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The Efflux Pump Inhibitor Timcodar Improves the Potency of Antimycobacterial Agents

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

Vertex Pharmaceuticals Incorporated, Boston, Massachusetts, USA[a]; Central New York Research Corporation, Veterans Affairs Medical Center, Syracuse, New York, USA[b]

Previous studies indicated that inhibition of 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 antituberculosis (anti-TB) drugs against *Mycobacterium tuberculosis* in *in vitro* and *in vivo* combination studies. When used alone, timcodar weakly inhibited *M. tuberculosis* growth in broth culture (MIC, 19 μg/ml); however, it demonstrated synergism in drug combination studies with rifampin, bedaquiline, and clofazimine but not with other anti-TB agents. When *M. tuberculosis* was cultured in host macrophage cells, timcodar had about a 10-fold increase (50% inhibitory concentration, 1.9 μg/ml) in the growth inhibition of *M. tuberculosis* 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 mouse model of long-term, chronic infection with treatment 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 the plasma exposure of bedaquiline. These data suggest that the antimycobacterial drug-potentiating activity of timcodar is complex and drug dependent and involves both bacterial and host-targeted mechanisms. Further study of the improvement of the potency of antimycobacterial drugs and drug candidates when used in combination with timcodar is warranted.

Tuberculosis (TB) is a major infectious disease killer due to HIV coinfection/immunosuppression, ineffective health care management, and widespread drug resistance. The prevalence of *Mycobacterium tuberculosis* infections remains very high, with approximately 2 billion latent infections, 10 million new cases, and 2 million deaths occurring each year (1). Tuberculosis is caused by *M. tuberculosis*, a slow-growing, acid-fast bacillus that withstands a harsh immunological assault by human host macrophages and effector cells as well as suboptimal chemotherapy by persisting in a semidormant state of replication. New drugs are urgently needed to shorten the treatment regimen and to combat increasing drug resistance.

The drug resistance and drug tolerance of TB are 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, the activities of antibiotic and anticancer agents can be potentiated (2–5). Similar to other bacteria, *M. tuberculosis* uses efflux pumps to export antibiotics and other toxins that would otherwise kill the 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 rifampin (RIF) in *M. tuberculosis*-infected THP-1 macrophage cells (9, 10). In addition, the efflux pump inhibitor piperine potentiated the antimycobacterial activity of RIF in broth cultures of *M. tuberculosis* by targeting Rv1258c (11), while verapamil accelerated the clearance of *M. tuberculosis* in C3HeB/FeJ mice when it was coadministered with agents used as the standard of care for the treatment of drug-sensitive *M. tuberculosis* infection (isoniazid [INH], RIF, and pyrazinamide [PZA]) (12). Furthermore, other efflux pumps have been described to play a more important role in drug resistance of *M. tuberculosis*, 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 antimycobacterial drugs *in vitro* against both extracellular and intracellular *M. tuberculosis* 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 anti-TB drugs can potentiate antimycobacterial activity *in vitro* and efficacy *in vivo*. Our findings indicate that TIM has a pronounced antimycobac-

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Address correspondence to Christopher P. Locher, Christopher_locher@hotmail.com.

* Present address: Trudy H. Grossman, Tetraphase Pharmaceuticals, Watertown, Massachusetts, USA; Peter L. Jones, Driftwood Kayak, Deer Isle, Maine, USA; Christopher P. Locher, Karaden Farms, Captain Cook, Hawaii, USA.

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terial adjuvant effect with antimycobacterial drugs when *M. tuberculosis* is cultured in macrophage-free broth or in *M. tuberculosis*-infected human macrophages. We also readily observed the potentiation of antibiotic activity by TIM in mouse 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), PZA, acriflavine (ACR), and ethidium bromide were purchased from Sigma Chemical Co. (St. Louis, MO). Gatifloxacin (GAT) was provided by Bristol-Myers Squibb. Linezolid (LZD) and PNU100480 (sutezolid) were provided by Pharmacia & Upjohn, levofloxacin (LVX) was provided by the R. W. Johnson Pharmaceutical Research Institute, and rifapentine (RFP) was provided by Marion Merrell Dow Chemical Company, Cincinnati, OH. All drugs were dissolved in 100% dimethyl sulfoxide (DMSO) and diluted in 7H10 broth with 10% oleic acid-albumin-dextrose-catalase (OADC; BBL Microbiology Systems, Cockeysville, MD) and 0.05% Tween 80.

Growth inhibition and MIC in broth culture. Compounds were prepared at 100 times the maximal concentration, added to the first wells of polystyrene 96-well round-bottom plates, and serially diluted 2-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 plates, picked, prepared at 1×10^8 CFU/ml, and diluted 200 times to a final concentration of 5×10^5 CFU/ml in 7H9 broth supplemented with 10% albumin-dextrose-catalase (ADC) as previously described (16). One hundred microliters of cells was added into each well containing drugs. The 96-well plates were then incubated at 37°C in ambient air.

For efflux pump inhibitor studies with *M. tuberculosis* (Erdman isolate), the compounds were prepared at 4 times the maximal concentration and 50 μl of 7H10 broth was added to each well containing serial dilutions of TIM diluted 2-fold in duplicate. Fifty microliters of 5×10^5 CFU/ml Erdman with or without TIM was added to each well. Isolates of strain Erdman were frozen at 5×10^7 CFU/ml and diluted to 5×10^5 CFU/ml. The plates were incubated for 18 to 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 with 10 μg/ml of TIM at 8 μg/ml to 0.008 μg/ml. ETA, ACR, and ethidium bromide were tested in 7H10 alone or 7H10 with 10 μg/ml of TIM at 128 μg/ml to 0.125 μg/ml. RIF and RFP were tested in 7H10 alone or 7H10 with 10 μg/ml of TIM at 1 μg/ml to 0.001 μg/ml.

For studies with the *M. tuberculosis* H37Ra isolate, 30 μl of resazurin detection buffer was added to each well after 9 days and the plate was returned to the incubator (17). After 24 h of incubation with resazurin, the fluorescence was read using a Biotek Synergy 2 plate reader at a gain of 35 with an excitation wavelength of 492 nm and an emission wavelength of 595 nm. For *M. tuberculosis* H37Ra, the MIC was defined as 90% growth inhibition, and this value was used for H37Ra when the fractional inhibitory index (FIC) was determined.

Susceptibility testing using *M. tuberculosis*-infected macrophage cultures. An *M. tuberculosis* strain (H37Ra) was transfected with pVV261 expressing firefly luciferase by methods similar to those previously described (18) and used to infect a human macrophage-like cell line (THP-1; ATCC TIB-202). Briefly, THP-1 cell stocks were maintained at a culture density of between 2×10^5 and 8×10^5 cells/ml in RPMI 1640 medium (with phenol red, 25 mM HEPES, and 2 mM L-glutamine; Gibco) supplemented with 10% fetal bovine serum (Gibco) and 0.05 mM β-mercaptoethanol (Invitrogen) in 96-well tissue culture plates (Costar 3903; Corning). A cell suspension of sonicated *M. tuberculosis* H37Ra expressing firefly luciferase (8×10^5 cells/ml) in RPMI 1640 cell culture medium was used to infect phorbol myristic acetate (PMA; 100 nM; Sigma Chemical Co.)-differentiated THP-1 cells (19) at a multiplicity of infection (MOI) of 2:1 for 2 h at 37°C. The supernatant containing cell-free *M. tuberculosis* was then carefully removed from each well and the *M. tuberculosis*-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 of fresh medium and 50 μl of medium containing test compounds. After 5 days of incubation, 100 μl of Steady-Glo (Promega) reagent was added to each well, the plate was incubated for 15 min at room temperature (RT) and covered with an adhesive top seal, and the luminescence was read using a Biotek Synergy 2 plate reader at a gain of 165 at the maximum integration time. THP-1 cell viability was determined by using the CellTiter-Glo cell viability assay per the manufacturer's instructions (Promega). The synergistic, additive, or interfering effects of compound combinations were determined in a checkerboard assay in 96-well tissue culture plates by calculating the FIC as previously described (20, 21): FIC = (MIC of compound A with compound B/MIC of compound A alone) + (MIC of compound B with compound A/MIC of compound B alone).

To measure the inhibition of *M. tuberculosis* growth in bone marrow-derived macrophages from C57BL/6 mice, the numbers of CFU were determined as previously described (22, 23) after coincubation of TIM with antimycobacterial agents (INH, RIF, or BDQ). Briefly, macrophage cells were cultured in 24-well tissue culture plates (1×10^6 per well) at 37°C in 5% CO_2. On day 7, the medium was removed and the primary macrophages were infected with *M. tuberculosis* H37Rv (ATCC 25618) at 1×10^6 CFU per well (MOI, 1). At 7 days postinfection with *M. tuberculosis*, the tissue culture medium was removed from the wells; the cells were washed twice with phosphate-buffered saline 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 1/1,000 dilution. The agar plates were then incubated at 37°C for 3 to 4 weeks, after which the bacterial colonies were counted and the numbers of CFU/ml of cell lysates were determined. The concentration of drug reducing the bacterial load by 2 log units (the minimal bactericidal concentration causing a 99% reduction [MBC_{99}] was recorded as a numerical readout.

Murine *M. tuberculosis* infection models. Six-week-old female C57BL/6 mice were purchased from The Jackson Laboratory, Bar Harbor, ME, and were maintained within the advanced biosafety level 3 facility at the Syracuse Veterans Affairs Medical Center's Veterinary Medical Unit, Syracuse, NY. All animal procedures were approved by the Subcommittee for Animal Studies (SAS). Mice were housed in microisolator 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; the 7H10 agar formulation with agar and malachite green omitted) with 10% OADC enrichment (BBL Microbiology Systems, Cockeysville, MD) and 0.05% Tween 80 for 5 to 10 days on a rotary shaker at 37°C. The culture was diluted to 100 Klett units (equivalent to 5×10^7 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 4 weeks. Mice were infected intranasally with *M. tuberculosis* Erdman at 10^6 CFU/mouse. At 1 week postinfection, the mice were treated by oral gavage in a 0.2-ml volume five times per week for 4 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 of body weight either once or twice a day, as indicated for each experiment in the figure legends; 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 the vehicle (data not shown). TIM was given in the morning, and drugs tested in combination were administered 5 to 6 h after the 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; mice in the LC group were moribund and needed to be euthanized at 14 days postinfection. At the end of the experiments, all surviving mice were euthanized by CO_2 inhalation. The right lungs were aseptically removed and ground in a sealed tissue homogenizer (IdeaWorks! Laboratory Devices, Syracuse, NY). The number of viable organisms was determined by serial dilution and titration on 7H10 agar plates. The plates were incubated at 37°C in ambient air for 4 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 (log_{10}) numbers of CFU. Due to the small sample size and the consequent need to protect against deviations from normality, an analysis of variance statistical analysis followed by a Dunnett's posttest adjustment was performed to determine whether statistically significant differences existed between the control and the 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 retroorbital bleeding (three mice per time point) at 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h after dosing, and plasma was obtained following centrifugation of blood samples at 3,000 × *g* for 2 min. Plasma samples were then stored at −20°C in sealed cluster tubes (Costar 4413 cluster tube system), and the samples were extracted using acetonitrile (4:1) containing an analytical internal standard. The tubes were mixed by vortexing for 5 min and centrifuged at 3,000 × *g* for 20 min, and the supernatants were transferred to fresh plates for quantitative liquid chromatography-mass spectrometry (MS). Calibration standards (1 to 5,000 ng/ml) and quality control (QC) samples (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 liquid chromatography system with a Waters Xterra C_{18} column, followed by MS analysis using a SCIEX API 4000 mass spectrometer (Applied Biosystems) in electrospray ionization mode and multiple-reaction-monitoring scan mode. The amounts in the standards, QCs, and samples were determined relative to the amount of the analytical internal standard. Pharmacokinetic parameters were determined by noncompartmental analysis of the plasma concentration data using WinNonlin software (Pharsight Corp., Mountain View, CA). In the pharmacokinetic studies where TIM was coadministered with other antimicrobials, TIM (200 mg/kg or 100 mg/kg) was orally administered to mice 6 h prior to the oral administration of RIF, MOX, or bedaquiline in their respective formulations. Blood sampling, plasma extraction, and quantification of RIF by liquid chromatography-MS analysis and pharmacokinetic analysis of the data were carried out as described above.

RESULTS

Activity of TIM with combinations of other drugs against *M. tuberculosis* in broth culture *in vitro*. 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 a similar potentiation could be observed when this efflux pump inhibitor was evaluated in cultures of *M. tuberculosis*. We found that TIM alone possessed weak growth-inhibitory activity using standard MIC assays of mycobacteria cultured in broth (MIC for the H37Ra isolate, 18.7 μg/ml [Table 1]; MIC for the Erdman isolate, 64 μg/ml). In assays with *M. tuberculosis* Erdman, TIM at 10 μg/ml combined with a diverse set of anti-TB antibiotics showed little potentiating effect in combination with any of the antibiotics with the exception of BDQ, where TIM conferred a 15-fold improvement in the MIC (MIC = 0.004 μg/ml with TIM versus 0.06 μg/ml without TIM; see Table S1 in the supplemental material). Furthermore, in broth cultures of *M. tuberculosis*, we found a 4-fold potentiating effect of TIM with the intercalator ethidium bromide, which is known to be a promiscuous efflux pump substrate in many organisms, suggesting that TIM had an antimycobacterial effect consistent with efflux inhibition when *M. tuberculosis* was cultured in broth medium. To further establish the synergy of TIM in combination with TB drugs *in vitro*, a checkerboard MIC assay was performed with TIM at higher concentrations together with antimycobacterial drugs, and the sum fractional inhibitory concentration (FIC) index was determined. In these assays, we found that combinations of TIM with RIF, BDQ, and CFZ showed synergy in broth cultures (Table 1).

TABLE 1 Synergistic and additive interactions of antimycobacterial compounds tested in combination with TIM in *M. tuberculosis* H37Ra broth cultures or in cultures of *M. tuberculosis*-infected THP-1 cells[a]

Compound	*M. tuberculosis* broth cultures		*M. tuberculosis*-infected THP-1 cells	
	MIC (μg/ml)	FIC	IC_{50} (μg/ml)	FIC
TIM	18.7	NA	1.87	NA
INH	0.21	1.00	0.01	0.63
RIF	0.03	0.25	0.01	0.50
MXF	0.16	0.75	0.12	0.50
LZD	0.53	1.00	0.36	1.00
BDQ	0.05	0.12	0.03	0.28
PA-824	0.14	0.63	0.05	2.0
CFZ	0.73	0.31	0.18	0.56

[a] MICs were determined at 90% inhibition by 2-fold serial dilution of compounds after 11 days of bacterial culture. A reporter strain of *M. tuberculosis* expressing firefly luciferase was used to infect THP-1 cells, and the IC_{50} for bacterial growth was measured at 5 days postinfection. FICs were determined for TIM plus each of the indicated compounds. For combinations of two compounds, the FIC was determined to be a synergistic interaction if the FIC was <0.5, an additive interaction if the FIC was from 0.5 to 2.0, or an antagonistic interaction if the FIC was >2.0. NA, not applicable.

Activity of TIM against *M. tuberculosis* in cultures of infected macrophages *in vitro*. Although TIM alone modestly inhibited *M. tuberculosis* growth in broth cultures, it was about 10 times more potent when growth inhibition was measured in *M. tuberculosis*-infected macrophages using a luciferase reporter strain (50% inhibitory concentration [IC_{50}], 1.9 μg/ml; Table 1). In *M. tuberculosis* H37Ra-infected THP-1 cell macrophage cultures, we found drug synergy with RIF, BDQ, and MXF. We also observed an additive effect with other antimycobacterial drugs tested, including INH and CFZ, although there were no antagonistic interactions.

Evaluation of timcodar in combination with antimycobacterial agents *in vivo*. TIM was first evaluated in a high-dose, acute infection model (10^6 CFU/mouse), and 4 weeks of oral compound administration was initiated on day 7 postinfection. TIM showed a dose-dependent reduction in the mycobacterial lung burden in a once-daily dosing regimen in combination with RIF (10 mg/kg/day); TIM was dosed 6 h before RIF and was found to be most active with RIF when used at a high dose (200 mg/kg/day in combination with RIF; Fig. 1A). TIM did not have an antimycobacterial effect when it was dosed alone and had a similar effect if it was dosed at 200 mg/kg once or twice per day with RIF dosed once daily (Fig. 1B). In each combination study of TIM with RIF, com-

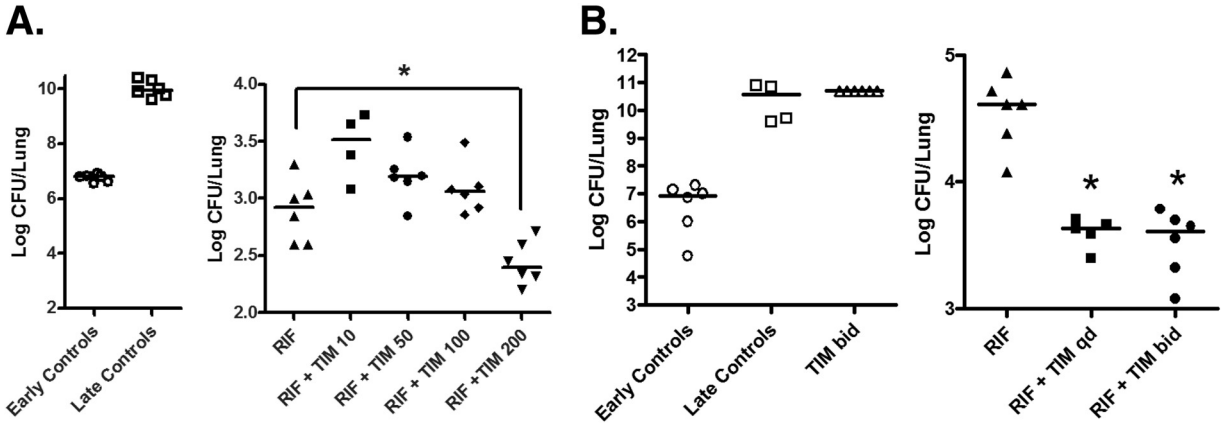

FIG 1 Codosing with TIM potentiates the activity of RIF in a high-dose, acute infection model of tuberculosis after 4 weeks of treatment. (A) Dose-response of TIM administered orally at 10, 50, 100, or 200 mg/kg/day with or without RIF at 10 mg/kg/day. TIM was dosed 6 h before RIF. Mice were infected with 2.0×10^6 CFU of *M. tuberculosis* Erdman per mouse and randomly assigned to groups of six mice each. Data for the early control and late control groups are shown. (B) Results for TIM at 200 mg/kg/day twice a day (TIM bid; left) and for RIF at 10 mg/kg/day once per day (RIF), RIF at 10 mg/kg/day once per day plus TIM at 200 mg/kg once per day (RIF + TIM qd), or RIF at 10 mg/kg/day once per day plus TIM at 200 mg/kg twice per day (RIF + TIM bid) (right) are shown. Scatter plots of the $\log_{10}$ number of 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 $0.01 > P > 0.001$ (**).

binations with TIM resulted in an approximately 10-fold reduction in the mycobacterial burden in lungs versus the reduction achieved with RIF alone (final count in lung, 4.5 $\log_{10}$ CFU in the RIF-treated group versus 3.5 $\log_{10}$ CFU in the RIF- and TIM-treated group; $P < 0.05$). A similar trend was observed regardless of whether TIM and RIF were either codosed simultaneously or dosed 6 or 8 h apart. In subsequent experiments, TIM was dosed 6 h before antibiotic treatment was administered (Fig. 2 and 3).

In further studies of TIM in mouse models of high-dose, acute TB infection, we evaluated TIM (200 mg/kg/day) in combination with PZA (150 mg/kg/day), RFP (10 mg/kg/day), MXF (100 mg/kg/day), PNU100480 (sutezolid; 100 mg/kg/day), BDQ (25 mg/kg/day), or GAT (100 mg/kg/day), but the inclusion of TIM did not lower the mycobacterial lung burden to a statistically significant level (see Table S2 in the supplemental material). However, there was an approximately one-quarter-log-unit reduction in the mycobacterial lung burden when TIM (200 mg/kg) was used in combination with INH (25 mg/kg/day; see Fig. S1 in the supplemental material) in the 4-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 8-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).

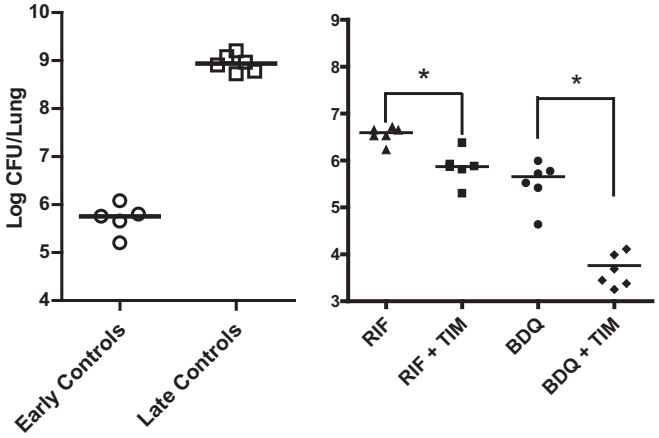

FIG 2 TIM in combination with BDQ in a high-dose, acute infection model of tuberculosis with 8 days of treatment. BDQ (25 mg/kg/day) and RIF (10 mg/kg/day) were administered alone or in combination with TIM (200 mg/kg/day). TIM was dosed 6 h before BDQ and RIF. Results for untreated early controls at 1 day postinfection and late controls infected with *M. tuberculosis* Erdman at 10^6 CFU are shown separately. Scatter plots of the $\log_{10}$ number of CFU recovered from the lungs of infected mice are shown, and statistical significance is noted on the graphs as *P* values of <0.05 (*).

Since coadministration of TIM improved the potency of RIF (and, to a lesser extent, that of INH), 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 9 or 12 weeks and held parallel groups of mice for an additional 8 weeks posttreatment to determine if they had a relapse of infection. At 9 weeks posttreatment, prior to the observation phase, fewer mice in the TIM-RIF-INH-treated group than the INH-RIF-treated group had detectable mycobacterial lung burdens (Fig. 3). After 12 weeks of treatment and 8 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 posttreatment, prior to the observation phase, there were also fewer mice in the TIM/RIF/INH-treated group (3 out of 6) than the INH/RIF-treated group (5 out of 6) that had detectable mycobacterial lung burdens, although the differences in the bacterial loads in lungs from these mice did not reach statistical significance.

Effect of TIM on pharmacokinetics of antimycobacterial drugs. Since TIM increased the potency of RIF and BDQ (and, to a lesser extent, that of INH) *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

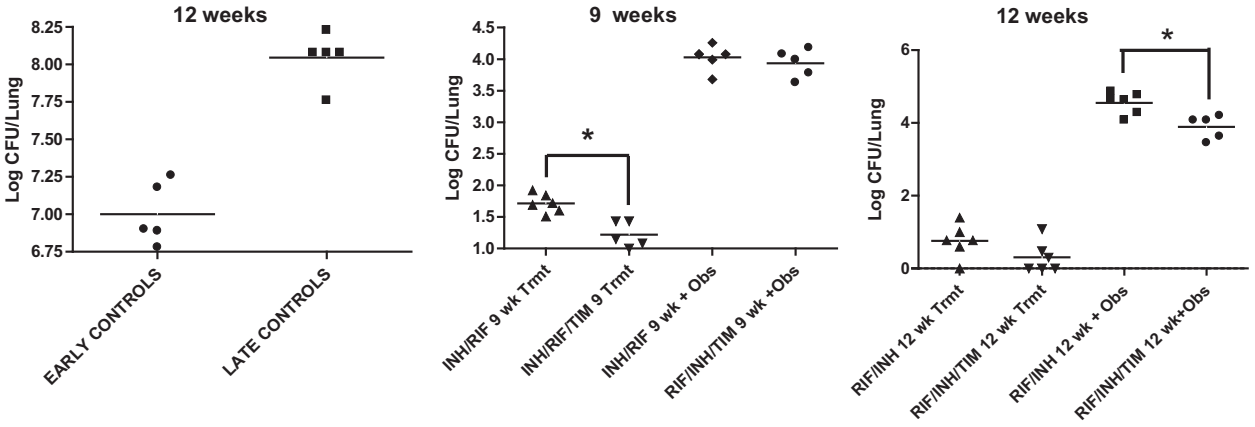

FIG 3 TIM reduced bacterial relapse after 12 weeks of treatment (Trmt) in a long-term, chronic infection model of *M. tuberculosis* infection. RIF at 10 mg/kg/day and INH at 25 mg/kg/day were dosed with TIM at 200 mg/kg/day, and mice were treated for either 9 weeks or 12 weeks after infection with 10^2 CFU of the *M. tuberculosis* Erdman isolate. TIM was dosed 6 h before RIF and INH were dosed; RIF and INH were dosed together. Separate groups of six mice each were used during each phase of treatment and were then left untreated for an additional 8 weeks during an observation phase (Obs) to determine the extent of relapse of infection. Scatter plots of the $\log_{10}$ number of CFU recovered from the lungs of infected mice are shown, and statistical significance is noted on the graphs as *P* values of <0.05 (*).

C57BL/6 mice was maintained at 5 to 15 μg/ml for over 16 h, which was similar to the concentrations in drug combinations showing synergy *in vitro*, and this dose of TIM provided a potentiating effect *in vivo*. The coadministration 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 by either the maximum concentration of drug (C_{max}) in plasma or the area under the concentration-time curve from time zero to infinity ($AUC_{0-\infty}$) (Table 2). However, the coadministration of TIM with BDQ (25 mg/kg) did increase the level of BDQ exposure by almost 3-fold.

DISCUSSION

The coadministration of efflux pump inhibitors with other drugs has been described to be a method of improving drug therapies targeting both mammalian and microbial cells (2–5, 24–28), including *M. tuberculosis* (8, 9, 12, 13, 29, 30). TIM was previously characterized to be 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 *M. tuberculosis* in broth culture, and this effect was more pronounced in the *M. tuberculosis*-infected macrophage assay using a luciferase reporter strain readout. The activity of TIM used in combination with RIF was synergistic against *M. tuberculosis*-infected macrophages, and the combination consistently improved the clearance of *M. tuberculosis* in infected mice in multiple experiments *in vivo*. The addition of TIM to the RIF regimen was as effective as the addition of another antimycobacterial drug to a RIF regimen *in vivo*. The efficacy of the TIM-RIF combination in *M. tuberculosis*-infected mice may be 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.

TABLE 2 TIM increases the exposure of BDQ in the plasma of C57BL/6 mice infected with *M. tuberculosis*

Drug(s) (dose [mg/kg])	No. of CFU in lung[a]	$AUC_{0-\infty}$ (μg · h/ml)	C_{max} (μg/ml)	$t_{1/2}$[b] (h)
TIM (10)	ND	3.4	2.7	1.6
TIM (100)	ND	60.4	8.9	2.4
TIM (200)	ND	137	14.7	2.4
RIF (10)	4.14 (0.13)	152.0	19.8	4.9
RIF (10) + TIM[c] (200)	3.39 (0.20)	153.0	14.9	6.1
MXF	3.09 (0.12)	23.7	7.17	2.87
MXF + TIM (200)	3.17 (0.21)	23.3	7.13	3.71
BDQ	3.52 (0.51)	7.94	0.64	7.14
BDQ + TIM (200)	3.15 (0.17)	21.6	1.51	7.46

[a] Data in parentheses represent standard deviation. ND, not determined.
[b] $t_{1/2}$, half-life.
[c] TIM was dosed 6 h prior to addition of RIF compounds, dosed in 0.5% methylcellulose in water.

We studied the pharmacokinetic parameters of selected antimycobacterial 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 codosing 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 *M. tuberculosis*-infected mice cotreated with TIM. Proinflammatory lymphocytes expressing P glycoprotein 1 (Pgp-1) surround sites of *M. tuberculosis* replication, and Pgp-1 inhibitors may help concentrate anti-TB drugs in the granuloma (35). Recent developments in granuloma imaging by using radioactive RIF or mass spectrometry (35–37) may allow

further elucidation of the role of efflux pump inhibition in models of *M. tuberculosis* infection. Furthermore, the use of C3HeB/FeJ mice or animal models in which more structured granulomas develop 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 *M. tuberculosis* broth culture 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 model (Fig. 2). Since there is evidence that TIM inhibits human cytochromes, this inhibitory mechanism may contribute to the increased exposure of BDQ in the mouse model, although inhibition of mouse cytochromes remains to be determined. TIM may also promote the uptake of an antimycobacterial agent into the infecting organism, increase the oral absorption and systemic distribution of an antimycobacterial agent in infected tissues, or modulate the metabolism of antimycobacterial 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 *M. tuberculosis* or in *M. tuberculosis*-infected macrophages, or both. This may explain why we saw differential effects depending on the culture conditions (broth versus *M. tuberculosis*-infected macrophages) and the drug with which TIM was combined (e.g., INH and BDQ). A specific *M. tuberculosis* target effect is supported by the observation that ethidium bromide efflux was inhibited by TIM in broth culture without host macrophage cells. In addition, TIM showed a substantial synergistic effect against *M. tuberculosis* in broth culture in combination with RIF, BDQ, and CFZ; however, in *M. tuberculosis*-infected macrophage cultures and in the mouse model, the potentiating adjuvant effect of TIM was the most pronounced with RIF (Table 1). *M. tuberculosis* infection is known to be primarily an intracellular macrophage infection in the mouse model, and since we observed the most synergy with RIF in *M. tuberculosis*-infected macrophages as well as in the mouse model, these results are consistent with TIM inhibiting an efflux mechanism that is induced in *M. tuberculosis*-infected macrophages. It is known that *M. tuberculosis* induces the protein expression of efflux pumps, such as Rv1258c, upon infection of macrophages and these efflux pumps are important for tolerance of RIF and survival during an intracellular infection (9, 10). Furthermore, a direct effect on the intracellular replication of *M. tuberculosis* or the virulence of *M. tuberculosis* 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).

M. tuberculosis 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 to try and reverse drug resistance using TIM and clinical isolates of *M. tuberculosis* and evaluation of these in broth and infected macrophage cultures, as well as *in vivo* studies, are warranted. TIM may also reduce the frequency of genetic mutations for resistance to INH or RIF when used in combination with these antimycobacterial drugs. Further studies of TIM with drugs and drug candidate combinations should be undertaken to identify the frequencies of resistance development and synergies with new compounds 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 12 weeks of treatment; however, since the relapse rate in the 9-week treatment was statistically significant after the treatment phase but not after 8 weeks in the relapse study (observation phase), larger experimental groups of animals and longer periods of time for the study of relapse would provide evidence on the beneficial effect of TIM in drug combinations more conclusive than that which we observed in the current studies. Furthermore, in each of the combination studies described above, the use of 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 *M. tuberculosis*-infected mice.

The utility of TIM in the treatment of human *M. tuberculosis* infection remains unknown. Clinical studies are under way 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 pharmacokinetic parameters within the granulomas of infected animals.

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