also show resistance to at least other three antibiotic classes were collected between May 2014 and October 2015 from separate patients in three tertiary hospitals of Beijing, China. Colistin, CCCP, phenylarginine- $\beta$ -naphthylamide (PA $\beta$ N), 1-(1-naphthylmethyl)-piperazine (NMP), omeprazole, and verapamil standards were purchased from Sigma-Aldrich (Shanghai, China). Reserpine standards were obtained from the National Institute for the Control of Pharmaceutical and Biological Products, China (Beijing, China).

MICs of EPIs, colistin, and colistin combined with EPIs were determined by the agar dilution method according to CLSI performance and interpretive guidelines (5). CCCP, NMP, and omeprazole were dissolved in dimethyl sulfoxide (DMSO) and

prepared as a 5-mg/ml stock solution, PA $\beta$ N was dissolved in sterile water and prepared as a 1.5-mg/ml stock solution, verapamil was dissolved in MeOH and prepared as a 50-mg/ml stock solution, and reserpine was dissolved in chloroform and prepared as a 1.5-mg/ml stock solution. Then Mueller-Hinton agar (MHA) plates containing a series of 2-fold-concentration increments of EPIs, colistin, or colistin in combination with either CCCP (10 mg/liter), PA $\beta$ N (25 mg/liter), NMP (25 mg/liter), omeprazole (20 mg/liter), verapamil (80 mg/liter), or reserpine (20 mg/liter) were freshly prepared. Strains were thawed and inoculated on agar plates with 5% sheep blood and incubated for 18 to 24 h at 35°C. Subsequently, drug plates were inoculated with a suspension of a 0.5 McFarland standard. The plates were incubated for 18 to 24 h at 35°C in ambient air before values were read. All MIC tests were carried out in triplicate on separate days.

To confirm the effect of EPIs on reversing colistin resistance, we randomly selected one isolate of each bacterial species to perform *in vitro* time-kill assays of colistin in the presence or absence of EPIs. In brief, strains were grown overnight in Mueller-Hinton II broth (MHB) medium. Then stationary-phase cells were reinoculated into fresh MHB containing 3 mg/liter of colistin with or without EPIs (at the same concentrations used in colistin MIC tests) to yield a density of  $\sim 5 \times 10^5$  CFU/ml. The cultures were incubated in a shaking bath at 35°C for 48 h. Samples were obtained at times zero, 3, 6, 12, 24, 36, and 48 h after inoculation and serially diluted in sterile 0.85% sodium chloride solution for determination of viable counts. The diluted samples, in 0.05-ml aliquots, were plated in duplicate on MHA plates. After the diluted samples were incubated at 35°C for 24 h in ambient air, formed colonies were counted, and the total bacterial log<sub>10</sub> CFU per mil-

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**TABLE 1** Intrinsic inhibitory effects of different EPIs on clinical isolates of 4 species of MDR Gram-negative bacteria

Colistin susceptibility/resistance by species ( <i>n</i> isolates)	MIC range (mg/liter)					
	CCCP	NMP	PAβN	Omeprazole	Verapamil	Reserpine
<b>Susceptible</b>						
<i>A. baumannii</i> (20)	40–80	250–>250	>200	>100	>400	>100
<i>K. pneumoniae</i> (20)	100–>100	>250	>200	>100	>400	>100
<i>P. aeruginosa</i> (26)	>100	>250	>200	>100	>400	>100
<i>S. maltophilia</i> (7)	40–100	>250	>200	>100	>400	>100
<b>Resistant</b>						
<i>A. baumannii</i> (4)	40–80	>250	>200	>100	>400	>100
<i>K. pneumoniae</i> (6)	50–80	>250	>200	>100	>400	>100
<i>P. aeruginosa</i> (5)	>100	>250	>200	>100	>400	>100
<i>S. maltophilia</i> (16)	30–80	>250	>200	>100	>400	>100

liliter of the original sample was calculated. All of the *in vitro* time-kill experiments were performed in triplicate on different days. The statistical analysis was conducted using GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA). Results are expressed as means ± standard deviation (SD).

**Table 1** shows the MIC range of EPIs for each bacterial species. The MICs of CCCP for various isolates were lower than those of other EPIs. Moreover, compared with colistin-susceptible *K. pneumoniae* and *S. maltophilia* strains, the MICs of colistin-resistant strains were relatively lower. However, generally, all EPIs had no intrinsic inhibitory activity against clinical isolates at the concentrations used for the colistin MIC and time-kill assays. **Table 2** shows the MIC range and the reduction in MICs of colistin for each species after the addition of EPIs. Particularly strong effects with CCCP were seen in colistin-resistant strains of all species. Besides, the addition of CCCP slightly reduced the MICs (MIC of ≤2 mg/liter) in colistin-susceptible strains of *S. maltophilia*. NMP had limited effects on MICs in both colistin-susceptible and colistin-resistant strains of *S. maltophilia*. In addition, PAβN could reduce the MICs by 4-fold or more in colistin-susceptible strains of *P. aeruginosa*, while having no effects on MICs in colistin-resistant strains. For the other three EPIs, no or minor effects on the MICs were observed, no matter whether in colistin-susceptible or colistin-resistant strains.

The results of *in vitro* time-kill assays are displayed in **Fig. 1**. For all colistin-susceptible strains, bacterial cell counts were reduced

in the first 6 h of testing when they were exposed to colistin alone, but then regrowth was observed. After exposure to both colistin and CCCP, the bacterial cell counts of colistin-susceptible *A. baumannii* and *S. maltophilia* strains fell below the lower threshold of detection at 6 h and remained so throughout the 48-h testing period. The bacterial cell counts of colistin-susceptible *K. pneumoniae* and *P. aeruginosa* strains also fell below the lower threshold of detection at 12 h, but regrowth occurred. For colistin-resistant *A. baumannii* strains, a single regimen of colistin showed no inhibitory effects on bacterial growth, while the addition of CCCP could completely suppress the growth. For colistin-resistant *K. pneumoniae*, *P. aeruginosa*, and *S. maltophilia* strains, reduction of bacterial cell numbers was observed after exposure to colistin and CCCP, but various degrees of regrowth were observed, and the effects of CCCP on *P. aeruginosa* were less pronounced, which was in accordance with the results of MIC tests. Besides, the addition of PAβN displayed inhibitory effects on colistin-susceptible *P. aeruginosa* strains but had no effects on the colistin-resistant strain. The effects of NMP on *S. maltophilia* were similar to those of PAβN for *P. aeruginosa*. After the addition of NMP, the bacterial cell counts of colistin-susceptible *S. maltophilia* strains could fall below the lower threshold of detection at 12 h, while the cell counts of colistin-resistant *S. maltophilia* strains were only slightly reduced in the first 3 h of testing, indicating the negligible inhibitory effects.

Our *in vitro* studies showed that the addition of low-dose

**TABLE 2** Effects of different EPIs on the MICs of colistin in clinical isolates of 4 species of MDR Gram-negative pathogens

Colistin susceptibility/resistance by species ( <i>n</i> isolates)	MIC range (mg/liter)	MIC range in mg/liter (fold MIC reduction) after addition of <sup>a</sup> :					
		CCCP	NMP	PAβN	Omeprazole	Verapamil	Reserpine
<b>Susceptible</b>							
<i>A. baumannii</i> (20)	0.25–1	0.25–1 (0–1)	0.25–1 (0–1)	0.25–1 (0)	0.25–1 (0)	0.25–1 (0)	0.25–1 (0)
<i>K. pneumoniae</i> (20)	0.125–1	0.125–1 (0–1)	0.125–1 (0–1)	0.125–1 (0)	0.125–1 (0)	0.125–1 (0)	0.125–1 (0)
<i>P. aeruginosa</i> (26)	0.5–2	0.5–2 (0)	0.5–2 (0)	<b>0.03–0.25 (4–16)</b>	0.5–2 (0)	0.5–2 (0)	0.5–2 (0)
<i>S. maltophilia</i> (7)	0.5–2	<b>0.125–0.5 (1–4)</b>	<b>0.25–1 (1–4)</b>	0.5–2 (0)	0.5–2 (0)	0.5–2 (0)	0.5–2 (0)
<b>Resistant</b>							
<i>A. baumannii</i> (4)	64–256	<b>0.25–0.5 (128–512)</b>	64–256 (0–1)	64–128 (0–1)	64–256 (0)	64–256 (0)	64–256 (0)
<i>K. pneumoniae</i> (6)	64–256	<b>0.25–1 (128–512)</b>	64–256 (0)	64–256 (0)	64–256 (0)	64–256 (0–1)	32–128 (0–1)
<i>P. aeruginosa</i> (5)	16–64	<b>0.5–2 (16–32)</b>	16–64 (0)	16–64 (0)	16–64 (0)	16–64 (0)	16–64 (0)
<i>S. maltophilia</i> (16)	16–256	<b>0.125–1 (64–1,024)</b>	<b>1–128 (1–16)</b>	16–256 (0)	8–256 (0–1)	16–256 (0–1)	4–256 (0–4)

<sup>a</sup> Particularly strong effects are highlighted in boldface.

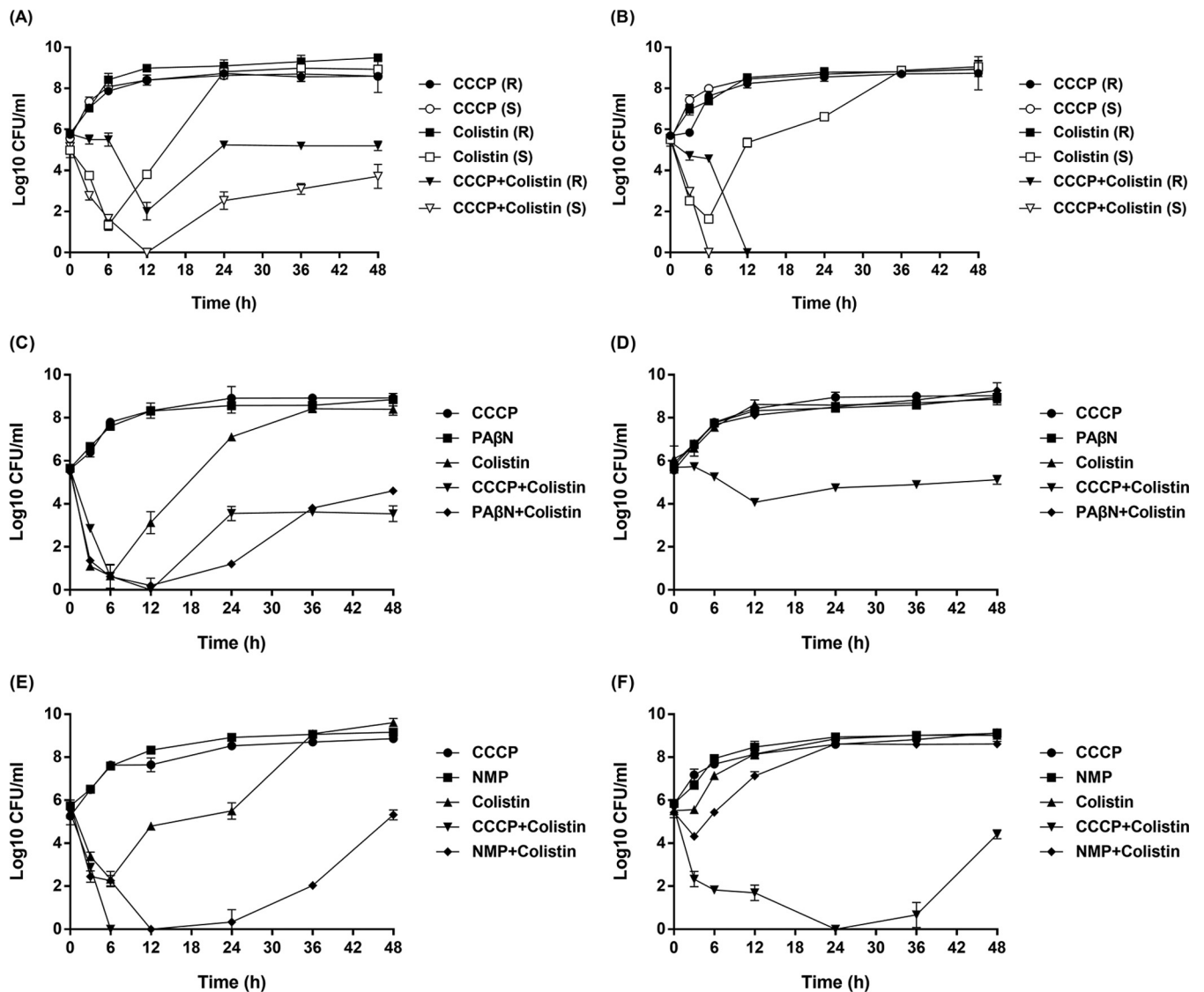


FIG 1 *In vitro* time-kill curves of colistin (3 mg/liter) alone and combined with EPIs against colistin-susceptible and colistin-resistant MDR Gram-negative bacteria. (A) Colistin-resistant *K. pneumoniae* (R) with MIC<sub>colistin</sub> of 256 mg/liter and MIC<sub>colistin+CCCP</sub> of 1 mg/liter and colistin-susceptible *K. pneumoniae* (S) with MIC<sub>colistin</sub> of 0.25 mg/liter and MIC<sub>colistin+CCCP</sub> of 0.25 mg/liter. (B) Colistin-resistant *A. baumannii* (R) with MIC<sub>colistin</sub> of 128 mg/liter and MIC<sub>colistin+CCCP</sub> of 0.25 mg/liter and colistin-susceptible *A. baumannii* (S) with MIC<sub>colistin</sub> of 0.5 mg/liter and MIC<sub>colistin+CCCP</sub> of 0.5 mg/liter. (C) Colistin-susceptible *P. aeruginosa* with MIC<sub>colistin</sub> of 64 mg/liter, MIC<sub>colistin+CCCP</sub> of 1 mg/liter, MIC<sub>colistin+PAβN</sub> of 64 mg/liter, and MIC<sub>colistin+PAβN+CCCP</sub> of 0.25 mg/liter. (D) Colistin-resistant *P. aeruginosa* with MIC<sub>colistin</sub> of 64 mg/liter, MIC<sub>colistin+CCCP</sub> of 2 mg/liter, and MIC<sub>colistin+PAβN</sub> of 64 mg/liter. (E) Colistin-susceptible *S. maltophilia* with MIC<sub>colistin</sub> of 1 mg/liter, MIC<sub>colistin+CCCP</sub> of 0.5 mg/liter, and MIC<sub>colistin+NMP</sub> of 0.5 mg/liter. (F) Colistin-resistant *S. maltophilia* with MIC<sub>colistin</sub> of 64 mg/liter, MIC<sub>colistin+CCCP</sub> of 0.5 mg/liter, and MIC<sub>colistin+NMP</sub> of 8 mg/liter.

CCCP to the test medium could significantly decrease the MICs of colistin-resistant strains and partially or completely inhibit the regrowth of the resistant subpopulation during the time-kill assays. Among other EPIs, only PAβN and NMP could partially inhibit the regrowth of the resistant subpopulation in *P. aeruginosa* and *S. maltophilia*, respectively. These findings suggest CCCP can suppress and reverse colistin resistance in MDR Gram-negative bacteria, especially in *A. baumannii* and *S. maltophilia*. CCCP is an uncoupler of oxidative phosphorylation, which can disrupt the proton gradient of the bacterial membranes (6). In addition, colistin with a positive charge targets lipopolysaccharide (LPS) of the membranes, displaces Mg<sup>2+</sup> or Ca<sup>2+</sup>, and binds the lipid A

component, resulting in the destabilization and disruption of the bacterial membrane (7). The most common mechanism of colistin resistance is its decreased binding to the bacterial outer membrane because of the decreased negative charge by LPS modifications (8). Therefore, CCCP may restore the negative charge of the membranes through the disruption of proton motive force. Nevertheless, the concrete mechanisms by which CCCP influences colistin resistance in Gram-negative bacteria require further investigation.

In summary, colistin resistance of Gram-negative bacteria can be suppressed and reversed by CCCP. Although CCCP cannot be directly applied in the clinic due to its intrinsic cytotoxicity, the

results of our study point out the way for the development of new agents to protect and potentiate the activity of colistin.

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