In Vitro/Vivo Activity of Potential MCR-1 Inhibitor in Combination With Colistin Againsts mcr-1-Positive Klebsiella pneumonia

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Carbapenem resistance among strains of the nosocomial pathogen Klebsiella pneumoniae is increasing worldwide, causing serious clinical infections and higher mortality rates. Polymyxins are some of the few “last resort” options for treatment of carbapenem-resistant Enterobacteriaceae, including K. pneumoniae, however, the emergence of plasmid-mediated colistin resistance gene mcr-1 has largely rendered polymyxin-class antibiotics ineffective in a clinical setting. We previously identified a natural compound, pterostilbene, which has a synergistic effect in combination with polymyxins. Here, we aimed to determine whether pterostilbene application can restore the bactericidal activity of polymyxins against mcr-1-positive K. pneumoniae. Checkerboard MIC studies confirmed that pterostilbene reduces the MIC of colistin against mcr-1-positive clinical K. pneumoniae isolates, with the bacteria going from resistant to sensitive, and also demonstrated a synergistic effect with colistin (FIC index = 0.11 ± 0.04 or 0.28 ± 0.00). Time-killing assays showed that individually, both pterostilbene and colistin failed to eradicate K. pneumoniae strains, while in combination, the two drugs effectively eliminated K. pneumoniae ZJ02 and K. pneumoniae ZJ05 by 1–3 h post-inoculation. The combined disk test also showed increases in the zones of inhibition only for mcr-1-positive Escherichia coli and K. pneumoniae isolates. A mouse infection model demonstrated that the survival rate of mice at 7 days post-intraperitoneal injection with a lethal dose of K. pneumoniae ZJ05 was significantly promoted from 0 to 67% following combination therapy. This is the first time a MCR-1 inhibitor has successfully been used in combination with colistin against human clinical MCR-1 producing K. pneumoniae ZJ05 isolate.

Keywords: K. pneumonia, MCR-1 inhibitor, pterostilbene, colistin, combination therapy
INTRODUCTION

The relentless increase in carbapenem-resistant *Enterobacteriaceae* (CRE) strains is now recognized as one of the most serious global threats to public health (Morrill et al., 2015). Carbapenem-resistant *K. pneumoniae* strains are especially worrying as they have higher morbidity and mortality rates, and treatment of these bacterial infections is frequently challenging because of the limited therapeutic options (Olaitan et al., 2014; Quan et al., 2017). *K. pneumoniae* is a common cause of pulmonary and bloodstream health care related infections and normally resides in the lower gastrointestinal tract, where it can acquire high-level antibiotic resistance (Hrabáč et al., 2011). This eventually forced a re-evaluation of the use of one of the earliest classes of antibiotics, polymyxins, for treatment of serious infections caused by carbapenem and multidrug resistant *K. pneumoniae* isolates often blaKPC or blaNDM- positive (Quan et al., 2017). In human clinical chemotherapy, polymyxin B and polymyxin E are usually used in combination, mainly because the dose escalation that is required to achieve sufficiently high concentrations under the currently recommended dosing protocols, risks the rapid onset of nephrotoxicity and neuromuscular blockade (Pogue et al., 2011).

Prior to the detection of the plasmid-mediated colistin resistance gene mcr-1, almost all studies of polymyxin resistance focused on the pmrAB and phoPQ two-component regulatory systems, inactivation of mgbB, or the lack of lipopolysaccharide (Halaby et al., 2013). mcr-1 encodes a phosphoethanolamine transferase that alters the charge on lipid A from electronegative to electropositive, thereby inhibiting the binding of polymyxins to target bacteria. mcr-1 determinant amongst CRE has almost eliminated their clinical susceptibility to polymyxin (Liu et al., 2016, 2017; Kieffer et al., 2017). Importantly, as mcr-1 is plasmid-mediated, resistance to polymyxins is no longer only associated with the chromosome, but can also be acquired by horizontal transmission (Giamarellou, 2016).

The loss of these last-line-of-defense antibiotics made necessary of the development of novel and effective strategies to deal with the serious challenges posed by MCR-1 expression, with the investment of large amounts of manpower and resources. It would also be useful to restore the efficacy of polymyxin to treat severe clinical bacterial infections caused by CRE (Bulman et al., 2017). Previously, we showed that a natural compound used in traditional Chinese medicine, pterostilbene, which has been extensively studied for its potent anti-cancer, anti-inflammatory, and anti-oxidant activities (Roupe et al., 2006), has a synergistic effect with polymyxin B against *E. coli* both in vitro and in vivo (Zhou et al., 2018). Because of its methoxyl substitution-induced hyperlipophilicity, pterostilbene may have higher bioactivity than resveratrol, making it potentially advantageous as a therapeutic agent (Cichocki et al., 2008; Kapetanovic et al., 2011). Here, we further characterized the efficacy of pterostilbene administrated together with polymyxins, and showed that it can help restore the bactericidal activity of polymyxins against mcr-1-positive *K. pneumoniae*.

MATERIALS AND METHODS

**Bacterial Strains and Chemicals**

Human clinical MCR-1 producing isolates *K. pneumoniae* ZJ02, *K. pneumoniae* ZJ05 and *E. coli* ZJ40 were collected in our previous study (Wang et al., 2017). And the mcr-1 gene was chromosomally located in *E. coli* ZJ40. *K. pneumoniae* E8.31, *K. pneumoniae* 13b5 and *K. pneumoniae* L18 were collected from food animals. We also used *E. coli* strain DH5α (pUC19-mcr-1) (Zhou et al., 2018), which carries a mcr-1 gene originating from *K. pneumoniae* ZJ05. Polymyxin-resistant mcr-1-negative *K. pneumoniae* isolate 16ZJJ19-19BC was obtained from a chicken cloacae sample collected in Zhejiang, China. *E. coli* ATCC25922, *K. pneumoniae* ATCC700603 and *K. pneumoniae* K7 were used as quality control strains. Pterostilbene (≥97% HPLC-pure) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Colistin sulfate, polymyxin B sulfate, penicillin, imipenem, gentamicin sulfate, and chloramphenicol were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Cephalothin sodium, streptomycin sulfate, kanamycin sulfate, erythromycin, and achemycin were purchased from Dalian Meilun Biotechnology Co. (Dalian, China). Stock solutions of pterostilbene were prepared in dimethyl sulfoxide (Sigma-Aldrich).

**MIC Determination and Growth Curves**

The MIC assays were used to identify synergies between pterostilbene and colistin against polymyxin-resistant strains (positive for mcr-1), polymyxin-resistant strains (negative for mcr-1), and polymyxin-sensitive strains (negative for mcr-1), and were carried out using the broth microdilution method following the guidelines of the Clinical and Laboratory Standards Institute (Wiegand et al., 2008; Espinel-Ingroff et al., 2016). The remaining nine antibiotics were also tested in combination with pterostilbene. The efficacies of the combinations were evaluated by calculating the fractional inhibitory concentration (FIC) index values (Ma et al., 2016). A growth curve assay was also performed to evaluate the effect of pterostilbene on the growth of the tested strains (Li et al., 2011). Briefly, *K. pneumoniae* ZJ02 and *K. pneumoniae* ZJ05 were cultured in Luria-Bertani (LB) medium at 37°C with shaking at 200 rpm to obtain an OD$_{600}$ value of 0.3. Aliquots (250 mL) of the culture were then transferred into six 50-mL Erlenmeyer flasks, and pterostilbene (or the dimethyl sulfoxide control) was added to the cultures at 0, 16, 32, 64, and 128 μg/mL, respectively. The bacteria were cultured at 37°C with shaking, and bacterial growth was estimated by measuring the OD$_{600}$ every 30 min.

**Time-Killing Assays**

The potential bactericidal effect of pterostilbene in combination with colistin was evaluated by time-killling assays (Petersen et al., 2006). Mid-logarithmic-phase bacterial cells were diluted to 5 × 10^6 CFU/mL in LB broth supplemented with colistin (4 μg/mL), pterostilbene (16 μg/mL), colistin (4 μg/mL) in combination with pterostilbene (16 μg/mL), or DMSO (normal control). Cultures were incubated at 37°C with shaking and samples were removed at 0, 1, 3, 5, and 7 h post-inoculation for bacterial counts.
Serial 10-fold dilutions of the samples were spread onto LB agar plates without antibiotics. Bacterial colonies were counted following incubation at 37°C for 24 h.

**Combined Disk Test**

The combined disk test (CDT) was carried out as described previously (Pournaras et al., 2013; Watts, 2013). Based on the results of the growth curve assay and checkerboard MIC studies, we selected pterostilbene concentrations of 0, 8, and 32 µg/mL, none of which resulted in an inhibitory effect against any of the screened strains. Colistin 10 µg disks (Oxoid Ltd., Basingstoke, United Kingdom) were first placed on Mueller-Hinton-Broth (MHB) agar plates inoculated with bacterial suspension at an OD₆₀₀ = 0.1. Ten-microliter aliquots of the different concentrations of pterostilbene solution were then directly added to the disks. The diameters of the inhibition zones around the colistin disks (with and without pterostilbene) were measured and compared following incubation for 18–24 h at 37°C.

**In Vivo Infection Model for K. pneumoniae ZJ05**

A mouse model of endonasal pulmonary infection was used to determine the synergistic effect of pterostilbene in combination with colistin in vivo. Eight-week-old female C57BL/6 mice weighing 20 ± 2 g were obtained from the Experimental Animal Centre of Jilin University (Changchun, China). Animal experiments were approved by and conducted in accordance with the Animal Care and Use Committee of Jilin University. Five mice were housed per cage in a pathogen-free environment maintained at 24 ± 2°C and 50% ± 10% relative humidity and subjected to a 12 h light/12 h dark cycle. All mice were rested for 5 days prior to use to allow acclimatization.

Pneumonia was induced in the mice as described previously (Bowers et al., 2015; Zhou et al., 2017). *K. pneumoniae* ZJ05 was grown to mid-logarithmic phase (OD₆₀₀ = 0.5) in LB medium at 37°C and then centrifuged at 5,000 × g for 5 min at 4°C. After washing three times with PBS, the bacteria were resuspended in PBS. The mice were divided randomly into five groups (solvent control for each treatment, pterostilbene alone, colistin alone, and pterostilbene in combination with colistin). Each experimental group contained 18 mice. For the survival experiments, the mice were lightly anesthetized by inhalation of isoflurane and then inoculated in the left nare with 20 µL of suspension containing 1 × 10⁸ CFU of the prepared *K. pneumoniae* ZJ05 cells. The infected mice were subcutaneously administered colistin (8 mg/kg), pterostilbene (50 mg/kg), a combination of pterostilbene (50 mg/kg) and colistin (8 mg/kg), or solvent on the same schedule at 2 h post-infection and then at 8-h intervals. Mice were monitored until day 7 post-infection.

For histopathological analysis of lung infection and calculation of the wet/dry weight ratio, mice were inoculated with 5 × 10⁷ CFU of prepared *K. pneumoniae* ZJ05 cells. The mice were killed with anesthesia followed by cervical dislocation at 48 h post-infection. Homogenates of lung tissue, which was collected from euthanized mice, were prepared in 1 ml of sterile PBS and used to calculate bacterial colony counts following serial dilution and smearing on LB agar plates. For histopathological analysis, the lungs were placed in 10% (v/v) formalin, followed by staining with hematoxylin and eosin and examination by light microscopy. The lungs were isolated to measure the wet weight, while the dry weight was measured after drying for 72 h at 70°C. The wet/dry weight ratio of the lung was then calculated.

**Statistical Analysis**

The IBM Statistical Program for Social Sciences (SPSS) version 19.0 (IBM Corp. Armonk, NY, USA) was used to analyze experimental data, and data are presented as the mean ± standard deviation. An independent Student’s t-test was used to determine significant differences, and differences were considered statistically significant when P-values were less than 0.05.

**RESULTS**

**Pterostilbene Showed a Synergistic Effect in Combination With Polymyxin Against mcr-1-Positive Bacteria**

We previously showed that pterostilbene (trans-3,5-dimethoxy-4′-hydroxystilbene) had a synergistic effect with polymyxin B and colistin against polymyxin-resistant *E. coli* strains (positive for MCR-1) (Zhou et al., 2018). In view of the clinical significance of *K. pneumoniae*, and to determine the synergistic effect of pterostilbene in combination with polymyxin alone, *mcr-1*-positive *K. pneumoniae* isolates ZJ02, ZJ05, E8.31, 13B5, and L18 were examined in this study. Our results confirmed the synergistic effect of pterostilbene only in combination with colistin against both *mcr-1*-positive clinical *K. pneumoniae* isolates (FIC = 0.11 ± 0.04–0.28 ± 0.00, respectively, in the presence of 16 µg/mL of pterostilbene) using the broth microdilution checkerboard method. No synergy was observed with any of the other nine tested antibiotics against either the *mcr-1*-positive or polymyxin-sensitive isolates. However, the synergistic effect of pterostilbene and polymyxin against *mcr-1*-negative polymyxin-resistant *K. pneumoniae* strain 16ZJJ9-19BC differed from that observed using *mcr-1*-positive isolates (Table 1). The growth curve showed that none of the concentrations of pterostilbene (0–128 µg/mL) affected the growth of *mcr-1*-positive *K. pneumoniae* isolates ZJ02 and ZJ05 (Figures 1A,B).

The combination of pterostilbene and colistin resulted in the lowest FIC index value, and thus was examined further via time-killing assays. The time-killing assays were performed using 16 µg/mL of pterostilbene and 4 µg/mL of colistin against *K. pneumoniae* grown in LB broth. When used alone, pterostilbene and colistin had little effect on bacterial growth. In contrast, the combination of pterostilbene and colistin resulted in the elimination of *K. pneumoniae* ZJ02 and *K. pneumoniae* ZJ05 at 1 h and 3 h post-administration, respectively (Figures 1C,D). Based on the results of the growth curve, pterostilbene concentrations of 0, 8, and 32 µg/mL were chosen for CDT assays. The results showed increases in the zones of inhibition only for *mcr-1*-positive *E. coli* and *K. pneumoniae* isolates (2.67...
**TABLE 1 | MIC values for the different antibiotics used alone or in combination with pterostilbene against each of the tested bacterial isolates.**

| Species                  | Source and mcr-1 confirmation                                                                 | Antibiotics          | MIC (μg/mL) | FIC Index |
|--------------------------|-----------------------------------------------------------------------------------------------|----------------------|-------------|-----------|
|                          |                                                                                               | Alone                | Combination |           |
| *K. pneumoniae* ZJ02     | *mcr-1*-carrying *K. pneumoniae* from clinical infections in Zhejiang                         | Colistin             | 16.00 ± 0.00 | 1.33 ± 0.58 | 0.11 ± 0.04 |
|                          |                                                                                               | Cefalotin sodium     | 1024.00 ± 0.00 | 1024.00 ± 0.00 | 1.03 ± 0.00 |
|                          |                                                                                               | Penicillin           | 1024.00 ± 0.00 | 1024.00 ± 0.00 | 1.03 ± 0.00 |
|                          |                                                                                               | Imipenem             | 2.67 ± 0.00    | 2.67 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Streptomycin         | 512.00 ± 0.00  | 512.00 ± 0.00  | 1.03 ± 0.00 |
|                          |                                                                                               | Kanamycin            | 1024.00 ± 0.00 | 1024.00 ± 0.00 | 1.03 ± 0.00 |
|                          |                                                                                               | Gentamycin           | 512.00 ± 0.00  | 512.00 ± 0.00  | 1.03 ± 0.00 |
|                          |                                                                                               | Chloramphenicol      | 512.00 ± 0.00  | 512.00 ± 0.00  | 1.03 ± 0.00 |
|                          |                                                                                               | Erythromycin         | 256.00 ± 0.00  | 256.00 ± 0.00  | 1.03 ± 0.00 |
|                          |                                                                                               | Acheomycin           | 213.33 ± 73.90 | 213.33 ± 73.90 | 1.20 ± 0.76 |
| *K. pneumoniae* ZJ05     | *mcr-1*-carrying *K. pneumoniae* from clinical infections in Zhejiang                         | Colistin             | 26.67 ± 9.24   | 2.67 ± 1.15    | 0.14 ± 0.04 |
|                          |                                                                                               | Cefalotin sodium     | 1024.00 ± 0.00 | 1024.00 ± 0.00 | 1.03 ± 0.00 |
|                          |                                                                                               | Penicillin           | 1024.00 ± 0.00 | 1024.00 ± 0.00 | 1.03 ± 0.00 |
|                          |                                                                                               | Imipenem             | 1.00 ± 0.00    | 1.00 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Streptomycin         | 21.33 ± 9.24   | 21.33 ± 9.24   | 1.03 ± 0.00 |
|                          |                                                                                               | Kanamycin            | 26.67 ± 9.24   | 26.67 ± 9.24   | 1.03 ± 0.00 |
|                          |                                                                                               | Gentamycin           | 3.33 ± 0.00    | 2.67 ± 1.15    | 0.86 ± 0.29  |
|                          |                                                                                               | Chloramphenicol      | 5.33 ± 2.31    | 5.33 ± 2.31    | 1.03 ± 0.00 |
|                          |                                                                                               | Erythromycin         | 128.00 ± 0.00  | 128.00 ± 0.00  | 1.03 ± 0.00 |
|                          |                                                                                               | Acheomycin           | 170.67 ± 73.90 | 170.67 ± 73.90 | 1.03 ± 0.00 |
| *E. coli* DH5α (pUC19-mcr-1) | Laboratory strain (carried a mcr-1 gene that originated from *K. pneumoniae* ZJ05) | Colistin             | 13.33 ± 4.62   | 2.00 ± 0.00    | 0.20 ± 0.07  |
|                          |                                                                                               | Cefalotin sodium     | 256.00 ± 0.00  | 256.00 ± 0.00  | 1.03 ± 0.00 |
|                          |                                                                                               | Penicillin           | 512.00 ± 0.00  | 512.00 ± 0.00  | 1.03 ± 0.00 |
|                          |                                                                                               | Imipenem             | 0.25 ± 0.00    | 0.25 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Streptomycin         | 2.00 ± 0.00    | 2.00 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Kanamycin            | 2.67 ± 1.15    | 2.67 ± 1.15    | 1.03 ± 0.00 |
|                          |                                                                                               | Gentamycin           | 1.67 ± 0.58    | 1.67 ± 0.58    | 1.03 ± 0.00 |
|                          |                                                                                               | Chloramphenicol      | 4.00 ± 0.00    | 4.00 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Erythromycin         | 16.00 ± 0.00   | 16.00 ± 0.00   | 1.03 ± 0.00 |
|                          |                                                                                               | Acheomycin           | 1.00 ± 0.00    | 1.00 ± 0.00    | 1.03 ± 0.00 |
| *E. coli* DH5α (pUC19)   | Laboratory strain (Polymyxin-sensitive mcr-1-negative)                                        | Colistin             | 0.83 ± 0.29    | 0.67 ± 0.89    | 0.86 ± 0.29  |
|                          |                                                                                               | Cefalotin sodium     | 256.00 ± 0.00  | 256.00 ± 0.00  | 1.03 ± 0.00 |
|                          |                                                                                               | Penicillin           | 512.00 ± 0.00  | 512.00 ± 0.00  | 1.03 ± 0.00 |
|                          |                                                                                               | Imipenem             | 0.25 ± 0.00    | 0.25 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Streptomycin         | 2.00 ± 0.00    | 2.00 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Kanamycin            | 2.00 ± 0.00    | 2.00 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Gentamycin           | 1.67 ± 0.58    | 1.67 ± 0.58    | 1.03 ± 0.00 |
|                          |                                                                                               | Chloramphenicol      | 4.00 ± 0.00    | 4.00 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Erythromycin         | 8.00 ± 0.00    | 8.00 ± 0.00    | 1.03 ± 0.00 |
|                          |                                                                                               | Acheomycin           | 1.00 ± 0.00    | 1.00 ± 0.00    | 1.03 ± 0.00 |
| *E. coli* ZJ40           | *mcr-1*-carrying *K. pneumoniae* from clinical infection in Zhejiang (mcr-1 located in chromosome) | Colistin             | 85.33 ± 36.95  | 3.33 ± 1.15    | 0.15 ± 0.05  |
|                          |                                                                                               | Polymyxin B          | 53.33 ± 18.48  | 2.67 ± 1.15    | 0.18 ± 0.02  |
TABLE 1 | Continued

| Species                     | Source and mcr-1 confirmation                  | Antibiotics          | MIC (µg/mL) | FIC Index |
|-----------------------------|------------------------------------------------|----------------------|-------------|-----------|
|                             |                                                | Alone                | Combination |           |
| K. pneumoniae-E8.31         | Polymyxin-resistant mcr-1-positive             | Colistin             | 21.33 ± 9.24| 3.33 ± 0.00| 0.20 ± 0.07|
|                             | K. pneumoniae from chicken cloacae in Shandong |                      | 16.00 ± 0.00| 2.67 ± 1.15| 0.20 ± 0.07|
| K. pneumoniae-L18           | Polymyxin-resistant mcr-1-positive             | Colistin             | 13.33 ± 4.62| 3.33 ± 1.15| 0.28 ± 0.00|
|                             | K. pneumoniae from chicken cloacae             |                      | 16.00 ± 0.00| 2.67 ± 1.15| 0.20 ± 0.07|
| K. pneumoniae-13b5          | Polymyxin-resistant mcr-1-positive             | Colistin             | 32.00 ± 0.00| 3.33 ± 1.15| 0.14 ± 0.04|
|                             | K. pneumoniae from chicken cloacae in Shanghai |                      | 26.67 ± 9.24| 2.67 ± 1.15| 0.14 ± 0.04|
| K. pneumoniae—16ZJJ9-19BC   | Polymyxin-resistant mcr-1-negative             | Colistin             | 32.00 ± 0.00| 10.67 ± 4.62| 0.36 ± 0.14|
|                             | K. pneumoniae from chicken cloacae in Zhejiang |                      | 26.67 ± 9.24| 10.67 ± 4.62| 0.45 ± 0.14|
| K. pneumoniae K7            | mcr-1-negative K. pneumoniae from clinical     | Colistin             | 1.33 ± 0.58 | 1.33 ± 0.58 | 1.03 ± 0.00 |
|                             | infection in Jilin                             |                      | 2.00 ± 0.00 | 2.00 ± 0.00 | 1.03 ± 0.00 |
| K. pneumoniae ATCC700603    | Laboratory strain                              | Colistin             | 0.67 ± 0.29 | 0.83 ± 0.29 | 1.36 ± 0.58 |
|                             |                                                | Polymyxin B          | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.03 ± 0.00 |

All MICs were determined in triplicate. According to the best synergistic effect, pterostilbene was used at a concentration of 16 µg/mL for K. pneumoniae and 32 µg/mL for E. coli, except E. coli ZJ40 (4 µg/mL). The FIC values of all mcr-1-positive isolates were indicated in bold.

FIGURE 1 | Pterostilbene in combination with colistin restores the in vitro sensitivity of K. pneumoniae to polymyxins. (A,B) Growth curves for K. pneumoniae ZJ02 (A) and K. pneumoniae ZJ05 (B) cultured in the presence of various concentrations (0–128 µg/mL) of pterostilbene. Values represent the averages of three independent experiments. (C,D) Time-killing curves for colistin, pterostilbene, colistin + pterostilbene, and control treatment (medium only) against K. pneumoniae ZJ02 (C) and K. pneumoniae ZJ05 (D). Values represent the averages of three independent experiments.

± 0.58 mm, 4.33 ± 0.29 mm, and 4.67 ± 0.29 mm) using disks containing 10 µg of colistin alone (Table 2 and Figure 2), and mcr-1-negative E. coli ATCC 25922 had little increases in the zones of inhibition with different concentrations of pterostilbene. We also confirmed
### TABLE 2 | Combined disk test for colistin in combination with pterostilbene for each of the tested bacterial isolates.

| Species | Assay | Colistin (10 µg) | Colistin (10 µg) + Pterostilbene (8 µg/mL) | Increase | Colistin (10 µg) + Pterostilbene (32 µg/mL) | Increase |
|---------|-------|-----------------|-------------------------------------------|----------|-------------------------------------------|----------|
| K. pneumoniae ZJ05 | Assay 1 | 8.5 | 11.5 | 3 | 13.0 | 4.5 |
|         | Assay 2 | 9.5 | 11.5 | 2.0 | 14.0 | 4.5 |
|         | Assay 3 | 9.0 | 11.0 | 2.0 | 14.0 | 5.0 |
|         | Mean | 9.0 ± 0.50 | 11.33 ± 0.29** | 2.33 ± 0.58 | 13.67 ± 0.58** | 4.67 ± 0.29 |
| K. pneumoniae ZJ02 | Assay 1 | 9.5 | 11.0 | 1.5 | 13.5 | 4 |
|         | Assay 2 | 9.0 | 10.5 | 1.5 | 13.5 | 4.5 |
|         | Assay 3 | 9.0 | 11.0 | 2 | 13.5 | 4.5 |
|         | Mean | 9.17 ± 0.29 | 10.83 ± 0.29** | 1.67 ± 0.29 | 13.50 ± 0.00** | 4.33 ± 0.29 |
| E. coli DH5α (pUC19-mcr-1) | Assay 1 | 10.5 | 12.0 | 1.5 | 13.5 | 3.0 |
|         | Assay 2 | 10.0 | 11.0 | 1.0 | 13.0 | 3.0 |
|         | Assay 3 | 11.0 | 11.5 | 0.5 | 13.0 | 2.0 |
|         | Mean | 10.0 ± 0.50 | 11.50 ± 0.50 | 1.00 ± 0.50 | 13.17 ± 0.29** | 2.67 ± 0.58 |
| E. coli ATCC 25922 | Assay 1 | 13.0 | 13.5 | 0.5 | 13.5 | 0.5 |
|         | Assay 2 | 12.5 | 13.5 | 1.0 | 12.0 | -0.5 |
|         | Assay 3 | 13.0 | 13.0 | 0.0 | 13.5 | 0.5 |
|         | Mean | 12.83 ± 0.89 | 13.33 ± 0.29 | 0.50 ± 0.50 | 13.00 ± 0.87 | 0.17 ± 0.58 |

The combined disk test method was performed in triplicate. Three 10-µg colistin disks with pterostilbene (0, 8, and 32 µg/mL) were used. **P < 0.01 compared with the colistin 10-µg disk alone based on two-tailed Student’s t-tests. The mean inhibition zone diameter of all isolates were indicated in italics, and the increased values were indicated in bold.

### FIGURE 2 | Zones of inhibition surrounding colistin disks supplemented with 0, 8, or 32 µg/mL of pterostilbene on lawns of K. pneumoniae ZJ02, K. pneumoniae ZJ05, and E. coli DH5α (pUC19-mcr-1) on MHB agar plates.
that pterostilbene in combination with colistin increased the size of the inhibition zones in a dose-dependent manner.

**Combination Therapy Had a Synergistic Effect in Vivo in Comparison With Monotherapy or the Control**

Based on the above results, we attempted to determine whether the synergistic effects could be replicated in vivo in a mouse model of pneumonia induced by *K. pneumoniae*. Mice were intranasally inoculated with *K. pneumoniae* ZJ05 and then treated with colistin (8 mg/kg), pterostilbene (50 mg/kg), pterostilbene (50 mg/kg) in combination with colistin (8 mg/kg), or PBS as a control at 2h post-infection, and bacterial burden was assessed at 24h post-infection. The combination of colistin and pterostilbene resulted in a significant reduction of the bacterial load in the lung compared with the monotherapy treatments (*P* < 0.01; Figure 3A), although the colistin-treated group also showed a significant decrease in CFU compared with the control group (*P* < 0.01).

We assessed the degree of pulmonary edema via the wet/dry weight ratio of the left lung. The results showed that mice treated with the combination therapy had a significant decrease in wet/dry weight ratio compared with the other groups (Figure 3B). Histopathological analysis of lung tissue was also performed to evaluate the treatment efficacy of pterostilbene in combination with colistin against pulmonary injury. Gross macroscopic inspection revealed that the lungs of infected mice that receiving either of the monotherapies or the control treatment were crimson and exhibited severe congestion and pulmonary edema. In contrast, the lung tissue of mice treated with combination therapy remained pink and fungous (Figure 3D). Examination of the pathologic manifestations (Figure 3E) revealed that the infected mice in either the untreated or monotherapy-treated groups exhibited severe tissue injury and inflammatory cell aggregation. In contrast, the tissue sections of the mice in the combination therapy group were similar to those of the normal mice.

The combination therapy was further tested using a mouse survival model. Following infection with $1 \times 10^8$ CFU of *K. pneumoniae* ZJ05, the majority of mice treated with a single agent or the control succumbed to infection within 168 h. However, as shown in Figure 3C, 67% (12/18) of the mice treated with a...
combination of pterostilbene and colistin survived until the end of the experiment.

DISCUSSION

Because of the significant burden of mcr-1-positive K. pneumoniae in a clinical setting, we investigated whether pterostilbene in combination with colistin could be used as a treatment for infections caused by colistin-resistant K. pneumoniae. Pterostilbene (trans-3,5-dimethoxy-4′-hydroxystilbene) is a naturally occurring phytoalexin found in several plant species. It has more favorable pharmacological properties than fellow phytoalexin resveratrol, including greater oral absorption efficiency, potential for greater cellular uptake, and a longer half-life. Moreover, it exhibits antibacterial activity against drug-resistant Staphylococcus aureus strains without inducing unacceptably high levels of cytotoxicity (0.125 mM) in mammalian cells. For example, administration of pterostilbene (3,000 mg/kg, daily, p.o.) for approximately 30 days did not result in remarkable local or systemic toxicity in mice. Another study showed that pretreatment of A/J mice with pterostilbene at doses of 50 and 250 mg/kg (i.p.) five times per week for 21 continuous weeks produced no signs of toxicity, such as changes in fur color, motor or behavioral abnormalities, or palpable masses (Chen et al., 2012). Pterostilbene is also generally safe for human consumption at doses of up to 250 mg per day, and is used as a dietary supplement to decrease the risk of coronary heart disease (Riche et al., 2013). Therefore, all studies confirm that pterostilbene has no measurable toxicity in animals or humans, regardless of the route of administration, and suggest that this natural compound is likely to be safe if applied in human clinical practice.

Although there is a significant synergistic effect of pterostilbene in combination with polymyxin, it is not enough to warrant the development of a therapeutic agent for clinical use. Therefore, it is necessary to study the molecular structure of pterostilbene, including modifications of the main chemical functional groups, which may be useful for reducing any potential side effects for clinical use. There are several limitations to the use of pterostilbene, including its low bioavailability and poor water solubility (Chen et al., 2012). However, compared with resveratrol, pterostilbene has a higher bioavailability and is processed more slowly (glucuronidated or sulfated) in vivo to warrant the development of a therapeutic agent for clinical use. Therefore, it is necessary to study the molecular structure of pterostilbene, including modifications of the main chemical functional groups, which may be useful for reducing any potential side effects for clinical use. There are several limitations to the use of pterostilbene, including its low bioavailability and poor water solubility (Chen et al., 2012). However, compared with resveratrol, pterostilbene has a higher bioavailability and is processed more slowly (glucuronidated or sulfated) in vivo, which may increase the functionality of pterostilbene when applied in systemic infections (Chiou et al., 2011).

The mechanisms of resistance to polymyxins, including mutations in the PmrAB/PhoPQ two-component regulatory systems, loss of lipopolysaccharide, MgrB inactivation, and plasmid-mediated colistin resistance, all involve the modification of lipid A, resulting in a reduction of polymyxin affinity (Ah et al., 2014; Antonelli et al., 2017). A variety of polymyxin resistance mechanisms are present in Enterobacteriaceae species, with some strains containing two or more pathways (Baron et al., 2016; Poirel et al., 2017). Therefore, we need to further explore the mechanism of resistance in mcr-1-negative colistin-resistant K. pneumoniae isolates. In the current study, we used several standard methods to determine the synergy of pterostilbene, including disk diffusion assays carried out as described by the Clinical and Laboratory Standards Institute. This technique is still used for in vivo susceptibility testing in many countries despite the fact that polymyxins do not readily diffuse in agar, resulting in reduced reliability of the method for measuring MIC (Boyen et al., 2010; Albur et al., 2014; Esposito et al., 2017). Despite the limitations of this assay method, we observed significant differences in MCR-1-producing isolates K. pneumoniae ZJ02, K. pneumoniae ZJ05, and E. coli DH5α (pUC19-mcr-1) compared with E. coli ATCC25922.

In summary, this study shows that a combination of polymyxins and pterostilbene could be a viable alternative treatment option for combating K. pneumoniae strains harboring mobile polymyxin resistance gene mcr-1. In addition, this alternative strategy provides potential opportunities to abate pathogenicity and its consequences without placing selective pressure on the target bacterium (Song et al., 2017). Furthermore, by reducing the amount of polymyxins used in clinical therapy, this strategy may also decrease the possibility of mutations arising in LPS modification pathways in K. pneumoniae, as can occur following long-term use of polymyxins. Further studies, including elucidation of the mechanism of inhibition of MCR-1 by pterostilbene, are needed to optimize the effects of combination therapy.

CONCLUSION

In this study, we identified a natural compound of a Traditional Chinese Medicine, pterostilbene, when used in combination with colistin, regain its bactericidal activity against the mcr-1-positive K. pneumoniae. The microdilution checkerboard method confirmed that the pterostilbene reduces the MIC of colistin in mcr-1-positive K. pneumoniae strains from resistance to sensitive. The time-killing assays showed that either pterostilbene or colistin failed to eradicate ZJ02 and ZJ05, but the combination eliminated ZJ02 and ZJ05 by 1–3 h post-inoculation. The mouse infection model demonstrated that the survival rate of mice following the infection with ZJ05 was significantly promoted from 0% in the group of the administrated as monotherapy to 67% in the group of combination therapy applied.

AUTHOR CONTRIBUTIONS

XD, YW, and YZ: Study design. YZ, TW, and YG: Experimental studies. SL, YS, and ST: Data analysis, interpretation. YZ and JW: Statistical analysis. XD, YW, and YZ: Manuscript preparation.

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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