Synergistic antibacterial activity of silver with antibiotics correlating with the upregulation of the ROS production

Lili Zou1,2, Jun Wang2, Yu Gao3, Xiaoyuan Ren1, Martin E. Rottenberg3, Jun Lu1,4 & Arne Holmgren1

Thiol-dependent enzymes, including the thioredoxin (Trx) and glutathione (GSH) systems, have recently been found as promising bactericidal targets in multidrug-resistant (MDR) bacteria. We previously discovered that silver acted synergistically with ebselen in the inhibition of the Trx system and also resulted in a fast depletion of GSH in Gram-negative bacteria. Silver has been found by others to improve the sensitivity of bacteria to certain conventional antibiotics. Here, we found that the synergistic antibacterial effects of silver with four conventional antibiotics was correlated with the blockage of bacterial Trx system by silver. The synergistic antibacterial effect came along with the production of reactive oxygen species. All these results suggested that silver primarily enhanced the bactericidal activities of conventional antibiotics towards Gram-negative strains through the upregulation of ROS production.

The worldwide ever-increasing of bacterial resistance to the conventional medical antimicrobial agents is currently one of the most serious health crisis for modern medicine1–5. This has serious negative impacts such as decreases of the effectiveness of existing treatments, and causes higher morbidity and mortality rates in patients with infections caused by multidrug-resistant (MDR) bacteria6,7. European Antimicrobial Resistance Surveillance Network (EARS-Net) latest data showed high and increasing resistance of Gram-negative bacteria, which are combined with resistance to third-generation cephalosporins, fluoroquinolones, and aminoglycosides for both Escherichia coli and Klebsiella pneumoniae8. Given the fact that even education for the public, controlled use in food animals, and prudent antibiotic prescribing9 cannot stop the growth and spread of bacterial resistance and can only slow and delay it10,11. An option to overcome bacterial resistance is the combination of selected antibiotics with each other and with other drugs to improve antibacterial efficacy1,8,10.

We and other groups previously found that 2-phenyl-1,2 benzisoselenazol-3(2H)-one (ebselen), is an effective compound against Gram-positive pathogens (including MDR mycobacteria tuberculosis) by targeting thioredoxin (Trx), which could transfer electrons from NADPH to their substrates via thioredoxin reductase (TrxR) that is critical for bacterial survival11,12. Further, the addition of silver acted synergistically with ebselen in treating infections caused by MDR Gram-negative pathogens, including five clinically most difficult-to-treat Gram-negative bacteria: Acinetobacter baumannii, Enterobacter cloacae, Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa13. The combination directly inhibited Trx, deplete glutathione (GSH), and induced striking ROS production13. Moreover, the possibility of selection of resistant mutants is very rare14. Thus, thiol-dependent antioxidant systems are promising targets for the development of antibiotics against MDR strains.

Dwyer et al.15 found that antibiotics (ampicillin, gentamicin, and norfloxacin) could alter bacterial redox physiology, and the lethality was accompanied by ROS generation, and Belenky et al.16 profiled the E. coli metabolome

1Division of Biochemistry, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, SE-171 77, Stockholm, Sweden. 2Translational Neuroscience & Neural Regeneration and Repair Institute/Institute of Cell Therapy, The People’s Hospital of China Three Gorges University, 443000, Yichang, China. 3Department of Microbiology, Tumour and Cell Biology, Karolinska Institutet, SE-171 77, Stockholm, Sweden. 4School of Pharmaceutical Sciences, Southwest University, 400715, Chongqing, China. Lili Zou and Jun Wang contributed equally to this work. Correspondence and requests for materials should be addressed to J.L. (email: junlu@swu.edu.cn) or A.H. (email: arne.holmgren@ki.se).
and showed that antibiotics (ampicillin, kanamycin, and norfloxacin)-induced metabolic alterations elevated redox state, nucleotide oxidation, and increased oxidative stress by tightly regulated glutathione pools. Kohanski et al.17 stated that all three classes of bactericidal drugs (aminoglycoside, quinolone, and beta-lactam) mediated hydroxyl radical damage. All these above reports pointed out that antibiotics can influence the redox balance in bacteria, but the exact mechanism is still not clear. Meanwhile, Collins and coworkers17,18 proved that ROS production is one of the lethal factors of bactericidal antibiotics, and silver can enhance their antibacterial efficacy.

Antibiotic-mediated cell death is a complex, multi-faceted process that cannot be fully accounted for by the direct interactions of antibiotics with specific cellular targets. Here, we try to figure out whether antibiotics representing five different functional categories (beta-lactams, aminoglycosides, synthesis, tetracycline, and macrolides) can work synergistically with silver against Gram-negative bacteria through targeting redox system as ebselen and silver do13. Our results showed that silver could enhance certain antibiotics antibacterial effects against Gram-negative bacteria which are correlated to reactive oxygen species (ROS) production.

Results

Silver and certain antibiotics in combination exhibited synergistic antibacterial effects against E. coli. The antibacterial effects of silver nitrate (AgNO₃) and nine antibiotics representing five different functional categories (beta-lactams, aminoglycosides, synthesis, tetracycline, and macrolides) on the growth of a model Gram-negative bacterium, E. coli, which were detected in the 96 wells microplates. Overnight cultured E. coli DHB4 cells were diluted 1:1000 times in Luria Bertani (LB) medium, and incubated with serial dilutions of ionic silver (Ag⁺) as a nitrate salt and 9 antibiotics in combinations, separately, for 24 h. Ebselen was used as the positive control, which acted synergistically with silver against Gram-negative bacteria in our recent report13. The results here showed that 4 (gentamicin, kanamycin, geneticin, tetracycline) out of 9 antibiotics had synergistic activity on E. coli DHB4 growth under the conditions we tested (Table S1). Further, the Bliss model was used to quantify the degree of synergy at 1 h and 4 h between Ag⁺ and 4 antibiotics (gentamicin, kanamycin, geneticin, tetracycline) in combinations, and the results showed that Ag⁺ and 4 antibiotics indeed had synergistic antibacterial effects against E. coli (Fig. 1). All the results pointed out that Ag⁺ could enhance the antibacterial effects of certain antibiotics against Gram-negative bacteria. In the following experiments, we used these 4 antibiotics for further studies.

ROS was one of the lethal factors for synergistic bactericidal effects of Ag⁺ and antibiotics. Ag⁺ and ebselen in combination could induce a high level of ROS production13, and the effects of Ag⁺ and antibiotics in combinations need further studies. We, therefore, detected the production levels of ROS in Ag⁺ and 4 antibiotics combinations treated cells, and Ag⁺ and ebselen in combination was used as the positive control. The results showed that treatment with 5μM Ag⁺ alone could not cause a ROS response, and 5μM Ag⁺ and 80μM antibiotics in combinations resulted in the upregulation of ROS (p < 0.0001) when compare with either antibiotics (Fig. 2A) or silver (Fig. S1A). Furthermore, the increased levels of H₂O₂ caused by the treatment with 5μM Ag⁺ and 80μM antibiotics in combinations were also notarized by Amplex Red assay (p < 0.0001) (Figs 2B and S1B). All the results demonstrated that ROS might be one of the determining factors for synergistic bactericidal effects of Ag⁺ and antibiotics in combinations against E. coli.
Ag⁺ could disrupt bacterial Trx system. One of the main functions of thiol-dependent antioxidant systems is to scavenge ROS to keep cellular redox homeostasis and protect against oxidative stress. The disruption of the Trx and GSH systems may responsible for the upregulation of ROS. Ag⁺ and ebselen in combination has been proven to target both bacterial Trx and GSH systems, while the effects of Ag⁺ and antibiotics in combinations need further studies. E. coli DHB4 cells grown to OD₆₀₀nm of 0.4 were incubated with 80μM antibiotics and 5μM Ag⁺ in combinations, and 80μM ebselen and 5μM Ag⁺ in combination was used as the positive control. (A) The production level of ROS was detected by flow cytometry (CyAnadp, Beckman coulter), and mean fluorescent intensity (MFI) ± s. d. of H₂DCF-DA-stained E. coli was detected. (B) The production level of H₂O₂ was detected by the Amplex® Red Hydrogen Peroxide/Peroxidase method (Invitrogen). Reaction buffer contains 50μM Amplex® Red reagent, 0.1 U/mL HRP, and the indicated amount of H₂O₂ in 50 mM sodium phosphate buffer (pH 7.4), which was incubated for 30 minutes at 25 °C and further verified with absorbance at 560 nm. The background absorbance was detected by a non-H₂O₂ control reaction, which has been subtracted from each value. Data that are presented here as means ± s. d. of 3 independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 (Student’s t-test).
showed that when targeting Trx system, silver and antibiotics in combinations were not acting as fast as silver and ebselen do, which could effect Trx system in 10 min\textsuperscript{13}.

**Ag**\textsuperscript{+} and antibiotics could not directly disrupt bacterial GSH system. In our previous work, 5\textmu M Ag\textsuperscript{+} and 80\textmu M ebselen in combination has been proven to deplete the GSH after 10 min treatment\textsuperscript{13}. In our study, only 5\textmu M Ag\textsuperscript{+} and 80\textmu M gentamicin or kanamycin in combinations could slightly deplete the total GSH amount in cell extracts when compared with antibiotics themselves (p < 0.05) (Fig. 4A), meanwhile other combinations showed no differences (Fig. 4A) (p > 0.05). Further, the protein S-glutathionylation was decreased only in Ag\textsuperscript{+} and ebselen in combination incubated bacteria\textsuperscript{13}, but not in those bacteria cells treated with 5\textmu M Ag\textsuperscript{+} and antibiotics in combinations for 10 min (Fig. 4B) or 60 min treatments (Fig. 5B).

All the results above suggested that silver and antibiotics had no direct effects on bacterial GSH system when acting against Gram-negative bacteria.

**Five most difficult-to-treat MDR Gram-negative clinical isolates were highly sensitive to Ag**\textsuperscript{+} and ebselen in combination. We have isolated five clinically most difficult-to-treat MDR Gram-negative pathogen strains: Acinetobacter baumannii, Enterobacter cloacae, Escherichia coli, Klebsiella pneumonia and Pseudomonas aeruginosa. Overnight cultures of five isolates were diluted 1:1000 times in LB medium, and incubated with serial concentrations of Ag\textsuperscript{+} and antibiotics in combinations for 24 h (Table 1). The results showed that Ag\textsuperscript{+} and antibiotics in combinations exhibited weak antibacterial effects on these MDR Gram-negative bacteria. Meanwhile, Ag\textsuperscript{+} and ebselen in combination might be the only effective antibiotic against a range of resistant bacteria.

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**Figure 3.** Silver could directly disrupt bacterial Trx system. *E. coli* DHB4 cells grown to OD\textsubscript{600nm} of 0.4 were incubated with antibiotics and Ag\textsuperscript{+} in combinations for 10 min, and ebselen and Ag\textsuperscript{+} in combination was used as the positive control. (A) Trx and (B) TrxR activities were detected by using DTNB reduction assay in the presence of TrxR or Trx in *E. coli* DHB4 cells extracts. (C) *E. coli* DHB4 cells grown to OD\textsubscript{600nm} of 0.4 were incubated with antibiotics and Ag\textsuperscript{+} in combinations for 60 min, and ebselen and Ag\textsuperscript{+} in combination was used as the positive control. *E. coli* DHB4 cells extracts were precipitated by 5% TCA, and further alkylated with 15 mM AMS and the redox state of Trx1 was analyzed by redox Western blot. The mean ± s. d. of 3 independent experiments was calculated. The t-test significances were calculated between control and rest groups, and \textstar p < 0.05, \textdoublestar p < 0.01, \texttriplestar p < 0.001.
bacteria. The results might be explained by the fact that the synergistic antibacterial effect of silver and antibiotics only dependent on the upregulation the ROS production.

Discussion
Recently a first new antibiotic in decades called Teixobactin was described inhibiting the peptidoglycan cell wall synthesis. However, Teixobactin only inhibits Gram-positive bacteria like Staphylococcus aureus. Gram-positive bacteria in general have several differences to Gram-negative one which have a cell wall with an extra membrane. Most of Gram-positive bacteria lack GSH and associated enzymes like glutaredoxins.

The current antibiotics principles are mainly based on disruption of protein synthesis, cell wall or cell membrane assembly, DNA or RNA replication. We recently showed that targeting bacterial thiol-dependent redox enzymes is a distinct antibacterial mechanism compared with traditional antibiotics.
Figure 5. Silver and antibiotics in combinations could not directly disrupt the bacterial GSH system. *E. coli* DHB4 cells grown to OD₆₀₀ nm of 0.4 were incubated with antibiotics and Ag⁺ in combinations for 60 min, and ebselen and Ag⁺ in combination was used as the positive control. (A) Total amounts of GSH were detected by GR-coupled DTNB reduction assay in *E. coli* DHB4 cells extracts. (B) Changes in protein S-glutathionylation in *E. coli*. The mean ± s. d. of 3 independent experiments was depicted. The t-test significances were calculated between control and rest groups, and *p < 0.05, **p < 0.01, ***p < 0.001.

Table 1. MIC of silver (μM) in the presence of different antibiotics against MDR Gram-negative bacteria. Gentamycin: Genta; Kanamycin: Kana; Geneticin: Gene; Tetracycline: Tetra; Ebselen: Ebse.
There are two prime thiol-dependent enzyme systems, namely, thioredoxin (Trx) system, and glutathione (GSH)/glutaredoxin (Grx) system. For the Trx system, electrons transfer occurs from NADPH to their substrates via thioredoxin reductases (TrxR) \(^{20-23}\), meanwhile, for GSH/Grx system, electron transfer from NADPH to their substrates via glutathione reductases (GR) \(^{21,24,25}\). Thiol-dependent enzymes are critical for ribonucleotide reductase (RNR) and DNA replication and repair, or defense against oxidative stress via peroxiredoxins (Prxs) and methionine sulfoxide reductases (MSRs) \(^{21,24,25}\). Therefore, these two systems are critical for cell viability and proliferation.

Trx systems are ubiquitous in all bacteria, whereas the GSH system, as mentioned above, has been found to be absent in many pathogenic bacteria, including nearly all Gram-positive species \(^{19,22,26}\). We previously reported that ebselen, which is a substrate of mammalian TrxR but a competitive inhibitor of bacterial TrxR, executes selective antibacterial effect toward Gram-positive bacteria \(^{4,12,27,28}\). Further, we recently presented that silver and ebselen in synergistic combination had a strong selective bactericidal effect against Gram-negative bacterial infections. The combination directly inhibited both bacterial Trx and TrxR, and depleted intracellular GSH \(^{13}\). Based on these new findings, and combined with other published reports about antibiotics having influence on redox balance \(^{15-17}\), and that silver could sensitize Gram-negative bacteria to conventional antibiotics, thus we asked whether they also targeted the redox systems.

Drug-drug interactions can be classified as synergistic, antagonistic, or additive (no interaction). Nine antibiotics used in this work represent five different functional categories (beta-lactams, aminoglycosides, synthesis, tetracycline, and macrolides). Among them, total synthesis and tetracycline are broad-spectrum, aminoglycosides used in this work represent five different functional categories (beta-lactams, aminoglycodies, synthesis, tetracycline, and macrolides). Among them, total synthesis and tetracycline are broad-spectrum, aminoglycosides are particularly effective against Gram-negative bacteria, and beta-lactams and macrolides are particularly effective against Gram-positive bacteria. In the work reported here, 4 out of 9 antibiotics acted synergistically with silver against E. coli, a model Gram-negative bacterium (Fig. 1), which might occur through inducing ROS production as one of the lethal strategies (Figs 2 and 3).

E. coli has one TrxR, two Trxs (Trx1, and Trx2), and three major thiol peroxidases (bacterioferritin in comigratory protein (BCP), thiol peroxidase (Tpx), and alkyl hydroperoxide peroxidase subunit C (AhpC)) \(^{29,30}\). Trx1 in E. coli is involved in protein repair by providing the electrons to E. coli methionine sulfoxide reductases (Msr) \(^{30}\), which participates in the protection of E. coli against oxidative damage from reactive nitrogen intermediates \(^{31}\). Trx1 in E. coli also acts as a specific reductase for homodimeric Tpx to scavenge ROS \(^{32}\). Thus, the decrease of Trx1 activity via its oxidization is at least partially responsible for the ROS production to cause E. coli cell death. Also the oxidative folding of disulfide containing membrane proteins is dependent on Trx and is possibly targeted \(^{33}\).

Meanwhile, although silver could inhibit Trx and TrxR (Fig. 3), yet the effect targeting GSH system is not universal (Figs 4 and 5). The presence of the GSH-Grx system in E. coli may be regarded as a backup for the Trx system. GSH/Grxs in E. coli participate in the antioxidant process by deglutathionylation and transfer electrons to ribonucleotide reductase. Silver and antibiotics in combinations showed no effects on GSH amount, and the S-glutathionylated proteins are not much different from that of in control group (Figs 4 and 5). Thus, this might be one of the reason to explain the anti-MDR-Gram-negative bacteria activities of silver and antibiotics in combinations were much weaker than silver and ebselen in combination (Table 1 and Fig. 6).

**Figure 6.** A graphic illustration that showed mechanisms for the synergistic antibacterial activity of Ag\(^{+}\) with antibiotics in combination. Antibiotics and Ag\(^{+}\) could result in the upregulation of ROS production, and Ag\(^{+}\) could directly inhibit E. coli TrxR and Trx, both of which determine cell death. (A) Ag\(^{+}\) was a strong inhibitor of both E. coli thioredoxin (Trx) and thioredoxin reductase (TrxR). (B) Antibiotics and Ag\(^{+}\) could result in upregulation of ROS production to determine cell death. (C) It is time-dependent. The inhibition is stronger in 60 min treatment than 10 treatment. Information: Trx, thioredoxin; TrxR, thioredoxin reductase; GSH, glutathione; GR, glutathione reductase; Grx: glutaredoxin; Prx, peroxiredoxins; MSR, methionine sulfoxide reductase; RNR, ribonucleotide reductase; ROS, reactive oxygen species.
All in all, silver enhanced the antibacterial effects of certain antibiotics against Gram-negative bacteria through upregulate the ROS production. In order to be more effective, the Trx and GSH systems must be targeted, as well, since both systems are particularly important for ribonucleotide reductase in bacteria which is essential for DNA replication and repair.

Materials and Methods

Bacterial strains. Escherichia coli (E. coli) DHB4 cells and multidrug-resistance (MDR) Gram-negative clinical isolates (Tables 2 and 3) were used in in vitro experiments. Five Clinical MDR Gram-negative isolates were obtained from clinical patients in People's Hospital of China Three Gorges University in Hubei Province, PRC, with all approvals and informed consents. All experiments were performed in accordance with relevant guidelines and regulations, and were subject to protocols approved by the Medical Care and Welfare Committee of China Three Gorges University.

Antibiotics and chemicals. Bacteria cells were cultured in Luria Bertani (LB) medium (EMD millipore). 2-Phenyl-1, 2-benzisoselenazol-3(2H)-one (ebselen) (Daiichi), 9 antibiotics (Table 4): ampicillin, carbenicillin, gentamicin, streptomycin, kanamycin, chloramphenicol, tetracycline, erythromycin, silver nitrate (Sigma-Aldrich), Methoxypolyethylene glycol maleimide (MeO-PEG-Mal) (Sigma-Aldrich), Iodoacetamide (IAM) (Sigma-Aldrich), protease inhibitor cocktails (Roche), N-acetylcycteine (NAC) (Sigma-Aldrich), DC™ protein assay (Bio-RAD), E. coli DHB4 TrxR, sheep anti-E. coli Trx1 antibody was from IMCO Corp. (Stockholm, Sweden; http://www.imcocorp.se), Rabbit anti-sheep IgG-HRP (Santa cruz), IgG2a mouse monoclonal antibody for glutathione-protein complexes (VIROGEN), 4–12% bolt Bis-Tris gel (VWR), all the other reagents were from Sigma-Aldrich.

Synergistic antibacterial activity of silver and antibiotics in combinations on the E. coli DHB4 growth. E. coli DHB4 cells from frozen stock were grown overnight at 37°C, 400 rpm. The overnight culture was diluted 1:100 with 5 ml of LB medium in 15 ml tubes and incubated at 37°C at 400 rpm. Overnight cultured DHB4 cells were grown until an OD600 nm of 0.4 and were used for antibiotic treatment. Briefly, DHB4 cells were diluted 1:1,000 into 100 µl of LB medium. Serial dilutions of antibiotics 100 µl (0, 1, 2, 4 µM) and silver nitrate

Table 2. Clinically isolated multidrug-resistant Gram-negative strains used in this work.

| Strain | Description | Source |
|--------|-------------|--------|
| KP-2   | K. pneumoniae subsp. pneumoniae 0322* | This work |
| AB-1   | Acinetobacter baumannii (A. baumannii) H1* | This work |
| PA-1   | Pseudomonas aeruginosa (P. aeruginosa) 1298* | This work |
| ECL-2  | E. cloacae 2301* | This work |
| ECO-1  | Escherichia coli (E. coli) 1139* | This work |

Table 3. Drug sensitivity of clinical isolated multidrug-resistant Gram-negative bacteria. *R: resistant; S: sensitive.
EDTA. The absorbance at 412 nm was detected for 5 min. The reaction mixture containing 50 nM GR, 200 μM NADPH, 50 mM Tris·HCl (pH 7.5), 1 mM DTNB and 1 mM EDTA was used for the positive control. Redox Western blotting was performed to detect the redox state of Trx1 in the DHB4 cell extracts was detected by the DTNB reduction activity assay 23. These experiments were performed in the 96 microwell plates that contains 50 μM Amplex ® Red reagent, and 0.1 U/mL HRP in 50 mM sodium phosphate buffer (pH 7.4) for 30 minutes at 25 °C that protected from light and measured with absorbance at 560 nm (Molecular Probes, Eugene, OR).

**Detection of Trx/TrxR activities and GSH amount in antibiotics and silver treated E. coli cell lysates.** E. coli DHB4 were grown until the OD 600 nm of 0.4 in 5 ml LB medium, and the DHB4 cells were incubated with silver and antibiotics in combinations for 10 min. Treated cells were harvested by centrifugation at 6,000 rpm for 5 min and thoroughly washed 3 times by PBS, and sonicated for 20 min. 50 μl samples were incubated with 50 mM Tris·HCl (pH 8.5) with 0.5% SDS containing 15 mM β-mercaptoethanol for 20 min. After the incubation, treated cells were spun down and re-suspended in PBS, and the amount of ROS was measured by flow cytometry (CyAnadp, Beckman coulter).

**Measurement of H2O2 Production.** The E. coli DHB4 were grown until the OD 600 nm of 0.4 in 5 ml LB medium, and the DHB4 cells were incubated with silver and antibiotics in combinations for 10 min. Treated cells were harvested by centrifugation at 6,000 rpm for 5 min and thoroughly washed 3 times by PBS, and sonicated for 20 min. The treated cells were collected by centrifugation at 13,000 rpm for 20 min and the protein concentrations were measured with the Lowry protein kit.

| Antibiotic | Abbreviation | Dose (μM) used | Primary target |
|------------|--------------|---------------|----------------|
| Ampicillin | Amp          | 0/1/2/4       | Cell wall formation |
| Carbenicillin | Car          | 0/1/2/4       | Cell wall formation |
| Chloramphenicol | Chl          | 0/1/2/4       | Protein synthesis, 30S ribosomal subunit |
| Erythromycin | Ery          | 0/1/2/4       | Protein synthesis, 30S ribosomal subunit |
| Gentamicin | Gent         | 0/1/2/4/80    | Protein synthesis, 30S ribosomal subunit |
| Gentamicin | Gent         | 0/1/2/4/80    | Protein synthesis, 30S ribosomal subunit |
| Kanamycin | Kan          | 0/1/2/4/80    | Protein synthesis, 30S ribosomal subunit |
| Streptomycin | Str          | 0/1/2/4       | Protein synthesis, 30S ribosomal subunit |
| Tetracycline | Tet          | 0/1/2/4/80    | Protein synthesis, 30S ribosomal subunit |

Table 4. Antibiotics used in the study with their dosages and primary targets.

(AgNO3, 0, 1.25, 2.5, 5, 10, 20, 40, 80μM) were added. The minimum inhibitory concentration (MIC) was verified as the lowest concentration of drugs that inhibited 90% of growth compared to the untreated cells after 24 hours culture at 37 °C. The cultures treated with the same serial dilutions of 100 μlebselen and silver nitrate were used as the positive control.

**Quantifying synergy of ebselenand silver using the bliss model.** Drug synergy was determined using the Bliss Independence Model, which calculates a degree of synergy using the following formula: S = (f XY / f00) − (fX+fY − f00), where f00 refers to the wild-type growth rate in the presence of the combined drugs at a concentration X, for one of the drugs, and Y for the other; fX and fY refer to the wild-type growth rates in the presence of the individual drugs at a concentration of X and Y, respectively; f00 refers to the wild-type growth rate in the absence of drugs; and S corresponds to the degree of synergy, a parameter that determines a synergistic interaction for positive values and an antagonistic interaction for negative ones. Growth rates at different time points are determined by calculating the slope of the growth or kill curve being analyzed 18.

**Detection the production of ROS.** The E. coli DHB4 were grown until the OD 600 nm of 0.4 in 5 ml LB medium, and the DHB4 cells were incubated with silver and antibiotics in combinations for 10 min. To measure the production level of ROS in the treated bacteria, DHB4 cells were harvested by centrifugation at 6,000 rpm for 5 min and thoroughly washed 3 times by PBS, and cells were further stained by 5 μM H2DCF-DA that protected from light for 20 min. After the incubation, treated cells were spun down and re-suspended in PBS, and the amount of ROS was detected by flow cytometry (CyAnadp, Beckman coulter).
MeO-PEG-Mal at 37 °C for 2 hours. Proteins were collected by centrifugation at 13,000 rpm for 20 min and the protein concentration was verified with the Lowry protein kit. Proteins were incubated with SDS-loading buffer at 90 °C for 10 min, and then separated on the 4–12% bolt Bis-Tris gel with MES running buffer (150 V, 40 min). The redox state of Trx1 was detected with NPSH anti-E. coli Trx1 antibody at 1:1000 dilution, followed by the detection of Chemiluminescence Reagent Plus.

Proteins S-glutathionylation in *E. coli* cells treated by silver and antibiotics in combinations. Total protein S-glutathionylation of antibiotics and AgNO₃ in combinations treated *E. coli* DHB4 cells was verified by Western blot. *E. coli* DHB4 were grown until the OD₆₀₀nm of 0.4 in LB medium, and the DHB4 cells were incubated with 80 µM antibiotics and 5 µM AgNO₃ for 10 and 60 min, respectively. The cells treated with 5 µM AgNO₃ and 80 µM ebselen were used as the positive control. Cells were washed 3 times, and re-suspended in lysis buffer (10 mM sodium pyrophosphate, 100 mM NaCl, 2.5 mM EDTA, 25 mM Tris-HCl (pH 7.5), 2.5 mM EGTA, 1 mM Na₂VO₄, 20 mM sodium β-glycerophosphate, 0.5% Triton X-100, 20 mM NaF and 50 mM IAM) containing protease inhibitor cocktail. After sonication, the treated cell lysates were collected by centrifugation at 13,000 rpm for 20 min. Protein concentration was measured with Lowry protein kit, and Western blot was performed as described above with IgG2a mouse monoclonal antibody (VIROGEN, 101-A/D8) for S-glutathione-protein complexes.

Antibacterial activities of antibiotics and silver on the growth of MDR Gram-negative clinical isolates. Five MDR Gram-negative clinical isolates were grown till an OD₆₀₀nm of 0.4, and were diluted 1:1,000 into 100 µL LB medium. Serial dilutions of 100 µl antibiotics (0, 1, 2, 4 µM) and AgNO₃ (0, 1.25, 2.5, 5, 10, 20, 40, 80, 160 µM) in combinations were added to the individual wells. The MBC was verified after 16 hours culture at 37 °C. The cells treated with the same serial dilutions of 100 µl ebselen and silver nitrate were used as the positive control.

Statistical analysis. Mean, Standard Deviation (SD) and t-test (two tails, unpaired) significances were calculated in GraphPad Prism Software. *p < 0.05, **p < 0.01, ***p < 0.001.

Data availability. The data sets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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**Author Contributions**

L.Z., J.L. and A.H. conceived the study. L.Z., J.W., J.L., A.H. designed experiments. L.Z., J.W., Y.G. performed experiments. L.Z., J.L., A.H. wrote the paper. All authors analyzed the results and approved the final version of the manuscript.

**Additional Information**

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