Lysocin E targeting menaquinone in the membrane of *Mycobacterium tuberculosis* is a promising lead compound for anti-tuberculosis drugs

Geberemichal Geberetsadik[1,10]*, Akane Inaizumi[1], Akihito Nishiyama[1,]*, Takehiro Yamaguchi[1,2], Hiroshi Hamamoto[3], Suresh Panthee[4,11], Aki Tamaru[5], Manabu Hayatsu[6], Yusuke Mizutani[6,7], Shaban Amina Kaboso[1], Mariko Hakamata[1,8], Aleksandr Ilinov[1,9], Yuriko Ozeki[1], Yoshitaka Tateishi[1], Kazuhisa Sekimizu[4], Sohkichi Matsumoto[1,]*

[1]Bacteriology, Niigata University School of Medicine, 1-757, Asahimachi-Dori, Chuo-ku, Niigata, 951-8510, Japan.

[2]Department of Bacteriology I, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo, Japan

[3]Institute of Medical Mycology, Teikyo University, 359 Otsuka, Hachioji, Tokyo, 192-0395, Japan.

[4]Drug Discoveries by Silkworm Models, Faculty of Pharma-Science, Teikyo University, 359 Otsuka, Hachio-ji shi, Tokyo, 192-0395, Japan

[5]Department of Infectious Diseases, Osaka Prefectural Institute of Public Health, 1-3-69, Nakamichi, Higashinari-ku, Osaka-shi, Osaka, 537-0025, Japan

[6]Division of Microscopic Anatomy, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachidori, Chuo-ku, Niigata, 951-8510, Japan

[7]Office of Institutional Research, Hokkaido University, 5 Kita 8-jonishi, Kita-ku, Sapporo, Hokkaido, 060-0808, Japan

[8]Department of Respiratory Medicine and Infectious Disease, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachidori, Chuo-ku, Niigata, 951-8510, Japan

[9]Department of General Surgery, Krasnoyarsk State Medical University, Partizan Zheleznyak str., 1, Krasnoyarsk, 660022, Russia

[10]Department of Biology, Natural and Computational Sciences Faculty, Assosa University, Assosa, Benishangul Gumuz, P.O. Box 18, Ethiopia

[11]Department of Medical Pathogenesis and Immunology, College of Medicine, Texas A&M University, 8447 Riverside Parkway Bryan, TX 77807, USA

*G.G., mikigebre73@gmail.com; A.N., anishi@med.niigata-u.ac.jp; S.M., sohkichi@med.niigata-u.ac.jp

Abstract:

Tuberculosis remains a public health crisis and a health security threat. There is an urgent need to develop new anti-tuberculosis drugs with novel modes of action to cure drug-resistant tuberculosis and shorten the chemotherapy period by sterilizing tissues infected with dormant bacteria. Lysocin E is an antibiotic that showed antibacterial activity against *Staphylococcus aureus* by binding to its menaquinone (commonly known as Vitamin K2). Unlike *S. aureus*, menaquinone is essential in both growing and dormant *Mtb*. This study aims to evaluate the anti-tuberculosis activities of lysocin E and decipher its mode of action. We show that lysocin E has high *in vitro* activity against both drug-susceptible and resistant *Mycobacterium tuberculosis* var. tuberculosis (*Mtb*), and dormant mycobacteria. Lysocin E is likely bound to menaquinone causing *Mtb* membrane disruption, inhibition of oxygen consumption and ATP synthesis. Thus, we have concluded that the high anti-tuberculosis activity of lysocin E is attributed to its synergistic effect of membrane disruption and respiratory inhibition. The efficacy of lysocin E against intracellular *Mtb* in macrophages was lower compared to its potent activity against *Mtb* in culture medium, probably due to its low ability to penetrate cells, but its efficacy in mice was still superior to that of streptomycin. Our findings indicate that lysocin E is a promising lead compound for the development of a new tuberculosis drug that cures drug-resistant and latent tuberculosis in a shorter period.

INTRODUCTION

Tuberculosis (TB) is one of the severe infectious diseases caused by *Mycobacterium tuberculosis* var. tuberculosis (*Mtb*) (*1*–*3*). The recent WHO report ranks TB above HIV as a leading cause of mortality by infectious diseases, with more than 1.5 million attributed deaths in 2020 (*1*). Moreover, it is estimated that a quarter of the world's population asymptomatically harbors *Mtb*, of which 5 to 10% will develop active disease in their lifetime (*3*). Although the current six-month therapy for drug-susceptible TB can achieve a cure rate of >90%, the issue of multidrug-resistant TB (MDR-TB) remains a public health crisis and a health security threat (*3*), and 10% increase was reported in

2019 as compared to 2018 (*4*). The increased number of MDR-TB and extensively drug resistant TB (XDR-TB) strains has created a great challenge for the treatment and control of TB and, the treatment involves the use of costly and less effective second-line drugs that have significant side effects (*3*). Moreover, both active and latent *Mtb* infections require lengthy treatments to achieve a durable cure. This problem has partly been attributed to the existence of nonreplicating *Mtb* “persisters” or dormant cells that are difficult to kill using conventional anti-TB treatments (*2*, *3*).

To support the efforts to fight this disease, the development of new drugs, which have a novel mode of action without cross resistance to any current first and second line drugs (*2*, *5*), help in shortening therapy duration for TB and cause optimal kill rate with fewer side effects, is an urgent priority (*2*, *5*–*7*).

Lysocin E is a recently reported antibiotic with a novel mode of action and potent bactericidal activity. It was isolated from *Lysobacter sp*. RH2180-5, and was structurally determined to be a 37-membered depsipeptide (molecular weight = 1618 Da). Lysocin E showed potent anti-bacterial activity against *Staphylococcus aureus* both *in vitro* (*8*, *9*) and *in vivo* using a silkworm model of bacterial infection (*10*) and a mouse systemic infection model (*8*). Its bacteriolytic activity against *S. aureus* is associated with membrane damage *(8-10)*. The target of lysocin E is Vitamin K2 (menaquinone (MK)), a membrane cofactor in the electron transport chain (ETC) specific to various kinds of bacteria such as *S. aureus* and *Mtb (7*–*9*).

Both *menA* and *menB*, encoding critical enzymes in MK biosynthesis, are not essential in *S. aureus*. Both Δ*menA* and Δ*menB* mutants survived and showed resistance to lysocin E (*8*). However, MK is facilitating transport of electrons in the ETC, which is essential for mycobacteria including *Mtb* (*2*, 11, *14*). Thus, we hypothesized that MK could be a more attractive target in *Mtb* than in *S. aureus*. In this study, we evaluated the anti-TB activities of lysocin E *in vitro* and *in vivo* and deciphered its mode of action against *Mtb*, as a possible candidate for a novel anti-TB drug.

RESULTS

Lysocin E shows potent antimicrobial activity against diverse *Mtb* strains

We tested the activity of lysocin E against a panel of replicating drug susceptible and resistant *Mtb* strains. Lysocin E exhibited antimycobacterial activity with a minimum inhibitory concentration (MIC) of 0.5 µg/ml against the replicating drug susceptible *Mtb* H37Rv (Table 1). The drug was also efficacious against a panel of extensively drug resistant clinical isolates (MIC ≤ 0.5 µg/ml (Table 1)). This showed the potential of the drug to be used in the treatment of drug resistant *Mtb* strains.

As shown in Fig. 1A, we also determined the bactericidal activity of lysocin E against *Mtb* within a concentration range of 0.5 (1x MIC) to 5 (10x MIC) µg/ml. As compared to time zero, the bacterial CFU count was reduced by more than 99.9% at all concentrations after seven days of drug treatment. The minimal bactericidal concentration (MBC) value against *Mtb* H37Rv was equal to the MIC value (0.5 µg/ml), and the bactericidal effect was time dependent. For example, 3 days of drug treatment with 1x MIC and 10x MIC reduced 1.3 $\log_{10}$ and 2.2 $\log_{10}$ CFU, respectively, while 7 days of treatment resulted in 2.4 $\log_{10}$, and 3.1 $\log_{10}$ CFU reduction respectively, relative to time-zero (Fig. 1A). This significant reduction in CFU count even at its MIC concentration showed the bactericidal nature of lysocin E against *Mtb*.

Lysocin E sterilizes *Mtb* culture better than front-line ant-TB drugs

Lysocin E was tested in combination with front-line anti-TB drugs using checkerboard assay against wild-type *Mtb* H37Rv. The combination of lysocin E with rifampicin (RFP) or bedaquilline (BDQ) showed no antagonistic effect. The interaction of lysocin E with either isoniazid (INH) or ethambutol (EMB) was additive with fractional inhibitory coefficient (FIC) index values of 1 and 0.56, respectively (Table 2). We also evaluated the 21 days kill kinetics of lysocin E compared to other anti-TB drugs by CFU count. Lysocin E was highly bactericidal against *Mtb in vitro* and reduced the CFU to undetectable level after 15 days of exposure (Fig. 1B). This activity, leading to 4 $\log_{10}$

CFU reduction over 7 days, was superior to that of INH, RFP and EMB alone, especially as the other drugs showed regrowth after 7-, 10-, and 15 days, respectively. In addition, the combination of lysocin E and INH showed a small additive effect (Fig. 1B). Taken together, lysocin E showed stronger bactericidal effect against *Mtb*, compared to front-line anti-TB drugs.

Lysocin E kills *Mtb* by binding to MK

We next assessed lysocin E's bactericidal mode of action in bactericidal efficacy to mycobacteria. In *S. aureus* (*8*, *12*), lysocin E binds to the membrane MK thus inducing its bactericidal effect via membrane disruption of staphylococcal cells, and the presence of exogenous MK in the culture increases the MIC of lysocin E against *S. aureus.* We also examined the effect of exogenous addition of MK in lysocin E-mediated anti-mycobacterial activity. The presence of MK in the culture medium increased the MIC of lysocin E by ten- and five-fold against *Mtb* and *Mycolicibacterium smegmatis* (formerly known as *Mycobacterium smegmatis* (*13*)), respectively (Table 1). This inhibition of lysocin E activity by exogenous MK was also confirmed by CFU count. In the presence of MK, lysocin E did not significantly reduce *Mtb* CFU (Fig. 1B). These data suggest that lysocin E could bind to MK present in the mycobacterial membrane to induce its bactericidal effect against mycobacteria.

To check if the reduction of MK in the mycobacterial membrane could increase bacterial resistance toward lysocin E, we constructed two conditional knockdown (CKD) *Mtb* strains of *menA* (MenA, Rv0534c), a gene encoding the essential enzyme (1, 4-dihydroxy-2-naphthoic acid prenyltransferase **(**MenA**))** in MK biosynthesis pathway (*14*), using TetR-regulated CRISPR interference system targeting two different non-template strands of *menA* (G^{8}GAGACCCACTGTGCGAAAC^{27} and T^{110}GTGGTGGAAAGCGCTGTTG^{129}, respectively). These strains are designated as A+8 and A+110, respectively. The *menA* CKD of A+8 and A+110 strains were induced by incubating the strains with anhydrotetracycline (aTc) and the CKD was confirmed by quantifying the mRNA of *menA* by real time PCR. Of the two strains considered in this

study, A+110 strain showed around 10-fold reduction in *menA* mRNA as compared to the vector control strain (*P*< 0.001, Fig. 2A).

To further confirm that the CKD resulted in the reduction of MK, we measured the amount of MK in each strain using high performance liquid chromatography (HPLC) after conditionally knocking down the strains for four weeks. The MK amount considered in this study is the sum of MK-8, MK-8 (H2), MK-9, and MK-9 (H2) which were collected at 8.8-, 9.9-,11.8- and 13.5-minutes retention times, respectively (Fig. S1A-E). The analysis of total MK showed a significant reduction of the MK amount in A+110 strain as compared to the vector control (*P*< 0.001, Fig. 2B). Following these observations, the susceptibility of the CKD strains to lysocin E was determined by CFU count. A+110 strain showed a threefold increment in CFU as compared to the vector control (*P*< 0.001, Fig. 2C). Susceptibility to INH, a control drug, was not altered significantly in all strains (Fig. 2D). These data suggest that MK is a target of lysocin E also in *Mtb*.

Lysocin E causes mycobacterial membrane disruption

Since lysocin E binding to MK has been suggested to be its mode of action against mycobacteria, we examined if this binding causes mycobacterial membrane damage as seen previously in *S. aureus* (*8*). The scanning electron microscope (SEM) micrographs showed the presence of a heavily damaged *Mtb* membrane. As the period of incubation in the presence of lysocin E increased, the bacterial damage was heavily increasing, i.e., the damage starts within 3 hours (Fig. 3A) and ends up with a completely damaged bacteria after 6 hours (Fig. 3B, C) whereas the no drug control maintained an intact membrane at all time points (Fig. 3D). These data support that the drug disrupts the bacterial membrane to kill bacteria.

To further corroborate these observations, we optimized the live/dead staining to detect the presence of membrane disrupted *M. tuberculosis* var. Bacille Calmette-Guérin (BCG, formerly known as *Mycobacterium bovis* Bacille Calmette-Guérin) (*13*) by FACS analysis. The staining of BCG with 0.15% v/v Syto9 in 0.9% v/v saline (0.334 mM stock solution) and 0.15% v/v Propidium iodide (PI) in saline (2 mM stock solution) provided

optimal concentration that gave consistent and distinct cell populations; a membrane intact (Syto9+/PI-) and two membrane disrupted populations (Syto9+/PI+ and Syto9-/PI+). This was clearly observed in the negative (live) and positive (heat-killed) control plots that showed 6.3% (Fig. 3E) and 86.3% (Fig. 3F) membrane disrupted cells, respectively. As for samples, BCG incubated with 10x MIC of lysocin E for three hours exhibited significantly more membrane disrupted cells (43%, Fig. 3G), compared to those incubated with 10x MIC of RFP (4.27%, Fig. 3H) and BDQ (5.05%, Fig. 3I). The membrane disrupted bacteria increased depending on lysocin E concentration and exposure time (Figs. 3J and S2A-C). In parallel to the SEM and FACS results, the CFU assay showed that more than 50% BCG died within 3 hours after lysocin E was added (Fig. 3K) and CFU further decreased at later time points (Fig. S2D-F). On the other hand, BCG treated with RFP and BDQ whose mode of action do not involve membrane disruption, significant bacterial death was observed prior to the increase of membrane disrupted cells (Fig. 3J, K). Then, the membrane disrupted bacteria increased at later time points as CFU further decreased (Figs. 3J and S2D-F). This suggests that the membrane disruption observed in RFP and BDQ was due to subsequent events that occurred after bacterial death rather than as a direct effect of the drugs on the membrane.

Lysocin E inhibits oxygen consumption and ATP synthesis

In the abovementioned experiments, we observed that lysocin E disrupted the mycobacterial membrane via binding to MK. Given that MK mediates electron transportation in bacterial energy production, we next investigated whether lysocin E could disrupt mycobacterial energy production by observing inhibition of oxygen consumption and measuring ATP synthesis in the presence or absence of lysocin E. Methylene blue oxidized by dissolved oxygen exhibits blue color. Therefore, we examined the oxygen consumption of mycobacterial culture by monitoring the decolorization of blue color of oxidized methylene blue. *Mtb* H37Rv was suspended in culture media containing methylene blue and various concentrations of the drugs. Glass tubes containing bacterial suspensions and drugs were sealed and incubated at 37 ˚C.

In the presence of 1/4x MIC (0.125 µg/ml) and 1/2x MIC (0.25 µg/ml) of lysocin E, the cultures retained their initial methylene blue color (Fig. 4A, tubes 1 and 2). As shown in Fig. 4A, *Mtb* incubated in the presence of 10x MIC (2.5 µg/ml) of BDQ (tube 3), 10x MIC (0.31 µg/ml) of INH (tube 4) and no drug control (tube 5) got decolorized. This suggests that lysocin E affects the rate of bacterial oxygen uptake which in turn affects proper energy production. To further verify this decolorization difference is not associated with the bacterial number, we checked the CFU count as shown in Fig 4B and, found that there was no significant difference between the decolorized cultures and the ones which retained their initial color (Fig. 4B, $P>0.05$). Similar observations were also made in *M. smegmatis* in terms of decolorization after 2 hrs of incubation in the presence of different drug concentrations (Fig. 4C). *M. smegmatis* culture incubated in the presence of 1/4x MIC (2.5 µg/ml) and 1/2x MIC (5 µg/ml) of lysocin E also retained the initial methylene blue color (Fig. 4C, tubes 2 and 3), whereas cultures incubated in the presence of 10x MIC (160 µg/ml) of INH (tube 4), 10x MIC (0.62 µg/ml) of BDQ (tube 5), and no drug (tube 6) control were decolorized.

We further evaluated the drug effect on ATP synthesis by incubating *Mtb* in the presence of different concentrations of lysocin E and control drugs. The result showed that the levels of intracellular ATP were significantly decreased by lysocin E treatment (Fig. 4D, $P \leq 0.05$). The effect of lysocin E on intracellular ATP production was even more pronounced than that of BDQ, a drug known for its ability to inhibit ATP synthesis by targeting ATP synthase. On the other hand, INH-treatment increased the production of ATP compared to the no drug control. Taken together, these results suggest that lysocin E treatment inhibits ATP synthesis in mycobacteria.

Lysocin E exhibits enhanced activity against a subpopulation of persisters

Since lysocin E disrupts the mycobacterial cell membrane and ATP synthesis, we hypothesized that it could kill non-replicating dormant mycobacteria. To test this hypothesis, hypoxic non-replicating (NRP) *M. smegmatis* and BCG were exposed to lysocin E. Although it did not sterilize the culture, 3x MIC of lysocin E and metronidazole

caused more than 3.6 $\log_{10}$ CFU reduction, which is by far higher than RFP and BDQ activity which caused only 0.66 $\log_{10}$ and 0.22 $\log_{10}$ CFU reduction respectively against *M. smegmatis,* after three days of incubation. However, INH, a drug known for killing only replicating mycobacteria, and the no drug control showed no reduction in CFU count compared to time zero CFU count (Fig. 5). The drug was also found to be successful in killing NRP BCG, but not as strong as its activity against *M. smegmatis* (Fig. S3B). This showed the potential utility of lysocin E in killing a subpopulation of persisters.

Lysocin E is noncytotoxic to THP1 cells and reduces infection burden in murine TB model

To assess the effects of lysocin E against intracellular *Mtb* we first checked its cytotoxicity to THP1 cell-derived human macrophages. As expected, it did not show toxicity since more than 98% of THP-1 cells survived after incubation with 300 µg/ml (600xMIC) of lysocin E (Fig. S4A).

We next tested the activity of lysocin E against *Mtb* infected THP-1 macrophages. As shown in Fig. 6A, lysocin E reduced intracellular *Mtb* in a dose-dependent manner compared to the untreated control cells, i.e., CFU reduction of ≈1.2 $\log_{10}$ and ≈2 $\log_{10}$ were observed at 10x and 50x MIC of lysocin E respectively at day-7. Lysocin E also showed better activity than streptomycin (STR) against intracellular *Mtb* which showed a CFU reduction of only 0.9 $\log_{10}$ at 50x MIC (Fig. 6A). However, its activity was lower than that of 10x MIC of INH and RFP which showed a CFU reduction of ≈2.5 $\log_{10}$ and ≈1.9 $\log_{10}$ respectively (Fig. 6A). These data suggest lower permeability of lysocin E into macrophages compared to INH and REF, which might explain its moderate inhibitory activity against intracellular *Mtb*.

We next evaluated the anti-TB activity of lysocin E *in vivo* using mouse *Mtb* infection model. The lysocin E dosing regimen used in the animal study was based on ED_{50} of 0.5 mg per kg bodyweight which was used against *S. aureus* in a previous study (*8*). As

shown in Fig. 6B, on day 1, the control mice had a bacterial load of ≈5 $\log_{10}$ CFU per lung. Twenty-four days post-infection, the control group had significantly higher bacterial load of ≈8.4 $\log_{10}$ CFU compared to that of day 1 CFU ($P \leq 0.05$).

After 2 weeks of treatment and compared to the control, the lysocin E treated group showed significant reduction in the lung CFU (≈7.2 $\log_{10}$), which was relatively higher than the effect of STR (≈7.6 $\log_{10}$) but lower than that of INH (≈ 6.2 $\log_{10}$) (Fig. 6B, P≤ 0.05). Lysocin E and INH treatment also significantly reduced the number of bacilli in the spleen by 0.62 $\log_{10}$ and 0.98 $\log_{10}$, respectively, relative to that of the untreated control group (Fig. 6C, P <0.005). Similar results were also obtained from the liver CFU count at 24 days post infection between the treated and untreated mice (Fig. 6D, P <0.001). Lysocin E treatment also significantly reduced the lung BCG burden by ≈1 $\log_{10}$ CFU relative to that of the untreated control group (Fig. S4B, P <0.001). Similar effects were observed against BCG in the spleen and liver (Fig. S4C and D, P <0.001). Most importantly, although not as strong as INH, lysocin E has shown activity against *Mtb in vivo*.

In concurrence with the CFU count, all the treated mice maintained their bodyweight whereas the untreated mice were moribund 2 weeks after treatment initiation as confirmed by the reduction in bodyweight (Fig. S4E, P< 0.001). The mononuclear cell count in the post-treatment lung also showed a significant reduction in all the treatment groups compared to the untreated control (Fig. S4F, P<0.001). Moreover, at lower magnification, we saw a clear difference in the presence of inflammatory cells from lung tissue images at 24 days post infection between the treated and untreated mice (Fig. 6E). These data indicate that the *in vivo* efficacy of lysosin E against TB is by suppression of bacterial burden and infection-related inflammation.

DISCUSSION

Lysocin E showed excellent *in vitro* activity against mycobacteria including drug resistant *Mtb* strains (Table1). The lack of cross-resistance with currently used anti-TB

agents indicates the potential utility of lysocin E and its derivatives that have the same mode of action against XDR and MDR strains. In addition, the bactericidal nature of the drug evidenced by the reduction of bacterial CFU to undetectable level by lysocin E alone in the 21 days kill kinetics assay are an important indication of its superior efficacy against persisters compared to other front-line drugs, which cannot kill persisters.

The binding of lysocin E to MK can disrupt the MK-containing membrane (*10*). As observed in *S. aureus* (*8*), lysocin E neutralization with free MK and the reduction of membrane MK levels by CKD of MK biosynthesis pathway enzyme MenA caused bacterial tolerance to lysocin E, indicating that *Mtb* membrane MK is a potential target of lysocin E. As observed in the SEM micrographs, maybe lysocin E binding to MK has caused *Mtb* membrane disruption as was seen with the loss of membrane integrity within 3 hrs of drug exposure. Unlike lysocin E, drugs like RFP that target DNA polymerase to kill bacteria (*15*) need more time to ultimately lead to membrane disruption, which was detected by Flow cytometry (Fig. 3J). Drugs like daptomycin and retinoids appear to insert into Gram-positive bacterial membrane, causing rapid permeabilization and cell death (*16*). Similarly, our data indicate that membrane disruption through direct binding to membrane MK is may have resulted in the uncontrolled movement of ions and molecules into and out of the bacterial cells which lead to cell death (*17*). This is a major mode of action of lysocin E in *Mtb*, which corroborates what was observed in *S. aureus* (*8*). Most importantly, bacterial membranes are attractive antipersister targets because they can be disrupted independent of their growth state (*16*). This quick action by lysocin E against the *Mtb* membrane may be a possible explanation for the rapid *Mtb* clearance from culture.

Unlike many bacteria, *Mtb* cannot support its energy needs through substrate-level phosphorylation. Instead, both actively growing, and nonreplicating persistent *Mtb* bacteria are dependent on the respiratory chain where MK functions as essential electron carriers to synthesize adequate amounts of ATP (*2*, *6*). Inhibitors of the electron transport system (ETS), including MK function, are expected to indirectly affect the respiratory ATP synthesis, consequently inhibiting bacterial growth (*2*, *6*). As

expected, lysocin E inhibited oxygen consumption and ATP synthesis at concentrations much less than its MIC against *Mtb*. This might be due to the direct binding of lysocin E to MK or the disruption of membrane integrity. The effect of lysocin E on intracellular ATP production was even higher than that of BDQ, a drug known for its ability to inhibit ATP synthase and kill non-replicating dormant bacteria. Thus, the disruption of the ETS and subsequent inhibition of ATP synthesis could also contribute to the rapid clearance of *Mtb* from the culture synergistically with its membrane disrupting activity.

Membrane integrity and the activity of the respiratory chain are also important for NRP mycobacteria with the latter being a major energy-generating pathway (*2*). Indeed, lysocin E showed activity against NRP hypoxia induced dormant *M. smegmatis* and BCG in this study. This is important given that the key to shortening the long TB treatment regimen lies in targeting this NRP persistent subpopulation (vide supra) (*2*, *14*, *17*). However, many of the current first-line and second-line anti-TB drugs are not effective against the NRP mycobacteria. Therefore, drugs that cause *Mtb*-membrane disruption can be promising candidates given that they target *Mtb* at any growth state and shorten the treatment duration.

MK biosynthesis is a unique and prime target for the development of new antimycobacterial targets (*2*). Various compounds have been reported to inhibit genes involved in MK biosynthesis in mycobacteria, including DG70 (*2*), menA inhibitors (*5*) and Aurachin RE (*6*). However, to our knowledge, lysocin E is the first antibiotic that kills mycobacteria via specific recognition of MK within the bacterial membrane. This suggests its potential role in tackling the appearance of drug resistant strains since lack of or the reduction of MK may decrease the potential pathogenicity of bacteria. Thus, we conclude that the antimycobacterial activity of the lysocin E could be attributed to the synergistic effect of MK associated membrane disruption and ETS inhibition.

So far, we tried to obtain lysocin E-resistant *Mtb* but we did not succeed. This suggests that the appearance rate of this population is extremely low because MK is critical for

Mtb survival. This unlikely appearance of lysocin E-resistant *Mtb* makes the drug beneficial for clinical use.

Natural products frequently affect the membrane integrity of eukaryotic cells. Lysocin E specifically binds to MK which is absent in the eukaryotic respiratory chain (*2*, *6*), but not ubiquinone, an electron transporter in the eukaryotic respiratory chain. As such, lysocin E did not exhibit any destruction of the eukaryotic cell membranes up to a concentration of 300 µg/ml and, its selective index was more than 1000x MIC. This high SI suggested that lysocin E might be pharmacologically safe as a candidate for further evaluation. In previous studies, lysocin E did not cause any organ toxicity after it was repeatedly administered to mice (*8*, *10*). However, it had moderate intracellular *Mtb* killing activity in macrophages, suggesting that its efficacy may be limited to the eradication of disseminated extracellular *Mtb* like STR. Similar results were observed *in vivo* where the efficacy of lysocin E was limited, compared to INH which is effective against intracellular *Mtb*. However, lysocin E's efficacy against *Mtb* was relatively higher than that of STR, causing a significant CFU reduction in mice and macrophage infection models, which showed its potential role as a choice for TB treatment. Our study results showed the superior characteristics of lysocin E as compared to STR, which is an approved anti-TB drug. However, as a proof-of-concept of the efficacy of lysocin E against *Mtb* infection, our *in vivo Mtb* infection study was limited to 24 days of infection and two weeks of treatment only. We consider this a limitation in our study design since extending the treatment and study duration might have provided insight into the efficacy of lysocin E over a longer duration.

The limited intracellular activity of lysocin E maybe because it cannot efficiently cross the host cell membrane due to its high molecular weight and low lipophilicity. The high-throughput strategy to prepare thousands of lysocin E analogues has already been attempted (*18*). Further studies may be able to improve the penetration of this drug into macrophages, tissues, and granulomas and drug efficacy against intracellular *Mtb* in addition to its potent killing activity against extracellular bacilli. This implies that there is a high possibility that some analogues might become candidate drugs considering

lysocin E's pharmacological safety, ability to kill mycobacteria at any growth state, its novel mode of action, and absence of antagonistic effect against anti-TB frontline drugs.

MATERIALS AND METHODS

Biosafety criteria and mycobacterial strains

All procedures involving *Mtb* specimens were carried out in the Biosafety level 3 containment facility located at the Graduate school of medical and dental sciences, Niigata University. Ethical approval to use recombinant *Mtb* and mice was obtained from both Niigata University school of medicine and the Japan Ministry of health, labor, and welfare.

Media, cultivation, and drug

Liquid cultures were grown in Middlebrook 7H9 broth (BD Difco) supplemented with 10% albumin-dextrose-catalase (BD Difco, MD, USA), 0.2% glycerol and 0.05% Tween-80 (Sigma–Aldrich MI, USA). Solid cultures were grown on Middlebrook 7H10 agar (BD Difco) supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase (OADC) (BD Difco). Lysocin E, purified from *Lysobater sp*. culture supernatant, used in this study was provided by Dr. Hamamoto (Teikyo University).

In vitro anti-*Mtb* activities

MIC determination and checkerboard assay for antibiotics

The MIC was evaluated by adding 50 µl bacterial suspension (OD_{600}, 0.00025-0.0006) to serially diluted 50 µl of drug containing Middlebrook 7H9 broth in a 96-well microtiter plate. We used the checkerboard assay to test for interactions between lysocin E and drugs with known anti-TB activities using the broth microdilution method in a 96-well microtiter plate. In the MIC and checkerboard assays, the microtiter plate was incubated

at 37 °C for 8 days for BCG and *Mtb,* and 3 days for *M. smegmatis.* The MIC for each drug was defined as the lowest concentration that inhibited bacterial growth as determined by the naked eye from the incubated palates. To evaluate the effect of each drug combination, the obtained MIC values were used to calculate the fractional inhibitory coefficient index (FICI) as follows: FICI= (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). The FIC index (FICI) calculated for each drug combination were categorized based on FICI: $\leq$0.5 as synergism, >0.5 to $\leq$1 as additivity; >1 to < 4 as no interaction, and >4 as antagonism (*7*, 19, 20).

Determination of MBC for lysocin E and its kill kinetics

The MBC for replicating *Mtb* culture was determined by adjusting exponentially growing bacteria to OD_{600} 0.001 and treating with 1-, 2-, 3-, 5- and 10x MIC of the drug. The MBC was defined as the lowest drug concentration that reduced the CFU by 99% relative to the time-zero inoculum on day 7 (*20*). Twenty-one-day kill kinetics was performed by adding 10x MIC of a single drug or in combination to the culture and incubated for 21 days at 37 °C while assaying CFU at different time points (*2*).

Bactericidal activities against nonreplicating mycobacteria

To prepare non replicating (NRP) mycobacteria, *M. smegmatis* and BCG culture were grown based on the Wayne hypoxia model (*15*). After incubation with different antibiotics in an anaerobic chamber, cultures were plated on 7H10 agar at selected time intervals to count CFU.

Intracellular activity of lysocin E against *Mtb*

To evaluate the efficacy of lysocin E against the intracellular *Mtb*, infected macrophages were treated by adding 100 µl DMEM containing 10- and 50x MIC of drugs. The cells were incubated at 37 °C in a humidified 5% CO_2 incubator. At indicated time points,

0.5% Triton X-100 was added to disrupt macrophages, and the resulting lysate and supernatant were spread on 7H10 agar for CFU counting *(2, 20)*.

Lysocin E mechanism of action in *Mtb*

Preparation of *menA*-CKD strains

Mtb menA-CKD strains were considered for this study. MenA gene (*menA*, Rv0534c) encodes isoprenyl diphosphate:1,4-dihydroxy-2-naphthoat (DHNA) isoprenyltransferase which is essential enzyme in MK biosynthesis. This catalyzes a critical reaction in MK biosynthesis that involves the conversion of cytosolic DHNA, to membrane bound demethylmenaquinone by transferring a hydrophobic 45-carbon isoprenoid chain (in the case of mycobacteria) to the ring nucleus of DHNA (5, 14). To construct *Mtb menA*-CKD strains TetR/*tetO*-inducible CRISPR-dCas9 interference specialized for mycobacteria was employed (*21*). pRH2502, a vector expressing an enzymatically inactive Cas9 (dCas9), and pRH2521, a vector expressing guide RNAs (sgRNAs), were kindly gifted by Dr. Robert N. Husson (*21*). Both dCas9 and sgRNA are expressed from TetR-regulated promoters (*uvtetO* and $P_{myc1\text{-}tetO}$, respectively).

We constructed *Mtb* H37Rv *menA*-CKD strains, as described previously (*22*). *Mtb* strain, which was already transformed with pRH2502 (for dCas9 expression), was transformed with these vectors or empty pRH2521, and then strains were selected on 7H10 agar plates containing 50 µg/ml HYG and 25 µg/ml KM (designated A+8, A+110, and vector control [VC]). For induction of dCas9 and sgRNA expression, aTc was supplemented in the bacterial culture at a final concentration of 200 ng/ml (*21*).

Checking MenA gene CKD

The *Mtb* MK CKD strains (A+110 and A+8) and VC cultures were grown in 7H9/ADC/Tw80 containing 25 µg/ml KM and 50 µg/ml HYG at a starting OD_{600} of 0.05 in 250 ml flasks. To induce and maintain the long-term expression of dCas9 and sgRNA, aTc was added to a final concentration of 200 ng/ml every two days for 4 weeks while

refreshing to starting OD_{600} of 0.05 every week. An equal amount of solvent used for dissolving aTc (DMSO) was also added to the VC. *menA* CKD was evaluated by measuring the *menA* transcription level by quantitative real time PCR using a primer set (forward 5'-GTGGTGGAAAGCGCTGTTG-3' and reverse 5'-TGCCGTCGGAGTAGTCATTG-3') (*21*, *23*).

Determination of MK level

After 4 weeks of maintaining CKD, MK was extracted from 1 ml of bacterial suspension. For MK extraction, 125 µl of extraction buffer (PBS containing 25 mM EDTA and 5 µM α-tocopherol [Merck]) was added to the bacterial pellet. Then, 625 µl of hexane/ethanol (5:2, v/v) was added to the pellet and the bacterial cells were suspended by vortex mixing for 2 min. The bacterial suspension was centrifuged at 5000 *g* at RT for 5 min, and the upper layer (hexane layer) was transferred to a new tube. This extraction step was repeated one more time. The hexane fractions were combined and dried using a vacuum centrifuge. After the dried fractions were dissolved in 100% EtOH, the obtained lipid components were analyzed using reverse phase high performance liquid chromatography (HPLC) to determine the MK levels as described previously (*8*).

Effect of lysocin E on oxygen consumption and ATP synthesis

To observe the effect of drugs on oxygen consumption, methylene blue was added to mycobacterial cultures grown to log phase (OD_{600}, around 0.5) to be a final concentration of 0.001%. Then, the tubes were sealed tightly by molten parafilm, and the *Mtb* and *M. smegmatis* cultures were incubated for 24 and 2 hours, respectively, in the presence of 1/4-, 1/2 x MIC of lysocin E, 10 x MIC of INH or 10 x MIC of BDQ. Oxygen consumption of *Mtb* and *M. smegmatis* cultures was indicated by methylene blue decolorization while viability was determined by bacterial CFU count (*11*).

To measure the effect of the drugs on ATP synthesis, *Mtb* grown to OD_{600} of 0.5 was treated with different concentrations of lysocin E, INH or BDQ and incubated at 37°C for

24 hours. Intracellular ATP was quantified using a BacTiter Glo microbial cell viability assay kit (Promega, Madison, WI, USA) (*2*). The relative light unit (RLU) was normalized by the CFU count.

Effect of lysocin E on membrane permeability

ACEA Flow cytometry was used to evaluate the effect of lysocin E on membrane permeability. BCG was grown to log phase and adjusted to OD_{600} of 0.05. The cultures were then treated with 1/4-, 1/2-, 1-, 10x MIC of lysocin E, 1/2-, 1-, 10x MIC of RFP or 1/2-, 1-, 10x MIC of BDQ, and incubated for 3, 6, 24 and 72 hours. After incubation, membrane permeability was determined by the Live/DEAD *Bac*Light Bacterial Viability kit (Thermo Fisher Scientific, Waltham, MA). Bacteria heat killed at 95 °C and no drug live samples were used as control groups (*17*).

In vivo activities of lysocin E

Infection of 42 female C57BL/6J mice (17 to 19 g), 6-7 weeks of age, was initiated by forced intratracheal instillation of *Mtb* H37Rv. The inoculum (≈5.5 $x10^5$ CFU per mouse suspended in 50 µl of PBS) was delivered to the lung through forced inhalation with a syringe on Day 0 (*24*). To evaluate the bactericidal activities of antibiotics, six infected mice were randomly assigned to one of the following treatment and control groups:

Group-1: No drug, early negative control

Group-2: No drug, late negative control

Group-3: No drug, final negative control treated with PBS

Group-4: Treated with lysocin E at 40 mg/kg/day

Group-5: Treated with INH at 25 mg/kg/day, a positive control

Group-6: Treated with STR at 100 mg/kg/day, a positive control.

From the 10th day, both treatment and control groups were treated for two weeks (5 days of treatment per week) with the stated drugs or vehicle. INH was administered in 200 µl volume by esophageal cannula (gavage), while lysocin E, STR, and saline were injected subcutaneously in 200 µl volume.

Twenty-four days post-infection (72 hrs after the last dose), all the remaining treatment and vehicle control groups were euthanized and sacrificed. Their lungs, livers and spleens were homogenized in 4.5 ml of sterilized water. 100 µl of the serially diluted suspensions were plated on Middlebrook 7H10 agar in duplicate. The plates were incubated at 37°C in a 5% CO_2–95% air atmosphere for 3-4 weeks, after which the number of *Mtb* colonies was enumerated and, the CFU per organ was calculated (*20*, *24*). In parallel, samples for the histopathological study were also prepared.

Statistical Analysis

The results are presented as means ± SD. The CFU counts were converted to logarithms, which were then statistically analyzed by one-way analysis of variance (ANOVA), followed by multiple comparison analysis of variance, Dunnett`s test, using SPSS. P values $\leq$ 0.05 were considered statistically significant.

REFERENCES

1. World Health Organization, Factsheet global tuberculosis report 2021.

2. P. Sukheja, P. Kumar, N. Mittal, S. Li, E. Singleton, R. Russo, A.L. Perryman, R. Shrestha, D. Awasthi, S. Husain, P. Soteropoulos, R. Brukh, N. Connell, J.S. Freundlich, D. A. Alland. A novel small-molecule inhibitor of the *Mycobacterium tuberculosis* demethylmenaquinone methyltransferase MenG is bactericidal to both growing and nutritionally deprived persister cells. 2017. *mBio* **8**, 1–15 (2017).

3. D. Machado, M. Girardini, M. Viveiros, M. Pieroni, Challenging the drug-likeness dogma for new drug discovery in Tuberculosis. *Front. Microbiol.* **9,** 1367 (2018),

doi:10.3389/fmicb.2018.01367.

4. World Health Organization, Factsheet global tuberculosis report 2020, 103 (2020).

5. B. J. Berube, D. Russell, L. Castro, S. Choi, P. Narayanasamy, T. Parish, Novel MenA Inhibitors Are Bactericidal against *Mycobacterium tuberculosis* and Synergize with Electron Transport Chain Inhibitors. *Antimicrob. Agents Chemother.* **63**, 1–7 (2019).

6. J. Debnath, S. Siricilla, B. Wan, D. C. Crick, A. J. Lenaerts, S. G. Franzblau, M. Kurosu, Discovery of selective menaquinone biosynthesis inhibitors against *Mycobacterium tuberculosis*. *J. Med. Chem.* **55**, 3739–3755 (2012).

7. L. Z. Montelongo-Peralta, A. León-Buitimea, J. P. Palma-Nicolás, J. Gonzalez-Christen, J. R. Morones-Ramírez, Antibacterial Activity of combinatorial treatments composed of transition-metal/antibiotics against *Mycobacterium tuberculosis*. *Sci. Rep.* **9**, 5–10 (2019).

8. H. Hamamoto, M. Urai, K. Ishii, J. Yasukawa, A. Paudel, M. Murai, T. Kaji, T. Kuranaga, K. Hamase, T. Katsu, J. Su, T. Adachi, R. Uchida, H. Tomoda, M. Yamada, M. Souma, H. Kurihara, M. Inoue, K. Sekimizu, Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. *Nat. Chem. Biol.* **11**, 127–133 (2015).

9. H. Hamamoto, S. Panthee, A. Paudel, K. Ishii, J. Yasukawa, J. Su, A. Miyashita, H. Itoh, K. Tokumoto, M. Inoue, K. Sekimizu, Serum apolipoprotein A-I potentiates the therapeutic efficacy of lysocin E against *Staphylococcus aureus*. *Nat. Commun.* **12,** 6364 (2021), doi:10.1038/s41467-021-26702-0.

10. H. Hamamoto, K. Sekimizu, Identification of lysocin E using a silkworm model of bacterial infection. *Drug Discov. Ther.* **10**, 24–29 (2016).

11. J. Puffal, J. A. Mayfield, Demethylmenaquinone Methyl Transferase Is a Membrane Domain-Associated Protein Essential for Menaquinone Homeostasis in *M. smegmatis* (2018), *Front. Microbiol.,* **9,** 1-12, (2018), doi:10.3389/ fmicb. 2018.03145.

12. A. Paudel, H. Hamamoto, S. Panthee, K. Sekimizu, Menaquinone as a potential target of antibacterial agents. *Drug Discov. Ther.* **10**, 123–128 (2016).

13. R. S. Gupta, B. Lo, J. Son, Phylogenomics and comparative genomic studies robustly support division of the genus Mycobacterium into an emended genus Mycobacterium and four novel genera. *Front. Microbiol.* **9**, 1–41 (2018).

14. M. A. Dejesus, E. R. Gerrick, W. Xu, S. W. Park, J. E. Long, C. C. Boutte, E. J. Rubin, D. Schnappinger, S. Ehrt, S. M. Fortune, C. M. Sassetti, T. R. Ioerger, Comprehensive essentiality analysis of the *Mycobacterium tuberculosis* genome via saturating transposon mutagenesis. *MBio*. **8**, 1–17 (2017).

15. L. G. Wayne, L. G. Hayes, An *In Vitro* Model for Sequential Study of Shiftdown of Mycobacterium tuberculosis through Two Stages of Nonreplicating Persistence. *Infect Immun,* **64**, 2062–2069 (1996).

16. W. Kim, G. Zou, T. P. A. Hari, I. K. Wilt, W. Zhu, N. Galle, H. A. Faizi, G. L. Hendricks, K. Tori, W. Pan, X. Huang, A. D. Steele, E. E. Csatary, M. M. Dekarske, J. L. Rosen, N. De Queiroz Ribeiro, K. Lee, J. Port, B. B. Fuchs, P. M. Vlahovska, W. M. Wuest, H. Gao, F. M. Ausubel, E. Mylonakis, A selective membrane-targeting repurposed antibiotic with activity against persistent methicillin-resistant *Staphylococcus aureus*. *Proc. Natl. Acad. Sci.* **116**, 16529–16534 (2019).

17. N. M. O'Brien-Simpson, N. Pantarat, T. J. Attard, K. A. Walsh, E. C. Reynolds, A rapid and quantitative flow cytometry method for the analysis of membrane disruptive antimicrobial activity. *PLoS One*. **11**, 1–15 (2016).

18. H. Itoh, K. Tokumoto, T. Kaji, A. Paudel, S. Panthee, H. Hamamoto, K. Sekimizu, M. Inoue, Development of a high-throughput strategy for discovery of potent analogues of antibiotic lysocin E. *Nat. Commun.* **10**, 1-11 (2019).

19. C. Temesszentandrási-Ambrus, S. Tóth, R. Verma, P. Bánhegyi, I. Szabadkai, F. Baska, C. Szántai-Kis, R. C. Hartkoorn, M. A. Lingerfelt, B. Sarkadi, G. Szakács, L. Orfi, V. Nagaraja, S. Ekins, Á. Telbisz, Characterization of new, efficient

Mycobacterium tuberculosis topoisomerase-I inhibitors and their interaction with human ABC multidrug transporters. *PLoS One*. **13**, 1–15 (2018).

20. W. Gao, J. Kim, J. R. Anderson, T. Akopian, S. Hong, Y. Jin, O. Kandror, J. Kim, I. Lee, S. Lee, J. B. Mcalpine, S. Mulugeta, S. Sunoqrot, Y. Wang, S. Yang, T. Yoon, A. L. Goldberg, G. F. Pauli, J. Suh, S. G. Franzblau, The Cyclic Peptide Ecumicin Targeting ClpC1 Is Active against *Mycobacterium tuberculosis In Vivo*. *Antimicrob. Agents Chemother.* **59**, 880–889 (2015).

21. A. K. Singh, X. Carette, L. P. Potluri, J. D. Sharp, R. Xu, S. Prisic, R. N. Husson, Investigating essential gene function in *Mycobacterium tuberculosis* using an efficient CRISPR interference system. *Nucleic Acids Res.* **44,** 1-13 (2016), doi:10.1093/nar/gkw625.

22. A. Savitskaya, A. Nishiyama, T. Yamaguchi, Y. Tateishi, Y. Ozeki, M. Nameta, T. Kon, S. A. Kaboso, N. Ohara, O. V. Peryanova, S. Matsumoto, C-terminal intrinsically disordered region-dependent organization of the mycobacterial genome by a histone-like protein. *Sci. Rep.* **8**, 1–15 (2018).

23. E. Choudhary, P. Thakur, M. Pareek, N. Agarwal, Gene silencing by CRISPR interference in mycobacteria. *Nat. Commun.* **6**, 1–11 (2015).

24. C. Chen, F. Ortega, J. Rullas, L. Alameda, I. Angulo-Barturen, S. Ferrer, U. S. Simonsson, The multistate tuberculosis pharmacometric model: a semi-mechanistic pharmacokinetic-pharmacodynamic model for studying drug effects in an acute tuberculosis mouse model. *J. Pharmacokinet. Pharmacodyn.* **44**, 133–141 (2017).

Acknowledgments:

GG is grateful for the financial aid provided by the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) scholarship. We acknowledge Tatsuo Ushiki for allowing us to use FE-SEM, S-4300, at the Department of Anatomy, Niigata University School of Medicine. We are also thankful to Niigata University School of

Medicine, Department of Pathology staff for their help in sample preparation for histopathological study.

Funding:

This work was supported by grants from the Japanese Ministry of Education, Culture, Sports, Science and Technology (SM, 21KK0136 & 20H03483; HH, 19K07140JP); and the Japan Agency for Medical Research and Development (AMED: Research Program on Emerging and Re-emerging Infectious Diseases (SM., J21J0075; 20fk0108090, A.N.), Drug Discovery Support Promotion Project (S.P. and K.S), TBRF and IFO fellowships to S.P. and K.S).

Author contributions:

Conceptualization: GG, AI, SM, HH, AN
Methodology: GG, AI, AN, SM, HH, MH, SAK, AI, YO, YT
Investigation: GG, AI, HH, AT, YM, KS, SP
Funding acquisition: SM, HH
Project administration: AN, SM, HH, KS
Supervision: AN, SM, HH
Writing–original draft: GG, AI
Writing–review & editing: GG, AI, AN, HH, SAK, SM

Competing interests: The authors declare no competing interests.

Data and materials availability: Requests for data and materials should be addressed to GG, AN or SM.

Table 1. MICs against Mycobacteria.

Mycobacteria[a]	**MIC (μg/ml)**[b]				
	Lysocin E	**Isoniazid**	**Rifampicin**	**Bedaquiline**	**Ethambutol**
Mtb (H37Rv)	0.5	0.031	0.002	0.25	0.75
MDR066 (MDR-*Mtb*)	0.5	8	>32	NE	2
MDR005 (XDR-*Mtb*)	≤ 0.13	8	16	NE	64
MDR044 (XDR-*Mtb*)	0.25	4	1	NE	64
Mtb + 400 μM MK4	5	NE	0.004	NE	NE
Mtb var. BCG	0.31	0.0625	0.00023	0.5	3
M. smegmatis	10	16	10	0.0625	0.25
M. smegmatis+ 200 μM MK4	50	NE	12.5	NE	NE

NE, not evaluated

[a]MDR005 (XDR-*Mtb*), resistant to INH, RFP, SM, EMB, KM, LVEX, SPFX, CPFX; MDR044 (XDR-*Mtb*), resistant to INH, RFP, SM, EMB, KM, LVEX, SPFX, CPFX; MDR066 (MDR-*Mtb*), resistant to INH and RFP.

[b] The MIC was evaluated by adding 50 μl bacterial suspension (OD_{600}, 0.00025-0.0006) to 50 μl of drug containing 7H9 broth in a 96-well microtiter plate. BCG and *Mtb* were incubated for 8 days, while *M. smegmatis* was incubated for 3 days at 37 °C. The MIC for each drug was defined as the lowest concentration that inhibited bacterial growth as determined by the naked eye from the incubated plate. These results are representative of three independent experiments. In all the following experiments described in this study, the drug concentrations used were indicated as fold of MIC values shown in this table.

Table 2. Combined activity of lysocin E with current TB drugs.

Drug combination	MIC (µg/ml)[a]				
	Drug	**Alone**	**In combination**	**FIC**	**FIC index, category [b]**
Lysocin E and INH	Lysocin E	0.25	0.125	0.5	1, Additive
	INH	0.031	0.0156	0.5	
Lysocin E and RFP	Lysocin E	0.25	0.25	1	1.5, No interaction
	RFP	0.004	0.002	0.5	
Lysocin E and EMB	Lysocin E	0.25	0.0156	0.06	0.56, additive
	EMB	0.75	0.375	0.50	
Lysocin E and BDQ	Lysocin E	0.25	0.25	1	2, No interaction
	BDQ	0.25	0.25	1	

[a]Checkerboard assay was used to evaluate the effect of lysocin E in combination with other front-line drugs against *Mtb*. Bacterial growth was determined as described in Table 1. [b]FIC index range of 0.5 to 4 is commonly used in drug combination studies. FICI: $\leq$0.5 as synergism, >0.5 to $\leq$1 as additivity; >1 to < 4 as no interaction, and >4 as antagonism (*7*, 19, 20). These results are representative of two independent experiments.

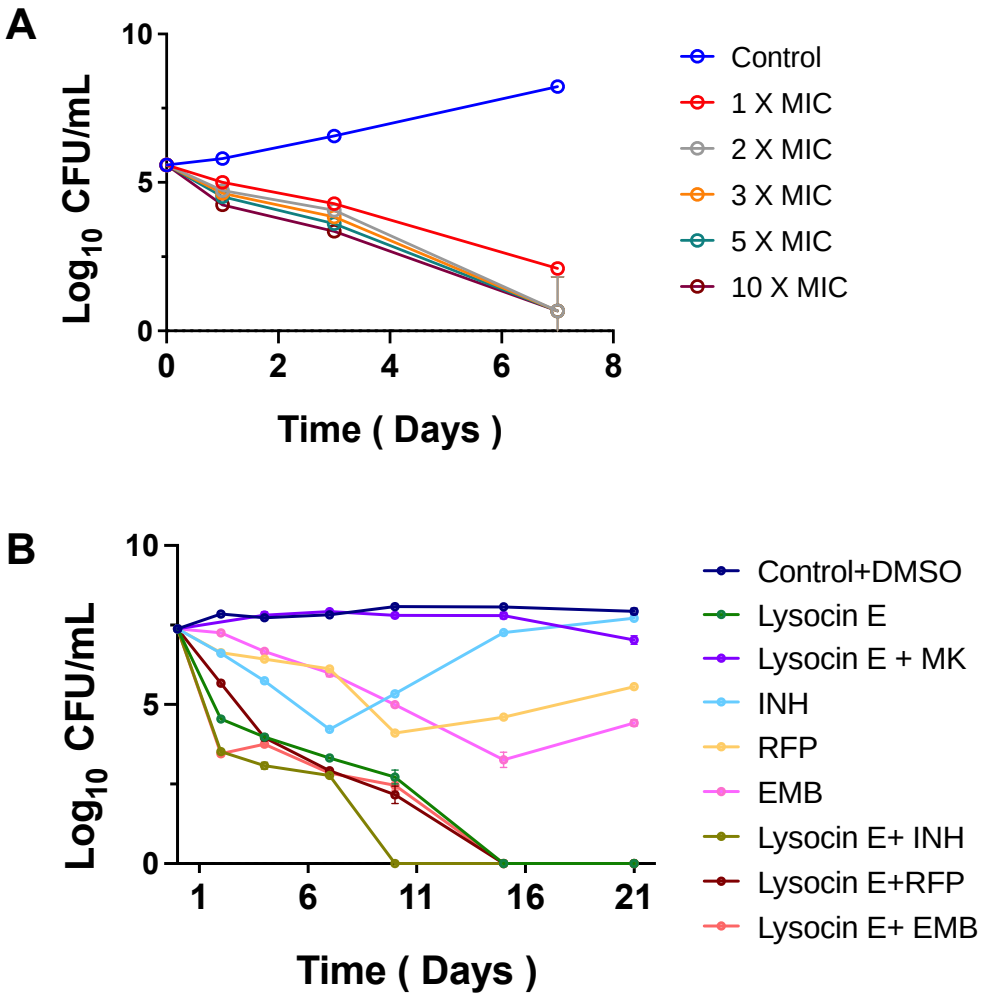


Fig. 1. Efficacy of lysocin E against mycobacteria *in vitro*. (A) MBC of lysocin E. *Mtb* harvested at exponential phase was adjusted to OD_{600} of 0.001, then exposed to different concentrations of lysocin E for 7 days at 37°C. The CFU was counted at the indicated time points. **(B) Twenty-one-days kill kinetics of lysocin E.** *Mtb* harvested at mid-log-phase and adjusted to OD_{600} 0.1 was incubated with 10x MIC of lysocin E, isoniazid (INH), rifampicin (RFP), and ethambutol (EMB). At the indicated time points, CFU with a detection limit of 2 log_{10}/ml was counted. Data represent the mean ±SD of triplicates. These results are representative of three independent experiments.

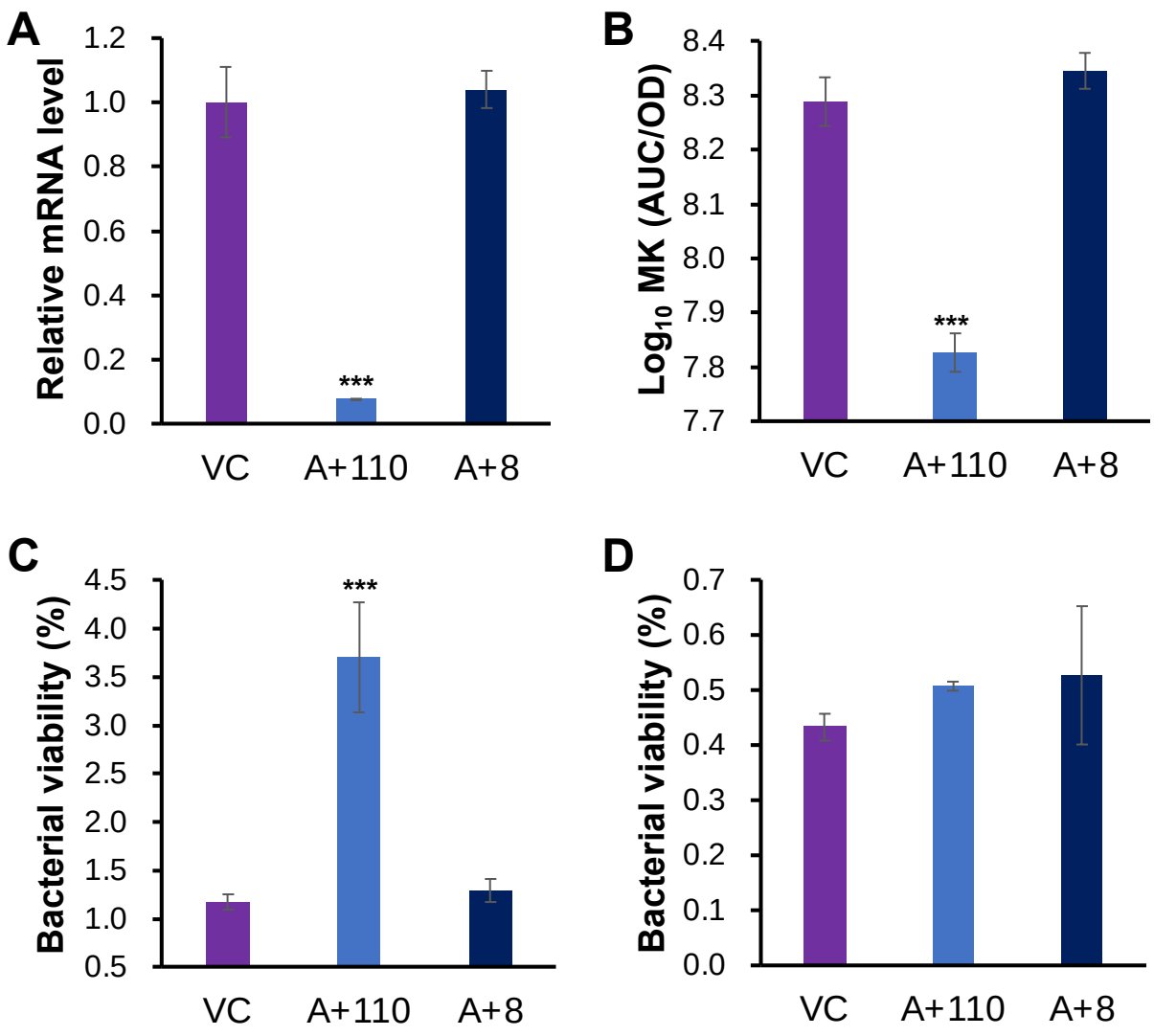


Fig. 2. The effect of MK biosynthesis CKD on the susceptibility of *Mtb* to lysocin E. The *menA* CKD strains, constructed using a TetR-regulated CRISPR interference system optimized for mycobacteria, were cultured in the presence of aTC which was supplied at final concentration of 200 ng/ml every two days. Four weeks later, the *menA* mRNA level was quantified by real-time PCR. VC, wild-type strain with empty vector. A+8 and A+110 strains, *Mtb* H37Rv *menA* CKD strains constructed using TetR-regulated CRISPR interference system targeting two different non-template strands of *menA* ($G^{8}GAGACCCACTGTGCGAAAC^{27}$ and $T^{110}GTGGTGGAAAGCGCTGTTG^{129}$, respectively). **(B)** Four weeks after achieving *menA* CKD, MK amount (AUC) was

quantified and normalized with bacterial OD_{600}. **(C** and **D)** Bacterial susceptibility to lysocin E and INH was also examined four weeks after *menA* CKD. To examine susceptibility, the bacteria (OD_{600} of 0.1) were incubated at 37 °C in the presence or absence of 50x MIC of lysocin E or 50x MIC of INH. After incubating the bacteria with the drugs for 24 hrs, bacterial CFU was determined in lysocin E-treated bacteria **(C)** or INH-treated bacteria **(D).** The bacterial viability (%) was calculated in comparison of the CFU counts of treated samples with those of their corresponding nontreated samples (same recombinant bacteria). Statistical differences were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test (***P<0.0001). Data represent the mean ±SD of triplicates. This experiment was repeated two times, and reproducible data was observed.

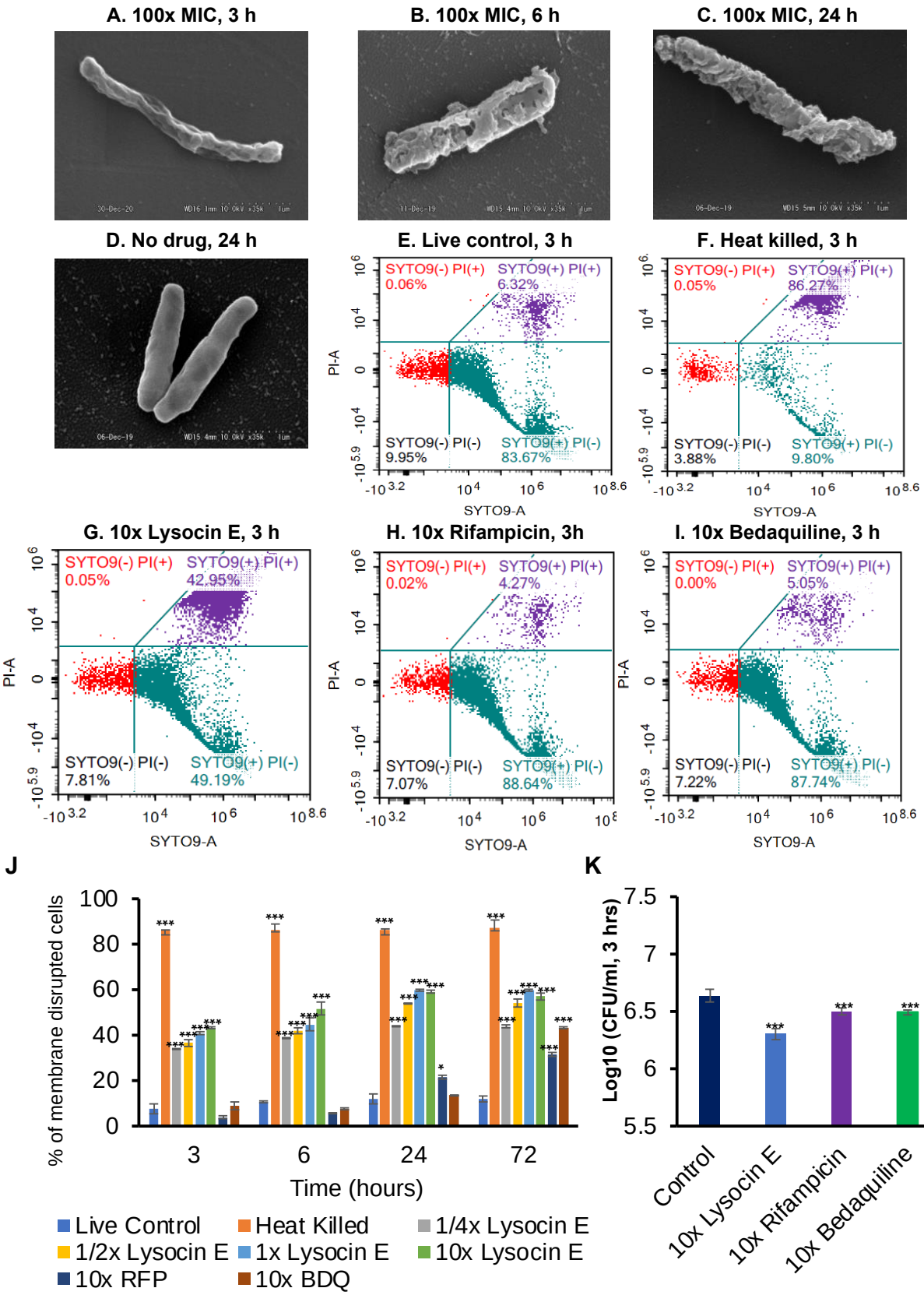

A. 100x MIC, 3 h
B. 100x MIC, 6 h
C. 100x MIC, 24 h
D. No drug, 24 h
E. Live control, 3 h
SYTO9(-) PI(+) 0.06%
SYTO9(+) PI(+) 6.32%
SYTO9(-) PI(-) 9.95%
SYTO9(+) PI(-) 83.67%
F. Heat killed, 3 h
SYTO9(-) PI(+) 0.05%
SYTO9(+) PI(+) 86.27%
SYTO9(-) PI(-) 3.88%
SYTO9(+) PI(-) 9.80%
G. 10x Lysocin E, 3 h
SYTO9(-) PI(+) 0.05%
SYTO9(+) PI(+) 42.95%
SYTO9(-) PI(-) 7.81%
SYTO9(+) PI(-) 49.19%
H. 10x Rifampicin, 3h
SYTO9(-) PI(+) 0.02%
SYTO9(+) PI(+) 4.27%
SYTO9(-) PI(-) 7.07%
SYTO9(+) PI(-) 88.64%
I. 10x Bedaquiline, 3 h
SYTO9(-) PI(+) 0.00%
SYTO9(+) PI(+) 5.05%
SYTO9(-) PI(-) 7.22%
SYTO9(+) PI(-) 87.74%
PI-A
SYTO9-A
J
% of membrane disrupted cells
Time (hours)
Live Control
Heat Killed
1/4x Lysocin E
1/2x Lysocin E
1x Lysocin E
10x Lysocin E
10x RFP
10x BDQ
K
Log10 (CFU/ml, 3 hrs)
Control
10x Lysocin E
10x Rifampicin
10x Bedaquiline

Fig. 3. Lysocin E causes mycobacteria membrane disruption. (**A–D**) SEM observation of *Mtb* exposed to 100x MIC of lysocin E for 3 hours (**A**), 6 hours (**B**), 24 hours (**C**), and untreated *Mtb* at 24 hours (**D**). All micrographs are at 35,000× magnification. **(E-I)** After treating BCG (OD_{600} of 0.05) with 1/4-, 1/2-, 1- and 10x MIC of lysocin E and 1/2-, 1- and 10x MIC of RFP and BDQ, the effect of drugs on membrane permeability was evaluated by the FACS analysis using Live/Dead (Syto9/PI) staining at 3, 6, 24 and 72 hrs. The representative flow cytometric dot plots of the live control **(E)**, heat killed control **(F)**, 10x MIC lysocin E **(G),** 10x MIC RFP **(H)** and 10x MIC BDQ **(I)** treated samples for three hours. **(J)** The percentage of membrane disrupted (PI-positive) cells were calculated and summarized as shown in the panel. **(K)** In parallel, the activity of drugs was evaluated by CFU count after treating BCG with 10x MIC of lysocin E, RFP and BDQ for 3 hrs. Statistical differences were analyzed by one-way ANOVA with Dunnett's multiple comparison test (*, $P<0.05$; ***, $P<0.0001$). Data represent the mean ±SD of triplicates and, each picture is representative of two reproducible biological experiments.

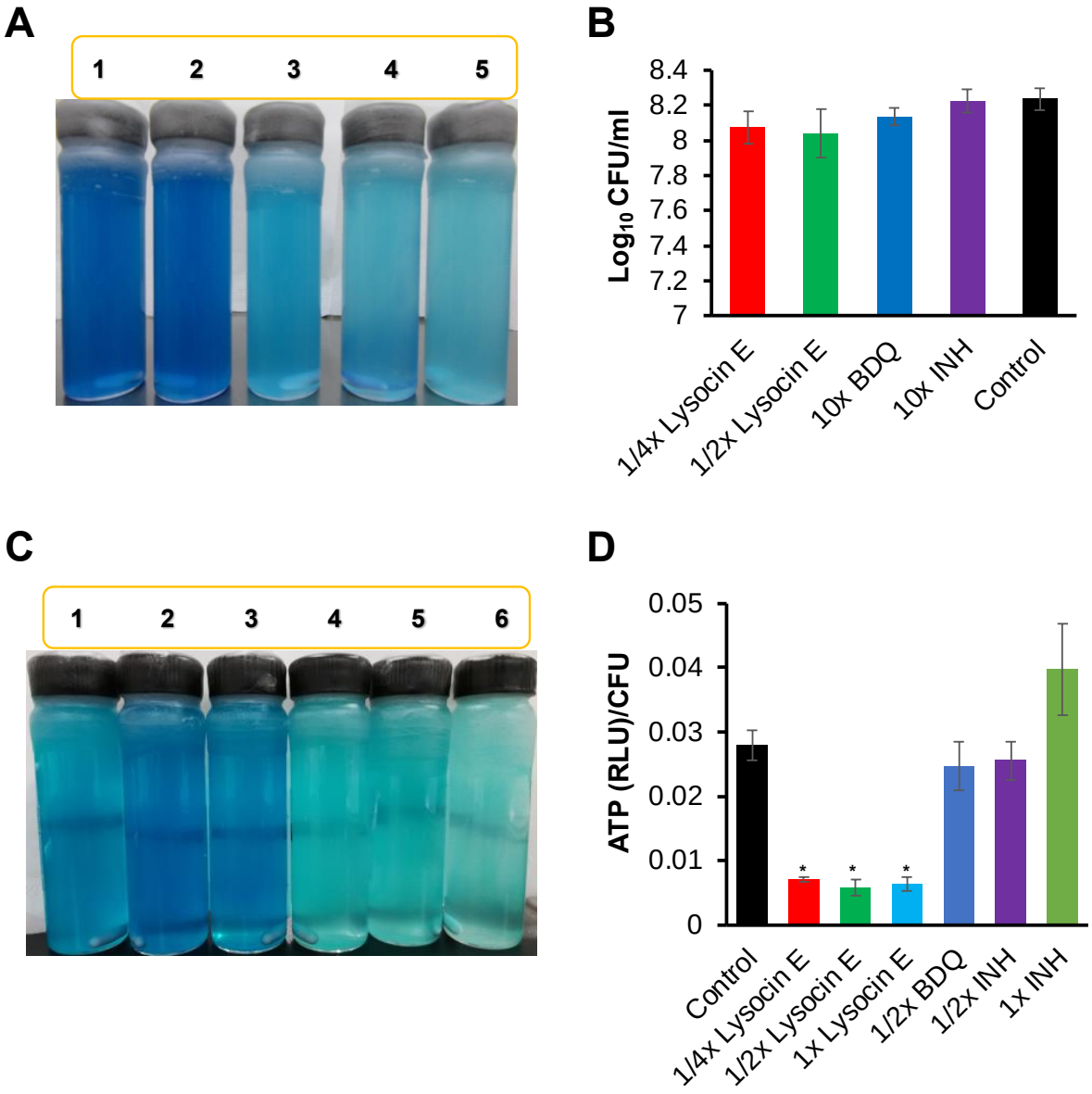


Fig. 4. Effect of lysocin E on mycobacteria energy production. (**A and B**) Oxygen consumption assay in *Mtb* H37Rv (OD_{600} of 0.5) was evaluated using 0.001% methylene blue as an oxygen sensor. BDQ, a drug that targets oxidative phosphorylation, and INH, a drug that targets cell wall synthesis but not cell membrane, were used as positive controls. **(A)** Tube 1, *Mtb* treated with 1/4x MIC (0.125 µg/ml) of lysocin E; tube 2, 1/2x MIC (0.25 µg/ml) of lysocin E; tube 3, 10x MIC (2.5 µg/ml) of BDQ; tube 4, 10x MIC (0.31 µg/ml) of INH and tube 5, control without drug. Cultures were incubated at 37°C for 24 hrs. **(B)** CFU of the cultures was also counted. (**C**) A similar assay was performed using *M. smegmatis* MC^2155 cultures. Tube 1, *M.*

smegmatis (OD_{600} of 0.5) treated with 1/8x MIC (1.25 μg/ml) of lysocin E; tube 2, 1/4x MIC (2.5 μg/ml) of lysocin E; tube 3, 1/2x MIC (5 μg/ml) of lysocin E; tube 4, 10x MIC (160 μg/ml) of INH; tube 5, 10x MIC (0.625 μg/ml) of BDQ; and tube 6, control without drug. Cultures were incubated at 37°C for 2 hrs. (**D**) The effect of the drug treatment on ATP synthesis. *Mtb* was treated with the indicated concentrations of lysocin E, INH and BDQ. After 24 hrs of incubation, the intracellular ATP was quantified using the BacTiter Glo microbial cell viability assay kit (Promega). The RLU was normalized by CFU. The statistical differences between the control and each treatment were analyzed by one-way ANOVA with Dunnett's multiple comparison test (*, $P < 0.01$). All experiments were repeated two times, and reproducible data was observed.

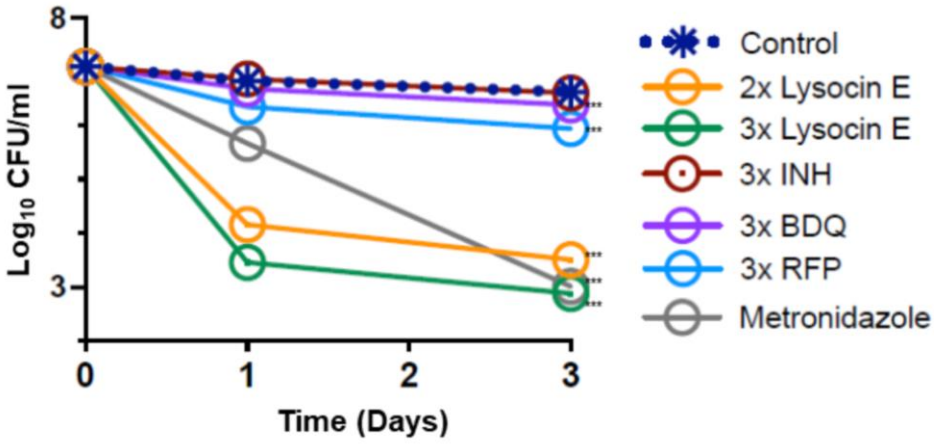


Fig. 5. Bactericidal activity of lysocin E against nonreplicating mycobacteria. The bactericidal activity against NPR bacteria was determined by exposing *M. smegmatis* subjected to growth in the Wayne hypoxia model, to the indicated antibiotics. Statistical differences between the wild type and each treatment were analyzed by one-way ANOVA with Dunnett's multiple comparison test (***, $P < 0.0001$). Data represent the mean ±SD of triplicates. This experiment was repeated two times, and reproducible data was observed.

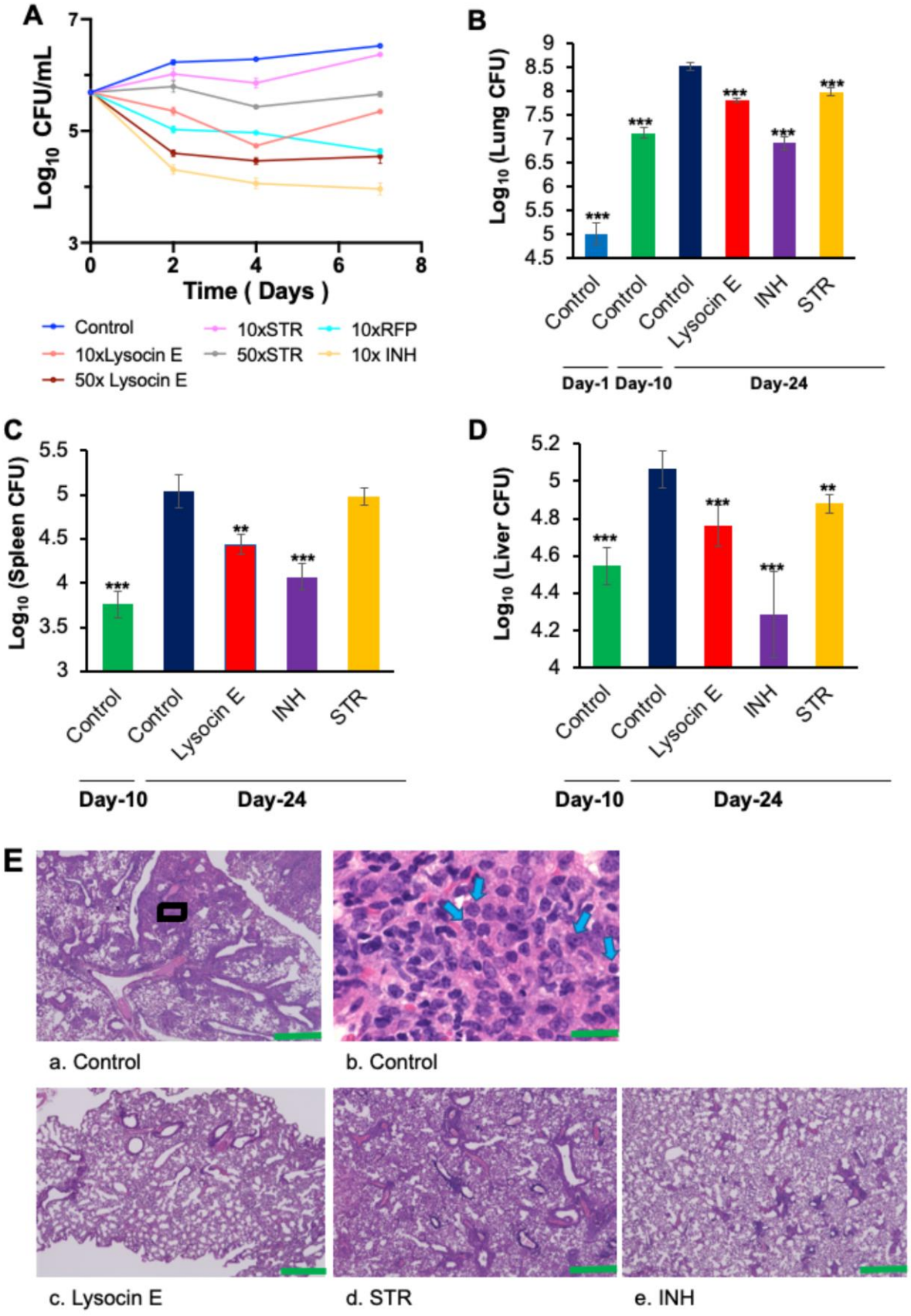

A
Log10 CFU/mL
Time (Days)
Control
10xLysocin E
50x Lysocin E
10xSTR
50xSTR
10xRFP
10x INH
B
Log10 (Lung CFU)
Control
Control
Control
Lysocin E
INH
STR
Day-1
Day-10
Day-24
C
Log10 (Spleen CFU)
Control
Control
Lysocin E
INH
STR
Day-10
Day-24
D
Log10 (Liver CFU)
Control
Control
Lysocin E
INH
STR
Day-10
Day-24
E
a. Control
b. Control
c. Lysocin E
d. STR
e. INH

Fig. 6. Efficacy of lysocin E against *Mtb* in macrophages and mice. (A) The efficacy of lysocin E against the intracellular *Mtb*. The THP 1 cells were infected with *Mtb* (MOI 1:1) for 4 hrs. Subsequently, the cultures were washed twice with a warm serum free DMEM. The infected macrophages were treated by adding 100 µl DMEM containing 10- or/and 50x MIC of lysocin E, INH, STR and RFP. The cells were incubated at 37°C in a humidified 5% CO_2 incubator. CFU on day-0, day-2, day-4 and day-7 were determined. Female C57BL/6J mice (17 to 19 g) infection was initiated by intratracheal instillation of *Mtb* H37Rv. For this purpose, *Mtb* was grown to an OD_{600} of 0.5. The inoculum ($5.5x10^5$ CFU per mouse suspended in 50 ul of PBS) was delivered to the lung through forced inhalation with a syringe at day 0. Six of the infected mice were then randomly assigned to one of the indicated groups to evaluate the bactericidal activities of antibiotics. Five mice from each group were sacrificed the day after infection, the day of treatment initiation, and after 14 days of treatment to determine the number of CFU implanted in the lungs, the pretreatment baseline CFU counts and the CFU counts after treatment, respectively. A mouse was also sacrificed from each group for histopathological study after treatment. **(B)** lung CFU **(C)** spleen CFU **(D)** liver CFU were counted. Data represent the mean ±SD of triplicates. Statistical differences were analyzed by one-way analysis of variance with Dunnett's multiple comparison test (**, $P<0.005$; ***$P < 0.001$). **(E)** Lung tissue images after treatment. Lung tissue sections of untreated control (**a** and **b**), lysocin E- (**c**), STR- (**d**), and INH- (**e**) treated mice were stained with hematoxylin and eosin (**a, c, d,** scale bars, 500 µm; **b**, a scale bar, 50 µm). **(b)** is an example of the magnified image (the area indicated as a black box in **a**) used for mononuclear cell counting. Arrows in **b** indicate the examples of mononuclear cell counted. Counted data of untreated and treated groups are shown and compared in Figure S4F.

Supplementary Materials for:

Lysocin E targeting menaquinone in the membrane of *Mycobacterium tuberculosis* is a promising lead compound for anti-tuberculosis drugs

Geberemichal Geberetsadik*, Akane Inaizumi, Akihito Nishiyama*, Takehiro Yamaguchi, Hiroshi Hamamoto, Suresh Panthee, Aki Tamaru, Manabu Hayatsu, Yusuke Mizutani, Shaban Amina Kaboso, Mariko Hakamata, Aleksandr Ilinov, Yuriko Ozeki, Yoshitaka Tateishi, Kazuhisa Sekimizu, Sohkichi Matsumoto*

Corresponding authors: *G.G., mikigebre73@gmail.com; A.N., anishi@med.niigata-u.ac.jp; S.M., sohkichi@med.niigata-u.ac.jp

Supplementary Materials include:

I. MATERIALS AND METHODS

Biosafety criteria and mycobacterial strains

All procedures involving *Mtb* specimens were carried out in the Biosafety level 3 containment facility located at the Graduate school of medical and dental sciences, Niigata University. Ethical approval to use recombinant *Mtb* and mice was obtained from both Niigata University school of medicine and Japan Ministry of health, labor, and welfare. In this study, drug-susceptible *Mtb* H37Rv strain, multidrug-resistant *Mtb* strains (MDR-*Mtb* 66), extensively drug-resistant *Mtb* strains (XDR-*Mtb* 5, XDR-*Mtb* 44), *Mtb* var. BCG Tokyo strain and *M. smegmatis* strain MC^2155 were used to evaluate the antimycobacterial activities of lysocin E.

Media & cultivation

Liquid cultures were grown in Middlebrook 7H9 broth (BD Difco) supplemented with 10% albumin-dextrose-catalase (BD Difco, MD, USA), 0.2% glycerol and 0.05% Tween-80 (Sigma–Aldrich MI, USA). Solid cultures were grown on Middlebrook 7H10 agar (BD Difco) supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose-catalase (OADC) (BD Difco). Liquid cultures were grown in 250 ml flasks or 50 ml round bottom tubes at 37°C. To generate hypoxic nonreplicating cultures, *Mtb* subspecies BCG Tokyo type 1 or *M. smegmatis* strain MC^2155 were grown in Dubos broth (0.5 g/l pancreatic digest of casein, 2 g/l asparagine, 1 g/l monopotassium phosphate, 2.5 g/l disodium phosphate, 50 mg/l ferric ammonium citrate, 10 mg/l magnesium sulfate, 0.5 mg/l calcium chloride, 0.1 mg/l zinc sulfate, 0.1 g/l copper sulfate, and 0.2% Tween 80; FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) supplemented with 0.5% Tween 80, 0.5% bovine serum albumin, 0.75% D-glucose, 0.085% sodium chloride, and 1.5 µg/ml methylene blue. RPMI-1640 medium containing 10% FCS plus penicillin-streptomycin solution) and D-MEM (High Glucose) (FUJIFILM Wako Pure Chemical Cooperation) was used for cell culture. All cultures were incubated at 37 °C.

Antimycobacterial drugs and other chemicals

Lysocin E, purified from *Lysobater sp*. culture supernatant, used in this study was provided by Dr. Hamamoto (Teikyo University). Hygromycin B (HYG) was purchased from Wako Pure Chemical Industries. Kanamycin (KM), isoniazid (INH), streptomycin (STR), bedaquiline (BDQ), ethambutol (EMB), rifampicin (RFP), metronidazole, CytoScanTM Water Soluble Tetrazolium Salts (WST-1), phorbol 12-myristate-13-acetate (PMA), and menaquinone 4 (Vitamin K2) were purchased from Merck (Darmstadt, Germany). Anhydrotetracycline (aTc) was purchased from Cayman Chemical (MI, USA).

In vitro anti-TB activities

Determination of MIC for antibiotics

To evaluate the MIC of drugs 50 µl bacterial suspension (OD, 0.00025-0.0006) was added to 50 µl of drug-containing Middlebrook 7H9 broth in a 96-well microtiter plate. First, 50 µl of Middlebrook 7H9 broth was aliquoted into all wells except the first column wells. Then, 100 µl of Middlebrook 7H9 broth containing antibiotics was aliquoted into the first column wells. After mixing column 1 wells 10-12 times using a multichannel pipettor, 50 µl was withdrawn and transferred to column 2 wells, followed by mixing and transferring of 50 µl from column 2 to column 3. This procedure was used to serially dilute the rest of the columns except column 12 which was used as a negative control.

To prepare the bacterial inoculum, the bacterial stock was transferred to 10 ml of Middlebrook 7H9 broth. The suspension density was adjusted to an optical density (OD_{600}) of 0.05 at 600 nm and incubated at 37°C with daily OD_{600} checking. When the OD_{600} reached 0.2 to 0.3, it was used as the original bacterial suspension to prepare mycobacterial suspension ($OD6_{00}$ of 0.00025-0.0006). Finally, 50 µl of the adjusted OD_{600} was added to the wells containing specified antibiotics. The microtiter plate was incubated at 37 °C for 8 days for BCG and *Mtb,* and 3 days for *M. smegmatis* to

determine MIC. The MIC for each drug was defined as the lowest concentration that inhibited bacterial growth as determined by the naked eye (*7*, 19).

Determination of compound interaction by checkerboard assay

We used the checkerboard assay to test for interactions between lysocin E and drugs with known anti-TB activities using the broth microdilution method in 96-well microtiter plates. In one plate, 100 µl of fresh Middlebrook 7H9 broth was aliquoted into all wells except the first column (A1-H1). Then vertically, 200 µl of lysocin E-containing medium was added to the first column wells of the plate (A1-H1). Then, 100 µl each of lysocin E-containing medium in all wells of column (A1-H1) was transferred to the wells in column (A2-H2) and then mixed well by sucking up and down 10 times using the multi-pipettor. This procedure was repeated up to the wells in column (A11-H11) and finally 100 µl each was withdrawn from column (A11-H11) and discarded. The wells in columns (A12-H12) were “No lysocin E” wells. This plate was prepared in duplicate to have enough serially diluted lysocin E-containing medium to be combined with the other four drugs. On another plate, the serial dilution of other drugs (INH, RFP, EMB or BDQ) was prepared. This was done by aliquoting 50 µl of fresh Middlebrook 7H9 broth into all wells except the first row (A1-A12). Then, horizontally, in each plate, 100 µl of the drug-containing medium was added to all first-row wells of the plate (A1-A12) and 50 µl each of drug-containing medium from wells in row A1-A12 was transferred to the wells in a row (B1-B12) and then mixed well by sucking up and down 10 times using the multi-pipettor. This procedure was repeated up to the wells in a row (G1-G12) and then 50 µl each was withdrawn from the row (G1-G12) and discarded. The wells in a row (H1-H12) were “No drug” wells.

For the checkerboard assay, 25 µl from each well of lysocin E plate and 25 ul from each well of another drug plate (INH, RFP, EMB or BDQ) were transferred to the corresponding wells of a new plate and mixed (final drug volume of each well was 50 µl). Then, like the MIC assay, 50 µl of mycobacterial suspension (OD_{600} of 0.0004-0.0006) was added to all wells of the plate. “No lysocin E” wells (A12-H12) were used to

determine the MIC of another drug (INH, RFP, EMB, or BDQ) when used alone. “No drug” wells (H1-H12) were used to determine the MIC of lysocin E when used alone.

The MIC for each drug was defined as the lowest concentration that inhibited *Mtb* growth as determined by the naked eye after incubating the plates at 37 °C for 8 days. To evaluate the effect of each combination, the obtained MIC values were used to calculate the fractional inhibitory coefficient index (FICI) as follows: FICI= (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination/MIC of drug B alone). The FIC index (FICI) calculated for each drug combination were categorized based on FICI: $\leq$0.5 as synergism, >0.5 to $\leq$1 as additivity; >1 to < 4 as no interaction, and >4 as antagonism (*7*, 19, 20).

Determination of MBC for lysocin E and its kill kinetics

MBC for replicating cultures was determined by adjusting exponentially growing *Mtb* to OD_{600} of 0.001 and treating them with 1x, 2x, 3x, 5x and 10x MIC of the drug. The cultures were washed with phosphate-buffered saline (PBS) twice by centrifugation at 10,000 rpm for 10 min to remove the drug prior to plating. Culture plating was done on 7H10 agar at day-0, -1, -3 and -7. The MBC was defined as the lowest concentration reducing CFU by 99% relative to the Time-zero inoculum on day 7 (*20*).

Twenty-one-day kill kinetics was performed to evaluate the bactericidal activity of lysocin E in combination with other drugs by CFU count. For this purpose, mid-log-phase *Mtb* culture was diluted in fresh medium (OD_{600} of 0.1). After aliquoting the culture to fresh tubes, 10xMIC of the drugs as a single drug or in combination were added and incubated for 21 days. Each culture dilution was plated at selected time intervals (day-0, -2, -4, -7, -10, -15 and -21) on 7H10 agar plates. The plates were incubated at 37 ^{O}C in a 5% CO_2–95% air atmosphere for 3-4 weeks, after which the number of *Mtb* CFU on each plate was enumerated (*2*).

Bactericidal activities against nonreplicating mycobacteria

To study the bactericidal activity of lysocin E against non-replicating mycobacteria (NRP), *M. smegmatis* and BCG culture were grown in Dubos broth to log phase. The cultures were adjusted to initial OD_{600} of 0.005 in a final volume of 250 in 300 ml flasks, the magnetic stirrers and methylene blue (1.5 µg/ml) were added. Then, the cultures were sealed tightly by molten parafilm and stirred gently at 160 rpm on magnetic stirring platforms to produce non replicating mycobacteria based on the Wayne hypoxia model (*15*). The non-replicating bacteria were obtained after incubating the *M. smegmatis* and BCG cultures for 7 days and 15 days respectively.

To study the bactericidal activity of lysocin E and to compare its activity with other first line drugs against NRP mycobacteria, *M. smegmatis* or BCG, were transferred to 15 ml test tubes inside an anaerobic chamber. *M. smegmatis* cultures were treated with 1-, 2- and 3x MIC of lysocin E, and 1- and 3x MIC of BDQ, RFP, and INH. BCG cultures were treated with 10x MIC of lysocin E, BDQ, RFP, and INH. 100 µg/ml and 50 µg/ml of metronidazole were added to BCG and *M. smegmatis* cultures, respectively, as a positive control. The cultures were washed with sterilized UPW twice by centrifugation at 10,000 rpm for 10 min to remove the drug prior to plating. The cultures were plated on 7H10 agar at selected time intervals and incubated at 37 °C for 3-4 days and 4 weeks for *M. smegmatis* and BCG, respectively.

Lysocin E mechanism of action in *Mtb*

Preparation of *menA*-CKD strains

Mtb menA-CKD strains were considered for this study. MenA gene (*menA*, Rv0534c) encodes isoprenyl diphosphate:1,4-dihydroxy-2-naphthoat (DHNA) isoprenyltransferase which is essential enzyme in MK biosynthesis. This catalyzes a critical reaction in MK biosynthesis that involves the conversion of cytosolic DHNA, to membrane bound demethylmenaquinone by transferring a hydrophobic 45-carbon isoprenoid chain (in the case of mycobacteria) to the ring nucleus of DHNA (5, 14). To construct *Mtb menA*-CKD

strains TetR/*tetO*-inducible CRISPR-dCas9 interference specialized for mycobacteria was employed (*21*). pRH2502, a vector expressing an enzymatically inactive Cas9 (dCas9), and pRH2521, a vector expressing guide RNAs (sgRNAs), were kindly gifted by Dr. Robert N. Husson (*21*). Both dCas9 and sgRNA are expressed from TetR-regulated promoters (*uvtetO* and $P_{myc1\text{-}tetO}$, respectively).

We constructed *menA*-CKD strains of *Mtb* H37Rv, as described previously (*22*). For the *menA* CKD, two sgRNAs were designed for targeting the non-template strand of *menA* at +8 - +27 (G^{8}GAGACCCACTGTGCGAAAC^{27}: synthesized oligonucleotides, 5'-GGGAGGAGACCCACTGTGCGAAAC-3' and 5'-AAACGTTTCGCACAGTGGGTCTCC-3') and at +110 - +129 (T^{110}GTGGTGGAAAGCGCTGTTG^{129}: synthesized oligonucleotides, 5'-GGGACAACAGCGCTTTCCACCACA-3' and 5'-AAACTGTGGTGGAAAGCGCTGTTG-3'). To minimize off-target effect, we confirmed that there was no gene which had similar sequence to the sgRNA with less than 5 mismatches in *Mtb* H37Rv by BLAST search. Each pair of designed oligonucleotides was annealed and then inserted into pRH2521 for sgRNA expression. *Mtb* strain, which was already containing pRH2502 (for dCas9 expression), was transformed with these vectors or empty pRH2521 and then strains were selected on 7H10 agar plates containing 50 μg/ml HYG and 25 μg/ml KM (designated A+8, A+110, and vector control [VC]). For induction of dCas9 and sgRNA expression, aTc was supplemented to the bacterial culture to a final concentration of 200 ng/ml (*21*).

Checking MenA gene CKD

The *Mtb menA*-CKD strains of (A+110 and A+8) and VC cultures were grown in 7H9/ADC/Tw80 containing 25 μg/ml KM and 50 μg/ml HYG at a starting OD_{600} of 0.05 in 250 ml flasks. To induce and maintain the long-term expression of dCas9 and sgRNA, aTc was added to a final concentration of 200 ng/ml every two days for 4 weeks while refreshing it to a starting OD_{600} of 0.05 every week. An equal amount of solvent used for dissolving aTc (DMSO) was also added to the VC. *menA* CKD was evaluated by measuring the *menA* transcription level by quantitative real time PCR using a primer set

(forward 5'-GTGGTGGAAAGCGCTGTTG-3' and reverse 5'-TGCCGTCGGAGTAGTCATTG-3') (*21*, *23*).

Mtb RNA extraction and quantification by Real-time PCR

After 4 weeks of maintaining *menA* CKD, 30 ml of *Mtb* cultures were pelleted at 9000 rpm for 20 min at 4 ℃ and washed with 10 ml cold ultra-pure water (UPW) once for RNA extraction. The pellets were suspended in 1 ml of triReagent by pipetting up and down. Samples were transferred to an O-ring screw cap containing 0.1 mm Zr/Si beads to approximately 0.25 mm Line of the screw cap. The cells were disrupted by bead beating for 30 seconds at a speed of 5000 rpm for 3 cycles, cooling on ice for 3-5 minutes between pulses followed by spin down steps at 14,000 rpm for 10 min at 4 ℃ twice.Then, the samples were harvested into a fresh RNase-free E-tube, one volume of ethanol (95-100%) was added directly to one volume sample (1:1) and mixed well by vortexing. The sample mixtures were then loaded into a Zymo-Spin Column in a collection tube, and centrifuge at 14,000rpm for 1 minute.

To clean up the RNA, the columns were washed with 400 µl RNA wash buffer by centrifuging for 30 seconds. To decontaminate DNA, DNase Ⅰ Reaction Mix was added directly to the column matrix and incubated the column at RT (20-30℃) for 15 minutes, followed by centrifugation for 30 seconds. Then, washed with 400 µl Direct-zol RNA PreWash twice by centrifuging for 1 minute. Again, washed by adding 700 µl RNA Wash Buffer to the column and centrifuging for 1 minute. Finally, 50 µl of Dnase/Rnase-Free Water was directly added to the column matrix and centrifuged for 1 minute. The eluted RNA can be used immediately or stored at -80℃.

To cheek the quality, RNA was measured with a spectrophotometer, and RNA samples having absorbance ratio of 1.8 or more at 260 nm to 280 nm (A260/280) were used for real-time PCR. The RNA integrity was also checked using Agilent 4200 TapeStation System. Further, DNA contamination was checked by running PCR amplification products on 1% agarose gel electrophoresis.

The Rever TraAce qPCR Master Mix (TOYOBO Co., LTD) kit was used to convert RNA to cDNA. First, RNA was incubated at 65 °C for 5 minutes and mixed with gDNA remover. The reaction mixture was mixed with 5x reverse transcriptase Master Mix II and no reverse transcriptase control mix as control. Then, the samples were incubated in the water bath for 15 minutes at 37 °C, 5 minutes at 50 °C and 5 minutes at 98 °C to anneal the primers, reverse transcribe RNA, and inactivate enzymes, respectively. Finally, the product was ready for real time PCR amplification or stocked at -30 °C.

Real-time PCR was performed by SYBR Green method using THUNDERBIRD SYBR qPCR Mix (TOYOBO). RNA polymerase sigma factor SigA gene (*sigA*) and 16S rRNA (16S) were used as an internal standard for quantification of gene expression.

Determination of MK level

The *Mtb menA* CKD strains and vector control cultures were grown in 7H9/ADC/Tw80 containing 25 μg/ml KM and 50 μg/ml HYG at a starting OD_{600} of 0.05 in 250 ml flasks. To induce and maintain the expression of dCas9 and sgRNA, aTc was added to a final concentration of 200 ng/ml every two days for 4 weeks while refreshing it to a starting OD_{600} of 0.05 every week. An equal amount of solvent used for dissolving aTc (DMSO) was added to VC.

After 4 weeks of CKD, 1 ml of bacterial suspension was centrifuged (12000 rpm, 4 ℃, 15 min). It was washed with UPW and, the collected bacteria stocked at -80℃. For MK extraction, 125 μl of extraction buffer (PBS containing 25 mM EDTA and 5 μM α-tocopherol [Merck]) was added to the bacterial pellet. Then 625 μl of hexane/ethanol (5:2, v/v) was added to the pellet and the bacterial cells were suspended by vortex mixing for 2 min. The bacterial suspension was centrifuged at 5000 *g* at RT for 5 min, and the upper layer (hexane layer) was transferred to a new tube. This extraction step was repeated one more time. The hexane fractions were combined and dried using vacuum centrifuge. After the dried fractions were dissolved in 100% EtOH, the obtained lipid components were analyzed using reverse phase high performance liquid

chromatography (HPLC) to determine the MK levels (*8*).

Determination of CKD strains susceptibility

Mtb CKD strains susceptibility to lysocin E and INH was also examined four weeks after *menA* CKD. The same bacteria used to extract RNA and MK, were refreshed to OD_{600} of 0.1 and incubated at 37 °C in the presence or absence of 50x MIC of lysocin E or 50x MIC of INH. After incubating the bacteria with the drugs for 24 hrs, bacterial CFU was determined in lysocin E-treated bacteria or INH-treated bacteria**.** The bacterial viability (%) was calculated in comparison of the CFU counts of treated samples with those of their corresponding nontreated samples (same recombinant bacteria) (*8*).

Effect of lysocin E on oxygen consumption and ATP synthesis

To observe the effect of drugs on oxygen consumption, methylene blue was added to mycobacterial cultures grown to log phase (OD_{600}, around 0.5) to be a final concentration of 0.001%. Then, the tubes were sealed tightly by molten parafilm, and the *Mtb* and *M. smegmatis* cultures were incubated for 24 and 2 hours, respectively, in the presence of 1/4-, 1/2 x MIC of lysocin E, 10 x MIC of INH or 10 x MIC of BDQ. Oxygen consumption of *Mtb* and *M. smegmatis* cultures was indicated by methylene blue decolorization while viability was determined by bacterial CFU count (*11*).

To measure the effect of the drugs on ATP synthesis, *Mtb* grown to OD_{600} of 0.5 was treated with different concentrations of lysocin E, INH or BDQ at 37°C for 24 hours. Intracellular ATP was quantified by the BacTiter Glo microbial cell viability assay kit (Promega, Madison, WI, USA) (*2*). The relative luminescence unit (RLU) measure for ATP was normalized by CFU count.

Effect of lysocin E on membrane permeability

Scanning electron microscopy (SEM) analysis of lysocin E effect on *Mtb*

Cultures of exponentially growing *Mtb* (OD_{600} of 0.5*)* were treated with 100x MIC of lysocin E. Samples were collected from both the control and treated cultures, after 3, 6, 12 and 24 hrs, and fixed with 1% glutaraldehyde in 50 mM cacodylate buffer pH 7.4 at 4°C overnight. Prefixed samples were postfixed with 1% osmium tetroxide in cacodylate buffer for 2 hours at 4 °C. The micrographs were viewed at a 35-kV accelerating voltage using SEM, and a secondary electron image of the cells for topography contrast was collected at several magnifications.

Effect of lysocin E on membrane permeability by Flow cytometry

To evaluate the effect of lysocin E on membrane permeability by Flow cytometry, BCG was grown to log phase and adjusted to OD_{600} of 0.05. Then, the cultures were treated with 1/4-, 1/2-, 1-, 10x MIC of lysocin E, 1/2-, 1, 10x MIC of RFP or 1/2-, 1-, 10x MIC of BDQ for 3, 6, 24 and 72 hours. After incubation, membrane permeability was determined by staining using the Live/DEAD *Bac*Light Bacterial Viability kit (Thermo Fisher Scientific, Waltham, MA). Bacteria heat killed at 95 ^{O}C and no drug live samples were used as control groups (*17*).

For staining, samples were pelleted by centrifugation at 10,000×g for 10 minutes. Then, the pellets were washed with 1 ml saline (0.85% NaCl) containing 0.05% Tween 80 twice by centrifugation at 10,000×g, RT, for 10 minutes. Finally, the OD_{600} of the resuspended pellet was adjusted to start OD_{600} (0.08). 100 µl aliquot of the bacteria was mixed with 1.5 µl of SYTO 9 (0.334 mM stock solution) and 1.5 µl of PI (2 mM stock solution) and incubated for 30 minutes at RT in the dark. Following incubation, 900 ul of 0.85% w/v saline containing 0.05% Tween 80 was added.

The bacterial cell samples were analyzed using ACEA NovoCyte flow cytometer. The fluorescence from SYTO 9 was measured through a 530-nm band-pass filter, and the red emission of PI was measured with a 615-nm long pass filter. A minimum of 10,000 bacterial events were collected and the multi-parametric data analyzed using the NoVo Express software *(17)*.

Cytotoxicity of lysocin E against THP 1 cells

To measure lysocin E cytotoxicity toward human THP 1 cell, cells were incubated with 10-, 50-, 100-, 200-, 300-, 400- 500- and 600x MIC of lysocin E for 48 hrs at 37℃. The percentage of cytotoxicity was evaluated using CytoScan™ WST-1 cell cytotoxicity assay in 96-well plates. Relative fluorescence unit was measured at OD_{450} using a plate reader. The cell viability percentage was calculated by dividing the test OD_{450} to control OD_{450} and multiplied by 100 *(2)*.

Intracellular activity of lysocin E against *Mtb*

To evaluate the efficacy of lysocin E against intracellular *Mtb*, THP 1 cells derived macrophages were infected with *Mtb* (multiplicity of infection [MOI] 1:1) for 4 hours. Subsequently, the cultures were washed twice with warm serum free DMEM. The infected macrophages were treated by adding 100 µl DMEM containing 10- or/and 50x MIC of lysocin E, STR, RFP and INH. The cells were incubated at 37 °C in a humidified 5% CO_2 incubator. At selected time intervals, 0.5% Triton X-100 was added and incubated at 37 °C for 5 minutes to disrupt macrophages. The resulting lysate and supernatant were spread onto 7H10 agar for CFU determination *(2, 20)*.

In Vivo activities of lysocin E

Infection of 42 female C57BL/6J mice (17 to 19 g), 6-7 weeks of age, was initiated by forced intratracheal instillation of *Mtb* H37Rv. For this purpose, *Mtb* was grown to an OD_{600} of 0.5. The inoculum (≈5.5 x10^5 CFU per mouse suspended in 50 µl of PBS) was delivered to the lung through forced inhalation with a syringe on Day 0 (*24*). To evaluate the bactericidal activities of the antibiotics, six infected mice were randomly assigned to one of the following treatment and control groups:

Group-1: No drug, early negative control

Group-2: No drug, late negative control

Group-3: No drug, final negative control treated with PBS

Group-4: Treated with lysocin E at 40 mg/kg/day

Group-5: Treated with INH at 25 mg/kg/day, a positive control

Group-6: Treated with STR at 100 mg/kg/day, a positive control

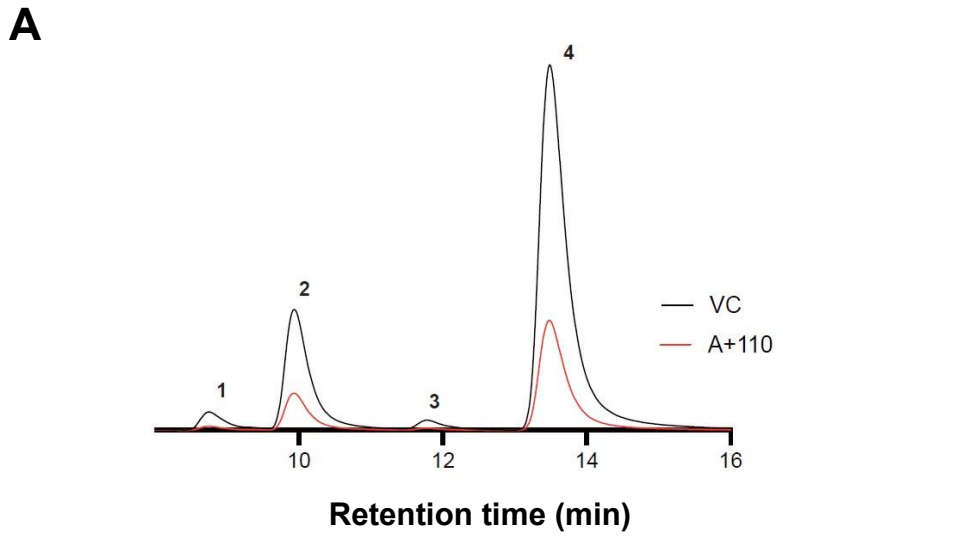
INH and STR doses represent the equipotent doses administered to humans.

On day 1, group-1 mice were sacrificed by cervical dislocation under anesthesia to determine the baseline bacterial load in lungs. On day 10, group-2 mice were sacrificed just before starting treatment to determine the pretreatment bacterial load. From day 10, both treatment and control groups were treated for two weeks (5 days of treatment per week) with the stated drugs or vehicle. INH was administered in a 200 µl volume by esophageal cannula (gavage), while lysocin E, STR and saline were injected subcutaneously in a 200 µl volume.

Twenty-four days post-infection (72 h after the last dose), all the remaining treatment and vehicle control groups were sacrificed following anesthesia. Their lungs, livers and spleens were homogenized in 4.5 ml of sterilized water. The homogenates were serially diluted, and 100 µl of the diluted suspensions were plated on Middlebrook 7H10 agar in duplicate. The plates were incubated at 37 °C in a 5% CO_2–95% air atmosphere for 3-4 weeks, after which the number of *Mtb* colonies was enumerated and CFU per organ calculated (*20*, *24*).

In parallel, a mouse from each group was sacrificed for histopathological study at day 24. Lungs, spleen, and liver were fixed with 4% formal aldehyde for 24 h before processing. Then, thin sections were stained with Haemotoxylin and Eosin (H&E) stain. The lung histopathological examination was done using florescence microscope at 4x and 40x magnification. Lung samples were also counted for mononucleoid cells under bright field florescence microscopy manually at 40x magnification. The mononucleoid cells were counted in $1x10^{-2}mm^2$ after randomly selecting about 30 areas on the lung.

II. Supplementary Figures



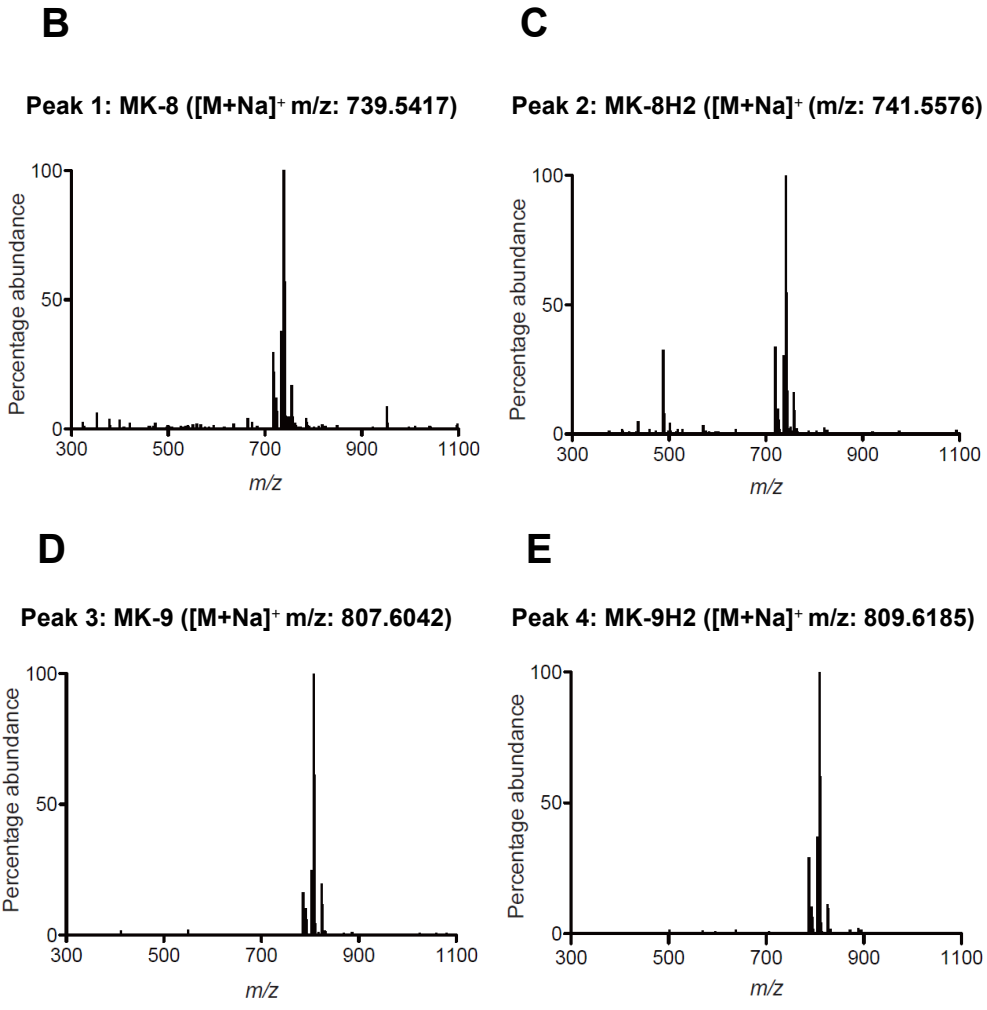
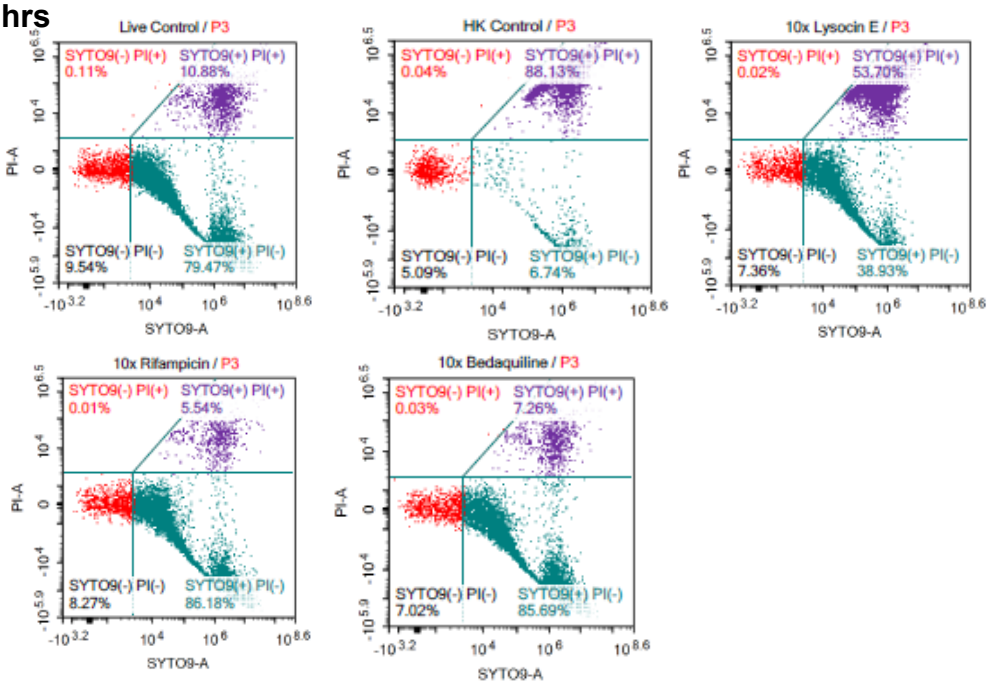
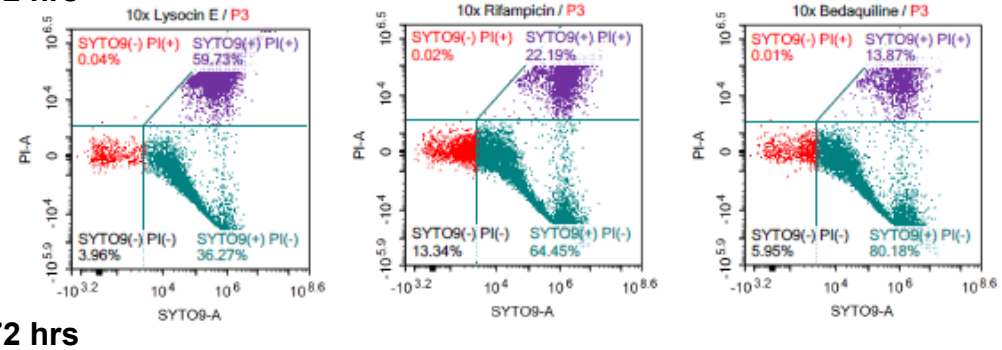


Fig. S1. MK analysis. In parallel to the quantification of gene expression, lipid fraction containing MK was extracted from 1 ml aliquot of each culture, as described in Materials and Methods, and analyzed by HPLC after the dried fractions were dissolved in 100% EtOH. (**A)** Representative HPLC chromatogram of MK peaks with their retention time (minutes). (**B**) MS/MS spectra of MK-8 (peak 1, ($[M+Na]^+$ m/z 739.5417), (**C**) MK-8H_2 (peak 2, ($[M+Na]^+$ m/z 741.5576), (**D**) MK-9 (peak 3, ($[M+Na]^+$ m/z: 807.6042), and (**E**) MK-9H_2 (peak 4, ($[M+Na]^+$ m/z 809.6185), respectively. The retention time and selected picks are representative of two reproducible experiments.

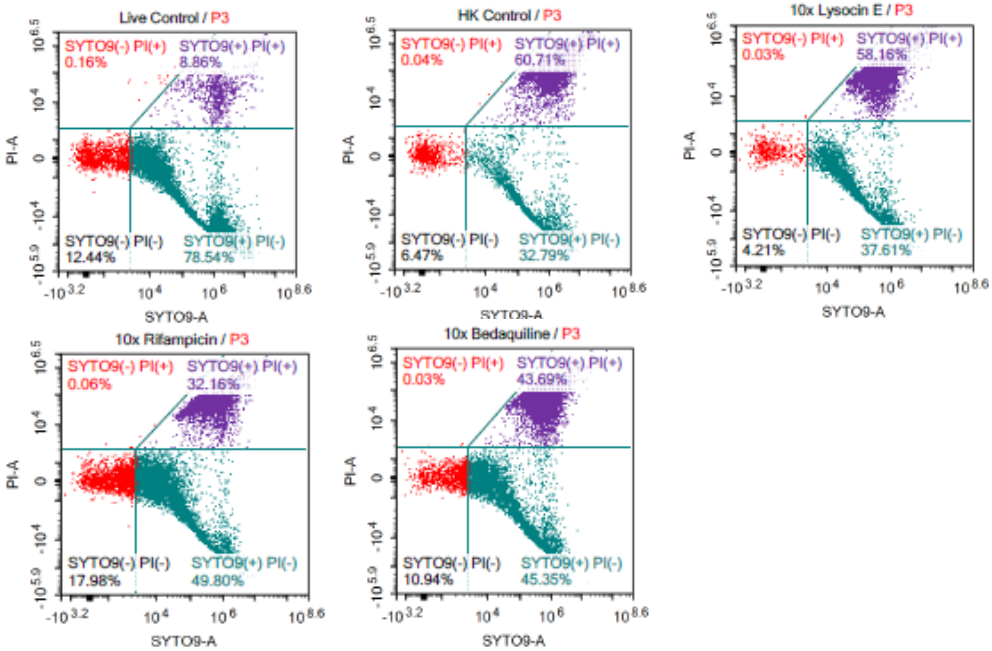
A. 6 hrs



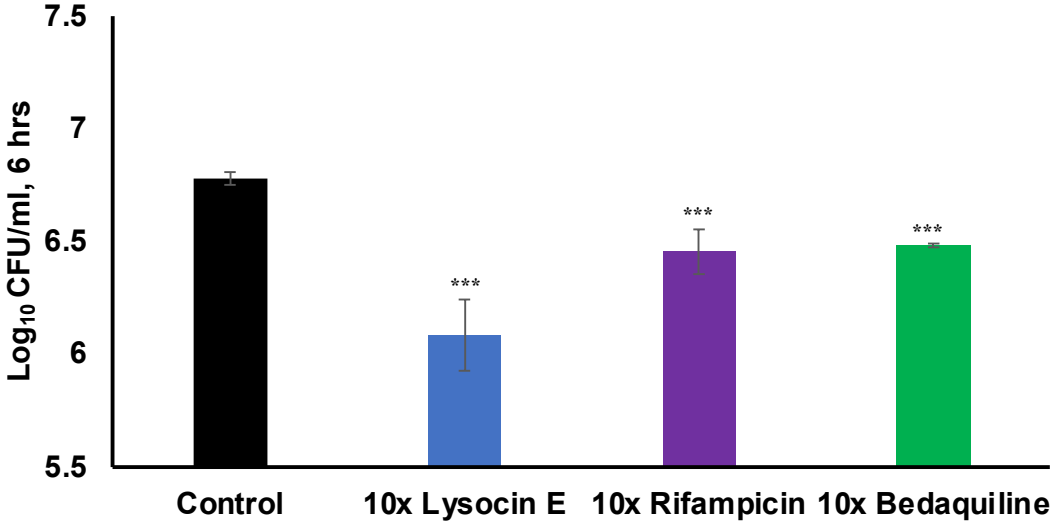
B. 12 hrs



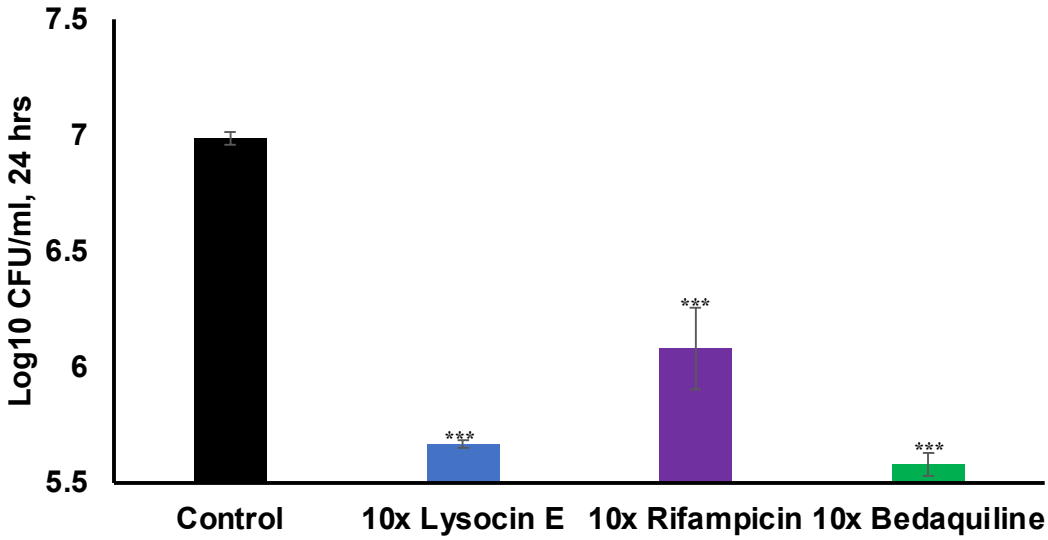
C. 72 hrs



D


7.5
7
6.5
6
5.5
Log10 CFU/ml, 6 hrs
***
***
***
Control
10x Lysocin E
10x Rifampicin
10x Bedaquiline

E


7.5
7
6.5
6
5.5
Log10 CFU/ml, 24 hrs
***
***
***
Control
10x Lysocin E
10x Rifampicin
10x Bedaquiline

F

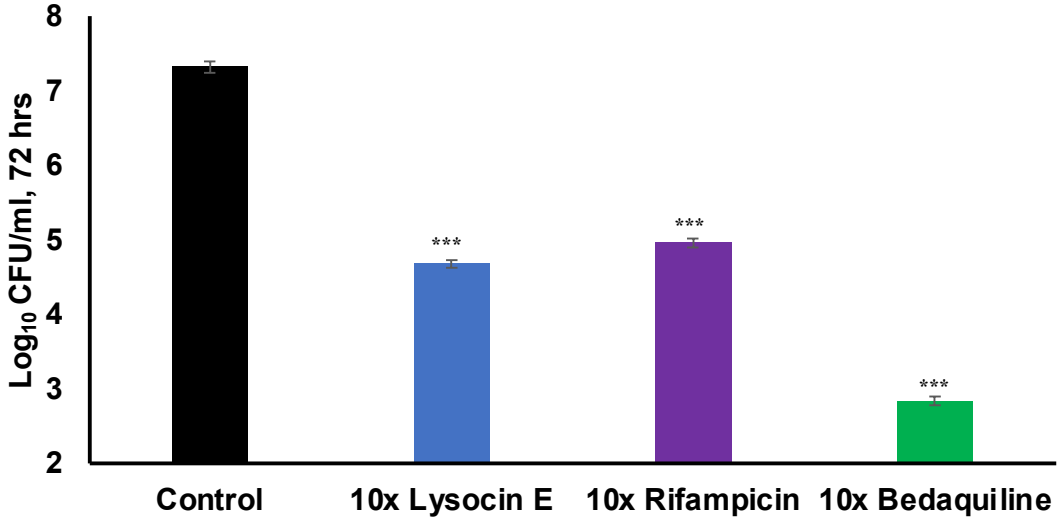
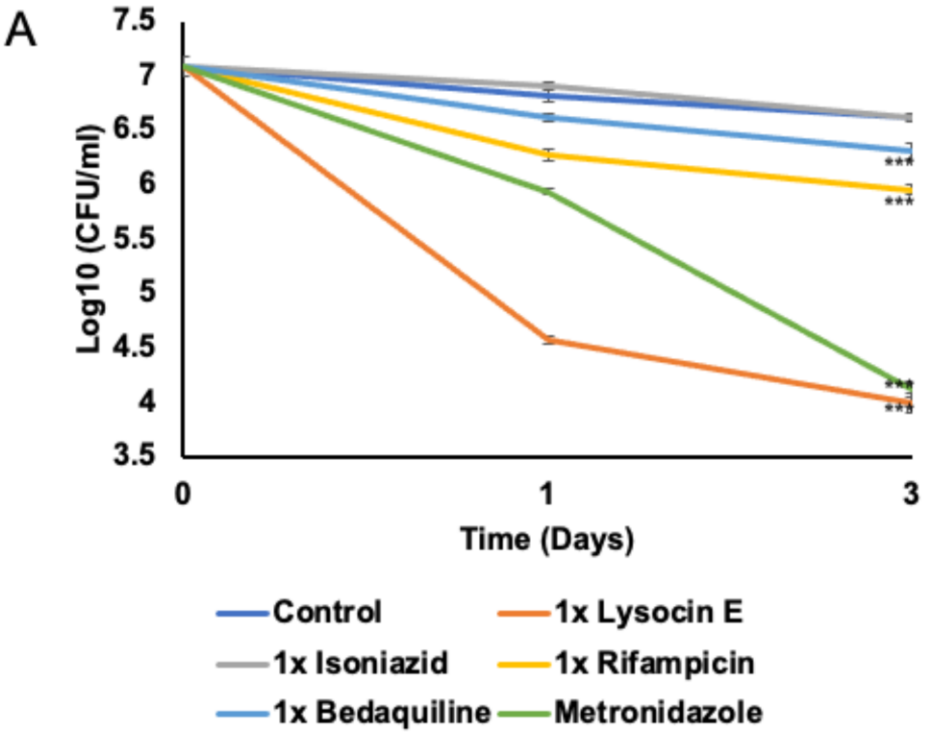

8
7
6
5
4
3
2
Log10 CFU/ml, 72 hrs
***
***
***
Control
10x Lysocin E
10x Rifampicin
10x Bedaquiline

Fig. S2. Lysocin E causes membrane disruption of mycobacteria

After treating BCG (OD_{600} of 0.05) with 1/4-, 1/2-, 1- and 10x MIC of lysocin E, 1/2-, 1- and 10x MIC of rifampicin or bedaquiline, the drug effect on membrane permeability was evaluated by FACS analysis using Live/Dead (Syto9/PI) staining at 6 (**A**), 24 (**B**), and 72 (**C**) hours. In parallel, the activity of drugs evaluated by CFU count after treating BCG with 10x MIC of lysocin E, rifampicin and bedaquiline for 6 (**D**), 24 (**E**), and 72 (**F**) hours. Statistical differences were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test (***, $P<0.001$). Data represent the mean ±SD of triplicates. This experiment was repeated two times.


A
7.5
7
6.5
6
5.5
5
4.5
4
3.5
Log10 (CFU/ml)
0
1
3
Time (Days)
***
Control
1x Lysocin E
1x Isoniazid
1x Rifampicin
1x Bedaquiline
Metronidazole

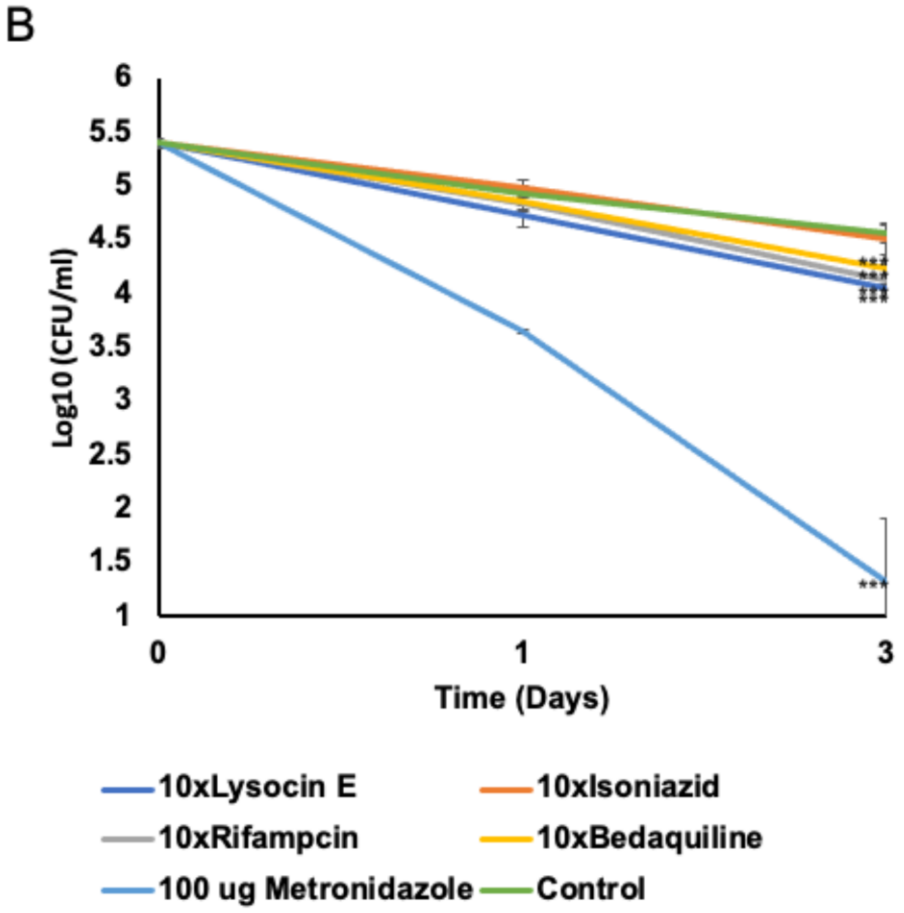
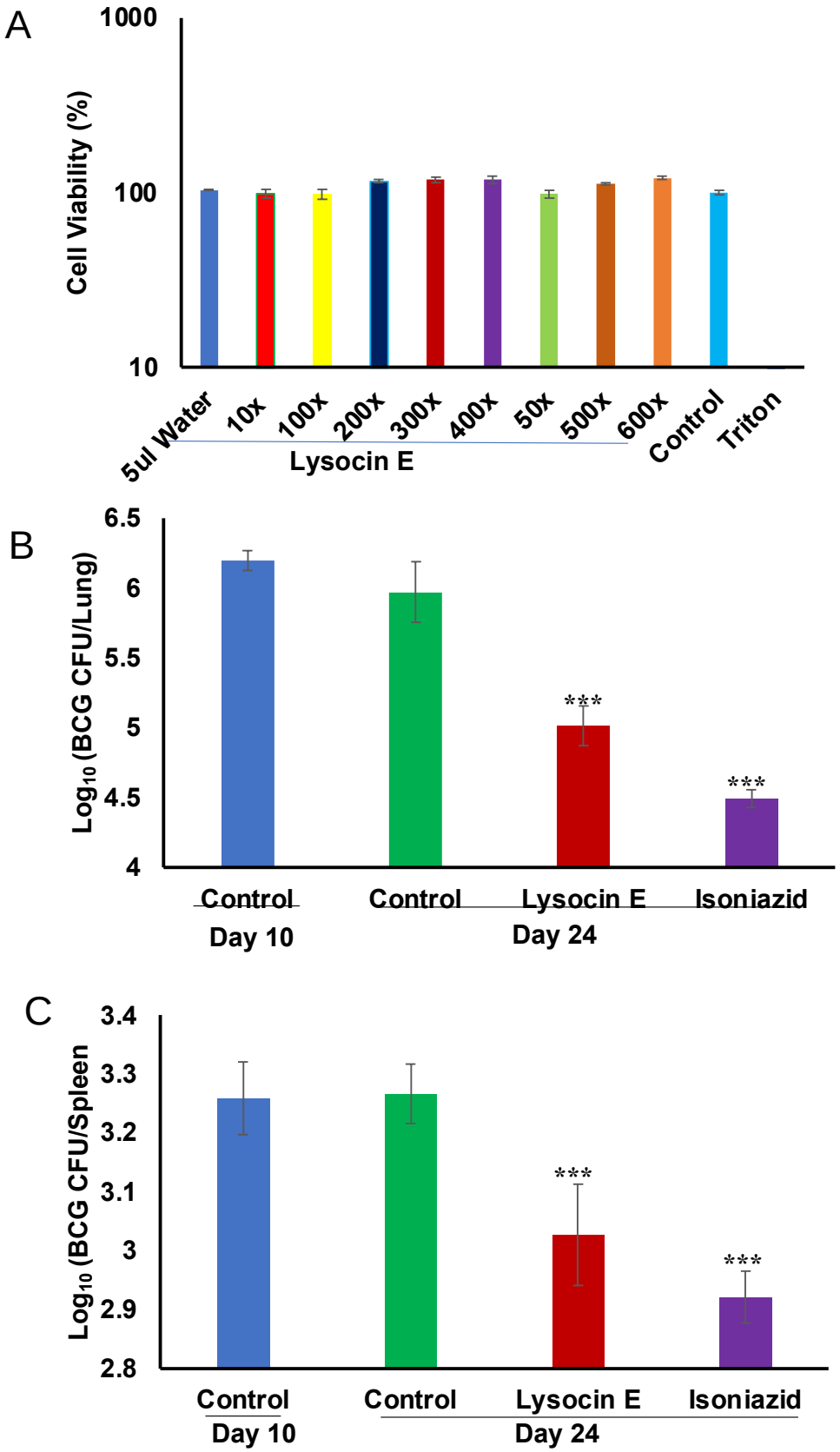
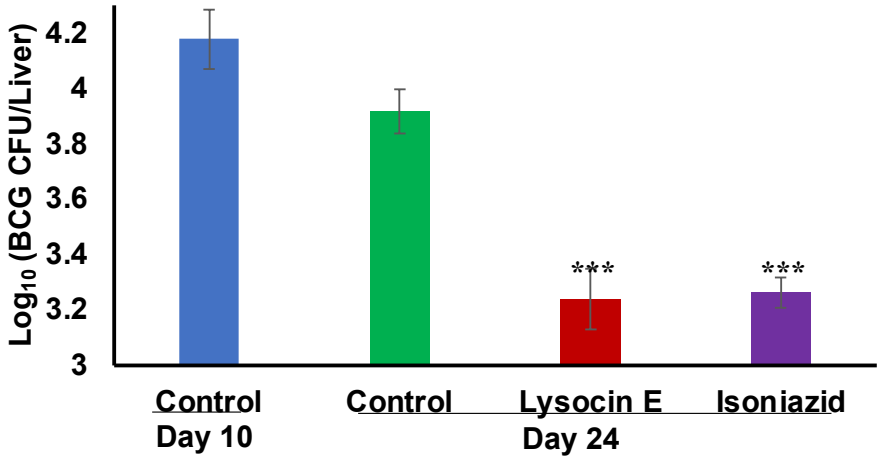

B
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
Log10 (CFU/ml)
0
1
3
Time (Days)
***
10xLysocin E
10xIsoniazid
10xRifampcin
10xBedaquiline
100 ug Metronidazole
Control

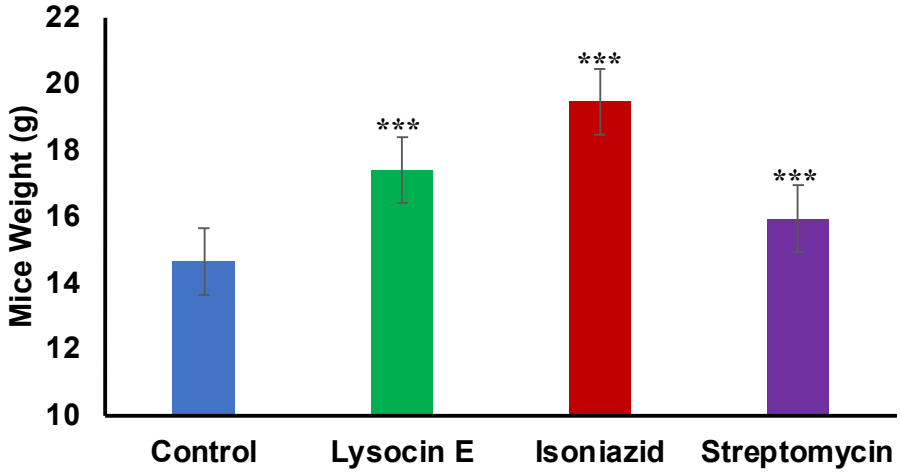
Fig. S3. Bactericidal activity of antibiotics against nonreplicating mycobacteria. The bactericidal activity against NRP bacteria was determined by exposing: **(A)** *M. smegmatis* and **(B)** BCG subjected to growth in the Wayne hypoxia model to different antibiotics. The detection limit was 2 $\log_{10}$ CFU. Statistical difference between control and each treatment was analyzed by one-way ANOVA with Dunnett's multiple comparison test (*, $P < 0.01$; ***, $P < 0.0001$). Data represent the mean ±SD of triplicates.


A
1000
100
10
Cell Viability (%)
5ul Water
10x
100x
200x
300x
400x
50x
500x
600x
Control
Triton
Lysocin E
B
6.5
6
5.5
5
4.5
4
Log10 (BCG CFU/Lung)
***
***
Control
Control
Lysocin E
Isoniazid
Day 10
Day 24
C
3.4
3.3
3.2
3.1
3
2.9
2.8
Log10 (BCG CFU/Spleen)
***
***
Control
Control
Lysocin E
Isoniazid
Day 10
Day 24

D


Log10 (BCG CFU/Liver)
4.2
4
3.8
3.6
3.4
3.2
3
***
***
Control
Day 10
Control
Lysocin E
Isoniazid
Day 24

E


Mice Weight (g)
22
20
18
16
14
12
10
***
***
***
Control
Lysocin E
Isoniazid
Streptomycin

F

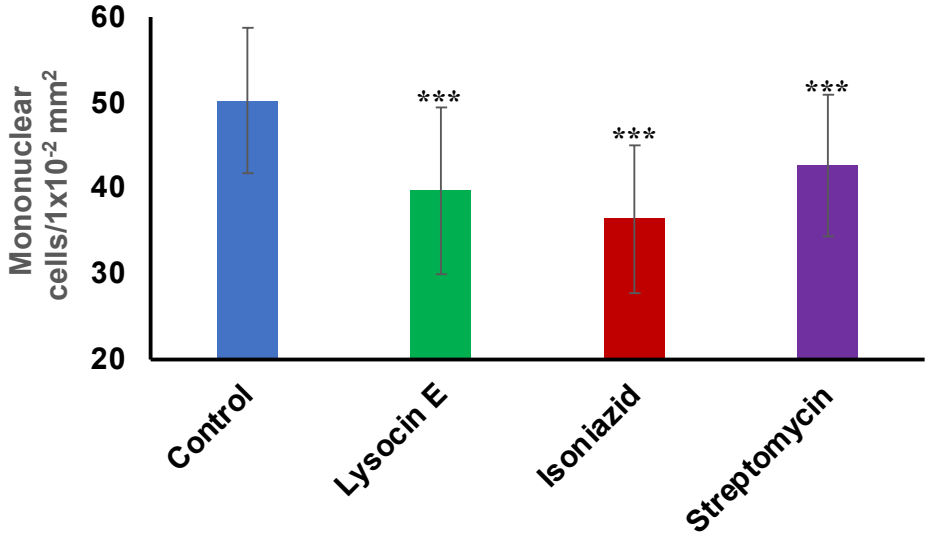

Mononuclear cells/1x10-2 mm2
60
50
40
30
20
***
***
***
Control
Lysocin E
Isoniazid
Streptomycin

Fig. S4. Cytotoxicity and efficacy of lysocin E against BCG and *Mtb* in mice model.

(**A**) Cytotoxicity of lysocin E against THP 1 cells. THP 1 cells were incubated with 10-, 50-, 100-, 200-, 300-, 400- 500- and 600x MIC of lysocin E for 48 hrs at 37 ℃. The percentage of cytotoxicity was evaluated using CytoScan™ WST-1 cell cytotoxicity assay in 96-well plates. Relative fluorescence units were measured at OD_{450} using a plate reader. The cell viability percentage was calculated by dividing the test OD_{450} to control OD_{450} multiplied by 100. Female C57BL/6J mice (17 to 19 g) infection was initiated by intratracheal instillation of *M. tuberculosis* H37Rv or subspecies BCG. The inoculum (10^6 CFU for BCG or 5.5 x10^5 *Mtb* per mouse suspended in 50 ul of PBS) was delivered to the lung through forced instillation with a syringe on Day 0. Five mice from each group were sacrificed on the day after infection, on the day of treatment initiation, and on day 14 after treatment to determine the numbers of CFU implanted in the lungs, pretreatment baseline CFU counts and the CFU counts after 14 days of treatment, respectively. A mouse was also sacrificed from each group for histopathological study. BCG CFU in the lung (**B**), BCG CFU in the spleen (**C**), BCG CFU the in liver (**D**), weight of the *Mtb* infected mice after treatment (**E**), and mononuclear cell count in the lung of *Mtb* infected mice after treatment (**F**) are shown. Statistical differences were analyzed by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test (**, $P<0.005$; ***, $P<0.001$). Data represent the mean ±SD of triplicates.

REFERENCES

1. World Health Organization, Factsheet global tuberculosis report 2021.

2. P. Sukheja, P. Kumar, N. Mittal, S. Li, E. Singleton, R. Russo, A.L. Perryman, R. Shrestha, D. Awasthi, S. Husain, P. Soteropoulos, R. Brukh, N. Connell, J.S. Freundlich, D. A. Alland. A novel small-molecule inhibitor of the Mycobacterium tuberculosis demethylmenaquinone methyltransferase MenG is bactericidal to both growing and nutritionally deprived persister cells. 2017. mBio 8, 1–15 (2017).

3. D. Machado, M. Girardini, M. Viveiros, M. Pieroni, Challenging the drug-likeness dogma for new drug discovery in Tuberculosis. Front. Microbiol. 9, 1367 (2018), doi:10.3389/fmicb.2018.01367.

4. World Health Organization, Factsheet global tuberculosis report 2020, 103 (2020).

5. B. J. Berube, D. Russell, L. Castro, S. Choi, P. Narayanasamy, T. Parish, Novel MenA Inhibitors Are Bactericidal against Mycobacterium tuberculosis and Synergize with Electron Transport Chain Inhibitors. Antimicrob. Agents Chemother. 63, 1–7 (2019).

6. J. Debnath, S. Siricilla, B. Wan, D. C. Crick, A. J. Lenaerts, S. G. Franzblau, M. Kurosu, Discovery of selective menaquinone biosynthesis inhibitors against Mycobacterium tuberculosis. J. Med. Chem. 55, 3739–3755 (2012).

7. L. Z. Montelongo-Peralta, A. León-Buitimea, J. P. Palma-Nicolás, J. Gonzalez-Christen, J. R. Morones-Ramírez, Antibacterial Activity of combinatorial treatments composed of transition-metal/antibiotics against Mycobacterium tuberculosis. Sci. Rep. 9, 5–10 (2019).

8. H. Hamamoto, M. Urai, K. Ishii, J. Yasukawa, A. Paudel, M. Murai, T. Kaji, T. Kuranaga, K. Hamase, T. Katsu, J. Su, T. Adachi, R. Uchida, H. Tomoda, M. Yamada, M. Souma, H. Kurihara, M. Inoue, K. Sekimizu, Lysocin E is a new antibiotic that targets menaquinone in the bacterial membrane. Nat. Chem. Biol. 11, 127–133 (2015).

9. H. Hamamoto, S. Panthee, A. Paudel, K. Ishii, J. Yasukawa, J. Su, A. Miyashita, H. Itoh, K. Tokumoto, M. Inoue, K. Sekimizu, Serum apolipoprotein A-I potentiates the therapeutic efficacy of lysocin E against Staphylococcus aureus. Nat. Commun. 12, 6364 (2021), doi:10.1038/s41467-021-26702-0.

10. H. Hamamoto, K. Sekimizu, Identification of lysocin E using a silkworm model of bacterial infection. Drug Discov. Ther. 10, 24–29 (2016).

11. J. Puffal, J. A. Mayfield, Demethylmenaquinone Methyl Transferase Is a Membrane Domain-Associated Protein Essential for Menaquinone Homeostasis in M. smegmatis (2018), Front. Microbiol., 9, 1-12, (2018), doi:10.3389/ fmicb. 2018.03145.

12. A. Paudel, H. Hamamoto, S. Panthee, K. Sekimizu, Menaquinone as a potential target of antibacterial agents. Drug Discov. Ther. 10, 123–128 (2016).

13. R. S. Gupta, B. Lo, J. Son, Phylogenomics and comparative genomic studies robustly support division of the genus Mycobacterium into an emended genus Mycobacterium and four novel genera. Front. Microbiol. 9, 1–41 (2018).

14. M. A. Dejesus, E. R. Gerrick, W. Xu, S. W. Park, J. E. Long, C. C. Boutte, E. J. Rubin, D. Schnappinger, S. Ehrt, S. M. Fortune, C. M. Sassetti, T. R. Ioerger, Comprehensive essentiality analysis of the Mycobacterium tuberculosis genome via saturating transposon mutagenesis. MBio. 8, 1–17 (2017).

15. L. G. Wayne, L. G. Hayes, An In Vitro Model for Sequential Study of Shiftdown of Mycobacterium tuberculosis through Two Stages of Nonreplicating Persistence. Infect Immun, 64, 2062–2069 (1996).

16. W. Kim, G. Zou, T. P. A. Hari, I. K. Wilt, W. Zhu, N. Galle, H. A. Faizi, G. L. Hendricks, K. Tori, W. Pan, X. Huang, A. D. Steele, E. E. Csatary, M. M. Dekarske, J. L. Rosen, N. De Queiroz Ribeiro, K. Lee, J. Port, B. B. Fuchs, P. M. Vlahovska, W. M. Wuest, H. Gao, F. M. Ausubel, E. Mylonakis, A selective membrane-targeting repurposed antibiotic with activity against persistent methicillin-resistant Staphylococcus aureus. Proc. Natl. Acad. Sci. 116, 16529–16534 (2019).

17. N. M. O'Brien-Simpson, N. Pantarat, T. J. Attard, K. A. Walsh, E. C. Reynolds, A rapid and quantitative flow cytometry method for the analysis of membrane disruptive antimicrobial activity. PLoS One. 11, 1–15 (2016).

18. H. Itoh, K. Tokumoto, T. Kaji, A. Paudel, S. Panthee, H. Hamamoto, K. Sekimizu, M. Inoue, Development of a high-throughput strategy for discovery of potent analogues of antibiotic lysocin E. Nat. Commun. 10, 1-11 (2019).

19. C. Temesszentandrási-Ambrus, S. Tóth, R. Verma, P. Bánhegyi, I. Szabadkai, F. Baska, C. Szántai-Kis, R. C. Hartkoorn, M. A. Lingerfelt, B. Sarkadi, G. Szakács, L. Orfi, V. Nagaraja, S. Ekins, Á. Telbisz, Characterization of new, efficient Mycobacterium tuberculosis topoisomerase-I inhibitors and their interaction with human ABC multidrug transporters. PLoS One. 13, 1–15 (2018).

20. W. Gao, J. Kim, J. R. Anderson, T. Akopian, S. Hong, Y. Jin, O. Kandror, J. Kim, I. Lee, S. Lee, J. B. Mcalpine, S. Mulugeta, S. Sunoqrot, Y. Wang, S. Yang, T. Yoon, A. L. Goldberg, G. F. Pauli, J. Suh, S. G. Franzblau, The Cyclic Peptide Ecumicin Targeting ClpC1 Is Active against Mycobacterium tuberculosis In Vivo. Antimicrob. Agents Chemother. 59, 880–889 (2015).

21. A. K. Singh, X. Carette, L. P. Potluri, J. D. Sharp, R. Xu, S. Prisic, R. N. Husson, Investigating essential gene function in Mycobacterium tuberculosis using an efficient CRISPR interference system. Nucleic Acids Res. 44, 1-13 (2016), doi:10.1093/nar/gkw625.

22. A. Savitskaya, A. Nishiyama, T. Yamaguchi, Y. Tateishi, Y. Ozeki, M. Nameta, T. Kon, S. A. Kaboso, N. Ohara, O. V. Peryanova, S. Matsumoto, C-terminal intrinsically disordered region-dependent organization of the mycobacterial genome by a histone-like protein. Sci. Rep. 8, 1–15 (2018).

23. E. Choudhary, P. Thakur, M. Pareek, N. Agarwal, Gene silencing by CRISPR interference in mycobacteria. Nat. Commun. 6, 1–11 (2015).

24. C. Chen, F. Ortega, J. Rullas, L. Alameda, I. Angulo-Barturen, S. Ferrer, U. S. Simonsson, The multistate tuberculosis pharmacometric model: a semi-mechanistic

pharmacokinetic-pharmacodynamic model for studying drug effects in an acute tuberculosis mouse model. J. Pharmacokinet. Pharmacodyn. 44, 133–141 (2017).