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ORIGINAL RESEARCH

Enhance the Antimycobacterial Activity of Streptomycin with Ebselen as an Antibiotic Adjuvant Through Disrupting Redox Homeostasis

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Purpose: Tuberculosis (TB) remains a major health threat worldwide, and the spread of drug-resistant (DR) TB impedes the reduction of the global disease burden. Ebselen (EbSe) targets bacterial thioredoxin reductase (bTrxR) and causes an imbalance in the redox status of bacteria. Previous work has shown that the synergistic action of bTrxR and sensitization to common antibiotics by EbSe is a promising strategy for the treatment of DR pathogens. Thus, we aimed to evaluate whether EbSe could enhance anti-TB drugs against *Mycobacterium marinum* (*M. marinum*) which is genetically related to *Mycobacterium tuberculosis* (*Mtb*) and resistant to many antituberculosis drugs.

Methods: Minimum inhibitory concentrations (MIC) of isoniazid (INH), rifampicin (RFP), and streptomycin (SM) against *M. marinum* were determined by microdilution. The Bliss Independence Model was used to determine the adjuvant effects of EbSe over the anti-TB drugs. Thioredoxin reductase activity was measured using the DTNB assay, and its effects on bacterial redox homeostasis were verified by the elevation of intracellular ROS levels and intracellular GSH levels. The adjuvant efficacy of EbSe as an anti-TB drug was further evaluated in a mouse model of *M. marinum* infection. Cytotoxicity was observed in the macrophage cells Raw264.7 and mice model.

Results: The results reveal that EbSe acts as an antibiotic adjuvant over SM on *M. marinum*. EbSe + SM disrupted the intracellular redox microenvironment of *M. marinum* by inhibiting bTrxR activity, which could rescue mice from the high bacterial load, and accelerated recovery from tail injury with low mammalian toxicity.

Conclusion: The above studies suggest that EbSe significantly enhanced the anti-*Mtb* effect of SM, and its synergistic combination showed low mammalian toxicity in vitro and in vivo. Further efforts are required to study the underlying mechanisms of EbSe as an antibiotic adjuvant in combination with anti-TB drug MS.

Keywords: Ebselen, antibiotic adjuvant, thioredoxin reductase, reactive oxygen species

Introduction

Tuberculosis (TB) treatment requires long-term multidrug therapy, and treatment failure frequently leads to the evolution and spread of drug resistance in *Mycobacterium tuberculosis* (*Mtb*).[1] Multidrug-resistant *Mtb* poses a substantial threat to public health and has long been at the top of the WHO pathogen priority list.[2] Multiple strategies have been tested to address antibiotic resistance in TB patients. Among these, antibiotic adjuvants offer an alternative approach to the development of new antibiotics. Antibiotic adjuvants are compounds that enhance antibiotic activity or inhibit bacterial

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resistance when administered in combination with antibiotics. This is a feasible and cost-effective way to reinvigorate old antibiotics with antibiotic adjuvants, as few novel antibiotics are currently under clinical development.

Ebselen (EbSe), a low molecular weight organo-selenium compound, is an inhibitor of *Mtb* antigen 85C (Ag85C).[3,4] It also possesses specific inactivation activity for bacterial thioredoxin reductase (bTrxR), which is the most recently recognized molecular target of this compound.[5,6] Inhibition of bTrxR blocks the disulfide reduction system in thioredoxin (Trx), which alters the biosynthesis of DNA, influences the redox status of bacterial cells, and induces ROS-related damage to *Mtb*[.[3,7] In addition, a previous study showed that the synergistic action of bTrxR and sensitization to common antibiotics, assisted by the use of EbSe, appears to be a promising direction in the treatment of drug-resistant (DR) pathogens.[7–10] Combined with the phenomenon that it is difficult for EbSe to induce DR,[3] making a case for evaluation of the combined activity of EbSe and anti-TB drugs in *Mtb*.

Streptomycin (SM) is one of the most effective drugs used for the treatment of DR TB.[11] SM was used as a monotherapy regimen to treat TB.[10] SM is also part of the first short-course TB therapy, which is combined with three first-line antibiotics: isoniazid (INH), rifampicin (RFP), and pyrazinamide (PZA).[12,13] These data suggest that there is a high demand for new effective antibiotics that can be used in combination with SM to augment bioactivity and impair tolerance.[14]

Mycobacterium marinum (*M. marinum*) is an environmental waterborne bacterium and opportunistic zoonotic pathogen that induces piscine mycobacteriosis and human cutaneous infections. Because of its genetic relatedness to *Mtb*, it grows faster than *Mtb* and is less pathogenic to humans, *M. marinum* is an experimentally tractable model organism for studying *Mtb*.

To the best of our knowledge, no in vitro and in vivo studies have reported the adjuvant activity of EbSe over classical anti-TB drugs in *Mtb*, and this subject deserves more attention. Thus, we set forth three goals: First, we investigated the antibacterial activities of EbSe + anti-TB drugs on *M. marinum* (genetically related to *Mtb*, a surrogate for *Mtb*) in vitro. Second, we evaluated the adjuvant efficacy of EbSe over anti-TB drugs by targeting *M. marinum* bTrxR. Third, we assessed the efficacy and toxicity of the EbSe + anti-TB drugs in vitro and in vivo. Our assays provide a facile screening platform for *M. marinum* bTrxR inhibitors by using EbSe, which inhibits DR and growth.

Materials and Methods

Mouse, Cell Line and Bacterial Strain

We obtained approval from the Medical Animal Care and Welfare Committee of China Three Gorges University for the use of C57BL/6J mice (male, 18–22 g) for research, and followed the policies of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. All mice were kept individually and fed normally in a conventional SPF animal house and were given free access to food and water.

Mycobacterium marinum (*M. marinum*) is a model organism for *Mtb*,[15] and *M. marinum* ATCC BAA-535 was a gift from Pro. Decheng Wang from the Institute of Infection and Inflammation of China Three Gorges University. All experiments were performed in a BSL2 laboratory.

Murine Raw264.7 macrophage cell line was purchased from Procell Life Science & Technology Co., Ltd.

Bacterial Growth Condition

M. marinum ATCC BAA-535 was routinely grown at 32°C, 100 rpm in a Difco Middlebrook 7H9 (with 10% OADC enrichment, 0.5% glycerol, and 0.05% Tween 80) or on Middlebrook 7H10 agar (with 10% OADC enrichment and 0.5% glycerol).

Inhibitory Effects of EbSe and SM/INH/RFP Against M. Marinum

M. marinum ATCC BAA-535 cells were cultured to A_{600} = 0.4, and then diluted 1000 times to be further treated with serial concentrations of EbSe (Daiichi) + anti-TB SM/INH/RFP (Selleck) drugs for 24 h, after which A_{600} was measured. The mean OD of quadruplicate wells in the indicated groups was used to calculate the percentage of bacterial viability as follows: percentage of bacterial viability = $(A_{treatment} - A_{blank}) / (A_{control} - A_{blank}) \times 100\%$.

The synergism of EbSe + anti-TB SM/INH/RFP drugs was determined using the Bliss Independence Model, which calculates the degree of synergy using the following formula: $S = (f_{X0}/ f_{00})(f_{0Y}/f_{00}) - (f_{XY}/f_{00})$, where f_{XY} refers to *M. marinum* growth rate in the presence of the EbSe + anti-TB drugs in combination at a concentration X for EbSe, and Y for anti-TB drugs; f_{X0} and f_{0Y} refer to *M. marinum* growth rates in the presence of the individual EbSe and anti-TB drugs at a concentration of X and Y, respectively; f_{00} refers to *M. marinum* growth rate in the absence of EbSe + anti-TB drugs, and S corresponds to the degree of synergy. All experiments were performed in triplicate.

The Inhibition of M. Marinum TrxR Activity by EbSe + INH/SM

M. marinum ATCC BAA-535 cells were cultured to A_{600} = 0.4, and further incubated with EbSe (80 μM) + INH (1900 μM) or SM (88 μM) for 30 min. The cells were then collected, washed three times with PBS, and resuspended in 50 mM Tris-EDTA buffer (pH 7.4) containing a cocktail. The cells were disrupted by sonication (240 W, 5 min) to obtain the supernatant and the protein concentration was measured using the Bradford assay.

25 μg obtained cell lysto were incubated with 2 mM EDTA and 200 μM NADPH at 37°C for 5 min, after which 5 μM Trx (IMCO) and 2 mM DTNB were added. A_{412} was determined using a VERSA microplate reader for 5 min, and the slope was used to represent TrxR activity. The activity in the untreated group was 100%.

Two-Dose Administration of EbSe + SM Against M. Marinum

M. marinum ATCC BAA-535 cells were cultured to A_{600} = 0.4 and diluted 1000 times to be treated with EbSe (80 μM) + SM (88 μM) for 24 h. 24 h post-incubation, the bacteria cells from one of the two plates were given a booster dose treatment [EbSe (80 μM) + SM (88 μM)]. A visible spectrophotometer (UV-Vis) was used to determine the A_{600} at different time points for another 24 h. All experiments were performed in triplicate.

Post-Antibiotic Effect of EbSe + SM Against M. Marinum

M. marinum ATCC BAA-535 cells were cultured to A_{600} = 0.4 and diluted 1000 times, and further treated with EbSe (80 μM) + SM (88 μM) for 24 h. Then, the drugs were washed three times with PBS (pH 7.4). The A600 of *M. marinum* cells was measured every 1 h for 6 h. All experiments were performed in triplicate.

The Effect of EbSe + SM on M. Marinum at Different Growth Stages

M. marinum cells were cultured to the lag phase (A_{600} = 0.2), log phase (A_{600} = 0.4), and stationary phase (A_{600} = 1.0) and diluted 1000 times to be treated with EbSe (80 μM) + SM (88 μM). UV-Vis spectroscopy was used to determine A_{600} at different time points for 24 h. All experiments were performed in triplicate.

Determination of the ROS Production in M. Marinum Treated with EbSe + SM

M. marinum ATCC BAA-535 cells were cultured to A_{600} = 0.4, and incubated with EbSe (80 μM) + SM (88 μM) for 30 min. collected *M. marinum* cells were washed three times with PBS and stained with 10 μM H_2DCFH-DA for 30 min at 37°C. After incubation, the cells were washed thrice, resuspended in PBS, and quantified using flow cytometry (Beckman Coulter, AW15093). All experiments were performed in triplicate.

The Rescue Effect of DTT Over EbSe + SM-Treated M. Marinum

M. marinum ATCC BAA-535 cells were cultured to A_{600} = 0.4, and diluted 1000 times to be treated with EbSe (80 μM) + SM (88 μM) after pre-incubation with 4 μM dithiothreitol (DTT) for 30 min at 26°C. The A600 of *M. marinum* cells was measured for 24 h. All experiments were performed in triplicate.

EbSe + SM on M. Marinum Bacterial Morphology

M. marinum ATCC BAA-535 cells were cultured to A_{600} = 0.4, and separately treated for 30 min with EbSe (80 μM), SM (88 μM), or PBS, or pre-incubated with 4 μM dithiothreitol (DTT) followed by EbSe (80 μM) + SM (88 μM) treatment. The bacterial cells were collected and fixed with 2.5% glutaraldehyde. The morphology and structure of *M. marinum* cells were observed using a transmission electron microscope (Hitachi H-7500, Japan).

Trx1 Protein Expression Level in in M. Marinum Treated with EbSe + SM

M. marinum ATCC BAA-535 cells were cultured to A_{600} = 0.4 and incubated with EbSe (80 μM) + SM (88 μM) for 30 min. Trx1 expression levels were detected by Western blotting with an Anti-Trx1 polyclonal antiserum (Coomassie Brilliant Blue-stained SDS-PAGE gel after electrophoresis as a reference). All experiments were performed in triplicate.

The Treatment of EbSe + SM Over M. Marinum-Caused Tail Vein Infectious Mice

23 healthy C57BL/6J male mice were pre-housed for 3 days and randomly divided into five groups: PBS (n=3), DMSO (n=5), EbSe (n=5), SM (n=5), and EbSe combined with SM (n=5). The PBS group was injected with PBS in the tail vein, and the other groups were injected with 3×10^8 CFU/mL of *M. marinum* ATCC BAA-535 in the tail vein. The mice were injected intraperitoneally with PBS, EbSe solvent (DMSO), EbSe (20 mg/kg b.w)., SM (15 mg/kg b.w)., or EbSe (20 mg/kg b.w). + SM (15 mg/kg b.w). once every other day for a total of seven doses, and then the mice were continuously observed for 3 days.

Tail tissues were fixed in formalin (10%) and embedded in paraffin. Anti-IL-6, anti-IL-10, anti-TNF-α, and anti-iNOS antibodies were used for immunohistochemical (IHC) analysis of cytokines in the tail tissue.

Tail, kidney, and liver sections from the mice were fixed in 10% phosphate-buffered formalin and embedded in paraffin. All tissues were sectioned, stained with hematoxylin and eosin (H&E) solution, and examined by light microscopy.

The Toxicity of EbSe + SM on Macrophage Raw264.7

Cells were seeded and cultured at a density of $5*10^3$/well, and treated with EbSe + SM for 24 or 48 h. Finally, CCK-8 reagent was added to detect the viability of Raw264.7 cells. All experiments were performed in triplicate.

The Toxicity of EbSe + SM on Mice

Twenty healthy male C57BL/6J mice were randomly divided into four groups: EbSe solvent (DMSO), EbSe (20 mg/kg body weight), SM (600 mg/kg body weight), and EbSe (20 mg/kg body weight) + SM (600 mg/kg body weight). All the groups received 14 doses once daily.

Blood samples were collected on the last day to determine serum alanine transaminase (ALT), aspartate aminotransferase (AST), serum albumin (ALB), urea nitrogen (BUN), creatinine (Cre), and uric acid (UA) levels using a fully automatic biochemical analyzer (Siemens, viva proE).

Group Size and Statistical Analysis

For in vitro experiments, a n=5 for each group, while along with triplicate biological replications. For in vivo experiments, a n=5 for each group, while along with duplicate biological replications. Statistical analyses were performed by GraphPad Prism 6.0 (GraphPad Software). The means of data between two groups were compared using an unpaired two tailed test Student's *t* test. Sample rates between the two groups were tested using chi-squared analysis. Statistical significance was set at $P < 0.05$.

Results

EbSe Enhance Antimycobacterial Activity of SM by Synergistic Targeting bTrxR

Inhibition of the bTrxR is a novel antibiotic approach that has been widely employed to effectively combat *Escherichia coli*,[7] *Yersinia pseudotuberculosis*,[9] and *Staphylococcus aureus*.[10] To determine the adjuvant effect of EbSe over SM/INH/RFP against *M. marinum*, a Bliss Independence Model was first determined. The synergisms of EbSe (80 μM) + RFP (1.2 μM), EbSe (80 μM) + INH (900 μM), and EbSe (80 μM) + SM (88 μM) were −0.138, 0. 25, and 0.43, respectively (Supplemental Figure 1), indicating that both EbSe + INH and EbSe + SM exerted synergistic inhibitory effects against *M. marinum*.

Further, following drug treatment, we measured bTrxR activity of *M. marinum* using the DTNB assay. The results indicated that, although EbSe (80 μM) + SM (88 μM) can synergistically inactivate bTrxR, EbSe (80 μM) + INH (900 μM) had no synergistic inhibitory impact on bTrxR (Figure 1A and B).

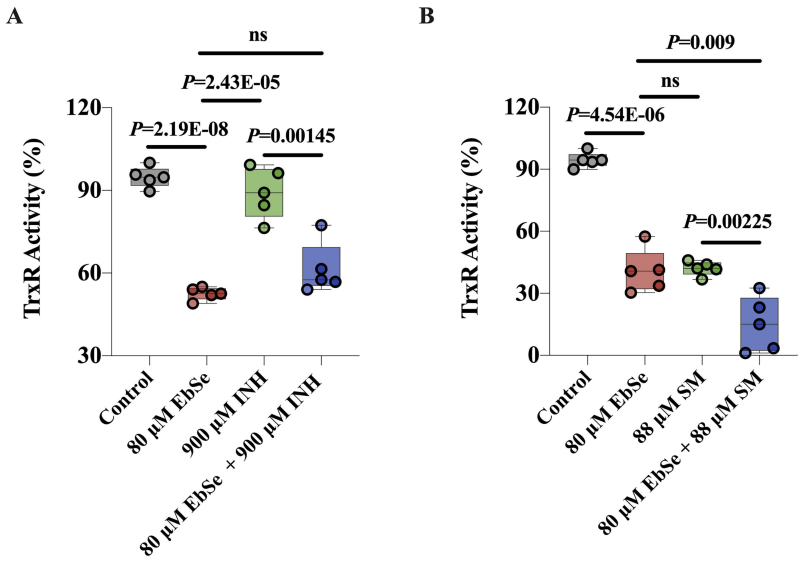


Figure 1 The detection of EbSe + anti-TB drugs disrupt bTrxR. *M. marinum* ATCC BAA-535 cells were cultured until an A_{600}= 0.4 and treated for 30 min with serial concentrations of EbSe + anti-TB drugs. TrxR activity endpoint was detected using DTNB reduction assay in the presence of Trx in *M. marinum* extracts. (**A**) EbSe + INH. (**B**) EbSe + SM. N = 5 independent observations per group, and triplicate biological replications were applied. Data are presented by Student's *t* test, $p<0.05$ constitutes statistical significance.

EbSe + SM Has a Bacteriostatic Effect Against M. Marinum in Its Different Growth Phases

Mycobacterium can enter a dormant state that displays phenotypic resistance to many of the commonly used drugs for treating TB[16] and lacks drugs that have activity against dormant bacilli.[17] To detect whether EbSe could assist SM against *M. marinum*, ATCC BAA-535 cells were cultured in the stationary phase (A_{600}=0.2), logarithmic growth phase (A_{600}=0.6), and stable phase (A_{600}=1.0) and detected by UV-Vis assay for 24 h. The results demonstrated that EbSe (80 μM) + SM (88 μM) had significant inhibitory effects on *M. marinum* in all three phases (Figure 2A–C).

The post-antibiotic effect (PAE) has been recognized as a pharmacodynamic parameter that may influence the optimal dosage intervals and can be used to define the vulnerability of bacterial targets.[18] Thus, the PAE of EbSe (80 μM) + SM (88 μM) was detected, and the results showed that the PAE of EbSe + SM against *M. marinum* was 4 h, which is longer than many clinical antibiotics, including quinolone (Figure 2D–F).

In addition, a booster dose was administered to evaluate whether EbSe could augment SM activity against *M. marinum*. The results showed that the bacteria were still able to grow under two-dose administration (Figure 2G–I). In conclusion, EbSe + SM exhibited bacteriostatic effects on *M. marinum*.

EbSe + SM Disrupted the Intracellular Redox Microenvironment of M. MarinuM

TrxR is the only known enzyme that catalyzes Trx reduction in *M. marinum*.[5] TrxR inhibition increases cellular oxidative stress, ultimately affecting bacterial survival, death, activation, and proliferation.[19,20]

The results showed that EbSe + SM significantly increased the intracellular ROS production in *M. marinum* (Figure 3A and B), causing cell damages (Supplemental Figure 2A). In addition, dithiothreitol (DTT), a small-molecule reducing agent, was added to verify that ROS accumulation is a key virulence factor. DTT effectively rescued the growth inhibition and cell damage of *M. marinum* caused by EbSe + SM (Figure 3C, Supplemental Figure 2B). Furthermore, Western blotting showed that EbSe + SM had no effect on Trx1 protein expression in *M. marinum* (Figure 3D and E).

All results revealed that EbSe + SM disrupted the intracellular redox microenvironment of *M. marinum* by inhibiting bTrxR activity instead of affecting the protein expression level of Trx1, and ROS accumulation was a virulence factor.

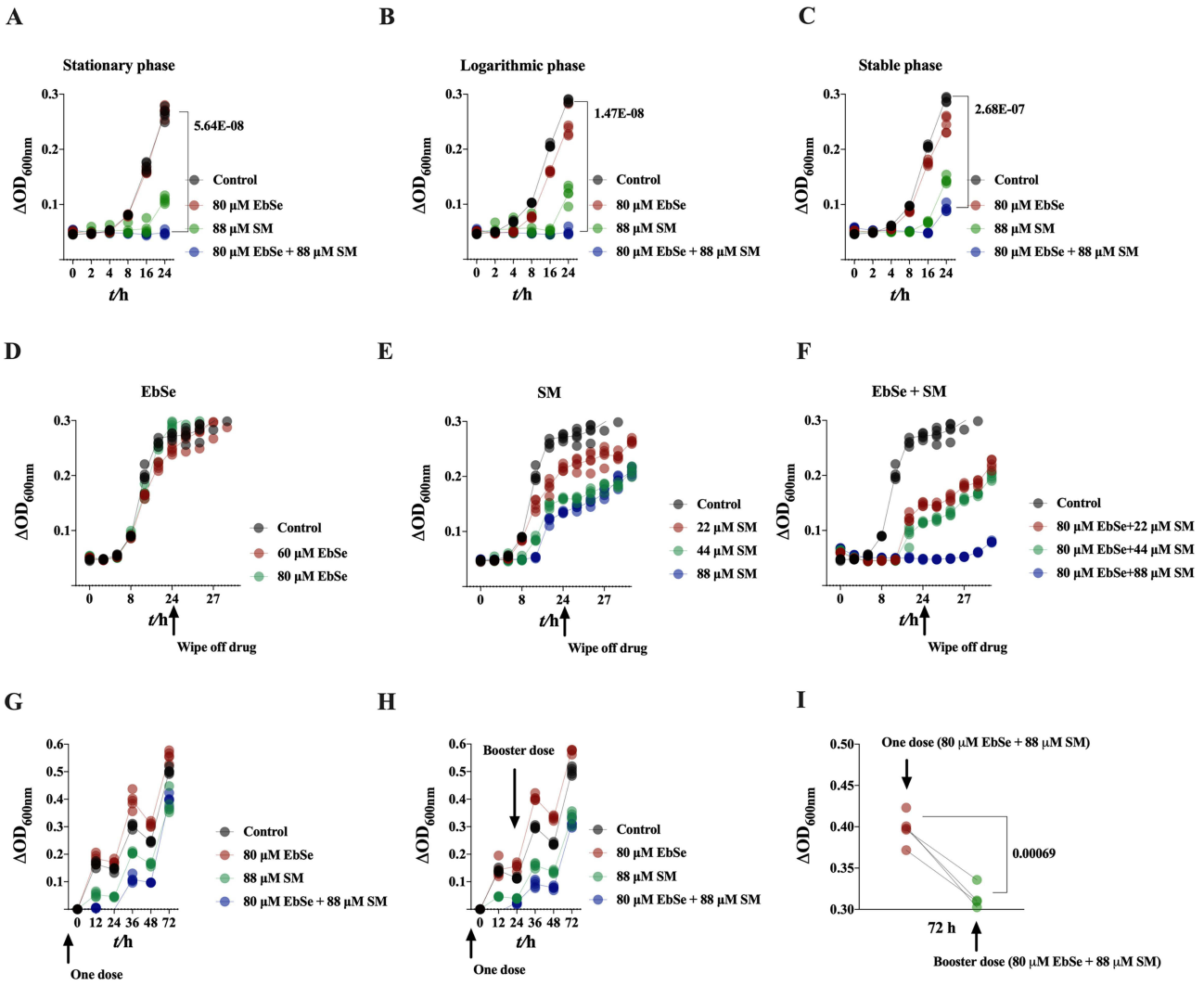


Figure 2 Antibacterial effect of EbSe + SM on *M. marinum*. *M. marinum* ATCC BAA-535 cells were cultured until an A_{600}= 0.4, diluted 1:1000 times, and treated with EbSe + SM. (**A–C**). Cell viability was represented by measuring A_{600}. The growth curves showed a synergistic antibacterial effect of EbSe + SM on *M. marinum*. Cell viability was represented by measuring A_{600}. (**A**) Stationary phase (A_{600}=0.2). (**B**) Logarithmic growth phase (A_{600}=0.6). (**C**) Stable phase (A_{600}=1.0). (**D–F**) The PAE effect of EbSe + SM against *M. marinum*. (**D**) EbSe. (**E**) SM. (**F**) EbSe + SM. (**G–I**) The bacteriostasis effect of EbSe + SM against *M. marinum*. (**G**) One-dose administration. (**H**) Two-dose administration. (**I**) One-dose VS two-doses. Cell viability was represented by measuring A_{600}. N = 5 independent observations per group, and triplicate biological replications were applied. Data are presented by Student's *t* test, $p<0.05$ constitutes statistical significance.

EbSe Enhance the Antimycobacterial Activity of SM Over M. Marinum-Infected Mice

The murine *M. marinum* infection model, in which the bacteria produce a local infection restricted to the tail tissue, has the advantage of mimicking some of the key hallmarks of human tuberculosis, such as the formation of granulomas with central caseating necrosis and the spontaneous development of a latency-like stage.[21] Thus, we constructed a mouse tail vein infection with *M. marinum* to see whether the drug remained effective in vivo. The results showed that EbSe + SM effectively assisted mouse tail recovery from infection (Figure 4A). In addition, we measured the damaged area of the mouse tail from days 3 to 24 and counted the bacterial load on day 24 (Figure 4B and C). Our results indicated that EbSe + SM reduced the bacterial load and accelerated recovery from injury.

Furthermore, we collected mouse tail tissues and performed hematoxylin-eosin (H-E) staining on day 24. Staining revealed that control mice had an increased number of inflammatory cells. Meanwhile, EbSe + SM-treated mice had fewer scattered lymphocytes (Figure 5A). We also measured the presence of the pro-inflammatory cytokines IL-6, iNOS, and TNF-α, and the anti-inflammatory cytokine IL-10 by immunohistochemistry (IHC). As shown in Figure 5B–E, EbSe

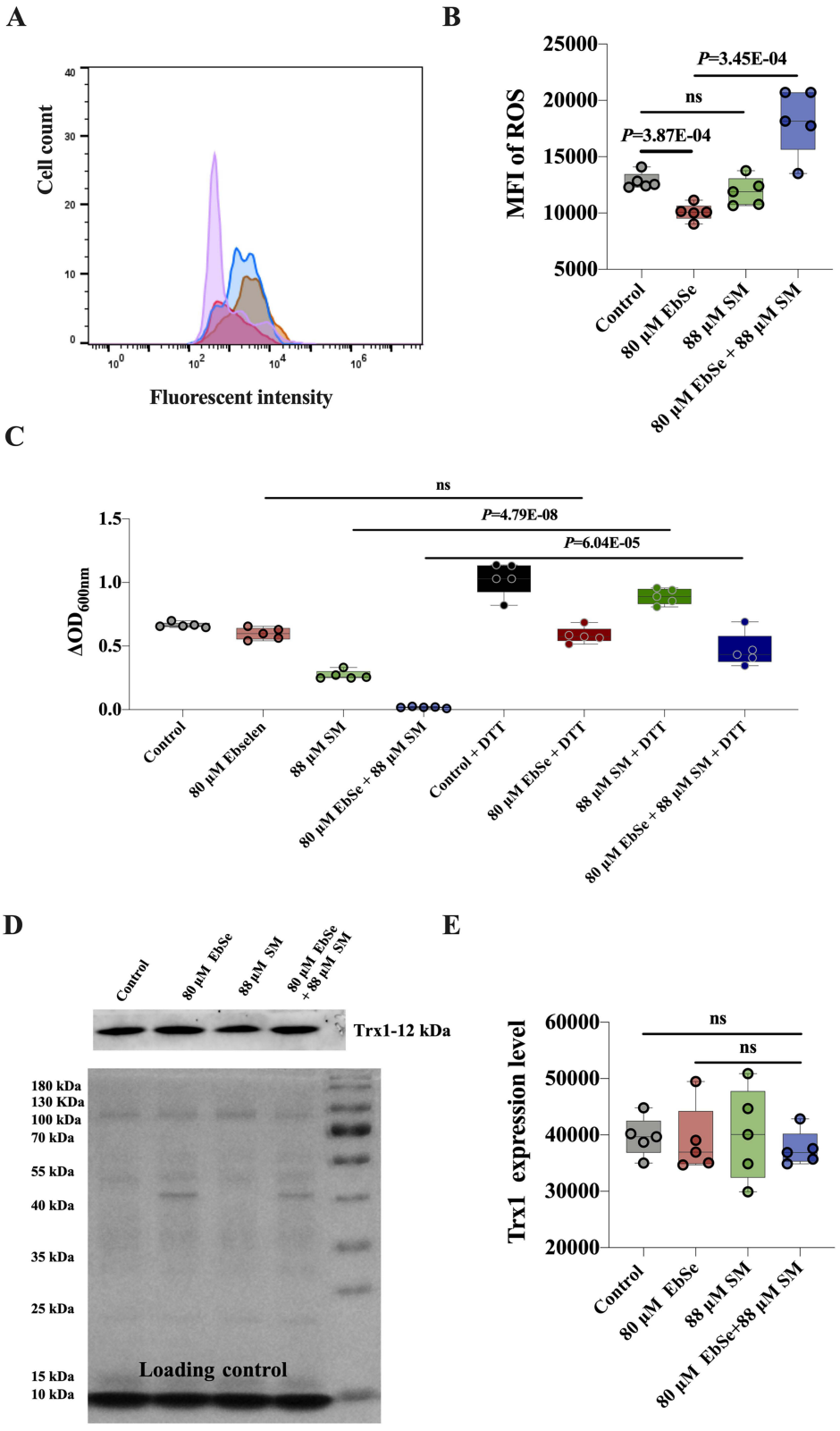
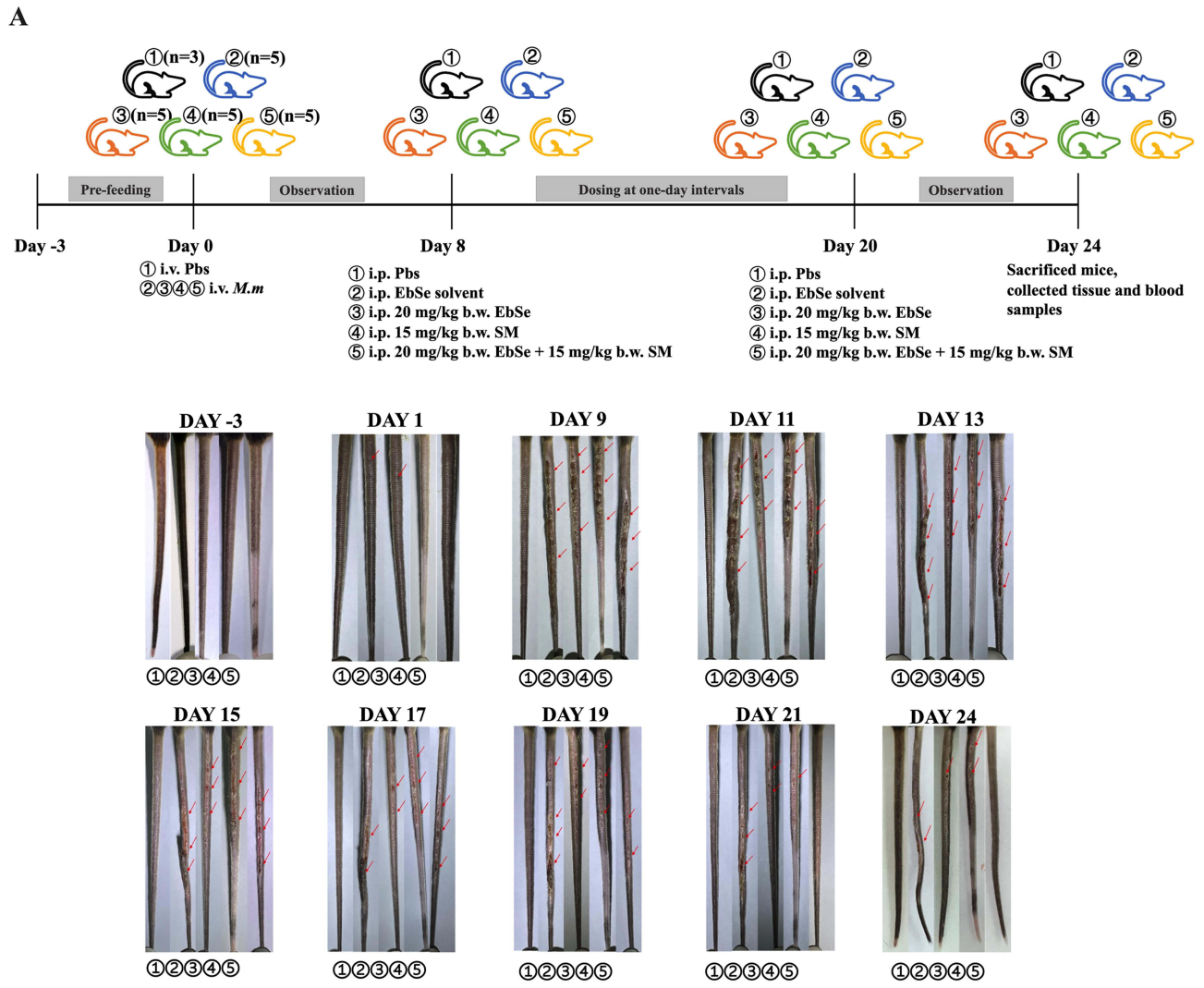
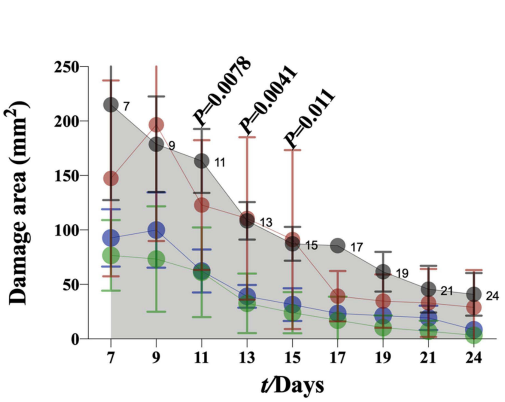


Figure 3 Antibacterial effect of EbSe + SM on *M. marinum* targeting TDRS system and producing of ROS. *M. marinum* ATCC BAA-535 cells were cultured until an A_{600}= 0.4 and treated for 30 min with EbSe + SM. (**A** and **B**) Mean fluorescent intensity (MFI) of H_2DCF-DA-stained *M. marinum* was used to detect ROS level by flow cytometry. (**C**) UV-vis at A_{600} has been used to present DTT could rescue *M. marinum* from EbSe + SM treatment. (**D** and **E**) Trx1 protein level was measured by Western blot. N = 5 independent observations per group, and triplicate biological replications were applied. Data are presented by Student's *t* test, p<0.05 constitutes statistical significance.





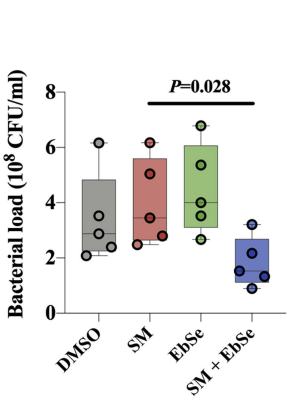


Figure 4 EbSe + SM has therapeutic effects over *M. marinum*-infected mice. (**A**) On day 0, mice infected with 100 μL 2×108 CFU/mL *M. marinum* to construct an acute tail infectious model. On day 8 and day 20, mice (n=5) were injected i.p. with DMSO, EbSe (20 mg/kg b.w.), SM (15 mg/kg b.w.), EbSe (20 mg/kg b.w.) + SM (15 mg/kg b.w.), respectively. (**B**) Damage area and (**C**). Bacterial load was observed. N= 5 independent observations per group, and duplicate biological replications were applied. Red arrows represent local infection on the tail tissue. Data are presented by log-rank (Mantel–Cox) test, $p<0.05$ constitutes statistical significance.

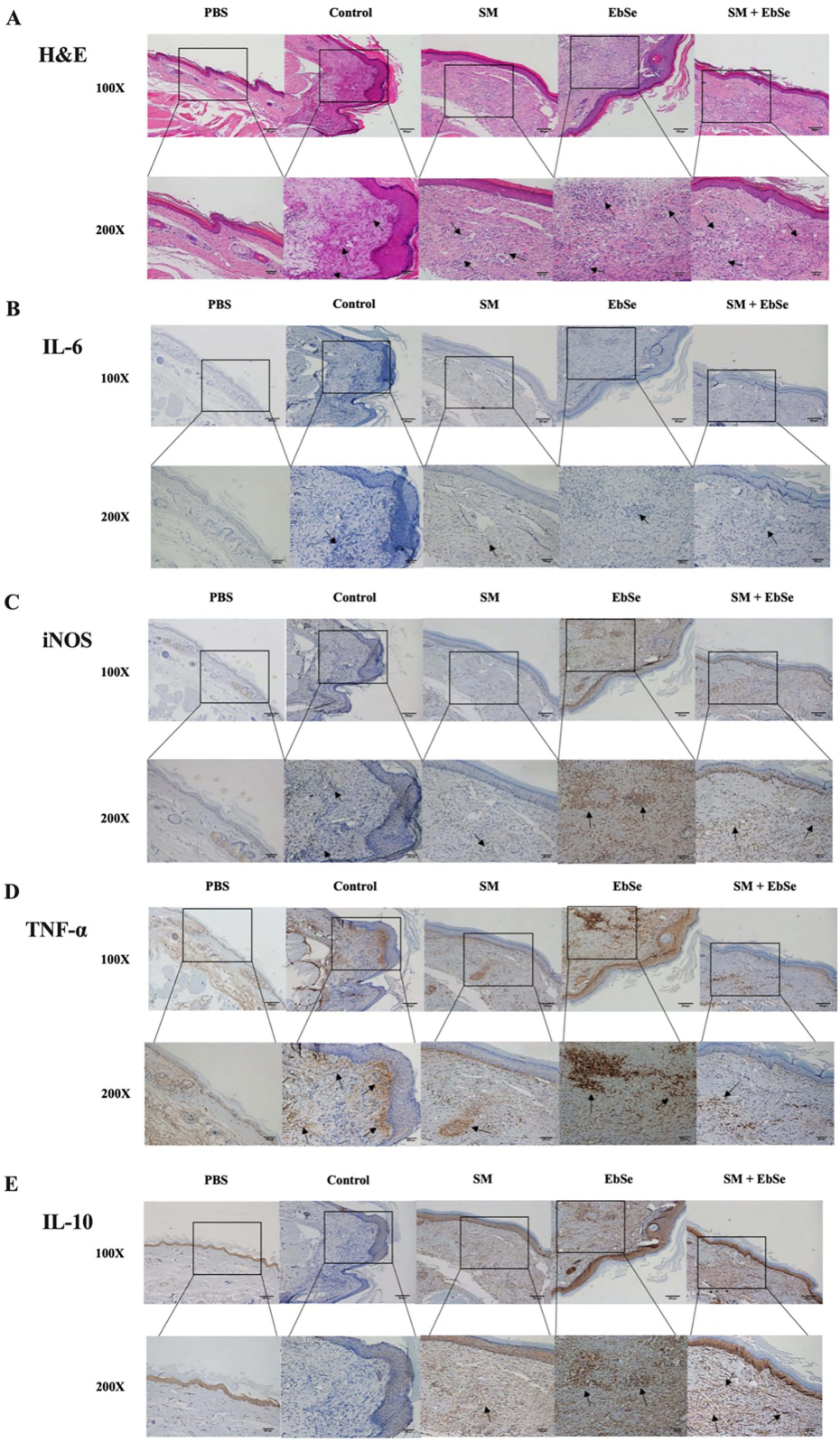


Figure 5 EbSe + SM has immune-modulatory property. The mouse tails were fixed and embedded. Paraffin sections were used to perform H&E staining (**A**) and to detect cytokines (IL-6, iNOS, TNF-α, and IL-10) levels using IHC analysis (**B-E**.). (**A**) H&E staining. (**B**) IL-6. (**C**) iNOS. (**D**) TNF-α. (**E**) IL-10. Black arrows represent inflammatory cells in tail tissue.

+ SM significantly downregulated the expression of IL-1β, iNOS, and TNF-α, and upregulated the expression of IL-10 compared to control mice (Figure 5B–E).

EbSe + SM Showed Non-Toxic at a Given Dosage

SM is widely accepted as a chronotoxic drug, which greatly restricts its application.[22] Thus, we used the acute infectious mice model and Raw264.7 model to evaluate the toxicity of EbSe + SM in vivo and in vitro. EbSe + SM had no significant effect on the body weight of the mice (Figure 6A). Peripheral blood samples from different groups of mice were collected and analyzed. The results showed that alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), blood urea nitrogen (BUN), Cre (creatinine), and uric acid (UA) levels in mouse blood serum were not significantly different from those in the SM treatment (Supplemental Figure 3). Furthermore, H-E staining revealed no features of necrosis, vacuolation, or other damage to the liver or kidney tissues (Figure 6B).

The effect of EbSe + SM on the survival and proliferation of Raw264.7, was detected using the CCK8 assay, and the results showed that EbSe (20 μM) + SM (88 μM) had no effect on cell viability at either 24 or 48 h post-drug treatment (Figure 6C).

Discussion

Antigen 85 complex and L, D-transpeptidases have been identified as attractive targets for the development of new antimicrobials against *Mtb*, as these proteins play important roles in the resistance and virulence of the bacterium.[3,4,23,24] EbSe has been shown to be a potent activator of both enzymes,[4,23] and our group previously demonstrated that it also possesses specific inactivation activity of bTrxR.[5] Furthermore, the recent use of EbSe as a bioenhancer has reduced the concentration of antibacterial agents used for the treatment of DR pathogens, which is effective and has the potential to achieve good clinical cure rates.[7–10,15,25] It's critical to ascertain whether EbSe inhibits mycobacterial development by specifically targeting the Ag85C or TrxR because its selenium atom is reactive toward any thiol functional group. Mycothiol is present in the cytoplasm of mycobacteria, and because of its functions in detoxification and redox equilibrium,[26] it seems improbable that the main EbSe target in *Mtb* is in the cytoplasm. In contrast, mycothiol synthesis in the mycobacterial cytoplasm is not expected to have an impact on the creation and stability of a selenenylsulfide bond between EbSe and Ag85C.[3]

Aminoglycoside SM was first used to treat TB in 1946. However, resistance to SM was observed in 1948, which is highly related to its sole use in TB treatment.[27] Our in vitro results showed that EbSe exerted potentiating enhancer effects on the activity of INH and SM, but not on RFP, against *M. marinum*, genetically related to *Mtb* (a surrogate for *Mtb*). The present study also provides additional novel findings in which the adjuvant effect of EbSe over SM was demonstrated in *M. marinum* by targeting bTrxR. This finding indicates a potential method for rejuvenating the anti-*Mtb* activity of SM in combination with EbSe.

As a naturally occurring pathogen of ectotherms, *M. marinum* is the closest genetic relative of the *Mtb* complex. The *M. marinum* genome is 85% identical to orthologous regions of the *Mtb* genome, and coding sequence amino acid identity averages 85% between orthologues. Rather than central mechanisms of disease, the loci linked to the *Mtb*-specific components of the genome are primarily relevant to variations in host transmission and organ specificity.[28] Besides, the synergistic action of EbSe and SM was tested over bTrxR, a housekeep gene coding enzyme.[6] Thus, the effect against *M. marinum* could be an appropriate reference for *Mtb* based on the genetic and physiological similarities of the two strains.

Using a well-established *M. marinum* infection model, in which the bacteria produce a local infection restricted to the tail tissue, offers an opportunity to evaluate preventive strategies for TB.[21] The *M. marinum* model has the advantage of mimicking some of the key hallmarks of human TB, which are not replicated in conventional murine *Mtb* models. Moreover, the model is non-lethal and enables longitudinal analysis of disease development in live animals.[21] In the current study, infected mice were intraperitoneally administered with EbSe + SM. The adjuvant effect of EbSe over SM was verified by a significant reduction in bacterial load. Furthermore, the pro-inflammatory cytokines TNF-α and IL-1β, which are highly related to inflammatory diseases,[5] were also detected. Our results showed that both TNF-α and IL-1β were downregulated by the EbSe + SM treatment, contributing to host defense

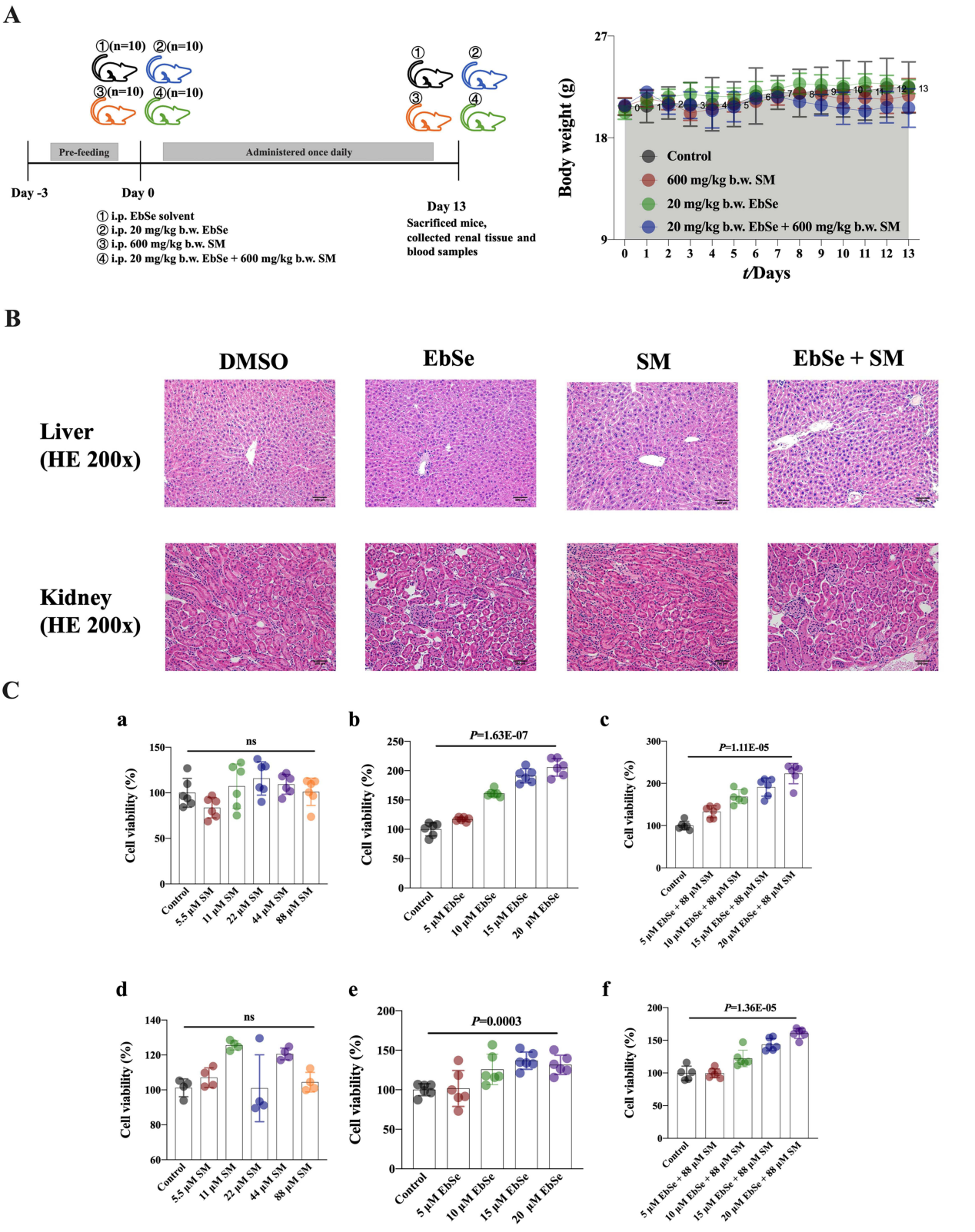


Figure 6 Toxicity analysis of EbSe + SM. On day 0, mice (n=5) were injected i.p. with DMSO, EbSe (20 mg/kg b.w.), SM (600 mg/kg b.w.), EbSe (20 mg/kg b.w.), and SM (600 mg/kg b.w.). (**A**) Body weight. (**B**). On day 13, mouse liver and kidney were fixed and embedded. Paraffin-embedded sections were stained with hematoxylin and eosin. (**C**). Effects of EbSe + SM on the growth of Raw264.7 cells. RAW264.7 cells were treated with serial concentrations of EbSe + SM for 24 h (**a**–**c**) and 48 h (**d**–**f**), and cell toxicity was assessed using a CCK8 assay. (**a** and **d**) SM; (**b** and **e**). EbSe; (**c** and **f**) EbSe + SM. N = 5 independent observations per group, and duplicate biological replications were applied. Data are presented by the chi-squared test, $p<0.05$.

during infection. Given that IL-10 is the central cytokine that maintains a delicate balance between effective immunity and tissue protection, it is not surprising that IL-10 is particularly important in preventing excessive inflammation during infection.[29] Our results showed that EbSe + SM upregulated IL-10, which provided a favorable outcome in host healing and might be highly related to its immunomodulatory, anti-inflammatory, and antioxidant activities.

To further evaluate the potential of EbSe + SM as a possible therapeutic combination to combat TB, we performed in vitro cytotoxicity assays using Raw264.7 macrophages.[30] Subsequent animal studies established the safety of EbSe + SM. In our animal trial, the peripheral blood of mice was collected, and the ALT, AST, and ALB levels were monitored; the results showed that the enzymes in the EbSe + SM group were not significantly different from those in the control group. BUN, Cre, and UA levels were also monitored, illustrating that the concentrations of EbSe + SM used in this experiment had no obvious nephrotoxicity in mice. This is particularly important considering the potential nephrotoxicity associated with SM treatment.[22] Our findings are in concordance with studies that emphasize the need for safer and more effective TB treatment regimens.[12,13]

The adjuvant effect of EbSe over SM observed in the present study raises the possibility that the addition of SM to EbSe could be, in some cases, useful as a treatment for *Mtb*. This highlights the need for further studies on the combinations of available drugs, including those that are no longer used for the treatment of TB. Reducing the time of treatment and the adverse effect of current treatment deserves attention in bringing additional light on SM activity, which may be a quick and economical strategy for helping patients infected with TB.

The current study, while providing valuable insights, also indicates the need for further research. The exact mechanisms by which EbSe disrupts the redox homeostasis of *M. marinum* warrant detailed investigation. Additionally, the potential of EbSe as an adjuvant for other anti-TB drugs should be explored to broaden the therapeutic arsenal against DR *Mtb*.

In conclusion, our study presents EbSe as a promising adjuvant to SM for the treatment of *Mt*b, offering a novel approach to combat drug-resistant TB. The findings highlight the potential of redox modulation in enhancing the efficacy of existing antibiotics and provide a foundation for future research into the therapeutic potential of EbSe in TB treatment. Therapeutic strategies that employ anti-TB drugs in combination could have some advantages over conventional therapies in special cases of resistance. In addition, it could be used to seek stronger or safer activities when anti-TB drugs are co-administered with EbSe.

Data Sharing Statement

The original contributions presented in the study are included in the article/supplementary material, and further inquiries can be directed to the corresponding author Lili Zou.

Statement

The animal study was reviewed and approved by the ethical permit approval of the Medical Animal Care and Welfare Committee of China Three Gorges University was obtained before using the mice for study.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflicts of interest in this work.

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