Discovery of novel oral protein synthesis inhibitors of

*Mycobacterium tuberculosis* that target leucyl-tRNA synthetase


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RUNNING TITLE: Antitubercular leucyl-tRNA synthetase inhibitors
ABSTRACT

The recent development and spread of extensively (XDR) and totally resistant (TDR) strains of *Mycobacterium tuberculosis*, highlights the need for new antitubercular drugs. Protein synthesis inhibitors have played an important role in the treatment of tuberculosis (TB) starting with the inclusion of streptomycin in the first combination therapies. Although the parenteral aminoglycosides are a key component in multidrug-resistant (MDR) TB therapy, the oxazolidinone, linezolid, is the only orally available protein synthesis inhibitor that is effective against TB. Herein, we show that small molecule inhibitors of aminoacyl-tRNA synthetases (AARS), known to be excellent antibacterial protein synthesis targets, can be designed that are orally bioavailable and effective against *M. tuberculosis* in TB mouse infection models. We applied the oxaborole tRNA trapping (OBORT) mechanism, which was first developed to target fungal cytoplasmic leucyl-tRNA synthetase (LeuRS), to *M. tuberculosis* LeuRS. X-ray crystallography was used to guide design of LeuRS inhibitors that have good biochemical potency and excellent whole cell activity against *M. tuberculosis*. Importantly, their good oral bioavailability translates into *in vivo* efficacy in both the acute and chronic mouse models of TB with comparable potency to the frontline drug isoniazid.
INTRODUCTION

The aminoacyl-tRNA synthetases (AARS) are a family of essential enzymes that are required for protein synthesis in all cells (38). Although various family members have been targeted for the design of novel antibacterial (37) only the isoleucyl-tRNA synthetase inhibitor, mupirocin, is an FDA-approved antibiotic (36). However, mupirocin is only approved for the topical treatment of staphylococcal and streptococcal skin infections (36) and *M. tuberculosis* is naturally resistant to this agent (31). Leucyl-tRNA synthetase (LeuRS) is a class I AARS that has two active sites separated by a distance of 30 Å; a synthetic site that aminoacylates tRNA^Leu^ and an editing site that ensures the fidelity of translation by a proofreading mechanism (7, 10, 19, 26). Recently boron-containing compounds known as oxaboroles have been shown to inhibit LeuRS by the oxaborole tRNA trapping (OBORT) mechanism (29), which exploits the ability of the boron atom to bond to the cis-diols of the 3′-terminal adenosine nucleotide, Ade76, of tRNA^Leu^. The resulting covalent adduct traps the 3′-end of tRNA^Leu^ in the editing site in a non-productive complex, inhibiting leucylation and thereby protein synthesis (29). Here we report the discovery of novel 3-aminomethyl derivatives that have potent antitubercular activity.
MATERIALS AND METHODS

Chemical Synthesis. Starting materials used were either available from commercial sources or prepared according to literature procedures and had experimental data in accordance with those reported. The syntheses of the compounds are described in detail in the Supplementary Material.

Expression, purification and crystallization of M. tuberculosis LeuRS editing domain. A DNA fragment coding for the region G309 to I513 of M. tuberculosis LeuRS (Uniprot P67510) was cloned into pETM-11 using the Ncol and XdeI restriction sites (EMBL). The protein containing an N-terminal six-histidine tag was prepared and purified following a similar protocol as for the E. coli LeuRS (26), except that the nickel affinity chromatography was conducted at pH 8.0. Protein was stored in buffer consisting of 20 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM MgCl2 and 5 mM 2-mercaptoethanol. Crystallization was performed at 20°C by the hanging drop vapor diffusion method. The solutions for the ternary complexes were prepared with 10 mg/mL LeuRS, 5 mM AMP and 1 mM of the corresponding benzoxaborole compound (provided by Anacor Pharmaceuticals, Palo Alto, CA). Initial crystals were obtained at 15 mg/mL LeuRS, 5 mM AMP and 1 mM of the corresponding benzoxaborole compound (provided by Anacor Pharmaceuticals, Palo Alto, CA). Crystals were obtained by mixing 2 μL of this solution with 2 μL of reservoir solution containing 0.1 M Bis-TRIS (pH 5.5), 22% (w/v) PEG 10000 and 0.2 M ammonium acetate. Quality and size of final diffracting crystal was improved by decreasing LeuRS concentration to 10 mg/mL and PEG 10000 to 17% (w/v). The crystals were frozen directly in liquid nitrogen in the mother liquor containing 15% (v/v) ethylene glycol as a cryoprotectant.

Structure determination and refinement. All diffraction data sets were collected at the European Synchrotron Radiation Facility (ESRF, Grenoble, France). Data were integrated and scaled with the XDS suite (15). Further data analysis was performed with the CCP4 suite (3). The structure of the LeuRS:AMP-compound 6 complex was initially solved by molecular replacement with PHASER (22) using the E. coli LeuRS editing domain structure(20) (PDB 2AJG) as a model. The model was improved by automatic building using ARP-WARP (28) and manual adjustments were made with COOT (9). The structures of the complexes with the compounds 14 and 16 were solved using the editing domain of M. tuberculosis LeuRS (described above) as a model. All models were refined using REFMAC5 with anisotropic B-factors. Structure quality was analyzed with MOLPROBITY(4) (http://molprobity.biochem.duke.edu/) and showed all residues in allowed regions (with 95.1-
98.0% of residues in favored regions) for the different models. Figures were drawn with PYMOL (http://www.pymol.org/).

**Aminoacylation assay.** An N-terminal six histidine-tagged LeuRS from *M. tuberculosis* H37Rv, which was codon-optimized for *E. coli* (GenScript, Piscataway NJ, USA), was overexpressed and purified according to Novagen (Madison, WI, USA) using an *E. coli* BL21(DE3) T7 RNA polymerase over-expression strain. Experiments were performed in 96-well microtiter plates, using 80 μL reaction mixtures containing 50 mM HEPES-KOH (pH 8.0), 30 mM MgCl₂, 30 mM KCl, 13 μM L-[¹⁴C]leucine (306 mCi/mmol, Perkin-Elmer), 15 μM total *E. coli* tRNA (Roche, Switzerland), 0.02% (w/v) BSA, 1 mM DTT, 0.2 pM LeuRS and 4 mM ATP at 30°C. Reactions were started by the addition of 4 mM ATP. After 7 minutes, reactions were quenched and tRNA was precipitated by the addition of 50 μL of 10% (w/v) TCA and transferred to 96-well nitrocellulose membrane filter plates (Millipore Multiscreen HTS, MSHAN4B50). Each well was then washed three times with 100 μL of 5% TCA. Filter plates were then dried under a heat lamp and the precipitated L-[¹⁴C]leucine tRNA<sub>Leu</sub> were quantified by liquid scintillation counting using a Wallac MicroBeta Trilux model 1450 liquid scintillation counter (PerkinElmer, Waltham, MA, USA).

**IC₅₀ determination.** To determine the inhibitor concentration, which reduces enzyme activity by 50% (IC₅₀), increasing concentrations of compound inhibitors that covered the IC₅₀ value were incubated with LeuRS enzyme, tRNA and L-leucine for 20 minutes. Reactions were initiated by the addition of 4 mM ATP. Reactions were stopped after 7 minutes then precipitated and counted to quantify radioactivity. IC₅₀ values were determined using a 4-parameter logistic nonlinear regression model (Graphpad Software Inc. (La Jolla, CA, USA).

**Isothermal titration calorimetry (ITC) experiments.** ITC experiments were performed at 25°C using an ITC200 system (MicroCal, GE Healthcare). The editing domain protein was dialyzed for 12 hours against the titration buffer (50 mM HEPES-KOH, 30 mM KCl and 30 mM MgCl₂, pH 8.0) at 4°C. Protein solutions at 50 μM plus AMP at 10 mM in the calorimetric cell were titrated with the appropriate compound dissolved in dialysis buffer. Compound solutions at 1-5mM plus AMP at 10 mM were incubated at 37°C during 1 hour before titrations. The heat evolved after each ligand injection was obtained from the integral of the calorimetric signal. The resulting binding isotherms were analyzed by nonlinear least-squares fitting of the experimental data to a single site model. Analysis of the data was performed using Microcal Origin software (OriginLab version 7). Experiments were
performed at least twice. The variability in the binding experiments was estimated to be 5% in the binding enthalpy; and 10% in both the binding affinity and the number of sites.

**Determination of minimum inhibitory concentration for M. tuberculosis.** MIC determinations were mainly determined using resazurin (Alamar Blue) as an indicator for cell growth (6) with additional determinations as described by Ollinger et al (25).

**Selection of M. smegmatis ATCC 700084 single-step mutants.** Resistant mutants to compound 1 were isolated on Middlebrook 7H10 medium plus 10% (v/v) oleic albumin dextrose catalase (OADC) supplement (Becton Dickinson) containing compound 1 at 4xMIC. Resistance was confirmed by measuring the mutants MIC value essentially as described by Collins et al (6).

**Selection of M. tuberculosis single-step mutants.** M. tuberculosis mutants resistant to compound 1 and 13 were isolated as described by Loerger et al (14). Mutants were isolated on Middlebrook 7H10 medium plus 10% (v/v) OADC (Becton Dickinson) containing compound 1 and 13 at 5x or 10x MIC90. Resistance was confirmed by measuring MIC90 on solid medium – defined as minimum concentration that inhibits 99% of CFU (34). Genome sequencing and identification of polymorphisms were essentially carried out as described by Loerger et al (14). In order to determine the rate of spontaneous resistant mutants, M. tuberculosis H37RV was grown at 37°C in fresh Middlebrook 7H9-ADC-Tween 80 to mid-exponential phase and then diluted in fresh Middlebrook 7H9-ADC-Tween 80 to 5x10^8 CFU/mL. Middlebrook 7H10-OADC plates with 4 and 10-fold MIC of each compound were inoculated with 10^8, 10^7, 10^6, and 10^5 CFU/plate, and the plates were incubated at 37°C for 3 to 4 weeks. The frequency of appearance of resistant mutants was calculated, and isolated colonies were restreaked onto Middlebrook 7H10-OADC agar containing the drugs and on plates without the drug.

**Time-kill assay.** Compounds were added at 20xMIC to a 10 mL exponential culture of M. tuberculosis H37Rv (~5x10^5 cfu/mL) in Middlebrook 7H9 with 10% (v/v) OADC and 0.05% (v/v) Tween-80. At specified time points, aliquots of cultures were withdrawn, serially diluted and plated on solid culture medium. Plates were then incubated at 37°C and CFU were counted after 3 to 4 weeks.

**In vitro cytotoxicity assay.** Vero epithelial cells (from African green monkey; ATCC CCL-81) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and maintained in a humidified incubator (37°C, 5% CO2). Cells were dislodged with a cell scraper, collected by centrifugation, resuspended in fresh medium at ~10^6 cells/mL, dispensed into 96-well microtitre plates (100mL/well) and incubated for...
18h at 37°C. Two-fold serial dilutions of test compounds (800–0.4 mg/L) in DMEM with 168 FBS were subsequently added and cells incubated for another 72h. From triplicate studies, the cytopathic effects of compounds were evaluated colorimetrically using the MTT cell proliferation assay (ATCC). IC50 data were obtained from dose–response curves plotted using Graphpad prism 5.

Mitochondrial protein synthesis assay. Human liver carcinoma derived HepG2 cell line was obtained from the ATCC (HB-8065). HepG2 cells were grown in Dulbecco's Modified Eagle's medium containing 10% Fetal Calf Serum, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids and 50 units/mL penicillin-streptomycin at 37°C with 5% CO2. HepG2 cells were seeded in 96-well plates at 3000 cells/200 μL/well in cell culture medium. Cells were then grown in the presence of the compounds at 37 °C in 10% CO2 for 7 days in at least duplicate concentrations, with the medium and compounds being replaced on the fourth day. After 7 days the levels of SDHA and COX1 were determined by using the MitoSciencesTM In-Cell ELISA kit. Janus Green staining was used to determined cell viability after 7 days (In-cell ELISA kit, cat # MS643).

Mouse pharmacokinetic analysis. The studies were conducted using female CD-1 for compounds 1, 11 and 12, while BALB/C mice were used for compound 13. Mice body weights were 19-28 g and on the morning of dosing, mice were split randomly into 3 dosing groups to receive test article by either tail-vein injection (IV) or oral gavage (PO). After dosing, blood samples were collected via cardiac puncture at specific time points (n=3 mice/time point) through 24 hours (K2EDTA as anticoagulant) and processed for plasma. Antibiotic concentrations in the plasma samples were analysed by LC/MS/MS. The LC/MS/MS analysis was conducted using analyte/internal standard peak area methods. The internal standard was AN3365 (13) and the instrument was a API4000 QTRAP (AB Sciex). The limit of quantititation (LOQ) was 1 or 2 ng/mL. Pharmacokinetic analyses of the mean plasma concentration-time profiles were performed using WinNonlin Pro version 5.2. A compartmental model was used for the IV data and non-compartmental model for PO data. The time-concentration curve after an IV dose showed a bi-exponential decline with first-order elimination. Compound 1 and 13 were formulated in saline (0.9% w/v NaCl) at 7.5 mg/mL and the pH adjusted to >5 by the addition of NaOH. Compound 11 was formulated to 6.5 mg/mL in Water/Dimethylacetamide/EtOH (76/19/5) and the pH adjusted to >5 by the addition of NaOH. Compound 12 was formulated to 7.5 mg/mL in PEG300/PG/water (55/25/20) and the pH adjusted to >5 by the addition of NaOH.
Mouse plasma protein binding determination. Compounds were added to 1.5-mL aliquots of mouse plasma and plasma ultrafiltrate to the following concentrations: 1 µg/mL and 10 µg/mL, and then incubated in a shaking water-bath at 37 °C for 15 minutes. Both samples were treated similarly and a 0.5-mL aliquot was removed from each tube and added to the filter reservoir of the Microcon® centrifugal filter devices (Ultracel YM-30, MWCO=30K Da, Bedford, MA). The devices were centrifuged at 1000 x g for 10 minutes and 100 µL of filtrate was transferred to the 96-well plate and diluted 5-fold. Ten-µL volumes of the samples were injected and analyzed with the LC/MS/MS system. All samples were analysed in duplicate. Quantitation was based on peak area ratio of analyte over internal standard and all integrations were performed with peak areas using Analyst version 1.4.1 (Applied Biosystems, Foster City, CA 94404, USA). Plasma protein binding was calculated, based on the following equation:

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\text{Plasma Protein Binding (\%)} = \left( \frac{\text{Peak Area}_{\text{Plasma Ultrafiltrate Spiked}} - \text{Peak Area}_{\text{Plasma Filtrate}}} {\text{Peak Area}_{\text{Plasma Ultrafiltrate Spiked}}} \right) \times 100
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Murine model of acute TB infection using C57/BL GKO IFNγ mice. Eight- to 10-week-old female specific-pathogen-free C57BL/6-Ifngtm1ts mice (IFNγ gene-disrupted [GKO] mice) were purchased from Jackson Laboratories, Bar Harbor, ME. The mice were infected via a low-dose aerosol exposure with \textit{M. tuberculosis} Erdman in a Middlebrook aerosol generation device (Glas-Col Inc., Terre Haute, IN) as described previously (18). One day post-aerosol, three mice were sacrificed to verify the uptake of 50 to 100 CFU of bacteria per mouse. Each treatment group consisted of five mice and treatment was started 10-13 days post-infection and continued for nine or fourteen consecutive days. Five infected mice were sacrificed at the start of treatment as pretreatment controls. Drugs were administered daily by oral gavage. Lungs were harvested 24 hours after the last administration and all lung lobes were aseptically removed, homogenized and frozen. Homogenates were plated on 10% OADC-7H11 medium for 21 days at 37°C. All animal studies strictly adhered to the protocols and regulations approved by their respective Animal Care and Use Committees of University of Illinois at Chicago and Colorado State University.

Murine model of acute and chronic TB infection using C57/BL6J mice. Specific pathogen-free, 8-10 week-old female C57BL/6 mice were purchased from Harlan Laboratories and were allowed to acclimate for one week. The experimental design for the acute assay has been previously described (30). In brief, for the acute assay, mice were intratracheally infected with 100,000 CFU/mouse (\textit{M. tuberculosis} H37Rv strain). Products
were administered for 8 consecutive days starting one day after infection. For the chronic
assay, mice were intratracheally infected with 100 CFU/mouse and the products administered
daily (7 days a week) for 8 consecutive weeks starting 6 weeks after infection. Lungs were
harvested 24 hours after the last administration. All lung lobes were aseptically removed,
homogenized and frozen. Homogenates were plated on 10% OADC-7H11 medium and
incubated for 21 days at 37°C. The viable colony forming units were converted to logarithms,
which were then evaluated by a one-way analysis of variance, followed by a multiple-
comparison analysis of variance by a one-way Tukey test (SigmaStat software program).
Differences were considered significant at the 95% level of confidence. All animal studies
were ethically reviewed and carried out in accordance with European Directive 2010/63/EU
and the GSK Policy on the Care, Welfare and Treatment of Animals.

Murine model of chronic TB infection using BALB/c mice. Six- to 8-week-old female
specific-pathogen-free immunocompetent BALB/c mice (Charles River, Wilmington, MA)
were infected via a low-dose aerosol exposure to *M. tuberculosis* Erdman as described (18).
One day post-aerosol, three mice from each run were sacrificed to verify the uptake of 50 to
100 CFU of bacteria per mouse. Each group consisted of five to six mice at each time point.
Treatment was started 3 weeks post-infection and continued for 12 weeks. Five infected mice
were sacrifice at the start of treatment as pretreatment controls. Drugs were administered 5
days per week by oral gavage, for four weeks. Lungs were harvested 72 hours after the last
administration. All lung lobes were aseptically removed, homogenized and frozen.
Homogenates were plated on 10% OADC-7H11 medium and incubated for 21 days at 37°C.
All animal studies strictly adhered to the protocols and regulations approved by Colorado
State University’s Animal Care and Use Committee.
RESULTS AND DISCUSSION

**Discovery of antitubercular LeuRS inhibitors.** A focus library of 20 benzoxaboroles was initially screened against *M. tuberculosis* H37Rv, which yielded AN3016 and AN3017 with minimal inhibitory concentration (MIC) of 1 µg/mL and 1.8 µg/mL and LeuRS half-maximal inhibitory concentration (IC_{50}) of 3.5 µM and 0.64 µM, respectively (Fig. 1). When we combined both the 3-aminomethyl and the 7-ethoxy substitutions in one moiety, compound 1, it gave significantly better activity with a LeuRS IC_{50} of 0.28 µM and an MIC of 0.26 µg/mL (Fig. 1). To confirm that compound 1 was targeting LeuRS in the cell, we obtained resistant mutants of *M. tuberculosis* H37Rv and *Mycobacterium smegmatis* ATCC 700084. The leuS gene, which codes for LeuRS, was sequenced from selected resistant mutants and mutations were found in both organisms, which was consistent for an OBORT LeuRS inhibitor (Fig. 2 and Table 1). Therefore, we progressed compound 1 into an *in vivo* mouse pharmacokinetic study to determine suitability for testing in an acute TB mouse model. Compound 1 was dosed by oral administration (PO) at 30 mg/kg, which yielded an area under the curve over 24 hrs (AUC_{0-24}) of 15 h*µg/mL with a maximum plasma level (C_{max}) of 4.33 µg/mL and oral bioavailability of 55% (data not shown). Since the existing *in vivo* efficacy mouse model used *M. tuberculosis* Erdman and not *M. tuberculosis* H37Rv, the MIC of compound 1 was confirmed against the Erdman strain as well as some drug-resistant isolates (Table 2). The *M. tuberculosis* Erdman MIC value was 0.127 µg/mL, which was not affected by resistance mechanisms to rifampin, isoniazid or streptomycin. Compound 1 was then tested in a BALB/c acute model of TB dosed at 100 mg/kg twice-a-day (BID) for 3 weeks with weekend drug holidays. The control drug, PA-824 (Pretomanid) (35), was dosed at 100 mg/kg once-a-day (QD), which gave a 1.9 log_{10} reduction in colony forming units (CFU) from mouse lungs compared to only a 0.4 log_{10} reduction in CFU for compound 1 (data not shown). Therefore, we separated the two enantiomers in compound 1 by chiral HPLC and determined their activities (Fig. 1). The active enantiomer was the (S)-isomer, compound 2, which had a LeuRS IC_{50} of 0.13 µM and an MIC of 0.13 µg/mL, while the (R)-isomer was barely active with an IC_{50} of 21 µM. The active enantiomer, compound 2, was then tested at 200 mg/kg BID for 9 days in the acute TB model using an IFN-γ gene knock-out (GKO) mouse (17), which showed a 2 log_{10} reduction in lung CFU and a 1.5 log_{10} reduction in spleen CFU compared to the control mice (Fig. 3). However, this was not as good as the frontline drug isoniazid (INH), which gave a 2.8 and 3.4 log_{10} reduction in CFU from lungs and spleen, respectively, when dosed at 25 mg/kg.
**M. tuberculosis** LeuRS inhibitor co-crystalization. In order to improve potency we performed structural and biophysical studies to understand the binding mode of these novel 3-aminomethyl benzoxaboroles to *M. tuberculosis* LeuRS. Crystallization trials with different editing domain constructs of *M. tuberculosis* LeuRS were attempted in the presence of compound 2 with either AMP or longer nucleotides such as CytAde or CytCytAde, which might act as surrogates for the 3’-end of the tRNA acceptor stem (32). An editing domain construct encompassing residues G309-I513 gave co-crystals with compound 2 and AMP that diffracted to 1.3 Å resolution, which permitted structure determination (Fig. 4A, 4B and Table S1). Compound 2 forms a bidentate covalent adduct with AMP (Fig. 4B), which mimics Ade76 of the tRNA acceptor end (13, 19, 26). The amino acid residues, T336-T337, of the threonine-rich region provides multiple H-bonding interactions to the covalent adduct, and L432 and Y435 of the AMP binding loop have extensive H-bonding and hydrophobic contacts with AMP (Fig. 4B). In addition, the amino group of compound 2 makes three key interactions with the carboxylic acid side chains of D447 and D450 and the carbonyl of M441. The 7-ethoxy enables not only a new interaction to R449 but also packs with the Ade76 ribose, thus further stabilizing the boron-tRNA adduct (Fig. 4B). Superposition of the compound 2 adduct bound structure with that of the *E. coli* LeuRS editing domain with methionine bound (20) shows that the 3-aminomethyl benzoxaborole moiety occupies the same position as the non-cognate amino acid (Fig. 4C). Although this moiety mimics the interactions established by the amino and the oxygen carbonyl groups of methionine, it lacks atoms at the positions of the Sδ-Cε atoms of methionine (Fig. 4C), which suggests that there is additional space to make further interactions.

**SAR of potent antituberculars.** Several derivatives were synthesized with different substitutions at position 4 as well as at positions 5 and 6 to explore this hypothesis (Fig. 1). The halogen substitutions 5-Cl (compound 5) and 6-F (compound 7) were not well tolerated with LeuRS IC₅₀ values worse than the original compound 1 and MIC values of 1.1 µg/mL or greater. The most potent analogs were compounds with halogen substitutions at position 4, bromo (compound 11), chloro (compound 4) and fluoro (compound 6), which improved MIC values more than 5-fold over compound 1 (Fig. 1). The phenyl (compound 10) substitution was not tolerated with LeuRS IC₅₀ values of 28 µM (Fig. 1). However, it must be noted that the significant improvements in MIC values for compounds 4, 6 and 11 were not fully reflected in their IC₅₀ values as determined using an aminoacylation assay with *M. tuberculosis* LeuRS, which could be due to the way that OBORT inhibitors indirectly inhibit...
aminoacylation by preventing Ade76 binding to the aminoacylation active site (29). We therefore decided to measure the direct binding of the compounds to the editing domain using isothermal titration calorimetry (ITC) and found that the 4-Cl and 4-Br substitutions significantly enhanced the affinity of the compounds to the *M. tuberculosis* LeuRS editing domain (Table 3). The increased affinity is due to a significant gain in the enthalpic contribution (3.1-4.5 Kcal mol\(^{-1}\)), which is consistent with additional favourable interactions being established by the halogen atoms in the editing site. To confirm whole cell activity was derived from inhibition of LeuRS we selected 6 *M. tuberculosis* mutants resistant to compound 13 and sequenced their *leuS* gene, while two additional mutants were selected for whole genome sequencing. All 8 resistant mutants had SNP in their *leuS* genes and the mutations were located in the editing domain as expected for OBORT LeuRS inhibitors (13, 24, 29) (Table S2). To further explore interactions at 4-position, we co-crystallised compounds with 4-Cl and 4-Br substitutions in the presence of AMP and solved the structures of the ternary complexes at 1.45 and 1.47 Å resolution, respectively (Table S1, Fig. S1). The structures showed that the halogenated compounds bind to the editing site without major structural changes and as predicted, the 4-Cl/Br atoms now occupy the position of the sulphur in bound methionine (Fig. S2A) allowing van der Waals interactions with the neighbouring protein atoms (Fig. S2B). These results confirmed the importance of the size and nature of the substitution at position 4, and agreed well with the *in vitro* activities and thermodynamic analysis (Fig. S2C).

We selected the three most potent compounds, 11, 12 and 13, for *in vivo* murine pharmacokinetic analysis and we dosed mice both intravenously (IV) and orally (PO). All three compounds showed improvements in plasma exposure as measured by AUC after oral administration over compound 2 (Table 4). Therefore, we tested them in a GKO mouse model of acute TB, which showed all to be very efficacious with the racemate compound 11 having similar efficacy to isoniazid (Fig. 5AB). In a chronic TB BALB/c mouse model, all compounds showed good efficacy (Fig. 5C) with compound 14, the \((S)\)-isomer of compound 11, being the most potent. In addition, we observed that compounds 13 and 14 did not show any cross-resistance against multidrug-resistant isolates (Table S3).

**Inhibition of mitochondrial protein synthesis.** Although protein synthesis inhibitors are validated TB drugs they are associated with some safety concerns, for example myelosuppression and neuropathy observed with linezolid (16) and deafness induced by aminoglycosides (12). The similarity between the bacterial and mitochondrial protein synthesis machinery (2) and their subsequent inhibition of mitochondrial protein synthesis is
thought to drive these toxicities. Therefore, we tested the ability of compound 14 and some close analogues to inhibit mitochondrial protein synthesis in the human liver carcinoma cell line HepG2 (Table 5). The ribosomal protein synthesis inhibitors linezolid, chloramphenicol and doxycycline inhibited the synthesis of the mitochondrial derived COX1 protein with EC50 values of between 23 and 31 µM, while erythromycin and compounds 12, 13 and 14 had EC50 values of >150 µM. Although this could be due to poor mitochondrial penetration, it is interesting to note that human mitochondrial LeuRS is known to be editing defective as it lacks key conserved amino acid residues in the AMP binding loop and amine binding pocket (21).

**Compound 14 in vitro and in vivo activity.** Since the racemate 11 had similar activity to isoniazid, which is an in vitro bactericidal compound (8), in the acute GKO mouse model (Fig. 5B) we tested the enantiomer pure compound 14 for in vitro bactericidal activity over 14 days at 20-fold its MIC (Fig. 6). The profile for compound 14 was very similar to the bacteriostatic protein synthesis inhibitor, linezolid, which was different from moxifloxacin a known bactericidal compound (33). Therefore, further tests of compound 14 in a murine chronic TB model (Fig. 7) were performed in parallel with the protein synthesis inhibitor linezolid. Compound 14 at 30 mg/kg QD showed good efficacy resulting in a 2.4 log10 reduction in CFU compared with a 2.6 log10 reduction in CFU for 100 mg/kg QD of linezolid.

In order to establish the optimal dosing regimen for compound 14 we tested it in the acute TB murine model and compared the efficacy from the following dosing regimes, BID, QD and q48h (Fig. 8). Similar to results in the chronic model, compound 14 was more active at lower doses than linezolid and dosing every other day (q48h) was as efficacious as QD or even BID, which suggests that the preliminary pharmacodynamic driver for efficacy was AUC/MIC.

**Resistance and LeuRS inhibitors.** Emergence of resistance during streptomycin monotherapy (1) and its noted reduction by the addition of p-aminosalicylic acid (11, 23) lead to the paradigm of combination TB drug therapy. The current core TB regimen calls for a four-drug combination of isoniazid, rifampin, ethambutol and pyrazinamide. Although compound 14 has a lower in vitro resistance frequency than isoniazid (Supplementary Table 7), the emergence of resistance to epetraborole (GSK2251052/AN3365), another 3-aminomethylbenzoxaborole LeuRS inhibitor, in a minority of patients in a complicated urinary tract infection trial might suggest some caution (24). However, the addition of trimethoprim to rifampicin, which has a similar resistance problem, in an urinary tract infection trial demonstrated the benefit of combination therapy in overcoming emergence of resistance.
rifampin-resistant strains (27). This suggests that the risk from emergence of resistance to
OBORT LeuRS inhibitors will likely be mitigated when used in combination therapy.

**Beneficial properties.** Since combination therapy necessitates a larger armamentarium than
regular monotherapy, the demonstration for the first time that an oral AARS inhibitor can be
a potent antitubercular adds a potential new tool to fight TB, which is timely noting the recent
onset of TDR-TB. In addition, the combination of low plasma protein binding, molecular
weight (207-285) and logD7.4 (-0.04-0.76), like the frontline TB drugs isoniazid,
pyrazinamide and ethambutol, suggests that this novel chemical class deserves further
optimisation and hopefully progression into clinical trials.
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ACCESSION CODES

Atomic coordinates and structure factors for the compound 2, 11 and 13 have been deposited in wwPDB with the following codes, 5AGR, 5AGS, 5AGT.
Table 1. MIC values for compound 1 resistant mutants.

<table>
<thead>
<tr>
<th>Organism/Mutant /SNP</th>
<th>Agar MIC (µg/mL)</th>
<th>Organism/Mutant /SNP</th>
<th>Liquid MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis H37Rv</td>
<td>0.6-1.3</td>
<td>M. smegmatis ATCC 700084</td>
<td>1</td>
</tr>
<tr>
<td>RM1leuS Y435C</td>
<td>21</td>
<td>RM1leuSΔ421-462</td>
<td>&gt;256</td>
</tr>
<tr>
<td>RM2leuS S311L</td>
<td>21</td>
<td>RM2leuS A428T</td>
<td>64</td>
</tr>
<tr>
<td>RM3leuS D450Y</td>
<td>21</td>
<td>RM3leuS R435C</td>
<td>8</td>
</tr>
<tr>
<td>RM4leuS S311L</td>
<td>21</td>
<td>RM4leuS A428T</td>
<td>64</td>
</tr>
</tbody>
</table>

The residues Y435 and D450 stabilize the adduct formed by compound 1 with AMP in the editing site of *M. tuberculosis* LeuRS (Figure 4b). The residue S311, like the equivalent *E. coli* LeuRS residue (20), interacts with phosphate of Ade76 thus stabilizing the adduct in the editing site. However, S311 is located at the flexible N-terminal part of our editing domain construct of *M. tuberculosis* LeuRS and thus is not visible in the crystal structure (Figure 4b).

SNP = single nucleotide polymorphism, RM = resistant mutant.
<table>
<thead>
<tr>
<th>Strain</th>
<th>Cmp 1</th>
<th>PA-824</th>
<th>RIF</th>
<th>INH</th>
<th>STR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em> Erdman</td>
<td>0.127</td>
<td>0.116</td>
<td>0.018</td>
<td>0.244</td>
<td>0.369</td>
</tr>
<tr>
<td>rRIF</td>
<td>0.120</td>
<td>0.128</td>
<td>&gt;4</td>
<td>0.383</td>
<td>0.216</td>
</tr>
<tr>
<td>rINH</td>
<td>0.059</td>
<td>≤0.063</td>
<td>0.037</td>
<td>&gt;8</td>
<td>0.202</td>
</tr>
<tr>
<td>rSTR</td>
<td>0.113</td>
<td>0.189</td>
<td>0.082</td>
<td>0.344</td>
<td>&gt;16</td>
</tr>
</tbody>
</table>

r, resistance to RIF = Rifampin, INH = Isoniazid or STR = Streptomycin
<table>
<thead>
<tr>
<th>Compound/parameter</th>
<th>$K_d^*$ (µM)</th>
<th>$\Delta G_{ap}$ (Kcal mol$^{-1}$)</th>
<th>$\Delta H_{ap}$ (Kcal mol$^{-1}$)</th>
<th>$-T\Delta S_{ap}$ (Kcal mol$^{-1}$)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3.7</td>
<td>-7.4</td>
<td>-1.1</td>
<td>-6.3</td>
<td>1.05</td>
</tr>
<tr>
<td>13</td>
<td>0.075</td>
<td>-9.7</td>
<td>-4.2</td>
<td>-5.5</td>
<td>1.19</td>
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<tr>
<td>11</td>
<td>0.040</td>
<td>-10.0</td>
<td>-5.5</td>
<td>-4.6</td>
<td>1.02</td>
</tr>
</tbody>
</table>

* The error in the thermodynamic binding parameters is about 5% for the apparent binding enthalpy, and 10% for the apparent binding constant and the number of sites (n). Values in the table are the average of at least 2 independent experiments.
Table 4. Murine Pharmacokinetic Parameters

<table>
<thead>
<tr>
<th></th>
<th>Cmp2</th>
<th>Cmp11</th>
<th>Cmp12</th>
<th>Cmp13</th>
<th>Cmp14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (µg/mL) @ 5 min</td>
<td>8.9</td>
<td>18.0</td>
<td>13.7</td>
<td>13.6</td>
<td>17.1</td>
</tr>
<tr>
<td>30 15 30 30 30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL (mL/h/kg)</td>
<td>2180</td>
<td>328</td>
<td>1119</td>
<td>582</td>
<td>687</td>
</tr>
<tr>
<td>Vss (mL/kg)</td>
<td>2116</td>
<td>968</td>
<td>3805</td>
<td>3142</td>
<td>3221</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>2.1</td>
<td>3.0</td>
<td>3.4</td>
<td>5.4</td>
<td>4.7</td>
</tr>
<tr>
<td>AUC_{0-∞} (h*µg/mL)</td>
<td>13.8</td>
<td>45.8</td>
<td>26.8</td>
<td>51.6</td>
<td>43.7</td>
</tr>
<tr>
<td>α-t_{1/2} (hr) [%AUC]</td>
<td>0.06 [5]</td>
<td>0.09 [2]</td>
<td>0.11 [7]</td>
<td>0.10 [2]</td>
<td>0.05 [5]</td>
</tr>
<tr>
<td>β-t_{1/2} (hr) [%AUC]</td>
<td>1.5 [95]</td>
<td>2.08 [98]</td>
<td>2.53 [93]</td>
<td>3.83 [98]</td>
<td>3.40 [95]</td>
</tr>
<tr>
<td>#PO (mg/kg)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>3.4</td>
<td>7.2</td>
<td>5.0</td>
<td>6.4</td>
<td>6.3</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.50</td>
<td>1.00</td>
<td>1.00</td>
<td>0.25</td>
<td>0.50</td>
</tr>
<tr>
<td>AUC_{24} (h*µg/mL)</td>
<td>13.2</td>
<td>35.9</td>
<td>23.8</td>
<td>47.5</td>
<td>57.6</td>
</tr>
<tr>
<td>Terminal t_{1/2} (h)</td>
<td>1.8</td>
<td>2.7</td>
<td>2.7</td>
<td>3.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Bioavailability (%)</td>
<td>96</td>
<td>39</td>
<td>89</td>
<td>92</td>
<td>100</td>
</tr>
<tr>
<td>Mouse PPB(%)</td>
<td>6</td>
<td>50</td>
<td>16</td>
<td>23</td>
<td>-</td>
</tr>
</tbody>
</table>

*WinNonlin two-compartment analysis iterative weighting.
#WinNonlin non-compartment analysis with uniform weighing.
Table 5. Mitochondrial protein synthesis inhibition

<table>
<thead>
<tr>
<th>Compound</th>
<th>COX1 EC₅₀ (µM)</th>
<th>SDHA EC₅₀ (µM)</th>
<th>Cell Viability EC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 12</td>
<td>&gt;150</td>
<td>39.5 ± 9.2</td>
<td>23.0 ± 1.4</td>
</tr>
<tr>
<td>Compound 13</td>
<td>&gt;150</td>
<td>20.5 ± 2.1</td>
<td>80.0 ± 5.7</td>
</tr>
<tr>
<td>Compound 14</td>
<td>&gt;150</td>
<td>21.5 ± 6.4</td>
<td>106 ± 37.5</td>
</tr>
<tr>
<td>Linezolid</td>
<td>27.3 ± 10.8</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>31.4 ± 23.2</td>
<td>&gt;150</td>
<td>110 ± 14.1</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>23.7 ± 6.4</td>
<td>109 ± 29</td>
<td>118 ± 35.5</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>&gt;150</td>
<td>&gt;150</td>
<td>&gt;150</td>
</tr>
</tbody>
</table>

COX1 is cytochrome c oxidase, which is a mitochondrial protein that is synthesized by mitochondrial ribosomes. SDHA is subunit A of succinate dehydrogenase complex, which is a mitochondrial protein that is synthesized by cytoplasmic ribosomes. Janus Green staining was used to determine cell viability after 7 days.
Table 6. *In vitro* resistance frequency

<table>
<thead>
<tr>
<th>Compound</th>
<th>4xMIC</th>
<th>10xMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 14</td>
<td>$4.6 \times 10^{-6}$</td>
<td>$3.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>Isoniazid</td>
<td>ND</td>
<td>$1.8 \times 10^{-5}$</td>
</tr>
<tr>
<td>Moxifloxacin</td>
<td>$1.7 \times 10^{-7}$</td>
<td>$1.1 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

The MIC values for compound 14, isoniazid, and moxifloxacin were determined on Middlebrook 7H10 agar as 0.2, 0.06 and 0.08 µg/mL, respectively.
FIGURE LEGENDS

Figure 1. In vitro structure-activity relationship. Mtb = M. tuberculosis, NT= not tested.

Figure 2. Compound 1 resistant mutants bear mutations in the editing domain of LeuRS. (A) Domain map of M. tuberculosis LeuRS. (B) Amino acid alignment of part of the editing domain of LeuRS from M. tuberculosis and M. smegmatis, identical residues are colored in blue, non-identical residues are colored in red with arrows indicating where the mutations were found.

Figure 3. In vivo efficacy of compound 2 in a murine GKO (C57BL/6-Ifngtm1ts) model of acute TB. Oral treatment was started 15 days (Start) after infection with a low dose aerosol of M. tuberculosis Erdman lux and continued for 9 consecutive daily treatments until day 23 when mice were euthanized on day 24 (End). CFU were determined from lungs (black) and spleens (grey) and means from five mice for drug treated groups and 6 mice per group for the untreated controls. *P < 0.001 by pairwise multiple comparison procedures (Tukey test) compared to control.

Figure 4. X-ray co-crystal structure of LeuRS with compound 2. (A) Crystal structure of M. tuberculosis LeuRS editing domain in complex with compound 2 (carbon atoms are colored in green)-AMP (carbon atoms are colored in magenta). Color code is the same throughout all figures with blue for nitrogen, red for oxygen, pink for boron, orange for phosphorus, yellow for sulfur. (B) Zoomed view into the editing site of M. tuberculosis LeuRS showing the compound 2-AMP adduct and the key residues establishing important hydrogen bonds (red dashed lines) with only the H-bond from the 3-aminomethyl to M441 being omitted for clarity. (C) Overlay of the LeuRS editing domain of M. tuberculosis and E. coli in complex with methionine colored in yellow (PDB: 2AJF). The 3-aminomethyl group of compound 2 mimics the amino group of methionine, including the interaction to the bacterial specific residue D447.

Figure 5. In vivo efficacy of compounds 11, 12, 13 and 14 in acute and chronic models of TB infection. (A) In vivo efficacy in a murine GKO (C57BL/6-Ifngtm1ts) model of acute TB. Compounds were dosed orally daily for 14 days after 10 days infection (Start) with a low dose aerosol of M. tuberculosis Erdman. Mean lung CFU were determined from five mice at End. (B) In vivo efficacy in a murine GKO (C57BL/6-Ifngtm1ts) model of acute TB. Oral treatment was started 13 days after infection (Start) with low dose aerosol of M. tuberculosis
Erdman lux and continued for 9 consecutive daily treatments until day 21 when mice were sacrificed on day 22 (End). Mean lung CFU were determined from five mice at End. (c) *In vivo* efficacy in a murine BALB/c model of chronic TB infection. Compounds were dosed orally 5 days a week for 4 weeks after infecting with *M. tuberculosis* Erdman with a low dose aerosol 21 days prior (Start). Lung (black) and spleen (grey) CFU were determined from six mice at End. **P < 0.01, *P < 0.001 by pairwise multiple comparison procedures (Tukey test) compared to control.

**Figure 6.** *M. tuberculosis* H37Rv *in vitro* kill kinetics. Cells were incubated with compounds at 20-fold their MIC values for different times over 14 days in 10 mL of 7H9 10% (v/v) ADC and 0.05% (v/v) Tween 80 medium. The MIC values used in this experiment were as follows, 0.013 µg/mL, 0.6 µg/mL and 0.06 µg/mL for compound 14, linezolid and moxifloxacin, respectively. The mean and the standard deviations of at triplicate cultures of each point are shown.

**Figure 7.** Efficacy of compound 14 in a mouse model of chronic TB infection. C57 BL/6J mice were infected with *M. tuberculosis* H37Rv intratracheally (~10^2 CFU) and were dosed once daily for 8 weeks starting 6 weeks after infection. Mice were sacrificed 24 hours after the last drug administration. Every column represents the mean value +/- SD of 7 mice per group for untreated and Linezolid treated groups and 3 mice for Compound 14 treated mice. *P < 0.001 by pairwise multiple comparison procedures (Tukey test) compared to control.

**Figure 8.** Efficacy of compound 14 in a mouse model of acute TB infection under different dosing regimes of once a day (QD), twice a day (BID) or every other day (q48h). C57 BL/6J mice were infected with *M. tuberculosis* H37Rv intratracheally (~10^3 CFU) and were dosed starting on the following day after infection for 8 days. Only one dose was administered on day 8 under the BID schedule. Mice were sacrificed at least 24 hours after the last drug administration. Every dot represents one mouse data point except for Linezolid (mean of 5 mice +/- SD).
FIG. 1

<table>
<thead>
<tr>
<th>Compound No</th>
<th>IC₅₀ (µM)</th>
<th>MIC (µg/mL)</th>
<th>EC₅₀ (µM)</th>
<th>M.tuberculosis LeuRS</th>
<th>M.tuberculosis H37RV</th>
<th>Vero Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>AN2679</td>
<td>21.3</td>
<td>7.5</td>
<td>NT</td>
<td></td>
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<td></td>
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<td>AN3016</td>
<td>3.5</td>
<td>1.0</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AN3017</td>
<td>0.64</td>
<td>1.8</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.28</td>
<td>0.26</td>
<td>&gt;50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.13</td>
<td>0.13</td>
<td>&gt;50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>NT</td>
<td>&gt;50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>0.09</td>
<td>0.04</td>
<td>&gt;50</td>
<td></td>
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</tr>
<tr>
<td>5</td>
<td>0.38</td>
<td>1.2</td>
<td>43</td>
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<tr>
<td>6</td>
<td>0.11</td>
<td>0.05</td>
<td>&gt;50</td>
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<tr>
<td>7</td>
<td>0.56</td>
<td>&gt;1.1</td>
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<td>0.20</td>
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<td>9</td>
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<td>0.29</td>
<td>&gt;50</td>
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<tr>
<td>10</td>
<td>28</td>
<td>17</td>
<td>&gt;50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>0.11</td>
<td>0.05</td>
<td>&gt;50</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>12</td>
<td>0.08</td>
<td>0.05</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>0.06</td>
<td>0.02</td>
<td>NT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0.08</td>
<td>0.02</td>
<td>NT</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
FIG. 2

A

- ZN Domain
- LS Domain
- Catalytic Domain
- AB/CT Domain
- Editing Domain

B

<table>
<thead>
<tr>
<th></th>
<th>Mtb 300</th>
<th>VKTQRNWIGRSTGAV A LFSARAASDDGF EVDIEVF TRPDTLFGATYLVLA</th>
<th>352</th>
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<tbody>
<tr>
<td></td>
<td>Msm 293</td>
<td>VKTQRNWIGRSTGASVL F-ATAA------------DIEVFTTRPD TLFQATYLVLA</td>
<td>337</td>
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<tr>
<td></td>
<td>Mtb 430</td>
<td>YVLAGVGT GAIMAVPG HDQDWDFA RAFGLP VEVI AGNISESAYTGDGIL</td>
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<tr>
<td></td>
<td>Msm 416</td>
<td>YVLAGVGT GAIMAVPG HDQDWDFA KEFGLP VEVVTGGDISEAAAYAGDGM</td>
<td>467</td>
</tr>
</tbody>
</table>

- S311L
- Y435C
- D450Y
- A428T
- R435C
- Δ421-462
FIG. 3

Start (Day 15) to End (Day 24) with INH 25 mg/kg QD

Cmp 1

200 mg/kg BID

Log_{10} CFU

Lung

Spleen

*
FIG. 5

A

Acute Model

Start (Day 10)
End (Day 24)
Rifampin
(10 mg/kg QD)
Cmp11
(100 mg/kg BID)
Cmp13
(100 mg/kg QD)

Log_{10} CFU/Lung

B

Acute Model

Start (Day 13)
End (Day 22)
Isoniazid
(25 mg/kg QD)
Cmp11
(100 mg/kg QD)
Cmp12
(100 mg/kg BID)

C

Chronic Model

Start (Day 21)
End (Day 48)
Cmp14
(10 mg/kg)
Cmp14
(33 mg/kg)
Cmp13
(33 mg/kg)
Cmp12
(90 mg/kg)
FIG. 7

6 Weeks  
No Treatment

14 Weeks  
No Treatment

Linezolid  
(100 mg/kg QD)

Compound 14 (10 mg/kg QD)  
Compound 14 (30 mg/kg QD)

Log_{10} CFU/Lung

*
FIG. 8