Phenothiazines are being repurposed for treatment of tuberculosis. We examined time-kill curves of thioridazine and first-line drugs against log-growth-phase and semidormant bacilli in acidic conditions and nonreplicating persistent Mycobacterium tuberculosis. While both the potency and the efficacy of first-line drugs declined dramatically as M. tuberculosi replication rates decreased, those of thioridazine improved. The mutation prevalence to 3 times the thioridazine MIC was $< 1 \times 10^{-11}$, better than for $\geq 2$ first-line drugs combined. Hollow fiber system studies revealed that the relationship between stabilizing effect and pharmacodynamic indices (PDI) was characterized by an $r^2$ of 0.88 for peak/MIC, an $r^2$ of 0.47 for the area under the concentration-time curve (AUC) to MIC, and an $r^2$ of 0.14 for the cumulative percentage of a 24-h period that the drug concentration exceeds the MIC under steady-state pharmacokinetic conditions ($\%T_{\text{MIC}}$) at the end of the first week. However, the PDI linked to effect “wobbled” as the duration of therapy increased, so that by the fourth week the $r^2$ was 0.88 for AUC/MIC, 0.78 for $\%T_{\text{MIC}}$, and 0.72 for peak/MIC. This “wobble” has implications on general pharmacokinetic/pharmacodynamic theory, whereby efficacy is linked to only one of the three PDIs in deterministic models. The potency changed 8.9-fold from the first to the fourth weeks. The non-protein-bound AUC/MIC associated with maximal kill at the end of therapy was 50.53 (protein binding = 99.5%). This thioridazine exposure was calculated to extinguish all three M. tuberculosis metabolic populations in human lungs in only 42.9 days of monotherapy. However, this concentration exceeds the 2- to 8-mg/liter thioridazine concentration in serum known to be lethal to humans. Therefore, the way forward for phenothiazine monotherapy that also reduces therapy duration is via synthesis of less toxic congeners.
identified the PDI linked to THI efficacy in our in vitro hollow fiber system model of tuberculosis (HFM-TB).

**MATERIALS AND METHODS**

**Bacteria.** *M. tuberculosis* H37Rv (ATCC 27294) stock cultures were stored at −80°C in 10% glycerol and 90% Middlebrook 7H9 broth with 10% oleic acid, albumin, dextrose, and catalase [OADC] (here termed “broth”). For each study, the *M. tuberculosis* stock was thawed and incubated in broth at 37°C for 4 days under shaking conditions and 5% CO₂ to facilitate exponential-phase growth.

**Drugs.** THI, isoniazid, rifampin, and pyrazinamide were purchased from Sigma-Aldrich. All drugs, except rifampin, were first dissolved in sterile water and subsequently diluted in broth (without 10% OADC). Rifampin was initially diluted in dimethyl sulfoxide and then back-diluted in broth to make a final dimethyl sulfoxide concentration of <1% as described in the past (24). All THI experiments were performed so as to avoid direct light, since phenothiazines are highly potent photosensitizers that cause photodynamic damage to cells, which would lead to exaggerated efficacy (31–33). All drugs were prepared fresh for each experiment.

**MICs and mutation frequencies.** THI MICs were identified using the agar dilution method (34). Mutation frequencies to 2/10⁵ CFU/ml of studies, agar was acidified to a pH of 5.8 prior to addition of the drug, and that cause photodynamic damage to cells, which would lead to exaggerated efficacy (31–33). All drugs were prepared fresh for each experiment.

**Growth rates of different *M. tuberculosis* metabolic populations.** Growth rates of different *M. tuberculosis* populations were measured using 4 different methods: changes in optical density monitored using absorbance at 600 nm (OD₆00), fluorescence in the Live/Dead cell viability assay (Invitrogen), quantification of CFU/ml on agar, and quantification of ribosomes (CFU) (35). RNA extraction was performed using the RNeasy minikit (Qiagen). Growth rates were examined for relative fluorescence units (RFU), CFU/ml, and OD₆00 by determining the slope per unit of time (hours or days).

For studies of log-growth-phase *M. tuberculosis* cultures, on the fourth day of log-phase growth were diluted to make a final concentration of 10⁶ CFU/ml and then coincubated with THI. The cultures were then incubated under ambient air at 37°C under shaking conditions and protected from light. In the first study, the bacilli were coincubated with 0, 0.1, 0.5, 1, 2.5, 5, 10, 20, 40, and 60 mg/liter of THI in duplicate and sampled on days 4 and 7. For all studies, samples were washed twice to remove THI and then serially diluted and cultured on agar. The cultures were incubated for 21 days, and colonies were counted.

The prevalence of mutants resistant to “critical” concentrations of rifampin, isoniazid, and pyrazinamide was also identified. The critical concentrations of rifampin, isoniazid, and pyrazinamide were chosen based on standard susceptibility breakpoints (34). Two hundred microliters of *M. tuberculosis* cultures on day 4 of log-phase growth was inoculated onto each of 20 agar plates to isolate and count resistant isolates in 8.0 log₁₀ CFU, 9.0 log₁₀ CFU, and 10.0 log₁₀ CFU. The cultures on agar were incubated for 4 weeks under 5% CO₂ at 37°C, after which colonies were counted.

Based on the results of this study, a follow-up study was performed with THI concentrations of 0, 0.5, 1, 5, 10, 20, 40, and 60 mg/liter, in triplicate, and sampled on day 7 for quantitative cultures. A third study, isoniazid concentrations of 0, 0.01, 0.03, 0.125, 0.5, 2, and 4 mg/liter, or rifampin concentrations of 0, 0.125, 0.250, 0.500, 0.750, 1, 1.25, 2, and 4 mg/liter, or pyrazinamide concentrations of 0, 3.125, 5, 6.25, 10, 12.5, 25, 30, 100, 150, and 200 mg/liter were coincubated in duplicate with *M. tuberculosis* cultures on day 4 of log phase growth, and cultures were incubated under 5% CO₂ and shaking conditions at 37°C. Cultures were sampled on day 7 for plating on agar for CFU/ml quantitation for all drugs, except those coincubated with pyrazinamide, which were incubated for 21 days prior to CFU quantitation.

Semidormant bacilli (SDB) under acidic conditions were generated in acidified Middlebrook 7H9 broth without OADC (here termed “acidified broth”), using methods described in prior studies (5, 26). Since progressively more acidic environments reduce the replication rates of the bacilli (25), we examined the effect of THI (pKₐ of 9.5) on bacilli growing slowly at three different pHs in acidified broth: 5.8, 6.0, or 6.8. Bacterial cultures on the 4th day of incubation at each pH were diluted in acidified broth to make a final bacillary density of 10⁶ CFU/ml. The SDB were then incubated with THI concentrations of 0, 5, 10, 20, 40, and 60 mg/liter at 37°C under shaking conditions. The cultures were sampled on day 7 and quantitative cultures performed as described above. Rifampin, isoniazid, and pyrazinamide concentrations and coincubation, as well as quantitative cultures, were performed as described for log-growth-phase cultures above.

**Nonreplicating persistent (NRP) *M. tuberculosis* cells were generated by inoculating 3.5 ml of 4.0 log₁₀ CFU/ml *M. tuberculosis* into a 7-ml Vacutainer, together with 3.5 ml of air, and incubated at 37°C under shaking conditions, based on the Wayne and Hayes model (36). Changes in oxygen concentration were monitored by changes in the color of 1.5 mg/liter methylene blue in multiple sentinel tubes. Then, 0, 0.1, 1, 2.5, 5, 10, 20, 40, or 60 mg/liter of THI was added (without adding air or methylene blue color change) to triplicate Vacutainers and then incubated at 37°C under slow shaking. Pyrazinamide coincubation concentrations were as described for log-phase growth cultures, except that all cultures were under anaerobic conditions until plating on agar. The rifampin concentrations were as described for log-phase growth cultures; however, the highest concentration used was 32 mg/liter instead of 8 mg/liter. Similarly, for isoniazid the highest concentration tested was 16 mg/liter. Cultures were sampled on day 7, except for pyrazinamide, as described above.

**HFM-TB studies.** Hollow fiber cartridges were purchased from Fibercell (Frederick, MD). Details of our HFM-TB model have been published in detail over the past decade (22–26). Two types of studies were performed, dose-effect and dose-scheduling studies, based on THI pharmacokinetics achieved in patients (37, 38). Dose-effect studies were performed with SDB *M. tuberculosis*. Each of seven hollow fiber systems (HFS) was dosed daily with one of seven THI exposures, shown in Table 1. To put this in context, 500 mg a day achieves a THI Cₘ₉₀ of 1.0 mg/liter and an AUC₉₀–₂₄ of 10.3 mg · h/liter; the pharmacokinetics were assumed to be linear. From these studies, the effective concentrations associated with either 20%, 50%, or 80% of maximal kill (EC₂₀, EC₅₀, and EC₈₀) were calculated. Next, a dose-scheduling study was performed using SDB in order to identify the PDI linked to THI efficacy. THI EC₂₀, EC₅₀, and EC₈₀ were administered as one of three dose schedules: a single dose once a week, the single dose equally divided into two and administered every 3.5 days, and the single dose equally divided into seven and administered daily (24, 26). The concentrations used for this study are shown in Table 2. The duration of therapy was 28 days. For each experiment, the central compartment was sampled 6 times over the first 24 h, and the samples were frozen at −80°C. The peripheral compartment of each HFM-TB was sampled on days 3, 7, 10, 14, 21, and 28 for the total *M. tuberculosis* population as well as the population growing on agar supplemented with 2X and 30 MIC of THI.

### Table 1 Daily dosing scheme in thioridazine dose-effect studies

<table>
<thead>
<tr>
<th>Dose no.</th>
<th>Cₘ₉₀ (μg/ml)</th>
<th>AUC₉₀–₂₄/MIC</th>
<th>%TₘIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>6.25</td>
<td>6.3</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>12.5</td>
<td>12.47</td>
<td>16.67</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>25.22</td>
<td>45.83</td>
</tr>
<tr>
<td>5</td>
<td>37.5</td>
<td>37.88</td>
<td>66.67</td>
</tr>
<tr>
<td>6</td>
<td>50</td>
<td>50.82</td>
<td>79.17</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>100.69</td>
<td>100</td>
</tr>
</tbody>
</table>
TABLE 2 Dosing scheme in thioridazine dose-scheduling studies

<table>
<thead>
<tr>
<th>Dosing schedule</th>
<th>$C_{\text{max}}$/MIC</th>
<th>$AUC_{0-24}$/MIC</th>
<th>%T$\text{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Daily</td>
<td>1.05</td>
<td>10.54</td>
<td>8</td>
</tr>
<tr>
<td>Daily</td>
<td>1.4</td>
<td>14.38</td>
<td>20.8</td>
</tr>
<tr>
<td>Once a week</td>
<td>10</td>
<td>14.38</td>
<td>15.46</td>
</tr>
<tr>
<td>Daily</td>
<td>2.5</td>
<td>25.22</td>
<td>45.83</td>
</tr>
<tr>
<td>Twice a week</td>
<td>8.75</td>
<td>25.22</td>
<td>29.76</td>
</tr>
<tr>
<td>Once a week</td>
<td>17.5</td>
<td>25.22</td>
<td>19.04</td>
</tr>
<tr>
<td>Daily</td>
<td>3.75</td>
<td>37.78</td>
<td>66.67</td>
</tr>
<tr>
<td>Daily</td>
<td>5.0</td>
<td>50.82</td>
<td>79.17</td>
</tr>
<tr>
<td>Twice a week</td>
<td>17.5</td>
<td>50.82</td>
<td>38.1</td>
</tr>
<tr>
<td>Once a week</td>
<td>35</td>
<td>50.82</td>
<td>23.21</td>
</tr>
<tr>
<td>Daily</td>
<td>10</td>
<td>100.69</td>
<td>100</td>
</tr>
</tbody>
</table>

Assays for identification of THI concentration. Samples from the HFM-TB central compartment were analyzed for THI content using a qualified liquid chromatography-tandem mass spectrometry method. Samples were diluted with methanol, and 10 μl was injected directly without further processing and separated using a YMC hydrosphere C18 guard column. The mobile phase consisted of 0.1% formic acid in methanol and 0.1% formic acid in deionized water. THI was detected using an API 3000 mass spectrometer, which was programmed in the multiple-reaction-monitoring mode and monitored the transition of the precursor ion m/z 371.2 to the product ion m/z 126.1. The method was accurate (±5%) and linear ($r^2 = 0.998$) between 0.1 ng/ml and 1,000 ng/ml. Within- and between-day variation was <5%.

Pharmacokinetic and PK/PD modeling. All THI concentrations were modeled using Adapt 5 software (39). THI concentrations were modeled using a one-compartment pharmacokinetic model with first-order input and elimination. Pharmacokinetic parameter estimates identified using the model were used to calculate exposures such as AUC, AUC/MIC, and %T$\text{max}$. The relationship between drug concentration and size of the M. tuberculosis population was analyzed using the inhibitory sigmoid $E_{\text{max}}$ model in both Adapt 5 and GraphPad 5 software. The inhibitory sigmoid $E_{\text{max}}$ model is a 4-parameter Hill-type equation described by equation 1:

$$\text{Effect} = \frac{E_{\text{max}}}{1 + \left(\frac{E_{\text{max}}}{\text{EC}_\text{50}}\right)^{H}}$$

where the effect is in CFU/ml, $E_{\text{max}}$ is the M. tuberculosis burden without drug treatment, $E_{\text{max}}$ is the maximal microbial kill of the drug in the log phase CFU/ml, EC$\text{50}$ is the drug concentration, and H is the Hill factor. EC$\text{50}$ is the concentration associated with 50% of $E_{\text{max}}$ and defines the potency of the drug.

RESULTS

The THI MIC for M. tuberculosis was 10 mg/liter based on the agar dilution method. There was a sharp cutoff point: 10 mg/liter THI agar plates had zero M. tuberculosis colonies compared to 4.98 ± 0.12 log$\text{10}$ CFU/ml on 5 mg/liter THI agar. When log-phase growth bacilli were incubated on agar with either 2× or 3× the THI MIC, none of 7.8 log$\text{10}$ CFU of bacilli grew on the agar in one experiment, none of 9 log$\text{10}$ CFU in the next experiment, and none of 11 log$\text{10}$ CFU in a third experiment. Thus, the prevalence of low-level THI-resistant mutants was $< 1 \times 10^{-11}$. On the other hand, the prevalence of mutants resistant to rifampin was 1.15 ± 10$^{-6}$ (confidence interval [CI], 1.12 × 10$^{-6}$ to 1.27 × 10$^{-6}$), to isoniazid it was 5.40 × 10$^{-6}$ (CI, 2.80 × 10$^{-6}$ to 7.99 × 10$^{-6}$), to 100 mg/liter of pyrazinamide it was 1.40 × 10$^{-5}$ (CI, 1.14 × 10$^{-5}$ to 1.67 × 10$^{-5}$), and to 300 mg/liter of pyrazinamide it was 1.40 × 10$^{-5}$ (CI, 1.30 × 10$^{-5}$ to 1.50 × 10$^{-5}$).

An examination of M. tuberculosis growth rates in our 3 different metabolic populations revealed the results shown in Table 3. The table demonstrates that log-growth-phase bacteria were growing at a rate multiplefold higher than that of SDB, that SDB growth at pH 5.8 differed only slightly from growth of NRP cells, and indeed that the last two were not statistically significant different from each other by some measures. These measures validate that our 3 metabolic populations grow at different rates, and measures such as the number of ribosomes/CFU demonstrate that the stable CFU/ml in SDB and NRP are not due to a balance of death and growth but to a shift down to reduced or no growth.

Next, we examined the microbial kill of the 3 different M. tuberculosis metabolic populations by the first-line antituberculosis drugs, as well as by THI, in the test tube. Inhibitory sigmoid $E_{\text{max}}$ parameters from these studies are shown in Table 4. First, maximal kill ($E_{\text{max}}$) of THI matched that of rifampin for NRP cells and pyrazinamide against SDB at low pH but was just slightly lower than that of isoniazid against log-growth-phase bacteria. Thus, THI is a drug that kills all 3 populations. Second, THI potency (EC$\text{50}$) was not altered by the different growth rates, as opposed to rifampin, isoniazid, and pyrazinamide, which were dramatically worse in NRP than in faster-growing bacteria. Third, the Hill factor or slope was no different by growth rate of the bacteria, suggesting that binding to drug targets of drug “receptors” does not change by growth rate or metabolic state.

Next, we performed PK/PD studies in the HFM-TB using SDB at pH 5.8. The THI pharmacokinetic parameters achieved in the HFM-TB were a systemic clearance of 0.61 ± 0.50 liter/h/kg, a volume of distribution of 3.61 ± 2.45 liter/kg and a $k_{\text{d}}$/ (absorption constant) of 0.94 ± 0.27/h. The relationship between drug exposure and microbial kill is shown in Fig. 1, with total drug exposure expressed as AUC/MIC. The EC$\text{50}$ increased 8.9-fold, from an AUC$\text{0-24}$/MIC of 4.84 ($r^2 = 0.96$) on day 3 to 42.86 ($r^2 = 0.91$) on day 28. Thus, the potency changed with time. The relationship between exposure and effect on day 28 can be described by equation 2 (where effect is in CFU/ml and $r^2$ is 0.91):

$$\text{Effect} = 6.5 - \left[7.6 \times \left(\frac{\text{AUC}_{0-24}/\text{MIC}}{1 + \sqrt{\text{AUC}_{0-24}/\text{MIC}}}ight)\right]$$

Next, we performed dose-scheduling studies using SDB at pH 5.8. The PDI linked to optimal efficacy is shown in Table 5. The table demonstrates that during the first 14 days, M. tuberculosis kill was most closely linked to THI $C_{\text{max}}$/MIC ratios. The relationship
TABLE 4 Inhibitory sigmoid $E_{\text{max}}$ parameters for different bacillary metabolic populations using static antibiotic concentrations

<table>
<thead>
<tr>
<th>Antibiotic and population</th>
<th>$E_{50}$ in mg/liter (95% CI)</th>
<th>$E_{\text{max}}$ in log_{10} CFU/ml (95% CI)</th>
<th>Hill factor (95% CI)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rifampin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log-phase growth cells</td>
<td>0.67 (0.37–0.56)</td>
<td>5.74 (5.00–6.48)</td>
<td>1.90 (1.29–2.52)</td>
<td>0.95</td>
</tr>
<tr>
<td>SDB</td>
<td>0.32 (0.29–0.36)</td>
<td>5.24 (4.85–5.62)</td>
<td>2.42 (1.91–2.93)</td>
<td>0.98</td>
</tr>
<tr>
<td>NRP cells</td>
<td>10.47 (0.26–5.55)</td>
<td>2.45 (0.90–4.00)</td>
<td>0.88 (0.29–1.46)</td>
<td>0.93</td>
</tr>
<tr>
<td>Isoniazid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log-phase growth cells</td>
<td>0.16 (0.06–0.26)</td>
<td>5.72 (4.73–6.71)</td>
<td>0.82 (0.49–1.15)</td>
<td>0.93</td>
</tr>
<tr>
<td>SDB</td>
<td>0.12 (0.04–0.20)</td>
<td>6.13 (5.20–7.05)</td>
<td>0.48 (0.35–0.61)</td>
<td>0.96</td>
</tr>
<tr>
<td>NRP cells</td>
<td>$&gt;16$</td>
<td>0</td>
<td>0</td>
<td>NC</td>
</tr>
<tr>
<td>Pyrazinamide</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log-phase growth cells</td>
<td>$&gt;200$</td>
<td>$---$</td>
<td>$---$</td>
<td>$---$</td>
</tr>
<tr>
<td>SDB</td>
<td>39.28 (0–87.56)</td>
<td>2.38 (0–4.83)</td>
<td>1.94 (0–5.42)</td>
<td>0.85</td>
</tr>
<tr>
<td>NRP cells</td>
<td>$&gt;200$</td>
<td>0</td>
<td>0</td>
<td>NC</td>
</tr>
<tr>
<td>Thioridazine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Log-phase growth cells</td>
<td>26.53 (22.67–30.39)</td>
<td>3.66 (3.22–4.16)</td>
<td>5.09 (2.93–7.25)</td>
<td>0.98</td>
</tr>
<tr>
<td>SDB pH 6.0</td>
<td>38.67 (12.84–60.0)</td>
<td>4.74 (0–7.00)</td>
<td>5.76 (40.84–52.35)</td>
<td>0.96</td>
</tr>
<tr>
<td>SDB pH 5.8</td>
<td>16.37 (12.01–20.73)</td>
<td>2.01 (1.55–2.47)</td>
<td>4.54 (0.21–8.87)</td>
<td>$&gt;0.99$</td>
</tr>
<tr>
<td>NRP cells</td>
<td>12.38 (5.27–19.49)</td>
<td>2.05 (1.20–2.91)</td>
<td>1.71 (0.13–3.29)</td>
<td>0.98</td>
</tr>
</tbody>
</table>

a Results are for 7-day incubation, except for pyrazinamide, for which incubation was 21 days. NC, not calculated.
b For pyrazinamide effect against log-phase growth cells, $E_{\text{max}}$ had not been achieved at the highest concentration tested, when kill was $\sim 1 \log_{10}$ CFU/ml.

demonstrated the change in thioridazine dose-effect as duration of therapy increases and a shift in EC_{50}.

between free, unbound fraction $C_{\text{max}}$ ($f_{\text{max}}$/MIC) and M. tuberculosis bacterial burden at the end of the first dosing interval (week 1) was as described by equation 3 (where effect is in CFU/ml):

\[
\text{Effect} = 6.0 - \left\{2.6 \cdot \left(\frac{C_{\text{max}}}{\text{MIC}}\right)^{5.3} / \left[(\frac{C_{\text{max}}}{\text{MIC}})^{5.3} + 20.8^{5.3}\right]\right\} (3)
\]

At the end of the second dosing interval (i.e., at the 2-week time point), the $r^2$ for $C_{\text{max}}$/MIC versus bacterial burden was already declining (Table 5), and the relationship was as described by equation 4:

\[
\text{Effect} = 6.1 - \left\{6.2 \cdot \left(\frac{C_{\text{max}}}{\text{MIC}}\right)^{1.3} / \left[(\frac{C_{\text{max}}}{\text{MIC}})^{1.3} + 36.5^{1.3}\right]\right\} (4)
\]

where effect is in CFU/ml. Unexpectedly, after day 14, the PDI that was best explanatory for M. tuberculosis kill was the AUC_0–24/MIC ratio and no longer the $C_{\text{max}}$/MIC ratio. Indeed, the $r^2$ for sterilizing effect and %T_MIC started to improve on day 21 and exceeded that for $C_{\text{max}}$/MIC by day 28, although AUC_0–24/MIC was still the most important (Table 5). This indicates a “wobble” between the PDIs as duration of therapy increases. The relationship between antimicrobial effect (in CFU/ml) and THI exposure on day 21 is described by equation 5:

\[
\text{Effect} = 6.6 - \left\{3.7 \cdot \left(\frac{\text{AUC}_0–24}{\text{MIC}}\right)^{2.3} / \left[(\frac{\text{AUC}_0–24}{\text{MIC}})^{2.3} + 34.9^{2.3}\right]\right\} (5)
\]

At the end of the study, on day 28, the relationship between THI exposure and effect (in CFU/ml) was as shown in Fig. 2 and as calculated by equation 6:

\[
\text{Effect} = 6.52 - \left\{6.506 \cdot \left(\frac{\text{AUC}}{\text{MIC}}\right)^{2.9} / \left[31.33^{2.9} + \left(\frac{\text{AUC}}{\text{MIC}}\right)^{2.9}\right]\right\} (6)
\]

From equation 6, we calculated the non-protein-bound EC_{50} as an AUC/MIC of 50.53, which we used to examine the time to complete sterilization.

For time to complete sterilization, the total bacterial burden expected in lung lesions was first calculated. Pyrazinamide, at 2 g per day, has a sterilizing effect rate (slope) of 0.114 (95% CI, 0.085 to 0.143) log_{10} CFU/ml/day, starting after 2 days of therapy, in patients and the HFM-TB (26, 40). Thus, the total bacillary burden of the subpopulation killed by pyrazinamide from days 2 to 56 in patients calculates to an upper limit estimate of 7.72 log_{10} CFU/ml.

**TABLE 5 Dose-scheduling study results for thioridazine**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>7 days</th>
<th>10 days</th>
<th>14 days</th>
<th>21 days</th>
<th>28 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$/MIC</td>
<td>0.88</td>
<td>0.86</td>
<td>0.79</td>
<td>0.52</td>
<td>0.72</td>
</tr>
<tr>
<td>AUC/MIC</td>
<td>0.47</td>
<td>0.46</td>
<td>0.50</td>
<td>0.83</td>
<td>0.88</td>
</tr>
<tr>
<td>%T_MIC</td>
<td>0.14</td>
<td>0.21</td>
<td>0.17</td>
<td>0.47</td>
<td>0.78</td>
</tr>
</tbody>
</table>

a Values in boldface indicate PDI linked to microbial kill.
ml. The THI EC\textsubscript{90} exposure, which is an AUC/MIC of 50.53 calculated from HFM-TB studies’ equation 6 above, had a sterilizing effect rate of 0.18 \log\textsubscript{10} CFU/ml/day. This drug exposure would lead to total sterilization of SDB population in 42.9 days, as opposed to the 56 days of pyrazinamide currently needed to eradicate this population. If, on the other hand, our calculation of the total microbial population was an underestimate by up to 2 \log\textsubscript{10} CFU/ml (i.e., if the total bacillary burden was 9.72 \log\textsubscript{10} CFU/ml), THI EC\textsubscript{90} would still be able to wipe out that bacterial burden of SDB in 54 days. Given that the efficacy rates and potency of THI against SDB are virtually similar to those against NRP cells in the time-kill studies in test tubes (Table 4) and the size of NRP is much less than SDB (41–43), NRP would be extinguished in a shorter time.

**DISCUSSION**

There are five important findings in our study. First, it has been known since the early days of penicillin and streptomycin use 60 years ago that rapidly dividing cells are easier to kill with chemotherapy than slowly growing ones and that NRP cells are the most difficult to kill (44–46). This is the principle that underlies the basis of current short-course chemotherapy. In reactivation pulmonary tuberculosis, *M. tuberculosis* organisms are believed to exist as one of three growth-rate-defined metabolic populations. Log-phase growth bacteria are rapidly killed by isoniazid within 2 to 5 days, and SDB are killed slowly by pyrazinamide over 8 weeks, while NRP cells are killed over 6 months by rifampicins (40, 41, 47–50). This necessitates multidrug therapy. Here, we found that the thioridazine effect was independent of these growth rates. For a drug whose mechanism of kill is inhibition of ATP synthesis and collapse of proton motive force, both necessary in replicating as well as NRP cells, it may matter little whether the bacillius is replicating or not (51). This suggests that the effectiveness of an antibiotic against *M. tuberculosis* is driven more by its own mechanism of effect than by rates of bacterial replication. Accordingly, antituberculosis agents such as isoniazid, pyrazinamide, ethambutol, streptomycin, and rifampicin that were discovered earlier happened to belong to pharmacophores whose mechanisms of effect involve inhibiting biochemical pathways required for bacterial replication. Their patterns of kill thus became the normative one, and with that came the historical categorization of *M. tuberculosis* metabolic populations as log-phase growth, “semidormant,” and “nonreplicating persistent” cells.

Second, during the early development of current antituberculosis regimens, acquired drug resistance was a major limitation of monotherapy, which necessitated the use of multiple-drug therapy (52–54). Thus, currently, “prevention of the emergence of drug resistance is defined as the ability of a drug to prevent selection of mutants resistant to the companion drug” (55). The explanation has been that the average mutation rates to each of the antituberculosis drugs range from $10^{-7}$ to $3 \times 10^{-10}$, which means that the probability of chromosomal mutations to a single drug arising is near certainty given total lung bacterial burdens of up to $10^9$ CFU. However, the probability of mutations arising to two or more drugs is very small given that it is the product of the mutation rates for each drug. We show, however, that the prevalence of mutants resistant to THI concentrations just above the MIC was better than that for combinations of some first-line antituberculosis drugs. Thus, THI and its congeners could offer an unusually high hurdle for acquired drug resistance, so that it should now be theoretically possible to design antituberculosis drugs that can suppress resistance for themselves without the need for companion drugs.

Third, the PDI linked to efficacy is often assumed to be a single one, often chosen based on the highest $r^2$ or Akaike information criteria for the inhibitory sigmoid $E_{\text{max}}$ model fit (28, 29, 56). It is these deterministic relationships that make PK/PD science a powerful tool for drug development. Nevertheless, these relationships are often derived based on short-term therapy in bacteria with doubling times of only 20 min, which are wiped out by antibiotics within a few days. *M. tuberculosis* grows “in slow motion” compared to these other bacteria (∼72 times slower than *Escherichia coli* when in log-phase growth and ∼720 times slower for semidormant *M. tuberculosis*). This low growth rate and the poor kill rates of antituberculosis drugs allowed us the practical interrogation of basic PK/PD theory. Moreover, the HFM-TB model has the major advantage of being amenable to repetitive sampling of the same microbial population at multiple points, difficult to achieve in other preclinical models in which sampling is a terminal procedure. This system eliminates the possibility that the change in PK/PD effect linked to efficacy could be due to different metabolic populations of *M. tuberculosis* predominating at different periods of the therapy, given that we inoculated only SDB whose status and growth rates we validated at each point in the repetitive sampling scheme. Moreover, since no resistant subpopulation arose, this cannot be due to a switch between the PDI linked to microbial kill and that associated with resistance emergence. Furthermore, we sampled the HFM-TB at the end of each 1-week dosing schedule; earlier sampling can introduce bias by examining effect prior to achievement of the full AUC in the least intermittently infected regimens. Thus, the switch from $C_{\text{max}}$/MIC linked sterilizing effect during the first 2 weeks to AUC/MIC is likely not an artifact. This brings up two obvious questions. First, if the PDI best linked to effect wobbles as the duration of therapy increases, when then is it the best time to identify that relationship? Second, at which time point is it best to calculate optimal drug exposures such as EC\textsubscript{50} and EC\textsubscript{mg}? This is because the concentration-effect curve changed with time such that the EC\textsubscript{50} increased 8.9-fold; values such as the EC\textsubscript{mg} would also change with time. Prudence would suggest choosing the inhibitory sigmoid $E_{\text{max}}$ at the point with the highest...
Different mechanisms of effect due to target promiscuity (57), it is possible that this concept of a wobbling PDI may apply to many more antibiotics used as chemotherapy characterized by long duration.

Fourth, we identified the relationship between THI concentration and efficacy, which enabled us to identify the optimal AUC/MIC exposure (EC_{opt}) of 50.53. If this concentration were achieved in tuberculosis lung lesions in patients, we calculate that relative kill rates, THI monotherapy would be able to wipe out all three *M. tuberculosis* metabolic populations in less than 8 weeks. We based these calculations on the size of the SDB population, which had an upper bound of 7.72 log_{10} CFU/ml, based on pyrazinamide kill rates as well as on the finding that both the EC_{50} and the E_{max} of THI against SDB and NRP cells were virtually the same. Moreover, even if the SDB population was 9.72 log_{10} CFU/ml, the bacterial population would still be wiped out in less than 8 weeks of therapy. Thus, phenothiazines represent an attractive pharmacophore for shortening therapy duration with a single drug, if patients can tolerate the optimal concentrations.

Finally, the EC_{opt} AUC/MIC of 50.53 was derived in the HFM-TB for sterilizing effect, which uses no protein in the broth; thus, this is a non-protein-bound concentration. THI protein binding in human serum is due to α1-glycoprotein and is 99.5% (58, 59). Thus, the EC_{opt} translates to a total AUC/MIC ratio of 10,106. In our laboratory *M. tuberculosis* strain, we found that the THI MIC was 10 mg/liter. If, on the other hand, one assumed a generously lower MIC of 0.1 mg/liter in clinical isolates, this would still be associated with an average concentration (C_{avg}) (C_{avg} = AUC/24 h) of 4.2 mg/liter. A THI C_{avg} of 1 mg/liter in serum is associated with toxicity to patients; 2 to 8 mg/liter is lethal (60). This means that doses that are optimal for *M. tuberculosis* kill will likely be toxic to patients. On the other hand, autopsy studies of patients who died of phenothiazine overdose, as well as pharmacokinetic studies in numerous animal species, demonstrate that concentrations in tissues such as lungs are multiplefold higher than in serum (61–63). Thus, it may be that the drug is concentrated at the site of pulmonary tuberculosis, so that the EC_{opt} could be achieved at lower doses than those that are lethal to humans. Nevertheless, the common MIC in clinical isolates is likely to be higher than 0.1 mg/liter and indeed has been reported as ranging between 6 and 32 mg/liter (64). Finally, the EC_{opt} target concentrations that we derived will also be useful as design specification of less toxic but equally efficacious congeners. Given the short time to extinction of SDB and NRP cells, such congeners would be able to shorten therapy to less than 2 months. We believe that it is in such congeners that greater prospects for use of phenothiazines as monotherapy antituberculosis agents lie.

There are several limitations to our study. First, we did not consider intracellular *M. tuberculosis* bacilli, which are 20% of those in pulmonary tuberculosis cavities (65). However, phenothiazines such as THI are known to be concentrated multiplefold inside mammalian cells (66). Thus, THI may be effective at concentrations lower than those that are toxic for extracellular bacilli. Second, our studies were performed using HFM-TB, which does not mimic many aspects of human physiology. Thus, the clinical meaning of our results could be limited. However, the quantitative predictions of the HFS model of tuberculosis have been shown to be fairly accurate as judged by clinical findings undertaken after the fact; they have also been shown to correlate well with findings in clinical studies performed prior to use of this technology (5–8, 23, 25, 67, 68). Thus, our results likely have clinical relevance. Third, as discussed above, if we had continued therapy beyond 28 days, say up to 2 months, it could be that a different PDI such as %T_{MIC} could have been identified for the latter period. This possibility cannot be discounted.

In summary, THI represents a pharmacophore that is equally effective whether *M. tuberculosis* is replicating or not, under hypoxia or not. The mutation frequency to even 5 × MIC is low, which means that the hurdle for acquired drug resistance is relatively high. THI exhibited an unusual phenomenon, which was that the PDI linked to sterilizing effect changed with time. Nevertheless, at optimal concentrations, THI would be expected to wipe out all 3 metabolic populations of *M. tuberculosis* in less than 2 months, as monotherapy. Unfortunately, the concentrations likely to achieve that would be fatal to patients.

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