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The Efflux Pump Inhibitor Timcodar (VX-853) Improves the Potency of Anti-Mycobacterial Agents

Trudy H. Grossman[1#], Carolyn M. Shoen[2], Steven M. Jones[1], Peter L. Jones[1*], Michael H. Cynamon[2], Christopher P. Locher[1]

[1] Vertex Pharmaceuticals Incorporated, 50 Northern Avenue, Boston, Massachusetts 02210

[2] Central New York Research Corporation, Veterans Affairs Medical Center, 800 Irving Avenue, Syracuse, New York 13210

Current address: #Tetraphase Pharmaceuticals, 480 Arsenal Street, Watertown, Massachusetts 02472; *Driftwood Kayak, Hardy's Hill Road, Deer Isle, Maine 04627

Corresponding author: Christopher Locher, Karaden Farms, P.O. Box 792, Captain Cook, Hawaii, 96704; email: Christopher_locher@hotmail.com

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Abstract

Previous studies indicated that inhibiting efflux pumps augments tuberculosis therapy. In this study, we used timcodar (formerly VX-853) to determine if this efflux pump inhibitor could increase the potency of TB drugs against *Mycobacterium tuberculosis* (Mtb) in *in vitro* and *in vivo* combination studies. When used alone, timcodar weakly inhibited Mtb growth in broth culture (MIC of 19 μg/mL); however, it demonstrated synergism in drug combination studies with rifampin, bedaquiline and clofazimine but not with other anti-TB agents. When Mtb was cultured in host macrophage cells, timcodar had about a 10-fold increase (IC_{50} of 1.9 μg/mL) in the growth inhibition of Mtb and demonstrated synergy with rifampin, moxifloxacin and bedaquiline. In a mouse model of tuberculosis lung infection, timcodar potentiated the efficacies of rifampin and isoniazid conferring 1.0, and 0.4, log_{10} reductions in bacterial burden in lung, respectively, compared to the efficacy of each drug alone. Furthermore, timcodar reduced the likelihood of a relapse infection when evaluated in a long term, chronic infection model of mice treated with a combination of rifampin, isoniazid and timcodar. Although timcodar had no effect on the pharmacokinetics of rifampin in plasma and lung, it did increase plasma exposure of bedaquiline. These data suggested that the anti-mycobacterial drug potentiating activity of timcodar is complex, drug dependent and involves both bacterial and host targeted mechanisms. Further study of the improvement of anti-mycobacterial drug and drug candidate potency in combination with timcodar is warranted.

Introduction

Tuberculosis (TB) is a major infectious disease killer due to HIV-co-infection/immunosuppression, ineffective health care management, and widespread drug resistance. The prevalence of TB infections remains very high with approximately two billion latent infections, ten million new cases, and two million deaths each year (1). Tuberculosis 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 combat increasing drug resistance.

Drug resistance and drug tolerance of TB is thought to be partly mediated by efflux pumps. When multidrug resistance (MDR) efflux pumps in bacterial pathogens, protozoa or mammalian cells are blocked by inhibitors of diverse chemotypes, antibiotic and anti-cancer agents can be potentiated (2-5). Similar to other bacteria, Mtb uses efflux pumps to export extracellular factors essential for growth and to remove antibiotics and other toxins that would otherwise kill mycobacteria (6-8). Mycobacteria are ingested by host phagocytic cells and within these cells, they resist killing by remaining viable under relatively harsh conditions that could otherwise kill other infectious bacteria. One of the mycobacterial mechanisms of survival and drug tolerance within the phagocytic cell is due to the activity of efflux pumps such as Rv1258c. It has been shown that verapamil improves the potency of the rifampicin (RIF) in Mtb-infected THP-1

macrophage cells (9, 10). In addition, the efflux pump inhibitor piperine potentiated the anti-mycobacterial activity of RIF in broth cultures of Mtb by targeting Rv1258c (11), while verapamil accelerated the clearance of Mtb in C3HeB/FeJ mice when it was co-administered with the standard of care agents for drug-sensitive Mtb treatment (isoniazid, INH, RIF and pyrazinamide, PZA) (12). Furthermore, other efflux pumps have been described to play a more important role in drug resistance of Mtb, including the small multidrug resistant (SMR) pump Rv1218c, the ATP-binding cassette (ABC) transporter Rv2459, and Rv3065 (12-14). Thus, efflux pump inhibitors can potentiate the effects of anti-mycobacterial drugs *in vitro* on both extracellular and intracellular Mtb in host macrophages and in a mouse model of TB.

We have previously shown that the mammalian efflux pump inhibitors biricodar (formerly VX-710) and timcodar (TIM, formerly VX-853) potentiated the activity of seven antibiotics in broth culture against the gram-positive bacteria *Staphylococcus aureus, Enterococcus faecalis,* and *Streptococcus pneumoniae* (15). In this study, we extended these findings to mycobacteria and show that TIM in combination with several TB drugs can potentiate anti-mycobacterial activity *in vitro* and efficacy *in vivo*. Our findings indicated that TIM had a pronounced anti-mycobacterial adjuvant effect with anti-mycobacterial drugs when Mtb was cultured in macrophage-free broth or in Mtb-infected human macrophages. We also found that the antibiotic potentiation of TIM was readily observed in mouse animal models *in vivo*.

Materials and Methods

Compounds. TIM, PA-824 and bedaquiline (BDQ/TMC-207/Sirturo) were prepared by Vertex Pharmaceuticals according to published methods, while moxifloxacin (MXF) was provided by Vertex Pharmaceuticals. INH, ethionamide (ETA), RIF, clofazimine (CFZ), pyrazinamide (PZA), acriflavin (ACR) and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Gatifloxacin (GAT) was provided by Bristol Myers Squibb. Linezolid (LZD), PNU100480 (sutezolid) and Levofloxacin (LVX) were provided by Pharmacia & Upjohn and RW Johnson Pharmaceutical Research Institute, respectively, while and rifapentine (RFP) was provided by Marion Merrell Dow Chemical Company, Cincinnati, Ohio. All drugs were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in 7H10 broth with 10% OADC (BBL Microbiology Systems, Cockeysville, Maryland) and 0.05% Tween 80.

Growth Inhibition and Minimal Inhibitory Concentration in broth culture. Compounds were prepared at 100X the maximal concentration, added to the first wells of polystyrene 96-well round bottom plates and serially diluted two-fold in duplicate by transferring 1 μl into each well of polystyrene 96-well flat bottom plates used for culturing mycobacteria (Corning Inc., Corning, NY). *M. tuberculosis* ATCC 35801 (strain Erdman) or ATCC 25177(strain H37Ra) colonies were cultured on 7H11 agar plate, picked and prepared at 1 X 10^8 colony forming units (CFU)/mL, and 200x diluted to a final concentration of 5 X 10^5 CFU/ml in 7H9 broth supplemented with 10% Albumin Dextrose Catalase

(ADC) as previously described (16). 100 µl of cells were added into each well containing drugs. The 96-well plates were then incubated at 37^0C in ambient air.

For efflux pump inhibitor studies with Mtb (Erdman isolate), the compounds were prepared at 4X the maximal concentration and 50 µl of 7H10 broth was added to each well containing serial dilutions of TIM diluted 2-fold in duplicate. 50 µl of 5 X 10^5 CFU/ml Erdman with or without TIM was added to each well. Erdman was frozen at 5 x 10^7 CFU/ml and diluted to 5 X 10^5 CFU/ml. The plates were incubated for 18-21 days and the MIC was defined as the lowest concentration of agents yielding no visible turbidity. LZD, GAT, MXF, LVX and INH were tested in 7H10 alone or 7H10 + 10 µg/ml of TIM at 8 µg/ml to 0.008 µg/ml. ETA, ACR ethidium bromide were tested in 7H10 alone or 7H10 + 10 µg/ml of TIM at 128 µg/ml to 0.125 µg/ml. RIF and RFP were tested in 7H10 alone or 7H10 + 10 µg/ml of TIM at 1 µg/ml to 0.001 µg/ml.

For studies with the Mtb H37Ra isolate, 30 µl of resazurin detection buffer was added to each well after nine days 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 Mtb H37Ra, the MIC was defined as 90% growth inhibition and this value was used for H37Ra when the fractional inhibitory index was determined.

Susceptibility testing using Mtb-infected macrophage cultures. An Mtb (H37Ra) strain was transfected with pVV261 expressing fire fly luciferase, similar to

methods previously described (18), and used to infect a human macrophage-like cell line (THP-1; ATCC TIB-202^{TM}). Briefly, THP-1 stocks were maintained at a culture density between 2x10^5 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 x 10^5 cells/ml) in RPMI-1640 cell culture medium was used to infect phorbol myristic acetate (PMA; Sigma Chemicals, 100 nM)-differentiated THP-1 cells (19) at a multiplicity of infection (MOI) of 2:1 for two hours at 37°C. The supernatant containing cell-free 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 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 determined by using Celltiter-Glo as per manufacturer's instructions (Promega). Synergy, additive or interfering effects of compound combinations were determined in a checkerboard assay in 96 well tissue culture plates by calculating the fractional inhibitory concentrations (FIC) as previously described (20, 21);

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

MIC Compound A alone MIC Compound B alone

To measure growth inhibition of Mtb in bone marrow-derived macrophages from C57BL/6 mice, CFUs were determined as previously described (22, 23) after co-incubation of TIM with anti-mycobacterial agents (INH, RIF or BDQ). Briefly, macrophage cells were cultured in 24 well tissue culture plates (1 x 10^6 per well) at 37º C at 5% CO_2 . On Day 7, the media was removed and the primary macrophages were infected with Mtb H37Rv (ATCC 25618) at 1 x 10^6 CFU per well (MOI of 1). Seven days post-infection with Mtb, tissue culture medium was removed from the wells; the cells were washed twice with PBS, and then lysed with sterile H_2O + 0.05% Tween 80. Cell lysates were serially diluted, 1:10, and plated on 7H11/OADC agar through the 1/1000 dilution. Agar plates were then incubated at 37º C for 3 - 4 weeks, after which the bacteria colonies were counted and CFU/mL of cell lysates were determined. The concentration of drug reducing the bacterial load by 2 log units (99% reduction [MBC_{99}] is recorded as a numerical readout.

Murine Mtb infection models. Six-week old female C57BL/6 mice were purchased from Jackson Laboratories, Bar Harbor, ME. and were maintained within the ABSL-3 at the Syracuse VA Medical Center's Veterinary Medical Unit, Syracuse, NY. All animal procedures were approved by the Subcommittee for Animal Studies (SAS). Mice were housed in micro-isolator cages (Lab Products Inc. Maywood, NJ) and maintained with water and Prolab RMH 3000 rodent chow (Purina, St. Louis, MO). *M. tuberculosis* ATCC 35801 (strain Erdman) was

obtained from the American Type Culture Collection (ATCC), Manassas, VA. The organism was grown in modified 7H10 broth (pH 6.6; 7H10 agar formulation with agar and malachite green omitted) with 10% OADC (oleic acid, albumin, dextrose, catalase) enrichment (BBL Microbiology Systems, Cockeysville, MD) and 0.05% Tween 80 for 5-10 days on a rotary shaker at 37°C. The culture was diluted to 100 Klett units (equivalent to 5 X 10^7 CFU/mL (Photoelectric Colorimeter; Manostat Corp., New York, NY). The culture was frozen at -70°C until use. On the day of infection the culture was thawed and sonicated. The final inoculum size was determined by titration, in triplicate, on 7H10 agar plates (BD Diagnostic Systems, Sparks, MD) supplemented with 10% OADC enrichment. The plates were incubated at 37°C in ambient air for four weeks. Mice were infected intranasally with *Mtb* Erdman at 10^6 CFU/mouse. One week post-infection mice were treated by oral gavage in a 0.2 ml volume five times per week for four weeks. All drugs were given orally, once daily, unless stated otherwise. For dose administration, TIM was dissolved in 0.5% methylcellulose (MC) and administered at a dose of 100 or 200 mg/kg either once or twice a day as indicated in each experiment in the figure legend; RIF was dissolved in 0.5% MC or 20% DMSO and administered at a dose of 10 mg/kg once a day; INH was dissolved in 0.5% MC and distilled water and administered at a dose of 25 mg/kg once a day, and BDQ was dissolved in acidified 20% hydroxypropyl β-cyclodextran and administered at a dose of 25 mg/kg once a day. RIF and INH efficacy was confirmed to be unaffected by vehicle (data not shown). TIM was given in the morning and drugs tested in combination were administered five to

six hours post-TIM treatment unless stated otherwise. An Early Control (EC) group was euthanized at the initiation of therapy to determine the infection load. A Late Control (LC) group was utilized to confirm virulence; LC mice were moribund and needed to be euthanized at 14 days post-infection. At the end of experiments, all surviving mice were euthanized by CO_2 inhalation. Right lungs were aseptically removed and ground in a sealed tissue homogenizer (IdeaWorks! Laboratory Devices, Syracuse, N.Y.). The number of viable organisms was determined by serial dilution and titration on 7H10 agar plates. Plates were incubated at 37°C in ambient air for four weeks prior to counting.

Statistical evaluation**.** To compare the viable cell counts recovered from the right lungs of mice, the numbers were first converted into log CFU (log_{10}). Due to the small sample size and the consequent need to protect against deviations from normality, an ANOVA statistical analysis followed by a Dunnett's post-test adjustment was performed to determine statistical differences between control and treatment groups.

Pharmacokinetic studies. In studies to determine oral exposure, TIM was administered by oral gavage to C57BL/6 mice (10 mL/kg) in aqueous solutions of 0.5% MC at doses of 10, 100 or 200 mg/kg. 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. 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 by

vortexing 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 TIM 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. Quantitation 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). In the pharmacokinetic studies where TIM was co-administered with other antimicrobials, TIM (200 mg/kg or 100 mg/kg) was orally administered to mice six hours prior to the oral administration of RIF, MOX or bedaquiline in their respective formulations. Blood sampling, plasma extraction and quantification of RIF by LC/MS analysis and pharmacokinetic analysis of the data were carried out as described above.

Results

Activity of TIM against Mtb in broth culture *in vitro* with drug combinations

Since we had observed a potentiating effect of the efflux pump inhibitor TIM in combination with antibiotics in broth cultures of Gram-positive bacteria (15), we sought to determine if similar potentiation could be observed when this efflux pump inhibitor was evaluated in cultures of Mtb. We found that TIM alone possessed weak growth inhibitory activity using standard MIC assays of

mycobacteria cultured in broth (H37Ra isolate, 18.7 µg/mL, Table 1; Erdman isolate, MIC of 64 µg/ml). In assays with Mtb Erdman, TIM at 10 µg/mL combined with a diverse set of TB antibiotics showed little potentiating effect with the exception of BDQ where TIM conferred a 15-fold improvement in MIC (MIC=0.004 µg/mL with TIM vs. 0.06 µg/mL without TIM; Table S1). Furthermore, we found a four-fold potentiating effect of TIM with the intercalator ethidium bromide in broth cultures of Mtb which is known to be a promiscuous efflux pump substrate in many organisms, suggesting that TIM had an anti-mycobacterial effect consistent with efflux inhibition when Mtb is cultured in broth medium. To further establish synergy of TIM in combination with TB drugs in vitro, a checkerboard MIC assay was performed with TIM at higher concentrations together with anti-mycobacterial drugs and the sum fractional inhibitory index (FIC) was determined. In these assays, we found synergy in combinations of TIM with RIF, BDQ, and CFZ in broth cultures (Table 1).

Activity of TIM against Mtb in cultures of infected macrophages *in vitro*

Although TIM alone had modest Mtb growth inhibition in broth cultures, it was about 10X more potent when growth inhibition was measured in Mtb-infected macrophages using a luciferase reporter strain (IC_{50} of 1.9 µg/mL; Table 1). In Mtb H37Ra-infected THP-1 cell macrophage cultures, we found drug synergy with RIF, BDQ and MXF. We also observed an additive effect with other anti-mycobacterial drugs tested including INH and CFZ although there were no antagonistic interactions.

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280 Evaluation of Timcodar in combination with anti-mycobacterial agents in vivo

281 TIM was first evaluated in a high dose acute infection model (10^6
282 CFU/mouse) and four weeks of oral compound administration was initiated on day
283 seven post-infection. TIM showed a dose-dependent reduction in the
284 mycobacterial lung burden in a once-daily dosing regimen in combination with
285 RIF (10mg/kg/day); TIM was dosed six hours before RIF and was found to be
286 most active with RIF when used at a high dose (200 mg/kg/day in combination
287 with RIF; Fig. 1A). TIM did not have an anti-mycobacterial effect when it was
288 dosed alone and had a similar effect if it was dosed once or twice per day at 200
289 mg/kg with RIF dosed once daily (Fig. 1B). In each combination study of TIM
290 with RIF, combinations with TIM resulted in approximately a 10-fold reduction in
291 the mycobacterial burden in lungs versus RIF alone (final lung CFUs were 4.5
292 $\log_{10}$ in the RIF-treated group vs. 3.5 $\log_{10}$ in the RIF and TIM treated group, $p <$
293 0.05). A similar trend was observed regardless of whether TIM and RIF were
294 either co-dosed simultaneously or dosed at six or eight hours apart. In
295 subsequent experiments, TIM was dosed 6 hours before antibiotic treatment was
296 administered (Figures 2-3 below).

297 In further studies of TIM in high dose, acute mouse models of TB infection,
298 we evaluated TIM (200 mg/kg/day) in combination with PZA (150 mg/kg/day),
299 RFP (10 mg/kg/day), MXF (100 mg/kg/day), PNU100480 (sutezolid, 100
300 mg/kg/day), BDQ (25 mg/kg/day), GAT (100 mg/kg/day) but the inclusion of TIM
301 did not lower the mycobacterial lung burden to a statistically significant level

(Table S2). However, there was approximately a one-quarter log reduction in the mycobacterial lung burden when TIM (200 mg/kg) was used in combination with INH (25 mg/kg/day; Fig. S1) in the four week treatment model although these data did not reach statistical significance. We also tested TIM in combination studies with BDQ in the mouse model in an eight day treatment model. We found that TIM in combination with BDQ (25 mg/kg/day) reduced the mycobacterial lung burden to a statistically significant extent (Fig. 2),

Since co-administration of TIM improved the potency of RIF (and of INH to a lesser extent), we sought to determine if TIM could also be effective in shortening the time to sterilization of infection in a chronic, long term infection model of TB. We treated mice with a combination of INH (25 mg/kg/day) and RIF (10 mg/kg/day) either with or without TIM (200 mg/kg/day) for either nine or 12 weeks and held parallel groups of mice for an additional eight weeks post-treatment to determine if they had a relapse of infection. At 9 weeks post-treatment prior to the observation phase, there were fewer mice that had detectable mycobacterial lung burdens in the TIM/RIF/INH-treated group compared to the INH/RIF-treated group (Fig. 3). After 12 weeks of treatment and eight weeks of observation (the relapse phase), we found that mice treated with TIM/RIF/INH had lower levels of mycobacterial lung burdens than mice treated with INH/RIF alone. Furthermore, at 12 weeks post-treatment prior to the observation phase, there were also fewer mice that had detectable mycobacterial lung burdens in the TIM/RIF/INH-treated group (3 out of 6)

compared to the INH/RIF-treated group (5 out of 6), although the differences in bacterial loads in lungs from these mice did not reach statistical significance.

The effect of TIM on the pharmacokinetics of anti-mycobacterial drugs

Since TIM increased the potency of RIF and BDQ (and INH to a lesser extent) in vivo, we sought to determine if TIM increased the exposure of TB drugs, which would help explain the reduction in the bacterial lung burdens in treated mice. The plasma exposure of TIM administered at a 200 mg/kg dose in C57/BL6 mice was maintained at 5 to 15 μg/ml for over 16 hours, which was similar to the concentrations showing drug combination synergy *in vitro* and this dose of TIM provided a potentiating effect *in vivo*. The co-administration of TIM (200 mg/kg) with INH (25 mg/kg), RIF (10 mg/kg), or MXF (100 mg/kg) had no effect on their overall exposure, as determined either by plasma C_{max} or AUC_{inf} (Table 3). However, the co-administration of TIM with BDQ (25 mg/kg) did increase BDQ exposure by almost three-fold.

Discussion

Co-administration of efflux pump inhibitors with other drugs has been described as a method of improving drug therapies targeting both mammalian and microbial cells (2-5, 24-28), including Mtb (8, 9, 12, 13, 29, 30). TIM was previously characterized as an inhibitor of several mammalian efflux pumps (25, 26, 31, 32) and enhanced the potency of antibiotics against gram-positive bacteria in broth cultures (15). The importance of evaluating efflux inhibitors in

multiple relevant disease models is represented by the work in this study where different efflux inhibitor/antibiotic combinations had different effects on mycobacterial growth in MIC assays, infected macrophage assays and mouse infections. When used as a single agent, the efflux pump inhibitor TIM had a modest effect on the growth of Mtb in broth culture and this effect was more pronounced in the Mtb-infected macrophage assay using a luciferase reporter strain readout. When used in combination, TIM was synergistic with RIF in Mtb-infected macrophages and consistently improved the clearance of Mtb in infected mice in multiple experiments *in vivo*. TIM was as effective as adding another anti-mycobacterial drug to a RIF regimen *in vivo*. The efficacy of the TIM/RIF combination in Mtb-infected mice may be due to the result of a combination of effects conferred by TIM, including inhibition of bacterial and mammalian cell efflux pumps involved in antibiotic efflux and possibly mycobacterial virulence.

We studied the pharmacokinetic parameters of selected anti-mycobacterial agents, including RIF, with and without TIM because it is known that transporters are involved in efflux mechanisms that can promote or limit the absorption of drugs in the gut and penetration into tissues (24, 28, 33, 34). Since the pharmacokinetic parameters of RIF in plasma (Table 2) and lung tissue (from uninfected mice, data not shown) were unaffected by the co-dosing of TIM, these findings suggest that the improved efficacy seen with the TIM/RIF combination is not due to enhanced RIF plasma or lung exposures. However, we cannot rule out the possibility that TIM works at the level of the granuloma and helps concentrate RIF at focal points of infection in the lung since we did not

quantify RIF in the lungs of Mtb-infected mice co-treated with TIM. Pro-inflammatory lymphocytes expressing Pgp-1 surround sites of Mtb replication and Pgp-1 inhibitors may help concentrate TB drugs in the granuloma (35). Recent developments in granuloma imaging by using radioactive RIF or mass spectrometry (35-37) may be able to further elucidate the role of efflux pump inhibition in models of TB infection. Furthermore, C3HeB/FeJ mice or animal models that develop more structured granulomas combined with quantitative methods to measure drug concentrations may provide further insight into the adjuvant effect of TIM in drug combination studies. Nevertheless, TIM did increase the plasma exposure of BDQ as well as improved its potency in Mtb broth and macrophage-infected cell culture, suggesting that increased exposure may partially explain the trend toward a beneficial effect of adding TIM to a regimen of BDQ in the mouse animal model (Fig. 2). Since there is evidence that TIM inhibits human cytochromes, this inhibitory mechanism may contribute to increase exposure of BDQ in the mouse model, although inhibition of mouse cytochromes remains to be determined. TIM may also promote uptake of an anti-mycobacterial agent into the infecting organism, increase oral absorption and systemic distribution of an anti-mycobacterial agent in infected tissues or modulate the metabolism of anti-mycobacterial agents via drug-drug interactions.

Although the specific target or mechanism of TIM is not currently known, its adjuvant effect may also be due to its interaction with several different efflux pumps, including those present in Mtb, Mtb-infected macrophages, or both. This may explain why we see a differential effect depending on the culture conditions

(broth vs. Mtb-infected macrophages) and the drug with which it is combined (e.g., INH and BDQ). A specific Mtb target effect is supported by the observation that ethidium bromide efflux is inhibited by TIM in broth culture without host macrophage cells. In addition, TIM showed a substantial synergistic effect against Mtb in drug combinations in broth culture with RIF, BDQ and CFZ; however, in Mtb-infected macrophage cultures and in the mouse model, the potentiating adjuvant effect of TIM was most pronounced with RIF (Table 1). Mtb is known to be primarily an intracellular macrophage infection in the mouse model and since we observed the most synergy with RIF in Mtb-infected macrophages as well as in the mouse model, these results are consistent with TIM inhibiting an efflux mechanism that is induced in Mtb-infected macrophages. It is known that Mtb induces the protein expression of efflux pumps such as Rv1258c upon infection of macrophages and these efflux pumps are important for drug tolerance of RIF and survival during an intracellular infection (9, 10). Furthermore, a direct effect on the intracellular replication of Mtb or virulence of Mtb cannot be completely ruled out since it has been established that efflux systems of unknown function are important for survival in the infected host (38-40).

Mtb drug-resistant strains have been described to increase the expression of efflux pumps to enable their survival and persistence in the presence of antibiotics (41-44). Although we did not explore drug resistant strains in this study, additional research using TIM to try and reverse drug resistance using clinical isolates of Mtb and evaluating these in broth and infected macrophage

cultures, as well as in vivo studies is warranted. TIM may also reduce the frequency of genetic resistance mutations to INH or RIF when used in combination with these anti-mycobacterial drugs. Further studies of TIM with drugs and drug candidate combinations should be undertaken to identify resistance frequencies and new compound synergies since efflux is an important mechanism in the development of drug resistance (45).

TIM reduced the relapse rate in combination studies of INH and RIF after twelve weeks of treatment; however, since the nine-week treatment was statistically significant after the treatment phase but not after eight weeks in the relapse study (observation phase), larger experimental groups of animals and longer periods of time to study relapse would provide more conclusive evidence on the beneficial effect of TIM in drug combinations than we observed in the current studies. Furthermore, in each of the above combination studies, longer treatment times or varying the timing of administration of the compounds may also demonstrate a more pronounced effect on the reduction in the lung burdens of Mtb-infected mice.

The utility of TIM in the treatment of human TB infection remains unknown. Clinical studies are underway to determine if verapamil, another known efflux pump inhibitor, can shorten the therapeutic regimen of the standard of care for drug sensitive TB treatment (www.wgnd.org). This planned clinical trial in India is also designed to determine a minimum effective dose of verapamil, which will be essential in elucidating its additive effect in TB patients. These studies should provide further guidance on the role of efflux pump inhibitors and the

treatment of TB. As a tool compound, TIM remains important in studying mycobacterial drug efflux, drug tolerance and drug resistance as well as a potential modulator of PK parameters within the granuloma of infected animals.

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REFERENCES

1. **(WHO) WHO.** 2013 Global tuberculosis report 2013.
2. **Martin SK, Oduola AM, Milhous WK.** 1987. Reversal of chloroquine resistance in Plasmodium falciparum by verapamil. Science (New York, N.Y.) **235:**899-901.
3. **Van Bambeke F, Pages JM, Lee VJ.** 2006. Inhibitors of bacterial efflux pumps as adjuvants in antibiotic treatments and diagnostic tools for detection of resistance by efflux. Recent patents on anti-infective drug discovery **1:**157-175.

4. **Li X-ZaHN.** 2004. Efflux-mediated drug resistance in bacteria. . Drugs **64:**159-204.

5. **Nobili S, I. Landini, B. Giglioni, E. Mini.** 2006. Pharmacological strategies for overcoming multidrug resistance. Curr. Drug Targets **7:**861-879.

6. **De Rossi E, Ainsa JA, Riccardi G.** 2006. Role of mycobacterial efflux transporters in drug resistance: an unresolved question. FEMS microbiology reviews **30:**36-52.

7. **Louw GE, Warren RM, Gey van Pittius NC, McEvoy CR, Van Helden PD, Victor TC.** 2009. A balancing act: efflux/influx in mycobacterial drug resistance. Antimicrobial agents and chemotherapy **53:**3181-3189.

8. **Viveiros M, Leandro C, Amaral L.** 2003. Mycobacterial efflux pumps and chemotherapeutic implications. International journal of antimicrobial agents **22:**274-278.

9. **Adams KN, Szumowski JD, Ramakrishnan L.** 2014. Verapamil, and Its Metabolite Norverapamil, Inhibit Macrophage-induced, Bacterial Efflux Pump-mediated Tolerance to Multiple Anti-tubercular Drugs. The Journal of infectious diseases.

10. **Adams KN, Takaki K, Connolly LE, Wiedenhoft H, Winglee K, Humbert O, Edelstein PH, Cosma CL, Ramakrishnan L.** 2011. Drug tolerance in replicating mycobacteria mediated by a macrophage-induced efflux mechanism. Cell **145:**39-53.

11. **Sharma S, Kumar M, Sharma S, Nargotra A, Koul S, Khan IA.** 2010. Piperine as an inhibitor of Rv1258c, a putative multidrug efflux pump of Mycobacterium tuberculosis. The Journal of antimicrobial chemotherapy **65:**1694-1701.

12. **Gupta S, Tyagi S, Almeida DV, Maiga MC, Ammerman NC, Bishai WR.** 2013. Acceleration of tuberculosis treatment by adjunctive therapy with verapamil as an efflux inhibitor. American journal of respiratory and critical care medicine **188:**600-607.

13. **Balganesh M, Dinesh N, Sharma S, Kuruppath S, Nair AV, Sharma U.** 2012. Efflux pumps of Mycobacterium tuberculosis play a significant role in antituberculosis activity of potential drug candidates. Antimicrobial agents and chemotherapy **56:**2643-2651.

14. **Rodrigues L, Villellas C, Bailo R, Viveiros M, Ainsa JA.** 2013. Role of the Mmr efflux pump in drug resistance in Mycobacterium tuberculosis. Antimicrobial agents and chemotherapy **57:**751-757.

15. **Mullin S, Mani N, Grossman TH.** 2004. Inhibition of antibiotic efflux in bacteria by the novel multidrug resistance inhibitors biricodar (VX-710) and timcodar (VX-853). Antimicrobial agents and chemotherapy **48:**4171-4176.

16. **Pucci MJ, Ackerman M, Thanassi JA, Shoen CM, Cynamon MH.** 2010. In vitro antituberculosis activities of ACH-702, a novel isothiazoloquinolone, against quinolone-susceptible and quinolone-resistant isolates. Antimicrobial agents and chemotherapy **54:**3478-3480.

17. **Franzblau SG, Witzig RS, McLaughlin JC, Torres P, Madico G, Hernandez A, Degnan MT, Cook MB, Quenzer VK, Ferguson RM, Gilman RH.** 1998. Rapid, low-technology MIC determination with clinical Mycobacterium tuberculosis isolates by using the microplate Alamar Blue assay. Journal of clinical microbiology **36:**362-366.

18. **Andreu N, Fletcher T, Krishnan N, Wiles S, Robertson BD.** 2012. Rapid measurement of antituberculosis drug activity in vitro and in macrophages using bioluminescence. The Journal of antimicrobial chemotherapy **67:**404-414.

19. **Murray JL, Mehta K, Lopez-Berestein G.** 1988. Induction of adenosine deaminase and 5' nucleotidase activity in cultured human blood monocytes and monocytic leukemia (THP-1) cells by differentiating agents. Journal of leukocyte biology **44:**205-211.

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

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. **Crowle AJ, Elkins N, May MH.** 1988. Effectiveness of ofloxacin against Mycobacterium tuberculosis and Mycobacterium avium, and rifampin against M. tuberculosis in cultured human macrophages. The American review of respiratory disease **137:**1141-1146.

530 23. **Keller C, Lauber J, Blumenthal A, Buer J, Ehlers S.** 2004. Resistance
531 and susceptibility to tuberculosis analysed at the transcriptome level:
532 lessons from mouse macrophages. Tuberculosis (Edinburgh, Scotland)
533 **84:**144-158.
534 24. **Brillault J, De Castro WV, Harnois T, Kitzis A, Olivier JC, Couet W.**
535 2009. P-glycoprotein-mediated transport of moxifloxacin in a Calu-3 lung
536 epithelial cell model. Antimicrobial agents and chemotherapy **53:**1457-
537 1462.
538 25. **Germann UA, Ford PJ, Shlyakhter D, Mason VS, Harding MW.** 1997.
539 Chemosensitization and drug accumulation effects of VX-710, verapamil,
540 cyclosporin A, MS-209 and GF120918 in multidrug resistant HL60/ADR
541 cells expressing the multidrug resistance-associated protein MRP. Anti-
542 cancer drugs **8:**141-155.
543 26. **Germann UA, Harding MW.** 1995. Chemosensitizers to overcome and
544 prevent multidrug resistance? Journal of the National Cancer Institute
545 **87:**1573-1575.
546 27. **Hodge G, Holmes M, Jersmann H, Reynolds PN, Hodge S.** 2013. The
547 drug efflux pump Pgp1 in pro-inflammatory lymphocytes is a target for
548 novel treatment strategies in COPD. Respiratory research **14:**63.
549 28. **Michot JM, Seral C, Van Bambeke F, Mingeot-Leclercq MP, Tulkens**
550 **PM.** 2005. Influence of efflux transporters on the accumulation and efflux
551 of four quinolones (ciprofloxacin, levofloxacin, garenoxacin, and

552 moxifloxacin) in J774 macrophages. Antimicrobial agents and
553 chemotherapy **49:**2429-2437.
554 29. **Amaral L, Martins M, Viveiros M.** 2007. Enhanced killing of intracellular
555 multidrug-resistant Mycobacterium tuberculosis by compounds that affect
556 the activity of efflux pumps. The Journal of antimicrobial chemotherapy
557 **59:**1237-1246.
558 30. **Singh M, Jadaun GP, Ramdas, Srivastava K, Chauhan V, Mishra R,**
559 **Gupta K, Nair S, Chauhan DS, Sharma VD, Venkatesan K, Katoch VM.**
560 2011. Effect of efflux pump inhibitors on drug susceptibility of ofloxacin
561 resistant Mycobacterium tuberculosis isolates. The Indian journal of
562 medical research **133:**535-540.
563 31. **Germann UA, Shlyakhter D, Mason VS, Zelle RE, Duffy JP, Galullo V,**
564 **Armistead DM, Saunders JO, Boger J, Harding MW.** 1997. Cellular and
565 biochemical characterization of VX-710 as a chemosensitizer: reversal of
566 P-glycoprotein-mediated multidrug resistance in vitro. Anti-cancer drugs
567 **8:**125-140.
568 32. **Mitchell AM, Tom M, Mortimer RH.** 2005. Thyroid hormone export from
569 cells: contribution of P-glycoprotein. The Journal of endocrinology **185:**93-
570 98.
571 33. **Dobson PD, Kell DB.** 2008. Carrier-mediated cellular uptake of
572 pharmaceutical drugs: an exception or the rule? Nature reviews. Drug
573 discovery **7:**205-220.

34. **Van Bambeke F, Michot JM, Tulkens PM.** 2003. Antibiotic efflux pumps in eukaryotic cells: occurrence and impact on antibiotic cellular pharmacokinetics, pharmacodynamics and toxicodynamics. The Journal of antimicrobial chemotherapy **51:**1067-1077.

35. **Dartois V, Barry CE, 3rd.** 2013. A medicinal chemists' guide to the unique difficulties of lead optimization for tuberculosis. Bioorganic & medicinal chemistry letters **23:**4741-4750.

36. **Davis SL, Nuermberger EL, Um PK, Vidal C, Jedynak B, Pomper MG, Bishai WR, Jain SK.** 2009. Noninvasive pulmonary [18F]-2-fluoro-deoxy-D-glucose positron emission tomography correlates with bactericidal activity of tuberculosis drug treatment. Antimicrobial agents and chemotherapy **53:**4879-4884.

37. **Kjellsson MC, Via LE, Goh A, Weiner D, Low KM, Kern S, Pillai G, Barry CE, 3rd, Dartois V.** 2012. Pharmacokinetic evaluation of the penetration of antituberculosis agents in rabbit pulmonary lesions. Antimicrobial agents and chemotherapy **56:**446-457.

38. **Bigi F, Gioffre A, Klepp L, Santangelo MP, Alito A, Caimi K, Meikle V, Zumarraga M, Taboga O, Romano MI, Cataldi A.** 2004. The knockout of the lprG-Rv1410 operon produces strong attenuation of Mycobacterium tuberculosis. Microbes and infection / Institut Pasteur **6:**182-187.

39. **Domenech P, Reed MB, Barry CE, 3rd.** 2005. Contribution of the Mycobacterium tuberculosis MmpL protein family to virulence and drug resistance. Infection and immunity **73:**3492-3501.

40. **Farrow MF, Rubin EJ.** 2008. Function of a mycobacterial major facilitator superfamily pump requires a membrane-associated lipoprotein. Journal of bacteriology **190:**1783-1791.

41. **Calgin MK, Sahin F, Turegun B, Gerceker D, Atasever M, Koksal D, Karasartova D, Kiyan M.** 2013. Expression analysis of efflux pump genes among drug-susceptible and multidrug-resistant Mycobacterium tuberculosis clinical isolates and reference strains. Diagnostic microbiology and infectious disease **76:**291-297.

42. **Chatterjee A, Saranath D, Bhatter P, Mistry N.** 2013. Global transcriptional profiling of longitudinal clinical isolates of Mycobacterium tuberculosis exhibiting rapid accumulation of drug resistance. PloS one **8:**e54717.

43. **Sheen P, Lozano K, Gilman RH, Valencia HJ, Loli S, Fuentes P, Grandjean L, Zimic M.** 2013. pncA gene expression and prediction factors on pyrazinamide resistance in Mycobacterium tuberculosis. Tuberculosis (Edinburgh, Scotland) **93:**515-522.

44. **Zimic M, Fuentes P, Gilman RH, Gutierrez AH, Kirwan D, Sheen P.** 2012. Pyrazinoic acid efflux rate in Mycobacterium tuberculosis is a better proxy of pyrazinamide resistance. Tuberculosis (Edinburgh, Scotland) **92:**84-91.

45. **Eldholm V. Norheim G., von der Lippe, B Kinander W, Dahle UR, Caugant DA, Mannsaker T, Mengshoel AT, Dyrrol-Rilse AM, Balloux F.** 2014. Evolution of extensively drug-resistant Mycobacterium

620 tuberculosis from a susceptible ancestor in a single patient. Genome
621 Biology 2014, **15**:490
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Table 1. Synergistic and additive interactions of anti-mycobacterial compounds tested in combination with timcodar (TIM) in *Mycobacterium tuberculosis* (Mtb; H37Ra) broth cultures or in cultures of a *M. tuberculosis*-infected human macrophage-like cell line (THP-1 cells).

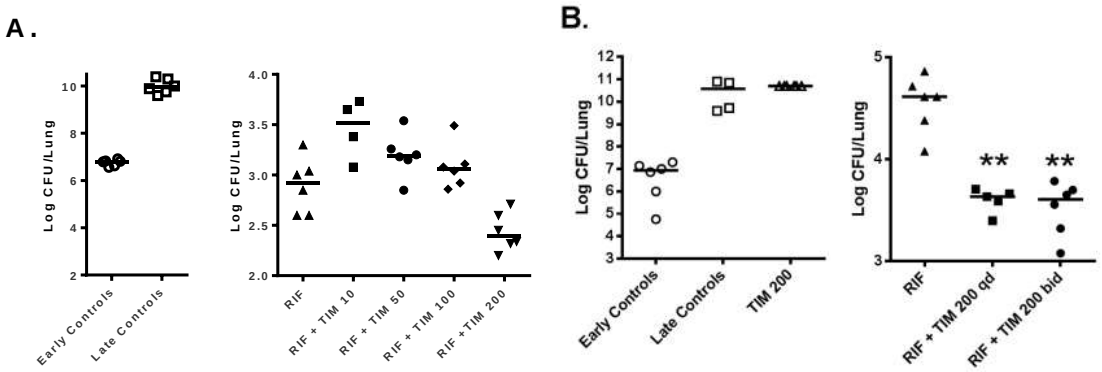
Compound	**MIC (µg/ml)**	**FIC**	**Mtb/THP-1 Cells (IC_{50})**	**FIC**
Timcodar	18.7	NA	1.87	NA
Isoniazid	0.21	1.00	0.01	0.63
Rifampin	0.03	0.25	0.01	0.50
Moxifloxacin	0.16	0.75	0.12	0.50
Linezolid	0.53	1.00	0.36	1.00
Bedaquiline	0.05	0.12	0.03	0.28
PA-824	0.14	0.63	0.05	2.0
Clofazimine	0.73	0.31	0.18	0.56

Minimal inhibitory concentrations (MICs) were determined at 90% inhibition (µg/ml) by two-fold serial dilution of compounds after 11 days of bacterial culture; concentrations in µg/mL are provided in parenthesis. A reporter strain of Mtb expressing firefly luciferase was used to infect THP-1 cells and measure bacterial growth a 50% inhibitory concentration at five days post-infection. The fractional inhibitory concentration (FIC) was determined for combinations of two compounds as a synergistic interaction (< 0.5), additive interaction (0.5-2.0) or antagonistic interaction (>2.0).

Table 2. Timcodar increases the exposure of bedaquiline in the plasma of C57BL/6 mice infected with *Mycobacterium tuberculosis*

Drug, dose	**Lung cfu**	**AUCinf** (µg.h/ml)	**Cmax** (µg/ml)	**T1/2 (**h)
TIM, 10 mg/kg	ND	3.4	2.7	1.6
TIM, 100 mg/kg	ND	60.4	8.9	2.4
TIM, 200 mg/kg	ND	137	14.7	2.4
RIF, 10 mg/kg	4.14 (0.13)	152.0	19.8	4.9
RIF, 10 mg/kg + TIM*, 200 mg/kg	3.39 (0.20)	153.0	14.9	6.1
Moxifloxacin	3.09 (0.12)	23.7	7.17	2.87
Moxifloxacin + TIM, 200 mg/kg	3.17 (0.21)	23.3	7.13	3.71
Bedaquiline	3.52 (0.51)	7.94	0.64	7.14
Bedaquiline + TIM, 200 mg/kg	3.15 (0.17)	21.6	1.51	7.46

* TIM dosed 6 hours prior to RIF # compounds dosed in 0.5% methyl cellulose in water



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697 **Figure 1. Co-dosing of Timcodar (TIM) potentiates the activity of RIF in a high dose, acute infection model of**
698 **tuberculosis after four weeks of treatment. A.** Dose response of TIM with co-dosed orally with RIF at 10 mg/kg/day,
699 once a day. TIM was co-dosed at 10, 50, 100 or 200 mg/kg/day, once a day X hours before RIF dosing. Mice were
700 infected with 2.0 X 10^6 CFU of Mtb Erdman per mouse and randomly assigned to groups of six mice. Early controls (EC)
701 and Late Control (LC) group data are shown. **B.** TIM 200 mg/kg/day once a day (left panel), RIF 10mg/kg/day once per
702 day, RIF 10 mg/kg/day once per day + TIM 200mg/kg dosed twice per day (bid) or RIF 10mg/kg/day once per day + TIM

200 mg/kg once per day (qd; right panel). Scatter plots of Log_{10} CFU recovered from the lungs of infected mice are shown and statistical significance is noted on the graphs as 0.05 >P>0.01 (*) or 0.01>P>.001 (**).

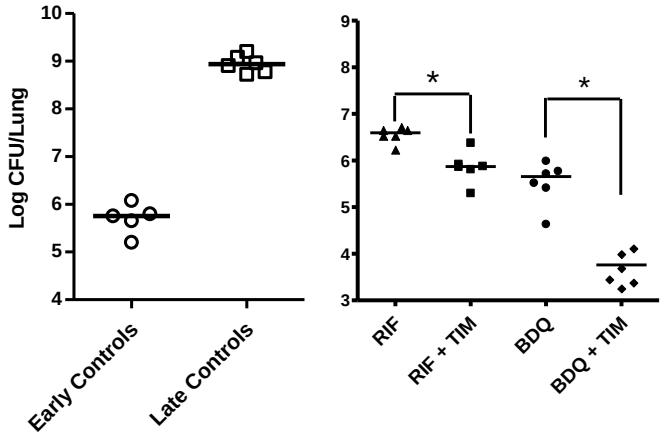

Figure 2. TIM in combination with bedaquiline (BDQ) in a high dose, acute infection model of tuberculosis with 8 days of treatment. BDQ (25mg/kg/day) and RIF (10mg/kg/day) was administered alone of in combination with TIM (200mg/kg/day). TIM was dosed six hours before BDQ and RIF. Untreated early controls (EC) one day post-infection and late controls (LC) of the Mtb Erdman infection (10^6 CFU) CFU are shown in the adjacent panel separately. Scatter plots

of Log_{10} CFU recovered from the lungs of infected mice are shown and statistical significance is noted on the graphs as P < 0.05 (*).

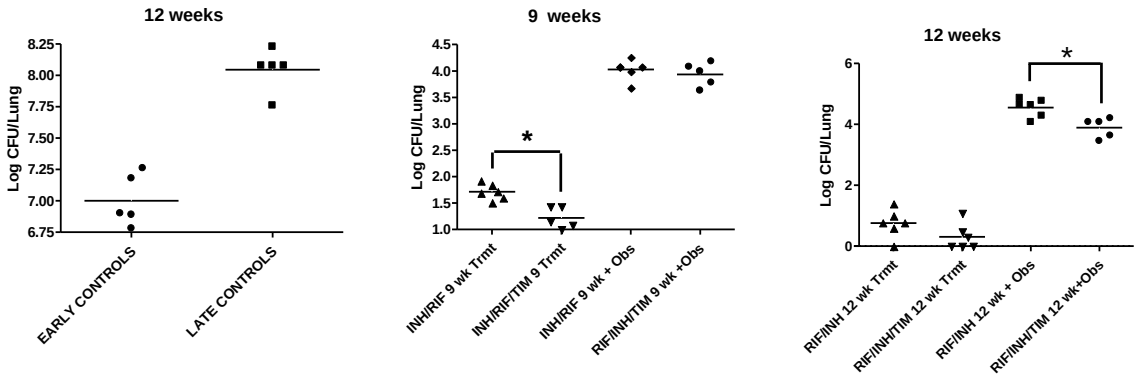

Figure 3. Timcodar (TIM) reduced bacterial relapse after 12 weeks of treatment in long term, chronic infection model of tuberculosis infection. RIF 10 mg/kg/day, INH) 25 mg/kg/day were dosed with TIM 200 mg/kg/day and mice were treated for either nine weeks or twelve weeks and infection with 10^2 CFU of the Mtb Erdman isolate. TIM was dosed six hours before RIF and INH; RIF and INH were dosed together. Separate groups of six mice were used during each

phase treatment and were then left untreated for an additional eight weeks during an observation phase (Obs) to determine the extent of relapse of infection. Scatter plots of Log_{10} CFU recovered from the lungs of infected mice are shown and statistical significance is noted on the graphs as $P < 0.05$ (*).

Figure 1

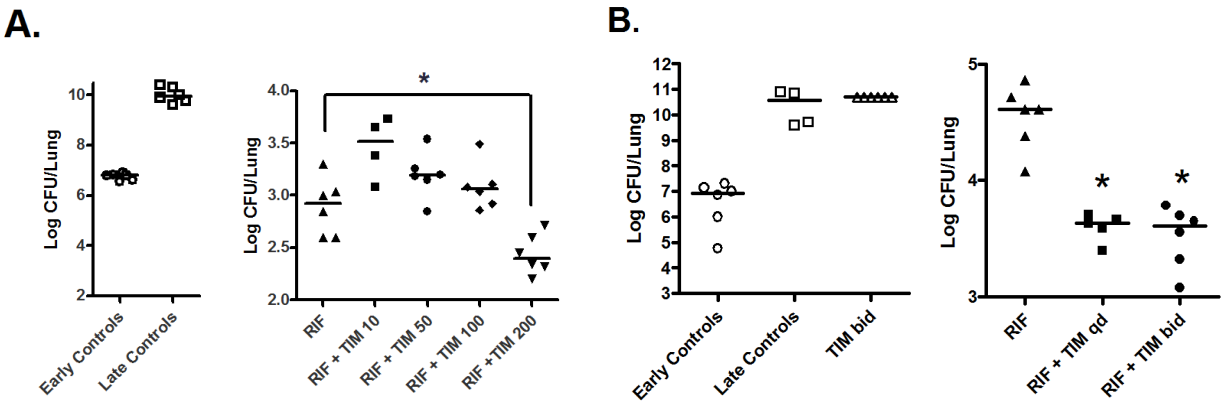

Figure 1. Co-dosing of Timcodar (TIM) potentiates the activity of RIF in a high dose, acute infection model of tuberculosis after four weeks of treatment. A. Dose response of TIM with co-dosed orally with RIF at 10 mg/kg/day, once a day. TIM was co-dosed at 10, 50, 100 or 200 mg/kg/day, once a day X hours before RIF dosing. Mice were infected with 2.0 X 10^6 CFU of Mtb Erdman per mouse and randomly assigned to groups of six mice. Early controls (EC) and Late Control (LC) group data are shown. **B.** TIM 200 mg/kg/day twice a day (left panel), RIF 10mg/kg/day once per day, RIF 10 mg/kg/day once per day + TIM 200mg/kg dosed twice per day (bid) or RIF 10mg/kg/day once per day + TIM 200 mg/kg once per day (qd; right panel). Scatter plots of Log_{10} CFU recovered from the lungs of infected mice are shown and statistical significance is noted on the graphs as 0.05 >P>0.01 (*) or 0.01>P>.001 (**).

Figure 2

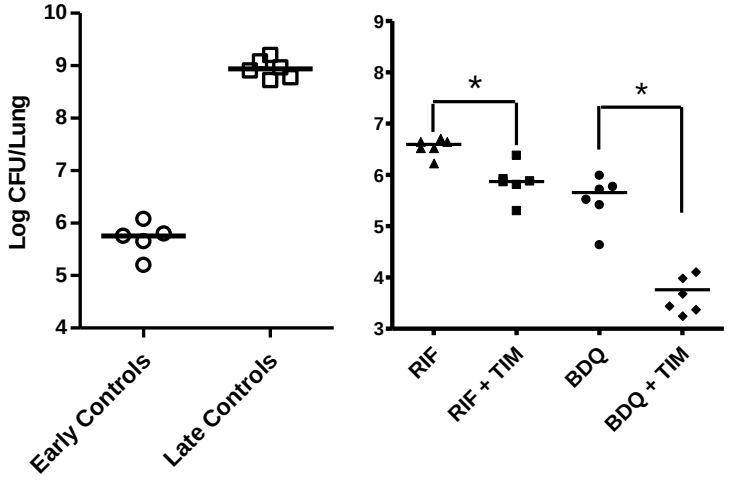

Figure 2. TIM in combination with bedaqualine (BDQ) in a high dose, acute infection model of tuberculosis with 8 days of treatment. BDQ (25mg/kg/day) and RIF (10mg/kg/day) was administered alone of in combination with TIM (200mg/kg/day). TIM was dosed six hours before BDQ and RIF. Untreated early controls (EC) one day post-infection and late controls (LC) of the Mtb Erdman infection (10^6 CFU) CFU are shown in the adjacent panel separately. Scatter plots of Log_{10} CFU recovered from the lungs of infected mice are shown and statistical significance is noted on the graphs as $P < 0.05$ (*).

Figure 3.

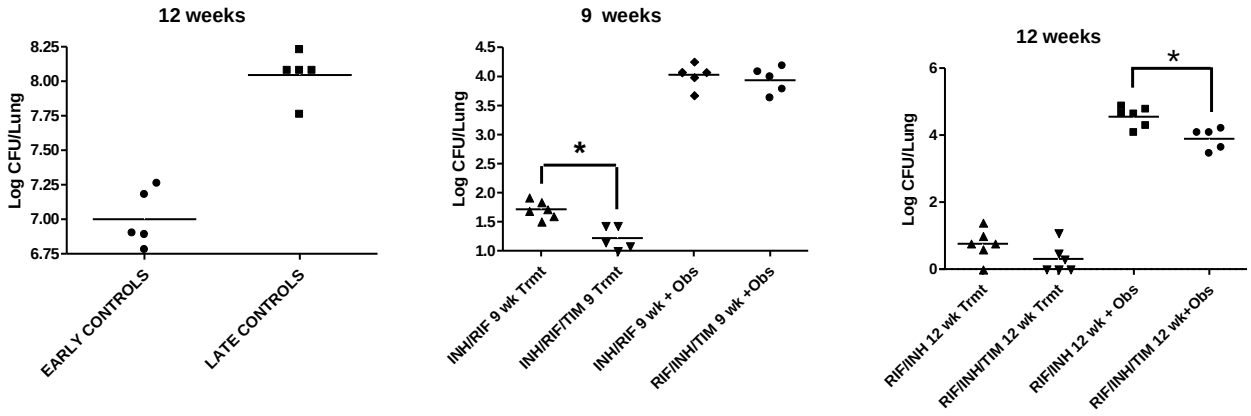

Figure 3. Timcodar (TIM) reduced bacterial relapse after 12 weeks of treatment in long term, chronic infection model of tuberculosis infection. RIF 10 mg/kg/day, INH) 25 mg/kg/day were dosed with TIM 200 mg/kg/day and mice were treated for either nine weeks or twelve weeks and infection with 10^2 CFU of the Mtb Erdman isolate. TIM was dosed six hours before RIF and INH; RIF and INH were dosed together. Separate groups of six mice were used during each phase treatment and were then left untreated for an additional eight weeks during an observation phase (Obs) to determine the extent of relapse of infection. Scatter plots of Log_{10} CFU recovered from the lungs of infected mice are shown and statistical significance is noted on the graphs as $P < 0.05$ (*).