Re-sensitization of mcr carrying multidrug resistant bacteria to colistin by silver
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Colistin is considered the last-line antimicrobial for the treatment of multidrug-resistant gram-negative bacterial infections. The emergence and spread of superbugs carrying the mobile colistin resistance gene (mcr) have become the most serious and urgent threat to healthcare. Here, we discover that silver (Ag+), including silver nanoparticles, could restore colistin efficacy against mcr-positive bacteria. We show that Ag+ inhibits the activity of the MCR-1 enzyme via substitution of Zn2+ in the active site. Unexpectedly, a tetra-silver center was found in the active-site pocket of MCR-1 as revealed by the X-ray structure of the Ag-bound MCR-1, resulting in the prevention of substrate binding. Moreover, Ag+ effectively slows down the development of higher-level resistance and reduces mutation frequency. Importantly, the combined use of Ag+ at a low concentration with colistin could relieve dermonecrotic lesions and reduce the bacterial load of mice infected with mcr-1-carrying pathogens. This study depicts a mechanism of Ag+ inhibition of MCR enzymes and demonstrates the potentials of Ag+ as broad-spectrum inhibitors for the treatment of mcr-positive bacterial infection in combination with colistin.

antimicrobial resistance | colistin | metalloenzymes | MCR-1 | silver

The emergence and spread of multidrug-resistant (MDR) or extensively drug-resistant (XDR) gram-negative bacteria have renewed interest in the use of polymyxins (polymyxin B and colistin). Polymyxins are a class of cationic polypeptide antibiotics, which kill gram-negative pathogens through disruption of membrane permeability via polar and hydrophobic interactions. Despite having serious adverse effects, polymyxin B and colistin have become the last-resort treatment options for MDR and XDR bacterial infections (1, 2). Colistin resistance was predominantly caused by phosphoethanolamine (pEA) transferases, which catalyze the addition of phosphoethanolamine (pEtN) to lipid A to reduce the electrostatic attraction between colistin and the gram-negative outer membrane (3–6). Such transmission has minimal clinical threats as it is chromosome mediated (2, 7, 8). However, the emergence and global spread of the first plasmid-borne transmissible colistin resistance gene, called mobile colistin resistance gene-1 (mcr-1), which was identified in 2015 (9), have significantly challenged the efficacy of this last-resort antibiotic. The MCR-1 enzyme encoded by the mcr-1 gene, belonging to the alkaline phosphatase (AP) metalloenzyme superfamily based on its structure (10, 11), contains an essential Zn(II) cofactor in its active site coordinated with Glu46, Asp465, His466, and phosphorylated Thr285 (TPO285) (11–14). It catalyzes the addition of pEtN moiety from those such as phosphatidylethanolamine (PE) to lipid A of the gram-negative membrane as shown in SI Appendix, Fig. S1. In addition to the mcr-1 gene, other families have also been identified, including mcr-2, mcr-3, mcr-4, and mcr-5, and each has its variants (1). These plasmid-borne resistances can be cross-spread rapidly via horizontal gene transfer between bacterial strains and species, resulting in the emergence of MDR superbugs (15, 16), which poses a significant challenge to clinicians.

Combination therapy comprising an available antibiotic and a nonantibiotic (as a resistance breaker) has been considered a safer, more economical and effective alternative than the development of new antibiotics (8, 17, 18). Such therapies have been successfully utilized clinically to treat infections caused by superbugs carrying serine-β-lactamases (SBLs) (19, 20). Metal-based agents have received increasing attention in coping with the current crisis of antimicrobial resistance (21–23). As resistance breakers, metal compounds have many advantages over organic compounds (24–26), in particular in the case that resistance is caused by a metalloenzyme such as metallo-β-lactams (MBLs), because metal ions can readily substitute the cofactors of metalloenzymes (8), resulting in broad-spectrum bactericidal activity (27, 28). Indeed, bismuth drugs such as colloidal bismuth subcitrate (CBS;
Silver Restores Colistin Activity against mcr-Positive Bacteria

**In Vitro.** By using Luria-Bertani (LB) broth, a nutritionally rich medium primarily used for the growth of bacteria, a panel of compounds, including 2 Zn^{2+} chelating agents, 4 bioligands, and 16 metal-based compounds, were initially screened against mcr-1–positive Escherichia coli (E. coli J53, denoted as MCR-1–J53 hereafter), which was confirmed to be resistant to colistin as judged by a minimal concentration to inhibit the 90% bacterial growth (MIC) of 8 μg mL^{-1} (6.93 μM) (9, 29). The growth inhibition was examined in the presence of compounds at a fixed concentration (1 μg mL^{-1}) and colistin at a subinhibitory concentration (1 μg mL^{-1}) (0.87 μM) for 16 h after bacterial inoculation. Primary screening results (*SI Appendix, Fig. S2 and Table S1*) showed that AgNO₃ exhibited the highest activity with over 90% growth inhibition being observed based on optical density (OD₆₀₀) reading.

Despite Ag⁺ exhibiting wide-spectrum antimicrobial activity through targeting multiple biological pathways via inactivation of key enzymes (27), there appears no report that Ag⁺ might serve as a resistant breaker rather than an antimicrobial agent. We first examined whether AgNO₃ could resensitize MCR-1 producers toward colistin. The standard checkerboard microdilution method (8, 30, 31) was used to monitor the interaction between colistin and AgNO₃ against MCR-1–J53 (Fig. 1A). A mcr-1–negative J53 strain with MICs of 0.5 μg mL^{-1} (0.43 μM) for colistin and 4 μg mL^{-1} (23.69 μM) for AgNO₃ individually (*SI Appendix, Fig. S3*) served as the negative control. AgNO₃ itself at the concentration of 1 μg mL^{-1} (5.92 μM) showed no or minor growth inhibition toward either mcr-1–positive (Fig. 1A) or -negative bacteria (*SI Appendix, Fig. S3*). However, when AgNO₃ and colistin were used in combination, AgNO₃ at a concentration as low as 0.5 to 1.0 μg mL^{-1} (2.96 to 5.92 μM) could resensitize mcr-1–positive bacterium to colistin with the MIC value of colistin dropped by 16-fold from 8 μg mL^{-1} (6.93 μM) to 0.5 μg mL^{-1} (0.43 μM) (Fig. 1A), which is susceptible according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (the MIC breakpoint of colistin for a resistant isolate is >2 μg mL^{-1} (≥1.73 μM) (29)). The fractional inhibitory concentration index (19) (FICI) was determined to be 0.375 (*SI Appendix, Fig. S4A*), indicative of the synergistic interaction between them (FICI of ≤0.5 is defined as synergism). In contrast, no such synergism was observed in *E. coli* J53 carrying no mcr-1 gene (FICI of 0.625; *SI Appendix, Fig. S4A*), suggesting that, at a concentration of silver used, such a synergism is attributable to inhibition of MCR-1 by Ag⁺. Time-kill curves further showed the time-dependent bactericidal effects of the drug combination against MCR-1–J53 and that the bacterial populations were lowered by over 10^{2}-fold after 24-h exposure to the combination of colistin (6 μg mL^{-1}, i.e., 5.19 μM) and AgNO₃ (2 μg mL^{-1}, i.e., 11.84 μM) in comparison to those in untreated or single-component groups (Fig. 1B).

In addition, the synergism between colistin and AgNO₃ was further observed in other mcr-1–carrying bacteria, including clinically isolated Salmonella typhimurium 0839, Klebsiella aerogenes 7014, Klebsiella pneumoniae 9607, Enterobacter kobei 4113, and engineering strain *E. coli* BL21 (DE3), with FICI values ranging from 0.25 to 0.37 (Fig. 1C). However, only partial synergies (0.5 ≤ FICI ≤ 1) were defined as partial synergism in this study. Other bacteria species (*SI Appendix, Fig. S4A*) exhibited almost no antibacterial activity. Silver restores the activity of colistin against MCR-1 producing bacteria, thus showing advantages for a broad-spectrum activity against MCR-1–positive bacteria. A synergistic effect only was observed when combined with silver nanoparticles (the size of 10 nm) under identical conditions (*SI Appendix, Fig. S4B*).

**Silver Inactivates MCR-1 by Displacement of the Zn^{2+} Cofactor.** MCR-1 catalyzes the addition of pEA to lipid A in the cell membrane to enhance the membrane potential of gram-negative bacteria. We selected *E. coli* J53 carrying five mcr-1 variants and six mcr family genes and examined their susceptibility to the colistin and AgNO₃ combination. We found that in the presence of 1 μg mL^{-1} AgNO₃ (0.25 MIC), at which concentration Ag⁺ exhibits almost no antibacterial activity, the MIC values of colistin were decreased by ca. 16-fold to a susceptible level of 0.5 μg mL^{-1} (0.43 μM) against the bacteria carrying almost all mcr genes except mcr-3 and mcr-4 (Fig. 1D). This is reasonable as both mcr-3 and mcr-4 have been demonstrated to function differently from mcr-1 (33, 34). This implies that the combination exhibits broad-spectrum antimicrobial activity against mcr-positive bacteria. A synergy was also observed when combined with silver nanoparticles (the size of 10 nm) under identical conditions (*SI Appendix, Fig. S4B*).

**Results and Discussion**

Silver Restores Colistin Activity against mcr-Positive Bacteria

AgNO₃ itself at the concentration of 1 μg mL^{-1} (5.92 μM) showed no or minor growth inhibition toward either mcr-1–positive (Fig. 1A) or -negative bacteria (*SI Appendix, Fig. S3*). However, when AgNO₃ and colistin were used in combination, AgNO₃ at a concentration as low as 0.5 to 1.0 μg mL^{-1} (2.96 to 5.92 μM) could resensitize mcr-1–positive bacterium to colistin with the MIC value of colistin dropped by 16-fold from 8 μg mL^{-1} (6.93 μM) to 0.5 μg mL^{-1} (0.43 μM) (Fig. 1A), which is susceptible according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) (the MIC breakpoint of colistin for a resistant isolate is ≥2 μg mL^{-1} (≥1.73 μM) (29)). The fractional inhibitory concentration index (19) (FICI) was determined to be 0.375 (*SI Appendix, Fig. S4A*), indicative of the synergistic interaction between them (FICI of ≤0.5 is defined as synergism). In contrast, no such synergism was observed in *E. coli* J53 carrying no mcr-1 gene (FICI of 0.625; *SI Appendix, Fig. S4A*), suggesting that, at a concentration of silver used, such a synergism is attributable to inhibition of MCR-1 by Ag⁺. Time-kill curves further showed the time-dependent bactericidal effects of the drug combination against MCR-1–J53 and that the bacterial populations were lowered by over 10^{2}-fold after 24-h exposure to the combination of colistin (6 μg mL^{-1}, i.e., 5.19 μM) and AgNO₃ (2 μg mL^{-1}, i.e., 11.84 μM) in comparison to those in untreated or single-component groups (Fig. 1B).

In addition, the synergism between colistin and AgNO₃ was further observed in other mcr-1–carrying bacteria, including clinically isolated Salmonella typhimurium 0839, Klebsiella aerogenes 7014, Klebsiella pneumoniae 9607, Enterobacter kobei 4113, and engineering strain *E. coli* BL21 (DE3), with FICI values ranging from 0.25 to 0.37 (Fig. 1C). However, only partial synergies (0.5 ≤ FICI ≤ 1) were defined as partial synergism in this study. Other bacteria species (*SI Appendix, Fig. S4A*) exhibited almost no antibacterial activity. Silver restores the activity of colistin against MCR-1 producing bacteria, thus showing advantages for a broad-spectrum activity against MCR-1–positive bacteria. A synergistic effect only was observed when combined with silver nanoparticles (the size of 10 nm) under identical conditions (*SI Appendix, Fig. S4B*).

**Silver Inactivates MCR-1 by Displacement of the Zn^{2+} Cofactor.** MCR-1 catalyzes the addition of pEA to lipid A in the cell membrane to enhance the membrane potential of gram-negative bacteria (7, 35, 36). We first examined whether AgNO₃ was able to affect membrane potential owing to inhibition of MCR-1 activity in live cells using a membrane potential assay kit according to a standard protocol (37–39). The green/red fluorescence ratios, which are inversely correlated to membrane potential, were reduced by ca. 30% in mcr-1–positive *Shigella flexneri* compared with the negative control. Treatment of mcr-1–positive *S. flexneri* with AgNO₃ at 2 μg mL^{-1} (12 μM) led to the recovery of the fluorescence ratio to the levels found for the negative control (from 4.1 ± 0.4 to 5.8 ± 0.3). Consistent with this, supplementation of carbonyl cyanide chlorophenylhydrazone (CCCP; 5 μM), a potent mitochondrial uncoupling agent to dissipate the transmembrane potential (38), to mcr-1–positive *S. flexneri* led to an increase in the fluorescence ratio (green/red) to comparable levels (6.1 ± 0.1; Fig. 1E), indicating the recovery of membrane potential is attributable to inhibition of MCR-1 by AgNO₃. The inhibition was further confirmed on full-length MCR-1 by a thin layer chromatography (TLC) plate assay (35). The full-length MCR-1 (40 μM) was pretreated with or without 10 molar equivalents of AgNO₃ and mixed with a fluorescent substrate, NBD-
The cleavage model for the chemical reaction catalyzed by MCR-1 (left) and the representative image of TLC plate (right) are shown here.

E. coli producing MCR variants

As shown in Fig. 2A, the addition of increasing amounts of AgNO₃ to Zn₃–MCR-1–S resulted in a gradual decrease in the stoichiometry of Zn²⁺ to MCR-1–S, accompanied by the increase in the binding stoichiometry of Ag⁺ to MCR-1–S, and eventually all Zn²⁺ (ca. 3.0 molar equivalents) were displaced, with ca. 10.2 molar equivalents of Ag⁺ bound. Supplementation of excess amounts of Zn²⁺ to Ag⁺-bound MCR-1–S resulted in negligible changes in the amounts of metals bound to MCR-1–S (SI Appendix, Fig. S6B), indicative of irreversible Zn²⁺ release under the experimental conditions.

We next measured the binding affinities of Ag⁺ to apo–MCR-1–S by isothermal titration calorimetry (ITC). The ITC data were fitted by a sequential-binding model, giving rise to an apparent dissociation constant (Kₐ) of 0.29 ± 0.09 μM and binding capacity (N) of 9.77 ± 0.43 (Fig. 2B). Such a high binding capacity of Ag⁺ was also observed in our matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF-MS) assay on Ag–MCR-1–S, where up to 9 Ag⁺ (m/z 39623.132) were clearly shown to bind into apo–MCR-1–S (m/z 38663.015) successfully in vitro (SI Appendix, Fig. S7A). Notably, for comparison, the apparent Kₐ of Zn⁺ to apo–MCR-1–S was also determined similarly to be 2.45 ± 0.93 μM (N= 2.87 ± 0.12; SI Appendix, Fig. S7B), suggesting that Ag⁺ binds the metalloenzyme around 10-fold stronger than Zn⁺ on the whole.

By using a cellular thermal shift assay (CETSA), we subsequently examined whether AgNO₃ binds to the enzyme in cellulo. E. coli BL21(DE3) cells expressing MCR-1–S after treatment with AgNO₃ at 1 μg mL⁻¹ (5.92 μM) for 16 h in LB medium were harvested and subjected to CETSA analysis according to a standard protocol (42–44). As shown in Fig. 2C,
Silver Binds to MCR-1 at an Atomic Resolution. We then investigated the inhibitory mode of action of Ag⁺ toward MCR-1 enzymes by X-ray crystallography. Coocrystallization of Ag(I)-bound MCR-1 or MCR-1–S was not successful, and thus crystal soaking with AgNO₃ was used. The Zn-bound MCR-1 was subjected to ICP-MS analysis (SI Appendix, Fig. S8A), verifying the binding of Ag⁺ to full-length MCR-1 in cells. The cell lysates from freeze-thawing in liquid nitrogen were subjected to ITC and ICP-MS measurements (SI Appendix, Fig. S8B), confirming the binding of Ag⁺ to full-length MCR-1 in cells.

Silver Binds to MCR-1 at an Atomic Resolution. We then investigated the inhibitory mode of action of Ag⁺ toward MCR-1 enzymes by X-ray crystallography. Coocrystallization of Ag(I)-bound MCR-1 or MCR-1–S was not successful, and thus crystal soaking with AgNO₃ was used. The Zn-bound MCR-1–S (Zn–MCR-1–S) crystal structure was firstly resolved at 1.83 Å as a homodimer in an asymmetric unit (Protein Data Bank [PDB] access code: 6L14). Only one Zn²⁺ was found in the active site of each chain (anomalous signals, ≥20σ), coordinating to Glu⁴⁶⁴, Asp⁴⁶⁵, His⁴⁶⁶, and TPO²⁸⁵ with a distorted tetrahedral geometry (SI Appendix, Fig. S9 A and B). The apo-form MCR-1–S crystals were obtained after soaking the native MCR-1–S crystals in a pool of cryosolution supplemented with ethylenediaminetetraacetate (EDTA-Na₂), and its structure was solved at a resolution of 1.82 Å (SI Appendix, Fig. S9 C and D). The Ag⁺-bound MCR-1–S crystal (Ag⁺–MCR-1–S, distarcted to 1.58 Å) was finally obtained after soaking the apo–MCR-1–S crystals into cryoprotectant solutions containing 1 mM AgNO₃ for 5 min in darkness.

The presence of Ag⁺ was evidenced by both the anomalous signals (≥20σ) and significantly positive peaks (≥20σ) in the miFo–DFc (difference Fourier) map. Occupancies of Ag⁺ were refined based on their B factors. On the electron density maps, 10 Ag⁺ ions were identified in 1 chain of each asymmetric unit, in consistence with ITC and ICP-MS measurements (SI Appendix, Fig. S10A). Superimposition of the structures of Ag-bound with Zn-bound or apo-form MCR-1–S showed negligible overall conformational changes with r.m.s.d. of 0.471 Å and 0.779 Å over all alpha carbon (Ca), respectively (SI Appendix, Fig. S10). In the original Zn²⁺ site, an Ag⁺ (Ag⁺ with occupancy of 0.7) was found to coordinate with Glu⁴⁶⁴, Asp⁴⁶⁵, His⁴⁶⁶, and TPO²⁸⁵, forming a distorted tetrahedral geometry (Fig. 2D). These bond lengths for Ag⁺ were longer than those for Zn²⁺ in general, probably owing to the larger ionic radius of Ag⁺ (1.15 Å) than Zn²⁺ (0.74 Å) (SI Appendix, Tables S2 and S3).

Besides, three additional Ag⁺ sites with occupancies ranging from 0.4 to 0.6 were also observed with either linear (Ag⁺) or trigonal (Ag⁺) and Ag⁺ geometries (Fig. 2 D and E). Six subsidiary Ag⁺ sites were located on the surface of MCR-1–S substrate-binding domain of MCR-1 in intact MCR-1–S producing E. coli BL21 (DE3) cells. The blotting is representative of three independent experiments. Ctrl, control. (D) The cartoon of tetra-nuclear silver center in the active-site pocket of the MCR-1–S enzyme. Ag⁺ shown as wheat spheres are bridged in a narrow pocket by either coordination bonds (red solid lines) or hydrogen bond (red dash line). (3-pEA, which gives rise to a UV absorbance at 460 nm, was used for the assay. The insets show the normalized absorbance at 280 nm for related the...
with linear geometry, and one of them (Ag$_{626}$) was only observed in one chain in the asymmetric unit with occupancy of ca. 0.5 (SI Appendix, Fig. S11), implying less accessibility of Ag$^+$ to this site. Considering their coordination environments, there appeared a faint possibility that the six Ag$^+$ showed direct inhibitory effects on MCR-1. Except for Ag$_{517}$, all the other Ag$^+$ possessed different metal-binding sites (SI Appendix, Table S3) from those of Zn$^{2+}$ based on previous reports (11–14). Their coordinating residues and corresponding bond lengths with the side chains are summarized in SI Appendix, Table S3. These crystallographic studies supported our biophysical data that Ag$^+$ inactivates the enzyme through the displacement of Zn$^{2+}$ of MCR-1.$^-$

Certain critical residues served as the μ$_2$-bridging ligands to yield an unprecedented tetra-nuclear silver center in the active-site pocket of MCR-1 (47–50) (Fig. 2 D and E and SI Appendix, Fig. S12). Specifically, in addition to the active site Ag$_{626}$ (Ag$_{417}$), Ag$_{619}$ was bridged with Ag$_{615}$ via O$_62$ of Asp$_{465}$ and O$_{61}$ of Glu$_{246}$ and further coordinated to Ne$_2$ of His$_{390}$ with a trigonal geometry. The bond lengths of Ag$_{619}$ to His$_{390}$-Ne$_2$, Asp$_{465}$-O$_{62}$, and Glu$_{246}$-O$_{61}$ varied from 2.30 to 2.77 Å, with a quasi-linear His$_{390}$-Ne$_2$-Ag$_{619}$-Asp$_{465}$ angle of 159.1$^\circ$ and planar His$_{390}$-Ne$_2$-Ag$_{619}$-Glu$_{246}$-O$_{61}$-Asp$_{465}$-O$_{62}$ dihedral of 161.9$^\circ$. Moreover, Ag$_{625}$ was bridged with Ag$_{619}$ via Ne$_1$ of His$_{390}$ and further bound to O$_{63}$ of Asp$_{327}$ and O of Asp$_{331}$ with a quasiplanar Ag$_{625}$-His$_{390}$-Ne$_1$-Ne$_2$-Ag$_{619}$ dihedral of 170.9$^\circ$ (Fig. 2 D). Interestingly, another Ag$^+$ (Ag$_{620}$) was also observed nearby which coordinated to Met$_{392}$-S$^-\delta\epsilon$ and His$_{395}$-N$^\epsilon$$_1$ by a linear geometry with a comparable bond length of 2.02 Å and a quasilinear S-Ag$_{620}$-N$_{16}$ angle of 170.7$^\circ$ (Fig. 2 D). Notably, a strong hydrogen bond was formed between Ne$_2$ of His$_{395}$ and O$_3$P of TPO$_{45}$, which was coordinated by Ag$_{617}$, with a bond length of 2.49 Å to stabilize the silver center (Fig. 2 D). Such a unique tetra-silver center in the active-site pocket of the MCR-1 enzyme was observed (12, 22), which illustrated the diversities of silver coordination environments and geometries in these proteins.

Notably, the binding of Ag$_{620}$ to Met$_{392}$ and His$_{395}$ induced ca. 66$^\circ$ rotation of the imidazole ring of His$_{395}$ and the shortening of the distance between Ne$_1$/His$_{395}$ and Ca/ethanolamine (ETA) from 3.94 to 1.32 Å in the substrate-binding site of MCR-1 (Fig. 2 E), in comparison to the MCR-1 complex with substrate analog ETA (PDB code: 5YLE). This possibly led to the prohibition of substrate binding to MCR-1 due to steric effects. To further validate the hypothesis, we investigated whether Ag$^+$ interfered with the binding of substrate (as NBD-glycerol-3-pEA) in MCR-1 and the MCR-1-M$_{390}$ variant. Ag-bound MCR-1, as well as Ag-bound MCR-1–M$_{390}$, was mixed with an equal molar equivalent of NBD-glycerol-3-pEA and subsequently subjected to size-exclusive chromatography analysis. The absorbance at 280 and 460 nm was monitored for the detection of protein and NBD-glycerol-3-pEA-bound protein, respectively. As shown in Fig. 2 F, the absorbance was only observed at 280 nm for the fraction of Ag-bound MCR-1, confirming the absence of substrate binding. In contrast, the absorbance was evident at both 460 and 280 nm for the fraction of MCR-1–M$_{390}$ under identical conditions, indicating the binding of NBD-glycerol-3-pEA to this mutant protein due to the lack of Ag$_{620}$ at Met$_{392}$.

Collectively, crystallographic and biophysical data substantially demonstrated that Ag$^+$ played dual roles in abolishing MCR-1 activity through replacing Zn$^{2+}$ with Ag$^+$ in the catalytic site and exhibiting steric effects in the substrate-binding site.

Silver Suppresses Resistance Evolution in E. coli-Carrying mcr-1. Considering the rapid evolution of mcr genes (51, 52), we therefore used an index of mutation prevention concentration (MPC) (53) to evaluate the effect of Ag$^+$ on the evolution of mcr genes against MCR-1–J53 in the absence and presence of different concentrations of Ag$^+$ (as AgNO$_3$). We found that colistin alone could not kill high-level resistant mutants even in the presence of 8-fold MIC (MPC = 16-fold MIC; Fig. 3 A and B). In contrast, with the increase in Ag$^+$ concentrations, the number of mutant colonies declined significantly as shown in the heat map (Fig. 3 B). The observed mutation frequency ranged from 1.4 × 10$^{-7}$ to 5.2 × 10$^{-7}$. However, no such reduction in mutant colonies was noted for the mcr-1–negative strain (SI Appendix, Fig. S13). The MPC of colistin was lowered to twofold MIC against MCR-1–J53 when Ag$^+$ (AgNO$_3$) was used at fivefold MIC, i.e., 20 μg mL$^{-1}$ (117.7 μM) (Fig. 3 A and B). In contrast, less mutation prevention was observed for AgNO$_3$ in an mcr-1–negative bacterial strain (SI Appendix, Fig. S13). Notably, the combination therapy significantly suppressed the evolution of high-level resistance over a period of 16 passages of MCR-1–J53 (Fig. 3 C). Gene sequencing of the 16th passage of mcr-1–positive bacteria upon treatment of colistin alone or in combination with AgNO$_3$ showed that no appearance of mutations occurred (SI Appendix, Fig. S14). Instead, the high level of resistance of the mcr-1–positive bacterium is likely attributable to the hyperproduction of the MCR-1 protein as revealed by a Western blotting assay, which showed a significant reduction (over 60%) in the level of MCR-1 in the bacterium treated by the combination therapy (Fig. 3 C, Insert).

Silver Restores Colistin Efficacy In Vivo. To further evaluate the potential utility of combination therapy in vivo, we first assessed their abilities to kill bacteria in mice infected by mcr-1–positive bacteria. Balb/c mice were injected intraperitoneally (i.p.) with a sublethal dose (2 × 10$^6$ colony-forming units [CFU] per mouse) of K. pneumoniae 9607, a clinical isolate producing MCR-1, and subsequently treated with a single dose of i.p. administration of vehicle control, Ag$^+$ (as AgNO$_3$ at 1.5 mg kg$^{-1}$), and colistin (2 mg kg$^{-1}$), and their combination (four mice in each group). After 48 h, all the mice were sacrificed, and bacterial loads in livers and spleens, which were severely infected by K. pneumoniae 9607 according to a pre-experiment, were examined by agar plating. In comparison to the bacterial load (at the level of 10$^5$ CFU mL$^{-1}$ g$^{-1}$) in both liver and spleen of mice treated by either AgNO$_3$ or colistin monotherapy, a significant reduction by >20-fold in the CFUs was observed in the combination therapy (Fig. 3 D and E and SI Appendix, Table S4).

Considering the urgency of treatment of skin infection caused by colistin-resistant bacteria producing MCR-1 (54–57), we further evaluated the efficacy of the combination therapy in the treatment of skin wound infected with mcr-1–positive bacteria. Similarly, a dose (2 × 10$^6$ CFU per mouse) of log-phased mcr-1–positive K. pneumoniae 9607 was applied to skin wounds of Balb/c mice under the treatment of full-thickness skin defect overlying the thoracic region. Then, all mice were administrated once daily with the monotherapy of vehicle control, colistin (2 mg kg$^{-1}$), and Ag$^+$ (as AgNO$_3$ at 1 mg kg$^{-1}$), or their combination. As shown in SI Appendix, Fig. S15, both AgNO$_3$ and colistin monotherapy at the concentration used have less protection in relieving the dermonecrotic lesions after 7 d postinfection. Surprisingly, the combination therapy significantly facilitated the infected mice to recover.
from the wound infection, and the morphology lesions had been negligible (SI Appendix, Fig. S15). Similarly, the bacterial loads from wound beds were also significantly reduced by over 10-fold under the treatment of combination therapy in comparison with other treatments (Fig. 3F).

Silver is hardly used internally as an antimicrobial agent due to potential toxicity. Nevertheless, no apparent toxicity of silver at an administered dose of 6 mg kg\(^{-1}\) was observed in mice in a previous report (21). In this study, a much lower dose of silver (1.5 mg kg\(^{-1}\)) was used in combination with colistin, which already significantly reduced the bacterial load of mcr-1-positive bacteria in infected mice. This suggests that the toxicity of silver should not be a major concern if it is used as an adjuvant of antibiotics instead of antimicrobial agents. Collectively, we demonstrate that the in vitro antimicrobial efficacy of a combination therapy of AgNO\(_3\) and colistin could be well translated into in vivo efficacy.

**Conclusion**

Our combined data show that AgNO\(_3\) can restore the efficacy of colistin through binding and functional disruption of MCR enzymes. Importantly, the in vitro antimicrobial efficacy of combination therapy could be successfully translated into in vivo efficacy. X-ray crystallographic data clearly reveal a dual function of silver in the inhibition of MCR-1 activity via kick-off of Zn\(^{2+}\) as well as interference of the substrate binding. Such a phenomenon might be attributable to the unique features of Ag\(^{+}\) coordination in proteins. Moreover, it should also be a complicated process which can be not only kinetically but also thermodynamically driven (40, 58), although it might be too rapid and tight to be accurately measured. The ability of silver as well as other metalloagents to inhibit key resistant determinants and to slow down the development of drug resistance (8, 42) offers a promising strategy for combating antimicrobial resistance when used in combination with antibiotics.

**Materials and Methods**

The construction of plasmids, gene mutations, compound screening, time-kill, drug resistance study, protein purification, membrane potential, enzyme activity, CETS, ITC, MALDI-TOF, Zn\(^{2+}\) release analysis, mouse peritonitis, and wound infection models are described in the SI Appendix. All animal experiments were approved by and performed in accordance with the guidelines approved by Committee on the Use of Live Animals in Teaching and Research (CULATR) (Ref Nos.: 4008-16 and 5079-19), The University of Hong Kong.

**X-Ray Crystallography.** Zn\(^{2+}\)-bound MCR-1-5 crystallization condition was screened using the sitting drop method by mixing equal volumes of protein and reservoir solution. The optimized reservoir solution contains 100 mM KSCN, 30 to 32% PEG 3350, and 100 mM Tris-HNO\(_3\) (pH 8.0). The crystals appeared after...
2 wk at 25 °C and were transferred into chelating buffer (32% PEG 3350, 100 mM Tris-HNO₃ [pH 8.0], 25% glycerol, and 10 mM EDTA-Na₂) for 12 h to prepare apo-MCR-1–S crystals. Next, these apo-crystals were washed three times in cryoprotectant solution (32% PEG 3350, 25% glycerol, and 100 mM Tri-HNO₃ [pH 7.5]) and then soaked in the buffer containing 1 mM AgNO₃, 32% PEG 3350, 25% glycerol, and 100 mM Tris-HNO₃ (pH 7.5) for different time lengths (from 1 min to 1 wk) in darkness. Then, the crystals were flash-frozen in liquid nitrogen. All data sets were collected using 0.979-Å synchrotron radiation at the BL17U1 beamline of Shanghai Synchrotron Radiation Facility (SSRF), Chinese Academy of Sciences (CAS). The diffraction data were processed with HKL2000 at SSRF. Molecular replacement was performed using the program Phaser suite, and Zn⁺⁺-bound MCR-1 (PDB code: SGRG) was used as the template model. The Ag⁺⁺ and Zn⁺⁺ occupancies were refined based on atomic B-factor. High Ag⁺⁺ occupancy (≥0.7) and absorbance signal (≥0.2) prompted the success in the replacement of Zn⁺⁺. Model rebuilding and refinement were performed by Refmac. TLS refinement was incorporated into late refinement stages. Solvents were added in Coat and refined by Refmac. The final models were validated with MolProbity. Details of the data collections and analysis and model refinement statistics are summarized in SI Appendix, Table S2.

Measurement of Metal Ions. Briefly, 10 μM apo-MCR-1–S was incubated with 100 μM ZnOAc₂ in dialysis buffer (50 mM Hepes [pH 7.4]) overnight at 4 °C to ensure that the metal ions were fully loaded onto these proteins. The unbound Zn⁺⁺ ions were removed by dialysis in a Zn⁺⁺-free buffer to ensure that the metal ions were fully loaded into these proteins. The concentration of MCR-1–S with or without treatment were quantified by a BCA kit (Thermal Fisher Scientific), and metal contents were determined by ICP-MS (Agilent 7500a; Agilent Technologies) after digestion with 65% HNO₃ overnight using 115In (5 ppb) as an internal standard. Considering the well-known similar chemical properties between Zn⁺⁺ and Co⁺⁺, the molecular ratio of Zn⁺⁺ in MCR-1–S was double confirmed by a Co⁺⁺-stimulation ICP-MS assay by using similar method. A similar protocol was also performed on full-length MCR-1 from E. coli BL21(DE3) by cutting and transferring the SDS-PAGE gel band into clear tubes with 150 mM HNO₃ to digest and analyze silver contents. 155In–S contents were also measured for further quantification of protein concentration. These assays were performed in triplicate, and results were expressed as average ± SD.

Substrate Inhibition Assay. The substrate inhibition assay was performed by gel filtration and ultraviolet (UV) spectroscopy. Briefly, the apo-form of the full-length MCR-1 was incubated with 10 molar equivalents of AgNO₃ at 4 °C overnight. The resulting Ag-bound MCR-1 was incubated with equal molar NBD-glycero-3-P-EA in a substrate-reaction buffer (50 mM Hepes, 100 mM NaOAc, and 0.4% DDM [pH 7.5]) for 20 h and then applied into gel filtration in substrate-reaction buffer. A similar procedure was performed on apo-MCR-1 with an M₉²⁹₂A mutation. All separated fractions were immediately subjected to UV–vis spectroscopy analysis in a range of 220 to 800 nm to record their intensities if present.

Data Availability. The coordinate and structure factor of Ag-MCR-1–S have been deposited in the PDB (accession code 7WIA). All other data are included in the article and/or SI Appendix.

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