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A Novel Inhibitor of Gyrase B is a Potent Drug Candidate for the Treatment of Tuberculosis and Non-Tuberculosis Mycobacteria

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Abstract

New drugs to treat drug resistant tuberculosis are urgently needed. Extensively drug resistant and probably totally drug resistant tuberculosis strains are resistant to fluoroquinolones such as moxifloxacin, which target gyrase A, and most people infected with these strains die within a year. In this study, we found that a novel aminobenzimidazole (VXc-486), which targets gyrase B, potently inhibits multiple drug sensitive isolates and drug resistant isolates of *Mycobacterium tuberculosis in vitro* (minimal inhibitory concentration 0.03-0.30 µg/mL and 0.08-5.48 µg/mL, respectively) and reduces mycobacterial burdens in lungs of infected mice *in vivo*. VXc-486 is active against drug resistant isolates, has bactericidal activity, and kills intracellular and dormant *M. tuberculosis* in a low oxygen environment. Furthermore, we found that VXc-486 inhibits the growth of multiple strains of *Mycobacterium abscessus*, *M. avium* complex and *M. kansasii* (0.1-2.0 µg/mL MIC) as well as several strains of *Nocardia* spp. (0.1-1.0 µg/mL MIC). We directly compared the parent compound VXc-486 and a phosphate prodrug of VXc-486 and showed that the prodrug of VXc-486 had more potent killing of *M. tuberculosis* than VXc-486 *in vivo*. In combination with other anti-mycobacterial drugs, the prodrug of VXc-486 sterilized *M. tuberculosis* infection when combined with rifapentine/pyrazinamide and bedaquiline/pyrazinamide in a relapse infection study in mice. Furthermore, the prodrug of VXc-486 appeared to perform at least as well as the gyrase A inhibitor moxifloxacin. These findings warrant further development of the prodrug of VXc-486 for the treatment of tuberculosis and non-tuberculosis mycobacterial infections.

Introduction

Tuberculosis (TB) is an important infectious disease threat with approximately two billion infections (mostly latent), ten million new cases, and two million deaths each year (1). HIV co-infection/immunosuppression, ineffective health care management, complex standard-of-care (SOC) regimens, and widespread drug resistance all contribute to the challenge of TB control. TB is caused by *Mycobacterium tuberculosis* (Mtb), a slow growing, acid-fast bacillus, that withstands a harsh immunological assault by human host macrophages and effector cells, as well as sub-optimal chemotherapy, by persisting in a semi-dormant state of replication. New drugs are urgently needed to shorten the treatment regimen and to more effectively treat drug-sensitive (DS) TB infections, and especially drug-resistant (DR) TB infections.

DNA gyrase and topoisomerase IV are two clinically validated drug targets for bacterial infections. They are highly conserved type II topoisomerases that are essential for DNA replication. Both targets are enzymes with A_2B_2 heterotetramers comprising the GyrA and GyrB subunits (DNA gyrase) and the ParC and ParE subunits (topoisomerase IV), respectively; however, a topoisomerase IV homolog has not been identified in Mtb. In an enzymatic reaction that is coupled with ATP hydrolysis, these enzymes break and re-join double stranded DNA. The catalytic subunits (GyrA/ParC) are clinically validated drug targets of the fluoroquinolones such as moxifloxacin (MXF) (2), while the ATPase subunits (GyrB/ParE) have not been as extensively exploited and may present a new option for treating DR strains of Mtb (3). Furthermore, several different chemical classes have been described as inhibitors of gyrase B with potent activity against DR bacteria including Mtb (4-10).

A novel class of antimicrobials that target the ATPase subunits (GyrB/ParE) are the aminobenzimidazoles, which were optimized using structure-guided design and SAR of potency against both Gram-positive and some Gram-negative bacterial species (11, 12). Further optimization of the metabolic profile led to the identification of VXc-486 (13) and its solubility was later improved using a phosphate prodrug approach (14). The purpose of this study was to explore the utility of VXc-486 and its phosphate prodrug (pVXc-486) against Mtb and non-tuberculosis Mycobacteria (NTM) *in vitro* and in Mtb mouse animal models *in vivo*.

Materials and Methods

Mycobacterium sp. Isolates

M. tuberculosis ATCC 35801 (strain Erdman), ATCC 25618 (H37Rv) or ATCC 25177(strain H37Ra) colonies were used for routine MIC analysis. The BK 35 (H37Rv) strain harbors the gyrase A G94A mutation, while the BK49 strain harbors the gyrase A A90V mutation and both were kindly provided by Dr. Nicolas Veziris of the Centre National de Référence des Mycobactéries et de la Résistance des Mycobactéries aux Antituberculeux, CHU Pitié-Salpêtrière, Paris. Fluoroquinolone-resistant variants Mtb Levo^{R} 2D, 2J, and 8D isolates were selected by plating Mtb Erdman on 7H10 agar containing 10% OADC and levofloxacin (LVX) at the Central New York Research Corporation. Several colonies were selected, grown in 7H10 broth with 10% OADC, tested for LVX resistance and frozen at -70°C. The LVX-resistant Mtb isolate, Levo/Gat^{R} 2C, was subsequently cultured on 7H10 agar containing gatifloxacin (GAT)

to further enhance its quinolone resistance and the following fluoroquinolone isolates were recovered: 2I1, 8D2, 2C, and 2H2. The XDR 5 isolate was provided by Dr. Tommie Victor; the TT135, R179, R884, X_3, X_27, X_60, X_61, X_131, and TT149 isolates were provided by Dr Paul Van Helden of Stellenbosch University (15); and the CDC1551, GN9, HH9, H13571, MC19062, H10460, Wg565, LL, W10, 210, W33, C913, AH517, Bw9, and AH13 isolates were provided by Dr. Barry Kreiswirth of the Public Health Research Institute, Newark, N.J.. The *M. abscessus* isolates were provided by Barbara Brown-Elliot of UT Health Northeast, Tyler, Tex. (6153, 6025, 6005, 5908, 6142, 5931, 5605, 5901, 5812, 5960, 5922, 6111, and 6126); Dr. Barbara Body of Lab Corp, Burlington, N.C. (BB1-BB9) ; and LT949 was provided by the Centers for Disease Control, Atlanta, Ga.. The *M. kansasii* isolates were obtained from the ATCC (35755 and 12478); Barbara Body (0008, 2242, 4302, 5075, 5983, 1673, 1701, 5076, 2610, 1295, 0009, 0164); and Dr. Betty-Ann Forbes of State University of New York, Syracuse, (W5219, PIC, RSL, SHP, 379, 258, and 399). The *M. avium* Far isolate was provided by Dr. Betty-Ann Forbes and 103 and 3404.4 isolates were provided by Leonid Heifets of National Jewish Hospital, Denver, Colo., while the *Nocardia i*solates were provided by Dr. Betty-Ann Forbes.

Compounds

VXc-486 and pVXc-486 were prepared by Vertex Pharmaceuticals Incorporated according to published methods, while other compounds or drugs were either purchased commercially or prepared by published methods. All compounds were dissolved in 100% dimethyl sulfoxide (DMSO) and stored as frozen stocks at a concentration of 10 mM or 1 mg/ml. Before evaluation *in vitro*, compounds were diluted

into DMSO that did not exceed 1% and then re-suspended into culture medium consisting of 7H10 broth supplemented with 10% OADC (BBL Microbiology Systems, Cockeysville, Maryland) and 0.05% Tween 80.

Enzyme Assay

The enzyme assay was performed as previously described (16). The fully assembled heterotetrameric Gyrase A_2B_2 (after expression of each separate subunit and subsequent reconstitution prior to assay) was used for inhibitor characterization. The Mtb *gyrA* (Rv0006) and *gyrB* genes (Rv0005) were cloned into a pET28b.1 vector, expressed as recombinant proteins and purified similarly as previously described for the *E. coli* enzymes. Enzymatic hydrolysis of ATP to ADP was coupled to the conversion of NADH to NAD+. The decrease in NADH absorbance was monitored at 340 nm for 20 min with a microtiter plate reader (Molecular Devices, Sunnyvale, Calif.).

Growth Inhibition and Minimal Inhibitory Concentration (MIC) in Broth Culture

For microdilution MIC assays, the Mtb strains were routinely cultured on 7H11 agar, picked and prepared at 1 X 10^8 CFU/ml, and diluted 200x to a final concentration of 5 X 10^5 CFU/ml in 7H9 broth supplemented with 10% ADC. Compound stock solutions were prepared in DMSO at 100-fold for each test concentration and 1 µl of each stock was dispensed into a microtiter well. Compounds were diluted by the addition of 100 µl of the bacterial cell-suspension in culture medium. The 96-well plates were then incubated at 37^0C in ambient air. For the bactericidal assay, both VXc-486 and MXF were used at 4-fold minimal inhibitory concentrations (MIC); VXc-486 was

used at a concentration of 0.24 µg/ml and MXF was used at a concentration of 0.12 µg/ml.

For MIC determination using the Mtb H37Ra isolate, 30µl of Resazurin detection buffer added into each well and the plate was returned to the incubator (17). After 24 h incubation with Resazurin, the fluorescence was read using a Biotek Synergy2, at a gain of 35 with excitation wavelength 492nm and emission wavelength 595 nm. For MIC of other isolates, wells were visually inspected after 9 days of incubation. The MIC was defined as the lowest concentration of a compound that inhibited reduction of Resazurin to its fluorescent (emission 595 nm) species by ≥90%. For the low oxygen recovery assay, compound growth inhibition against H37Rv was evaluated as previously described (18). For *M. kansasii* testing, Middlebrook 7H9 broth**,** pH6.6, supplemented with 10% Middlebrook albumin-dextrose-catalase enrichment was used. *M. avium* was tested in 7H10 broth supplemented with 10% OADC, while for *M. abscessus* and *Nocardia* sp. testing, Mueller-Hinton broth was utilized.

For drug resistant strains from South Africa, mycobacterial growth was measured by using mycobacterial growth indicator tubes (MGIT®). Mycobacterial inocula were prepared from cultures of all strains grown on Lowenstein Jensen (LJ) slants. Cell suspensions were prepared in saline and the turbidity adjusted to 0.5 McFarland units. A 1:5 dilution of the bacterial suspension was prepared and 0.5 ml of the suspension was inoculated into MGIT tubes containing test and control compounds. For mycobacterial growth evaluation, the MGIT 960 system (Becton Dickinson, Sparks, Md.) was used where Mtb growth is observed through fluorescent changes due to oxygen consumption during mycobacterial growth (19, 20). 0.1 ml of serially diluted compound

was added to the 7H9 culture medium containing MGIT tube with the final DMSO concentration not exceeding 1.2%. Incubation at 37^0C was continued in the MGIT system and the growth units (GU) were monitored daily. For MIC_{99} evaluations, a 1% bacterial control culture was prepared in a drug free MGIT tube and the MIC_{99} of the compound determined relative to the growth units of the control (GU=400). When the GU of the growth control was 400 and the GU of the drug-containing tube was more than 100, the results were defined as resistant and when the GU of the drug-containing tube was equal to or less than 100, the results were considered susceptible.

Growth Inhibition using Mtb-infected macrophage cultures

THP-1 stocks were maintained at a culture density between 2 and $8x10^5$ cells/ml in RPMI-1640 media (with phenol red, 25 mM HEPES and 2mM L-glutamine; Gibco) supplemented with 10% FBS (Gibco) and 0.05 mM β-mercaptoethanol (Invitrogen) in 96 well tissue culture plates (Costar 3903, Corning). A cell suspension of sonicated Mtb H37Ra expressing firefly luciferase (8×10^5 cells/ml) in RPMI-1640 cell culture medium was used to infect PMA (Sigma Chemicals, 100 nM)-differentiated THP-1 cells at a multiplicity of infection (MOI) of 2:1 for two hours at 37°C. The supernatant containing un-ingested Mtb was then carefully removed from each well and the Mtb-infected THP-1 cells were replenished with 100 µl of fresh cell culture medium. The cells were then washed a second time and replaced with 50 µl fresh media and 50 µl of media containing test compounds. After five days of incubation, 100 µl of Steady-Glo (Promega) reagent was added to each well, incubated for 15 minutes at room temperature (RT), covered with adhesive top seal and the luminescence was read using a Biotek Synergy2, at a gain of 165 at maximum integration time. THP-1 viability was

180 determined by using Celltiter-Glo as per manufacturer's instructions (Promega).
181 Synergy (FIC ≤ 0.5), additive (FIC 0.5 to 4.0) or interfering (FIC > 4.0) effects of
182 compound combinations were determined by calculating the fractional inhibitory
183 concentrations (FIC) as previously described (21, 22).

184 $$FIC = \frac{\text{MIC Compound A with B}}{\text{MIC Compound A alone}} + \frac{\text{MIC Compound B with A}}{\text{MIC Compound B alone}}$$

186 Selection of VXc-486 Resistant Clones

187 Four resistant clones were selected at 2x and 4x MIC using 10e8 to 10e9 cfu of
188 the H37Ra laboratory strain of *M. tuberculosis* plated on supplemented 7H11 agar
189 plates. The clones were then sub-cultured in broth medium and MIC shifts (about 20-
190 fold) were verified in repeated MIC assays and then cultures were then selected for
191 gene amplification using nested PCR primers as follows: primers to amplify gyrB
192 ORF:1. gyrB_1 For: 5'-cgacacctacggataacacg -3' (base 183-202); 2. gyrB _2 Rev: 5'-
193 catctcctgctcgatgtcaa-3' (base 2574-2556) and internal primers 3. gyrB_2 For: 5'-
194 cttcgccaacaccatcaac -3' (base 1349-1367) and 4. gyrB_1 Rev: 5'-ttcagtagcttgcggtcctt
195 -3'(base 1456-1437). The same primer set was used to amplify the gyrB gene of
196 TT149 XDR-TB clinical isolate. The PCR was carried out for 35 cycles with denaturation
197 at 95^{o}C for 15 min, annealing at 52^{o}C for 1 min, and extension with Pyrobest DNA
198 polymerase (Takara, Otsu, Japan) at 72^{o}C for 1.5 min. The PCR products were gel
199 purified using a gel extraction kit (Tianjen, Beijing, China) and sequenced using
200 standard automated methods to determine the genetic variants. The variants and
201 corresponding MICs are detailed in Table S3.

202

203 Murine *Mtb* Infection Model

204 Six-week old female BALB/c mice were purchased from Charles River
205 Laboratories (Wilmington, DE), and C57BL/6 mice were purchased from Jackson
206 Laboratories (Bar Harbor, ME). All animal procedures were approved by the Institutional
207 Animal Care and Use Committee (Johns Hopkins University) or the Subcommittee for
208 Animal Studies (SAS; Central New York Research Corporation). Aerosol and intranasal
209 infections were performed as previously described (23, 24) . Mycobacterial stocks were
210 stored frozen at -70°C until use and on the day of infection, the culture was thawed and
211 sonicated. The final inoculum size was determined by titration, in triplicate, on 7H10
212 agar plates (BD Diagnostic Systems, Sparks, MD) supplemented with 10% OADC
213 enrichment. An Early Control (EC) group was euthanized at the initiation of therapy to
214 determine the infection load and a Late Control (LC) group was used to determine the
215 infection load at the end of an experiment and to confirm virulence.

216 A paucibacillary model of latent tuberculosis infection was used as previously
217 described by using BALB/c mice immunized with the *M. bovis* Bacille Calmette-Guérin
218 (BCG) vaccine (Pasteur strain) (25) using a recombinant BCG strain that overexpresses
219 the 30 kDa major secretory protein (rBCG30) (26) with minor modifications (27).
220 Quantitative cultures of lung homogenates were performed in parallel on selective agar
221 plates (7H11 supplemented with 10% OADC) and 2-thiophenecarboxylic acid hydrazide
222 (TCH, 40 µg.ml, Sigma, St. Louis, MO) to select for Mtb or hygromycin (40 µg/ml, Roche
223 Diagnostics, Indianapolis, IN) to select for rBCG30.

224 Chemotherapy.

Mice were block randomized by run to experimental arms of each study. Three weeks post-infection or as indicated otherwise, treatment was typically administered by oral gavage in a 0.2 ml volume 5 times per week for 4 weeks at the following dosages: VXc-486 and pVXc-486 (100 mg/kg in all experiments unless indicated otherwise), moxifloxacin (MXF, 100 mg/kg), rifampin or rifapentine (RIF or RPT, 10 mg/kg), isoniazid (INH, 10 or 25 mg/kg), pyrazinamide (PZA, 150 mg/kg), bedaquiline (BDQ, 25 mg/kg), linezolid (LZD, 50 mg/kg), PA-824 (50 mg/kg), clofazimine (20 mg/kg), ethambutol (EMB, 100 mg/kg), amikacin (AMK, 30 mg/kg). For dose administration, VXc-486 was dissolved in Vitamin E TPGS (d-alpha tocopheryl polyethylene glycol 1000 succinate), while the VXc-486 prodrug and all other single agent compounds administered individually were dissolved in 0.5% carboxymethylcellulose (CMC) in distilled water. In three-compound combination studies, VXc-486 was dissolved with the other compounds in 50% polyethylene glycol 400, while the INH, RIF, and PZA combination was dissolved in 20% DMSO and BDQ was dissolved in acidified 20% hydroxyl-propyl beta cyclodextrin. At the end of experiments, mice were euthanized by CO_2 inhalation and the lungs were aseptically removed, homogenized and the number of viable organisms was determined by serial dilution and titration on 7H10 agar plates. All animal studies were carried out in accordance with the approval of the Institutional Animal Care and Use Committee and according to the Guide for Care and Use of Laboratory Animals.

Statistical Evaluation

Colony-forming unit counts were log-transformed before analysis. Group means were compared by one-way analysis of variance with Dunnett's *post hoc* test

(experimental groups vs. each control group). Group proportions were compared using Fisher's exact test, adjusting for multiple comparisons. All analyses were performed with GraphPad Prism version 4.01 (GraphPad, San Diego, CA).

Pharmacokinetic Studies

In studies to determine oral and lung exposure, VXc-486 and pVXc-486 were administered by oral gavage to uninfected or Mtb-infected (Erdman) mice. Whole blood was sampled by retro-orbital bleeding (three mice per timepoint) at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hours after dosing and plasma was obtained following centrifugation of blood samples at 3000 x g for 2 minutes. Lung homogenates were prepared from Mtb-infected mice at one and six hours at the termination of the four-week treatment study. Plasma samples were then stored at -20^{o}C in sealed cluster tubes (Costar, 4413) and the samples were extracted using acetonitrile (4:1) containing an analytical internal standard. The tubes were mixed thoroughly for 5 minutes, centrifuged at 3,000 x g for 20 minutes, and the supernatants were transferred to fresh plates for quantitative LC/MS. Calibration standards (1 to 5000 ng/mL) and QCs (20 to 2,000 ng/mL) of VXc-486 were prepared in plasma matrix and extracted as described above. Samples, standards and QCs were analyzed using an Agilent 1100 LC system with a Waters Xterra C18 column followed by MS analysis using a SCIEX API 4000 (Applied Biosystems) Mass Spectrometer in ESI Ionization Mode and MRM Scan Mode. Quantification of standards, QCs and samples were determined relative to the analytical internal standard. Pharmacokinetic parameters were determined by non-compartmental analysis of the plasma concentration data using WinNonlin software (Pharsight Corp., Mountain View, CA).

Results

Growth Inhibition of *M. tuberculosis in vitro*

VXc-486 was observed to have potent anti-mycobacterial activity against genetically diverse strains of DS- and DR-Mtb, with MICs ranging from 0.03-0.25 µg/mL (Table 1). The mycobacterial cell potency as measured by MIC was consistent with Mtb gyrase B enzyme inhibition using a cell-free assay with the purified enzyme (IC_{50} < 0.16 µg/mL; Ki <0.39 µg/mL), the potency in this enzymatic assay reached the lower limit of biochemical detection. In a biochemical assay for human topoisomerase II using the alpha isoform, VXc-486 had negligible activity, indicating inhibition of this host enzyme is highly unlikely. In addition, VXc-486 did not have potency against 66 mammalian kinases in enzymatic assays (data not shown). No cross-resistance to VXc-486 was observed in H37Rv laboratory strains resistant to fluoroquinolones (e.g., BK35, BK49; Table 1). Furthermore, clinical isolates that were defined as multidrug resistant (MDR-TB) and extensively drug resistant (XDR-TB, resistant to fluoroquinolones; genetic profiles are summarized in Table S1) were also susceptible to VXc-486, confirming the activity of the compound against drug-resistant and clinically relevant Mtb isolates (Table 2).

The bactericidal activity of VXc-486 was also examined *in vitro* against actively growing cells of *M. tuberculosis* strain Erdman (MIC of 0.06 µg/mL). VXc-486 achieved the threshold for bactericidal activity (≥ 3 log reduction of the colony forming units, CFU) after 14 days and a clear trend toward slow killing of Mtb was observed (Figure 1a). As a comparator compound, MXF (MIC of 0.03 µg/mL) was not as bactericidal as VXc-486

but also achieved 3 logs of killing after 14 days. Specifically, the Log CFU mean value for VXc-486 was 3.991, while for MXF it was 4.885 after 14 days.

VXc-486 has potent activity against DS-Mtb in an *in vitro* model system of quiescent bacterial survival, the Low Oxygen Recovery Assay (LORA; Mtb H37Rv strain). In this model, the MIC of VXc-486 was 0.26 μg/ml and more active than the fluoroquinolones, MXF and GAT with LORA MICs of 1.60 μg/ml and 8.81 μg/ml, respectively, Table 3 and as previously reported (18).

VXc-486 is also active against Mtb growing within macrophages. The compound inhibited the growth of strain H37Ra cultured in the human macrophage-like cell line, THP-1 cells (IC_{50}, 0.013 μg/mL, IC_{90}, 0.3 μg/mL), as well as the CDC1551 strain cultured in the mouse macrophage-like cell line, J774 cells (IC_{50} of 0.15 μg/mL). To assess drug combinations *in vitro*, a checkerboard MIC assay was used to determine the FIC. We found borderline drug synergy (FIC =0.5) with linezolid in broth culture and bedaquiline (BDQ) and clofazimine (CFZ) in Mtb-infected THP-1 cells (Table S2). The remaining anti-mycobacterial combinations evaluated with VXc-486 showed an additive effect and none of the combinations tested showed an antagonistic effect in these assays.

To identify the amino acid residues important in determining target-based resistance to VXc-486, resistant clones were selected in the presence of VXc-486 (2x and 4x MIC) for more than one month on agar plates. Four resistant variant clones were confirmed by MIC testing and the colonies were expanded and genomic DNA for the entire *gyrB* gene was amplified for gene sequencing. Similar to a previous report that

evaluated benzimidazoles against Mtb (28), we also found that the A92S variant was associated with up to a 20-fold MIC shift (Figure S1a). In addition, we found an S208A variant in one of the resistant clones with a similar shift in MIC. In order to understand the impact of this mutation, we performed docking studies into the published crystal structure of Mtb gyrase B to generate a 3D model of VXc-486 in complex with the target. The predicted binding mode resulting from these studies was consistent with the experimental binding modes reported for closely related compounds in complex with the highly homologous gyrase B from *S. aureus* (13). The model suggests that the S208A mutation negatively affects the binding of VXc-486 to Mtb gyrase B by altering the hydrogen bonding network and destabilizing the catalytic water present in the active site (Figure S1b).

Growth Inhibition of Non-tuberculosis Mycobacteria (NTM) *in vitro*

Non-tuberculosis mycobacteria (NTM; *Mycobacterium abscessus*, *M. avium* and *M. kansasii*) and *Nocardia* spp. are responsible for respiratory infections, localized abscesses and disseminated diseases in immunocompromised individuals and cystic fibrosis patients. Infections caused by these organisms are difficult to treat because of naturally occurring poor responsiveness to classic anti-tuberculosis drugs and also to most other antibiotics. The potency of VXc-486 and MXF was tested against multiple isolates of *M. abscessus* and *M. kansasii*. We found that VXc-486 potently inhibited multiple strains of *M. abscessus* (MIC_{50} of 1.0 µg/mL, MIC_{90} 4.0; Table S3) and *M. kansasii* (MIC_{50}/MIC_{90} of 0.06 and 0.5 µg/ml for VXc-486 and MXF, respectively; Table S4), while the potency for *M. smegmatis* was substantially less (MIC of 2.4 µg/mL). The activity against *M. kansasii* was bactericidal and comparable to that of MXF (Figure 1b).

We also tested the *in vitro* activity of VXc-486 against a small subset of *M. avium* (MIC of 0.12 – 0.23 µg/mL) and *Nocardia* isolates (MIC of 0.1 - 1.0 µg/mL), demonstrating that VXc-486 had significant inhibitory effects against these NTMs as well (Table S5).

Growth Inhibition of Mtb *in vivo*

We next sought to determine if VXc-486 could provide a protective effect in a low dose, chronic tuberculosis model in mice. pVXc-486 which has improved solubility and is rapidly converted to VXc-486 after oral administration (Table S6), was directly compared to VXc-486 in a dose response study *in vivo*. We found that pVXc-486 had dose-dependent bactericidal activity that was more potent than that of VXc-486 and comparable to that of MXF (Figure 2). Compared with the initial mycobacterial burden prior to treatment (early control, EC), the median mycobacterial lung burdens after 27 days of treatment with the two higher doses (30 and 100 mg/kg) of VXc-486 and pVXc-486 were significantly reduced (at least -1 log10 difference versus the EC group of mice), indicating that VXc-486 and pVXc-486 had potent bactericidal activity *in vivo*. We also found substantially higher levels of VXc-486 in the lungs of infected mice after administration of pVXc-486, compared to when dosing the parent compound, suggesting that the improved protection may be due to improved exposure at the site of TB infection in the treated mice (Figure 3).

In vivo Activity of pVXc-486 in Combination with Known Anti-Mycobacterial Agents.

Since tuberculosis is treated with drug combinations and new combinations are needed to treat drug-resistant strains, we sought to determine if pVXc-486 could be used in combination with known TB drugs to sterilize an Mtb infection in mice. The

incorporation of pVXc-486 was evaluated with standard of care (SOC) drugs in first- and second-line regimens for DS and DR TB, respectively. When added to the first-line regimen of rifampin (RIF), isoniazid (INH), and pyrazinamide (PZA) or when substituted for INH, pVXc-486 significantly increased the activity of the combination ($p < 0.001$) over the first two months of treatment (Figure 4). The proportion of mice relapsing after four months of treatment was 1 of 15 mice treated with two months of RIF/INH/PZA followed by two months of RIF/INH; 2 of 15 mice treated with two months of RIF/INH/PZA/pVXc-486 followed by two months of RIF/INH/pVXc-486; and 1 of 15 mice treated with two months of RIF/PZA/pVXc-486 followed by two months of RIF/pVXc-486. No relapse occurred in any of these 3 groups after 5 months of treatment (data not shown). In the second line regimen, the substitution of pVXc-486 for amikacin (AMK) or ethambutol (EMB) also increased the bactericidal activity of MXF, EMB, PZA and AMK during the initial phase of therapy ($p<0.001$), but neither substitution promoted greater sterilizing activity during the relapse phase after PZA was discontinued (Table S7).

In another experiment evaluating two-compound combinations with pVXc-486, we found that pVXc-486 was able to replace MXF in combination with RFP or BDQ without loss of efficacy; (Figure 5A). There was only one relapse in the pVXc-486/RFP combination group (1/6) compared to three relapses in the MXF/RFP combination group (3/6). In addition, there were two relapses in the pVXc-486/BDQ group (2/6) compared to one relapse in the MXF/BDQ combination group (1/6). When each of these four groups are compared with each other, the results do not reach statistical significance; larger groups of mice would be needed to achieve statistically significant results that quantify the *in vivo* performance of pVXc-486 compared to that of MXF in these two-

drug combinations. However, based on this study, pVXc-486 and MXF seem to contribute similar activity to these combinations *in vivo*.

In a separate experiment, Mtb bacilli were not recovered from lungs of mice when treated in a three compound combination of pVXc-486 with the oxazolidinone linezolid (LZD) and PZA; PA-824 and PZA; or MXF and rifapentine (RFP). However, bacilli were recovered from lungs of mice treated with pVXc-486 combined with PZA and MXF or PZA and CFZ (Figure 5B). The results from the observation phase (eight weeks post treatment) showed a similar trend although these findings did not reach statistical significance because of experimental challenges. In a separate study, we found that pVXc-486 sterilized the infection in this mouse model when used in a three compound combination with PZA and RFP or BDQ. Similarly, MXF also sterilized the infection in mice when used in three compound combinations with PZA and RFP or BDQ (Figure 5C).

pVXc-486 in a Murine Latent TB Chemotherapy Model

To compare potency of pVXc-486 (100 mg/kg) monotherapy with INH and RIF in a model of chemotherapy of latent TB infection, mice were first immunized with *M. bovis* (BCG vaccine strain) and then challenged with Mtb (H37Rv strain) to establish a stable paucibacillary infection. After both one and two months of treatment, all monotherapy regimens were ostensibly superior to no treatment (late control) ($p<0.01$ for pVXc-486 and INH; $p<0.001$ for RIF) and pVXc-486 demonstrated activity comparable to INH in this model (Figure 6). However, despite a trend towards improved activity with the addition of pVXc-486 to RIF, no statistically significant benefit of combining pVXc-486 with either INH or RIF was demonstrated.

Discussion

Gyrase B is an essential and underexploited enzyme that is an important drug target for eliminating DS and DR bacterial infections. VXc-486 is highly active against Mtb (0.05 µg/mL to 0.5 µg/mL for most isolates tested) under various mycobacterial culture conditions including in broth, in macrophages, and in a model of Mtb dormancy. The potent anti-mycobacterial activity of VXc-486 against genetically diverse and MDR-TB/XDR-TB Mtb and NTM strains suggests that this compound has potential utility in the treatment of DR TB (Tables 1 and 2) and NTM (Tables S3-S5). One isolate, TT149 from South Africa, was intrinsically resistant; likely either by decreased compound permeability or increased compound efflux, since genetic sequencing of this isolates showed no *gyrB* mutations or variations (Table 2). Importantly, the MXF-resistant isolates of Mtb and *M. kansasii* were susceptible to VXc-486, indicating that fluoroquinolone-resistant strains will remain susceptible to antimicrobials targeting gyrase B (Table S4). The observed potency in the Mtb gyrase biochemical enzyme assay is consistent with an antibacterial mechanism where DNA replication is inhibited. While the Mtb killing kinetics of VXc-486 *in vitro* appears to be relatively slow (Figure 1), the observed potent activity against quiescent bacteria in the low oxygen model of dormant Mtb may also distinguish the aminobenzimidazole series from the fluoroquinolone series (Table 3).

The resistance frequency to VXc-486 was about 10^{-8} indicating a single enzyme is targeted similar to fluoroquinolones that target Mtb gyrase A. Among the VXc-486 resistant clones, we found A92S and S208A variants that had been reported previously by other investigators researching compounds targeting Mtb gyrase B (Figure S1). The

A92S variant identified in Mtb was consistent with another study describing aminobenzimidazole GyrB inhibitors (28). The A92S variant introduces a hydroxyl group into the ATP binding site and would predictably cause a conformational change, but would not completely obliterate the interaction of VXc-486 with the GyrB target enzyme, which is consistent with a 10-20-fold MIC shift. Although *M. abscessus* possesses a natural A92S mutation in its *gyrB* gene (28), we found the potency against this NTM and other NTMs in the MIC assay was more potent compared to MXF. The S208A variant is consistent with a study describing the pyrroloamides targeting gyrase B of Mtb (29). Ser 208 is located in proximity to the urea of VXc-486 in the model of the inhibitor bound to the active site of gyrase B. It does not make a hydrogen bond to the compound but it makes one to the catalytic water, which in turn is hydrogen bonded to the inhibitor. The switch from serine to alanine alters the hydrogen bonding network and destabilizes the catalytic water.

VXc-486, and its phosphate prodrug, pVXc-486, also protected mice in a low dose, chronic tuberculosis infection model using the Mtb Erdman strain and administration of pVXc-486 demonstrated activity similar to MXF at 100 mg/kg, once daily dosing regimen. At a dose of 30 mg/kg, pVXc-486 was superior to VXc-486 (Figure 2). Previously, several other Vertex aminobenzimidazoles targeting gyrase B were evaluated for their anti-mycobacterial tuberculosis activity in the high dose acute infection model using the Erdman strain but did not provide protection to a statistically significant level (unpublished studies). The difference may be due to the slow mycobacterial killing kinetics of VXc-486 and other similar compounds of the aminobenzimidazole class. These studies highlight the importance of using a chronic

infection model rather than an acute infection model to evaluate this compound series. The slow bacterial killing kinetics of this compound series was also noted in time-kill experiments of Mtb (Figure 1) and other bacteria *in vitro* (unpublished data). The acute infection model is most appropriate for compounds with rapid mycobacterial killing kinetics as single agents, such as INH, MXF or PA-824. Although the acute infection model can provide a relatively rapid experimental result (about two months, depending on the length of time for treatment) as compared to the low dose, chronic model (between three and four months), the acute model is not ideal for compounds with slower mycobacterial killing kinetics, such as PZA (30).

The exposure to VXc-486 at the doses used was adequate to enable pharmacology studies in mouse models of infection. Both the extent and rate of conversion of pVXc-486 were high and there was no observable exposure to pVXc-486 following oral administration of the prodrug in mice (Table S6). The AUC of VXc-486 increased supra-proportional to dose from 3 to 100 mg/kg and the Cmax of VXc-486 increased proportional to dose from 3 to 100 mg/kg. The mechanisms of conversion of pVXc-486 to VXc-486 are not fully understood; however, preliminary studies suggest bioconversion is likely to occur in the intestinal lumen via epithelial/luminal phosphatases following active uptake (14).

When pVXc-486 was added to a RIF/INH/PZA combination in a mouse model of DS SOC agents, we found that it increased the bactericidal activity of the regimen (Figure 4). In addition, the substitution of pVXc-486 for INH increased the bactericidal activity of RIF/PZA, indicating that there was an antagonistic effect of INH on the RIF/PZA (with and without pVXc-486) combination. A treatment-shortening effect was not observed in

this experiment since there was a lower than expected relapse rate after RIF/INH/PZA followed by RIF/INH. Similarly, in a mouse of model of DR SOC agents, substitution of the pVXc-486 for AMK or EMB increased the bactericidal activity of the initial phase of therapy, but neither substitution promoted greater sterilizing activity during the continuation phase, after PZA was discontinued (Table S7).

Since we saw encouraging sterilization results with pVXc-486 combined with LZD and PZA in a three drug combination study (Figure 5B), but not when PZA was omitted from the drug combination (Figure 5A), it may be that another oxazolidinone such as sutezolid (PNU-100480) or AZD5847 would be a better substitute since they may be more potent and/or better tolerated than LZD (31-34). One could envision a three-drug combination of pVXc-486 with BDQ or RFP combined with an oxazolidinone as a new combination that may provide a sterilizing effect in the mouse model and the former combination should be effective against DR strains as well.

In the mouse model of the chemotherapy of latent TB infection (Figure 6), we found that pVXc-486 was at least as effective as INH and its activity approached that of RIF. Furthermore, pVXc-486 had an additive activity when it was combined with INH or RIF.

In summary, the *in vivo* data demonstrate the effectiveness and potency of VXc-486 administered as a phosphate prodrug, pVXc-486. Importantly, pVXc-486 performed well in combination with RFP, BDQ, LZD and PZA and improved the bactericidal activity of combination regimens in models of DS and DR TB treatment *in vivo*. pVXc-486 could potentially be used in place of MXF, especially against DR

499 variants and XDR-TB. The results from these studies suggest that further development
500 of pVXc-486 is warranted.

501

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References

1. **(WHO) World Health Organization.** 2013 Global tuberculosis report 2013.
2. **Woodcock JM, Andrews JM, Boswell FJ, Brenwald NP, Wise R.** 1997. In vitro activity of BAY 12-8039, a new fluoroquinolone. Antimicrobial agents and chemotherapy **41:**101-106.
3. **Mdluli K, Ma Z.** 2007. Mycobacterium tuberculosis DNA gyrase as a target for drug discovery. Infectious disorders drug targets **7:**159-168.
4. **Kale MG, Raichurkar A, P SH, Waterson D, McKinney D, Manjunatha MR, Kranthi U, Koushik K, Jena L, Shinde V, Rudrapatna S, Barde S, Humnabadkar V, Madhavapeddi P, Basavarajappa H, Ghosh A, Ramya VK, Guptha S, Sharma S, Vachaspati P, Kumar KN, Giridhar J, Reddy J, Panduga V, Ganguly S, Ahuja V, Gaonkar S, Kumar CN, Ogg D, Tucker JA, Boriack-Sjodin PA, de Sousa SM, Sambandamurthy VK, Ghorpade SR.** 2013. Thiazolopyridine ureas as novel antitubercular agents acting through inhibition of DNA Gyrase B. Journal of medicinal chemistry **56:**8834-8848.
5. **Kale RR, Kale MG, Waterson D, Raichurkar A, Hameed SP, Manjunatha MR, Kishore Reddy BK, Malolanarasimhan K, Shinde V, Koushik K, Jena LK, Menasinakai S, Humnabadkar V, Madhavapeddi P, Basavarajappa H, Sharma S, Nandishaiah R, Mahesh Kumar KN, Ganguly S, Ahuja V, Gaonkar S, Naveen Kumar CN, Ogg D, Boriack-Sjodin PA, Sambandamurthy VK, de Sousa SM, Ghorpade SR.** 2014. Thiazolopyridone ureas as DNA gyrase B inhibitors: optimization of antitubercular activity and efficacy. Bioorganic & medicinal chemistry letters **24:**870-879.

6. **Shirude PS, Madhavapeddi P, Tucker JA, Murugan K, Patil V, Basavarajappa H, Raichurkar AV, Humnabadkar V, Hussein S, Sharma S, Ramya VK, Narayan CB, Balganesh TS, Sambandamurthy VK.** 2013. Aminopyrazinamides: novel and specific GyrB inhibitors that kill replicating and nonreplicating Mycobacterium tuberculosis. ACS chemical biology **8:**519-523.

7. **Basarab GS, Manchester JI, Bist S, Boriack-Sjodin PA, Dangel B, Illingworth R, Sherer BA, Sriram S, Uria-Nickelsen M, Eakin AE.** 2013. Fragment-to-hit-to-lead discovery of a novel pyridylurea scaffold of ATP competitive dual targeting type II topoisomerase inhibiting antibacterial agents. Journal of medicinal chemistry **56:**8712-8735.

8. **Trzoss M, Bensen DC, Li X, Chen Z, Lam T, Zhang J, Creighton CJ, Cunningham ML, Kwan B, Stidham M, Nelson K, Brown-Driver V, Castellano A, Shaw KJ, Lightstone FC, Wong SE, Nguyen TB, Finn J, Tari LW.** 2013. Pyrrolopyrimidine inhibitors of DNA gyrase B (GyrB) and topoisomerase IV (ParE), Part II: development of inhibitors with broad spectrum, Gram-negative antibacterial activity. Bioorganic & medicinal chemistry letters **23:**1537-1543.

9. **Tari LW, Li X, Trzoss M, Bensen DC, Chen Z, Lam T, Zhang J, Lee SJ, Hough G, Phillipson D, Akers-Rodriguez S, Cunningham ML, Kwan BP, Nelson KJ, Castellano A, Locke JB, Brown-Driver V, Murphy TM, Ong VS, Pillar CM, Shinabarger DL, Nix J, Lightstone FC, Wong SE, Nguyen TB, Shaw KJ, Finn J.** 2013. Tricyclic GyrB/ParE (TriBE) inhibitors: a new class of broad-spectrum dual-targeting antibacterial agents. PloS one **8:**e84409.

10. **Jeankumar VU, Renuka J, Kotagiri S, Saxena S, Kakan SS, Sridevi JP, Yellanki S, Kulkarni P, Yogeeswari P, Sriram D.** 2014. Gyrase ATPase domain as an antitubercular drug discovery platform: structure-based design and lead optimization of nitrothiazolyl carboxamide analogues. ChemMedChem **9:**1850-1859.

11. **Charifson PS, Grillot AL, Grossman TH, Parsons JD, Badia M, Bellon S, Deininger DD, Drumm JE, Gross CH, LeTiran A, Liao Y, Mani N, Nicolau DP, Perola E, Ronkin S, Shannon D, Swenson LL, Tang Q, Tessier PR, Tian SK, Trudeau M, Wang T, Wei Y, Zhang H, Stamos D.** 2008. Novel dual-targeting benzimidazole urea inhibitors of DNA gyrase and topoisomerase IV possessing potent antibacterial activity: intelligent design and evolution through the judicious use of structure-guided design and structure-activity relationships. Journal of medicinal chemistry **51:**5243-5263.

12. **Grossman TH, Bartels DJ, Mullin S, Gross CH, Parsons JD, Liao Y, Grillot AL, Stamos D, Olson ER, Charifson PS, Mani N.** 2007. Dual targeting of GyrB and ParE by a novel aminobenzimidazole class of antibacterial compounds. Antimicrobial agents and chemotherapy **51:**657-666.

13. **Anne-Laure Grillot, Arnaud Le Tiran, Dean Shannon, Elaine Krueger, Yusheng Liao, Hardwin O'Dowd, Qing Tang, Steve Ronkin, Tiansheng Wang, Nathan Waal, Pan Li, Dave Lauffer, Emmanuelle Sizensky, Jerry Tanoury, Emanuele Perola, Trudy H. Grossman, Tim Doyle, Brian Hanzelka, Steven Jones, Vaishali Dixit, Nigel Ewing, Shengkai Liao, Brian Boucher, Marc Jacobs, Youssef Bennani and Paul Charifson.** 2014 Second-Generation

582 Antibacterial Benzimidazole Ureas: Discovery of A Preclinical Candidate with
583 Reduced Metabolic Liability. Submitted for publication.
584 14. **H. O'Dowd DS, K. Chandupatla, Y. Bennani, J. Engtrakul, Z. Ye, S. Yeola, S.**
585 **Liao, N. Ewing, P. Jones, H. Tsao, E. Kolaczkowski, S. Donahue, R.**
586 **Seliniotakis, N. Bao, A. Tsai, R. Shawgo, V. Dixit, S. Jones, C. F. McNeil, B.**
587 **Song, D. Macikenas, A-L. Grillot, P. Charifson.** 2014. Discovery and
588 Characterization of a Water- soluble Phosphate Prodrug of a Dual Inhibitor of
589 Bacterial DNA Gyrase and Topoisomerase IV. Manuscript in preparation. .
590 15. **Klopper M, Warren RM, Hayes C, Gey van Pittius NC, Streicher EM, Muller**
591 **B, Sirgel FA, Chabula-Nxiweni M, Hoosain E, Coetzee G, David van Helden**
592 **P, Victor TC, Trollip AP.** 2013. Emergence and spread of extensively and totally
593 drug-resistant tuberculosis, South Africa. Emerging infectious diseases **19:**449-
594 455.
595 16. **Mani N, Gross CH, Parsons JD, Hanzelka B, Muh U, Mullin S, Liao Y, Grillot**
596 **AL, Stamos D, Charifson PS, Grossman TH.** 2006. In vitro characterization of
597 the antibacterial spectrum of novel bacterial type II topoisomerase inhibitors of
598 the aminobenzimidazole class. Antimicrobial agents and chemotherapy **50:**1228-
599 1237.
600 17. **Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A,**
601 **Degnan MT, Cook MB, Quenzer VK, Ferguson RM, Gilman RH.** 1998. Rapid,
602 low-technology MIC determination with clinical Mycobacterium tuberculosis
603 isolates by using the microplate Alamar Blue assay. Journal of clinical
604 microbiology **36:**362-366.

18. **Cho SH, Warit S, Wan B, Hwang CH, Pauli GF, Franzblau SG.** 2007. Low-oxygen-recovery assay for high-throughput screening of compounds against nonreplicating Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy **51:**1380-1385.

19. **Rusch-Gerdes S, Pfyffer GE, Casal M, Chadwick M, Siddiqi S.** 2006. Multicenter laboratory validation of the BACTEC MGIT 960 technique for testing susceptibilities of Mycobacterium tuberculosis to classical second-line drugs and newer antimicrobials. Journal of clinical microbiology **44:**688-692.

20. **Company BDa.** May, 1999. Bactec MGIT 960 System User's Manual. . Catalog 4405876

21. **Chou TC.** 2006. Theoretical basis, experimental design, and computerized simulation of synergism and antagonism in drug combination studies. Pharmacological reviews **58:**621-681.

22. **Heifets LB.** 1996. Clinical mycobacteriology. Drug susceptibility testing. Clinics in laboratory medicine **16:**641-656.

23. **Almeida D, Nuermberger E, Tasneen R, Rosenthal I, Tyagi S, Williams K, Peloquin C, Grosset J.** 2009. Paradoxical effect of isoniazid on the activity of rifampin-pyrazinamide combination in a mouse model of tuberculosis. Antimicrobial agents and chemotherapy **53:**4178-4184.

24. **Shoen CM, DeStefano MS, Sklaney MR, Monica BJ, Slee AM, Cynamon MH.** 2004. Short-course treatment regimen to identify potential antituberculous agents in a murine model of tuberculosis. The Journal of antimicrobial chemotherapy **53:**641-645.

628 25. **Nuermberger EL, Yoshimatsu T, Tyagi S, Bishai WR, Grosset JH.** 2004.
629 Paucibacillary tuberculosis in mice after prior aerosol immunization with
630 Mycobacterium bovis BCG. Infection and immunity **72:**1065-1071.
631 26. **Zhang T, Zhang M, Rosenthal IM, Grosset JH, Nuermberger EL.** 2009. Short-
632 course therapy with daily rifapentine in a murine model of latent tuberculosis
633 infection. American journal of respiratory and critical care medicine **180:**1151-
634 1157.
635 27. **Zhang T, Li SY, Williams KN, Andries K, Nuermberger EL.** 2011. Short-
636 course chemotherapy with TMC207 and rifapentine in a murine model of latent
637 tuberculosis infection. American journal of respiratory and critical care medicine
638 **184:**732-737.
639 28. **Chopra S, Matsuyama K, Tran T, Malerich JP, Wan B, Franzblau SG, Lun S,**
640 **Guo H, Maiga MC, Bishai WR, Madrid PB.** 2012. Evaluation of gyrase B as a
641 drug target in Mycobacterium tuberculosis. The Journal of antimicrobial
642 chemotherapy **67:**415-421.
643 29. **Hammeed SP, Solapure S, Mukherjee K, Nandi V, Waterson D, Shandil R,**
644 **Balganesh M, Sambandamurthy VK, Raichurkar AK, Deshpande A, Ghosh**
645 **A, Awasthy D, Shanbhag G, Sheikh G, McMiken H, Puttur J, Reddy J,**
646 **Werngren J, Read J, Kumar M, R M, Chinnapattu M, Madhavapeddi P,**
647 **Manjrekar P, Basu R, Gaonkar S, Sharma S, Hoffner S, Humnabadkar V,**
648 **Subbulakshmi V, Panduga V.** 2014. Optimization of Pyrrolamides as
649 Mycobacterial GyrB ATPase Inhibitors: Structure-Activity Relationship and In

650 Vivo Efficacy in a Mouse Model of Tuberculosis. Antimicrobial agents and
651 chemotherapy **58:**4993-4994.
652 30. **Rullas J, Garcia JI, Beltran M, Cardona PJ, Caceres N, Garcia-Bustos JF,**
653 **Angulo-Barturen I.** 2010. Fast standardized therapeutic-efficacy assay for drug
654 discovery against tuberculosis. Antimicrobial agents and chemotherapy **54:**2262-
655 2264.
656 31. **Wallis RS, Dawson R, Friedrich SO, Venter A, Paige D, Zhu T, Silvia A,**
657 **Gobey J, Ellery C, Zhang Y, Eisenach K, Miller P, Diacon AH.** 2014.
658 Mycobactericidal activity of sutezolid (PNU-100480) in sputum (EBA) and blood
659 (WBA) of patients with pulmonary tuberculosis. PloS one **9:**e94462.
660 32. **Shaw KJ, Barbachyn MR.** 2011. The oxazolidinones: past, present, and future.
661 Annals of the New York Academy of Sciences **1241:**48-70.
662 33. **Balasubramanian V, Solapure S, Iyer H, Ghosh A, Sharma S, Kaur P,**
663 **Deepthi R, Subbulakshmi V, Ramya V, Ramachandran V, Balganesh M,**
664 **Wright L, Melnick D, Butler SL, Sambandamurthy VK.** 2014. Bactericidal
665 activity and mechanism of action of AZD5847, a novel oxazolidinone for
666 treatment of tuberculosis. Antimicrobial agents and chemotherapy **58:**495-502.
667 34. **Werngren J, Wijkander M, Perskvist N, Balasubramanian V,**
668 **Sambandamurthy VK, Rodrigues C, Hoffner S.** 2014. In Vitro Activity of
669 AZD5847 against Geographically Diverse Clinical Isolates of Mycobacterium
670 tuberculosis. Antimicrobial agents and chemotherapy **58:**4222-4223.

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675 **Table 1** Antimicrobial activity of VXc-486 against *M. tuberculosis* laboratory strains
676 and clinical isolates

Strain		MIC (μg/ml)	
	Phenotype[1]	VXc-486	Moxifloxacin
Erdman	DS	0.06-0.125	0.015-0.03
H37Rv	DS	0.12-0.25	0.06
CDC1551	DS	0.015	0.03
HN878 (Beijing-Type)	DS	0.015	0.015-0.03
GN9	DS	0.03	0.015-0.03
HH9	DS	0.008-0.015	0.015
H13571	DS	0.03-0.06	0.03
MC19062	DS	0.06-0.125	0.015-0.03
H10460	MDR	0.5	0.25
Wg565	MDR	0.008-0.015	1.00
LL	MDR	0.015	0.008-0.15
W10	MDR	0.125	0.03
210	MDR	0.03-0.06	0.015
W33	MDR	0.015-0.06	0.5
C913	MDR	0.06	0.03
AH517	MDR	0.06	0.03
BW9	MDR	0.06-0.125	0.008

AH13	MDR	0.06	0.125-0.25
Levo^{R} 2J	FQ^{R}	0.03	1.0
Levo^{R} 2D	FQ^{R}	0.015	2.0
Levo^{R} 8D	FQ^{R}	0.125	4-8
Levo/Gat^{R} 2I1	FQ^{R}	0.125-0.25	16
Levo/Gat^{R} 8D2	FQ^{R}	0.03	8-16
Levo/Gat^{R} 2C	FQ^{R}	0.06	8.0
Levo/Gat^{R} 2H2	FQ^{R}	0.125	1.0
BK35	FQ^{L}	0.03	0.25-0.5
BK49	FQ^{L}	0.03	2.0
XDR 5	XDR	0.125	4-8

[1]Drug sensitive (DS), multi-drug resistant (MDR; resistant to INH and RIF) and extensively drug resistant (XDR) isolates were evaluated. Mtb Levo^{R} 2D, 2J, and 8D isolates were selected by plating Mtb Erdman on 7H10 agar with containing levofloxacin (LVX). LVX and gatifloxacin (GAT) originally had an MIC of 0.06 µg/ml. After selection, the MIC for LVX was 4 µg/ml and GAT was 1 µg/ml for the Levo 2D isolate. The LVX-resistant Mtb isolate, Levo/Gat^{R} 2D, was subsequently cultured on 7H10 agar containing GAT to further enhance its quinolone resistance and the following fluoroquinolone isolates were recovered 2I1, 2C, 8D2, and 2H2. The Mtb 2C isolate MIC for LVX was 2 µg/ml and GAT was 8 µg/ml.

687 **Table 2.** Antimicrobial activity of VXc-486 against drug resistant clinical isolates of
688 *M. tuberculosis* from South Africa.

Isolate	Genotype	Susceptibility status	Drug Resistance Profile	VXc-486 MIC, µg/mL
TT135	Atypical Beijing	XDR	H;R;E;Z;ET;S;A;C;O;M;PAS	0.08
R179	Atypical Beijing	MDR	H;R;E;ET;S	0.08
R884	Low copy	Pre-XDR	H;R;E;Z;ET;S;O	0.08
X_3	Beijing	XDR	H;R;E;K;S;C;O;	0.68
X_27	Beijing	XDR	H;R;ET;O;A	0.68
X_60	Beijing	XDR	H;R;E;A;ET;O;S;C;K	0.68
X_61	Beijing	XDR	H;R;ET;A;O;K;S;	0.68
X_131	F11	Pre-XDR	H;R;K;S;O	0.17
TT149	Atypical Beijing	XDR	H;R;ET;S;A;C;O;M	5.48

689

690 Mycobacterial growth was measured by using mycobacterial growth indicator tubes
691 (MGIT®) and the minimal inhibitory concentration (MIC, µg/mL) of VXc-486 was
692 determined. The growth units were recorded once the 1:100 controls reached a GU
693 (gas unit) of 400 and the MIC is regarded as a concentration that gives a zero GU. The
694 isolates grew well in the presence of isoniazid (INH), confirming resistance to this drug.
695 Abbreviations, H: isoniazid; R: rifampicin; E: ethionamide; ET: ethambutol; S:
696 streptomycin; A: amikacin; C: capreomycin; O: ofloxacin; M: moxifloxacin; PAS: *para*-
697 aminosalicylic acid; Z: pyrazinamide.

698 **Table 3.** VXc-486 inhibits Mtb replication in macrophages and low oxygen IC_{50}
699 (µg/ml).

Isolate	VXc-486	RIF	MXF	GAT	Reporter Gene
H37Ra	0.012	0.008	0.120	0.530	Luciferase in macrophages
CDC-1551	0.115	0.040	0.216	2.500	M-cherry in macrophages
H37Rv (LORA)	0.252	0.160	1.600	8.807	Green Fluorescent Protein

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701 Rifampin (RIF) and moxifloxacin (MXF) were used as comparator compounds. The low
702 oxygen recovery assay (LORA) was carried out in broth culture without macrophages.

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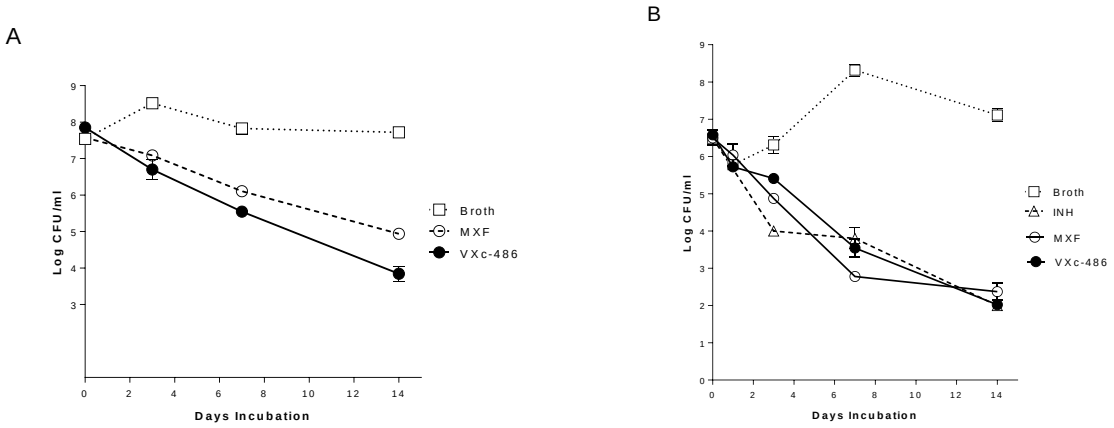

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709 Figure 1A. Bactericidal activity of VXc-486 and moxifloxacin (MXF) against
710 *Mycobacterium tuberculosis* (Erdman strain). Representative results are shown from
711 two independent experiments cultured in triplicate. Both VXc-486 and MXF were used
712 at 4-fold minimal inhibitory concentrations (MIC); VXc-486 was used at a concentration
713 of 0.24 µg/ml and MXF was used at a concentration of 0.12 µg/ml. (B). Bactericidal
714 activity of VXc-486, isoniazid (INH) and moxifloxacin (MXF) against *Mycobacterium*
715 *kansasii* (strain 0008).

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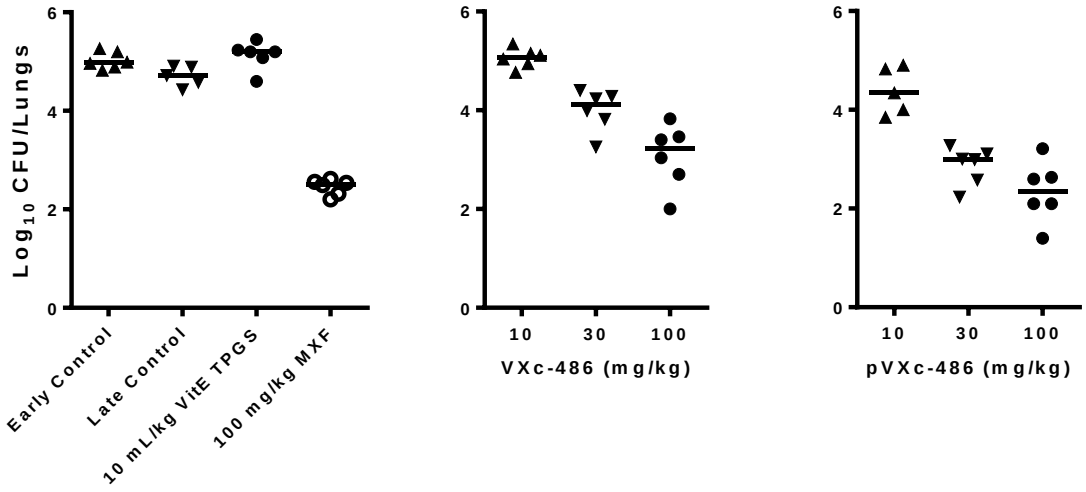

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721 Figure 2. VXc-486 and the pVXc-486 reduced the mycobacterial burden in a model of
722 chronic tuberculosis infection in mice (Erdman isolate). The potency of VXc-486 and
723 pVXc-486, which target gyrase B were compared with moxifloxacin (MXF), which
724 targets gyrase A. VXc-486 and pVXc-486 were administered twice a day, while MXF
725 was administered once a day by oral gavage for four weeks at the doses indicated.
726 Compared to the early and late control groups, VXc-486 (at 100 mg/kg), the pVXc-486
727 (at 30 and 100 mg/kg), and MXF (at 100 mg/kg) reduced the mycobacterial burden to a
728 statistically significant extent ($p < 0.05$). Mycobacterial burdens were measured by
729 colony forming unit (CFU) counts from lung homogenates of Mtb-infected mice at the
730 end of four weeks of treatment.

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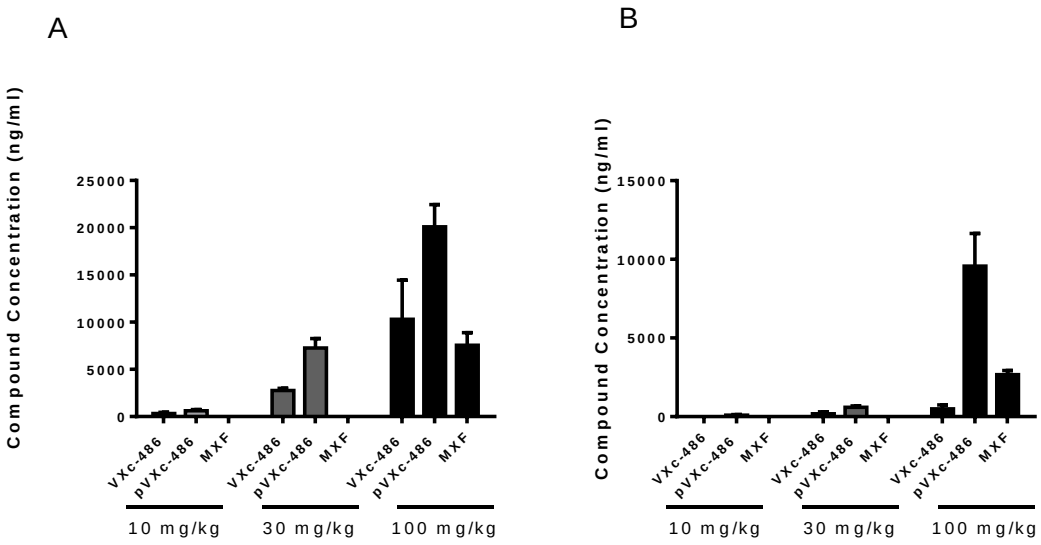
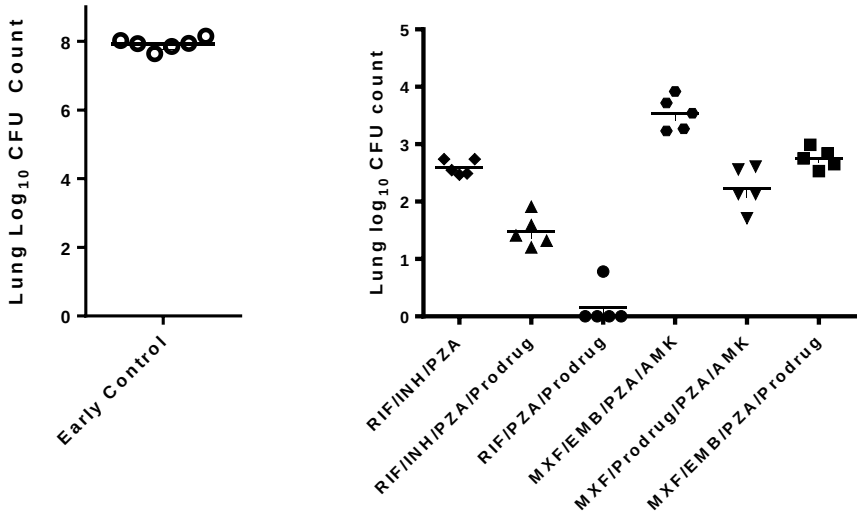


Figure 3. pVXc-486 (pVXc-486) has increased exposure in the lungs of infected mice compared to the parent (mesylate salt) VXc-486 and moxifloxacin (MXF). Administered dose is noted at the bottom of each group. Samples were collected one hour (A) and six hours (B) post-administration of the compounds at the termination of the four-week treatment model (described in Figure 2) and lung concentrations of VXc-486 were determined.



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751 Figure 4. pVXc-486 improved the bactericidal activity of anti-mycobacterial drugs.
752 Mycobacterial burdens were measured by colony forming unit (CFU) counts from lung
753 homogenates of Mtb-infected mice (H37Rv) and were assessed at the end of eight
754 weeks of treatment. Each compound was administered by the oral route once per day,
755 five days per week, for eight weeks at the following doses: pVXc-486, 100 mg/kg;
756 isoniazid (INH, 10 mg/kg), rifampin (RIF, 10 mg/kg); pyrazinamide, (PZA, 150 mg/kg);
757 moxifloxacin (MXF, 100 mg/kg); ethambutol (EMB, 100 mg/kg), and amikacin (AMK, 30
758 mg/kg).

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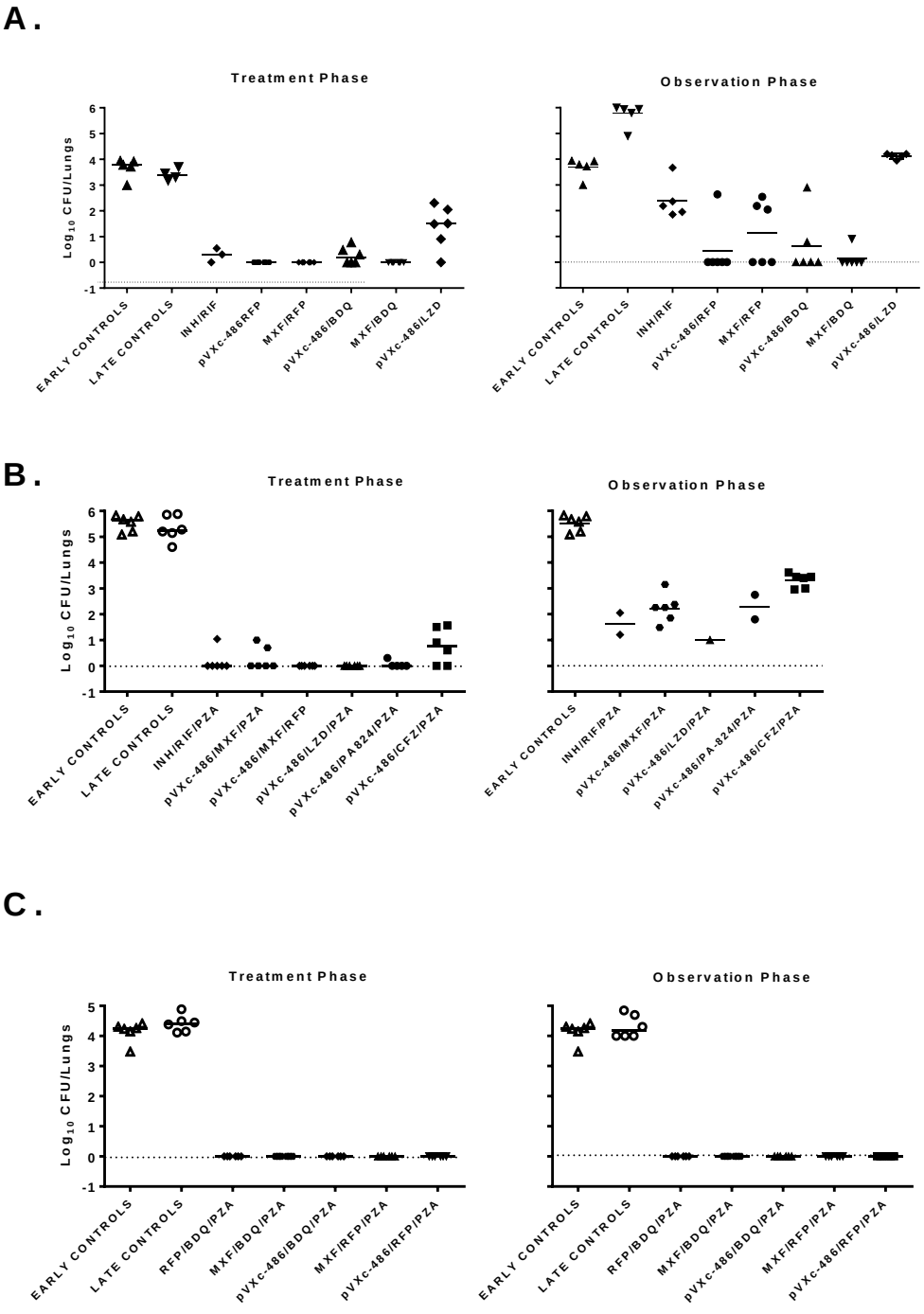

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762 Figure 5. pVXc-486 was administered to Mtb-infected mice (100 mg/kg, five days per
763 week) in combination with one or two other anti-mycobacterial drugs at the following
764 doses: isoniazid (INH, 10 mg/kg), rifampin or rifapentine (RIF or RFP, 10 mg/kg),
765 pyrazinamide (PZA, 150 mg/kg), moxifloxacin (MXF, 100 mg/kg), bedaquiline (BDQ, 25
766 mg/kg), linezolid (LZD, 50 mg/kg), PA-824 (50 mg/kg), and clofazimine (20 mg/kg).
767 Mycobacterial burdens were measured by colony forming unit (CFU) counts from lung
768 homogenates of Mtb-infected mice at the end of the first eight weeks of treatment
769 (treatment phase) and after an additional eight weeks without treatment (observation
770 phase). Each compound was administered by oral gavage once per day, five days per
771 week, for eight weeks. A two drug combination was used with pVXc-486 and the
772 potency was compared directly to MXF (A). A three drug combination in regimens
773 containing PZA was used in two separate experiments (B and C).

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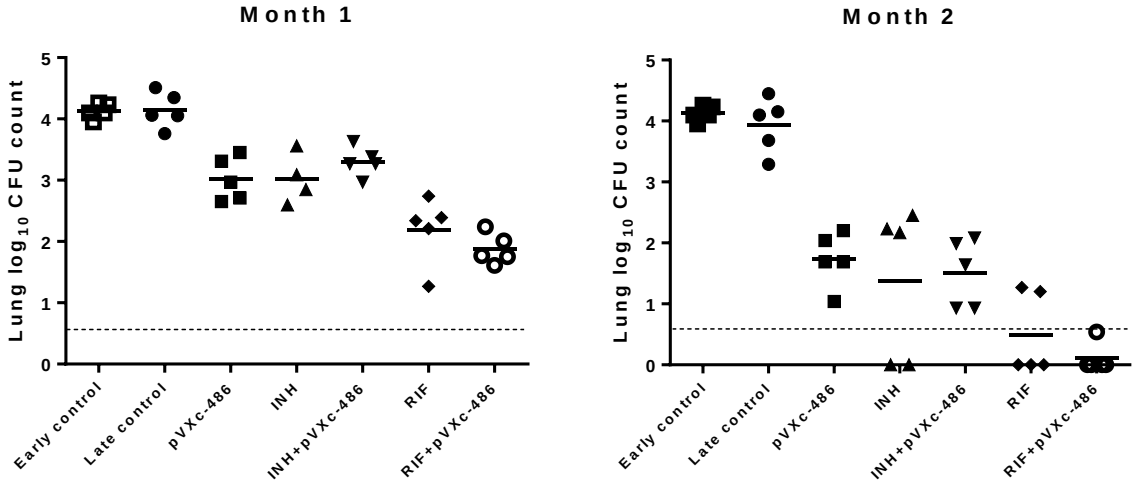

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778 Figure 6. pVXc-486 has comparable activity to isoniazid (INH) when used as a single
779 agent and renders mice culture-negative in combination with rifampin (RIF) in a mouse
780 model of latent tuberculosis (TB) chemotherapy. BALB/c mice were immunized with
781 *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) five weeks prior to being infected
782 with Mtb (H37Rv). Mice were then treated five weeks later for one (M1) or two (M2)
783 months with the pro-drug of VXc-486 and control drugs. The Mtb H37Rv mean lung
784 $\log_{10}$ CFU counts were 4.13 ± 0.13 at the start of drug treatment (early control). The
785 dotted line represents the lower limit of detection of the assay.

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