Reduced Emergence of Isoniazid Resistance with Concurrent Use of Thioridazine against Acute Murine Tuberculosis

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The repurposing of existing drugs is being pursued as a means by which to accelerate the development of novel regimens for the treatment of drug-susceptible and drug-resistant tuberculosis (TB). In the current study, we assessed the activity of the antipsychotic drug thioridazine (TRZ) in combination with the standard regimen in a well-validated murine TB model. Single-dose and steady-state pharmacokinetic studies were performed in BALB/c mice to establish human-equivalent doses of TRZ. To determine the bactericidal activity of TRZ against TB in BALB/c mice, three separate studies were performed, including a dose-ranging study of TRZ monotherapy and efficacy studies of human-equivalent doses of TRZ with and without isoniazid (INH) or rifampin (RIF). Therapeutic efficacy was assessed by the change in mycobacterial load in the lung. The human-equivalent dose of thioridazine was determined to be 25 mg/kg of body weight, which was well tolerated in mice. TRZ was found to accumulate at high concentrations in lung tissue relative to serum levels. We observed modest synergy during coadministration of TRZ with INH, and the addition of TRZ reduced the emergence of INH-resistant mutants in mouse lungs. In conclusion, this study further illustrates the opportunity to reevaluate the contribution of TRZ to the sterilizing activity of combination regimens to prevent the emergence of drug-resistant *M. tuberculosis*.

Multidrug-resistant (MDR) and extensively drug-resistant (XDR) tuberculosis (TB) are becoming increasingly prevalent in many parts of the world, requiring the urgent development of novel strategies for its control (1). One potential solution is the repurposing of nonantibiotic compounds with antibacterial properties (2, 3), such as enhancement of antibiotic activity (4, 5) reversal of antibiotic resistance (6–8), and inhibition of drug efflux pumps (9–12).

The new use of the old antipsychotic phenothiazine thioridazine (TRZ) for therapy of MDR and XDR TB is now being seriously considered (5, 13–15). The potential advantages of TRZ include its multiple bacterial targets, greatly reducing the risk of drug resistance (16–19), its low cost and the availability of generic formulations, and its documented efficacy against drug-resistant strains both *in vitro* (20, 21) and *in vivo* (22–24). TRZ exhibits synergy with rifampin (RIF) and streptomycin *in vitro* (20) and appears to target both replicating and nonreplicating bacilli (25).

TRZ is known to have significantly higher intracellular than extracellular accumulation, as its MIC against *Mycobacterium tuberculosis* within macrophages is 100-fold lower than that against extracellular bacilli (8, 26). We recently reported that TRZ lacks bactericidal activity in a guinea pig model of predominantly extracellular TB, perhaps due to limited drug penetration into the necrotic cores of guinea pig granulomas as a result of the relatively high protein binding of the drug (95% in humans) (27–30). We hypothesized that the efficacy of TRZ would be greater in the murine model of TB, in which the bacilli are almost exclusively located in the intracellular compartment (31).

In the current study, we conducted single-dose and steady-state pharmacokinetic studies in BALB/c mice to establish human-equivalent doses of TRZ. Next, we performed three separate studies to determine the bactericidal activity of TRZ in BALB/c mice with chronic TB, including a dose-ranging study of TRZ monotherapy, and efficacy studies of human-equivalent doses of TRZ with and without isoniazid (INH) and rifampin (RIF).

MATERIALS AND METHODS

Pharmacokinetics studies. TRZ was purchased from Sigma (St. Louis, MO). Separate groups of three mice were given a single dose of TRZ at 12.5 mg/kg, 25 mg/kg, 37.5 mg/kg, or 50 mg/kg. Mice were anesthetized with isoflurane and exsanguinated by cardiac puncture at 1, 3, 5, 7, 9, and 24 h after TRZ dosing. To generate the steady-state data, mice were treated once daily for 14 successive days, and samples were collected before the 14th dose (24 h after the 13th dose; 0-h time point) followed by collections at 1 h, 3 h, 6 h, 9 h, 12 h, 15 h, 18 h, 21 h, 24 h, 29 h, 32 h, and 48 h. Blood was collected in microcentrifuge tubes, held for 30 min at room temperature, and centrifuged at 6,500 rpm for 10 min to obtain serum. Whole lungs and sera at each time point were frozen at −20°C. Serum and lung homogenate concentrations of TRZ were measured using liquid chromatography-mass spectrometry and liquid chromatography-tandem mass spectrometry, and data were entered into a WinNonlin worksheet (WinNonlin version 4.0; Pharsight, Mountain View, CA) and analyzed using standard noncompartmental techniques in order to determine the relevant pharmacokinetic parameters as described earlier (27).

***Mycobacterium tuberculosis* strains.** An *M. tuberculosis* H37Rv strain, which has been passaged twice in mice (H37Rv-JHU), was used for this study (27). Prior to aerosol infection, cultures were grown to log phase (optical density at 600 nm [OD_{600}], ~0.6 and ~1.0 for chronic and active infections, respectively) in Erlenmeyer flasks containing Middlebrook 7H9 broth (Difco Laboratories) supplemented with 10% OADC (Becton, Dickinson), 0.05% Tween, and 0.1% glycerol on a shaker at 37°C.

Animals. All animals were maintained under pathogen-free conditions and fed water and chow *ad libitum*. Protocols/procedures ap-

TABLE 1 Basic experimental scheme[a]

Expt, group, and dose (mg/kg)	No of mice per time point				
	Day 28	Day 0	1 mo	2 mo	Total
1					
Untreated	5	5	5		15
INH, 10			5		5
TRZ, 12.5			5		5
TRZ, 25			5		5
TRZ, 50			5		5
TRZ, 100			5		5
TRZ, 200			5		5
Total	5	5	35		45
2					
Untreated	5	5	5	5	20
INH, 10			5	5	10
TRZ, 25			5	5	10
INH, 10, + TRZ, 25			5	5	10
RIF, 10			5	5	10
RIF, 10, + TRZ, 25			5	5	10
Total	5	5	30	30	70
3					
Untreated	5	5	5		15
INH, 10			5	5	10
TRZ, 25			5	5	10
INH, 10, + TRZ, 25			5	5	10
RIF, 10			5	5	10
RIF, 10, + TRZ, 25			5	5	10
Total	5	5	30	25	65

[a] Doses of each drug were determined to be equivalent to human exposures based on area under the serum concentration-time curve (AUC) and were given daily (5/7) by gavage.

proved by the Institutional Animal Care and Use Committee at the Johns Hopkins University School of Medicine were followed. Female BALB/c mice aged 4 to 6 weeks were purchased from Charles River Labs (Wilmington, MA).

Aerosol infections and study endpoints. The basic experimental scheme is shown in Table 1. Briefly, we examined activity of TRZ against *M. tuberculosis* in a low-dose, chronic-infection model, and in a high-dose, acute-infection model. A total of 180 mice were aerosol infected with *M. tuberculosis* H37Rv using an inhalation exposure system (Glas-Col, Terre Haute, IN) calibrated to deliver ~10^2 CFU/mouse lung for the chronic-infection studies (experiments 1 and 2) and ~10^4 CFU/mouse lung for the acute-infection studies (experiment 3). After aerosol infec-

tion, mice were randomized into different treatment groups as outlined in Table 1. Five mice per group were sacrificed on the day after infection, on the day of treatment initiation, and at the indicated time points after treatment in order to determine the numbers of CFU implanted in the lungs, the pretreatment baseline CFU counts, and bactericidal activity of each regimen, respectively (Table 1). For assessing efficacy of treatment groups, animals were sacrificed 3 days after the last anti-TB drug dose, to prevent possible carryover of anti-TB drugs onto culture plates. Animal body weights were recorded on a weekly basis, and lung and spleen weights were recorded at the time of sacrifice. The lungs of sacrificed animals were examined grossly for visible lesions, and small, randomly selected sections were formalin fixed for histopathology. The remainder of each lung was homogenized in 2.5 ml PBS. Lung homogenates were plated on 7H11 plates containing cycloheximide (50 μg/ml), carbenicillin (100 μg/ml), polymyxin B (200 U/ml), and trimethoprim (20 μg/ml) for CFU enumeration after 4 weeks of incubation at 37°C.

In addition, undiluted and diluted lung homogenates were plated on 7H11 antibiotic-containing plates plus 4× MIC of TRZ, INH, and RIF in order to quantify the number of drug-resistant colonies. The MICs used for INH, RIF, and TRZ were 0.06 μg/ml, 0.25 μg/ml, and 10 μg/ml, respectively. The proportion of resistant mutants was calculated as the ratio of the CFU count obtained on antibiotic-containing medium to that obtained on antibiotic-free plates.

Antibiotic therapy. INH, RIF, and TRZ were purchased from Sigma and were either dissolved or suspended in distilled water before oral gavage (single dose given once daily, 5 times weekly). Stock solutions were prepared weekly and stored at 4°C. The following doses were used: INH, 10 mg/kg (33); RIF, 10 mg/kg (33); and TRZ, 12.5 mg/kg, 25 mg/kg, 50 mg/kg, 100 mg/kg, and 200 mg/kg.

Statistical analysis. CFU data were derived from five mice per group. Log-transformed CFU were used to calculate means and standard deviations. Comparisons of data among experimental groups were performed by *t* test. *P* values of <0.05 were considered to be statistically significant.

RESULTS

Identification of human-equivalent exposures of thioridazine in mice. Prior to undertaking drug efficacy studies, we sought to determine the human-equivalent dose of TRZ in mice (Table 2) (34). After single-dose oral administration of TRZ at 25 mg/kg, 37.5 mg/kg, and 50 mg/kg, the mean peak serum concentrations (C_{max}) were 79.2 ng/ml, 89.3 ng/ml, and 100.9 ng/ml, respectively. The values for the area under the concentration-time curve from 0 to infinity (AUC$_{0\to\infty}$) for serum were 931.5 ng · h/liter, 1,693.1 ng · h/liter, and 1,306.1 ng · h/liter following TRZ doses of 12.5 mg/kg, 25 mg/kg, and 50 mg/kg, respectively. At steady state, 25 mg/kg TRZ yielded a C_{max} of 86.2 ng/ml and an AUC$_{0\to\infty}$ of 852 ng · h/ml, closely approximating the corresponding values in hu-

TABLE 2 Pharmacokinetics of thioridazine in mice[a]

Test species	Drug dose[b]	C_{max} (ng/ml)	T_{max} (h)	$t_{1/2}$ (h)	AUC$_{0\to\infty}$ (ng · h/ml)
Mouse (serum)	25 mg/kg (SD)	79.2	9.0	3.4	931.5
	37.5 mg/kg (SD)	89.3	9.0		1,693.1
	50 mg/kg (SD)	100.9	9.0		1,306.1
	25 mg/kg(SS)	86.2	1	5.43	852
Mouse (lung)	25 mg/kg (SS)	3,326	1	2.37	26,112
Guinea pig[c]	5 mg/kg (SD)	404 ± 154.86	1.50 ± 0.71	3.19 ± 1.44	2,187.95 ± 9.98
Human[d]	25 mg (SD)	110.83 ± 51.07	1.77 ± 0.81	6.82 ± 1.67	554.86 ± 276.04
Human[d]	50 mg (SD)	197.13 ± 101.86	1.36 ± 0.39	8.24 ± 1.53	1,084.30 ± 582.75
Human[d]	100 mg (SD)	371.80 ± 137.10	1.48 ± 0.38	9.25 ± 1.91	2,639.30 ± 859.20

[a] Data are means ± standard deviations for a minimum of 3 animals.
[b] SD, single dose; SS, steady state.
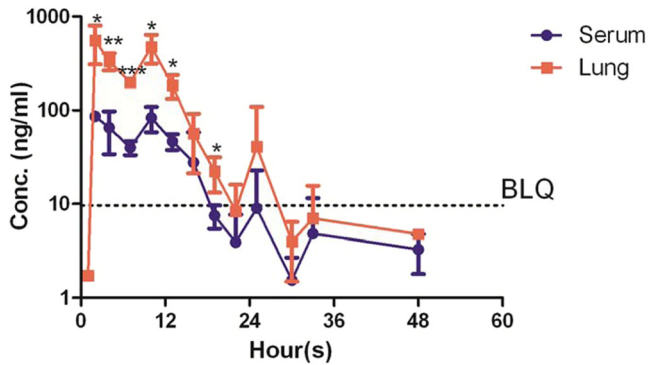[c] See reference 27.
[d] See reference 34.

FIG 1 Serum and lung homogenate concentration profile following repeated dosing of 25 mg/kg thioridazine in mice (3 to 4 per time point). The data are medians ± standard deviations. Statistically significant differences were noted between serum and lung concentrations (*, $P < 0.01$; **, $P < 0.001$; ***, $P < 0.0001$). BLQ, below the limit of quantitation.

mans (C_{max}, 110 ng/ml, and AUC$_{0 \to \infty}$, 554 ng · h/ml) following daily dosing with 25 mg TRZ. The steady-state C_{max} and AUC$_{0 \to \infty}$ in the lungs of mice following daily dosing with 25 mg/kg TRZ were 3,326 ng/ml and 26,112 ng · h/ml, respectively, indicating that the drug concentrates more than 30-fold in the lungs relative to the serum (Table 2 and Fig. 1).

Morbidity and mortality during treatment. We found that 25 mg/kg TRZ was well tolerated in mice, and normalized mean body weights showed increasing trends over time (data not shown). In

the chronic-TB model, all mice treated with 200 mg/kg TRZ died within 3 days and all mice treated with 100 mg/kg TRZ died within 1 week of the initiation of treatment. All remaining mice survived throughout the course of the experiment. Although the cause of death was not determined in the high-dose treatment groups, we hypothesize that it may have been related to the well-known cardiac toxicity of TRZ, which is dose related (15). In the acute-infection model, untreated mice became moribund by 3 weeks after aerosol infection and were euthanized in accordance with animal care regulations, while treated groups of mice survived until the predetermined time points, except for TRZ monotherapy-treated groups.

Therapeutic efficacy. In the chronic-infection model, a total of 1.77 ± 0.04 log$_{10}$ bacilli were implanted in mouse lungs on day 27, and the organisms multiplied to a peak lung burden of 6.32 ± 0.13 log$_{10}$ CFU on day 0 (treatment initiation). Bacillary growth was controlled in the lungs of untreated mice, which maintained a relatively stable plateau of ~6 log$_{10}$ CFU until the end of the study (Fig. 2A). Positive-control mice received 10 mg/kg INH, which showed significant ($P = 0.001$) killing activity against *M. tuberculosis*. However, TRZ given daily at doses ranging from 12.5 mg/kg to 50 mg/kg for a total of 1 month showed minimal bactericidal activity.

In the second experiment using the above model, 2 months of treatment with 10 mg/kg INH or 10 mg/kg RIF reduced the lung bacillary burden by 2.3 log$_{10}$ and 1.4 log$_{10}$ CFU, respectively, compared to the mean lung CFU at the end of the experiment in the untreated control group (Fig. 2B). Treatment with 25 mg/kg TRZ for 2 months reduced lung CFU by 0.41 log$_{10}$. However, TRZ in
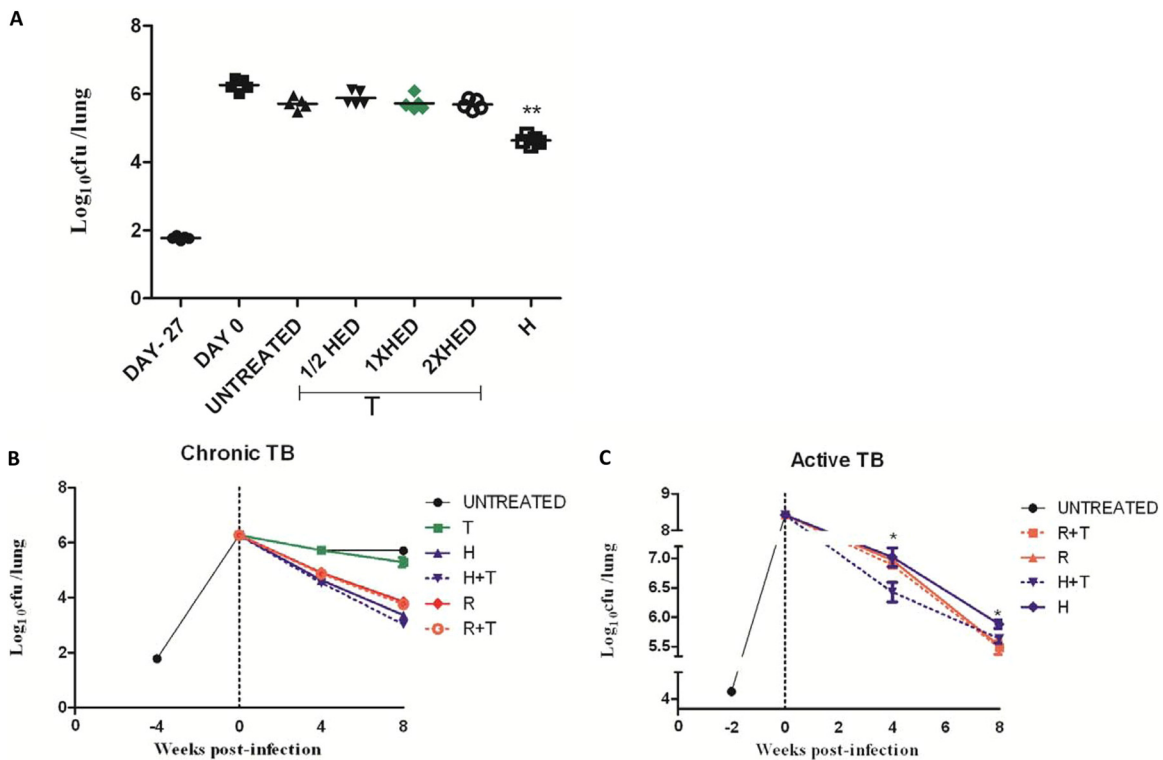

FIG 2 Antitubercular activity of thioridazine in infected mice. Animals were infected via aerosol with ~10^2 (A and B) and ~10^4 (C) CFU of *M. tuberculosis* H37Rv and were either left untreated or treated with drugs daily (5 days/week, once daily) beginning 4 or 2 weeks after infection, respectively. R, rifampin at 10 mg/kg; H, isoniazid at 10 mg/kg; T, thioridazine; HED, human-equivalent dose (25 mg/kg). **, $P < 0.001$ between untreated and INH groups; *, $P < 0.05$ between INH and INH+TRZ groups.

combination with INH or RIF did not show any additive effect (Fig. 2B).

In the third experiment designed to evaluate the activity of TRZ in an acute-TB model, the mean lung CFU count on the day after aerosol infection was 4.37 ± 0.09 $\log_{10}$, and 13 days later, at the initiation of treatment (day 0), the mean lung CFU count was 8.41 ± 0.08 $\log_{10}$ (Fig. 2C). All mice treated with INH or RIF survived, but all mice treated with TRZ alone died in the same time period as untreated mice. Both INH and RIF showed significant bactericidal activity. Coadministration of TRZ with RIF had no additive effect on lung bacillary killing; however, TRZ given in combination with INH showed very modest synergistic activity and a significantly reduced bacterial burden compared to INH alone after 1 month (7.0 ± 0.3 and 6.4 ± 0.4, respectively; $\Delta\log_{10} = 0.59$; $P = 0.03$) and 2 months (5.8 ± 0.1 and 5.6 ± 0.1, respectively; $\Delta\log_{10} = 0.24$; $P = 0.04$) of treatment.

In untreated mice, the total frequency of INH-resistant mutants was 1.3×10^{-6}, as previously reported (35). After 2 months of treatment, INH-resistant mutants were recovered from mice treated with INH alone at a frequency of 3.4×10^{-4}, whereas the addition of TRZ reduced the frequency of INH-resistant mutants 7-fold (2.1×10^{-5}).

DISCUSSION

Our study is the first to explore the pharmacokinetics of the phenothiazine TRZ in BALB/c mice, the validated mouse model used for preclinical TB drug screening. We found that TRZ is lethal in mice at concentrations above 50 mg/kg but that the human-equivalent dose of 25 mg/kg is well tolerated. We also found that TRZ accumulates in murine lung tissue relative to serum. However, TRZ monotherapy did not exhibit greater activity during acute or chronic infection in the murine model, which is characterized by intracellular infection, than in the guinea pig model, in which the majority of organisms are located in the extracellular compartment (28, 33, 36). Indeed, monotherapy with human-equivalent doses of TRZ had very limited activity against chronic murine TB infection, and dose response activity was not observed. On the other hand, an important finding of this study is that, when given in combination with INH, TRZ displayed modest synergy and prevented the selection of INH-resistant mutants.

Our findings expand upon prior studies reporting efficacy of TRZ *in vivo*. Martins et al. showed a >0.7 $\log_{10}$ reduction in lung CFU when TRZ at 0.5 mg daily was initiated 30 days after intraperitoneal infection of BALB/c mice with 10^6 CFU of *M. tuberculosis* (22). van Soolingen et al. showed that monotherapy with TRZ at 32 mg/kg and TRZ at 70 mg/kg reduced the lung bacillary burden by 0.2 to 0.4 $\log_{10}$ compared with untreated controls in mice infected with drug-susceptible *M. tuberculosis*. In mice infected with MDR *M. tuberculosis*, treatment with TRZ alone at daily doses of 32 and 70 mg/kg reduced CFU counts by 0.1 to 0.2 $\log_{10}$ (23).

Interestingly, Viveiros et al. reported that TRZ enhances the activity of RIF and streptomycin when used in combinations at concentrations that are minimally effective when employed separately against clinical strains of *M. tuberculosis* resistant to two or more antibiotics (poly-drug-resistant *M. tuberculosis*) (20). The phenothiazines had no effect on the activity of INH against poly-drug-resistant bacilli. The discrepancy between our findings and those of Viveiros et al. may be due to the different model systems (*in vivo* and *in vitro*, respectively), the use of poly-drug-resistant

M. tuberculosis strains, or the significantly lower concentrations of INH employed in their studies (20).

Phenothiazines inhibit the activity of calcium-dependent ATPase, contributing to the process of acidification of the phagolysosome and the subsequent activation of its hydrolases, thereby inhibiting the replication of the bacterium (10). TRZ also has been reported to act as a drug efflux pump inhibitor in *M. tuberculosis* (37), perhaps via the *emrE*-encoded efflux pump (38). Interestingly, the expression of the *Rv3065* gene, which encodes the multidrug-transport integral membrane protein EmrE, and another putative efflux pump gene, *Rv1634*, is induced following TRZ treatment. In *Mycobacterium smegmatis*, deletion of the *Rv3065* homolog causes increased susceptibility to a number of drugs, including ethidium bromide, acriflavine, and fluoroquinolones.

Several mycobacterial efflux pumps and their regulators are induced during macrophage infection (39). Bacterial efflux pumps that are required for intracellular growth mediate this macrophage-induced tolerance (11). Machado and colleagues have demonstrated that overexpression of such efflux pumps favors accumulation of mutations in INH targets and that INH resistance can be reduced by means of the efflux pumps inhibitors TRZ, chlorpromazine, and verapamil in *M. tuberculosis* (18). Efflux pump inhibitors are thought to act by enhancing the killing of intracellular *M. tuberculosis* by nonkilling macrophages, inhibiting the expression of *M. tuberculosis* efflux pumps responsible for extruding antibiotics prior to reaching their intended targets, and by inhibiting the activity of existing efflux pumps that contribute to the MDR phenotype of *M. tuberculosis*. Therefore, this class of drug should be seriously considered in the development of new therapeutic strategies for preventing the emergence of MDR-TB during treatment (18).

Accumulating evidence suggests that TRZ has the potential to be used as an adjunct to standard therapy that might improve the treatment costs and outcomes of active, latent, and drug-resistant TB. Future studies should focus on whether TRZ can improve the sterilizing activity of combination regimens against persistent bacilli *in vivo*, with the goal of shortening the duration of treatment for drug-susceptible and MDR-TB. Although long-term administration of the phenothiazines is limited by their toxicity, the synthesis of less toxic congeners appears promising (40). Structural modification of the phenothiazine core is possible in a manner that does not affect the ability of the phenothiazine derivatives to inhibit *M. tuberculosis* but abolishes undesirable dopamine and serotonin receptor binding (41).

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