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Pterostilbene, a Potential MCR-1 Inhibitor That Enhances the Efficacy of Polymyxin B

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ABSTRACT We characterized the synergistic effect produced between pterostilbene and polymyxin B (fractional inhibitory concentration [FIC] index = 0.156 or 0.188) against MCR-producing *Escherichia coli* strains of both human and animal origins. The time-killing assays showed that either pterostilbene or polymyxin B failed to eradicate the *mcr-1*- and NDM-positive *E. coli* strain ZJ487, but the combination eliminated the strain by 1 h postinoculation. The survival rate of mice after intraperitoneal infections was significantly enhanced from 0% to 60% in the group in which combination therapy was applied.

KEYWORDS MCR-1, *Enterobacteriaceae*, polymyxin B, inhibitor, pterostilbene

The increasing incidence of carbapenem-resistant *Enterobacteriaceae* (CRE) in human clinical settings is now recognized as one of the most serious global threats to public health (1). Polymyxins, including polymyxin B and polymyxin E (colistin), are a class of multicomponent polypeptide antibiotics that differ by only one amino acid but display similar pharmacodynamics *in vitro* (2). They are now considered to be one of the "last-line" treatments for serious infections caused by CRE, most of which carry bla_{NDM} or bla_{PKC} (3). The important role of polymyxins in human clinical medicine has led to the reconsideration and restriction of its extensive usage in livestock (4, 5).

The spread of the plasmid-mediated colistin resistance gene *mcr-1* is breaking down the previous vulnerability of CRE infections to polymyxin administration (6). To date, the *mcr-1* gene has mostly been identified in *Escherichia coli* isolates from animals, humans, and food samples, but it has also been found in isolates belonging to other enterobacterial species from more than 40 countries (7). Critically, the emergence of *mcr-1* makes the development of pan-drug resistance by *Enterobacteriaceae* and the subsequent global dissemination of these "superbugs" highly likely (8). Therefore, a novel and effective strategy is urgently needed to deal with the serious challenges posed by MCR-1. Herein, we describe the identification of a novel MCR-1 inhibitor, pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene), which is obtained from fresh leaves or fruits and has been extensively studied for its potent anticancer, anti-inflammatory, and antioxidant activities (9), that enhanced the therapeutic effect of polymyxins both *in vitro* and *in vivo* experiments.

We applied a broth microdilution checkerboard method (10) to identify potential synergies between different natural compounds (n = 115) (see Table S1 in the supplemental material) and polymyxin B, and we examined the antibacterial activities of these combinations against both polymyxin-resistant strains (positive for MCR-1) and polymyxin-sensitive strains (negative for MCR-1) after 24 h of incubation at 37°C. Four of the *mcr-1*-positive *E. coli* isolates, namely, ZJ478, ZJ69, ZJ378, and DZ2-12R, were collected during our previous studies (11, 12). The *mcr-1*-negative *Klebsiella pneumoniae* strain K7 (13), the *mcr-1*-negative *Salmonella enterica* serovar Typhimurium

Received 18 October 2017 **Returned for modification** 29 November 2017 **Accepted** 4 January 2018

Accepted manuscript posted online 16 January 2018

Citation Zhou Y, Liu S, Wang T, Li H, Tang S, Wang J, Wang Y, Deng X. 2018. Pterostilbene, a potential MCR-1 inhibitor that enhances the efficacy of polymyxin B. Antimicrob Agents Chemother 62:e02146-17. https://doi.org/10.1128/AAC.02146-17.

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TABLE 1 MIC values of the polymyxin B and pterostilbene combination therapy for each of the tested bacterial isolates

Strain	Source	Reference	*mcr-1* confirmation[a]	Antibiotic compound	MIC (μg/ml [fold change])[b]		FIC index
					Alone	Combination	
E. coli ZJ478	Human intraabdominal fluid (bla_{NDM-1} carrying)	11	+	Polymyxin B	8	1 (8)	0.156
				Pterostilbene	1,024	32	
E. coli ZJ69	Human urine	11	+	Polymyxin B	8	1 (8)	0.188
				Pterostilbene	512	32	
E. coli ZJ378	Human feces	11	+	Polymyxin B	8	1 (8)	0.156
				Pterostilbene	1,024	32	
E. coli DZ2-12R	Chicken cloacae	12	+	Polymyxin B	8	1 (8)	0.156
				Pterostilbene	1,024	32	
E. coli DH5α(pUC19-*mcr-1*)	Laboratory strain (carries an *mcr-1* gene that originated from ZJ478)		+	Polymyxin B	8	2 (4)	0.313
				Pterostilbene	512	32	
E. coli DH5α(pUC19)	Laboratory strain		−	Polymyxin B	0.5	0.25 (2)	0.563
				Pterostilbene	512	32	
E. coli W3110(pUC19-*mcr-1*)	Laboratory strain (carries an *mcr-1* gene that originated from ZJ478)		+	Polymyxin B	8	1 (8)	0.156
				Pterostilbene	1,024	32	
E. coli W3110(pUC19)	Laboratory strain		−	Polymyxin B	0.5	0.25 (2)	0.531
				Pterostilbene	1,024	32	
E. coli ATCC 25922	Laboratory strain		−	Polymyxin B	0.5	0.25 (2)	0.531
				Pterostilbene	1,024	32	
S. Typhimurium SL1344	Derived from the virulent strain SL1344	14	−	Polymyxin B	1	1 (1)	1.031
				Pterostilbene	1,024	32	
A. baumannii ATCC 19606	Laboratory strain		−	Polymyxin B	2	1 (2)	0.531
				Pterostilbene	1,024	32	
K. pneumoniae K7	Human	13	−	Polymyxin B	2	2 (1)	1.031
				Pterostilbene	1,024	32	
E. coli ZJ478	Human intraabdominal fluid (bla_{NDM-1} carrying)		+	Colistin	8	2 (4)	0.266
				Pterostilbene	1,024	16	
E. coli W3110(pUC19-*mcr-1*)	Laboratory strain (carries an *mcr-1* gene that originated from ZJ478)		+	Colistin	16	2 (8)	0.141
				Pterostilbene	1,024	16	

[a]+, *mcr-1*-positive strain; −, *mcr-1*-negative strain.
[b]All MICs were determined in triplicates.

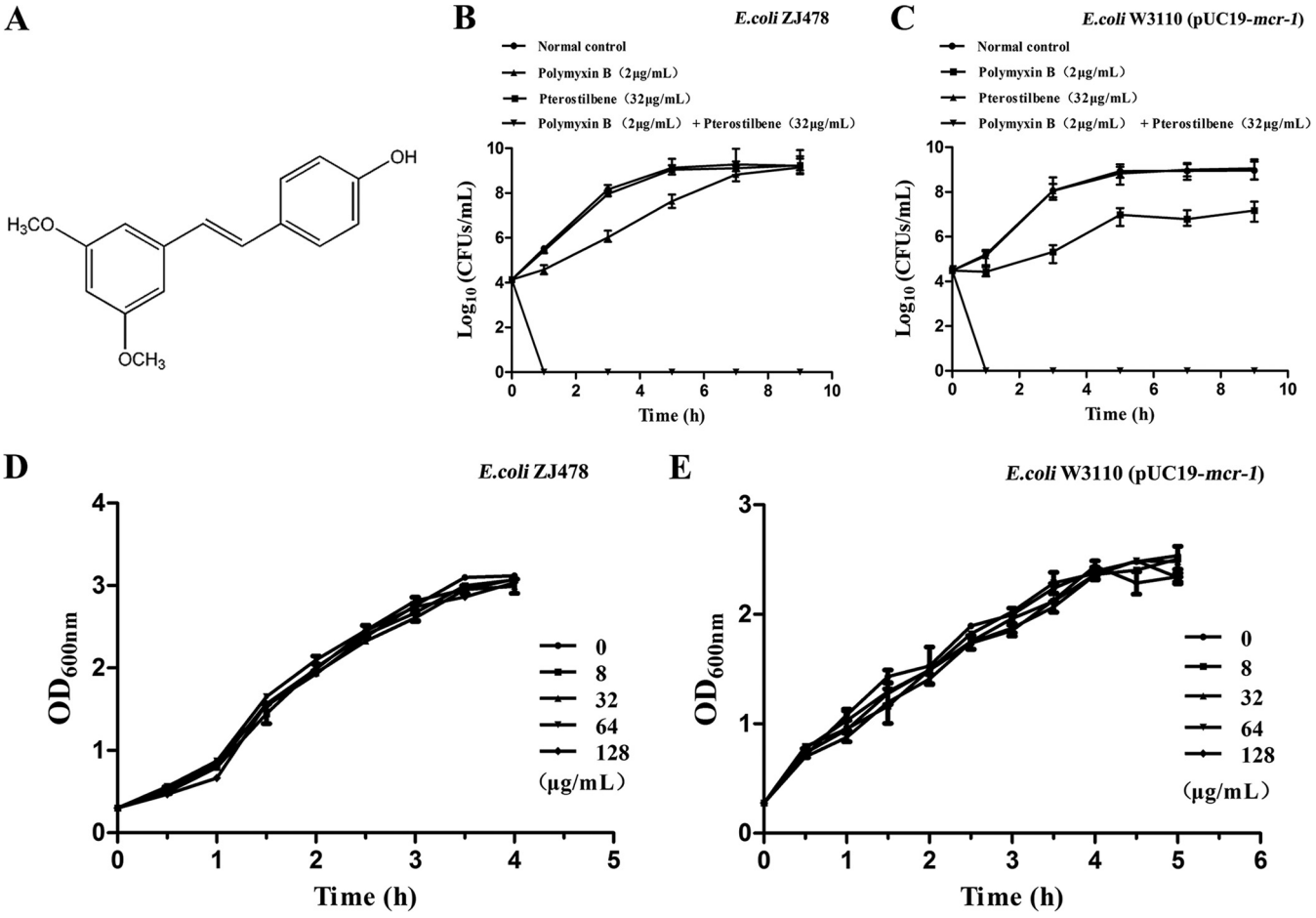

FIG 1 Effects of pterostilbene on carbapenem-resistant *Escherichia coli* ZJ478 and *E. coli* W3110(pUC19-*mcr-1*) *in vitro*. (A) Chemical structure of pterostilbene. Time-killing curves of polymyxin B, pterostilbene, polymyxin B plus pterostilbene, and a control treatment (only medium without drug or natural compound) against *E. coli* ZJ478 (B) and *E. coli* W3110(pUC19-*mcr-1*) (C). Growth curves for *E. coli* ZJ478 (D) and *E. coli* W3110(pUC19-*mcr-1*) (E) cultured with various concentrations (0 to 128 μg/ml) of pterostilbene. The data are the means plus standard errors from three independent experiments.

strain SL1344 (14), and *E. coli* strain ATCC 25922 were used as negative-control strains (Table 1).

The MIC assay using the CRE strain ZJ478 was performed to screen the efficacies of all tested compounds, and a fractional inhibitory concentration (FIC) value of $\leq$0.5 was defined as indicating a synergistic effect for the combination of the tested natural compound and polymyxin B (15). One of the tested natural compounds, pterostilbene, was identified as having a synergistic effect with polymyxin B (Fig. 1A). The results from an assay applying the broth microdilution checkerboard method further confirmed the synergistic effect between pterostilbene and polymyxin B in *mcr-1*-positive clinical strains (FIC = 0.156 or 0.188), with an 8-fold decrease in the MIC of polymyxin B (from 8 to 1 μg/ml) in the presence of 32 μg/ml pterostilbene, whereas no synergy was observed in any of the tested polymyxin-sensitive strains (Table 1). Additionally, a synergistic effect between pterostilbene and colistin was also observed in *mcr-1*-carrying strains (Table 1). The potential bactericidal effect of pterostilbene (32 μg/ml) combined with polymyxin B (2 μg/ml) was then evaluated using time-killing assays (16). This combination had an efficient bactericidal effect against clinical strain ZJ478, killing the bacteria by 1 h postinoculation (Fig. 1B and C). The results from a growth curve assay (17) show that pterostilbene with various concentrations from 0 to 128 μg/ml did not affect the growth of the original *mcr-1*-positive strain ZJ478 or of W3110 carrying pUC19-*mcr-1* (Fig. 1D and E). These findings suggest that pterostilbene could affect the function of MCR-1 and restore the antibacterial activity of polymyxin B.

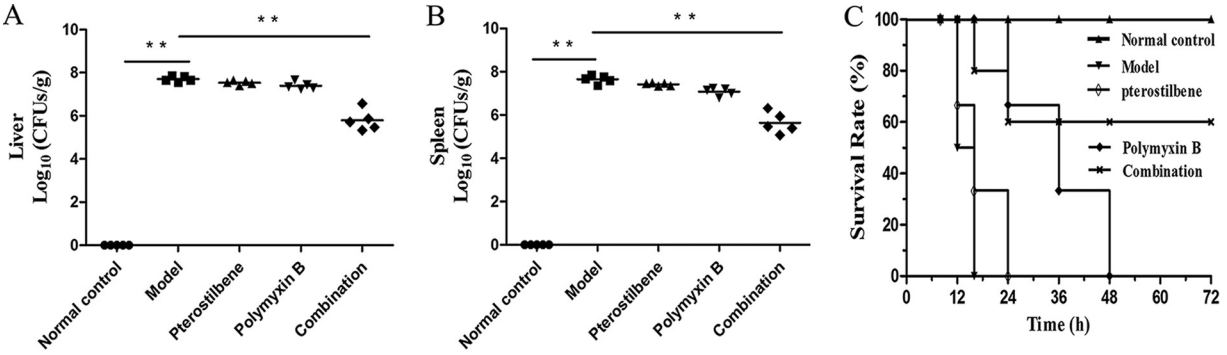

FIG 2 Effects of pterostilbene and polymyxin B combination therapy *in vivo*. Mice were infected with carbapenem-resistant *Escherichia coli* ZJ478 after treatment with pterostilbene, polymyxin B, pterostilbene combined with polymyxin B (combination), or a control solvent (model) or were left uninfected (normal control). Bacterial loads in the livers (A) and spleens (B). **, $P < 0.01$. (C) The survival of the mice was monitored for 3 days postinfection. The data are the means from three separate experiments using 5, 6, and 6 mice for the first, second, and third assays, respectively, for a total of 17 mice used for each group.

The results of *in vivo* treatment experiments further illustrate the synergy of pterostilbene in combination with polymyxin B. A total of 85 female 6- to 8-week-old BALB/c mice were infected intraperitoneally with a lethal dose of *E. coli* ZJ478 (2×10^8 CFU) to cause a systemic infection, and the effects of pterostilbene or polymyxin B monotherapy or of the combination therapy were evaluated. The 68 infected mice were subcutaneously administered polymyxin B (5 mg/kg of body weight, $n = 17$), pterostilbene (80 mg/kg, $n = 17$), a combination ($n = 17$) of pterostilbene (80 mg/kg) and polymyxin B (5 mg/kg), or solvent ($n = 17$) on the same schedule at 30 min postinfection. An additional 17 mice were used for a blank control. All these mice were monitored for survival until day 3 postinfection. To determine the liver and spleen bacterial loads and perform histopathological experiments, the mice were infected intraperitoneally with a dose of 1×10^8 CFU of *E. coli* ZJ478 and treated with the same therapeutics. The spleens and livers were harvested at 24 h postinfection. The combination treatment led to significant remission of liver and spleen pathological damage, as demonstrated by histopathology (see Fig. S1, black arrows). Additionally, the combination therapy significantly reduced the bacterial loads in the spleen and liver following subcutaneous administration ($P < 0.01$). A reduction in CFU counts of more than one order of magnitude was observed, on average, in the livers and spleens of the mice in the combination therapy group compared with those in the organs of groups treated with pterostilbene or polymyxin B alone (Fig. 2A and B). The administration of the combination therapy significantly improved the survival rate, with survival increasing from 0% (solvent-treated controls) to 60% (combination therapy group) (Fig. 2C). Interestingly, neither pterostilbene nor polymyxin B alone was able to prevent lethality following infection with *E. coli* ZJ478. Together, these results indicate that the combination of polymyxin B and pterostilbene may be efficient for treating infections caused by *mcr-1*-positive and carbapenem-resistant *Enterobacteriaceae*.

Here, we propose the use of pterostilbene, an MCR-1 inhibitor, in combination with polymyxins as a treatment against colistin-resistant *Enterobacteriaceae* (18). Pterostilbene reportedly exhibits antibacterial activity against drug-resistant *Staphylococcus aureus* without exerting unacceptable cytotoxicity (0.01, 0.05, and 0.125 mM) on mammalian cells (19), and the oral administration of a high dose (3,000 mg/kg/day) of pterostilbene for approximately 30 days is not associated with significant local or systemic toxicity in mice (20). Pterostilbene is also generally safe for human consumption at doses of up to 250 mg per day and functions as a dietary supplement to decrease the risk of coronary heart disease (21). All these results indicate that this natural compound is likely to be safe if applied in human clinical practice. However, the exact mode of MCR-1 inhibition by pterostilbene still needs to be further examined.

In addition to the global spread of *mcr-1*, another widely disseminated mobile

colistin resistance gene, *mcr-3*, has been found in European countries and China (22, 23). Our preliminary experiments in one *mcr-3*-positive strain demonstrated that pterostilbene is equally effective in combination with polymyxin B against this novel *mcr*-carrying strain (see Table S2), suggesting the potential inhibitory function of the combination therapy on other mcr variant-carrying isolates. However, further studies on the synergistic mechanism and the potential effect of this combination therapy on other MCR-3-producing strains are still needed.

In conclusion, this study shows that the combination of polymyxins with pterostilbene is a promising alternative treatment option for combating infections caused by MCR-positive carbapenem-resistant *Enterobacteriaceae*. Selectively targeting MCR using the natural compound pterostilbene is an attractive strategy, as this approach may not exert the direct selective pressure associated with current antimicrobial agents. Further studies, including preclinical investigations of inhibitor-antibiotic combinations, are warranted to evaluate the efficacy of this combination against additional MCR-producing bacterial species.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02146-17.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

This work was supported by the National Key R&D Program of China (2017YFD0501500), the National Natural Science Foundation of China (31422055 and 81661138002), and the National Key Technology R&D Program (no. 2016YFD 05013).

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