Discovery of a capuramycin analog that kills nonreplicating *Mycobacterium tuberculosis* and its synergistic effects with translocase I inhibitors

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Capuramycin (1) and its analogs are strong translocase I (MurX/MraY) inhibitors. In our structure–activity relationship studies of capuramycin analogs against *Mycobacterium tuberculosis* (Mtb), we observed for the first time that a capuramycin analog, UT-01320 (3) killed nonreplicating (dormant) Mtb at low concentrations under low oxygen conditions, whereas selective MurX inhibitors killed only replicating Mtb under aerobic conditions. Interestingly, 3 did not exhibit MurX enzyme inhibitory activity even at high concentrations, however, 3 inhibited bacterial RNA polymerases with the IC50 values of 100–150 nM range. A new RNA polymerase inhibitor 3 displayed strong synergistic effects with a MurX inhibitor SQ 641 (2), a promising preclinical tuberculosis drug.

*The Journal of Antibiotics* (2015) 68, 271–278; doi:10.1038/ja.2014.133; published online 1 October 2014

INTRODUCTION

One-third of the 42 million people living with HIV/AIDS worldwide are coinfected with *Mycobacterium tuberculosis* (Mtb).1,2 The WHO (World Health Organization) estimated that 650 000 new cases of multidrug-resistant-Mtb emerge each year.3 An outbreak of extensively drug resistant-Mtb threatens tuberculosis (TB) control and prevention efforts.4 Treatment duration for multidrug-resistant-Mtb strains is at least 20–28 months. TB chemotherapy for extensively drug resistant-TB takes substantially longer than multidrug-resistant-TB, and extensively drug resistant-Mtb strains are responsible for very high mortality rate.5 Therefore, it is very important to discover new drugs that can shorten current TB drug regimens. Mechanisms that enter nonreplicating (or dormant) state of Mtb are accounted for a significant factor that requires long-term chemotherapy.6 Wayne et al.7 reported that oxygen starvation is linked to TB drug resistance; upon depletion of oxygen in culture, Mtb terminates growth and develops into a characteristic dormant form.8 Significantly, the dormant form of Mtb was found to be resistant to most of the clinically utilized antimycobacterial agents.8 Thus, new drugs targeting nonreplicating Mtb are likely to revolutionize TB chemotherapy.

The cell wall of Mtb offers many unique targets for drug development.9 However, most of the drugs associated with cell wall biosynthesis have proven difficult to reduce treatment time of TB drug regimens due to the facts that the dormant bacteria are not actively synthesizing cell walls.10 On the contrary, it was recently reported that a peptidoglycan biosynthesis inhibitor, meropenem (a carbapenem) was effective in killing nonreplicating Mtb in combination with clavulane (a β-lactamase inhibitor).11 Although a mechanism of action of their bactericidal effect against dormant Mtb cells is not known, it is one of the few examples that peptidoglycan biosynthesis inhibitors kill dormant form of Mtb. Because several translocase I (MurX/MraY, hereafter referred to as MurX for *Mtb* translocase I) inhibitors kill Mtb much faster than other TB drugs under aerobic conditions (Figure 1),12 we commenced structure–activity relationship studies of capuramycin (1), a known MurX inhibitor antibiotic, to improve efficacy of its antimycobacterial activity in *vitro* and *in vivo* (Figure 2).13–15 Daiichi-Sankyo and Sequella reported several capuramycin analogs in which *in vivo* MraY enzyme and antimycobacterial activity could be improved via the modification of the carboxylic group of the capuramycin biosynthetic intermediate, A-500359.16–19 We have synthesized new capuramycin analogs via our total synthetic scheme,15 in which all analogs are structurally different from the reported molecules and they are difficult to access from A-500359. In screening of new capuramycin analogs against replicating and nonreplicating (dormant) Mtb, it was found that a 2'-methylated capuramycin analog, UT-01320 (3) killed both replicating and nonreplicating Mtb in microplate alamar blue assay (MABA) and low oxygen recovery assay (LORA), respectively.20 To the best of our knowledge, it is the first observation that a capuramycin analog exhibited bactericidal activity against nonreplicating Mtb at low concentrations. Herein, we report *in vitro* and *in vivo* biological evaluations of 3, synergistic effect with known MurX inhibitors 1 or 2, and insights into a molecular target of 3 (Figure 2).
RESULTS

Effectiveness of UT-01320 against nonreplicating M. tuberculosis

To date, we have synthesized capuramycin (1), SQ 641 (2), and over 100 new capuramycin analogs in which their structures were diversified with optically active amines (R₃) and the four uridyl templates, 3’O-methyl (natural form), 2’O-methyl, 2’3’O-dimethyl, and 3’O-demethylated (unnatural forms) uridine-mannopyranuronate derivatives (Figure 2). The syntheses of capuramycin analogs with these templates were accomplished via the synthetic scheme reported previously.¹⁴,¹⁵ As the results of in vitro evaluation of these molecules in MurX enzyme inhibition²¹ and Mtb (H₃₇Rv) growth inhibitory activities against replicating and nonreplicating (dormant) Mtb via the MABA and LORA assays,²⁰ it was identified that UT-01309 (4) possessing 3-amino-1,4-benzodiazepine-2-one exhibited increased...
MurX enzyme and Mtb growth inhibitory activity (MIC: 2.5 μg ml⁻¹ vs 12.0 μg ml⁻¹ for 1; IC₅₀ 0.095 μM vs 0.127 μM for 1). The analogs synthesized from the 3’O-methyluridyline-mannopyranuronate (natural form) did not exhibit bactericidal activity against nonreplicating Mtb even at 400 μg ml⁻¹ concentrations. The isocapuramycin (2’O-methyl) analogs (R₁=Me and R₂=H in Figure 2) showed equal MurX inhibitory and antimycobacterial activities to the corresponding analogs derived from the natural form (R₁=H and R₂=Me in Figure 2). The antimycobacterial MurX inhibitors, isocapuramycin analogs (2’O-methyluridyline-mannopyranuronate derivatives) were not capable of killing nonreplicating Mtb in the LORA assay. The 3’O-demethyl capuramycin analogs (R₁, R₂=H in Figure 2) did not exhibit activities in MurX enzyme inhibitory and antimycobacterial assays. Remarkably, 2’O-methylated capuramycin, UT-01320 (3) killed both replicating and nonreplicating Mtb at low concentrations with the MIC₁₅⁰₉₋₉₅₀₉ value of 1.72, whereas the MIC₉₀₉₋₉₅₀₉ value for rifampicin was 7.35 (Table 1). The MIC₉₀₉₋₉₅₀₉ value close to 1 or below 1 is considered to be ideal TB drug lead molecules in our programs.

Table 1 MICs (Mtb) and IC₅₀ₐₐₐ (MurX enzyme and mammalian cells) for capuramycin (1) and its analogs, 2–4

| Molecule             | IC₅₀ₐₐₐ | MIC₉₀₉₋₉₅₀₉ | MIC₉₀₉₋₉₅₀₉ | MIC₉₀₉₋₉₅₀₉ |
|----------------------|--------|-------------|-------------|-------------|
| Capuramycin (1)      | 0.127  | 12.5        | >100        | >400        |
| SQ 641 (2)           | 0.098  | 1.56        | >100        | >400        |
| UT-01320 (3)         | >100   | 1.50        | 2.58        | >400        |
| UT-01309 (4)         | 0.095  | 2.50        | >100        | >400        |
| RFP                  | —      | 0.20        | 1.47        | >400        |
| INH                  | —      | 0.04-0.10   | >100        | >400        |
| EMB                  | —      | 0.78        | >100        | >400        |

Abbreviations: EMB, ethambutol; INH, isoniazid; MABA, microplate alamar blue assay; Mtb, Mycobacterium tuberculosis; LORA, low oxygen recovery assay; RFP, rifampicin.
*Cytotoxicity against Vero monkey kidney cells was >400 μg ml⁻¹.

Bactericidal activity of 3 against dormant Mtb was confirmed via the assays using a modified Wayne model under low oxygen conditions and a nutrient-deficient Mtb cell model; in both assays, no CFU was observed after 15 days’ incubation of the Mtb cultures treated with 3 at 100 μg ml⁻¹, 10 μg ml⁻¹ and 2.58 μg ml⁻¹ (MIC₁₅⁰₉), respectively (Figure 3).

Rapid antimycobacterial activity of UT-01320

Because of alteration of molecular target, we were interested in knowing whether a nonMurX inhibitor 3 remains rapidly bactericidal against replicating Mtb under aerobic conditions. The time-kill experiments were performed at two and fourfold the MIC of 1, 3 and a few first-line TB drugs (rifampicin (RFP) and isoniazid (INH)). Viable cell counting was performed at every 24 h for 14 days. CFUs were counted after 15 days of incubation at 37 °C. The rate of killing of 3 against Mtb was compared directly with the reference molecules and the time-kill assessments at 2x and 4x MIC concentrations are shown in Figure 4. The MurX inhibitor, capuramycin (1) yielded log₁₀ CFU ml⁻¹ reduction of 3.0 or greater for Mtb in 1 day and 1 killed Mtb completely in 2 days at 24.0 μg ml⁻¹, albeit, RFP and INH required over 7 days to kill 50% of Mtb at 0.4 μg ml⁻¹ and 1.0 μg ml⁻¹, respectively (Figure 4a). UT-01320 (3) killed >50% of Mtb within 3 days and 100% within 5 days at 3.0 μg ml⁻¹ (2x MIC), and killed 100% of Mtb within 3 days at 6.0 μg ml⁻¹ (4x MIC) (Figure 4b). Thus, the fast killing profile of 3 was similar to that of capuramycin; 3 killed Mtb faster than RFP and INH, but was less effective than capuramycin.

Synergistic effect of UT-01320 with MurX inhibitors

The synergistic or antagonistic activities of MurX inhibitors (capuramycin (1) or SQ 641 (2)) and UT-01320 (3) were assessed in vitro via micro dilution broth checkerboard technique. In the checkerboard analyses of a combination of SQ 641 and UT-01320, the FIC index range of 0.06 to 4 was observed for 47 combinations of two molecules out of 64 different concentrations. Table 2 summarizes the results of

![Wayne Model](image)

Figure 3 Bactericidal activity of UT-01320 against nonreplicating Mtb evaluated in Wayne and nutrient-deficient models. M. tuberculosis that entered dormant states were cultured with the inhibitors. After 96h under anaerobic (Wayne model) or nutrient-deficient conditions, 10-, 100-, 1000- and 10 000-fold serial dilutions from each culture tube was sampled, and 20 μl from each dilution was plated on 7H10 agar plates supplemented with OADC enrichment. Plates were incubated under aerobic conditions for 15 days in a static incubator at 37 °C and colonies were counted. CFU counted for each plate is shown relative to control (100). UT-01320 (3) killed M. tuberculosis at the MIC₁₅⁰₉ concentrations in both assays. (a) Wayne model; (b) a nutrient-deficient model. A full color version of this figure is available at The Journal of Antibiotics journal online.
that killed Mtb at much lower concentrations than the MIC A and MICB.

3 A: capuramycin (1) exhibited synergistic combination (ΣFIC < 1). It was demonstrated that UT-01320 (3) had a very narrow spectrum of activity against Gram-positive and negative bacteria including Mycobacterium spp., killed Mtb strains selectively. It is interesting to note that unlike other capuramycin analogs, UT-01320 (3) did not kill a fast-growing M. smegmatis at >200 μg ml⁻¹ concentrations (for example, MICs 6.5 μg ml⁻¹ and 25 μg ml⁻¹ for 1 and 4, respectively).

Effectiveness of the collected molecules against nonreplicating Mtb has been evaluated in the Wayne model as well as the low oxygen recovery assay. Several electron transport system inhibitors, menaquinone biosynthesis inhibitors, a F1F0-ATPase inhibitor, nitroimidazoles, a RNA polymerase inhibitor (for example, RFP), protein biosynthesis inhibitors (for example, amikacin, capreomycin, etc.), and bacterial DNA gyrase inhibitors were effective in killing nonreplicating Mtb, however, in most cases their MIC values against nonreplicating Mtb cells are significantly higher than those against aerobically growing Mtb cells. In preliminary assay screening of UT-01320 (3) against the potential drug targets for nonreplicating Mtb, it was observed that 3 inhibited Escherichia coli, Staphylococcus aureus and M. smegmatis RNA polymerases with the IC50 values of the 100–150 nM range (vs IC50 120 nM for RFP against E. coli RNA polymerase) (Figure 3b), whereas, 3 did not inhibit electron transport systems, menaquinone biosynthesis, Mtb serine/threonine kinases.

### Spectrum of activity of UT-01320

As summarized in Table 3, UT-01320 (3) has a very narrow spectrum of activity; in growth inhibitory assays against Gram-positive and

| Combination of A and B | MIC A (μg ml⁻¹) | MIC B (μg ml⁻¹) | ΣFIC |
|------------------------|---------------|----------------|------|
| A: SQ 641 (2)          | 1.56          | 0.10           | 0.12 |
| B: UT-01320 (3)        | 1.50          | 0.08           | —    |
| A: SQ 641 (2)          | 1.56          | 0.10           | 0.17 |
| B: UT-01320 (3)        | 1.50          | 0.16           | —    |
| A: SQ 641 (2)          | 1.56          | 0.10           | 0.28 |
| B: UT-01320 (3)        | 1.50          | 0.32           | —    |
| A: SQ 641 (2)          | 1.56          | 0.10           | 0.49 |
| B: UT-01320 (3)        | 1.50          | 0.645          | —    |
| A: SQ 641 (2)          | 1.56          | 0.10           | 0.92 |
| B: UT-01320 (3)        | 1.50          | 1.29           | —    |
| A: capuramycin (1)     | 12.5          | 1.563          | 0.18 |
| B: UT-01320 (3)        | 1.50          | 0.08           | —    |
| A: capuramycin (1)     | 12.5          | 1.563          | 0.23 |
| B: UT-01320 (3)        | 1.50          | 0.16           | —    |
| A: capuramycin (1)     | 12.5          | 0.78           | 0.28 |
| B: UT-01320 (3)        | 1.50          | 0.32           | —    |
| A: Capuramycin (1)     | 12.5          | 0.78           | 0.49 |
| B: UT-01320 (3)        | 1.50          | 0.645          | —    |
| A: capuramycin (1)     | 12.5          | 0.78           | 0.92 |
| B: UT-01320 (3)        | 1.50          | 1.29           | —    |

**Table 2 Fractional inhibitory concentration of a combination of two inhibitor molecules**

**Table 3 Spectrum of activity of UT-01320 (3)**

| Species and strain                  | MIC (μg ml⁻¹) |
|-------------------------------------|--------------|
| Mycobacterium tuberculosis H37Rv   | 1.50         |
| M. tuberculosis H37Rv INH          | 2.50         |
| M. tuberculosis H37Rv RFP          | 2.45         |
| M. smegmatis ATCC 607              | >200         |
| Staphylococcus aureus ATCC 6538D-5 | >200         |
| Escherichia faecium ATCC 349       | >200         |
| E. coli ATCC 25019                 | >200         |
| Klebsiella pneumoniae ATCC 8047    | >200         |
| Pseudomonas aeruginosa ATCC 27853  | >200         |

**Abbreviations:** INH, isoniazid; RFP, rifampicin.

*The broth dilution method was used.

**Abbreviation:** FIC, fractional inhibitory concentration.

*FIC index for the wells at growth—no growth interface.

**MIC A** and **MIC B** are the MIC value of molecule A or B against Mtb (see Table 1).

**ΣFIC** is the sum of fractional inhibitory concentration calculated by the equation ΣFIC = FICₐ + FICₐ/Cₐ/Cₐ/Cₐ/Cₐ.

**Annexure**

**Figure 4** In vitro time-kill assessment of capuramycin (1), UT-01320 (3), and the first-line TB drugs (RFP and INH). Log-killing reduction in log₁₀ CFU ml⁻¹ at the concentrations of the molecules at 2x and 4x MIC. (a) 2x MIC of RFP (0.4 μg ml⁻¹), INH (0.2 μg ml⁻¹), UT-01320 (3.0 μg ml⁻¹) and capuramycin (24.0 μg ml⁻¹); (b) 4x MIC of RFP (0.8 μg ml⁻¹), INH (0.4 μg ml⁻¹), UT-01320 (6.0 μg ml⁻¹) and capuramycin (48.0 μg ml⁻¹). A full color version of this figure is available at The Journal of Antibiotics journal online.
Methylation at the C2'-hydroxyl group. Our total chemical synthetic scheme enables the modification of the C2'- and C3'-hydroxyl groups of capuramycin with chemically stable ether groups. As a result of reliable MurX enzyme assays of a small optimized library of capuramycin analogs followed by anticymbobacterial growth inhibitory assays, several new analogs were identified as improved MurX inhibitors (for example, UT-01304 (4)) and nonMurX inhibitors having anticymbobacterial activity (Figure 2). Methylation at the C2'-hydroxyl group of capuramycin (R1 in Figure 2) alters the molecular target of capuramycin. UT-01302 (3) did not interfere with MurX, but exhibited bactericidal activity against nonreplicating (dormant) Mtb with the MICLORA/MICMABA value of 1:72 (MICLORA/MICMABA = 7.35 for RFP). Effectiveness of 3 in nonreplicating Mtb was confirmed via assays using a modified Wayne model under low oxygen conditions and a nutrient-deficient Mtb cells at the MICLORA concentrations (2.58 μg ml⁻¹) (Figure 3). Cytotoxicity (IC₅₀) of 3 was evaluated in vitro using Vero monkey kidney cells and HepG2 human hepatoblastoma cells. As observed for other capuramycin analogs, 3 did not show cytotoxicity even at 400 μg ml⁻¹ concentrations (Table 1); the selectivity index (IC₅₀ in mammalian cells per MIC against Mtb) of 3 was determined to be >266.

### DISCUSSION

It is important to discover effective TB drugs that kill nonreplicating Mtb at low concentrations for shortening the treatment duration of current TB chemotherapies. UT-01320 (3) killed nonreplicating Mtb at 2.58 μg ml⁻¹ concentrations (Table 1). Based on the antibacterial activities tested, it was determined that UT-01320 has an antimicrobial spectrum focused against Mtb (Table 3). Selective anticymbobacterial agent is preferable for TB chemotherapy due to the fact that TB chemotherapy requires a long regimen, so that broad-spectrum antiTB agents may cause resistant to other bacteria during TB chemotherapy.

To date, structure-activity relationship studies of capuramycin (1) have been performed with the biosynthesis intermediate, A-500359, and the C2'-hydroxyl group of capuramycin has been modified with the limited functional groups (that is, acyl groups). Capuramycin has not been developed into a pursuable lead by acyl modification at the C2'-hydroxyl group. Our total chemical synthetic scheme enables the modification at the C2'- and C3'-hydroxyl groups of capuramycin with chemically stable ether groups. As a result of reliable MurX enzyme assays of a small optimized library of capuramycin analogs followed by anticymbobacterial growth inhibitory assays, several new analogs were identified as improved MurX inhibitors (for example, UT-01304 (4)) and nonMurX inhibitors having anticymbobacterial activity (Figure 2). Methylation at the C2'-hydroxyl group of capuramycin (R1 in Figure 2) alters the molecular target of capuramycin. UT-01302 (3) did not interfere with MurX, but exhibited bactericidal activity against nonreplicating (dormant) Mtb with the MICLORA/MICMABA value of 1:72 (MICLORA/MICMABA = 7.35 for RFP). Effectiveness of 3 in nonreplicating Mtb was confirmed via assays using a modified Wayne model under low oxygen conditions and a nutrient-deficient Mtb cells at the MICLORA concentrations (2.58 μg ml⁻¹) (Figure 3). Cytotoxicity (IC₅₀) of 3 was evaluated in vitro using Vero monkey kidney cells and HepG2 human hepatoblastoma cells. As observed for other capuramycin analogs, 3 did not show cytotoxicity even at 400 μg ml⁻¹ concentrations (Table 1); the selectivity index (IC₅₀ in mammalian cells per MIC against Mtb) of 3 was determined to be >266.

Several TB drugs and drug leads that have the potential to kill nonreplicating Mtb in vitro were identified. Bedaquiline is an Mtb ATPase inhibitor that showed effectiveness in killing nonreplicating Mtb in vitro. Capreomycin and streptomycin that target protein biosynthesis by binding to the bacterial ribosomes and bacterial topoisomerase II inhibitors (for example, fluoroquinolones) killed nonreplicating Mtb in vitro. It was reported that nitric oxide-mediated unique immune responses by metronidazole and PA-824 (a phase II clinical drug) killed nonreplicating Mtb under hypoxic conditions. However, in most cases their MIC values against nonreplicating Mtb cells are significantly higher than those against Mtb grown under aerobic conditions. Therefore, it is considered to be very important to discover new TB drugs that effectively kill nonreplicating Mtb at the same MIC concentrations as those under aerobic conditions. Table 4 summarizes the potential molecular targets whose inhibitors were effective in killing nonreplicating Mtb. Recently, Ishizaki et al. reported that caprazamycin (an MraY inhibitor) analog, CPZEN-45 inhibited mycobacterial WecA, a phosphotransferase responsible for capsular polysaccharide synthesis. Although much studies are required to understand WecA in dormant Mtb, CPZEN-45 killed nonreplicating Mtb in vitro. As such the alternation of molecular target of a uridine-glycosyl peptide MraY inhibitor by chemical modifications has observed. While the exact mechanism of action of UT-01320 (3) is far from completely understood, 3 is a new pharmacophore that inhibits the polymerization of RNA at low concentrations in a dose-response manner (Figure 5). UT-01320 (3) killed RFP-resistant-Mtb at low concentrations. This fact

### Table 4 Potential targets for nonreplicating M. tuberculosis

| Electron transport systems | Menaquinone biosynthesis | F.F₁ ATP synthase | Activation via reductase(s) to form cytotoxic species | Protein kinase (for example, PknA, PknB and PknG) | DosS/DosR systems | Glyoxylate pathway | Methyltransferase | Protein biosynthesis | DNA gyrase |
|---------------------------|--------------------------|------------------|-------------------------------------------------|-----------------------------------------------|------------------|------------------|------------------|-------------------|----------|

**Figure 5** MurX and RNA polymerase inhibitory activities of UT-01320, capuramycin and SQ 641. (a) MurX containing P-60 was used; (b) Data summarized here were obtained with E. coli RNA polymerase. UT-01320 (3) inhibited RNA polymerases from M. smegmatis and S. aureus with similar IC₅₀ values. A full color version of this figure is available at The Journal of Antibiotics journal online.
may imply that 3 inhibits RNA polymerases by interfering with the catalytic sites that are different from the RFP binding site(s). The structure of UT-01320 (3) is closely related to the MurX inhibitor SQ 641 (2). The PK/PD parameters of 3 may be correlated with those of 2, and thus, a combination of two capuramycin analogs that have distinct drug targets is a great interest in evaluation of their synergistic interactions. The nonMurX inhibitor 3 exhibited strong synergistic effect with a promising preclinical TB drug, SQ 641 (3); a combination of two molecules significantly improved in vitro antimycobacterial activity (Table 2). In addition, UT-01320 (3) retained a fast-bacterial characteristic observed for capuramycin (Figure 4). These forward-looking biological characteristics for UT-01320 promise that new TB drug leads will be discovered by extensive structure–activity relationship studies of 2′-O-methylated capuramycin analogs. We are currently optimizing UT-01320 (3) to be more effective against both replicating and nonreplicating Mtb by modifying the R3 group (Figure 2). Through in vitro biological evaluation of nonMurX inhibitors identified from capuramycin analogs and in vivo evaluation of promising molecules will be reported elsewhere.

**MATERIALS AND METHODS**

**General/chemicals and reagents**

All chemicals were purchased from commercial sources and used without further purification unless otherwise noted. Difco Middlebrook 7H10 agar, Middlebrook 7H9 broth, Tryptic soy broth, MOPS, tris (hydroxymethyl)aminomethane, 2-mercaptoethanol, sucrose and triton-X 100 were purchased from Sigma-Aldrich (St Louis, MO, USA). E. coli H37Rv (BEI Resources, NIAID). A single colony of a bacterial strain (M. smegmatis (ATCC 607) and M. tuberculosis H37Rv cells were transformed by mixing at least 1 μg of purified the plasmid, pFCA-luxB and incubating at room temperature for 30 min, followed by electroporation. 24 M. tuberculosis pFCA-luxB strain cultured was diluted in Middlebrook 7H12 broth, and sonicated for 15 s. The cultures were diluted to obtain an A595 of 0.03 to 0.05 and 3000 to 7000 RLUs per 100μl. Twofold serial dilutions of antimicrobial agents were prepared in black 96-well microtiter plates (100μl) and 100μl of the cell suspension was added. The microplate was placed under anaerobic conditions (oxygen concentration, <0.16%) by using an Anoxomat model WS-8080 (MART Microbiology, Drachten, the Netherlands) and three cycles of evacuation and filling with a mixture of 10% H2, 5% CO2 and 85% N2. Incubation was continued for 10 days, and transferred to an ambient gaseous condition (5% CO2-enriched air) incubator for a 24 h recovery. 100μl culture was transferred to white 96-well microtiter plates for determination of luminescence.

**MIC on nutrient starved M. tuberculosis.** M. tuberculosis H37Rv was grown in conical flasks with air filter, containing Middlebrook 7H9 medium supplemented with OADC at 37°C. Exponentially growing Bacilli at an OD600 of 0.2 were transferred to PBS supplemented with 0.025% Tween 80 and diluted to a final OD600 of 0.1. 50 ml of this suspension was transferred into a conical flask and starved for 14 days at 37°C. 100 μl of the culture was transferred to a 96- well plate and MIC experiment was performed according to the procedure described above. 22

**Kill-curve graph: Determination of CFU per milliliter.** After the treatment of M. tuberculosis culture with the inhibitors, 10-, 100-, 1000- and 10,000-fold serial dilutions from each culture tube was sampled every day and 20μl from each dilution was plated on 7H10 agar plates supplemented with OADC enrichment. Plates were incubated for 15 days in a static incubator at 37°C and colonies were counted.

**MurX assay**

MurX assay substrates, Park’s nucleotide-N′-dansylthiourea, neryl phosphate, were chemically synthesized according to the reported procedures. 23-25 M. tuberculosis cells were harvested by centrifugation (4700 RPM) at 4°C followed by washing with 0.9% saline solution (trishe) and ~ 5 g of pellet (wet weight) was collected. The cell pellets were suspended in homogenization buffer (containing 50 mM MOPS of pH = 8, 0.25 M sucrose, 10 mM MgCl2 and 5 mM 2-mercaptoethanol) and disrupted by probe sonication on ice (10 cycles of 60 s on and 90 s off). The resulting suspension was centrifuged at 10000 x for 10 min at 4°C to remove unbroken cells. The supernatant was centrifuged at 25000 x for 40 min at 4°C (three to four times). All pellets in each tube were pooled and a second sonication was performed (10 cycles of 60 s on and 90 s off). The lysate was centrifuged once at 25 000 x for 1 h and the supernatant
was subjected to ultracentrifugation at 60,000 x g for 1 h at 4 °C. The supernatant was discarded and the membrane fraction containing MurX enzyme (P-60) was suspended in the TRIS-HCl buffer (pH 7.5, containing 2-mercaptoethanol). Total protein concentrations are about 8 – 10 mg/ml. Aliquots were stored in Eppendorf tubes at –80 °C. Similarly, the membrane fractions containing MrATP enzyme (P-60) were prepared from M. smegmatis, S. aureus and E. coli, respectively.

Park’s nucleotide-N-dansylhydrazone (2 ms stock solution; 3.75 μl (75 μM), MgCl2 (0.5 M; 10 μl (50 mmoles)), KCl (2 M, 10 μl (200 mmoles)) and triton X 100 (0.5%; 11.25 μl), trius-buffer (pH 8; 50 μl, 2.5 μl), neryl phosphate (10 μmol) and inhibitor (0–100 μM, in DMSO (2.5 μl)) were placed in a 500 μl Eppendorf tube. To a stirred-reaction mixture, P-60 (15 μl) was added (the total volume of the reaction mixture=100 μl). The reaction mixture was incubated for 1 h at room temperature (26 °C), and quenched with CHCl₃.

The 1x reaction mixture was kept 30 μl, tris-buffer (pH 7.5; 25 μl), MgCl₂ (0.5 M; 10 μl (50 mmoles)), neryl phosphate (10 μmol) and inhibitor (0–100 μM, in DMSO (2.5 μl)) were placed in a 500 μl Eppendorf tube. To a stirred-reaction mixture, P-60 (15 μl) was added (the total volume of the reaction mixture=100 μl). The reaction mixture was incubated for 1 h at room temperature (26 °C), and quenched with CHCl₃.

The 1x solution. Temperature was maintained at 4 °C for the rest of the procedure.

The water phase (10 μl) was injected into HPLC (solvent: CH₃CN:0.05 Maq. NH₄HCO₃=25:75, UV: 350 nm, flow rate: 0.5 ml min⁻¹, column: Kinetex 5 μ 100 Å, 150 x 4.60 mm) and the area of the peak for lipid I-neryl derivative was subjected to ultracentrifugation at 60,000 x g. The supernatant was treated slowly with 10% v/v glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol) and 0.5 M NaCl and centrifuged for 30 min at 6000 r.p.m. The aqueous phase was assayed via reverse-phase HPLC. The supernatant was resuspended in TGED and was subjected to DNA cellulose chromatography as described in Burgess and Jendrisak. Fractions were pooled and aliquots were suspended in TGED and was subjected to DNA cellulose chromatography as described in Burgess and Jendrisak. Fractions were pooled and aliquots were suspended in TGED and was subjected to DNA cellulose chromatography as described in Burgess and Jendrisak. Fractions were pooled and aliquots were suspended in TGED and was subjected to DNA cellulose chromatography as described in Burgess and Jendrisak. Fractions were pooled and aliquots were suspended in TGED and was subjected to DNA cellulose chromatography as described in Burgess and Jendrisak. Fractions were pooled and aliquots were suspended in TGED and was subjected to DNA cellulose chromatography as described in Burgess and Jendrisak. Fractions were pooled and aliquots were suspended in TGED.

RNA polymerase assays
M. smegmatis RNP was prepared according to the procedures described previously by Burgess and Jendrisak. M. smegmatis ATCC 607 was cultured to log phase, harvested by centrifugation and washed thrice with 0.9% saline solution. Temperature was maintained at 4 °C for the rest of the procedure unless mentioned otherwise. Cells were suspended in a lysis buffer (0.05 M Tris, 5% v/v glycerol, 2 x 10⁶ cpm/μg, ETA, 0.1 M dithiothreitol, 1 x 2-mercattothiol, 0.233 M NaCl, 130 μg ml⁻¹ lysisoyme, 23 μg ml⁻¹ PhCH₂SO₄F and 4% v/v sodium deoxycholate) and disrupted by probe sonication on ice (10 cycles of 60 s on and 90 s off). The resulting suspension was then centrifuged at 8000 r.p.m. for 45 min at 4 °C.

The supernatant was subjected to polyethylene-nemamine fractionation. The supernatant was treated with 10% v/v solution of polyethylene-nemamine (pH 7.9) to a final concentration of 0.35%, stirred for 5 min and centrifuged for 15 min at 6000 r.p.m. Supernatant was discarded. Pellet was suspended in TGEB buffer (0.01 M Tris, pH 7.9, 5% v/v glycerol, 0.1 M EDTA, 0.1 M dithiothreitol) and 0.5 M NaCl and centrifuged for 15 min at 6000 r.p.m. Supernatant was discarded and pellet was resuspended in TGED and 1 x NaCl. Centrifuged for 30 min at 6000 r.p.m. Supernatant was treated with solid ammonium sulfate to 50% saturation, stirred for 20 min and centrifuged for 45 min at 8000 r.p.m. Pellet was suspended in TGED and was subjected to DNA cellulose chromatography as described in Burgess and Jendrisak. Fractions were pooled and aliquots were stored at –80 °C. The RNA-DNA, RNA-NTP mix and 10x fluorescence dye was diluted 10-fold with water and the RNAP enzyme was diluted 10-fold with 1x assay buffer. The assay mixture was then prepared in a black-bottom 96-well plate. Control of compounds with known toxicity such as RFP or INH was included on each plate. The plates were incubated and cytotoxic effects were determined via the MTT assay.

Cytotoxicity assays
Cytotoxicity assays will be performed using Vero monkey kidney (ATCC CCL-81) and HepG2 human hepatoblastoma cell (ATCC HB-8065) lines. Vero or HepG2 cells are cultured in 96-well cell culture plates using ATCC-formulated Eagle’s minimum essential medium. Serially diluted aliquots of each test compound at concentrations ranging from 1–25 X of the MIC were added to the cells. Control of compounds with known toxicity such as RFP or INH was included on each plate. The plates were incubated and cytotoxic effects were determined via the MTT assay.

6-{(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-4-hydroxy-3-methoxy-tetrahydro-furan-2-yl}-methoxy}-4,5-dihydroxy-5,6-dihydro-4H-pyran-2-carboxylic acid (3,4-difluoro-phenyl)-amide (SQ 641, 2). [α]⁺[c]⁺= +105 (c 0.1 in H₂O); 1H NMR (400 MHz, CD₃OD) δ 7.87 (d, J = 8.4 Hz, 1H), 7.68 (m, 1H), 7.34 (m, 1H), 7.19 (m, 1H), 6.07 (d, J = 4.4 Hz, 1H), 5.74 (s, 1H), 5.63 (d, J = 8.0 Hz, 1H), 5.08 (d, J = 7.2 Hz, 1H), 4.65 (s, 1H), 4.42 (d, J = 8.8 Hz, 1H), 4.30 (t, J = 4.8 Hz, 1H), 4.21 (m, 1H), 3.79 (m, 1H), 3.71 (t, J = 1.2, 1H), 3.48 (s, 3H); HRMS (ESI) calcd for C₂₅H₃₆N₅NaO₁₂ ([M+Na⁺]⁺): 593.1307, found: 593.1312.

Synergistic effect of UT-01320 (3) with SQ 641 or capuramycin. The synergistic or antagonistic activities of MurX inhibitors (capuramycin (1) or SQ 641 (2) and UT-01320 (3) were assessed in vitro via micro dilution broth checkerboard technique.24,42 The FIC index was calculated to according to the following equation. ΣFIC = FICₐ + FICₐₙ if Sₐ + Sₐₙ ≥ 1 or Sₐ + Sₐₙ ≥ 2, where Sₐ and Sₐₙ are the MIC of drugs A and B, Cₐ and Cₐₙ are concentrations of drugs A and B used in combination. In these interaction studies, ΣFIC of less than 1 represents synergistic activity.

ACKNOWLEDGEMENTS
The National Institutes of Health is greatly acknowledged for financial support of this work (AI084411). We also thank University of Tennessee for generous financial support. NMR data were obtained on instruments supported by the NIH Shared Instrumentation Grant. The following reagent was obtained through BEI Resources, NIAID, NIH: M. tuberculosis, strain H37Rv and gamma-irradiated M. tuberculosis, NR-18419. The authors gratefully acknowledge Drs William Clemons (California Institute Technology) and Crick (Colorado State University) for useful discussions.

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