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## Discovery of a capuramycin analog that kills non-replicating *Mycobacterium tuberculosis* and its synergistic effects with translocase I inhibitors

Shajila Siricilla<sup>†</sup>, Katsuhiko Mitachi<sup>†</sup>, Bajoie Wan<sup>‡</sup>, Scott G. Franzblau<sup>‡</sup>, and Michio Kurosu<sup>†</sup>

<sup>†</sup>Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, 881 Madison Avenue, Memphis, TN 38163, United States

<sup>‡</sup>Institute for Tuberculosis Research, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood Street, Chicago, Illinois 60612, United States

### Abstract

Capuramycin (**1**) and its analogs are strong translocase I (MurX/MraY) inhibitors. In our SAR studies of capuramycin analogs against *M. tuberculosis* (Mtb), we observed for the first time that a capuramycin analog, UT-01320 (**3**) killed non-replicating (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 IC<sub>50</sub> 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 TB drug.

### Keywords

Translocase I; MraY (MurX); RNA polymerase; Non-replicating (dormant) *M. tuberculosis*; Synergistic effect; TB drug

## INTRODUCTION

One-third of the 42 million people living with HIV/AIDS worldwide are co-infected with *Mycobacterium tuberculosis* (Mtb).<sup>1,2</sup> The WHO estimated that 650,000 new cases of multidrug-resistant (MDR)-Mtb emerge each year.<sup>3</sup> An outbreak of extensively-drug resistant (XDR)-Mtb threatens TB control and prevention efforts.<sup>4</sup> Treatment duration for MDR-Mtb infections is at least 20-28 months. Tuberculosis chemotherapy for XDR-TB takes substantially longer than MDR-TB, and XDR-Mtb strains are responsible for very high mortality rate.<sup>5</sup> Therefore, it is very important to discover new drugs that can shorten current TB drug regimens. Mechanisms that enter non-replicating (or dormant) state of Mtb are

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Address correspondence to Michio Kurosu, mkurosu@uthsc.edu.

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accounted for a significant factor that requires long-term chemotherapy.<sup>6</sup> Wayne et. al. 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.<sup>7,8</sup> Significantly, the dormant form of Mtb was found to be resistant to most of clinically utilized antimycobacterial agents.<sup>8</sup> Thus, new drugs targeting non-replicating Mtb are likely to revolutionize TB chemotherapy.

The cell-wall of Mtb offers many unique targets for drug development.<sup>9</sup> However, most of 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.<sup>10</sup> On the contrary, it was recently reported that a peptidoglycan biosynthesis inhibitor, meropenem (a carbapenem) was effective in killing non-replicating Mtb in combination with clavulanate (a  $\beta$ -lactamase inhibitor).<sup>11</sup> Although a mechanism of action of their bactericidal effect against dormant Mtb cells is not known, it is one of few examples that peptidoglycan biosynthesis inhibitors kill dormant form of Mtb. Because several translocase I (MurX/MraY, hereafter referred to as Mur X for *Mtb* translocase I) inhibitors kill Mtb much faster than other TB drugs under aerobic conditions (Figure 1),<sup>12</sup> we commenced SAR studies of capuramycin (**1**), a known MurX inhibitor antibiotic, to improve efficacy of its antimycobacterial activity *in vitro* and *in vivo* (Figure 2).<sup>13,14,15</sup> Daiichi-Sankyo and Sequella reported several capuramycin analogs in which *in vitro* MraY enzyme and antimycobacterial activity could be improved *via* the modification of the carboxylic group of the capuramycin biosynthetic intermediate, A-500359.<sup>16,17,18,19</sup> We have synthesized new capuramycin analogs *via* our total synthetic scheme,<sup>15</sup> 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 non-replicating (dormant) Mtb, it was found that a 2'-methylated capuramycin analog, UT-01320 (**3**) killed both replicating and non-replicating Mtb in microplate alamar blue assay (MABA) and Low-oxygen recovery assay (LORA), respectively.<sup>20</sup> To the best of our knowledge, it is the first observation that a capuramycin analog exhibited bactericidal activity against non-replicating Mtb at low concentrations. Herein, we report *in vitro* biological evaluations of **3**, synergistic effect with known MurX inhibitors **1** or **2**, and insights into a molecular target of **3** (Figure 2).

## 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 agar, Tryptic soy broth, MOPS, tris(hydroxymethyl)aminomethane, 2-mercaptoethanol, sucrose and triton-X 100 were purchased from Sigma-Aldrich. ADC enrichment was purchased from Fisher Scientific. Magnesium chloride and potassium chloride were obtained from VWR. RNA polymerase assay kit (*S. aureus* and *E. coli*) was purchased from Profoldin. The kit contained 10 $\times$  assay buffer, 100 $\times$  DNA template, 100 $\times$  NTP mix, 100 $\times$  *S. aureus* or *E. coli* RNA polymerase (RNAP) enzyme and 10 $\times$  fluorescence dye.

Tetrahydrofuran (THF), methylene chloride (CH<sub>2</sub>Cl<sub>2</sub>), dimethylformamide (DMF) were purified *via* MBRAUN Solvent Purification Systems (MB-SPS) under an Argon atmosphere. Reactions were monitored by thin-layer chromatography (TLC) performed with 0.25 mm coated commercial silica gel plates (EMD, Silica Gel 60F<sub>254</sub>) using UV light for visualization at 254 nm, or developed with ceric ammonium molybdate or anisaldehyde or copper sulfate or ninhydrin solutions by heating on a hot plate. Reactions were also monitored by using SHIMADZU LCMS-2020 with solvents: A: 0.1% formic acid in water, B: acetonitrile. When necessary, reactions were monitored by SHIMADZU prominence HPLC using Phenomenex Kinetex 1.7 μ XB-C18 100A column (150 × 2.10 mm) and detected at 220, 254 nm. Flash chromatography was performed with Whatman silica gel (Purasil 60 Å, 230-400 Mesh). Proton magnetic resonance (<sup>1</sup>H-NMR) spectral data were recorded on 400, and 500 MHz instruments. Carbon magnetic resonance (<sup>13</sup>C-NMR) spectral data were recorded on 100 and 125 MHz instruments. For all NMR spectra, chemical shifts (δ H, δ C) were quoted in parts per million (ppm), and *J* values were quoted in Hz. <sup>1</sup>H and <sup>13</sup>C NMR spectra were calibrated with residual undeuterated solvent (CDCl<sub>3</sub>: δ H =7.26 ppm, δ C =77.16ppm; CD<sub>3</sub>CN: δ H=1.94ppm, δ C =1.32ppm; CD<sub>3</sub>OD: δ H =3.31ppm, δ C =49.00 ppm; DMSO-d<sub>6</sub>: δ H=2.50ppm, δ C =39.5ppm; D<sub>2</sub>O: δ H=4.79 ppm) as an internal reference. The following abbreviations were used to designate the multiplicities: s=singlet, d=doublet, dd=double doublets, t=triplet, q=quartet, quin=quintet, hept=heptet, m=multiplet, br=broad.

### Bacterial strains

The strains used were *Mycobacterium smegmatis* (ATCC 607) and *Mycobacterium tuberculosis* H<sub>37</sub>Rv, H<sub>37</sub>Rv INHr, H<sub>37</sub>Rv RFPPr, *E. coli* (ATCC 25019), *S. aureus* (ATCC 6538D-5), *E. faecium* (ATCC 349), *K. pneumonia* (ATCC 8047), and *P. aeruginosa* (ATCC 27853). These bacteria were obtained from ATCC except for *Mycobacterium tuberculosis* H<sub>37</sub>Rv (BEI Resources, NIAID).

### MIC assays

**Log phase bacterial culture**—A single colony of a bacterial strain (*M. smegmatis* or *M. tuberculosis*) was grown on a Difco Middlebrook 7H10 nutrient agar (enriched with OADC). Seed cultures and larger cultures were obtained using Middlebrook 7H9 broth enriched with OADC. Single colonies of *S. aureus*, *E. faecium*, *E. coli*, *K. pneumonia* and *P. aeruginosa* strains were grown on tryptic soy agar. The flasks were incubated overnight in a shaking incubator at 37 °C with a shaking speed of 200 rpm and cultured to mid-log phase (Optical density - 0.5). The optical density was monitored at 600 nm using a 96 well microplate reader.

**Determination of minimum inhibitory concentration (MIC)**—The inhibitors were dissolved in DMSO (a final concentration of 1 mg per 100 μL). This concentration was used as the stock solution for all studies. Bacterial cultures at 0.5 optical density, was treated with serial dilutions of inhibitors in aerobic conditions and incubated at 37 °C for 48 hours and 15 days for *M. smegmatis* and *M. tuberculosis* respectively. Incubation time was 24 hours for *S. aureus*, *E. faecium*, *E. coli*, *K. pneumonia* and *P. aeruginosa* strains. 20 μL of alamar blue was added and incubated on a shaking incubator at 37 °C for 2 h. The lowest concentration

at which the color of alamar blue was completely retained as blue was read as the MIC (Pink = Growth, Blue = No growth). The absorbance measurements were also performed using a Biotek Synergy XT, 96 well plate reader at 570 nm and 600 nm.

**Luminescence-based low-oxygen-recovery assay (LORA) assay**—These assays were performed according to the reported procedures.<sup>20</sup> In brief, *M. tuberculosis* H<sub>37</sub>Rv cells were transformed by mixing at least 1 µg of purified the plasmid, pFCA-luxAB and incubating at room temperature for 30 min, followed by electroporation.<sup>21</sup> *M. tuberculosis* pFCA-luxAB strain cultured was diluted in Middlebrook 7H12 broth, and sonicated for 15s. The cultures were diluted to obtain an A<sub>570</sub> of 0.03 to 0.05 and 3,000 to 7,000 RLU per 100 µl. Twofold serial dilutions of antimicrobial agents were prepared in black 96-well microtiter plates (100 µl), and 100 µl of the cell suspension was added. The microplate was placed under anaerobic conditions (oxygen concentration, less than 0.16%) by using an Anoxomat model WS-8080 (MART Microbiology) and three cycles of evacuation and filling with a mixture of 10% H<sub>2</sub>, 5% CO<sub>2</sub>, and 85% N<sub>2</sub>. Incubation was continued for 10 days, and transferred to an ambient gaseous condition (5% CO<sub>2</sub>-enriched air) incubator for a 28h “recovery.” 100 µl culture was transferred to white 96-well microtiter plates for determination of luminescence.

**MIC on nutrient starved *M. tuberculosis***—*M. tuberculosis* H<sub>37</sub>Rv was grown in conical flasks with air filter, containing Middlebrook 7H9 medium supplemented with OADC at 37 °C. Exponentially growing bacilli at an OD<sub>600</sub> of 0.2 were transferred to PBS supplemented with 0.025% Tween 80 and diluted to a final OD<sub>600</sub> 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.<sup>22</sup>

**Kill-curve graph: Determination of colony forming units 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*<sup>ε</sup>-dansylthiourea, neryl phosphate, were chemically synthesized according to the reported procedures.<sup>23,24,25,26</sup> *M. tuberculosis* cells were harvested by centrifugation (4,700 RPM) at 4 °C followed by washing with 0.9% saline solution (thrice) and ~5g 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 MgCl<sub>2</sub> and 5 mM 2-mercaptoethanol) and disrupted by probe sonication on ice (10 cycles of 60s on and 90s off). The resulting suspension was centrifuged at 1,000 ×g for 10 min at 4 °C to remove unbroken cells. The supernatant was centrifuged at 25,000 ×g for 40 min at 4 °C (3 to 4 times). All pellets in each tube were pooled and a second sonication was performed (10 cycles of 60s on and 90s off). The lysate was centrifuged once at 25,000 ×g for 1h and the supernatant was subjected to ultracentrifugation at 60,000 ×g for 1h 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).<sup>27</sup> Total protein concentrations are about 8~10 mg/mL.<sup>28</sup> Aliquots were stored in Eppendorf tubes at  $-80^{\circ}\text{C}$ . Similarly, the membrane fractions containing MraY enzyme (P-60) were prepared from *M. smegmatis*, *S. aureus*, and *E. coli*, respectively.

Park's nucleotide-*N*<sup>ε</sup>-dansylthiourea (2 mM stock solution; 3.75  $\mu\text{L}$  (75  $\mu\text{M}$ )),  $\text{MgCl}_2$  (0.5 M; 10  $\mu\text{L}$  (50 mM)), KCl (2 M, 10  $\mu\text{L}$  (200 mM)), triton X100 (0.5%; 11.25  $\mu\text{L}$ ), tris-buffer (pH = 8; 50mM, 2.5  $\mu\text{L}$ ), neryl phosphate (10 mM, 45  $\mu\text{L}$ ), and inhibitor (0-100  $\mu\text{M}$ , in DMSO (2.5  $\mu\text{L}$ )) were placed in a 500  $\mu\text{L}$  Eppendorf tube. To a stirred reaction mixture, P-60 (15  $\mu\text{L}$ ) was added (the total volume of the reaction mixture: 100  $\mu\text{L}$ ). The reaction mixture was incubated for 1h at room temperature ( $26^{\circ}\text{C}$ ), and quenched with  $\text{CHCl}_3$  (200  $\mu\text{L}$ ). Two phases were mixed *via* vortex and centrifuged at 25,000  $\times g$  for 10 min. The upper aqueous phase was assayed *via* reverse-phase HPLC. The water phase (10  $\mu\text{L}$ ) was injected into HPLC (solvent:  $\text{CH}_3\text{CN}$ : 0.05 M aq.  $\text{NH}_4\text{HCO}_3 = 25: 75$ , UV: 350 nm, flow rate: 0.5 mL/min, Column: Kinetex 5u  $\text{C}_8$  100 $\text{\AA}$ , 150  $\times$  4.60mm), and the area of the peak for lipid I-neryl derivative was quantified to obtain the  $\text{IC}_{50}$  value. The  $\text{IC}_{50}$  values were calculated from plots of the percent product inhibition versus the inhibitor concentration.<sup>26</sup>

**RNA polymerase assays**—*M. smegmatis* RNAP was prepared according to the procedures described previously by Burgess and Jendrisak.<sup>29</sup> *M. smegmatis* ATCC 607 was cultured to log phase, harvested by centrifugation and washed thrice with 0.9% saline solution. Temperature was maintained at  $4^{\circ}\text{C}$  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 mM EDTA, 0.1 mM dithiothreitol, 1 mM 2-mercaptoethanol, 0.233 M NaCl, 130  $\mu\text{g}/\text{mL}$  lysozyme, 23  $\mu\text{g}/\text{mL}$   $\text{PhCH}_2\text{SO}_2\text{F}$  and 4% w/v sodium deoxycholate) and disrupted by probe sonication on ice (10 cycles of 60s on and 90s off). The resulting suspension was then centrifuged at 8,000 rpm for 45 min at  $4^{\circ}\text{C}$ . The supernatant was subjected to polyethylenamine (PEI) fractionation. The supernatant was treated slowly with 10% v/v solution of PEI (pH 7.9) to a final concentration of 0.35%, stirred for 5 min and centrifuged for 15 min at 6000 rpm. Supernatant was discarded. Pellet was suspended in TGED buffer (0.01 M Tris, pH 7.9, 5% v/v glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol) and 0.5 M NaCl and centrifuged for 15 min at 6000 rpm. Supernatant was discarded and pellet was resuspended in TGED and 1 M NaCl. Centrifuged for 30 min at 6000 rpm. Supernatant was treated with solid ammonium sulfate to 50% saturation, stirred for 20 min and centrifuged for 45 min at 8000 rpm. 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  $-80^{\circ}\text{C}$ . The 100 $\times$  DNA, 100 $\times$  NTP mix and 10 $\times$  fluorescence dye was diluted 10 fold with water and the RNAP enzyme was diluted 10 fold with 1 $\times$  assay buffer. The assay mixture was then prepared in a black-bottom 96 well plate by the addition of 15  $\mu\text{L}$  water, 3  $\mu\text{L}$  of 10 $\times$  buffer, 3  $\mu\text{L}$  of 10 $\times$  DNA template, 3  $\mu\text{L}$  of 10 $\times$  NTP mix, 3  $\mu\text{L}$  of 10 $\times$  RNA polymerase enzyme (*S. aureus* or *E. coli*) or RNAP from *M. smegmatis* (obtained as described above) and 3  $\mu\text{L}$  of inhibitor (final concentration, 0-10  $\mu\text{M}$ ). The total volume of the reaction mixture was kept 30  $\mu\text{L}$  and was incubated for 1h at room temperature. 30  $\mu\text{L}$  of the 1 $\times$  fluorescent dye was added and incubated for 5 min. The fluorescence intensity was

measured at an excitation wavelength of 485 nm and emission wavelength of 528 nm using a Biotek Synergy XT, 96 well plate reader.

**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 rifampicin or INH was included on each plate. The plates were incubated and cytopathic effects were determined via the MTT assay.

**6-{Carbamoyl-[5-(2,4-dioxo-3,4-dihydro-2H-pyrimidin-1-yl)-3,4-dimethoxy-tetrahydro-furan-2-yl]-methoxy}-4,5-dihydroxy-5,6-dihydro-4H-pyran-2-carboxylic acid (2-oxo-azepan-3-yl)-amide (UT-01320, 3)**—UT-01320 (**3**) was synthesized according to the scheme reported previously.<sup>14,15</sup>  $[\alpha]_D^{20} = +90$  (*c* 0.1 in H<sub>2</sub>O); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  7.79 (d, *J* = 8.4 Hz, 1H), 5.90 (d, *J* = 4.0 Hz, 1H), 5.88 (d, *J* = 6.4 Hz, 1H), 5.70 (d, *J* = 8.0 Hz, 1H), 5.12 (d, *J* = 6.0 Hz, 1H), 4.46 (d, *J* = 11.2 Hz, 1H), 4.41 (d, *J* = 3.2 Hz, 1H), 4.32 (t, *J* = 4.0 Hz, 1H), 4.25 (t, *J* = 3.2 Hz, 1H), 4.08 (m, 2H), 3.78 (m, 1H), 3.35 (s, 3H), 3.31 (s, 3H), 1.90-1.57 (m, 6H), 1.32 (m, 2H); <sup>13</sup>C NMR (101 MHz, CD<sub>3</sub>OD)  $\delta$  176.5, 173.7, 166.0, 162.1, 152.3, 144.3, 142.7, 109.0, 103.6, 103.1, 82.1, 81.0, 79.5, 78.2, 68.8, 63.9, 58.6, 58.1, 55.4, 53.5, 42.5, 29.9, 27.5; HRMS (ESI) calcd for C<sub>24</sub>H<sub>33</sub>N<sub>5</sub>NaO<sub>12</sub> ([M + Na]<sup>+</sup>): 606.2023, found: 606.2031.

**6-{Carbamoyl-[5-(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)**— $[\alpha]_D^{20} = +105$  (*c* 0.1 in H<sub>2</sub>O); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  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 (dd, *J* = 8.8 Hz, 1H), 4.30 (t, *J* = 4.8 Hz, 1H), 4.21 (m, 1H), 3.79 (m, 1H), 3.71 (d, *J* = 1.2, 1H), 3.48 (s, 3H); HRMS (ESI) calcd for C<sub>23</sub>H<sub>24</sub>F<sub>2</sub>N<sub>4</sub>NaO<sub>11</sub> ([M + Na]<sup>+</sup>): 593.1307, found: 593.1312.

**6-{Carbamoyl-[5-(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 (2-oxo-azepan-3-yl)-amide (Capuramycin, 1)**—Capuramycin was resynthesized according the reported procedure.<sup>15</sup>  $[\alpha]_D^{20} = +98$  (*c* 0.1 in H<sub>2</sub>O); <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  7.71 (d, *J* = 8.0 Hz, 1H), 5.97 (s, 1H), 5.82 (d, *J* = 8.0 Hz, 1H), 5.73 (s, 1H), 5.35 (s, 1H), 4.59 (d, *J* = 11.2 Hz, 2H), 4.47 (s, 1H), 4.44 (d, *J* = 4.8 Hz, 1H), 4.34 (s, 1H), 4.15 (s, 1H), 3.71 (t, *J* = 4.8 Hz, 1H), 3.26 (s, 3H), 1.94-1.57 (m, 6H), 1.32 (m, 2H); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O)  $\delta$  176.3, 173.0, 166.1, 161.4, 151.2, 141.5, 141.0, 109.4, 101.8, 99.3, 90.1, 81.6, 78.1, 75.5, 71.9, 64.7, 61.7, 57.8, 52.2, 41.4, 30.3, 27.3; HRMS (ESI) calcd for C<sub>23</sub>H<sub>31</sub>N<sub>5</sub>NaO<sub>12</sub> ([M + Na]<sup>+</sup>): 592.1867, found: 592.1864.

**Synergistic effect of UT-01320 (3) with SQ641 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.<sup>30,31</sup> The FIC (Fractional Inhibitory Concentration) index was calculated according to the following equation.  $\Sigma FIC = FIC_A + FIC_B = C_A/MIC_A + C_B/MIC_B$  where,  $MIC_A$  and  $MIC_B$ : MIC of drugs A and B,  $C_A$  and  $C_B$  = concentrations of drugs A and B used in combination. In these interaction studies,  $\Sigma FIC$  of less than 1 represents synergistic activity.

## RESULT

### Effectiveness of UT-01320 against non-replicating *M. tuberculosis*

To date, we have synthesized capuramycin (1), SQ641 (2), and over 100 new capuramycin analogs in which their structures were diversified with optically active amines ( $R_3$ ) and the four uridyl templates, 3'-O-methyl (natural form), 2'-O-methyl, 2',3'-O-dimethyl-, and 3'-O-demethylated (unnatural forms) uridine-manno-pyranuronate derivatives (Figure 2). The syntheses of capuramycin analogs with these templates were accomplished *via* the synthetic scheme reported previously.<sup>14,15</sup> As the results of *in vitro* evaluation of these molecules in MurX enzyme inhibition<sup>26</sup> and Mtb (H<sub>37</sub>Rv) growth inhibitory activities against replicating and non-replicating (dormant) Mtb *via* the microplate alamar blue assay (MABA) and low oxygen recovery assay (LORA) assays<sup>20</sup>, 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  $\mu$ g/mL vs 12.0  $\mu$ g/mL for 1, IC<sub>50</sub> 0.095  $\mu$ M vs 0.127  $\mu$ M for 1).<sup>15</sup> The analogues synthesized from the 3'-O-methyluridine-manno-pyranuronate (natural form) did not exhibit bactericidal activity against non-replicating Mtb even at 400  $\mu$ g/mL concentrations. The isocapuramycin (2'-O-methyl) analogs ( $R_1 = \text{Me}$ ,  $R_2 = \text{H}$  in Figure 2) showed equal MurX inhibitory and antimycobacterial activities to the corresponding analogs derived from the natural form ( $R_1 = \text{H}$ ,  $R_2 = \text{Me}$  in Figure 2). The antimycobacterial MurX inhibitors, isocapuramycin analogs (2'-O-methyluridine-manno-pyranuronate derivatives) were not capable of killing non-replicating Mtb in the LORA assay. The 3'-O-demethyl capuramycin analogs ( $R_1, R_2 = \text{H}$  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 non-replicating Mtb at low concentrations with the MIC<sub>LORA/MABA</sub> value of 1.72, whereas the MIC<sub>LORA/MABA</sub> value for rifampicin was 7.35 (Table 1). The MIC<sub>LORA/MABA</sub> value close to 1 or below 1 is considered to be ideal TB drug lead molecules in our programs.

Bactericidal activity of 3 against dormant Mtb was confirmed *via* the assays using a modified Wayne model under low-oxygen conditions<sup>7,32</sup> and a nutrient deficient Mtb cells<sup>33</sup>; in both assays, no colony-forming unit (CFU) was observed after 15 days' incubation of the Mtb cultures treated with 3 at 100  $\mu$ g/mL, 10  $\mu$ g/mL, and 2.58  $\mu$ g/mL (MIC<sub>LORA</sub>), respectively (Figure 3).

### Rapid antimycobactericidal activity of UT-01320

Because of alteration of molecular target, we were interested in whether a non-MurX inhibitor 3 remains rapid bactericidal activity against replicating Mtb under aerobic conditions. The time-kill experiments were performed at two- and four-fold the MIC of 1, 3, and a few 1<sup>st</sup>-line TB drugs (rifampicin and isoniazid (INH)). Viable cell counting was

performed at every 24 h for 14 days. Colony forming units (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 2× and 4× MIC concentrations are shown in Figure 4. The MurX inhibitor, capuramycin (**1**) yielded log<sub>10</sub> CFU/mL reduction of 3.0 or greater for Mtb in 1 day and **1** killed Mtb completely in 2 days at 24.0 µg/mL, albeit, rifampicin and INH required over 7 days to kill 50% of Mtb at 0.4 and 1.0 µg/mL, respectively (Figure 4A).<sup>12</sup> UT-01320 (**3**) killed >50% of Mtb within 3 days and 100% within 5 days at 3.0 µg/mL (2× MIC), and killed 100% of Mtb within 3 days at 6.0 µg/mL (4× MIC) (Figure 4B). Thus, the fast killing profile of **3** was similar to that of capuramycin; **3** killed Mtb faster than rifampicin 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.<sup>30,34</sup> 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 FIC index analyses for a combination of **2** plus **3** and **1** plus **3** that showed synergistic combination ( $\Sigma$ FIC < 1). It was demonstrated that UT-01320 (**3**) exhibited synergy with SQ 641 (**2**) and capuramycin (**1**) that killed Mtb at much lower concentrations than the MIC<sub>A</sub> and MIC<sub>B</sub>.

### 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 -negative bacteria including *Mycobacterium spp.*, **3** killed Mtb strains selectively. It is interesting to note that unlike other capuramycin analogs, **3** did not kill a fast-growing *M. smegmatis* at >200 µg/mL concentrations (e.g. MICs 6.5 and 25 µg/mL for **1** and **4**, respectively).

### A potential molecular target for UT-01320

Effectiveness of the collected molecules against non-replicating Mtb has been evaluated in the Wayne model<sup>32</sup> as well as the low oxygen recovery assay<sup>20</sup>. Several electron transport system inhibitors, menaquinone biosynthesis inhibitors,<sup>35</sup> a F<sub>1</sub>F<sub>0</sub>-ATPase inhibitor, nitroimidazoles, a RNA polymerase inhibitor (e.g. rifampicin), protein biosynthesis inhibitors (e.g. amikacin, capreomycin), and bacterial DNA gyrase inhibitors were effective in killing non-replicating Mtb, however, in most cases their MIC values against non-replicating Mtb cells are significantly higher than those against aerobically growing Mtb cells.<sup>35</sup> In preliminary assay screening of UT-01320 (**3**) against the potential drug targets for non-replicating Mtb, it was observed that **3** inhibited *E. coli*, *S. aureus*, and *M. smegmatis* RNA polymerases with the IC<sub>50</sub> values of the 100-150 nM range (vs IC<sub>50</sub> 120 nM for rifampicin against *E. coli* RNA polymerase) (Figure 5B), whereas, **3** did not inhibit electron transport systems, menaquinone biosynthesis, *Mtb* serine/threonine kinases (PKnA and B), and DNA gyrase.<sup>36,37,38,39</sup> Capuramycin (**1**) and SQ 641 (**2**) did not inhibit bacterial RNA polymerases even at 100 µM concentrations. Figure 5 summaries MurX and RNA polymerase inhibitory activities of Capuramycin (**1**), SQ 641 (**2**), and UT-01320 (**3**).



## DISCUSSION

It is important to discover effective TB drugs that kill non-replicating Mtb at low concentrations for shortening the treatment duration of current TB chemotherapies.<sup>35</sup> UT-01320 (**3**) killed non-replicating Mtb at 2.58 µg/mL 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 antimycobacterial agent is preferable for TB chemotherapy due to the fact that TB chemotherapy requires a long regimen, so that broad-spectrum anti-TB agents may cause resistant to other bacteria during TB chemotherapy.<sup>35</sup>

To date, SAR studies of capuramycin (**1**) have been performed with the biosynthesis intermediate, A-500359, and the C2'-hydroxy group of capuramycin has been modified with the limited functional groups (i.e. acyl groups).<sup>16,19</sup> Capuramycin has not been developed into a pursuable lead by acyl modification at the C2'-hydroxy group. Our total chemical synthetic scheme enables the modification at the C2'- and C3'-hydroxy groups of capuramycin with chemically stable ether groups. As the result of reliable MurX enzyme assays of a small optimized library of capuramycin analogs followed by antimycobacterial growth inhibitory assays,<sup>26</sup> several new analogs were identified as improved MurX inhibitors (e.g. UT-01304 (**4**)) and non-MurX inhibitors having antimycobacterial activity (Figure 2).<sup>15,26</sup> Methylation at the C2'-hydroxy group of capuramycin (R<sub>1</sub> in Figure 2) alters the molecular target of capuramycin. UT-01320 (**3**) did not interfere with MurX, but exhibited bactericidal activity against non-replicating (dormant) Mtb with the MIC<sub>LORA</sub>/MIC<sub>MABA</sub> value of 1.72 (MIC<sub>LORA</sub>/MIC<sub>MABA</sub> = 7.35 for rifampicin). Effectiveness of **3** in non-replicating Mtb was confirmed *via* assays using a modified Wayne model under low-oxygen conditions and a nutrient deficient Mtb cells at the MIC<sub>LORA</sub> concentrations (2.58 µg/mL) (Figure 3). Cytotoxicity (IC<sub>50</sub>) 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 concentrations (Table 1); the selectivity index (IC<sub>50</sub> in mammalian cells/MIC against Mtb) of **3** was determined to be >266.

Several TB drugs and drug leads that have the potential to kill non-replicating Mtb *in vitro* were identified. Bedaquiline is an Mtb ATPase inhibitor that showed effectiveness in killing non-replicating Mtb *in vitro*. Capreomycin and streptomycin that target protein biosynthesis through binding to the bacterial ribosomes and bacterial topoisomerase II inhibitors (e.g. fluoroquinolones) killed non-replicating Mtb *in vitro*. It was reported that nitric oxide-mediated unique immune responses by metronidazole and PA-824 (a phase II clinical drug) kill non-replicating Mtb under hypoxic conditions.<sup>40</sup> However, in most cases their MIC values against non-replicating 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 non-replicating Mtb at the same MIC concentrations as those under aerobic conditions. Table 4 summarizes the potential molecular targets whose inhibitors were effective in killing non-replicating Mtb. Recently, Ishizaki et al. reported that caprazamycin (an MraY inhibitor) analogue, CPZEN-45 inhibited mycobacterial WecA, a phosphotransferase responsible for capsular polysaccharide synthesis.<sup>41</sup> Although much

studies are required to understand WecA in dormant Mtb, CPZEN-45 killed non-replicating Mtb *in vitro*.<sup>42</sup> 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).<sup>38</sup> UT-01320 (**3**) killed rifampicin-resistant Mtb at low concentrations. This fact may imply that **3** inhibits RNA-polymerases by interfering with the catalytic sites that are different from the rifampicin binding site(s).<sup>43</sup> 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 non-MurX inhibitor **3** exhibited strong synergistic effect with a promising preclinical TB drug, SQ 641 (**2**); a combination of two molecules significantly improved *in vitro* antimycobacterial activity (Table 2). In addition, UT-01320 (**3**) retained a fast-bactericidal 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 SAR studies of 2'-O-methylated capuramycin analogs. We are currently optimizing UT-01320 (**3**) to be more effective against both replicating and non-replicating Mtb by modifying the R<sub>3</sub> group (Figure 2). Through *in vitro* biological evaluation of non-MurX inhibitors identified from capuramycin analogs and *in vivo* evaluation of promising molecules will be reported elsewhere.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

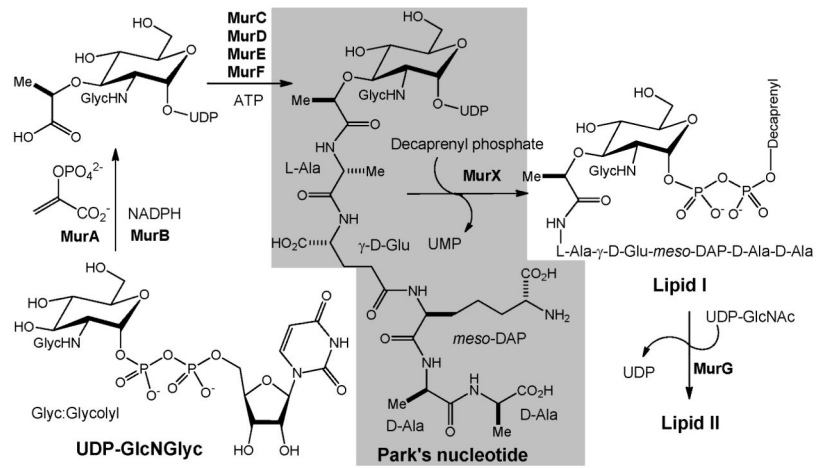
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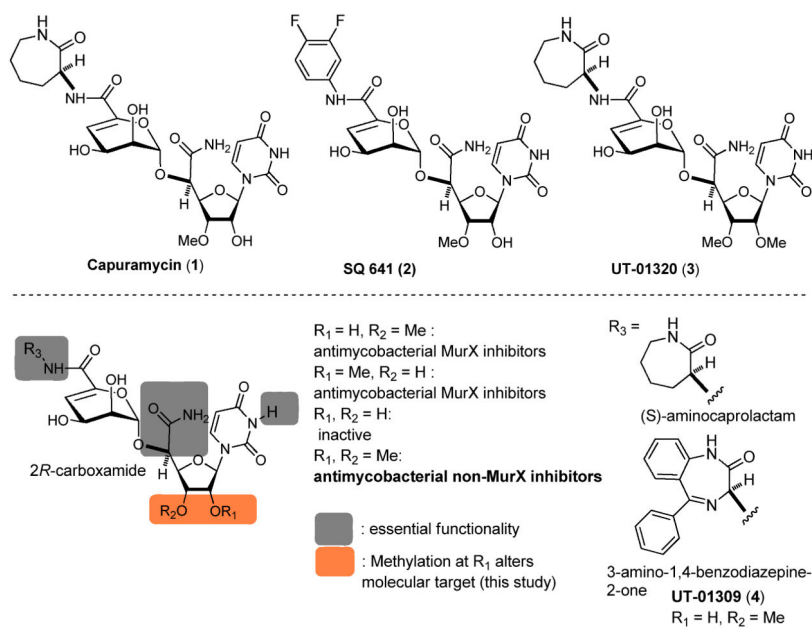
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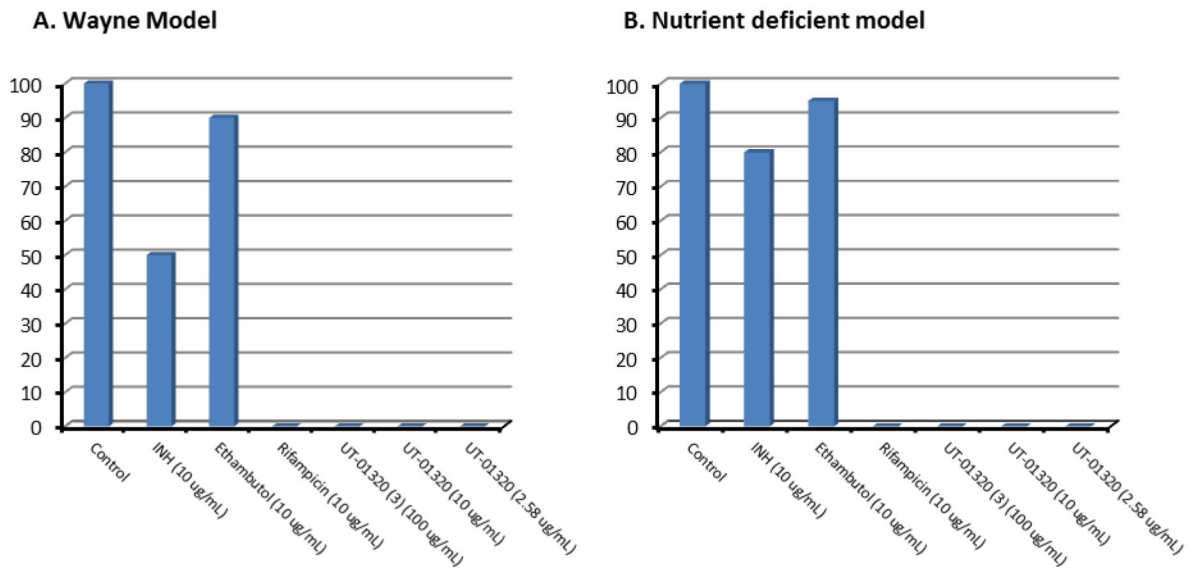
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**Figure 1.** Biosynthesis of lipid II in *M. tuberculosis*. Translocase I (MurX) catalyzes the transformation from Park's nucleotide to lipid I.



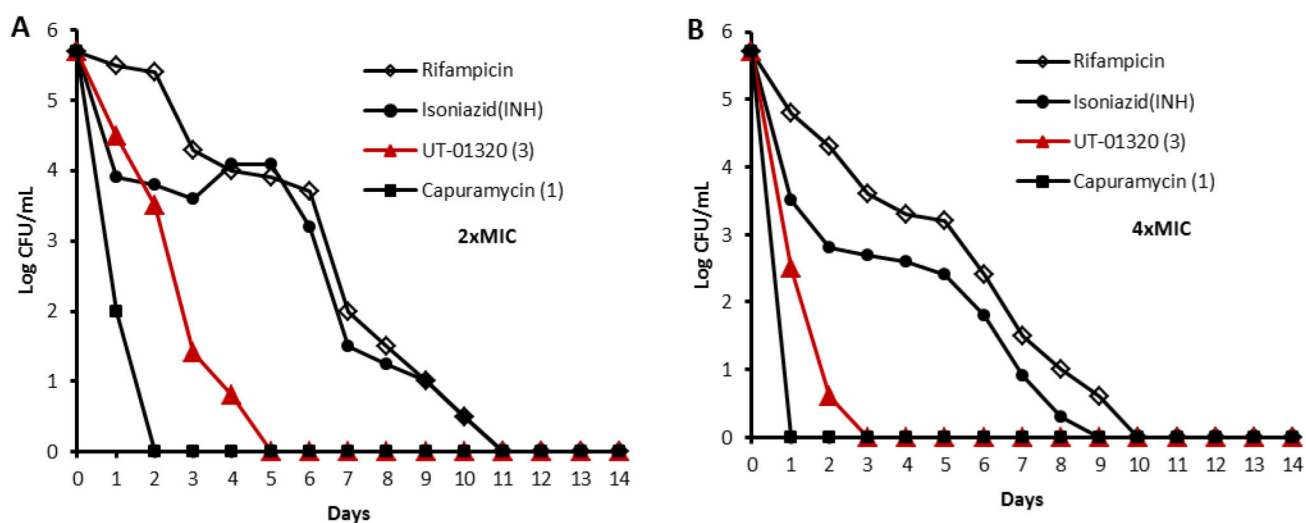
**Figure 2.** Structures of capuramycin (1), SQ 641 (2), UT-01320 (3), and UT-01309 (4) and summary of SAR obtained from a 100-membered library.



**Figure 3.**

Bactericidal activity of UT-01320 against non-replicating Mtb evaluated in Wayne and nutrient deficient models.

*M. tuberculosis* entered dormant states were cultured with the inhibitors. After 96h under anaerobic (Wayne model) or nutrient deficient conditions, 10-, 100-, 1,000- and 10,000-fold serial dilutions from each culture tube was sampled, and 20 $\mu$ L 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 MIC<sub>LORA</sub> concentrations in both assays. **A:** Wayne model.; **B:** a nutrient deficient model.

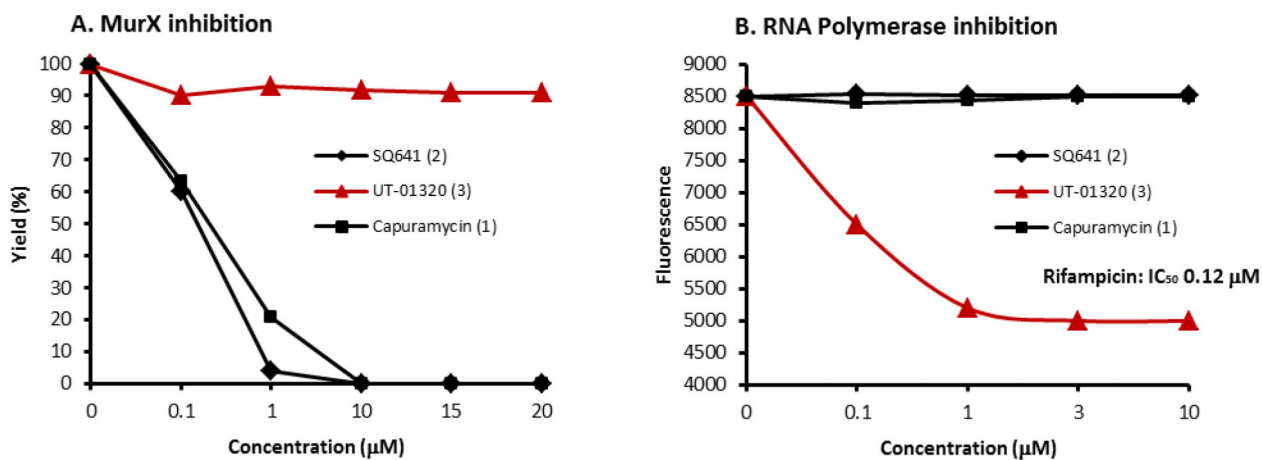


**Figure 4.**

*In vitro* time-kill assessment of capuramycin (1), UT-01320 (3), and the first-line TB drugs (rifampicin and INH).

Log-kill reduction in  $\log_{10}$  CFU/mL at the concentrations of the molecules at 2 $\times$  and 4 $\times$  MIC. **A:** 2 $\times$  MIC of rifampicin (0.4  $\mu$ g/mL), INH (0.2  $\mu$ g/mL), UT-01320 (3.0  $\mu$ g/mL), capuramycin (24.0  $\mu$ g/mL).; **B:** 4 $\times$  MIC of rifampicin (0.8  $\mu$ g/mL), INH (0.4  $\mu$ g/mL), UT-01320 (6.0  $\mu$ g/mL), capuramycin (48.0  $\mu$ g/mL).





**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 IC<sub>50</sub> values.

**Table 1**MICs (Mtb) and IC<sub>50</sub>s (MurX enzyme and mammalian cells) for capuramycin (**1**) and its analogues, **2-4**.

Molecule	IC <sub>50</sub> $\mu$ M MurX	MIC $\mu$ g/mL (MABA) <sup>a</sup>	MIC $\mu$ g/mL (LORA) <sup>b</sup>	IC <sub>50</sub> $\mu$ g/mL HepG2 cells <sup>c</sup>
Capuramycin ( <b>1</b> )	0.127	12.5	>100	>400
SQ 641 ( <b>2</b> )	0.098	1.56	>100	>400
UT-01320 ( <b>3</b> )	>100	1.50	2.58	>400
UT-01309 ( <b>4</b> )	0.095	2.50	>100	>400
Rifampicin (RFP)	-	0.20	1.47	>400
Isoniazid (INH)	-	0.04-0.10	>100	>400
Ethambutol (EMB)	-	0.78	>100	>400

<sup>a</sup>MABA: microplate alamar blue assay.<sup>b</sup>LORA: low-oxygen recovery assay.<sup>c</sup>Cytotoxicity against Vero monkey kidney cells was >400  $\mu$ g/mL.

**Table 2**Fractional inhibitory concentration of a combination of two inhibitor molecules.<sup>a</sup>

Combination of A and B	MIC <sub>A</sub> (µg/mL) MIC <sub>B</sub> (µg/mL) <sup>b</sup>	MIC (µg/mL) C <sub>A</sub> and C <sub>B</sub> <sup>c</sup>	ΣFIC <sup>d</sup>
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	

<sup>a</sup>ΣFIC index for the wells at growth–no growth interface.<sup>b</sup>MIC<sub>A</sub> and MIC<sub>B</sub> are the MIC value of molecule A or B against Mtb (see Table 1).<sup>c</sup>C<sub>A</sub> and C<sub>B</sub> are concentrations of A and B.<sup>d</sup>ΣFIC is the sum of fractional inhibitory concentration calculated by the equation  $\Sigma FIC = FIC_A + FIC_B = C_A/MIC_A + C_B/MIC_B$ .

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

Species and strain	MIC ( $\mu\text{g/mL}$ ) <sup>a</sup>		
	UT-01320 ( <b>3</b> )	RFP <sup>d</sup>	INH <sup>e</sup>
<i>M. tuberculosis</i> H37Rv	1.50	0.20	0.04-0.10
<i>M. tuberculosis</i> H37Rv INHr <sup>b</sup>	2.50	0.20-0.10	>8.0
<i>M. tuberculosis</i> H37Rv RFP <sup>c</sup>	2.45	>4.0	0.05
<i>M. smegmatis</i> ATCC607	>200	>200	>200
<i>S. aureus</i> ATCC6538D-5	>200	-	-
<i>E. faecium</i> ATCC 349	>200	-	-
<i>E. coli</i> ATCC 25019	>200	-	-
<i>K. pneumonia</i> ATCC 8047	>200	-	-
<i>P. aeruginosa</i> ATCC 27853	>200	-	-

<sup>a</sup>The broth dilution method was used.<sup>b</sup>INH-resistant *M. tuberculosis*.<sup>c</sup>RFP-resistant *M. tuberculosis*.<sup>d</sup>RFP: rifampicin.<sup>e</sup>INH: isoniazid.

**Table 4**Potential targets for non-replicating *M. tuberculosis*.

- 
- Electron transport systems
  - Menaquinone biosynthesis
  - F<sub>o</sub>F<sub>1</sub> ATP synthase
  - Activation *via* reductase(s) to form cytotoxic species.
  - Protein kinase (e.g. PknA, PknB, and PknG)
  - DosS/DosR systems
  - Glyoxylate pathway
  - Methyltransferase
  - Protein biosynthesis
  - DNA gyrase
- 

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